INFECTION AND IMMUNITY, Nov. 2008, p. 5429–5435 Vol. 76, No. 11
0019-9567/08/$08.00+0 doi:10.1128/IAI.00866-08

Functional Characterization and Novel Rickettsiostatic Effects of a Kunitz-Type Serine Protease Inhibitor from the Tick *Dermacentor variabilis*▽

Shane M. Ceraul,[1]* Sheila M. Dreher-Lesnick,[1] Albert Mulenga,[2] M. Sayeedur Rahman,[1] and Abdu F. Azad[1]

Department of Microbiology and Immunology, University of Maryland School of Medicine, 660 West Redwood Drive, Baltimore, Maryland 21201,[1] and Department of Entomology, Texas A&M University, 412 Heep Center, College Station, Texas 77843-2475[2]

Received 14 July 2008/Returned for modification 12 August 2008/Accepted 28 August 2008

Here we report the novel bacteriostatic function of a five-domain Kunitz-type serine protease inhibitor (KPI) from the tick *Dermacentor variabilis*. As ticks feed, they release anticoagulants, anti-inflammatory and immunosuppressive molecules that mediate the formation of the feeding lesion on the mammalian host. A number of KPIs have been isolated and characterized from tick salivary gland extracts. Interestingly, we observe little *D. variabilis* KPI gene expression in the salivary gland and abundant expression in the midgut. However, our demonstration of *D. variabilis* KPI's anticoagulant properties indicates that *D. variabilis* KPI may be important for blood meal digestion in the midgut. In addition to facilitating long-term attachment and blood meal acquisition, gene expression studies of *Drosophila*, legumes, and ticks suggest that KPIs play some role in the response to microbial infection. Similarly, in this study, we show that challenge of *D. variabilis* with the spotted fever group rickettsia, *Rickettsia montanensis*, results in sustained *D. variabilis* KPI gene expression in the midgut. Furthermore, our in vitro studies show that *D. variabilis* KPI limits rickettsial colonization of L929 cells (mouse fibroblasts), implicating *D. variabilis* KPI as a bacteriostatic protein, a property that may be related to *D. variabilis* KPI's trypsin inhibitory capability. This work suggests that anticoagulants play some role in the midgut during feeding and that *D. variabilis* KPI may be involved as part of the tick's defense response to rickettsiae.

The success of ticks as long-term arthropod hosts and vectors to *Rickettsia* spp. is due, in part, to the defense response elicited upon detection of the threat of rickettsial colonization. As ticks feed, saliva rich with immunosuppressants and anticoagulants is released at the bite site (8). The pharmacologically active saliva creates a feeding lesion on the host and promotes microbial transmission and acquisition by the tick (16). Once imbibed, rickettsiae must first evade the immunologically active tick midgut to establish themselves as endosymbionts. Irrespective of evasion and colonization, studies show that *Rickettsia montanensis* elicits antimicrobial gene expression in the midgut and fat body of *Dermacentor variabilis* (2). Related studies demonstrate that insect-derived antimicrobial peptides effectively reduce the viability of *Rickettsia peacockii* in vitro (1), alluding to the possibility that rickettsiae may be sensitive to tick-derived antimicrobials.

*K*unitz-type *p*rotease *i*nhibitors (KPIs) are secreted with tick saliva into the feeding lesion where they prevent blood coagulation, helping to ensure acquisition of a blood meal (6, 7, 14). In addition to their anticoagulant properties, several studies of different model systems suggest that KPIs have a role as part of the response to microbial challenge. Stimulation of *Drosophila melanogaster* with bacteria or fungi results in an increase in gene expression for two KPIs (3). Also, KPIs are expressed in plants as part of the hypersensitive response (HR) activated toward both pathogenic and nonpathogenic endosymbionts (10, 11, 21). Interestingly, the HR is shown to control the growth and spread of nodulating endosymbionts (21). Recently, expression of a KPI from the southern cattle tick, *Rhipicephalus* (*Boophilus*) *microplus*, was found to be upregulated in response to *Babesia bovis* infection (18).

Our studies reveal that *Dermacentor variabilis* KPI is highly expressed in the midgut and is induced upon feeding. Additionally, rickettsial challenge elicits sustained gene expression of *D. variabilis* KPI in the midgut. Results from our studies, as well as others, suggest that *D. variabilis* KPI may have bacteriostatic as well as anticoagulant properties. We tested the hypothesis that *D. variabilis* KPI is a bacteriostatic protease inhibitor that limits rickettsial colonization of host cells. Upon further experimentation, we observed that *D. variabilis* KPI limits rickettsial colonization of host cells. These findings indicate that rickettsiae must evade the rickettsiostatic effects of *D. variabilis* KPI to colonize the tick.

MATERIALS AND METHODS

Ticks. Female *D. variabilis* ticks fed for 4 days were a generous gift from Daniel E. Sonenshine (Department of Biological Sciences, Old Dominion University). Tick colony maintenance and animal husbandry were carried out according to approved protocols of Old Dominion University's Institutional Animal Care and Use Committee.

Tick challenge. Our method of tick challenge is described by Ceraul et al. (2). Ticks fed for 4 days were used for all tick challenge experiments. Briefly, *R. montanensis*-infected L929 cells or uninfected L929 cells (control) were resuspended in whole sheep's blood and delivered to each tick using artificial capillary feeding. Ticks were allowed to imbibe the blood meal and were incubated at 22°C

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Maryland School of Medicine, 660 West Redwood Drive, Baltimore, MD 21201. Phone: (410) 706-3337. Fax: (410) 706-0282. E-mail: scera001@umaryland.edu.

▽ Published ahead of print on 8 September 2008.

