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Cloning and expression of a gene encoding gallerimycin, a cysteine-rich antifungal peptide, from eri-silkworm, *Samia cynthia ricini*

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Abbreviations: PGN, Peptidoglycan; PBS, phosphate buffered saline; NF-κB, nuclear factor κB; C/EBP, CCAAT/enhancer binding protein; CRE-BP1, cAMP-responsive element binding protein 1;

Abstract

A cDNA clone encoding gallerimycin was isolated from larval fat body of immunized *Samia cynthia ricini* and named as *Scr-gallerimycin*. In naive larvae, no gene expression was detected, but strongly induced in fat body and hemocytes following immune challenge with bacteria or entomopathogenic fungus *Beauveria bassiana*. Strong expression of the gene was also induced by injection of peptidoglycan and zymosan, but very weakly by non-pathogenic fungus *Aspergillus oryzae*. Analysis of the sequence upstream from the cDNA shows the presence of motifs homologous to binding sites for NF-κB, C/EBP and CRE-BP1.

Keywords: Insect immunity; Antifungal peptide; Gallerimycin; NF-κB; C/EBP; Eri-silkworm; *Samia cynthia ricini*

1. Introduction

Insects have an effective humoral defense system against microorganisms, in which inducible antimicrobial peptides play a major role to eliminate invading microorganisms (Hetru et al., 2003). Defensins and defensin-like peptides are the most widely distributed family of defense molecules against microbial infections (Dimarcq et al., 1998), which have been found not only in insects, but also in vertebrates and plants (Hoffmann et al., 1999). Insect defensins are cationic peptides with six conserved cysteine residues, and most of them act against Gram-positive bacteria but with little effect on Gram-negative bacteria and fungi (Dimarcq et al., 1998). In lepidopteran insects, typical defensin has not been described so far, but inducible defensin-like peptides with antifungal activity have been identified; heliomicin from *Heliothis virescens* (Lamberty et al., 1999), and gallerimycins from *Galleria mellonella* (Schuhmann et al., 2003) and *Spodoptera frugiperda* (Volkoff et al., 2003). These antifungal peptides have the six conserved cysteine residues as in defensins, but rather low sequence homology to insect defensins, and are more related to drosomycin, the antifungal peptide from *Drosophila melanogaster* (Fehlbaum et al., 1994).

Gallerimycin has first been identified as a cDNA clone coding for a defensin-like peptide in the hemocytes of LPS-injected *G. mellonella* larvae, and the antimicrobial activity was tested with the recombinant protein (Schuhmann et al., 2003). The recombinant protein exhibited activity against *Metarhizium anisoplie*, an entomopathogenic fungus, but not against Gram-positive and negative bacteria or yeast. In this study, we cloned a cDNA encoding

gallerimycin from immunized larval fat body of wild silkmoth, *Samia cynthia ricini*, and studied for the first time the induction of the gene expression in some detail. We also analyzed the upstream region of the gene from the transcription start in order to identify *cis* regulatory elements involved in the regulation of the gene.

2. Materials and methods

2.1 Insect and microorganisms

Eri-silkworms (*S. cynthia ricini*) were reared on an artificial diet (Silkmate L4M, Nihon Nosan Kogyo) at 27ºC under aseptic conditions as previously described (Fujimoto et al., 2001). The larvae on the third day of the fifth instar were used for the experiments. *Escherichia coli* K12 (IFO3301) and *Bacillus licheniformis* (IFO12195) were obtained from Institute of Fermentation Osaka. Bacteria were cultured as previously described (Morishima, 1998), and killed by irradiating UV-light (254 nm) in a cross-linker CL-1000 (UVP Inc.) at 1 J/cm^2 for 5 min. The bacterial cells were extensively washed with PBS and immediately used for the experiments. *Aspergillus oryzae* and *Beauveria bassiana* were from the stock culture of Microbial Technology Laboratory, Tottori University, and cultured on potato dextrose agar at 25ºC.

