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A Defensin-Like Gene Expressed in the Black-Legged Tick, Ixodes scapularis (Acari: Ixodidae)

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A defensin-like gene expressed in the black-legged tick, *Ixodes scapularis*.


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Abstract. The black-legged tick *Ixodes scapularis* Linnaeus (Acari: Ixodidae) is an important vector of microbial pathogens. Knowledge of the tick’s innate immune response, particularly defensin and other antimicrobial peptides, is important for understanding how microbes survive in this tick. A defensin gene (*slnA*) from *I. scapularis* was obtained by RT-PCR using mRNA extracted from tissues of female ticks. RT-PCR indicated the gene was expressed in the midgut, hemocytes, and fat-body, although no evidence of a peptide was found. Sequencing a cloned cDNA fragment revealed a 225 bp ORF encoding a 74 amino acid preprodefensin, including the putative 38 amino acid mature peptide. Similarity between the defensin amino acid sequences of *I. scapularis* and *Dermacentor variabilis* (Say) (Acari: Ixodidae) was 62.2% for the preprodefensin region; for the mature defensins from these two species the similarity was 78.9%, with the 6 cysteine residues being located in the same relative position. PCR amplification and sequencing of chromosomal DNA suggests that *slnA*, along with *vsnA*, the defensin gene from *D. variabilis*, does not contain any introns. This is in contrast to the defensins described for the soft tick, *Omithodoros moubata* (sensu Walton) (Acari: Argasidae). The role of defensin in the innate immune response of *I. scapularis* following microbial invasions is discussed.
Introduction

Defensins are small peptides, approximately 4 kDa in size, usually containing six cysteine residues that form disulfide bridges. These peptides kill microorganisms by forming membrane-penetrating channels in their cell (Bulet et al., 1999; Ganz, 2003; Gillespie et al., 1997). Defensins are best known for their antimicrobial activity against gram-positive bacteria, but defensins that inhibit gram-negative bacteria, fungi (Gillespie et al., 1997) and even protozoan parasites, e.g., Plasmodium ookenetes are also known (Richman et al., 1997; Shahabuddin et al., 1998).

Defensins have been identified in several species of ticks. A cationic defensin (varisin) was identified from the hemocytes of the hard tick, Dermacentor variabilis (Say) (Ceraul et al., 2003; Johns et al., 2001a). Varisin has similarity to members of the insect family of defensins. In addition to antimicrobial activity against gram-positive bacteria, varisin is also active against Borrelia burgdorferi. A defensin-like molecule from Ixodes ricinus Linnaeus was recently described and found to be induced following microbial challenge (Rudenko et al., 2005). Two non-cationic defensin-like isoforms with antimicrobial activity against gram-positive and gram-negative bacteria have been identified in the metastriate hard tick Amblyomma hebraeum Koch (Lai et al., 2004). Two cysteine-rich antimicrobial peptides, one a novel 10.2 kDa polypeptide, the other a 4.29 kDa peptide, were identified in the hard tick Boophilus microplus (Canestrini). Although both molecules contained six cysteine residues, only the smaller one was characteristic of the insect family of defensins (Fogaca et al., 2004). Defensins have also been identified in a soft tick, Ornithodoros moubata (sensu Walton), where four
different isoforms have been characterized from different tissues (Nakajima et al., 2001; Nakajima et al., 2002b).

The blacklegged-tick (also known as the deer tick), *Ixodes scapularis*, is the primary vector of *B. burgdorferi*, the causative agent of Lyme disease, in the United States. This tick is also the primary vector of *Anaplasma phagocytophilum*, the agent of Human Granulocytic Anaplasmosis, and *Babesia microti*, the causative agent of a malaria-like illness in humans. Thus, knowledge of the antimicrobial peptides of *I. scapularis* is important for understanding innate immunity in this vector tick and the role of this response in vector competence.

The aim of this study was to sequence the preprodefensin gene amplified from the tissues of blood-fed female blacklegged-ticks and compare the obtained sequence to that of other tick defensins.