TABLE 1. List of primers

Primer	Sequence (5′–3′)[a]
For-Dv-KPI	ATGAAATCTCAGACATACATC
Rev-GeneRacer poly-T	GCTGTCAACGATACGCTACGTA ACGGCATGACAGTG$(T)_{24}$
For-GeneRacer	CGACTGGAGCACGAGGACA CTGA
Rev-Dv-KPI	TGGATTGAACCTGTAGCCCCTA AATGCTGCGCGGCAAATTT
Dv-KPI-For pcDNA	CACCATG**G**AATCTCAGACATAC ATCGCAGTACTC
Dv-KPI-Rev pcDNA	GTCGACTATATTTTGGCCCAAGT
qRT-PCR For-Dv-KPI	CGAAGAATCAGAGTGCTGGA GAAC
qRT-PCR Rev-Dv-KPI	CCGAGGTGGTTTTTAGGTCCTG
qRT-PCR For-Actin	CCGGTTCAGCCCTCGTTCT
qRT-PCR Rev-Actin	TTGAGGCCAGGGATGGAGC
qRT-PCR For-GAPDH	TCAACGACCCCTTCATTGAC
qRT-PCR Rev-GAPDH	ATGCAGGGATGATGTTCTGG
qRT-PCR For-*16S rRNA*	GTTCGGAATTACTGGGCGTA
qRT-PCR Rev-*16S rRNA*	AATTAAACCGCATGCTCCAC

[a] The underlined bases on Dv-KPI-For pcDNA were incorporated to facilitate directional Topo cloning as suggested by the manufacturer (Invitrogen). The bold residue represents a switch from an adenine to a guanine to create a Kozak sequence.

and 90% humidity for 24, 48, or 72 h postchallenge. The appropriate blood meal (infected or uninfected) was supplied daily using artificial capillary feeding until each group of ticks was collected for midgut dissection.

Cell culture and rickettsia. Murine fibroblasts (L929; ATCC CCL-1) were used for routine propagation of *R. montanensis* and for transfection experiments. Unless otherwise noted, L929 cells were grown in T-150 150-cm^3 flasks (Corning, Corning, NY) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) at 34°C and 5% CO_2. For propagation, rickettsia-infected L929 cells were grown to 80% infection, at which time the rickettsiae were purified from host cells using a Renografin procedure. Infected L929 cells were washed with fresh medium, scraped, and lysed by five passages through a 3-ml syringe fitted with a 27-gauge needle. Large particulates of host material were removed by low-speed centrifugation at 500 × *g* for 5 min at 4°C. The clarified supernatant was layered onto a 25% Renografin solution (in 218 mM sucrose, 3.8 mM KH_2PO_4, 7.2 mM K_2HPO_4, 4.9 mM L-glutamate [pH 7.2]) at a ratio of 1:1 of supernatant to Renografin. Each sample was centrifuged at 17,000 × *g* for 10 min at 4°C. The supernatant-Renografin gradient was removed from the pelleted rickettsiae. Rickettsiae were resuspended in fresh DMEM plus 5% FBS and counted using the BacLight Live/Dead assay (Molecular Probes, Carlsbad, CA) on a hemocytometer at ×400 magnification. Rickettsiae were stored at −80°C until use in aliquots containing 1×10^6 to 1×10^7 rickettsiae.

Sequence identification and analysis. *D. variabilis* KPI was discovered as part of a serine protease inhibitor homology cloning project. The full-length sequence was amplified from total tick RNA using a GeneRacer rapid amplification of cDNA ends kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The primer combination For-*D. variabilis* KPI and Rev-GeneRacer poly-T was used to obtain the 3′ end, and the combination For-GeneRacer and Rev-*D. variabilis* KPI (Table 1) was used to obtain the 5′ end of the sequence. The deduced amino acid sequence was submitted for BLAST analysis (http://www.expasy.org/) using the arthropod database. The following amino acid sequences were retrieved by their Swiss-Prot accession numbers (corresponding organisms are in parentheses), aligned using Muscle (4), and edited using GeneDoc (15) and Adobe Illustrator (Adobe Systems Incorporated): A9YPC4 (*D. variabilis*), Q4PMU5 (*Ixodes scapularis* 1), Q8MVC4 (*I. scapularis* 2), Q4PML9 (*I. scapularis* 3), Q4PMM5 (*I. scapularis* 4), Q6B8C7 (*Ixodes pacificus*), and Q3HYC9 (*Rhipicephalus* [*Boophilus*] *microplus*). The Kunitz domains were identified using the SMART database accessed through the InterProScan link on the European Bioinformatics Institute website (http://www.ebi.ac.uk/). The percent similarities and identities were taken from the BLAST results.

Stable transfection and protein purification. The open reading frame for *D. variabilis* KPI was amplified with primer combination *D. variabilis* KPI-For pcDNA and *D. variabilis* KPI-Rev pcDNA (Table 1) and cloned into pcDNA 3.1D TOPO-V5-HIS (Invitrogen) to generate pcDNA 3.1D TOPO-DvKPI-V5-HIS. Sequence integrity was confirmed by DNA sequencing. One day prior to transfection, 4×10^5 L929 cells were plated in six-well plates and allowed to incubate at 34°C and 5% CO_2 for 24 h. Two micrograms of pcDNA 3.1D TOPO-DvKPI-V5-HIS or pcDNA 3.1D TOPO-LacZ-V5-HIS control plasmid was transfected into L929 cells using SuperFect transfection reagent (Qiagen, Valencia, CA). Transfected L929 cells were grown in DMEM (5% FBS) supplemented with 400 μg/ml Geneticin (Gibco, Carlsbad, CA) for growth selection of transfected cells. Medium was harvested from *D. variabilis* KPI-transfected cells and clarified by centrifugation at 3,200 × *g* for 25 min, and production of recombinant *D. variabilis* KPI (r *D. variabilis* KPI) was confirmed by Western blotting with mouse anti-V5 (Invitrogen) using standard conditions. Once expression was confirmed, the medium was exchanged for NPI-10 buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole [pH 8.0]) using Amicon Ultra 5000 nominal-molecular-weight filters (Millipore, Billerico, MA). r *D. variabilis* KPI was purified using nickel-nitrilotriacetic acid (Ni-NTA)-charged agarose gravity flow columns (Qiagen) under native conditions. Briefly, *D. variabilis* KPI in NPI-10 was loaded onto the Ni-NTA column. The column was washed with 10 ml each of NPI-10 and NPI-20 (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole [pH 8.0]). The protein was eluted with 3 ml of NPI-250 (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole [pH 8.0]) and collected in 0.5-ml fractions. The purity of each purification was assessed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, followed by staining with Imperial protein stain (Pierce). The presence of r *D. variabilis* KPI was confirmed by Western blotting with anti-V5. r *D. variabilis* KPI-containing fractions was combined, and the buffer was exchanged with 0.18% NaCl using Amicon Ultra 5000 nominal-molecular-weight filters (Millipore) to a 1-ml volume, after which point the sample was lyophilized. The r *D. variabilis* KPI sample was reconstituted with 0.2 ml of deionized water (a final NaCl concentration of 0.9%), the protein concentration was estimated with the bicinchoninic acid assay (Pierce, Rockford, IL), and the protein was stored at −20°C until use for functional assays.

