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# Selective Cysteine Protease Inhibition Contributes to Blood-feeding Success of the Tick *Ixodes scapularis**

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*Ixodes scapularis* **is the main vector of Lyme disease in the eastern and central United States. Tick salivary secretion has been shown as important for both blood-meal completion and pathogen transmission. Here we report a duplication event of cystatin genes in its genome that results in a transcription-regulated boost of saliva inhibitory activity against a conserved and relatively limited number of vertebrate papain-like cysteine proteases during blood feeding. We further show that the polypeptide products of the two genes differ in their binding affinity for some enzyme targets, and they display different antigenicity. Moreover, our reverse genetic approach employing RNA interference uncovered a crucial mediation in tick-feeding success. Given the role of the targeted enzymes in vertebrate immunity, we also show that host immunomodulation is implicated in the deleterious phenotype of silenced ticks making** *I. scapularis* **cystatins attractive targets for development of anti-tick vaccines.**

Among the differences that make a relationship between two organisms parasitic rather than symbiotic is the lack of mutual benefit; the parasite manages to continuously receive valuable resources from the host without returning this favor; in addition, sometimes it triggers catastrophic conditions to the host such as disease transmission. Hard ticks can be considered another case of efficient ectoparasites that are able to suck blood, a rich source of nutrients, from their vertebrate host(s) for several days (1). If the "blood donor" is aware of the tick attack to the skin/blood circulation, given that a tick cannot fly, rejection could be the best scenario and death the worst for the arthropod. Consequently, ticks have developed a series of mechanisms to gain undisturbed access to their nutritious meal, including saliva injection in biting sites (2). Tick salivary glands regulate water and ion excretion by saliva secretion that in addition reduces the volume of the blood bolus in the tick digestive tract as feeding progresses. Furthermore, they deliver a repertoire of pharmacologic compounds in the site of infestation that affects among other things, hemostasis and host immunity, thus facilitating the completion of a good quality meal for the tick (3). Unluckily for the host, saliva has also been shown to enhance tick vector competence, *e.g.* its capability to transmit pathogens (4).

The black-legged tick *Ixodes scapularis* is among the most successful arthropod blood feeders; after hatching from the egg, larvae and nymphs feed normally on small rodents, whereas adults feed on larger animals (5). This species transmits the Lyme disease etiologic agent *Borrelia burgdorferi* as well as *Anaplasma phagocytophilum*, *Babesia microti* (causing the diseases anaplasmosis and babesiosis respectively), and viruses within the tick-borne encephalitis complex (6). To enhance our knowledge about saliva constituents that account for its unpleasant (for the host) biologic properties, two massive sequencing projects of salivary expressed sequence tags (ESTs)[2] were completed in our laboratory (7, 8). Analysis using bioinformatic tools revealed gene family expansions in salivary gland secretome. This finding created, as is almost always the case, more questions than answers. Is the expansion due to polymorphisms or gene duplications, and if the last is the case, are they maintained stable in the genome to achieve antigenic variation, redundancy in biochemical pathways, or a combination of these?

In an attempt to bridge this gap between genomics and the function of the secretome, we focused on characterizing two cystatins with high amino acid (aa) identity to each other that are secreted from salivary glands of *I. scapularis*. Cystatins are present in vertebrates, invertebrates, plants, and protozoa, and all of them form tight, equimolar, and reversible inhibitory complexes with papain-like cysteine proteases. This holds true for the first cystatin we expressed, which we named sialostatin L because of its affinity for cathepsin L (9). We further showed that saliva indeed displays inhibitory activity against cathepsin L *in vitro* that could be partially attributed to the presence of sialostatin L. Consistent with the role of its target enzymes in immunity, we finally discovered an antiinflammatory and immunosuppressive action of the protein to the vertebrate host (9). Here we report the characterization of the second cystatin, which we named sialostatin L2 to emphasize its redundant inhibitory activity against cathepsin L. Other than their high aa identity and similar affinity for cathepsin L, we show that the two cystatins are not equally potent in inhibition of other target enzymes and that they also differ in antigenicity. Furthermore,

[2] The abbreviations used are: EST, expressed sequence tag(s); aa, amino acid(s); dsRNA, double-stranded RNA; IC$_{50}$, concentration of inhibitor at which 50% enzyme inhibition is achieved; $K_i$, inhibition constant(s); $K_i$*, apparent dissociation constant(s); RNAi, RNA interference; RT, reverse transcription; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; PIPES, piperazine-*N*,*N*-bis-2-ethane sulfonic acid; contig, group of overlapping clones.