2.2. Chemicals

Peptidoglycan (PGN) was prepared from *B. licheniformis* cell wall, and solubilized by hydrolysis with hen egg lysozyme as previously described (Morishima, 1998). The purity of the PGN preparations was confirmed by amino acid analysis. LPS from *E. coli* K235, laminarin from *Laminaria digitata* and zymosan from *Saccharomyces cerevisiae* were purchased from Sigma Chemicals. Oligo-chitin (hexa-*N*-acetylchitohexaose) was purchased from Seikagaku Kogyo. Curdlan was purchased from Wako Pure Chemicals.

2.3. Injection and collection of tissues

The *Samia* larvae on the third day of the 5th instar were anesthetized on ice for 30 min, and injected either with 2 x 10^6 cells of UV-killed bacteria, 2 x 10^4 fungal conidia or 10 μg of various cell wall components in 10 μL of PBS (137 mM NaCl, 3 mM KCl in 6 mM phosphate buffer, pH 7.4; prepared with Milli-Q water and filtered through a 0.22 μm membrane) into hemocoel with a syringe. The larvae were dissected on ice, and the tissues were collected and immediately frozen on dry ice and kept at -80ºC.

2.4. cDNA cloning and nucleotide sequencing

cDNA fragments with high homology to *G. mellonella* gallerimycin cDNA were selected from subtracted cDNA library enriched with the genes differentially expressed in immunized fat body (Bao et al., 2003), and used as a probe to screen a cDNA library of immunized *Samia* larval fat body (Bao et al., 2005).

The upstream region from the gallerimycin cDNA was cloned by means of inverse PCR. Genomic DNA extracted from the fat body was digested with the restriction enzyme *Pst* I, and recircularized. The ligation product was used as template for a PCR with the primers 5'-GCGAACACCAAACAAGCC-3' and 5'-CTCCATTCCCGAGATGTGTG-3', and the resulting PCR product was cloned. The nucleotide sequence was determined using Big Dye Terminator Cycle Sequencing Kit and a DNA sequencer (Applied Biosystems). The nucleotide sequence and deduced amino acid sequence were compared with the sequences available at DDBJ (www.ddbj.nig.ac.jp) using the FASTA program. Transcription factor binding consensus sites in the putative promoter regions were identified using MOTIF Search program at KEGG (motif.genome.jp).

2.5. Northern blotting

Total RNA was extracted from tissues with guanidine thiocyanate. Three to ten μg of the RNA was denatured and electrophoresed on a 1% agarose-formaldehyde gel. After transferring the RNA to Hybond-N^+ membrane (Amersham), the membrane was hybridized with ^{32}P-labeled cDNA probe as previously described (Bao et al., 2003). The membrane was exposed to Imaging Plate (Fuji Film) for 3 h, and the image was visualized and analyzed with a fluoroimage analyzer (Fuji Film FLA-5000).

3. Results

3.1. Isolation of cDNA clone encoding gallerimycin

A full length cDNA clone encoding gallerimycin was isolated by screening a cDNA library of immunized *Samia* larval fat body, and named *Scr-gallerimycin*. The nucleotide sequence and deduced amino acid sequence are shown in Fig. 1 (DDBJ/GenBank/EMBL accession no. **AB366558**). The cDNA had a single open reading frame encoding 74 amino acid residues. The encoded protein had a predicted signal peptide of twenty residues (Bendtsen et al., 2004). The calculated molecular mass and pKa of the mature protein were 6.21 kDa and 7.6, respectively.

3.2. cDNA upstream region analysis of Scr-gallerimycin gene

Partial promoter sequence was analyzed for *Scr-gallerimycin* gene (Fig. 2). A putative TATA box is located 25 bp upstream from the transcription start site. NF-κB binding site was found 275 bp upstream from the transcription start site. C/EBP-like binding site was identified 166 bp upstream from the NF-κB binding site, and proximal to this site, a GATA binding site was identified. cAMP-responsive element binding protein 1 (CRE-BP1) binding site was also identified 111 bp upstream from the transcription start site.