aPTT. Activated partial thromboplastin time (aPTT) reagents are marketed under Amax Alexin and were purchased from Fisher Scientific (A 1801/A). Alexin and $CaCl_2$ were warmed to 37°C for 15 min. r *D. variabilis* KPI (2.8 μM final concentration) was incubated with rabbit plasma (Sigma) and Alexin reagent at 37°C for 3 min. Calcium chloride was added to a final concentration of 6.67 mM, and the optical density at 405 nm (OD_{405}) was measured for 3 min at room temperature. A blank (0.9% NaCl) was run to serve as a buffer control for r *D. variabilis* KPI. OD was plotted against time in Excel (Microsoft Corporation). Time to initiation of coagulation was defined as the point of greatest initial change in OD on the plotted sigmoidal curve. The mean time to initiation of coagulation and the standard deviation of three separate experiments were plotted using SigmaPlot 10.0 (Systat, San Jose, CA). Statistical significance was tested using Student's *t* test.

Trypsin inhibitor assay. All reagents were purchased from Sigma Chemical Company (St. Louis, MO). Trypsin (5 nM; Sigma T1426) was incubated with 2.8 μM of r *D. variabilis* KPI in trypsin inhibitor assay buffer (106 mM triethanolamine, 10.6 mM $CaCl_2$) for 15 min at room temperature. The trypsin substrate *N-p*-tosyl-Gly-Pro-Arg *p*-nitroanilide acetate salt was added to a final concentration of 125 μM. OD_{405} readings were measured for 5 min at room temperature. Aprotinin (Sigma A1153) replaced r *D. variabilis* KPI at 0.012 U/ml to serve as a positive control. A blank (no trypsin or inhibitor) and an uninhibited reaction (no inhibitor) were also run as controls. Percent inhibition was calculated as follows: 1 − (inhibited/uninhibited) × 100. The mean and standard deviation of three separate experiments were plotted using SigmaPlot 10.0. One-way analysis of variance followed by the least significant difference multiple comparison procedure was used to test for significance between groups at the 5% level.

Protein quantification, SDS-polyacrylamide gel electrophoresis, and Western blotting. Protein samples were quantified using the bicinchoninic acid protein assay kit (Pierce). Samples were diluted with 1× SDS sample buffer and electrophoresed on 4 to 20% Bis-Tris SDS gels according to the manufacturer's instructions (Invitrogen). Proteins were transferred to 0.45-μm polyvinylidene difluoride (Invitrogen) using standard conditions. r *D. variabilis* KPI was detected using mouse anti-V5 monoclonal antibody (Invitrogen), and blots were developed using the Western Breeze chemiluminescent detection kit according to the manufacturer's instructions (Invitrogen).

qRT-PCR. For gene expression, one-step quantitative reverse transcription-PCR (qRT-PCR) was performed using 0.5 to 1 μg total RNA and the Brilliant II Sybr green qRT-PCR 1-Step kit (Stratagene) on an Mx3000P real-time thermal cycler (Stratagene). The following qRT-PCR primers were used: qRT-PCR For-*D. variabilis* KPI and qRT-PCR Rev-*D. variabilis* KPI, qRT-PCR For-Actin and qRT-PCR Rev-Actin, qRT-PCR For-GAPDH and qRT-PCR Rev-GAPDH, and qRT-PCR For-*16s rRNA* and qRT-PCR Rev-*16s rRNA*. All primers are listed in Table 1. Data were exported for estimation of the amplification effi-

