Developmental and Comparative Immunology 34 (2010) 638–647

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Functional analysis of four processing products from multiple precursors encoded by a lebocin-related gene from *Manduca sexta*

Subrahmanyam Rayaprolu [a], Yang Wang [a], Michael R. Kanost [b], Steven Hartson [c], Haobo Jiang [a,*]

[a] Department of Entomology and Plant Pathology, Oklahoma State University, Stillwater, OK 74078, USA
[b] Department of Biochemistry, Kansas State University, Manhattan, KS 66506, USA
[c] Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater, OK 74078, USA

ARTICLE INFO

Article history:
Received 7 October 2009
Received in revised form 7 January 2010
Accepted 12 January 2010
Available online 28 January 2010

Keywords:
Insect immunity
Antimicrobial peptide
Convertase
Variable repeats
Hemolymph protein

ABSTRACT

Antimicrobial peptides (AMPs) are a crucial component of the natural immune system in insects. Five types of AMPs have been identified in the tobacco hornworm *Manduca sexta*, including attacin, cecropin, moricin, gloverin, and lebocin. Here we report the isolation of lebocin-related cDNA clones and antibacterial activity of their processed protein products. The 17 cDNA sequences are composed of a constant 5′ end and a variable 3′ region containing 3–16 copies of an 81-nucleotide repeat. The sequence of the corresponding gene isolated from a *M. sexta* genomic library and Southern blotting results indicated that the gene lacks introns and exists as a single copy in the genome. The genomic sequence contained 13 complete and one partial copy of the 81-nucleotide repeat. Northern blot analysis revealed multiple transcripts with major size differences. The mRNA level of *M. sexta* lebocin increased substantially in fat body after larvae had been injected with bacteria. The RXXR motifs in the protein sequences led us to postulate that the precursors are processed by an intracellular convertase to form four bioactive peptides. To test this hypothesis, we chemically synthesized the peptides and examined their antibacterial activity. Peptide 1 killed Gram-positive and Gram-negative bacteria. Peptide 2, similar in sequence to a *Galleria mellonella* AMP, did not affect the bacterial growth. Peptide 3 was inactive but peptide 3 with an extra Arg at the carboxyl terminus was active against *Escherichia coli* at a high minimum inhibitory concentration. Peptide 4, encoded by the 81-bp repeat, was inactive in the antibacterial tests. The hypothesis that posttranslational processing of the precursor proteins produces multiple bioactive peptides for defense purposes was validated by identification of peptides 1, 2, and 3 from larval hemolymph via liquid chromatography and tandem mass spectrometry. Comparison with the orthologs from other lepidopteran insects indicates that the same mechanism may be used to generate several functional products from a single precursor.

1. Introduction

Antimicrobial peptides (AMPs) participate in innate immunity of various organisms ranging from prokaryotes to plants, invertebrates, and vertebrates [1,2]. Most of them are less than 5 kDa, hydrophobic, membrane-active, and carry positive net charge at physiological pH. These peptides are either absent or present at low constitutive levels in naïve insects. Upon microbial infection, association of host recognition molecules and microorganisms triggers extracellular serine proteases to activate a spätzle precursor via limited proteolysis [3]. Spätzle then binds to the Toll receptor to initiate an intracellular pathway that relays the signal into nucleus, where transcription factors of the Rel family induce the AMP gene expression [4]. Identification of orthologous genes in the *Anopheles gambiae*, *Aedes aegypti*, *Apis mellifera*, *Tribolium castaneum*, and *Bombyx mori* genomes suggests that similar signaling pathways exist in other insects to induce the production of defense proteins by fat body and hemocytes [5–9]. Fat body, a tissue analogous to liver, synthesizes AMPs and secretes these heat-stable compounds into the plasma to kill the invading microbes.