3.3. Induction of Scr-gallerimycin gene expression

Time course of *Scr-gallerimycin* gene expression in larval fat body was analyzed by Northern blotting using *Scr-gallerimycin* cDNA as a probe after injecting the larvae with UV-killed *E. coli* or *B. licheniformis* cells, or live conidia of pathogenic *B. bassiana* or non-pathogenic *A. oryzae* (Fig. 3). The expression of attacin gene (Kishimoto et al, 2002) was also examined for comparison. The transcript of *Scr-gallerimycin* gene was not detected in non-injected control, but detectable 1 h after the injection of microorganisms, and reached the maximum levels 6 to 9 h after the injection. Among the microorganisms tested, *E. coli* was the most effective elicitor. The *B. licheniformis* and *B. bassiana* also induced strong expression of *Scr-gallerimycin* gene, but *A. oryzae* induced only weak expressions slightly above the levels caused by PBS injection. The expression pattern of *Scr-gallerimycin* gene was totally similar to that of attacin gene.

3.4. Tissue specific expression of Scr-gallerimycin gene

The expression of *Scr-gallerimycin gene* was studied in major tissues, that have been known to express various immune-related genes (Kishomoto et al., 2002; Bao et al., 2003; 2005; Hashimoto et al., 2007). In naive larva, *Scr-gallerimycin* transcript was not detected in any tissue tested, but upon injection with bacteria or *B. bassiana*, the gene expression was strongly induced in hemocytes and fat body, weakly in Malpighian tube and almost none in mid gut (Fig. 4). The expression levels of *Scr-gallerimycin* gene in hemocytes were much higher than the levels in fat body, in contrast to attacin gene, which expressed most strongly in fat body.

3.5. Elicitor specificity for induction of Scr-gallerimycin gene expression

The elicitor specificity was analyzed by injecting *Samia* larvae with UV-killed bacteria and various microbial cell wall components, and the levels of *Scr-gallerimycin* and attacin gene

expressions in the fat body were detected by Northern blotting (Fig. 5). Gram-positive and negative bacterial cells, *B. licheniformis* PGN and zymosan induced strong expression, but LPS, oligo chitin, laminarin and curdlan induced only very low levels of expression. The expression pattern was very similar to that of attacin gene.

4. Discussion

A cDNA clone encoding gallerimycin was isolated from the fat body of immunized *S. ricini* larvae, and designated as *Scr-gallerimycin*. Amino acid sequence of the deduced protein was compared with two gallerimycins available on the data base (Fig. 6). The three gallerimycins have high homology with each other; the identities of the whole sequence of *Scr-gallerimycin* to *Spodoptera* and *Galleria* proteins are 59 and 63%, respectively. Each of the proteins contains six cysteine residues in the C-terminal part that are common for arthropod defensins and possibly involved in intramolecular disulfide bonds as suggested by Volkoff et al. (2003), and the amino acid residues in this region are highly conserved among three gallerimycins.

In the upstream sequence of *Scr-gallerimycin* gene, several putative binding sequences for transcription factors, such as NF-κB, C/EBP, GATA and CRE-BP1, were identified (Fig. 2). Recently, in mosquito *Defensin* promotors, three pairs of putative NF-κB and C/EBP binding sequences were identified, and shown to be essential for the immune regulation of the defensin genes (Meredith et al, 2006). They also found that many insect antimicrobial gene promoters contained putative C/EBP binding site within 20 base pairs of NF-κB motifs, and suggested that a close association between NF-κB and C/EBP binding sites could be important in the expression of the majority of insect antimicrobial genes (Meredith et al, 2006). In *Scr-gallerimycin* promoter, putative NF-κB and C/EBP binding sites are apart from each other by 327 nucleotides, and only one each of them was found within 582 bp upstream from the transcription start site, but a GATA factor binding site was found at five nucleotides downstream from C/EBP binding site (Fig. 2). GATA factor binding site was clustered with NF-κB motif in *Drosophila Cecropin A1* gene, and suggested to modulate the NF-κB response (Kadalayil et al, 1997). In *Spodoptera* gallerimycin gene, GATA and NF-κB sites are separated by 268 nucleotides, and no clear C/EBP binding site is found (Volkoff et al, 2003). Although these binding sites have been found in many insect immune genes, a common rule for the interrelationship of their positions in a promoter is not clear at this moment.