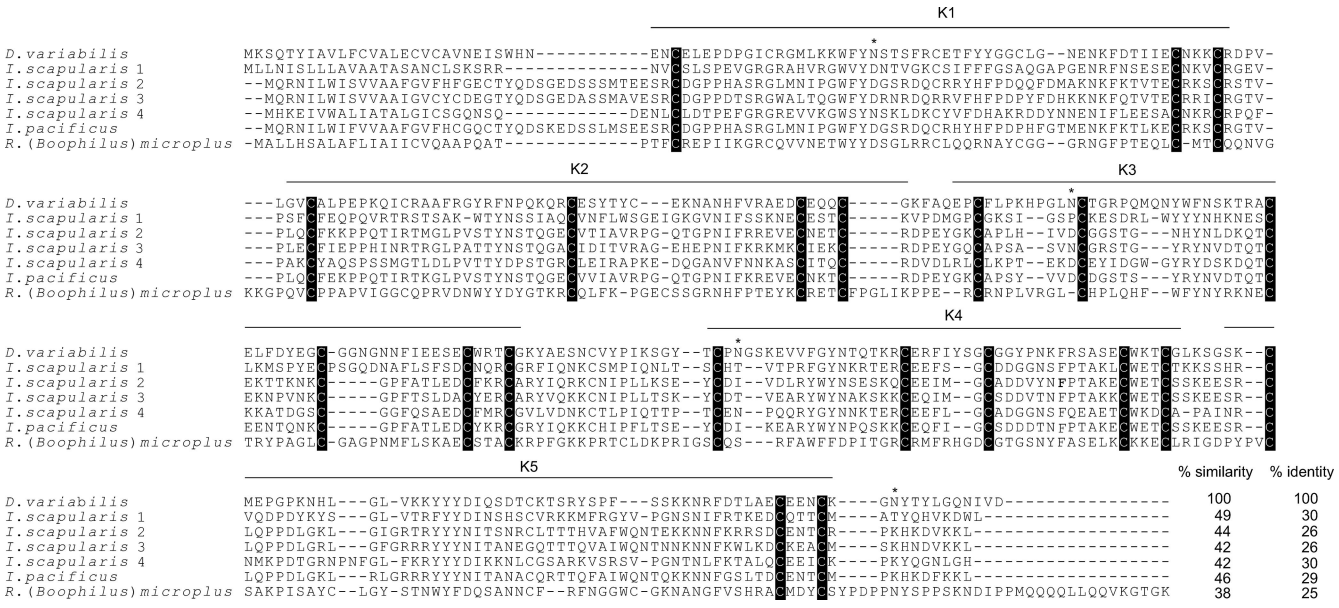

FIG. 1. *D. variabilis* KPI shares highly conserved cysteine with other tick Kunitz domain-bearing proteins. To determine the conservation of structure between *D. variabilis* KPI and other reported Kunitz-bearing protease inhibitors from ticks, we performed an alignment at the protein sequence level. Even though *D. variabilis* KPI shares no greater than 49% similarity with the other Kunitz-type protease inhibitors, the cysteine positions are conserved. This suggests that *D. variabilis* KPI has a similar disulfide-bonding pattern and therefore tertiary structure to that reported for penthalaris from *I. scapularis* (6). Kunitz domains were predicted for *D. variabilis* KPI using the SMART database (accession number 00131). The level of similarity between *D. variabilis* KPI and the other Kunitz-type serine protease inhibitors may suggest additional functions for *D. variabilis* KPI as is presented in this study. Swiss-Prot accession numbers are as follows: A9YPC4 (*D. variabilis*), Q4PMU5 (*I. scapularis* 1), Q8MVC4 (*I. scapularis* 2), Q4PML9 (*I. scapularis* 3), Q4PMM5 (*I. scapularis* 4), Q6B8C7 (*I. pacificus*), and Q3HYC9 (*Rhipicephalus* [*Boophilus*] *microplus*). Asterisks denote predicted N-linked glycosylation sites.

ciency for each primer set using LinRegPCR (19). The efficiencies and cycle threshold (C_T) values from the experiments were imported into Q-Gene for calculation of normalized expression (12). To calculate normalized *D. variabilis* KPI expression, the efficiency-corrected C_T values for *D. variabilis* KPI were divided by those for actin. To calculate burden, the efficiency-corrected C_T values for a rickettsial housekeeping gene (16S rRNA) were divided by that for a host housekeeping gene (*GAPDH*). The difference (*n*-fold) between experimental and control samples is reported for all normalized relative gene expression data. The median values are reported for all experiments. A nonparametric randomized permutation test was performed to derive *P* values and 95% confidence intervals as described by Ceraul et al. (2).

***D. variabilis* KPI inhibition of rickettsial burden.** Six-well plates were plated with 4×10^5 nontransfected, LacZ-transfected, or *D. variabilis* KPI-transfected cells and incubated for 72 h at 34°C and 5% CO_2 without Geneticin (Gibco). To confirm the presence of *D. variabilis* KPI in the culture medium from *D. variabilis* KPI-expressing cells and its absence in our two control cell lines (untransfected L929 and LacZ-expressing cells), we harvested medium from each cell line, clarified the medium by centrifugation at 3,200 × *g* for 25 min, and confirmed the production of r *D. variabilis* KPI by Western blotting with mouse anti-V5 (Invitrogen) using standard conditions (see Fig. 5B). We began our experiments after 72 h of cell growth because pilot studies demonstrated that increases in *D. variabilis* KPI concentrations in the medium were negligible after 72 h of growth as steady-state levels were reached. Each cell type was infected with a multiplicity of infection of 10 and incubated at 34°C and 5% CO_2 for 24 h. After 24 h, the cells were washed with 1× phosphate-buffered saline and lysed by resuspension in 700 μl of RLT (with β-mercaptoethanol) for RNA isolation using the RNeasy Micro kit (Qiagen). Rickettsial burden was measured by qRT-PCR using the rickettsial 16S rRNA and the mouse housekeeping gene *GAPDH*. The median of at least three separate experiments, run in duplicate, was plotted using SigmaPlot 10.0.

Sequence accession numbers. *D. variabilis* KPI has been deposited with NCBI and ExPASY under accession numbers EU265775 and A9YPC4, respectively.

RESULTS

***D. variabilis* KPI possesses five Kunitz domains with similarity to other tick KPIs.** Kunitz domains are prevalent across tick species and well characterized as anticoagulants (Fig. 1). We observe between 38 and 49% similarity between *D. variabilis* KPI and other Kunitz domain-bearing protease inhibitors from ticks. *D. variabilis* KPI shares conserved cysteine residues with all of the analyzed protease inhibitors, most notably penthalaris, for which the tertiary structure has been predicted. NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/) predicted four N-linked glycosylation sites at amino acid residues 50, 154, 218, and 319, which are depicted in Fig. 1.

***D. variabilis* KPI inhibits trypsin activity and possesses anticoagulant properties.** KPIs are reported to inhibit coagulation as tissue factor pathway inhibitors and thrombin inhibitors (8). Using this precedent, we wanted to test the function of *D. variabilis* KPI as both an anticoagulant and as a general trypsin inhibitor. Using the aPTT test, we observe a twofold delay ($P < 0.049$) in coagulation (Fig. 2A). The predominant midgut gene expression (see below) and the anticoagulant properties of *D. variabilis* KPI suggest a role for anticoagulants in the midgut during the feeding process. Additionally, *D. variabilis* KPI possessed robust antitrypsin activity (52%) very similar to the inhibition observed for aprotinin (63%) (Fig. 2B). *D. variabilis* KPI's role as a trypsin inhibitor indicates its versatility that could prove to be important to the inhibition of rickettsial colonization (22).

