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Sarcotoxin II from the flesh fly *Sarcophaga crassipalpis* (Diptera): A comparison of transcript expression in diapausing and nondiapausing pupae

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Abstract. Many stress-induced genes, including those related to the insect humoral immune response, are upregulated during diapause even in the absence of stress. We further test the relationship between stress genes and diapause in *Sarcophaga crassipalpis* by cloning *sarcotoxin II*, a member of the attacin family, and examining its expression pattern in relation to pupal diapause. Unlike several other stress-related genes, *sarcotoxin II* is not developmentally upregulated during diapause, but it remains fully responsive to immune challenge. Interestingly, the elevation of *sarcotoxin II* mRNA in response to body wall injury, but not immune challenge, is initiated more slowly and persists longer in diapausing pupae than in nondiapausing individuals.

INTRODUCTION

Entry into diapause is accompanied both by a shut down in the expression of many genes as well as the upregulation of a select group of genes (Joplin et al., 1990; Flannagan et al., 1998). Among the genes that are upregulated in the pupal diapause of the flesh fly, *Sarcophaga crassipalpis,* are genes that encode two of the heat shock proteins, hsp23 (Yocum et al., 1998) and hsp70 (Rinehart et al., 2000), and an AP endonuclease that is most likely involved in DNA repair (Craig & Denlinger, 2000). This pattern is not unique to flesh flies: the stress protein hsp70 is upregulated in the Colorado potato beetle as well (Yocum, 2001). In addition, recent data indicates that coupling of diapause with stress-related proteins extends to stress of an immunological nature. *Defensin* is upregulated during larval diapause in the spruce budworm *Choristoneura fumiferana* (Palli et al., 2001), *drosomycin* is upregulated during the adult diapause of *Drosophila triauraria* (Diabo et al., 2001), a peptide with potential anti-fungal activity is uniquely present during diapause in adults of the leaf beetle *Gastrophysa atrocyanea* (Tanaka et al., 1998), antimicrobial peptides are present in diapausing larvae of the blow fly *Calliphora vicina* (Chernysh et al., 2000), and *hemolin* is upregulated in the midgut of diapausing pharate larvae of the gypsy moth *Lymantria dispar* (Lee et al., 2002). Thus, diverse genes encoding proteins associated with stress and immune responses appear to be upregulated during diapause. This prompted us to investigate the expression of another immune response gene, *sarcotoxin II*, during pupal diapause in flesh flies.

Proteins of the Sarcotoxin II group are antibacterial proteins in the attacin family that have been well characterized from *Sarcophaga peregrina* (Natori et al., 1999). To test the possibility that this defense molecule might be among those that are upregulated during diapause in flesh flies we isolated a partial clone that encodes *sarcotoxin II* in *S. crassipalpis* and examined its expression in relation to diapause and in response to physical injury or an injection of lipopolysaccharides. Our results indicate that this gene is not developmentally upregulated during diapause but can be quickly upregulated in diapausing pupae in response to stress. Interestingly, the elevated levels of *sarcotoxin II* mRNA in response to physical injury persist longer in diapausing pupae than in pupae that are not in diapause.

MATERIALS AND METHODS

Insects

All flies used in these experiments were from a laboratory colony of *Sarcophaga crassipalpis* Macquart. Nondiapausing flies were reared under long daylength (15 : 9 light : dark) at 25°C, while diapause was induced by rearing the parental adults in short daylength (12L : 12D) at 25°C, with the resulting larvae and diapausing pupae reared in short daylength conditions at 20°C as previously described (Denlinger, 1972). When conducting experiments that compared diapausing and nondiapausing individuals, both groups were held at 20°C while maintaining respective light regimes.

