Differential Activation of the Lectin and Antimicrobial Peptide Genes in *Sarcophaga peregrina* (the Flesh Fly)

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*Sarcophaga* lectin is an immune defense protein which is transcriptionally induced upon immune challenge in the flesh fly, *Sarcophaga peregrina*. So far, we have revealed that the *Sarcophaga* lectin gene has multiple NF-κB -binding motifs in its promoter. Here we showed that the nuclear extracts from *Sarcophaga*-derived culture cells, NIH-Sape-4, and larval fat bodies have binding activity to the multiple κB motifs in the lectin gene promoter, some of which were responsive to immune stimuli. We also compared the expression profiles of the lectin gene with those of the antibacterial peptide genes from the point of view of inducers, expression tissues and local induction in digestive tracts. In each case, the lectin gene was activated in different manners from other inducible defense genes. These results indicate the complex regulation of the lectin gene, possibly by NF-κB-related transcription factors. Arch. Insect Biochem. Physiol. 69:189–198, 2008. © 2008 Wiley-Liss, Inc.

**KEYWORDS:** *Sarcophaga* lectin; insect immunity; NF-κB

**INTRODUCTION**

Insects synthesize a variety of defense proteins to protect themselves from microbial infection (Bulet et al., 2004; Christophides et al., 2004; Ferrandon et al., 2007; Kanost et al., 2004; Lemaitre and Hoffmann, 2007; Tanji and Ip, 2005). These proteins include antibacterial ones, antifungal ones, and lectins. Macromolecules derived from microbial cell walls, such as peptidoglycans and β-1,3-glucans, trigger activation of the genes for these insect defense proteins.

In *Drosophila melanogaster*, it is well known that the two distinct signaling pathways, the Toll and immune deficiency (IMD) pathways, are activated by different microbial components respectively to induce the expression of antimicrobial peptide genes systemically; peptidoglycans from Gram-positive bacteria and β-1,3-glucans from fungi activate the Toll pathway, while peptidoglycans from Gram-negative bacteria activate the IMD pathway. The signal from each pathway recruits adequate NF-κB -related transcription factors (Dorsal, DIF and Relish), which bind to NF-κB -binding motifs in the promoters of antimicrobial peptide genes with different binding preferences depending on the sequences (Senger et al., 2004). The preferences confer specificity to the innate immunity to some extent. For example, the infection of fungi, which activates Dorsal/DIF through the Toll pathway, leads to the activation of the antifungal peptide *Drosomycin* gene via the Dorsal/DIF-binding site in its promoter. On the other hand, the infection of Gram-negative bacteria, which activates Relish through the IMD pathway, leads to the activation of the anti-Gram-negative bacterial peptide *Diptericin* gene via the Relish-binding site in

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its promoter. Recently, several NF-κB-related transcription factors have also been cloned from other insect species including mosquitoes, beetle and silk-worm, and their functions in immunity have been characterized (Barillas-Mury et al., 1996; Sagisaka et al., 2004; Shin et al., 2002; Shin et al., 2005; Tanaka et al., 2007; Tanaka et al., 2005). These reports demonstrated that the selective activation of immune defense by multiple NF-κB-related transcription factors is common among insects.

Not only the systemic but also the local immune response is an important factor for the fight against the pathogen at the site of infection. There, the activation of the antimicrobial peptide genes is also regulated in a different manner in Drosophila (Tzou et al., 2000).

We have been studying the defense proteins of Sarcophaga peregrina (flesh fly). In addition to many antimicrobial peptides, such as Sarcotoxin I (Sarcophaga homologue of Cecropin), Sarcotoxin II (that of Attacin), and Sapecin (that of Defensin) (Ando et al., 1987; Matsuyama and Natori, 1988; Okada and Natori, 1983), a lectin was found to be produced upon immune challenge (Komano et al., 1980). They are induced at the transcriptional level like many antimicrobial peptides in Drosophila (Ando and Natori, 1988; Matsumoto et al., 1986; Matsuyama and Natori, 1988; Takahashi et al., 1985). However, the profiles of the induction were yet to be investigated in details.