**Materials and Methods**

*Ixodes scapularis* ticks were collected from the Pine Ridge State Park, near Armonk, New York, USA. Ticks were reared on New Zealand white rabbits (*Oryctolagus cunniculus*); spirochete-free progeny were maintained as described previously (Johns et al., 2001a). *Dermacentor variabilis* was colonized from specimens collected near Suffolk Virginia, USA and maintained as described previously (Sonenshine, 1993). All use of animals in this research was done in accordance with protocol 01-007 approved by the Old Dominion University Institutional Animal Care and Use Committee, February 12, 2002.
For RNA extractions, female ticks were detached from rabbits after feeding for 6 days, washed in RNAZap (Ambion, Austin, TX) and RNase-free 1x PBS before dissection. Total tissues, as well as isolated midgut, fat-body, and hemocytes were collected. To amplify defensin transcript, tissues were collected into extraction buffer and homogenized on wet ice; isolated tissues were washed prior to homogenization.

mRNA was isolated from the tissues using the QuickPrep™ Micro mRNA Purification Kit in accordance with the manufacturers instructions (Amersham Biosciences, Piscataway, NJ). Oligonucleotide primers used throughout this study were either obtained with the kits or made by IDT (Coralville, IA). First-strand synthesis of the 3' region was performed using 25ng of mRNA in the lmProm-II Reverse Transcription System (Promega, Madison, WI) with 500ng of cDNA cloning primer (5'-GAAGAATTCTCGAGCGGCCGC I I I I I I I I I I I I I I I I I I I V-3').

Amplification of the 3' region was accomplished using a primer based on a consensus amino acid sequence for the start of mature defensin peptides; the sequence was adjusted to fit codon usage for I. scapularis genes. The primer, designated ls-2b (5'-GGATACGGATGCCCCTICAACC-3'), was used with the cDNA cloning primer to amplify the cDNA using Hi-Fi Platinum Supermix (Invitrogen Corp., Carlsbad, CA). Cycling parameters were an initial denaturation at 95°C for 5 min followed by 35 cycles of 95°C for 30 sec, 58°C for 1 min, 68°C for 1 min; cycling was followed by a final extension at 68°C for 10 min. The resultant amplicon (~250bp) was isolated from a gel using the Qiagen gel extraction kit (Qiagen, Valencia, CA), re-amplified, then cloned into the pCR4 TOPO vector (Invitrogen, Carlsbad, CA) and sequenced. Sequencing of cloned cDNA was carried out using the ABI Prism BigDye
Terminator v3.0 Ready Reaction Kit, with samples run on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

The 5' region of the cDNA fragment of the defensin gene was obtained using the GeneRacer kit as described by the manufacturer (Invitrogen). A GeneRacer RNA Oligo (5'-CGACUGGAGCAGGACACUGACAUUGACUGAAGGAGGAAGAAA-3') containing the 5' nested primer sequence (5'-GGACACTGACATGGACTGAAGTAGA-3') was ligated to the isolated mRNA prior to the reverse transcription reaction. This provided a known priming site at the 5' end of the cDNA. First strand synthesis was carried out with the ImProm-II Reverse Transcription System with 20 pmol of the gene-specific (lx-GSP-1) primer (5'-GGAGTTTATTGAACAGCCGCCGGAAGGA-3') derived from the sequence of the 3' region. The 5' region cDNA was amplified using Hi-Fi Platinum Supermix (Invitrogen) and 10µM GeneRacer 5' nested primer and lx-GSP-1. PCR conditions were an initial denaturation at 95°C for 5 min followed by 30 cycles of 95°C for 30 sec, 58°C for 1 min, 68°C for 1 min followed by a final extension at 68°C for 10 min. The resulting amplicon (~420bp) was cloned into pCR4 TOPO following gel extraction and re-amplification, then sequenced as described for the 3' region.