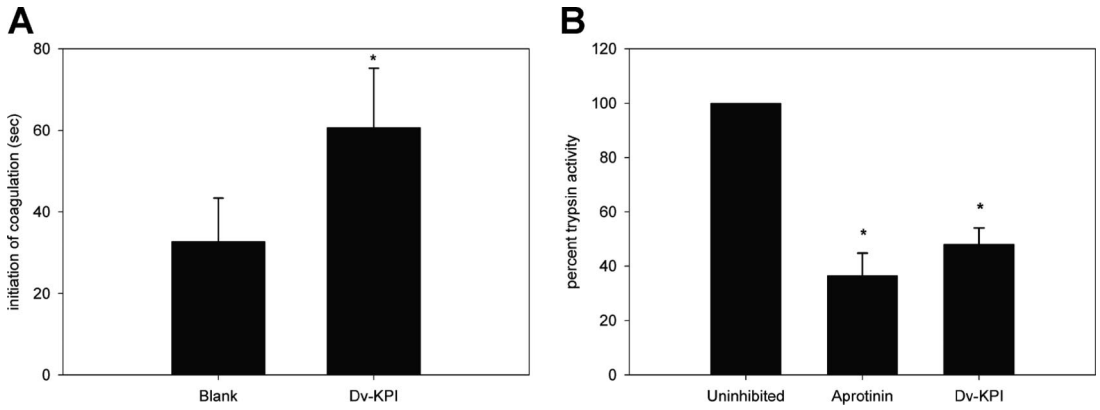

FIG. 2. *D. variabilis* KPI inhibits coagulation and trypsin activity. We tested *D. variabilis* KPI for its predicted activity as an anticoagulant and its role as a general trypsin inhibitor. (A) We used the aPTT test to determine if *D. variabilis* KPI caused a delay in the initiation of clot formation, indicating anticoagulant activity. Compared to the control, *D. variabilis* KPI delayed initiation of coagulation by 1.9-fold ($P = 0.03$). (B) r *D. variabilis* KPI inhibited trypsin activity similarly to that of aprotinin (canonical Kunitz-type protease inhibitor). Aprotinin inhibited trypsin activity by 63%, whereas *D. variabilis* KPI inhibited activity by 52%. These observations demonstrate the potential in vivo significance of *D. variabilis* KPI in relation to feeding or as a general trypsin inhibitor. Each experiment was repeated three times. The results are represented as the mean ± the standard deviation. Asterisks denote significant differences. Significance was determined by one-way analysis of variance followed by the least significant difference multiple comparison procedure to test for significance between groups at the 5% level.

***D. variabilis* KPI is abundantly expressed in the tick midgut and induced upon feeding.** As most KPIs are secreted with the saliva, we wanted to determine the tissue distribution for gene expression. We found that *D. variabilis* KPI is abundantly expressed in the midgut with little expression in the salivary gland from ticks fed for 4 days (Fig. 3A). As KPIs are involved with feeding success, we tested for the effects of feeding on gene expression in the midgut and salivary gland of fed and unfed ticks. We observe a 10.8-fold ($P < 0.001$) increase in *D. variabilis* KPI expression upon feeding, which is consistent with the predominant midgut expression (Fig. 3B). In contrast to other tick KPIs expressed from salivary glands that are vital as anticoagulants during feeding, *D. variabilis* KPI expression in *D. variabilis* salivary glands appears to be unaffected by feeding (Fig. 3B). This finding suggests that anticoagulants, specifically *D. variabilis* KPI, are important in the midgut, in addition to the feeding lesion at the tick-host interface, during feeding.

***D. variabilis* KPI expression is sustained in the presence of the spotted fever group rickettsia.** Interestingly, bacterial and fungal challenge of *D. melanogaster* induced gene expression for two KPIs, suggesting an immunological function beyond their commonly designated hemostatic properties (3). Recent studies report an increase in transcription of a KPI in *Rhipicephalus* (*Boophilus*) *microplus* ovaries in response to *B. bovis* infection (18). These studies led us to examine the role that *D. variabilis* KPI may play during the response to rickettsial in-

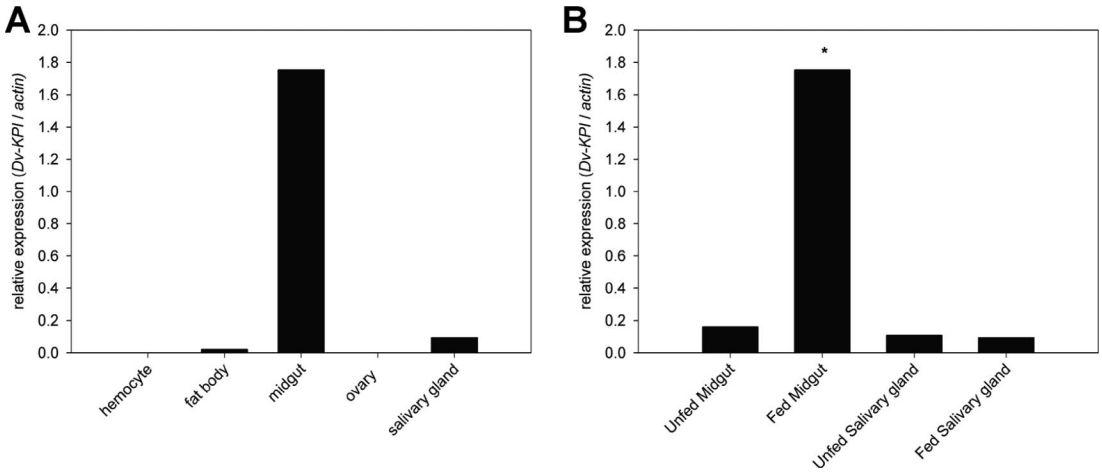

FIG. 3. *D. variabilis* KPI is highly expressed in the midgut in response to feeding. We performed real-time qPCR to determine tissue distribution and the effects of feeding on *D. variabilis* KPI expression. (A) Gene expression of *D. variabilis* KPI was greatest in the midguts from ticks fed for 4 days. (B) Kunitz-type protease inhibitors are known anticoagulants that maintain the fluidity of blood at the feeding lesion. We tested whether gene expression was induced in the midgut and the salivary gland as a result of imbibing a blood meal. Surprisingly, expression in the salivary gland from fed ticks decreased 1.15-fold relative to that of the unfed controls ($P > 0.111$). Expression in the midgut from ticks fed for 4 days increased 10.8-fold compared to that of the unfed controls ($P < 0.001$). Results represent the median of four to six individual tick replicates. Asterisk denotes significant difference.