In naive *S. ricini* larvae reared under aseptic conditions, *Scr-gallerimycin* transcripts were not detected in any tissue tested. The results are somehow different from those with

Sf-gallerimycin, whose transcripts are detected at low levels in *S. frugiperda* larvae in absence of immune challenge (Volkoff et al., 2003). The apparent difference in observed results may be due to the differences in rearing conditions. *Scr-gallerimycin* gene was strongly induced by an injection of larvae with entomopathogenic fungi, *B. bassiana*, but very weakly by non-pathogenic fungi, *A. oryzae* (Fig. 3 and 4). The induction of the gene transcription by fungi was rather quick phenomenon, reaching the maximum levels 6 h post-injection, then gradually diminished (Fig. 3). This suggests that the gene induction was triggered directly by injected conidia, and possibly not related to the development, or pathogenicity, of the fungi. Apparent difference in elicitor activities of *B. bassiana* and *A. oryzae* is interesting to note, but the reason for this is unknown so far. The *Scr-gallerimycin* gene expression was also strongly induced by UV-killed bacteria, PGN and zymosan, and the induction patterns including time course were totally similar to those of attacin gene, suggesting that both genes are simultaneously induced after recognizing common elicitors.

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Legend to figures

Fig. 1. Nucleotide and deduced amino acid sequences of *Scr-gallerimycin* cDNA. Nucleotides are numbered on the right of each line. Deduced amino acid sequence is shown below the nucleotide sequence and numbered from the first methionine. Initiation and termination codons are shown in bold face. The preceding possible signal peptides (Bendtsen et al., 2004) are shown by underline.

Fig. 2. Upstream sequence of *Scr-gallerimycin* gene. Start of cDNA sequence is indicated under the sequence. Putative TATA box is boxed. Predicted NF-κB binding site is shadowed. Predicted CRE-BP1 binding site and GATA-like binding site are underlined with bold and

dotted lines, respectively. Predicted C/EBP binding site is double-underlined.

Fig. 3. Time course of *Scr-gallerimycin* gene expression in fat body. Total RNA was extracted from larval fat body at indicated time after injection of UV-killed *E. coli* K12 or *B. licheniformis* (2 x 10^6 cells/10 μL PBS), conidia of *B. bassiana* or *A. oryzae* (2 x 10^4 conidia/10 μL PBS) or PBS alone (10 μL; 9 and 24 h). Ten μg each of RNA was analyzed by Northern blot hybridization using ^{32}P-labeled *Scr-gallerimycin* (A) or attacin (B) cDNAs as a probe. The blotted membrane was exposed to an imaging plate, and the radioactive spots were visualized with a fluoro-image analyzer. None injected control (N) is shown at the left end.

Fig. 4. Tissue specific expression of *Scr-gallerimycin* gene. Total RNA was extracted from fat body (Fb), hemocytes (Hc), mid gut (Mg) and Malpighian tube (Mt) of non-treated larvae (N) or the larvae 9 h after injection of *E. coli*, *B. licheniformis*, *B. bassiana* or *A. oryzae* (I) as described in Fig. 3, and analyzed by Northern blot hybridization using ^{32}P-labeled *Scr-gallerimycin* (A) or attacin (B) cDNAs as a probe.