Once the sequence of the 5' and 3' regions were obtained, primers were designed to amplify the gene-coding region. The cDNA transcript of the defensin gene from the midgut, fat-body, and hemocytes was obtained following first strand synthesis with the ImProm-II Reverse Transcription System using an oligo dT primer (IDT). The cDNA was then amplified using Platinum Supermix (Invitrogen) with lx-ATG (5'-ATGAGGGGTCATTGCTGTAC-3') and lx-TAG (5'-CTAGTTTGGTACATGTGC-3') primers using the following cycle conditions: an initial denaturation at 94°C for 1 min
followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min followed by a final extension at 72°C for 10 min.

Amplification of the gene-encoding region from chromosomal DNA was carried out on DNA extracted from total tick tissue using the DNeasy tissue kit according to the manufacturer’s instructions (Qiagen, Valencia, CA). DNA was extracted from total tissue removed from the tick. Amplification was carried out using Hi-Fi Platinum Supermix with either lx-ATG and lx-TAG for *I. scapularis* or Vsn-ATG (5'-ATGCGCGGACTTTGCATCT-3') and Vsn-TAA (5'-TTAATTCCTGTAGCAGGTGCA-3') for *D. variabilis*. Cycle conditions were an initial denaturation at 94°C for 1 min followed by 30 cycles of 94°C for 30 sec, 56°C (*Dermacentor*) or 58°C (*Ixodes*) for 1 min, 72°C for 1 min followed by a final extension at 72°C for 10 min.

Sequence analysis and alignments were carried out using the Vector NTI Suite of programs (Invitrogen). The resultant sequence containing the defensin gene, designated *slnA*, was submitted to GenBank under accession number AY660970.

**Results and Discussion**

In a previous study, defensin was detected in the hemolymph plasma of *B. burgdorferi*-injected *D. variabilis* females, but not in sham-inoculated controls (Johns *et al.*, 2001a). In this species, the band with the rMW previously identified as varisin became visible within 1 h after bacterial challenge. Such rapid availability of defensin in the hemolymph is similar to that found in many insects (Bulet *et al.*, 1999; Gillespie *et al.*, 1997). However, when hemolymph plasma from *I. scapularis* females injected with *B. burgdorferi, B. subtilis,* or *E. coli* was analyzed by the same SDS-PAGE method, no
evidence of a band that co-migrated with the *D. variabilis* defensin was found (not shown).

The reason for the apparent absence of the peptide in the hemolymph of *I. scapularis* is currently unknown, but it is possible that the peptide is either not processed or that this tick fails to detect the presence of the bacteria. In *D. variabilis* defensin is released into the hemolymph following microbial challenge (Ceraul et al., 2003); it is possible that in *I. scapularis* the signal that results in the release is either not present or does not get activated. Therefore, there is no release of defensin into the hemolymph. Failure of *I. scapularis* to detect *B. burgdorferi* could also result in the absence of defensin in the hemolymph. The signals and possible receptors involved in detection of pathogens, and/or triggering release of antimicrobial peptides are currently unknown in ticks and are in need of further investigation. Differences between the ticks in these receptors or accessory proteins may explain why ticks carry and transmit different microorganisms.