Insect AMPs can be categorized into the following groups: α-helical peptides (e.g. cecropin and moricin), disulfide-stabilized peptides (e.g. defensin and drosomycin), proline-rich peptides (e.g. lebocin and drosocin), glycine-rich peptides (e.g. gloverin and diptericin), and others [2]. In the tobacco hornworm *Manduca sexta*, at least five types of AMPs have been identified, including attacin, cecropin, moricin, gloverin, and lebocin [10–13]. As a step

* Corresponding author at: Department of Entomology and Plant Pathology, 127 Noble Research Center, Oklahoma State University, Stillwater, OK 74078, USA. Tel.: +1 405 744 9400; fax: +1 405 744 6039.
E-mail address: haobo.jiang@okstate.edu (H. Jiang).

0145-305X/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.dci.2010.01.008

towards understanding the role of AMPs in humoral immune responses of *M. sexta*, we have characterized a lebocin-like protein, its processing products, and corresponding gene in this work.

Lebocins are proline-rich AMPs first purified from the silkworm, *B. mori* [14]. These 32-residue peptides are glycosylated at Thr^{15} and this modification is important for the antimicrobial activity against Gram-negative bacteria. cDNA and gene cloning indicates that the active peptide is located near the carboxyl terminus, after a signal peptide and a 102-residue pro-segment [15,16]. Lebocins increase the permeability of liposomes at a low ionic strength and have weak antibacterial activities under physiological conditions. They seem to function as synergists by reducing the minimum inhibitory concentration of cecropin D [17]. Lebocin cDNAs have been isolated from *Trichoplusia ni* [18] and *Pseudoplusia includens* [19]. In lebocin homologs of *Samia cynthia* [20], the pro-segment aligns well with the *B. mori* sequences, but the part corresponding to the mature lebocin differs significantly. Two peptides, purified from hemolymph of *Helicoverpa armigera* and *Galleria mellonella*, are similar to a region in the pro-segment of *B. mori* lebocins [21,22]. The 42-residue anionic peptide-1 from the greater wax moth *G. mellonella* is active against *Micrococcus luteus*, *Listeria monocytogenes* and filamentous fungi, but neither of these peptides inhibits the growth of Gram-negative bacteria (e.g. *Escherichia coli*). It is unclear how these peptides are derived from their protein precursors (pro-lebocins). Here we report the cDNA and genomic cloning of lebocin-related proteins from *M. sexta*, which suggests a conserved mechanism in Lepidoptera to generate structural/functional diversity in products derived from pro-lebocins. This mechanism is validated by identification in hemolymph of three processing products by mass spectrometry. For simplicity, we use "lebocin" in parts of the paper to describe the entire gene, cDNA, and protein that are related but not the same as the mature lebocin peptide.

2. Materials and methods

2.1. Insect rearing and bacterial challenge

M. sexta (eggs purchased from Carolina Biological Supply) were reared on an artificial diet [23]. Day 2, fifth instar larvae were injected with formalin-killed *E. coli* (2×10^8 cells/larva). Alternatively, day 2, fifth instar larvae or day 1, male adults were injected with 100 μg *M. luteus* (Sigma) or with sterile water as a control. At various time points after injection, hemocytes and fat body were collected for RNA preparation. Muscle tissues or gut-removed carcasses were used for genomic DNA isolation.

2.2. Screening for full-length lebocin cDNA clones

A 280 bp *M. sexta* lebocin cDNA fragment [11] was labeled with [α-^{32}P]-dCTP using Multiprime DNA Labeling System (GE Healthcare Life Science). A *M. sexta* induced fat body cDNA library [24] was screened according to Sambrook and Russell [25]. Positive plaques were purified to homogeneity via secondary and tertiary screening. Plasmids, *in vivo* excised from the positive bacteriophages, were sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). Sequences were assembled using MacVector Sequence Analysis Software (Oxford Molecular Ltd.).

