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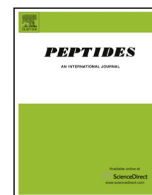

Paralytic peptide activates insect humoral immune response *via* epidermal growth factor receptor

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ABSTRACT

Paralytic peptide (PP) activates innate immunity of silkworm *Bombyx mori*, inducing production of anti-microbial peptides (AMPs) and phagocytosis-related proteins; however the signal pathways of PP-dependent immune responses are not clear. In present study, we characterized BmE cells as a PP-responsive cell line by examining the expression of AMP genes and activation of p38 mitogen-activated protein kinase (p38 MAPK) under PP stimulation, and we also found PP directly binds to BmE cell membrane. Then we found that PP-dependent expression of AMP genes is suppressed by tyrosine kinase inhibitor (genistein) both in BmE cells and in fat body of silkworm larvae. Moreover, the specific tyrosine kinase epidermal growth factor receptor (EGFR) inhibitor (AG1478) attenuates PP-induced expression of AMP genes in BmE cells and fat body of silkworm and RNA interference (RNAi) to BmEGFR also suppresses PP-induced expression of AMP genes. Furthermore, the PP-induced p38 MAPK phosphorylation is inhibited by AG1478. Our results suggest that BmE cells can be used as a cell model to investigate the signal pathway of PP-dependent humoral immune response and receptor tyrosine kinase EGFR/p38 MAPK pathway is involved in the production of AMPs induced by PP.

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Introduction

The innate immune system consists of humoral and cellular immune response. As the major effector molecules in insect humoral immunity, anti-microbial peptides (AMPs) are usually induced by pathogen infections. The best-characterized signaling pathway regulating AMP production is Toll and IMD pathway, both of which are responsible for transducing pathogen recognition signal to activate NF-κB-like transcriptional factors [2,8,9]. Insect cellular defense, including phagocytosis, nodulation, and encapsulation of invading microorganisms, requires cytoskeleton remodeling. Studies have proved that insect immune system has many features highly conserved with vertebrate [4]. For instance, insect Toll and IMD signaling pathway are evocative in several aspects of mammalian TLR and TNF-α receptor pathway [19], and insect plasmatocytes function similar to mammalian macrophages by surrounding and engulfing microorganisms [7]. However, no orthologs of mammalian cytokine have been identified in insect, and the mechanism of communication between different types of cells during immune response or the modulation between humoral and cellular immune response in insect is less well understood.

A family of peptides, first characterized in Lepidoptera then identified in at least five insect orders, has been defined as "insect cytokine" based on their diverse functions in immune response [10]. All of these peptides are synthesized as precursor proteins consisting of 73–152 amino acids, and processed into active peptides about 19–30 amino acids long with sequence homology. The active peptides were originally referred as ENF peptides in Lepidoptera, named after their common N-terminal sequence, E-N-F [21]. Further studies found a signature motif, C-X(2)-G-X(4,6)-G-X(1,2)-C-[KR] present in all ENF peptide as well as their homologs from other insect orders. Interestingly, mammalian EGF also possess this motif at its C-terminus, and NMR analysis showed that the tertiary structure of ENF peptide, composed of a well-structured core stabilized by a disulfide bond and a short antiparallel β-sheet together with unstructured N- and C-terminus, closely resembles C-terminal domain of EGF [1,3]. The active peptides display similar functions in different species, including stimulating aggregation and spreading of phagocytic hemocytes, and inducing the expression of AMPs [5,12,13].

Abbreviations: PP, paralytic peptide; AMP, anti-microbial peptide; p38 MAPK, p38 mitogen-activated protein kinase; EGFR, epidermal growth factor receptor; RNAi, RNA interference; MAPKs, mitogen-activated protein kinases; GBP, Growth-blocking peptide; qRT-PCR, quantitative RT-PCR; FITC-PP, FITC-labeled active PP.

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Efforts taken to understand the molecular mechanism discover several molecules are involved in the signaling pathway activated by those cytokines. Mitogen-activated protein kinases (MAPKs) participate in ENF peptide-dependent induction of AMP genes in Lepidoptera [5]. And *Drosophila* peptide mediates acute innate immune reactions *via* JNK pathway [23]. Recently, an plasma membrane protein p77, which has a trans-membrane domain and an ITAM-like sequence along with SH2/SH3 domain-binding motifs in the cytoplasmic tail, has been characterized as the adaptor of Growth-blocking peptide (GBP), an ENF peptide identified in *Pseudaletia separata* [14]. However, p77 homologs could be only found in the family of Noctuidae but no other Lepidopteran insects. Surprisingly, GBP can also promote the proliferation of human keratinocytes, and compete with EGF in binding to the cytoplasmic membrane of keratinocytes as well as Sf9 cells [15]. Whether any receptor tyrosine kinase, like EGFR participates in transducing insect cytokine-activated signals is still unknown.

As a model organism of Lepidoptera, *B. mori* has been widely studied in search of general physiological and molecular mechanisms. The purpose of this work was to identify whether paralytic peptide (PP), the ENF peptide in silkworm, activates immune response through receptor tyrosine kinase EGFR. Both inhibitor assay and RNAi experiment proved that EGFR is required for PP-induced production of AMPs in fat body as well as in BmE cells, which would be a more consistent and sustainable system for further studies of ENF peptide-activated signaling pathway.

Materials and methods

Animals, cell lines and active PP

Silkworm larvae (DaZao P50 strain) were reared on fresh mulberry leaves at 25 °C and relative humidity of 80%. BmE cells [17] were cultured in Grace medium supplemented with 10% fetal calf serum at 27 °C. PP was chemically synthesized as described previously [12] and the C-terminal were modified by Biotin (ENFVGGCATGFKRTADGRCRPIF-K-biotin, Biotin-PP).

