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BmEts upregulates promoter activity of *lebocin* in *Bombyx mori*

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ABSTRACT

The Ets family protein BmEts is assumed to be implicated in determination of diapause in the embryogenesis of *Bombyx mori*. In this study, we found that expression of *BmEts* was increased in the fat body and other tissues of the 5th instar larvae in response to *Escherichia coli* injection. Cotransfection experiments using a silkworm cell line revealed that overexpression of BmEts significantly elevated the activity of *lebocin* promoter but not of *cecropin B1*, *cecropin D*, *attacin*, and *moricin* promoters. Activation of the *lebocin* promoter by BmEts was dependent on at least two κB elements and the most proximal GGAA/T motif located on the 5′-upstream region. BmEts further synergistically enhanced *E. coli* or BmRelish1-d2 (active form)-stimulated *lebocin* promoter activation. Two κB elements were also found to be involved in promoter activation by BmRelish1-d2 and in synergistic promoter activation by BmEts and BmRelish1-d2 in the silkworm cells. Specific binding of recombinant BmEts to the proximal κB element and the most proximal GGAA/T motif and interaction between BmEts and BmRelish1 were also observed. To our knowledge, this is the first report of an Ets family protein directly regulating immune-related genes in invertebrates.

1. Introduction

Insects have advanced immune systems that defend against invading pathogens (Brennan and Anderson, 2004; Kanost et al., 2004; Lemaitre and Hoffmann, 2007; Osta et al., 2004; Royet et al., 2005). Elimination of these pathogens by antimicrobial peptides (AMPs) is one of the main humoral defense reactions (Engström, 1999; Imler and Bulet, 2005). Insect AMPs are rapidly produced in specific tissues, such as the fat body and hemocytes, in response to bacterial infection and subsequently secreted into the hemolymph. The expression of insect AMP genes is mainly regulated at the transcriptional level, and several transcription factors, such as Rel/NFκB family proteins (Dushay et al., 1996; Gross et al., 1996; Ip et al., 1993), GATA transcription factors (Petersen et al., 1999; Senger et al., 2006), and C/EBP transcription factors (Meredith et al., 2006), have been shown to regulate transcription of AMP genes in insects. In *Drosophila melanogaster*, three Rel/NFκB family transcription factors, Dorsal, dorsal-related immunity factor (Dif), and Relish are known to be involved in promoter activation of AMP genes (Engström, 1999; Lemaitre and Hoffmann, 2007). These factors possess the conserved Rel homology domain (RHD) and recognize the so-called κB elements located on the 5′-upstream region of AMP genes. In normal conditions, Dorsal and Dif form an inactive complex with Cactus in the cytoplasm. Infection by Gram-positive bacteria or fungi leads to activation of the Toll pathway and subsequent translocation of Dorsal and Dif into the nuclei by dissociation from Cactus (Valanne et al., 2011). Relish, generally present in the cytoplasm, possesses an inhibitor κB domain with ankyrin repeats at the C-terminal region. The activation of the Imd pathway by Gram-negative bacteria initiates endoproteolytic cleavage of Relish, and the N-terminal RHD of Relish translocates into the nuclei and subsequently increases promoter activities of target AMP genes by binding to the κB elements (Kaneko and Silverman, 2005). Dorsal and Relish orthologs are found in other insects, such as *Anopheles gambiae* (Christophides et al., 2002), *Aedes aegypti* (Waterhouse et al., 2007), *Tribolium castaneum* (Zou et al., 2007), *Apis mellifera* (Evans et al., 2006), and *Bombyx mori* (Tanaka et al., 2008).

Ets family proteins are a super family of transcription factors possessing a highly conserved Ets domain that comprises approximately 85 amino acids. Ets proteins bind to specific DNA sequences with the core motif “GGAA/T” and mediate cellular proliferation, differentiation, and tumorigenesis by regulating the expression of

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key genes involved in these phenomena (Oikawa and Yamada, 2003; Wasylyk et al., 1993). In addition, Ets proteins are known to be involved in immunity in vertebrates (Gallant and Gilkeson, 2006). Myeloid Elf-1-like factor (MEF), a member of the Ets protein family, increases the transcription of human *β-defensin 2* (Lu et al., 2004), *lysozyme* (Kai et al., 1999), and a couple of cytokine genes (Hedvat et al., 2004; Miyazaki et al., 1996). Other Ets proteins, such as Ets1 (Blumenthal et al., 1999; Ho et al., 1990), Ets2 (Blumenthal et al., 1999; Ma et al., 1997), Elf-1 (Rellahan et al., 1998), GA-binding protein α (GABPα) (Xue et al., 2004), epithelial-specific Ets (ESE) (Rudders et al., 2001), and Purine-rich box 1 (PU.1) (Rivera et al., 1993) are also reported to regulate the expression of immune-related genes in mammals. However, to our knowledge, Ets proteins that directly regulate the expression of immune-related genes in invertebrates have not yet been reported.

In *B. mori*, six different families of AMPs (cecropin, attacin, lebocin, moricin, gloverin, and defensin) have been identified, and all of them are known to be upregulated in response to bacterial injection (Tanaka and Yamakawa, 2011). κB elements are also conserved in the 5′-upstream region of these genes (Tanaka and Yamakawa, 2011). Furthermore, BmRels (Tanaka et al., 2005) and BmRelishes (Tanaka et al., 2007), putative orthologs of Dorsal and Relish, respectively, in *D. melanogaster* are reported to play a role in activation of the promoter of these genes by recognizing κB elements.

In this study, we found that gene expression of BmEts, which belongs to the Ets family of proteins and appears to be related to diapause in *B. mori*, was upregulated in response to bacterial injection in the silkworm larvae. We further confirmed that BmEts enhanced promoter activity of *lebocin* by recognizing κB elements. To our knowledge, this is the first report of an Ets protein directly regulating immune-related genes in invertebrates.