Fig. 5. Induction of *Scr-gallerimycin* gene expression by bacterial cells and various microbial cell wall components. Fifth instar larvae were injected with 10 μL of PBS (lane 2), 10 μg of solubilized PGN from *B. licheniformis* (lane 3), 10 μg of LPS (lane 4), 2 x 10^6 cells of UV-killed *B. licheniformis* and *E. coli* K12 (lane 5 and 6), 10 μg of oligo chitin, zymosan, laminarin and curdlan (lane 7, 8, 9 and 10), respectively. Non injected control is shown in lane 1. Total RNA was extracted from fat body 9 h after the injection and analyzed by Northern blot hybridization using ^{32}P-labeled *Scr-gallerimycin* (upper) or attacin (middle) cDNAs as a probe as described in Fig. 2. Ethidium bromide staining of 18S rRNA is shown in the bottom panel.

Fig. 6. Amino acid alignment of gallerimycin. The CLUSTALW program was used for alignments. Predicted signal peptide sequences are underlined. Identical residues are in bold face. Conserved cysteine residues are shadowed. The DDBJ/GenBank/EMBL accession numbers for the sequences used are as follows: *Scr-gallerimycin* (**AB366558**), *Galleria mellonella* gallerimycin (**AF453824**), *Sf-gallerimycin* (**AY238440**).

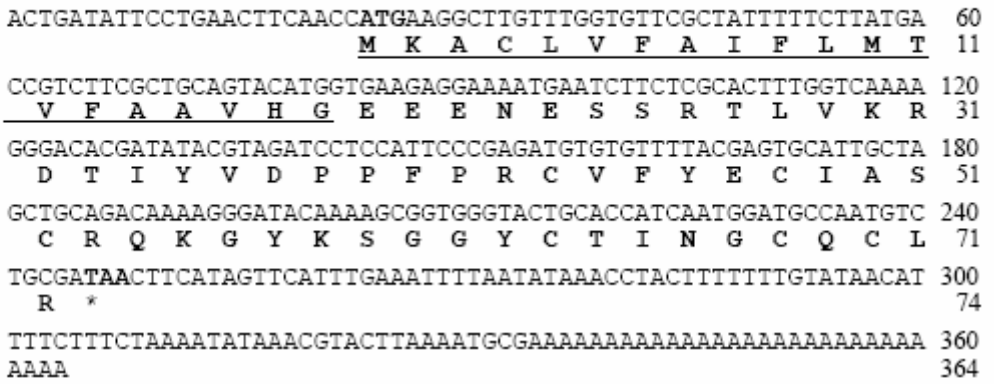

Fig. 1

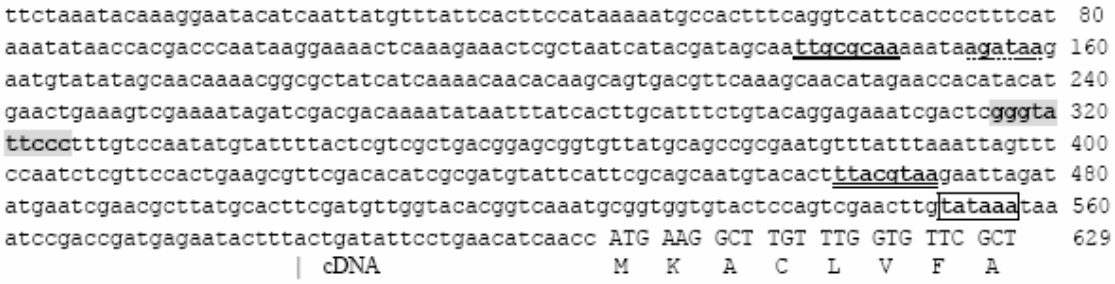

Fig. 2.