Immune challenges

All immune challenges were conducted on nondiapausing pupae or pupae that had been in diapause for 20 days. Prior to challenge, the head capsule, consisting of the first three segments of the puparium, was carefully removed to expose the pupal head. In the first set of experiments, challenge consisted solely of body wall injury, which has been shown to elicit *sarcotoxin II* expression in *S. peregrina* (Kanai & Natori, 1990). In our experiments, body wall injury consisted of piercing the pupal head with a sterile 26 gauge hypodermic needle. After injury, samples were harvested at regular intervals for RNA isolation. Our second set of experiments consisted of injection of the pyrogen lipopolysaccharide (LPS) (Sigma, Inc.), an agent that dramatically increases the induction of immune responses (Nanbu et al., 1988). In our studies, 0.5 μg of LPS in 1 μl of

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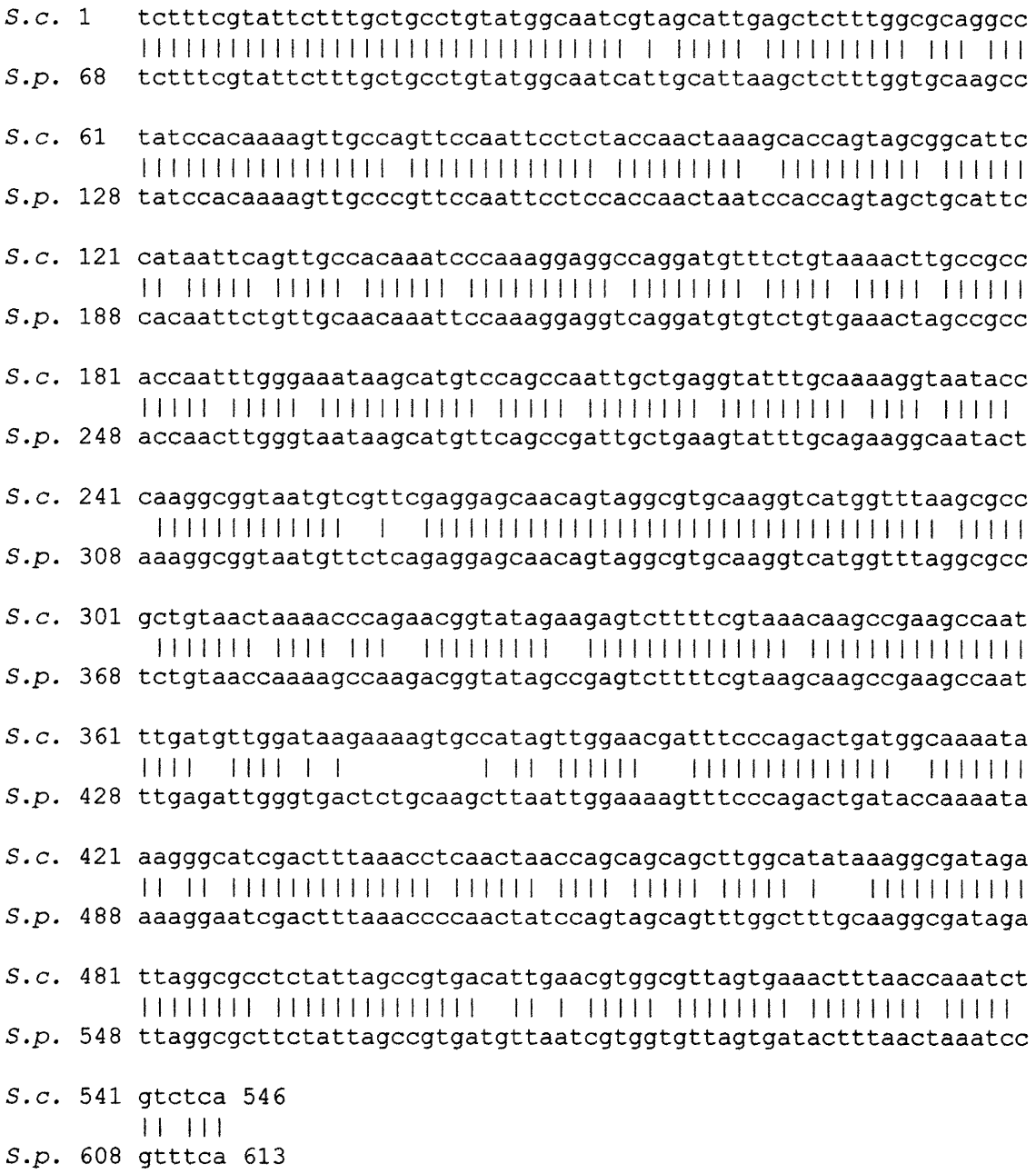

Fig. 1. Comparision of sequences of *sarcotoxin II* from *S. crassipalpis* (S.c.) and *S. peregrina* (S.p.)

insect saline was injected into pupae using a sterile 26 gauge needle.