we report major differences in their transcript abundance during tick infestation; sialostatin L2 transcripts greatly accumulate in the salivary glands as feeding to the host progresses, whereas sialostatin L transcripts slightly decrease at the same time. Given this transcriptional induction of sialostatin L2 and the absence of classical genetic approaches to this non-model arthropod vector, we undertook a reverse genetic approach to silence sialostatin L2 by RNA interference (RNAi). This well established technique (10) led to reduction of cystatin transcripts in the salivary glands followed by feeding inhibition, reduced tick size, and number of eggs laid. Moreover, normal ticks when exposed to a rabbit previously infested with silenced ticks exhibited significant feeding impairment due to an enhanced host immune response.

Because of its stringent and unique specificity, sialostatin L2 can be useful for studying the role of certain papain-like proteases in various biologic phenomena. In addition it can provide a starting point for potent pharmaceutical interventions that target the key role of those enzymes in human diseases. Besides their limited number of targets, we reveal the crucial mediation of *I. scapularis* cystatin salivary constituents in blood-meal uptake through control of their targets' proteolytic activity. Taking into account their role in the success of parasitism, they should be considered in the development of antiparasitic vaccines; they may be additional candidate ingredients in the mixture of antigens that will potentially lead to achievement of this difficult goal.

## EXPERIMENTAL PROCEDURES

Unless otherwise indicated, protocols followed standard procedures (11), and all experiments were performed at room temperature ($25 \pm 1$ °C). All materials were obtained from Sigma, and the water used was of 18-megaohm quality, produced by a MilliQ apparatus (Millipore, Bedford, MA).

*Bioinformatic Tools*—To obtain genomic information relative to the cystatin transcripts, raw trace FASTA files from shotgun genomic sequences of *I. scapularis* (found in ftp://ftp.ncbi.nih.gov/pub/TraceDB/ixodes_scapularis) representing nearly 24 million sequences were downloaded and removed of vector and primer sequences using a homemade tool written in Visual Basic. Sequences with average quality values below 20 were excluded. Sialostatins L and L2 coding sequences (NCBI accession gi:22164282 and gi:67083499, respectively) were blasted against these genomic sequences using blastn with a word size of 80 ($-$W 80 switch). The resulting matches were assembled using the cap3 assembler (12), and the produced consensus sequences were in turn blasted against the two cystatin transcripts. All other sequence comparisons reported here were done using the BLAST server at the NCBI (http://www.ncbi.nlm.nih.gov/BLAST) and the ClustalW Service at the European Bioinformatics Institute, whereas protein secretion signals were revealed in the SignalP 3.0 server of the Technical University of Denmark.

*Expression, Purification, and Sequence Verification of Sialostatin L2*—We followed the same procedure as described before for sialostatin L (9) except that sialostatin L2 cDNA was PCR-amplified using high fidelity Taq polymerase from a λTriplEx2 cDNA clone, described in our previous work (8),

with gene-specific primers (forward, 5′-GCC CAT ATG GAA CTG GCA CTG CGT GGC GGT TAC CGC GAG CG-3′; reverse, 5′-GCC CTC GAG TTA TGC GGC CGC ACA CTC AAA GGA GCT-3′) designed for subcloning into the pET17b bacterial expression vector.

*Enzymatic Assays*—Apparent inhibition constants of sialostatin L2 or L for various proteases were obtained as described earlier (9) by measuring loss of enzymatic activity at increasing concentrations of inhibitor in the presence of a fluorogenic enzyme substrate in large excess.

*Production of Polyclonal Sera*—Female Swiss Webster mice, 6–8 weeks old, were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in the NIAID Animal Care Facility (Twinbrook 3 Bldg., NIH) under pathogen-free conditions in temperature-controlled rooms and received water and food *ad libitum*. Groups of six mice each received intradermal injections of 10 μg of pure recombinant protein in each ear and four boosts followed at 2-week intervals. Preimmune sera were taken from each mouse before vaccination, whereas control groups received buffer (vehicle) vaccination in parallel. All treatments were performed in accordance with *The Guide for Care and Use of Laboratory Animals* (NIH).