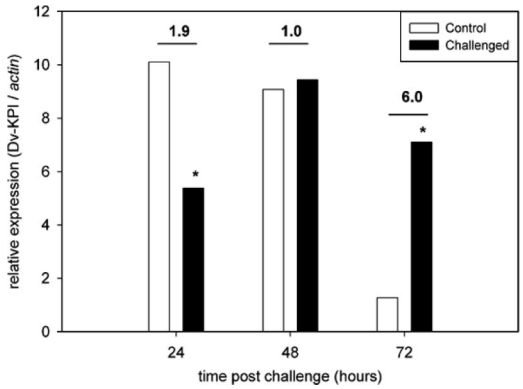

FIG. 4. *R. montanensis* induces *D. variabilis* KPI gene expression. Midguts from *R. montanensis*-challenged *D. variabilis* ticks shows sustained levels of *D. variabilis* KPI transcript over a 72-h time period. Results are reported as the median. The following 95% confidence intervals (in parentheses) were calculated as described previously (2, 17): 24 h (0.511, 0.553), 48 h (0.999, 1.08), 72 h (5.36, 5.80). The measurements are the result of two independent trials and four to six individual tick replicates. Asterisks indicate significant differences between groups.

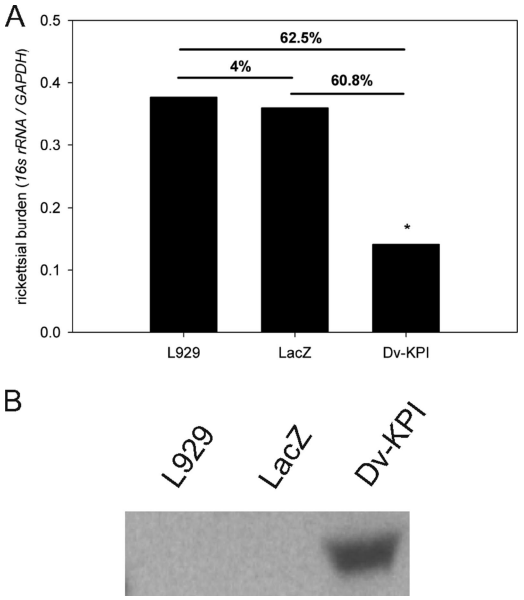

FIG. 5. *D. variabilis* KPI limits rickettsial colonization of host cells. (A) Rickettsial burden is reduced in *D. variabilis* KPI-expressing cells compared to that in nontransfected and LacZ-transfected cells. Results are reported as the median. (A) The 95% confidence intervals (in parentheses) for each comparison are as follows: L929 versus LacZ (0.043, 0.046), L929 versus *D. variabilis* KPI (0.317, 1.24), and LacZ versus *D. variabilis* KPI (0.318, 1.16). Asterisk indicates significant differences between the groups. Each experiment was repeated at least three times in duplicate. (B) *D. variabilis* KPI protein expression is not detected in nontransfected L929 or LacZ-transfected control cell lines by Western blot assays. *D. variabilis* KPI was detected using mouse anti-V5 monoclonal antibody.

fection. We performed qRT-PCR on midguts from ticks challenged with rickettsia to determine if *D. variabilis* KPI expression could be induced by *R. montanensis* infection (Fig. 4). It is unclear why there is an initial 1.9-fold reduction in *D. variabilis* KPI expression at 24 h postchallenge ($P < 0.006$). Similar trends occur early in the time course for defensin and lysozyme expression in *R. montanensis*-challenged tick midguts (2). *D. variabilis* KPI gene expression in the control 72 h after rickettsial challenge is reduced to levels observed in ticks that have fed for 4 days (compare Fig. 3 to 4). In contrast, *D. variabilis* KPI transcript levels in rickettsia-challenged ticks are sixfold greater ($P < 0.001$) than those of the controls 72 h postchallenge.

Interestingly, transcript levels in the control ticks at 24 h after rickettsial challenge are greater than those observed in midguts from ticks fed for 4 days (compare Fig. 3A to 4). We speculate that the increase in transcript abundance for *D. variabilis* KPI shown in Fig. 4 results from the fresh blood that is imbibed during artificial feeding. As mentioned above, transcript abundance for *D. variabilis* KPI in midguts from control ticks 72 h postchallenge does return to levels comparable to those observed for ticks fed for 4 days. Leveling off of *D. variabilis* KPI transcription may represent what happens in the tick during the later stages of feeding on the animal. Sustained *D. variabilis* KPI expression in rickettsia-challenged ticks suggests that *D. variabilis* KPI is involved in the response to rickettsia challenge.

***D. variabilis* KPI limits rickettsial colonization of host cells.** KPIs are expressed as part of the HR mounted to *Rhizobium* endosymbionts, which is hypothesized to limit growth and spread of microbes throughout the host plant (10, 11). To determine if *D. variabilis* KPI affected rickettsial growth, we performed an in vitro antimicrobial assay using *D. variabilis* KPI-expressing L929 fibroblasts. *D. variabilis* KPI-expressing L929 cells were permitted to grow for 72 h postplating to allow for the accumulation of *D. variabilis* KPI in the medium. We challenged the *D. variabilis* KPI-expressing cells after 72 h of growth because pilot experiments indicated negligible increases in *D. variabilis* KPI concentrations after this time point. Rickettsial burden was measured by qRT-PCR 24 h postinfection. The burden from the two control cell lines (nontransfected and LacZ-expressing cells) was not different ($P = 0.207$) (Fig. 5A); however, the burden from the *D. variabilis* KPI-expressing cells was 62.5% ($P = 0.0079$) and 60.8% ($P = 0.0082$) less than those from the nontransfected and LacZ-expressing cells, respectively (Fig. 5A). The observation of reduced burden at 24 h postinfection indicates that *D. variabilis* KPI possesses some bacteriostatic function that limits rickettsial colonization either at the point of entry or during early replication.