Clone development

The partial clone of *S. crassipalpis sarcotoxin IIa* was developed via rt-PCR conducted on RNA from immunologically challenged individuals, with unchallenged individuals serving as controls. Primers were constructed from conserved regions of the gene as determined by analysis of sequences from *S. peregrina*, resulting in a 5' primer of TCTTTCGTATTCTTTGCTGC and a 3' primer of ACTGTGACCCACCAGCATTG. PCR was conducted for 40 cycles, with denaturing at 94°C for 30 secs, annealing at 55°C for 30 secs, and extension at 72°C for 1 min, using standard protocol (Rinehart et al., 2000). Resolution of PCR products on a 1.5% agarose, ethidium bromide stained gel revealed a single band of approximately 550bp present in the challenged individuals but absent in unchallenged controls. The product was excised from the gel, ligated into a PcR2.1 vector, which was then used to transform cells with a TA cloning kit (Invitrogen, Inc.). Sequencing was conducted at the University of Georgia using an Applied Biosystems 373A automatic sequencer.

RNA isolation and northern blot hybridization

To isolate RNA for rt-PCR and northern blot hybridization, whole pupae were ground in TRIzol Reagent (Invitrogen, Inc.), with total RNA being isolated using standard protocol. Three samples from each treatment group and time point were then pooled, and 20 μg of this total RNA were loaded onto a 1.5% agarose, 0.41M formaldehyde gel for separation by electrophoresis. Following electrophoresis, a turboblotter apparatus (Schleicher and Schull, Inc.) was used to transfer the samples to a Magnacharge + nylon membrane (Micon Separations, Inc.) via

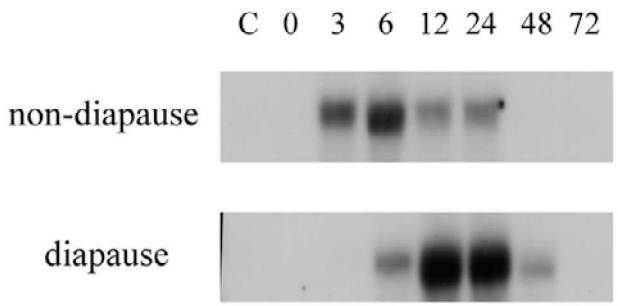

Fig. 2. *Sarcotoxin II* transcription in response to body wall injury. In nondiapausing individuals, upregulation was seen within three hours of injury, and lasted through 24 hours of recovery. In contrast, diapausing individuals exhibited delayed induction, with no transcription seen until 6 hours after injury. In addition, transcription can still be detected in diapausing individuals 48 hours after injury. C = untreated controls, 0 = immediately after injury, 3, 6, 12, 24, 48 and 72 = hours after injury.

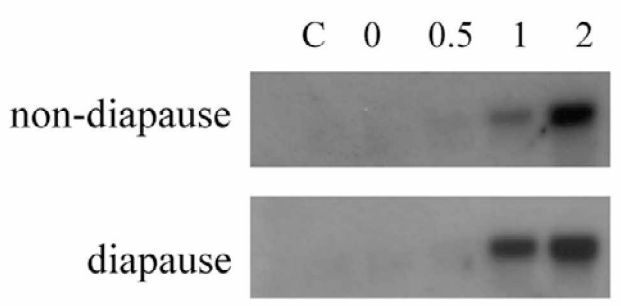

Fig. 3. *Sarcotoxin II* transcription in response to LPS injection. When injected with 0.5 μg of LPS, flesh fly pupae increased *sarcotoxin II* transcription within 1 hr. In contrast to upregulation by body wall injury alone, no differences were discernable in the time of upregulation in diapausing individuals as compared to their nondiapausing counterparts. The minor differences in the 1hr expression levels were not consistent among three replications. C = untreated controls, 0 = immediately after injection, 0.5, 1, and 2 = hours after injection.

downward capillary action. Ultraviolet irradiation was then used to crosslink the samples to the membrane prior to hybridization.