Inducible lectins have also been found from other insects such as hornworm (Yu et al., 1999; Yu and Kanost, 2000), which supports the idea that the existence of an inducible lectin is not specific to flesh fly but general in insects. Although the activation of the antimicrobial peptide genes has been well characterized in Drosophila, the activation of the lectin genes has been poorly analyzed, since no lectin genes are known to be induced intensively upon infection in this model animal. Therefore, to characterize the expression profiles of the Sarcophaga lectin gene will provide us a novel insight into the expression manner of an unexplored set of inducible lectins involved in immune defense. In this work, we compared the expression of the Sarcophaga lectin gene with the antimicrobial peptide genes and showed that it is regulated in different manners in many aspects.

MATERIALS AND METHODS

Cells and Animals

The Sarcophaga embryonic cell line, NIH-Sape-4, was routinely cultured in M-M medium at 25°C as described in (Komano et al., 1987).

Flesh flies, Sarcophaga peregrina, were kept at 27°C with dry milk, sugar cubes and fresh water. Larvae were reared on pork liver, and when they crawled upward at the third instar, they were collected, washed and kept in a plastic container with a small amount of water. Larvae pupated 16 h after their transfer to dry conditions (Ohtaki, 1966).

Electrophoretic Mobility Shift Assay

A nuclear extract from NIH-Sape-4 cells was prepared as described in (Kobayashi et al., 1993). For the nuclear extract from fat body of the third instar larvae, larvae were pricked with a thin needle and dissected 6 h later. Fat bodies from 30 larvae were rinsed in Grace's Insect Cell Culture Medium (Invitrogen), kept in five volumes of hypotonic buffer for 10 min on ice and centrifuged at 1,000 × g for 10 min. The pellet was resuspended in two volumes of hypotonic buffer and homogenized with a Teflon homogenizer. Then the homogenate was centrifuged at 1,000 × g for 10 min. The pellet was centrifuged again at 25,000 × g for 20 min after the resuspension in 500 μl of hypotonic buffer and nuclei was collected as pellet. The nuclear extract was prepared as that from NIH-Sape-4 cells.

The nuclear extract (10 μg protein) was subjected to electrophoretic mobility shift assay as described in (Kobayashi et al., 1993).

The sequences of synthetic oligodeoxyribonucleotides used as probes were listed in Figure 1 with a few flanking bases. The mutated NF-κB-binding motif (5’-CATATTAATACCCTG-3’) was used as a nonspecific competitor.
Infection of the Third Instar Larvae

As foreign substances, *Escherichia coli* (K-12 594), *Staphylococcus aureus* (Cowan I strain), spores of *Beauveria bassiana* (IFO No. 31676) and NIH-Sape-4 cells were used for the injection. They were rinsed with insect saline (130 mM NaCl, 5 mM KCl and 1 mM CaCl₂) and resuspended at the final concentration of 20%, 2% and 0.2% (v/v) before injection. The suspension (5 μl) was subjected into the body cavity with an injection needle.

For feeding experiments, lipopolysaccharide from *Escherichia coli* O55: B5 (Sigma-Aldrich), peptidoglycan from *Micrococcus luteus* (Sigma-Aldrich), and zymosan from *Saccharomyces cerevisiae* (Sigma-Aldrich) were also used at the final concentration of 0.125 mg/ml. Larvae were soaked in distilled water containing microorganisms or microbial components for 6 h. Feeding time period was determined by confirming that India ink reached the midgut after soaking in the ink.

Northern Blot Analysis

Total RNA was prepared from dissected tissues and 20 μg of RNA was subjected to Northern blot hybridization as described in (Kobayashi et al., 1993).

RESULTS

The *Sarcophaga* Lectin Gene Has Multiple NF-κB-Binding Motifs in the Promoter

Many antimicrobial peptide genes in *Drosophila* have multiple NF-κB-binding motifs in their promoters (Engström, 1997; Hultmark, 1993). Recently, it has been shown that, not only multiple κB motifs are necessary, but cooperative regulation...
by these motifs are required for the maximum and adequate induction (TANJI et al., 2007).

The Sarcophaga lectin gene is produced upon injury of body cavity, mainly in fat body (Komano et al., 1983). Before, one κB motif was found at about 0.3 kb 5′-upstream of the lectin gene. This motif is required for the activation of the lectin gene promoter at least in NIH-Sape-4 cells, which are an embryonic cell line of Sarcophaga and known to express the lectin gene under the normal culture condition (Kobayashi et al., 1993; SHIRAISHI et al., 2000). Later, we sequenced up to 3.1 kb of the 5′-upstream sequence of the lectin gene (the sequence deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB054644), and found many putative κB motifs in the distal region of its promoter (TANJI et al., 2002).