*Tick Rearing*—For most experiments ticks were harvested after detaching from mice (nymphs) or rabbits (adults). Engorged nymphs were maintained at 23 °C and >90% relative humidity under a 14-h light/10-h dark photoperiod until enough time elapsed for them to molt into the adult stage. In all feeding experiments involving adult ticks, we placed an equal number of female and male ticks on the ears of New Zealand White rabbits. Ears were covered with cotton ear bags, and an Elizabethan collar was placed around the neck of each rabbit to prevent grooming. Engorged adult ticks were held under similar conditions as nymphs until enough time elapsed for them to lay eggs. For harvesting tick tissues, partially fed females were dissected within 4 h of being removed from hosts.

*Harvesting Tick Tissues*—Tick tissues (salivary glands and midguts) were dissected in ice-cold 100 mM MOPS buffer containing 20 mM ethylene glycol/EGTA, pH 6.8. After removal, glands were washed gently in the same ice-cold buffer. Dissected tissues were stored immediately after dissection in RNAlater (Ambion, Austin, TX) before isolating total RNA. Tissues were used immediately after dissection or stored at −70 °C in 0.5 M PIPES, pH 6.8, containing 20 mM EGTA, 1× Complete™ Mini Protease inhibitor mixture (Roche Applied Science). All other manipulations were carried out at 4 °C.

*Synthesis of Tick Salivary Gland cDNA and Reverse Transcription (RT)-PCR*—Total RNA was isolated using an RNAqueous™ total RNA isolation kit (Ambion) from dissected partially fed female salivary glands/midguts and unfed female adult salivary glands/midguts. Concentration of total RNA was determined spectrophotometrically, separated into aliquots, and stored at −70 °C before use. Total RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase according to manufacturer's protocol. For each gene, cDNA was PCR-amplified using gene-specific primers; sialostatin L2, forward 5′-CTA TGC GGC TTC CTC GAA GGG GCT-3′, and reverse, 5′-GGC TAC AGC GAG AGG GCG AAC CAC CAA-3′; tick salivary gland Isac, forward,

5′-AGC GAA GAC GGT CTC GAG CAA GAT-3′, and reverse, 5′-TCG GCA CAC GAT GCC TCA GGG AAT-3′; β-actin forward, 5′-GAA GAT CTT GAG AAG ATG GCC CAG-3′, and reverse, 5′-CGG TAC CGT CGA TGG TCA CC-3′, as the control. The PCR program we used included the following cycles: 75 °C for 3 min, 94 °C for 2 min, and 22 cycles of 94 °C for 1 min, 49 °C for 1 min, and 72 °C for 1.20 min followed with 10 min at 72 °C.

*Real-time Quantitative PCR*—Real-time quantitative PCR was performed using the Mx4000 or Mx3005P Multiplex Quantitative PCR system and the Brilliant SYBR Green Single-Step QRT-PCR Master Mix kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. A standard curve ($10^0$–$10^7$ copies per reaction) was generated using purified sialostatin L and L2 PCR products as the template. The following primers were used for all reactions; sialostatin L, forward 5′-TCG CGA TCG CTA GCA TCA CAC TT-3′, and reverse, 5′-AGC AGA AGG ACC AAA GCG AAG GTA-3′; sialostatin L2, forward 5′-AAG TCC ATT AGC TCC TTC GAG TGT G-3′, and reverse, 5′-ATC ATT CCG CGA CGT ACA GTG AGA-3′. Reactions (25-μl final volume) contained 10 ng of total RNA and were run under the following conditions; 1 cycle of 50 °C for 30 min and 95 °C for 15 min followed by 40 cycles of 95 °C for 30 s and 55 °C for 30 s. Fluorescence was measured every cycle at the end of the 55 °C step. Samples were run in triplicate as well as in the absence of reverse transcriptase or template as negative controls. The copy number of sialostatin L and L2 mRNA in each sample was determined using the Mx4000 or Mx3005P data analysis software based on the standard curve.

*Double-stranded RNA (dsRNA) Synthesis, Tick Injections, and Feeding*—Sialostatin L2 RT-PCR product was joined to the Block-iT T7 TOPO linker. This TOPO linking reaction was used in two PCR reactions with gene-specific and T7 PCR primers to produce sense and antisense linear DNA templates. These sense and antisense DNA templates were used to generate sense and antisense transcripts using the BLOCK-iT RNA TOPO transcription kit. The resulting dsRNA was analyzed by agarose gel electrophoresis to verify its size. Subsequently, unfed female ticks were injected with 0.5 μg of cystatin dsRNA or with 1 μl of TS.MOPS (vehicle) using a 35-gauge needle. After injection of dsRNA or buffer alone, ticks were kept at 37 °C overnight under high humidity to observe tick survival. Surviving ticks were exposed to a naïve (never tick-bitten) rabbit and allowed to blood-feed to repletion. Their feeding success was determined by total engorged weight, survival, and egg lying. The ears of the rabbits exposed to dsRNA sialostatin L2 or water-injected ticks were cleaned by the end of the experiment; the animals were kept for 14 days and then re-exposed to normal unfed ticks, and feeding success evaluation was performed as described above.