RNA interference (RNAi) in vivo

Template for dsRNA synthesis was generated by PCR using primer pair specific for BmEGFR: forward 5′-TAATACGACTCACTATAGGGACCTGTCGCTATAAAGTGCTAA-3′ and reverse 5′-TAATACGACTCACTATAGGGCATCGGGATTCAGTTGATTGT-3′. Then dsRNA was synthesized *in vitro* using T7 RiboMAX Large Scale RNA Production System (Promega) following the manufacturer's instruction. The dsRNA was diluted in nuclease-free water to the desired concentration (final volume 10 μl) and injected into the abdomen of larvae at the first day of the 5th instar. 24 h later, 50 ng PP (final volume 10 μl) was injected into the hemocoel. After 3 h, silkworm larvae were dissected and fat body tissues were collected on ice.

Quantitative RT-PCR

BmE cells were seeded at 1×10^5 per well in a 12-well culture plate and treated with Biotin-PP (200 ng/ml) in serum-free medium either in the presence or absence of tyrosine kinase inhibitor genistein (Sigma) or EGFR inhibitor AG1478 (Cell Signaling Technology) for 6 h before harvesting. To test the effect of inhibitors *in vivo*, genistein or AG1478 was injected into larvae 30 min before injection of Biotin-PP, followed by additional 3 h incubation at 27 °C. Then the fat body tissues were collected on ice.

Total RNA was extracted from cells or tissue samples using Total RNA Kit (Omega). cDNAs were synthesized by GoScript reverse transcription reagents (Promega) according to the manufacturer's instructions. Primers used for quantitative RT-PCR (qRT-PCR) were listed in Table 1. qRT-PCR was performed using SYBR Premix ExTaq II (Takara) on StepOne™ Plus Real-Time PCR System (Applied Biosystems) with a program consisting of an initial denaturing step of 30 s at 95 °C and 40 amplification cycles consisting of 5 s at 95 °C followed by 30 s at 60 °C. The expression level of AMPs was normalized to the control (SilkDB Probe number: sw22934). All data from the analysis were expressed as means ± standard deviation (S.D.). The statistical significance of differences was determined using Student's *t*-test.

Table 1
Sequence of primers used in qRT-PCR.

Gene	SilkDB/Genebank number	Sequence of primers
BmCecropin A	BGIBMGA014285	F 5′-TTGAGCTTCGTCTTCGCGTT-3′ R 5′-TTGCGTCCCACTTTCTCAATT-3′
BmMoricin	BGIBMGA011495	F 5′-CCGCTCCAGCAAAATACCT-3′ R 5′-TTGAAAACATCGTTGGCTGT-3′
BmAttacin	BGIBMGA002747	F 5′-GTGTAGCGTTGTTGTTGTT-3′ R 5′-AGGTTCCATCCGAGTTCA-3′
BmGloverin B	BGIBMGA013863	F 5′-GGCTGCTATTGACTTGAAC-3′ R 5′-TCTGTGACCGAACTCCTT-3′
BmEGFR	BGIBMGA000602	F 5′-TGTGTTATGGTATCATGTTGGATGTT-3′ R 5′-CGCCATTTCAGCGAATGTATC-3′
SfDefension	gi|32394731	F 5′-AGGTAATAAATGTGTTTCTGCTGA-3′ R 5′-GTGACTGACGCAGATGCCGTATGTG3′
SlCecropin A	gi|4762019	F 5′-TTCTTCGTGTTCGCGTGTCTGCT-3′ R 5′-TCCAGCCTTGATGATACCGTCTCTG-3′
SlCecropin B	gi|4762021	F 5′-ATCTTGTCCTTCGTGTTCG-3′ R 5′-CCTTTACGATACCGTCTCGGATGTT-3′
SlLysozyme	gi|206598481	F 5′-GCGCTTGTGGCGTTTTGCTTGCATT-3′ R 5′-GTTCCCATCTTGTCCGTCTTCCTAC-3′

Western blot analysis

Cell membrane protein was extracted from BmE cell according to the method described by Wang et al. [24]. Fat body tissues were dissected and homogenized in the lysis buffer described in previous report [14]. After centrifugation at 17,000 × *g* for 15 min at 4 °C, the supernatant was collected on ice for western blot analysis. Concentration of cell membrane protein or whole cell lysate was determined by BCA assay. 20 μg proteins per sample were resolved on 12% SDS-PAGE. After transferred to PVDF membrane (GE Health Care), samples were immuno-blotted with antibodies as indicated in graphs.

Confocal microscopy

FITC-labeled active PP (FITC-PP) were chemically synthesized and purified by reverse-phase HPLC. BmE cells seeded on confocal dish were blocked with 1% BSA/Grace medium at 4 °C for 10 min prior to 30 min incubation with FITC-PP (20 ng/ml). Then cells were washed three times with PBS and fixed in 4% formaldehyde at 4 °C for 15 min. Finally, cells were stained with DAPI (Sigma) in PBS at 4 °C for 5 min and washed twice. Fluorescence was observed under a confocal microscope (Olympus FV1000) and the images were edited using FV10-ASW1.1 software.

Results

PP induces production of AMPs and activation of p38 MAPK in BmE cells

To investigate PP activated signaling pathway in a more sustainable and stable system, we examined the response of different cell lines to active PP. After treating Sf9, Spli221, BmNs [16] or

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