As a first step, we performed electrophoretic mobility shift assay using nuclear extract from NIH-Sape-4 to find out whether it has the binding activity to these putative κB motifs; the binding activity to 11 out of 13 putative κB motifs was detected (Fig. 1A,B). Then we moved on to nuclear extract from the fat body of the third instar larvae and it also showed binding activity to those 11 κB motifs (Fig. 1C). Among them, the binding activity to two sites, 1.5 kb and 0.6 kb 5′-upstream of the lectin gene respectively, increased modestly when the extract from injured larvae was subjected, suggesting that these κB motifs participate in the activation of the lectin gene upon injury and infection.

Differential Induction of the Immune Defense Genes Expression by Various Pathogens and Tumorous Cells

In Drosophila, it has been known that the infection of Gram-negative bacteria activates Relish via the IMD pathway, and the infection of Gram-positive bacteria and fungi activates other NF-κB-related transcription factors Dorsal and DIF via the Toll pathway. Hence, different kinds of pathogens induce the antimicrobial peptide genes in different manners (LEMAITRE et al., 1997).

To investigate the expression profile of the Sarcophaga lectin gene, we injected various kinds of pathogens into the hemocoel of the third instar larvae, and investigated the induction of the Sarcophaga lectin gene 20 hours after injection by Northern blot. It has been reported that the induction by injury itself and/or saline injection almost disappears by then; the gene expression induced by foreign substances still persists (Takahashi et al., 1986). We subjected Escherichia coli as Gram-negative bacteria, Staphylococcus aureus as Gram-positive bacteria, and Beauveria bassiana as fungi. In addition to them, we also examined the effect of NIH-Sape-4 cells as tumorous cells from the identical species; tumor cells would be harmful as well as pathogenic microorganisms, and they should be eliminated when they are inside the body. Then the expression of the lectin gene was compared with that of sarcotoxin I and sarcotoxin II genes. Both sarcotoxin I and II genes have been shown to be tandemly aligned in the genome (KANAI and NATORI, 1989, 1990), and the sequences for sarcotoxin IA and sarcotoxin II unit 3 were used for the probes as representatives of each gene cluster. These probes can cross-react with the other transcripts expressed from the respective clusters.

Though body injury and injection of saline itself induced the lectin gene expression, all kinds of the examined stimulants increased the expression level (Fig. 2). Especially, E. coli and B. bassiana increased the expression in a dose-dependent manner. When compared with the antimicrobial peptide genes, the induction of sarcotoxin II genes expression was relatively indiscriminate with low induction efficiency. On the contrary, the induction of the sarcotoxin I genes expression was rather specific with high induction efficiency; i.e., it was not induced by the infection of fungi B. bassiana well except the relatively weak induction at the lowest concentration. Furthermore, the concentration of foreign substances that saturates the induction was different among those immune defense genes. Sarcotoxin I genes were regulated in a dose-dependent manner when induced by S. aureus and NIH-Sape-4 cells. These results indicate that the induction of the Sarcophaga lectin
gene expression is similar to that of sarcotoxin II genes expression, and sarcotoxin I genes are regulated in a different manner.

**Differential Activation of Immune Defense Genes at Various Tissues**

Though a fat body and hemocytes are tissues responsible for systemic immune response including the production of antimicrobial peptides, it has been known in *Drosophila* that the epithelial tissues also participate in local immune response (Tzou et al., 2000). We analyzed the expression of the *Sarcophaga* lectin gene at various tissues 6 h after body injury by Northern blot, and compared it with the expression of the antimicrobial peptide genes.

As reported before, the induction of the *Sarcophaga* lectin and sarcotoxin II genes expression was detected in fat body, while that of the sapecin gene expression was detected in hemocytes. But some other tissues also expressed these genes after injury (Fig. 3). The expression of the *Sarcophaga* lectin gene was also induced strongly in epidermis and/or muscle, and modestly in digestive tracts and tracheae. The expression of sarcotoxin II genes was induced in tracheae and hemocytes at the intermediate level, and modestly in digestive tracts and epidermis and/or muscle. The expression of sapecin gene was induced in cuticle and/or muscle, tracheae and fat body. These results indicate that these defense genes are induced in many tissues to participate in local immunity, but with the different distribution from one another. It should also be noted that the lectin gene expression was not detectable in hemocytes like sarcotoxin II and sapecin, suggesting that the relatively extended induction was not quite ubiquitous, but was under restricted regulation. Furthermore, the constitutive expression of the *Sarcophaga* lectin and sarcotoxin II genes was detected at the cephalic portion though the exact expression tissue was not identifiable.