*Statistics*—All data are expressed as the mean ± S.E. Statistical significance was determined by Student's *t* test; differences in multiple comparisons among different experimental groups were determined by analysis of variance using the Tukey test.

## RESULTS

*The Two Cystatin Transcripts Are Encoded by Two Different Genes*—Several *I. scapularis* transcripts were revealed to be of salivary origin during our most recent massive EST sequencing project (8) including a novel cystatin that shows 75% identity at the protein level to sialostatin L, a secreted cystatin previously characterized in our laboratory (9). When the secretion signal was removed from this polypeptide, multiple alignment with sialostatin L showed a clustering of aa substitutions in two regions of the protein; of a total of 27 aa substitutions throughout the 115-residue polypeptide, 12 were located in the first 22 amino-terminal residues, whereas another 12 substitutions gathered in the last 33 carboxyl-terminal aa of the protein (Fig. 1*A*). This raised the possibility that the two proteins could be allelic products of the same gene. To test this hypothesis, a bioinformatic approach was undertaken. cDNA sequences of both transcripts were compared by BLAST analysis to the publicly available shotgun genomic sequences from the *I. scapularis* genome project. The resulting matches were assembled into contigs that were in turn compared by BLAST to both cystatin transcripts. The result showed clearly that the two cystatins are encoded by two different genes (data not shown). The sialostatin L locus consists of three exons, whereas only two exons coding for parts of the amino terminus and carboxyl terminus of the second cystatin could be revealed (data not shown). Possibly the third exon was not detected due to the limited DNA sequence available. In both genes, intronic sequences were partial but unique; their high numbers of repeating sequences made impossible their successful extension due to the very large number of matches with repetitive sequences from intronic regions found in the shotgun genomic sequences.