DISCUSSION

Here we report the bacteriostatic nature of a Kunitz-type serine protease inhibitor, *D. variabilis* KPI, with the capacity to limit *R. montanensis* colonization of host cells. Controlling the growth of potentially harmful microbes that are ingested during feeding is critical to the survival of any hematophagous arthropod vector. To this end, the influx of rickettsiae experienced during feeding may require detection and subsequent action on the part of the tick's immune response to control rickettsial burden. We have previously shown that defensin and lysozyme are activated in the midgut of *D. variabilis* as *R. montanensis* is acquired through feeding (2). Our observation

that *D. variabilis* KPI was induced in the midgut following *R. montanensis* challenge was surprising given that KPIs are predominately found in the salivary gland and function as anticoagulants. Interestingly, this is not the first report of KPI gene induction in response to infection. An increase in gene expression for two KPIs in *D. melanogaster* following challenge with the nonpathogenic bacteria *Escherichia coli* and *Micrococcus luteus* or the pathogenic fungus *Beauveria bassiana* (3) further implicates their roles as immune-responsive proteins. Strong evidence for KPI involvement in the response to infection has come from proteomic studies of tick ovaries. *B. bovis* infection of *Rhipicephalus* (*Boophilus*) *microplus* resulted in an increase in protein expression for the KPI BmTI-A (18) and offers the possibility that KPIs play a role in the response to infection in the ovary.

The correlation between feeding and immune activation is well documented in arthropod vectors. Feeding alone causes an increase in defensin expression in the midgut of the stable fly *Stomoxys calcitrans* (9) and both defensin and lysozyme expression in the midgut of *D. variabilis* (2). *D. variabilis* KPI expression may be induced as a component of a genetic network commonly regulated during transcription and translation or posttranslationally in response to feeding and microbial challenge. For example, *Thor*, encoding a member of the 4E-binding protein family responsible for preventing the formation of the translation initiation complex, is induced for expression in response to both starvation and microbial challenge (24). A recent report draws a connection between nutritional stress and the immune response by demonstrating that the serine/threonine kinase encoded by *ird1*, a Vps15 homologue known for its role in starvation-induced autophagy, also modulates starvation-induced immune activation within the immune deficiency pathway (*imd*) cascade in *D. melanogaster* (23). It is also feasible that *D. variabilis* KPI was coadapted to dual functionality as both an anticoagulant and an immunological effector. Coadaptation in hematophagy is well exemplified in the hard ticks *Rhipicephalus* (*Boophilus*) *microplus* and *D. variabilis* and the soft tick *Ornithodoros moubata*, where β-hemoglobin fragments generated in the midgut as a byproduct of digestion were identified as being antimicrobial (5, 13, 20).

Because *D. variabilis* KPI is identified as having antimicrobial properties and demonstrates trypsin inhibitory activity, our data suggest that *D. variabilis* KPI functions as a rickettsiostatic serine protease inhibitor. This finding is biologically relevant, as trypsin inhibitors have been shown to reduce host cell colonization and growth by *Rickettsia rickettsii*. The synthetic amidine-type trypsin inhibitor, bis(5-amidino-2-benzimidazolyl)-methane, was shown to inhibit or reduce plaque formation by *R. rickettsii* in vitro and delay the onset of fever and death in *R. rickettsii*-infected guinea pigs (22). The exact mechanism of bacteriostatic action of *D. variabilis* KPI is still unclear. Mounting evidence indicates that Kunitz domain-bearing proteins limit bacterial metastases. This is nicely illustrated by studies of the HR elicited in plants toward both compatible (endosymbiont) and incompatible (pathogen) infections (10, 11, 21). *Rhizobium* spp. invade root hairs and induce the formation of infection threads that the bacteria follow on their way to the root cortex (21). Not all infection threads are successful and terminate before reaching the root cortex (21). Signs of an HR are observed at the terminated ends of the failed infection threads (21) and are also associated with senescent nodules, characterized by necrotic host tissue and dead bacteroids (11). Ultrastructural and gene expression studies indicate that a 21-kDa KPI identified in senescent nodules may limit the spread of *Rhizobium* spp. (bacteroids) to uninfected portions of the plant (11). Even though the symbiosis between rhizobia and legumes is mutualistic, the endosymbiont is not permitted to spread unabated for risk of physiologic stress and disease to the host (10, 11, 21).

Given the findings in *Rhipicephalus* (*Boophilus*) *microplus*, it will be important to assess the effect that *D. variabilis* KPI has on transovarial transmission of rickettsiae. Currently, we cannot predict how *D. variabilis* KPI affects rickettsial acquisition in the midgut of the tick. The evidence in the literature and the data from the present study suggest that if *D. variabilis* KPI was rendered inactive, an increase in rickettsial burden may be the result. We are currently testing this idea in vivo using RNA interference. The current study suggests that as a rickettsiostatic serine protease inhibitor, *D. variabilis* KPI is one factor that may control the growth of rickettsiae, thereby contributing to the success of endosymbioses and the vector competency of ticks.

ACKNOWLEDGMENTS

The project described was supported by award number R01AI04320016 and from R01AI017828 from the National Institute of Allergy and Infectious Diseases.

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Allergy and Infectious Diseases or the National Institutes of Health.

We acknowledge Daniel E. Sonenshine for providing the ticks used in this project.