2. Materials and methods

2.1. Biological materials

B. mori larvae (Tokai × Asahi) were reared on an artificial diet (Nihon Nosan Kogyo) at 25 °C under a controlled environment of 11 h of light and 13 h of darkness. Fifth instar larvae (4 days old) were used in the experiments. The *B. mori* cell lines NIAS-Bm-aff3 and NIAS-Bm-oyanagi2 (Imanishi et al., 2002) were maintained at 25 °C in IPL-41 medium (Invitrogen) containing 10% fetal bovine serum (Equitech-Bio). *Escherichia coli* K12 strain JM109 was grown in Luria–Bertani medium (Difco Laboratories). *Staphylococcus aureus* and *Bacillus subtilis* were grown in 3% tryptic soy broth medium (Difco Laboratories). These bacteria were heated at 121 °C for 1 min, collected by centrifugation (1400× g), and then resuspended in physiological saline (Otsuka Pharmaceutical).

2.2. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted, first-strand cDNA was synthesized, and qRT-PCR was performed on a LightCycler 480 (Roche Diagnostics) according to the method of Tanaka et al. (2010). Standard curves were generated by serial dilutions of the plasmid DNA including the PCR fragments. The mRNA levels were quantified using the second derivative maximum method of LightCycler Software (Roche Diagnostics). Primers used were as follows: 5′-CGGTTCCAACAAGAAGAC-3′ and 5′-TCGAAGAGACACCGTGAG-3′ (forward and reverse primers, respectively, for *ribosomal protein (RP) 49*, GenBank accession number BP179699), 5′-ACCGAGATGATTGGAAGTA-3′ and 5′-CCAGTCACTCTGTAGTTAAA-3′ (forward and reverse primers, respectively, for *BmEts*, GenBank accession number NM_001043437). Gene-specific amplification was confirmed by a single peak in the melting curve analysis (data not shown). Statistical differences were determined by Student's *t*-test. Differences with $P < 0.05$ were considered significant.

2.3. Construction of luciferase reporter, expression, and short hairpin RNA expression plasmids

The 5′-upstream region of *cecropin B1* (GenBank accession number D25320), *attacin*, (GenBank accession number D76418) *lebocin* (GenBank accession number AB003035 equivalent to *lebocin 3*), and luciferase fusion gene constructs were described previously (Tanaka et al., 2005). The 5′-upstream regions of *cecropin D* (GenBank accession number AB701752, −479 to −11) and *moricin* (GenBank accession number AB019538, −585 to −1) (Furukawa et al., 1999), where +1 denotes the translation start site, were amplified by PCR, and then each PCR product was ligated to the pGL3-basic vector containing the luciferase gene (Promega). Site-specific deletion constructs of the *lebocin* promoter were prepared using the KOD-Plus-Mutagenesis kit (Toyobo, Osaka, Japan). For constructing the internal control plasmid pOpIE2-core-Rluc, the region corresponding to −531 to −42 of the *immediate-early 2* gene promoter was deleted from pOpIE2-Rluc (Tanaka et al., 2009b). For construction of pIEx-4-BmEts, the segment from nucleotide no. 25 to 1194 of BmEts cDNA was amplified by PCR and subcloned into the *Nco*I and *Xho*I sites of pIEx-4 (Novagen). For short hairpin RNA expression plasmids targeting BmRelishes (GenBank accession numbers AB298441 and AB298442 for *BmRelish1* and *BmRelish2*, respectively) (pSH-BmRelishes) and BmRels (GenBank accession numbers AB096087 and AB096088 for *BmRelA* and *BmRelB*, respectively) (pSH-BmRels), inverted repeated DNAs 5′-AGGATGAATGCCTCATGTTGTGTGTGCTGTCCACAACATGAGGCATTCATCCT-3′ and 5′-GTACACTTCCAGCTGAAGAGGGTGTGCTGTCCCCTCTTCAGCTGGAAGTGTAC-3′ were inserted between the *Nco*I and *Stu*I sites of pIEx-4-BmU6M (Tanaka et al., 2009a), respectively, according to the method of Tanaka et al. (2009a). pSH-EGFP corresponds to "EGFP-L1" described by Tanaka et al. (2009a).

2.4. Transfection of NIAS-Bm-aff3 cells

A mixture of the transfection reagent, containing 1.35 μl of Polyfect (Qiagen), 0.2 μg of AMP gene promoter-luciferase reporter gene constructs, 0.25 μg of pOpIE2-core-Rluc, and, in some cases, the expression plasmid, was added to NIAS-Bm-aff3 cells (7×10^4). Firefly luciferase activity from each cell extract was measured 48 h after transfection and was normalized against *Renilla* luciferase activity. Both luciferase activities were measured by a lumicounter (Nition) with the dual-luciferase assay system (Promega). Statistical differences were determined with Student's *t*-test. Differences with $P < 0.05$ were considered significant.

2.5. Recombinant protein production

The region between nucleotide no. 25 and 1194 of BmEts cDNA (accession number_001043437) was amplified by PCR and subcloned into the *Nco*I and *Xho*I sites of the glutathione S-transferase (GST) expression vector pGEX-4T-1 (Pharmacia). The fusion protein was expressed in *E. coli* BL21 and purified by affinity chromatography using glutathione agarose beads (Sigma) according to the method of Ip et al. (1992).

2.6. Electrophoresis mobility shift assay (EMSA)

EMSA was performed using the digoxigenin (DIG) gel shift kit, 2nd generation (Roche), according to the manufacturer's instructions. In brief, double-stranded DNA fragments corresponding to