*The Polypeptide Products of the Two Genes Differ in Their Target Specificity and Display Different Antigenicity*—We next proceeded to the expression and purification of the protein encoded by the novel transcript, which was subsequently used in inhibition assays of various commercially available purified proteases. Only four cysteine proteases of seven tested were affected by the presence of the protein in the assay, namely cathepsins L, V, S, and C (Fig. 1*B*). No inhibition was observed for cysteine proteases cathepsin X/Z/P, B, or H (Table 1), aspartic proteases cathepsin D and legumain, or serine proteases cathepsin G and elastase (data not shown). We next compared this novel cystatin with sialostatin L for efficiency in inhibiting their overlapping target enzymes. The results are shown in Fig. 1*C* and are summarized in Table 1. Briefly, the two inhibitors are equally potent for inhibition of cathepsins L and V (Fig. 1*C*, *upper panel*) but displayed major differences in inhibition of cathepsins S and C (Fig. 1*C*, *lower panel*). To further evaluate those findings, we tested whether this novel cystatin is a tight inhibitor for cathepsin L, as is the case for sialostatin L (9). Indeed, when we used decreasing amounts of cathepsin L in our assays, less cystatin was necessary to achieve the same percentage of enzymatic inhibition (Fig. 2*A*), which is a typical characteristic of tight inhibition. The decrease in the concentration of the inhibitor at which 50% enzymatic inhibition ($IC_{50}$) is achieved was actually analogous to the reduction of the amount
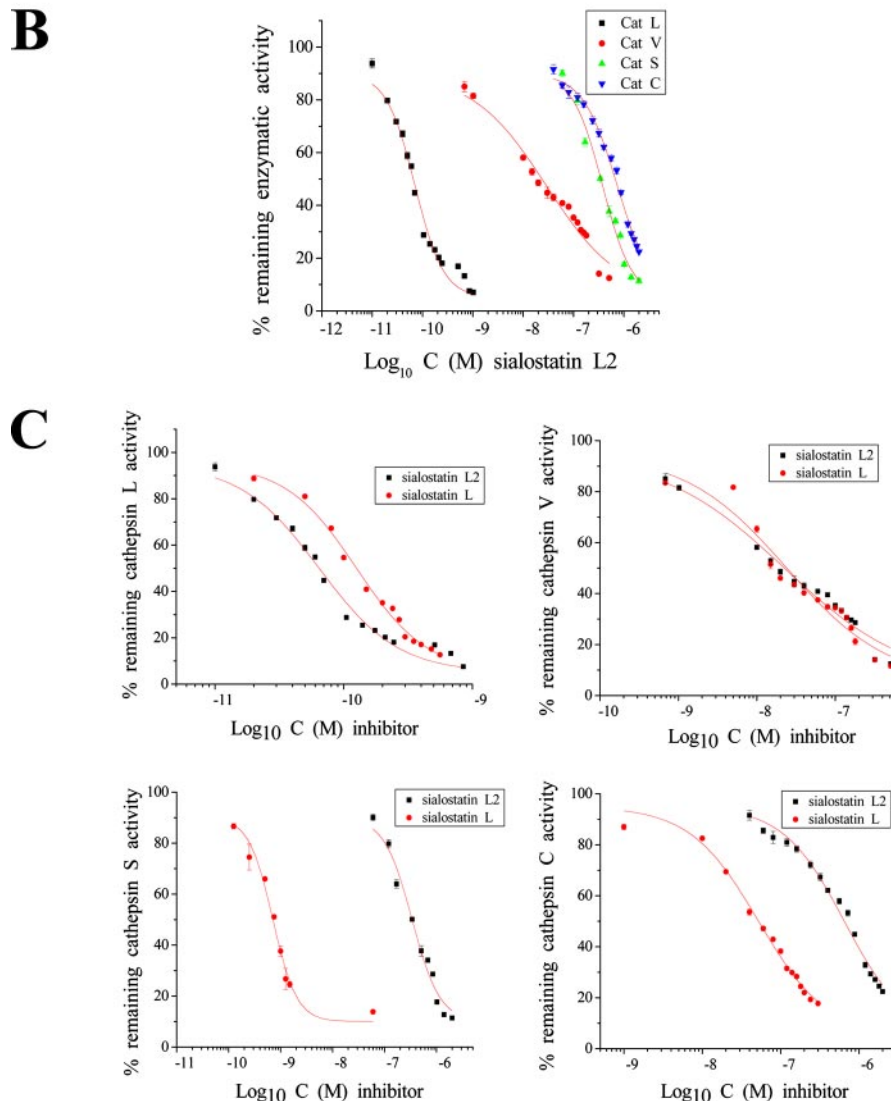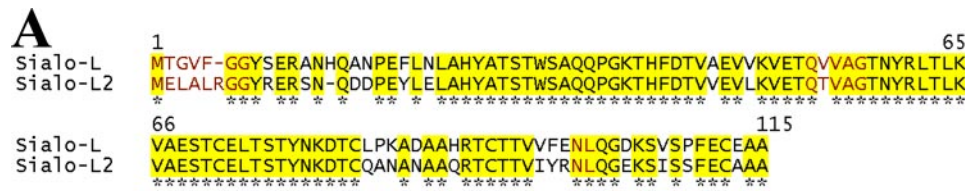
FIGURE 1. *A*, aa sequence alignment of the two secreted cystatins from *I. scapularis*. *Asterisks* and a *yellow color* denote conserved, common residues in both proteins. *Red*, aa predicted to play a role in inhibition of cysteine proteases by forming the interaction interface with the active site of the enzyme. *B*, proteolytic enzymes targeted by sialostatin L2. The *abscissa* represents sialostatin L2 concentration (*M*) in $\log_{10}$ scale, and the *ordinate* shows the percentage of remaining enzymatic activity in the presence of sialostatin L2. Each experiment was performed in triplicate. Additional details can be found in Table 1. *C*, sialostatin L2 differs in affinity for two of their common enzymatic targets when compared with sialostatin L. The two inhibitors were allowed to interact with the same amount of enzyme under the same assay conditions. The resulting reduction of enzymatic activity was plotted against the corresponding inhibitor concentration. The *abscissa* represents inhibitor concentration (*M*) in $\log_{10}$ scale, and the *ordinate* shows the percentage of remaining enzymatic activity in the presence of the inhibitor. Each experiment was performed in triplicate. *Red*, results for sialostatin L; *black*, results for sialostatin L2. Additional details can be found in Table 1.

was plotted against the substrate concentration, indicating a *y* intercept of 65.5 ± 23.1 pM that is the inhibition constant ($K_i$) of this novel cystatin for cathepsin L. The sialostatin L $K_i$ for the same enzyme is 95.3 ± 7.3 pM (9), demonstrating a similar affinity of the two inhibitors for cathepsin L. To emphasize this similarity, we assigned the name sialostatin L2 to this second salivary cystatin.