REFERENCES

1. **Baldridge, G. D., T. J. Kurtti, and U. G. Munderloh.** 2005. Susceptibility of *Rickettsia monacensis* and *Rickettsia peacockii* to cecropin A, ceratotoxin A, and lysozyme. Curr. Microbiol. **51:**233–238.
2. **Ceraul, S. M., S. M. Dreher-Lesnick, J. J. Gillespie, M. S. Rahman, and A. F. Azad.** 2007. New tick defensin isoform and antimicrobial gene expression in response to *Rickettsia montanensis* challenge. Infect. Immun. **75:**1973–1983.
3. **De Gregorio, E., P. T. Spellman, G. M. Rubin, and B. Lemaitre.** 2001. Genome-wide analysis of the *Drosophila* immune response by using oligonucleotide microarrays. Proc. Natl. Acad. Sci. USA **98:**12590–12595.
4. **Edgar, R. C.** 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics **5:**113.
5. **Fogaca, A. C., P. I. da Silva, Jr., M. T. Miranda, A. G. Bianchi, A. Miranda, P. E. Ribolla, and S. Daffre.** 1999. Antimicrobial activity of a bovine hemoglobin fragment in the tick *Boophilus microplus*. J. Biol. Chem. **274:**25330–25334.
6. **Francischetti, I. M., T. N. Mather, and J. M. Ribeiro.** 2004. Penthalaris, a novel recombinant five-Kunitz tissue factor pathway inhibitor (TFPI) from the salivary gland of the tick vector of Lyme disease, *Ixodes scapularis*. Thromb. Haemost. **91:**886–898.
7. **Francischetti, I. M., J. G. Valenzuela, J. F. Andersen, T. N. Mather, and J. M. Ribeiro.** 2002. Ixolaris, a novel recombinant tissue factor pathway inhibitor (TFPI) from the salivary gland of the tick, *Ixodes scapularis*: identification of factor X and factor Xa as scaffolds for the inhibition of factor VIIa/tissue factor complex. Blood **99:**3602–3612.
8. **Hovius, J. W., M. Levi, and E. Fikrig.** 2008. Salivating for knowledge: potential pharmacological agents in tick saliva. PLoS Med. **5:**e43.
9. **Lehane, M. J., D. Wu, and S. M. Lehane.** 1997. Midgut-specific immune molecules are produced by the blood-sucking insect *Stomoxys calcitrans*. Proc. Natl. Acad. Sci. USA **94:**11502–11507.
10. **Lievens, S., S. Goormachtig, and M. Holsters.** 2004. Nodule-enhanced protease inhibitor gene: emerging patterns of gene expression in nodule development on *Sesbania rostrata*. J. Exp. Bot. **55:**89–97.
11. **Manen, J. F., P. Simon, J. C. Van Slooten, M. Osteras, S. Frutiger, and G. J. Hughes.** 1991. A nodulin specifically expressed in senescent nodules of winged bean is a protease inhibitor. Plant Cell **3:**259–270.
12. **Muller, P. Y., H. Janovjak, A. R. Miserez, and Z. Dobbie.** 2002. Processing of gene expression data generated by quantitative real-time RT-PCR. Biotechniques **32:**1372–1374, 1376, 1378–1379.

13. **Nakajima, Y., K. Ogihara, D. Taylor, and M. Yamakawa.** 2003. Antibacterial hemoglobin fragments from the midgut of the soft tick, Ornithodoros moubata (Acari: Argasidae). J. Med. Entomol. **40:**78–81.
14. **Nazareth, R. A., L. S. Tomaz, S. Ortiz-Costa, G. C. Atella, J. M. Ribeiro, I. M. Francischetti, and R. Q. Monteiro.** 2006. Antithrombotic properties of Ixolaris, a potent inhibitor of the extrinsic pathway of the coagulation cascade. Thromb. Haemost. **96:**7–13.
15. **Nicholas, K. B., and H. B. J. Nicholas.** 1997. GeneDoc: a tool for editing and annotating multiple sequence alignments. http://www.nrbsc.org/gfx/genedoc/index.html/.
16. **Nuttall, P. A., and M. Labuda.** 2004. Tick-host interactions: saliva-activated transmission. Parasitology **129**(Suppl.)**:**S177–S189.
17. **Price, R. M., and D. G. Bonett.** 2002. Distribution-free confidence intervals for difference and ratio of medians. J. Stat. Comput. Simul. **72:**119–124.
18. **Rachinsky, A., F. D. Guerrero, and G. A. Scoles.** 2007. Differential protein expression in ovaries of uninfected and *Babesia*-infected southern cattle ticks, *Rhipicephalus* (*Boophilus*) *microplus*. Insect Biochem. Mol. Biol. **37:** 1291–1308.
19. **Ramakers, C., J. M. Ruijter, R. H. L. Deprez, and A. F. M. Moorman.** 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci. Lett. **339:**62–66.
20. **Sonenshine, D. E., W. L. Hynes, S. M. Ceraul, R. Mitchell, and T. Benzine.** 2005. Host blood proteins and peptides in the midgut of the tick *Dermacentor variabilis* contribute to bacterial control. Exp. Appl. Acarol. **36:**207–223.
21. **Vasse, J., F. de Billy, and G. Truchet.** 1993. Abortion of infection during the *Rhizobium meliloti*-alfalfa symbiotic interaction is accompanied by a hypersensitive reaction. Plant J. **4:**555–566.
22. **Walker, D. H., R. R. Tidwell, T. M. Rector, and J. D. Geratz.** 1984. Effect of synthetic protease inhibitors of the amidine type on cell injury by *Rickettsia rickettsii*. Antimicrob. Agents Chemother. **25:**582–585.
23. **Wu, J., K. E. Randle, and L. P. Wu.** 2007. *ird1* is a Vps15 homologue important for antibacterial immune responses in *Drosophila*. Cell. Microbiol. **9:**1073–1085.
24. **Zinke, I., C. S. Schutz, J. D. Katzenberger, M. Bauer, and M. J. Pankratz.** 2002. Nutrient control of gene expression in *Drosophila*: microarray analysis of starvation and sugar-dependent response. EMBO J. **21:**6162–6173.

Editor: W. A. Petri, Jr.