2.3. Multiple sequence alignment and phylogenetic analysis

Based on BLAST search of GenBank (http://www.ncbi.nlm.nih.gov/) and ButterflyBase (http://butterflybase.ice.mpg.de/) using *M. sexta* lebocin as query, homologous protein sequences were retrieved and compared using ClustalX 1.83 (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/) [26]. A Blosum 30 matrix [27], with a gap penalty of 10 and an extension gap penalty of 0.1 were selected for multiple sequence alignment and unrooted phylogenetic tree was constructed based on neighbor-joining algorithm. Treeview (http://taxonomy.zoology.gla.ac.uk/rod/treeview. html) [28] was used to display phylogram.

2.4. Genomic library screening, subcloning, and DNA sequencing

The insert from a full-length lebocin cDNA clone (NC2), obtained after digestion with EcoRI and XhoI, was labeled with [α-^{32}P]-dCTP and used as a probe to screen a *M. sexta* genomic library in λGEM11, kindly provided by Dr. Yucheng Zhu at the Southern Insect Management Research Unit (USDA ARS). Following plaque purification and amplification, phage DNA was isolated using Wizard Lambda Preps DNA Purification System (Promega). To determine its restriction map, the DNA was digested with one, two or three of the enzymes (XhoI, ApaI, EcoRI, HindIII, KpnI, SacI, SalI and XbaI) and separated by 0.8% agarose gel electrophoresis. After transferring onto a GenScreen Plus membrane (NEN Life Science Products), the DNA fragments were hybridized with the full-length lebocin cDNA, labeled by DIG-High Prime DNA Labeling Detection Kit (Roche Applied Science). Fragments of the lebocin gene were subcloned, sequenced, and assembled as described above.

2.5. Genomic Southern blot analysis

M. sexta genomic DNA was extracted from muscles of a single fifth instar larva using a DNeasy Blood and Tissue Kit (Qiagen). The genomic DNA was also isolated from two larval carcasses according to Bradfield and Wyatt [29]. About 10 μg of DNA was digested with XhoI and ScaI at 37 °C overnight and separated by 1% agarose gel electrophoresis. After capillary transfer onto a nitrocellulose membrane, hybridization was carried out using ^{32}P-labeled XhoI–ScaI fragment of the lebocin cDNA.

2.6. Northern blot and RT-PCR analysis

Fat body RNA samples were prepared from the individual insects using Micro-to-Midi Total RNA Purification System (Invitrogen) or the method described by Chinzei et al. [30]. Denatured total RNA samples were separated on a 1% agarose gel containing 2.2 M formaldehyde. After electrophoresis, RNA was transferred to a membrane and hybridized with ^{32}P-labeled XhoI–ScaI fragment of the cDNA. Similarly, the time course of lebocin expression in larvae and adults were examined. For RT-PCR analysis, hemocyte and fat body total RNA samples were prepared from naïve and injected larvae. In each reaction, RNA (2–4 μg), oligo(dT) (0.5 μg) and dNTPs (1 μl, 10 mM each) were mixed with DEPC-treated H_2O in a final volume of 12 μl and denatured at 65 °C for 5 min. M-MLV reverse transcriptase (1 μl, 200 U, Invitrogen), 5× buffer (4 μl), 0.1 M dithiothreitol (2 μl), and RNase OUT (1 μl, 40 U) were added to the RNA for cDNA synthesis at 37 °C for 50 min. The *M. sexta* ribosomal protein S3 mRNA was used as an internal control to normalize the cDNA samples in a PCR using primers j501 (5′-GCCGTTCTTGCCCTGTT-3′) and j504 (5′-CGCGAGTTGACTTCGGT-3′). The lebocin cDNA fragment was amplified using forward (5′-CTGATTTTGGGCGTTGCGCTG-3′) and reverse (5′-GCGCGTATCTTCTATCTGGA-3′) primers under conditions empirically chosen to avoid saturation: 30 cycles of 94 °C, 30 s; 50 °C, 30 s; 72 °C, 30 s. The relative levels of lebocin mRNA in the normalized samples were determined by 1.5% agarose gel electrophoresis.