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Developmental regulation and antifungal activity of a growth-blocking peptide from the beet armyworm *Spodoptera exigua*

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ABSTRACT

Insect cytokine growth-blocking peptides (GBPs) are involved in growth regulation and the innate immune response. However, the microbial binding and antimicrobial activities of GBPs remain unclear. Here, we investigate the developmental role and antifungal activity of a GBP from the beet armyworm *Spodoptera exigua* (SeGBP). Sequence analysis predicted that mature SeGBP consists of 24 amino acid residues, including 2 cysteine residues. During *S. exigua* development, *SeGBP* is constitutively expressed in the fat body during the larval and adult stages but not in pupae. *SeGBP* expression is up-regulated by 20-hydroxyecdysone and down-regulated by juvenile hormone analog. Recombinant SeGBP purified from baculovirus-infected insect cells retards the growth of *S. exigua* larvae. Additionally, *SeGBP* expression is acutely induced in the fat body after injection with *Escherichia coli*, *Bacillus thuringiensis*, or *Beauveria bassiana*. Recombinant SeGBP can bind to *B. bassiana* but not to *E. coli* or *B. thuringiensis*. Consistent with these findings, SeGBP shows antifungal activity against *B. bassiana*. Therefore, these results provide insight into the role of SeGBP during the innate immune response following microbial infection, and furthermore, they suggest a novel function for SeGBP as a direct antifungal agent against entomopathogenic fungi, such as *B. bassiana*.

1. Introduction

Since growth-blocking peptide (GBP) was first discovered in armyworms (*Pseudaletia separata*) parasitized by the parasitoid wasp *Cotesia kariyai* (Hayakawa, 1990, 1991, 1995), homologous peptides have also been identified in several other insect species (Kamimura et al., 2001; Matsumoto et al., 2012). Structurally, these GBP peptides are composed of 23–25 amino acids and contain disulfide bonds (Aizawa et al., 1999, 2001; Yoshida et al., 2004; Miura et al., 2002). Functionally, GBPs show a range of biological activities, such as growth retardation, paralytic effects, induction of cell proliferation, and immune cell stimulation (Hayakawa, 1990, 1991; Clark et al., 1997, 1999; Hayakawa and Ohnishi, 1998; Furuya et al., 1999; Wang et al., 1999; Strand et al., 2000; Kamimura et al., 2001; Ohnishi et al., 2001). Furthermore, GBPs belong to the larger family of insect cytokines known as multifunctional ENF peptides, which share the consensus N-terminal amino acid sequence Glu-Asn-Phe (Strand et al., 2000; Hayakawa, 2006; Matsumoto et al., 2012; Tsuzuki et al., 2012).

The silkworm *Bombyx mori* possesses an ENF peptide family member homologous to GBP known as paralytic peptide, which plays diverse roles in the defense reaction and growth regulation (Kamimura et al., 2001; Miura et al., 2002), and a recent publication reported that *Drosophila* GBP mediates acute innate immune reactions under various stress conditions (Tsuzuki et al., 2012). Furthermore, it has been shown that GBP induces antimicrobial peptide (AMP) expression in insects, indicating that GBP affects the humoral immune response (Tsuzuki et al., 2012). Similarly, GBP has been shown to act as a cytokine in insects, in which it up-regulates the expression of immune-related genes and contributes to host defense (Ishii et al., 2008, 2013). To date, at least 16 members of the insect ENF cytokine family have been identified (Matsumoto et al., 2012). Although three variants of GBP (referred to as paralytic peptide (PP) I–III) have been have been identified in the hemolymph of the beet armyworm *Spodoptera exigua* larvae (Skinner et al., 1991), molecular studies of *S. exigua* GBP (SeGBP) are limited. In addition, the functions of GBPs with respect to microbial binding and antimicrobial activity have yet to be determined.

In this study, we report the developmental regulation and antifungal activity of SeGBP. First, we cloned *SeGBP* cDNA and expressed recombinant SeGBP in baculovirus-infected insect cells.

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Next, we characterized the expression and hormonal regulation of *SeGBP* during *S. exigua* development, and measured the transcriptional induction of *SeGBP* in response to microbial challenge. Finally, recombinant SeGBP was assayed for microbial binding and antimicrobial activities, and we provide the first evidence that SeGBP possesses direct antifungal activity, specifically against the entomopathogenic fungus *Beauveria bassiana*.

2. Materials and methods

2.1. Experimental insects

The beet armyworm *S. exigua* used in this study was reared on an artificial diet (Kwon et al., 2006; Wan et al., 2012) at 25 °C under a 16 h light/8 h dark photoperiod with a relative humidity of 60 ± 5%.

2.2. cDNA library screening, nucleotide sequencing and data analysis

The clone containing the GBP cDNA of interest was isolated from an expressed sequence tag (EST) collection generated from a whole larval *S. exigua* cDNA library (Wan et al., 2012). Plasmid DNA was extracted using the Wizard mini-preparation kit (Promega, Madison, WI, USA) and sequenced using an ABI 310 automated DNA sequencer (Perkin–Elmer Applied Biosystems, Foster City, CA, USA). The sequences were compared using the DNASIS and BLAST software programs from NCBI (http://www.ncbi.nlm.nih.gov/BLAST), and MacVector (ver. 6.5, Oxford Molecular Ltd.) was used to align the SeGBP amino acid sequences.