A biotinylated probe was constructed from our partial clone of *sarcotoxin II* by use of the NEBlot phototope kit following the manufacturer's protocol (New England Biolabs, Inc.). The resulting probe was then applied to our membranes at 68°C for an overnight incubation. Following a series of stringency washes, signal was developed by using the Phototope-Star Detection Kit, again following the manufacturer's protocol (New England Biolabs, Inc.). The resulting chemiluminescent signal was detected by exposing x-ray film to the membranes at room temperature.

RESULTS

Cloning of *Sarcotoxin II*

Sequence analysis of our clone revealed a 546bp fragment with high sequence similarity to *sarcotoxin IIA* from the flesh fly *Sarcophaga peregrina* (Fig. 1). Our clone lies at the 5' end of the transcript, entirely within the ORF of *sarcotoxin IIa*. It exhibits 85% identity at the nucleic acid level, and 82% identity, with 90% positives at the amino acid level. The sequence of *S. crassipalpis sarcotoxin II* was deposited into Genbank, and has been designated accession number AY130768.

Expression after body wall injury

In nondiapausing individuals, *sarcotoxin II* transcripts were undetectable in untreated controls and immediately after body wall injury, but were upregulated by 3 hrs after body wall injury, with peak expression exhibited 6 hrs after injury (Fig. 2). Expression continued through 24 hrs post injury, and levels returned to below detection by 48 hrs. As in nondiapausing pupae, *sarcotoxin II* transcripts in diapausing pupae were not detectable in untreated controls, but the gene remained responsive to body wall injury. However, the dynamics of the response were altered by diapause, with no transcripts being detected until 6 hrs after injury, and detectable levels persisted 48 hrs after the cuticle was pierced (Fig. 2).

Expression after LPS injection

Both diapausing and nondiapausing flesh flies rapidly responded to an injection of LPS by elevating the expression of *sarcotoxin II*. In both cases, substantial transcript levels were discernable within 1hr after LPS injection (Fig. 3). There were no apparent differences in the duration of the response: only trace amounts of expression were noted by day three (data not shown).

CONCLUSIONS AND DISCUSSION

Previous studies have indicated that the induction of several stress responses, including the heat shock proteins and elements of the immune response, are key molecular elements of diapause in many species (Denlinger, 2002). This study indicates that the same is not true for *sarcotoxin II* during the pupal diapause of *S. crassipalpis*. It is interesting to note that previous studies investigating the regulation of immune proteins in *S. peregrina* showed no developmental control of Sarcotoxin II, even though Sarcotoxin I, a member of the cecropin family, showed transient upregulation during the embryonic and pupal stages (Nanbu et al., 1988).

Although this gene is not upregulated as a function of diapause, it remains responsive to immunological challenge. In this respect it is much like heat shock protein 90, which is not upregulated by diapause, but remains responsive to stress (Rinehart & Denlinger, 2000). The sarcotoxin response indicates that a key signaling pathway remains intact during diapause. Binding motifs for NF-kappa-B are located in the 5' upstream region of *sarcotoxin II* in *S. peregrina* (Kobayashi et al., 1993), and the NF-kappa-B signal cascade has been shown to regulate many insect humoral responses. The response we note with *sarcotoxin II* in response to immunological challenge suggests that this pathway remains intact during diapause.

We also note that when elicited by body wall injury, the *sarcotoxin II* mRNA upregulation is initiated later and

persists longer in diapausing pupae than in nondiapausing counterparts. This may simply be a reflection of the overall suppression of the metabolic rate that characterizes diapause, or possibly the expanded expression period offers additional protection during diapause. Alternatively, other immune functions could be suppressed during diapause, leading to a delay in clearing of the foreign substances, thereby resulting in longer expression times for the humoral responses. No such differences in *sarcotoxin* expression, however, were noted in response to an LPS injection.

Clearly, the immune responses of *S. crassipalpis* and other species during diapause deserve further investigation. Investigating the expression patterns of other immunological gene families during diapause will provide vital information on the underlying mechanism of immune challenge survival and other stressors as they relate to diapause.

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