Having in hand both pure and active cystatins, we then examined their antigenicity, *i.e.* their capability to induce production of specific polyclonal sera in a vertebrate host, in this case female Swiss Webster mice. Sialostatin L or L2 was administered (20 μg) in each mouse five times at 2-week intervals; 2 weeks post the last vaccination, their sera were tested by enzyme-linked immunosorbent assays for recognition of vaccination antigen (sialostatin L or L2) and potential for cross-reaction with the second cystatin (sialostatin L2 or L, respectively). The results are shown in Fig. 3. Although both proteins were immunogenic, only sera from mice vaccinated with sialostatin L2 cross-reacted with sialostatin L. In a step further we estimated the mean antibody titer in the sera of the mice in both experimental groups using standard methods (11); for the sialostatin L-vaccinated mice the mean antibody titer was 4100 ± 400 for sialostatin L and 200 ± 35 for sialostatin L2, whereas the mean antibody titer in the sera of the sialostatin L2 vaccinated mice was 4000 ± 450 for sialostatin L2 and 1070 ± 136 for sialostatin L.

To sum up, the 27 different amino acids between the two cystatin molecules apparently results in changes in their interaction interface with some of the targeted enzymes (and, therefore, their

of enzyme used in the assay (Fig. 2*B*). Because conventional Michaelis-Menten kinetics do not hold true for tight binding inhibition, we applied Morrison's equation (13) to obtain apparent dissociation constants ($K_i^*$) in the presence of varying substrate concentrations. Fig. 2*C* shows the linear regression line ($r^2 = 0.9918$) when $K_i^*$ for several substrate concentrations

binding affinity). These primary structure changes and, more interestingly, the observed different affinity of the two inhibitors for cathepsin S, a critical enzyme for antigen processing and presentation, can account for the observed differences in their recognition from the vertebrate immune system as well.

**TABLE 1**

**Sialostatin L2 affinity changes for proteolytic enzymes when compared with sialostatin L**

Repertoire of cysteine proteases tested for inhibition by sialostatins L and L2 and the concentration of inhibitor at which 50% inhibition of the activity of the targeted proteolytic enzymes is achieved ($IC_{50}$) $\pm$ S.E. Enzyme concentration used in the assays is also given for all their targets. NI, no inhibition, *i.e.*, inhibition of the enzyme was not observed in the presence of 10 $\mu$M inhibitor.

| Enzyme | Enzyme concentration | Sialostatin L2 $IC_{50}$ | Sialostatin L $IC_{50}$ |
|---|---|---|---|
| Cathepsin L | 20 pM | 70.9 $\pm$ 3.4 pM | 125.8 $\pm$ 3.7 pM |
| Cathepsin V | 1 nM | 28.8 $\pm$ 2.8 nM | 24.1 $\pm$ 2.7 nM |
| Cathepsin S | 60 pM | 378.1 $\pm$ 23 nM | 0.7 $\pm$ 0.01 nM |
| Cathepsin C | 10 nM | 740.4 $\pm$ 22.3 nM | 52.2 $\pm$ 2.3 nM |
| Cathepsin X/Z/P | 16 nM | NI | 937 $\pm$ 14 nM |
| Cathepsin B | | NI | NI |
| Cathepsin H | | NI | NI |
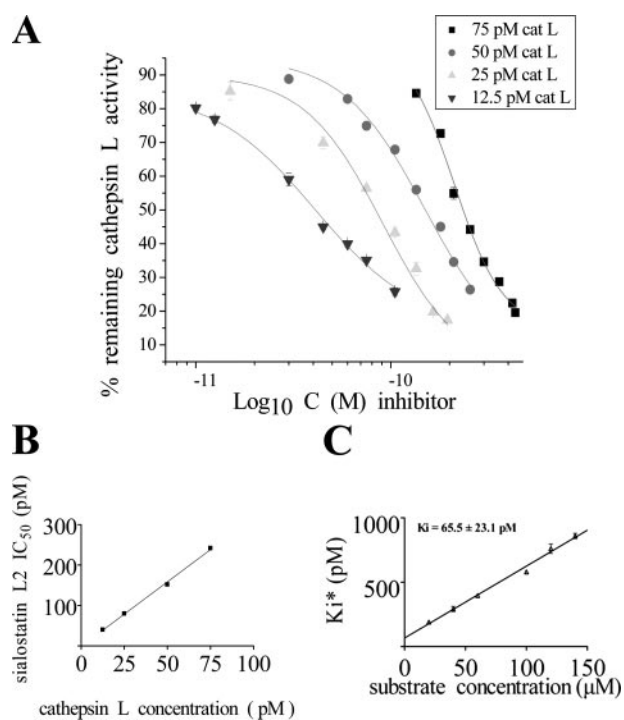