In order to determine whether or not *I. scapularis* has a defensin gene, molecular techniques were used since protein analysis had not suggested the presence of the peptide. Sequencing the RT-PCR products gave a 410 bp region that contains a 225 bp ORF encoding a 74 amino acid preprodefensin, which includes the 38 amino acid region presumed to be the mature peptide. The putative protein includes six cysteine residues in the mature peptide, a common characteristic of described tick defensins. Thus far, as with *D. variabilis* (Ceraul et al., 2003), only a single isoform has been found, referred to as scapularisin, in *I. scapularis*. However, a GenBank homology search indicated that a gene identified as encoding a putative secreted salivary gland
protein (Accession number AY775825) is in fact a defensin gene. The translated sequence of this gene is the same as the sequence determined in this paper except for amino acid 17. The sequence obtained from the mRNA of hemocytes, fat body and midgut, as well as the genomic DNA indicates a phenylalanine at this position, whereas the peptide translated from the salivary gland gene (AY775825) has a leucine. This change occurs within the signal sequence region of the prepropeptide and should not affect the properties of the signal peptide region associated with secretion. Whether this suggests a different isoform present in the salivary gland remains to be determined. In addition, a sequence having similarity to other defensins was identified in the sialome of an *Ixodes scapularis* salivary gland (Valenzuela *et al.*, 2002); the cloned sequence was truncated but had less than 40% similarity to mature scapularisin at the amino acid level. These differences do not indicate this peptide to be an isozyme of scapularisin but may represent a different defensin molecule. In contrast to the one or perhaps two isoforms in *I. scapularis*, the soft tick *O. moubata* has four different isoforms, including one specifically found in the midgut (Nakajima *et al.*, 2002a). The tissue site(s) where scapularisin is synthesized was not determined, but transcript for *slnA* was amplified from mRNA isolated from midgut, hemocytes and fat-body (Fig. 1). Whether defensin is found in the salivary glands as suggested by the presence of transcripts of the secreted salivary gland peptide and the alternative defensin reported by Valenzuela (Valenzuela *et al.*, 2002) is currently unknown. In *D. variabilis*, defensin peptide was identified in the hemocytes (Ceraul *et al.*, 2003). More recently, transcript for varisin was found in the midgut and fat-body of these ticks suggesting that perhaps a low level of expression occurs in many tissues of the tick (Ceraul, 2005).
Unlike the defensin genes of the soft tick *O. moubata*, the defensin genes from neither of the hard ticks, *I. scapularis* or *D. variabilis*, show the presence of introns. Rather, the amplified DNA fragment is the same size (225 bp) as that obtained by RT-PCR from mRNA, with the genes from both ticks being the same size (Fig. 2). Sequencing of the amplified fragments gave the same sequence as that obtained from mRNA indicating that no introns were present in the open reading frame for either defensin gene. Whether the presence/absence of introns has any impact on the biological significance of the tick defensins is unknown, but it is interesting to note that with *Omithodoros* there are introns and multiple isoforms (Nakajima et al., 2001; Nakajima et al., 2002b), whereas with *Dermacentor* and *Ixodes* there are no introns and there appears to be only a single isoform. It will be of interest to see if other tick defensins have or lack introns and what this tells us about the biology of the organism.

Comparison of the translated amino acid sequence of the scapularisin gene to the defensins from other species of hard and soft ticks indicated that the gene encodes a peptide with the potential to act as a defensin-like molecule. Similarity between the *I. scapularis* and *D. variabilis* defensins was 66.5% at the nucleotide level and 62.2% at the amino acid level. However, when similarities in the mature regions of the defensins from these two species were compared, the amino acid similarity was 78.9%, with all 6 cysteine residues being found in the same relative locations. When the complete translated scapularisin sequence was aligned with other tick defensins, similarities ranged from as high as 62.2% for *B. microplus* to as low as 26.1% for *A. hebraeum*. Of special interest was the finding that the similarity with the sequence for *I. ricinus* was only 55%, even though *I. scapularis* and *I. ricinus* are very closely related species within
the same genus. The peptide encoded by the ORF of *I. ricinus* is 76 amino acids in length, as compared to only 74 for the *I. scapularis* sequence. As determined by the alignment, one of the extra amino acids occurs at the cleavage site of the mature defensin from the propeptide. The *I. ricinus* defensin also lacks high levels of similarity with the defensin identified in the sialome of *I. scapularis*. Phylogenetic analysis of the *I. ricinus* complex, which includes *I. scapularis*, indicates that *I. scapularis* and *I. ricinus* are not sister taxa (Xu *et al.*, 2003). This may explain the lack of similarity with the defensin genes. Alignments of the mature defensin regions showed higher similarities, ranging from as high as 86.8% for *B. microplus* to as low as 44.7% for *A. hebraeum*; again, only 57.9% similarity was seen with the *I. ricinus* defensin. The *A. hebraeum* defensin shows the greatest difference, not only to the scapularisin sequence but also to that of the other tick defensins. The *Amblyomma* defensin is produced as an 84 amino acid peptide, with the mature defensin being comprised of 41 amino acids (Lai *et al.*, 2004) versus only 38 in the other hard ticks (Ceraul *et al.*, 2003; Fogaca *et al.*, 2004), and 36 in the soft ticks (Nakajima *et al.*, 2001; Nakajima *et al.*, 2002b). These results show a high degree of variability among the tick defensins, which may have been influenced by the types of microbes the different tick species have encountered during their evolutionary development, as well as their geographic isolation.