**Feeding on Microorganisms and Microbial Components Induces the Expression of the Immune Defense Genes**

For insects, natural infection from digestive tract is the most likely way to get an infection in the wild, and induction of immune defense genes in the tract by bacterial diet has been reported in other insect species (Freitak et al., 2007; Vodovar et al., 2005). From the tissue expression profiles, immune defense genes including the *Sarcophaga* lectin gene were turned out to be induced upon body injury not only in the fat body and/or hemocytes but also in epithelial tissues like digestive tracts. Therefore, these genes were expected to participate in local immunity in the digestive tract. To mimic the natural infection, we fed the third instar larvae on microorganisms or microbial compounds, and examined the expression of the *Sarcophaga* lectin gene in the midgut along with the antimicrobial peptide genes by Northern blot. We subjected *Escherichia coli* (Gram-negative bacteria), *Staphylococcus aureus* (Gram-positive bacteria) and *Beauveria bassiana* (fungi) as microorganisms. As microbial compounds, we examined lipopolysaccharide (Gram-negative compound), peptidoglycan from Gram-positive bacteria and zymosan (fungal compound).
Though the expression of sarcotoxin I and sarcotoxin II genes were not detectable under any conditions (data not shown), the expression of the Sarcophaga lectin and sapecin gene was detected (Fig. 4). Essentially, the expression of both genes was induced by all kinds of inducers examined, but the preferences were different. The expression of the Sarcophaga lectin gene was strongly induced by E. coli but not by B. bassiana. On the other hand, the sapecin gene expression was induced by B. bassiana as strongly as by E. coli. As for microbial compounds, the induction of the sapecin gene expression was stronger than that of the Sarcophaga lectin gene, especially by peptidoglycan from Gram-positive bacteria. These results indicate that the local induction of the Sarcophaga lectin gene in midgut is regulated in a different manner from that of the antimicrobial peptide genes.

**DISCUSSION**

From electrophoretic mobility shift assay, it was suggested that the Sarcophaga lectin gene expression was regulated by multiple NF-κB-binding motifs in its promoter. The Drosophila genome encodes three NF-κB-related transcription factors, Dorsal, DIF, and Relish. As for Sarcophaga, its genome probably encodes at least three NF-κB-related transcription factors. The partial cDNA for the Dorsal homologue was cloned from NIH-Sape-4 cDNA library, and the Sarcophaga fat body and
NIH-Sape-4 cells have a protein that cross-reacts with an anti-Drosophila DIF antibody with the equivalent molecular weight (unpublished data). Whereas, the existence of the Relish homologue is still in question. Besides, Sarcophaga has another transcription factor, SRAM (Sarcophaga-derived Rel/Ankyrin Molecule), of which no homologues have been identified in any other species (Shiraishi et al., 2000). SRAM is a sole factor detected to bind to the most proximal κB motif of the lectin gene specifically (Kobayashi et al., 1993). Some of these three factors may cooperatively regulate the expression of the lectin gene utilizing multiple κB motifs.

In Drosophila, the binding specificity of three NF-κB-related transcription factors was extensively analyzed (Senger et al., 2004). Though the DIF SELEX assay showed ambiguous sequences following the first three G’s, Dorsal (GGGWWWHCBN) and Relish (GGGAHNYMYN) have more restrictive specificity. The sequence of the κB motif at 1.5 kb 5’-upstream of the lectin gene is close to the Dorsal binding consensus. The other one at 0.6 kb 5’-upstream of the gene is close to both Dorsal and Relish binding consensus. We also found out that the recombinant SRAM can bind to these two κB motifs (unpublished data). Although the conservation of binding consensus between Drosophila and Sarcophaga is not clear, it is likely considering the similarity of amino acid sequences of Dorsal from these two species. Our speculation is that multiple NF-κB-related transcription factors contribute to the expression of the lectin gene.