2.3. Production and purification of recombinant SeGBP

A baculovirus/Sf9 insect cell expression system (Je et al., 2001) was used to produce recombinant SeGBP. The *SeGBP* cDNA was amplified with PCR using the plasmid *pBluescript-SeGBP* as a template with the forward primer 5′-GGATCCATGAAATTAACAATCGCC-3′ and the reverse primer 5′-GGTACCTTAATGATGATG ATGATGATGTCCAAAGGTGGGTTT-3′; the reverse primer was designed to include a His-tag sequence. The PCR cycling conditions were as follows: 94 °C for 3 min; 30 cycles of 94 °C for 30 °s, 55 °C for 30 s, and 72 °C for 1 min, and 72 °C for 5 min. The PCR products were sequenced using the BigDye Terminator Cycle Sequencing Kit and an automated DNA sequencer (Perkin–Elmer Applied Biosystems). The amplified *SeGBP* fragment was inserted into the *pBacPAK8* vector (Clontech, Palo Alto, CA, USA) to generate an expression vector under the control of the *Autographa californica* nucleopolyhedrovirus (AcNPV) polyhedrin promoter. For the expression experiments, 500 ng *pBacPAK8-SeGBP* and 100 ng AcNPV viral DNA (Je et al., 2001) were co-transfected into 1.0–1.5 (10^6 Sf9 cells for 5 h using Lipofectin transfection reagent (Gibco BRL, Gaithersburg, MD, USA). Transfected cells were cultured in TC100 medium (Gibco BRL) supplemented with 10% fetal bovine serum (FBS; Gibco BRL) at 27 °C for 5 days. Recombinant baculoviruses were propagated in Sf9 cells cultured in TC100 medium at 27 °C. Recombinant proteins were purified using the MagneHis™ Protein Purification System (Promega). Protein concentrations were determined using the Bio-Rad Protein Assay Kit. To produce and purify mature SeGBP, purified recombinant proSeGBP was digested with bovine factor Xa according to the manufacturer's instructions (Novagen). Briefly, 10 mg recombinant proSeGBP was treated with 10 units of Xa in cleavage buffer at room temperature. After the reaction was complete, mature SeGBP was purified using the MagneHis™ Protein Purification System (Promega). SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analyses were performed as previously described (You et al., 2010).

2.4. RNA isolation and Northern blot analysis

Northern blotting and image analyses were performed as previously described (You et al., 2010). Total RNA was isolated from the epidermis, fat body, and midgut using the Total RNA Extraction Kit (Promega). Total RNA (5 μg/lane) was separated through electrophoresis on a 1.0% formaldehyde/agarose gel, transferred onto a nylon blotting membrane (Schleicher & Schuell), and hybridized at 42 °C with a radioactive cDNA probe diluted in hybridization buffer, which consisted of the following: 5× SSC (1× SSC consists of 0.15 M sodium chloride and 0.15 M sodium citrate), 5× Denhardt's solution (0.1% each of BSA, Ficoll, and polyvinylpyrrolidone), 0.5% SDS, and 100 mg/ml denatured salmon sperm DNA. The radioactive probe was created by labeling the *SeGBP* cDNA with [α-^{32}P] dATP (Amersham Biosciences) using the Prime-It II Random Primer Labeling kit (Stratagene, La Jolla, CA, USA). Following hybridization, the membrane filter was washed three times for 30 min each time with 0.1% SDS and 0.2× SSC at 65 °C and exposed to autoradiography film.

2.5. Growth assay

S. exigua larvae were injected with sample solution between the first and second abdominal segments using 10 μl syringes (Hamilton). To investigate the growth-blocking activity of SeGBP, *S. exigua* larvae were injected on the first day of the fifth-instar stage with 100–1000 ng of purified mature SeGBP diluted in PBS. After injection, the larvae were fed an artificial diet in plastic containers and maintained at 25 °C under a 16 h light/8 h dark photoperiod with a relative humidity of 60 ± 5%. Larvae were weighed and monitored at 24 h intervals.

2.6. Hormonal treatment

Twenty-hydroxyecdysone (20E; Sigma) was dissolved in distilled water and stored at −20 °C until later use. Briefly, 2 μg 20E dissolved in 5 μl distilled water was injected into *S. exigua* larvae on the first day of the fifth-instar stage (Gui et al., 2006). Then, 5 ng of the juvenile hormone analog (JHA) fenoxycarb (Sankyo, Japan) was dissolved in 5 μl acetone and applied topically along the dorsal midline of larvae using a micropipette (Gui et al., 2006). Each assay was carried out in triplicate using three independent tissue preparations. Total RNA was isolated from these tissues as described above.

2.7. Microorganism injection

Injected control *S. exigua* larvae were injected with PBS on the first day of the fifth-instar stage (5 μl/larva). Experimental larvae were inoculated with either bacteria (*Escherichia coli* or *Bacillus thuringiensis*) or fungus (*B. bassiana*). The bacterial cells were isolated through centrifugation from 10 ml overnight cultures, washed with PBS, and resuspended in PBS, and *S. exigua* larvae were injected with a 5 μl solution containing 5.5×10^3 bacterial cells. Fungal spores collected from a culture plate were resuspended in PBS, and larvae were injected with a 5 μl solution containing 5.5×10^3 spores (Hwang and Kim, 2011). Larval tissues were collected and washed twice with PBS. Total RNA was isolated from these tissues as described above.