FIGURE 2. **Sialostatin L2 is a tight binding inhibitor for cathepsin L.** *A*, lower inhibitor concentration is necessary for the same percentage of cathepsin L inhibition to be achieved, as the concentration of the enzyme used in the assays decreases from 75 to 12.5 pM. Each experiment was performed in triplicate. The *abscissa* represents sialostatin L2 concentration (*M*) in $\log_{10}$ scale, and the *ordinate* represents the percentage of remaining cathepsin L activity in the presence of sialostatin L2. *B*, the reduction in sialostatin L2 concentration at which 50% inhibition of cathepsin L activity is achieved ($IC_{50}$) is analogous to the reduction of cathepsin L concentration used in the assay. The *abscissa* represents ($IC_{50}$) $\pm$ S.E. of triplicates, and the *ordinate* represents cathepsin L concentration. *C*, relationship of the apparent dissociation constant $K_i^*$ to substrate concentration when reactions were initiated by the addition of cathepsin L. Values for $K_i^*$ were calculated as described under "Results." Linear regression of the data yields a $K_i$ of 65.5 $\pm$ 23.1 pM ($r^2 = 0.992$). Each point in the graph is the mean $K_i^* \pm$ S.E. of four independent experiments.

*Sialostatin L2 Transcription Increases as Feeding to the Host Progresses*—To shed light on the transcriptional control of the two genes during *I. scapularis* feeding on the vertebrate host, we employed real-time quantitative RT-PCR using RNA isolated from unfed or partially fed adult female tick salivary glands or midguts. Expression levels were first normalized using the constitutively expressed actin transcript as a standard (14). Similar accumulation of sialostatin
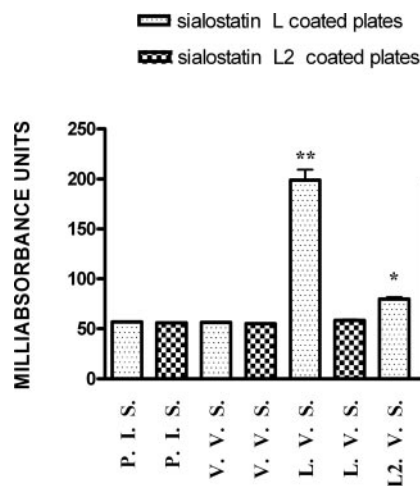


FIGURE 3. **The two proteins differ in antigenicity.** Female Swiss Webster mice 6–8 weeks old were vaccinated as described under "Experimental Procedures." Plates (96 well) were coated with either sialostatin L or L2 followed by enzyme-linked immunosorbent assays using preimmune sera (*P.I.S.*), sera from vehicle-vaccinated mice (*V.V.S.*), sera from sialostatin L-vaccinated mice (*L.V.S.*), or sera from sialostatin L2-vaccinated mice (*L2.V.S.*). Each group consisted of six mice. The *ordinate* shows mean milliabsorbance units of the enzyme-linked immunosorbent assays read ($\lambda = 405$ nM) for each sample serum $\pm$ S.E. **, statistically significant difference ($p < 0.001$); *, statistically significant difference ($p < 0.05$) in the absorbance read when corresponding sera were tested.

**TABLE 2**

**Transcriptional regulation of sialostatins L and L2 in the midgut and salivary glands during the onset of tick blood feeding**

The table shows the difference in accumulation of transcripts for the two tick cystatins both in the midgut and in the salivary glands as feeding continues and when compared with the corresponding transcript abundance in tissues from unfed ticks. Similar levels of sialostatin L transcripts were revealed in unfed salivary glands and midguts, 80 and 20 times higher when compared with those of sialostatin L2 in the corresponding tissues.

| Feeding period | Sialostatin L2) | | Sialostatin L | |
|---|---|---|---|---|
| | Midguts | Salivary glands | Midguts | Salivary glands |
| | *-fold difference* | | *-fold difference* | |
| Unfed | 1 | 1 | 1 | 1 |
| 24 h | 1.3 | 29 | 0.3 | 0.3 |
| 48 h | 1.2 | 273 | 0.1 | 0.1 |
| 72 h | 0.3 | 232 | 0.1 | 0.2 |
| 96 h | 1.3 | 940 | 0.03 | 0.1 |