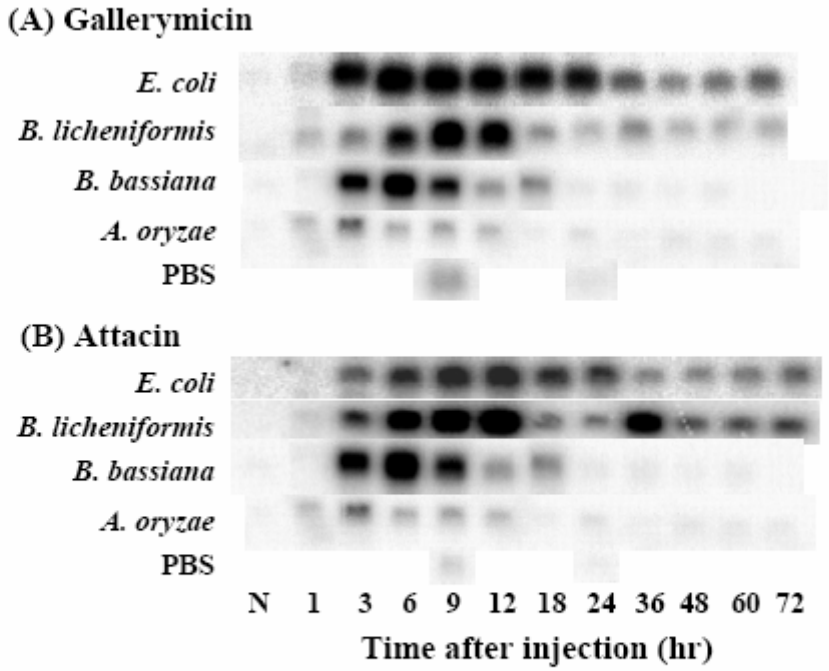

Fig. 3

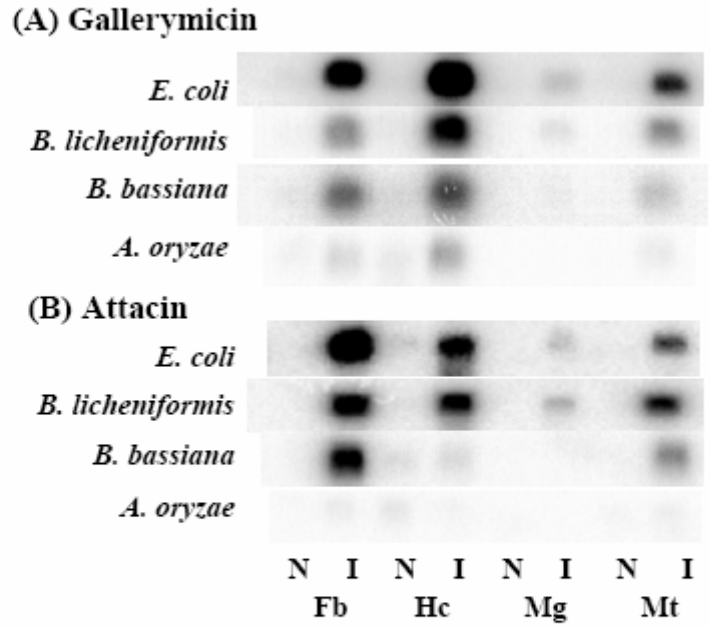

Fig. 4

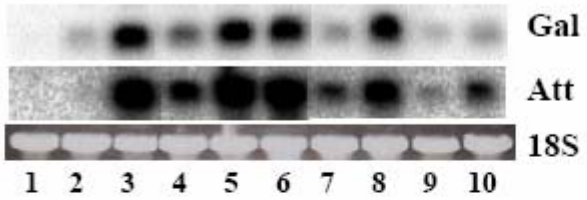

Fig. 5

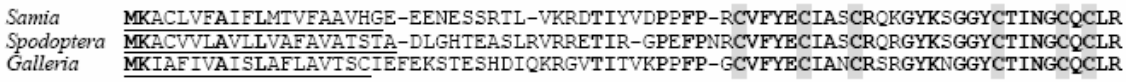

Fig. 6.