In the fat body, the Sarcophaga lectin gene expression was induced by many pathogens and tumorous cells. Because specificity of the induction is different among immune defense genes, it is reasonable to think that more than one signaling pathway participates in the response, like the Toll and IMD pathways in Drosophila. More restricted expression of sarcotoxin I genes than that of the Sarcophaga lectin and sarcotoxin II genes suggests that the former gene is activated by one pathway and the latter genes are activated by either another pathway or multiple pathways.

In Drosophila, the gene encoding Cecropin, the homologue of sarcotoxin I, is regulated by both the Toll and IMD pathways (Corbo and Levine, 1996; Lemaître et al., 1996). On the other hand, the gene encoding Attacin, the homologue of sarcotoxin II, is preferably regulated by the IMD pathway (Georgel et al., 2001). Cecropin is both antibacterial and antifungal (Ekengren and Hultmark, 1999; Samakovlis et al., 1990) and Attacin homologue in other insect species are specific to Gram-negative bacteria (Ando et al., 1987; Hultmark et al., 1983). Combined with this knowledge, Drosophila selectively produces antimicrobial peptides which attack infected pathogens.

The expression pattern of sarcotoxin I and sarcotoxin II genes doesn’t seem to correspond to each antimicrobial spectrum. However, this paradox may be explainable considering the environment where flesh flies live in the wild; it is probably enriched with Gram-negative bacteria, and once they are injured or infected, the defense against Gram-negative bacteria by anti-Gram-negative bacterial peptides like sarcotoxin II is required. Possibly, Sarcophaga lectin is also among those immune defense proteins; or the lectin is induced with wide spectrum and acts against a variety of pathogens.

Interestingly, the injection of NIH-Sape-4 also increased the expression of these immune defense genes. Cecropin is known to have antitumor activity both in vitro and in vivo (Moore et al., 1994; Winder et al., 1998). Sarcophaga lectin activates murine macrophages, resulting in production of tumor necrosis factor-α and lysis of tumor cells (Itoh et al., 1984, 1985; Yamazaki et al., 1983). These immune defense proteins may function in the elimination of tumor cells in the fly.

Contrary to the similarity in the induction by foreign substances in fat body, the expression pattern in each tissue is not similar between the Sarcophaga lectin and sarcotoxin II genes. Although they were both mainly induced in fat body, the sarcotoxin II genes but not the Sarcophaga lectin gene were induced in hemocytes, another main immune defense tissues. Furthermore, the expression of the Sarcophaga lectin gene is abundant in
epidermis or muscle after body injury while that of sarcotoxin II genes is more abundant in tracheae. Sarcotoxin II may play a role in local immune response in tracheae. One interesting possibility is that Sarcophaga lectin is excreted through cuticle upon injury and infection, and acts on pathogens surrounding the animals. The biological meaning of this differential expression is yet to be elucidated.

The Northern blot analysis using Sarcophaga tissues also showed that the cephalic portion expressed the Sarcophaga lectin and sarcotoxin II genes constitutively. Though many tissues such as imaginal discs and brain are included in this portion, we consider salivary glands the most likely candidate of the expression tissue, for we detected the activation of the lectin gene promoter in Drosophila salivary glands by reporter assay (Tanji et al., 2002). Salivary glands are also important tissues for the first line immune defense against pathogens invading through mouth with foods by secreting antimicrobial substances. For example, the constitutive activation of the antifungal peptide Drosomycin gene promoter has been reported in both larval and adult stages of Drosophila salivary glands (Ferrandon et al., 1998; Tzou et al., 2000). Furthermore, the secretion of antimicrobial peptides, such as histatins and β-defensins, in vertebrate saliva has been reported (MacKay et al., 1984; Mathews et al., 1999; Pollock et al., 1984).

Finally, we showed that the expression of the Sarcophaga lectin gene was also induced in larval midgut by feeding on microorganisms and microbial components as well as sapecin gene. Previously, we discussed the possibility of the participation of the lectin in digestive tracts (Tanji et al., 2002). We assumed that the constitutive activation of the lectin gene promoter in the transgenic Drosophila digestive tracts was caused by food components. Drosophila standard food contains yeast. The induction of the lectin gene by zymosan from S. cerevisiae supports the idea that the yeast activated the promoter. In summary, the expression of the Sarcophaga lectin gene is induced by various stimulants in many tissues, and the induction is rather indiscriminate than that of some antimicrobial peptide genes. Possibly, Sarcophaga lectin functions in many occasions including both systemic and local immune defenses.

**LITERATURE CITED**


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