Although recognized as a defensin because of its similarity to other tick defensins, scapularisin's role as an antimicrobial peptide is unclear. No expression or secretion of a peptide similar to the known tick defensin varisin was found following challenge (Ceraul, 2005). Also noteworthy is the report by Johns *et al* (Johns *et al.*, 2001b) showing that *B. burgdorferi* spirochetes remained intact when inoculated directly
into the hemolymph of fed *I. scapularis* females and remained viable for up to 24h post-inoculation. Another report (Johns *et al.*, 2001c) noted tick mortality ranging from 44 to 55% in fed female *I. scapularis* injected with *E. coli*, *B. subtilis*, and *Micrococcus luteus* but virtually no mortality when these same bacteria were injected into fed female *D. variabilis*. Injection of other bacteria, *Corynebacterium xerosis*, *Serratia marcesans*, and *Enterobacter aerogenes*, that had 40% mortality in *D. variabilis* were substantially more harmful when injected into *I. scapularis*, showing 55% mortality. However, no mortality was observed when either *I. scapularis* or *D. variabilis* females were injected with *B. burgdorferi* (Johns, 2003). Currently we do not know what would occur if the ticks, in particular *I. scapularis*, were challenged with organisms such as *B. burgdorferi* via an oral route. In the case of *D. variabilis* spirochetes appear to be destroyed in the midgut, whereas with *I. scapularis* intact spirochetes were detected (Sonenshine *et al.*, 2002). If such a challenge results in midgut expression of defensin, similar to that shown with *I. ricinus* (Rudenko *et al.*, 2005), it does not appear to result in destruction of the organism. It is unlikely that such an oral challenge would result in release of defensin into the hemolymph if a more direct inoculation fails to stimulate release of the peptide, however passage through the midgut may stimulate defensin expression or release; this remains to be determined. Many questions regarding expression of these antimicrobial peptides in both the midgut and hemolymph are still to be answered.

Defensins are an important component of the innate immune system and are believed to contribute to the ability of most animals to destroy invading microbes (Gillespie *et al.*, 1997). In *D. variabilis*, defensin was found to contribute to the elimination of *B. burgdorferi* (Johns *et al.*, 2001a) and is believed to be effective against
other microbial pathogens. However, detection of the pathogen, perhaps by some unknown mechanism involving pathogen associated molecular patterns (PAMPs) on the invading spirochetes, must occur before defensin can be upregulated and/or secreted. Such a mechanism is required for release of defensin by NK cells, a component of the human innate response (Chalifour et al., 2004). If the appropriate triggers are not present in I. scapularis, defensin may not be produced or released. The finding that I. scapularis has a defensin, scapularisin, but does not appear capable of controlling B. burgdorferi implicates other factors, presently unknown, in this tick's antimicrobial defense.

Further study is needed to determine whether the I. scapularis defensin is effective against B. burgdorferi and other tick-borne microbial pathogens, and if so, under what conditions, e.g., alone or in combination with lysozyme. In D. variabilis, lysozyme was found to enhance the action of varisin (Johns et al., 2001a). Other studies are needed to determine the tissue(s) involved in production, as well as the rate and sustainability of defensin expression. Such studies are essential to understand why some tick species are competent vectors of pathogens while others are not.

Acknowledgements

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References


Figure legends

Fig. 1. RT-PCR products showing the presence of transcript in I. scapularis midgut (MG), hemocytes (Hc), and fatbody (FB). The first lane in each pair had RT included in the reaction (+), the second lane had no RT in the reaction (-). The 50bp molecular weight marker is in MW; band sizes are in bp.

Fig. 2. RT-PCR product (cDNA) and PCR product of chromosomal DNA fragments from I. scapularis. The 100bp molecular weight marker is in MW; band sizes are in bp.