L transcripts was revealed in unfed salivary glands and midguts, which were 80 and 20 times higher when compared with sialostatin L2 expression levels in the corresponding tissues. Furthermore, the difference in transcript abundance for the two tick cystatins, both in the midgut and in the salivary glands, as feeding continues and when compared with the corresponding transcript abundance in tissues from unfed ticks was estimated, and it is presented in Table 2. Briefly, as feeding starts, sialostatin L transcript levels decrease in both the midgut and salivary glands. On the other hand, sialostatin L2 transcripts slightly fluctuate in the midgut but drastically accumulate in salivary glands. Our bioinformatic approach uncovered that the 600-bp of the 5'-untranslated region of the two genes do not show any similarity when compared with BLASTN (data not shown), indicating that the differences in the transcription regulation of the two genes can be partially or fully attributed to their different 5'-untranslated region nucleotide sequences.
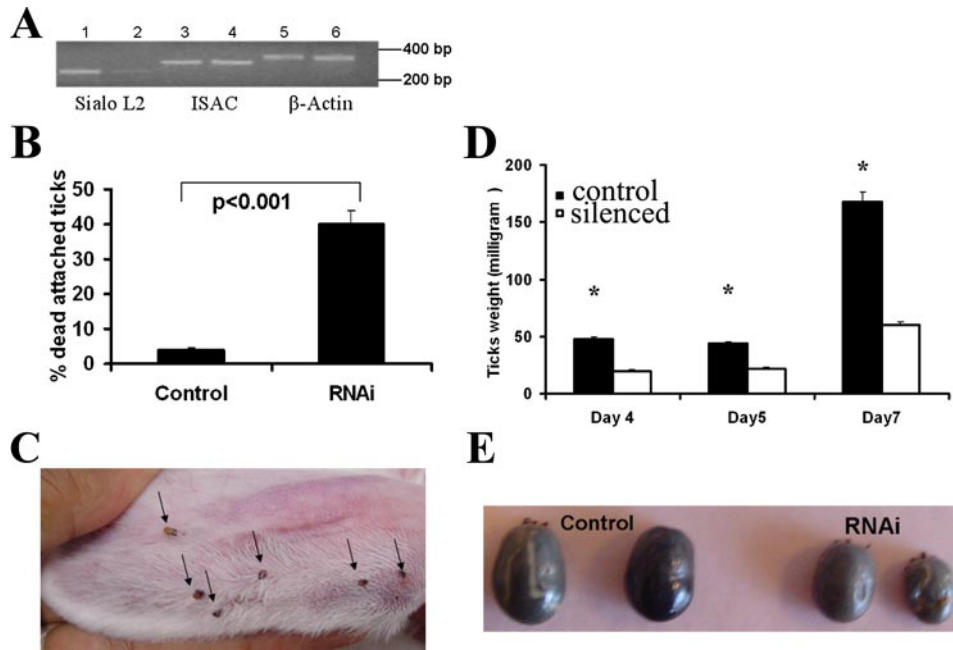
FIGURE 4. ***In vivo* sialostatin L2 RNAi in the salivary glands of *I. scapularis.*** *A*, RT-PCR with total RNA prepared from water-injected control salivary glands (*lanes 1, 3,* and *5,* respectively) or sialostatin L2 RNAi salivary glands (*lanes 2, 4,* and *6,* respectively) using sialostatin L2 (*lanes 1* and *2*), Isac (*lanes 3* and *4*), and β-actin (*lanes 5* and *6*) gene-specific primers for transcript amplification. *B*, sialostatin L2 RNAi ticks were unable to feed successfully; three experiments were performed on different dates during the active adult tick feeding period using different batches of ticks and New Zealand rabbits. Each experiment was carried out with water-injected control (*n* = 50) and sialostatin L2 dsRNA-injected (*n* = 50) ticks. *C*, the percentage of feeding inhibition was calculated by counting dead ticks attached to rabbit ear (*arrows*) during the first 24–48 h of infestation. *D*, partially fed female adult ticks were pulled from the rabbit on days 4, 5, and 7 (*n* = 10) and weighed during each experiment. The *ordinate* shows the average tick weight (in mg) of three replicate experiments; *, statistically significant difference (*p* < 0.05). *E*, fully engorged female adult ticks, representing the control and the experimental group that dropped off and were kept for egg mass recovery.
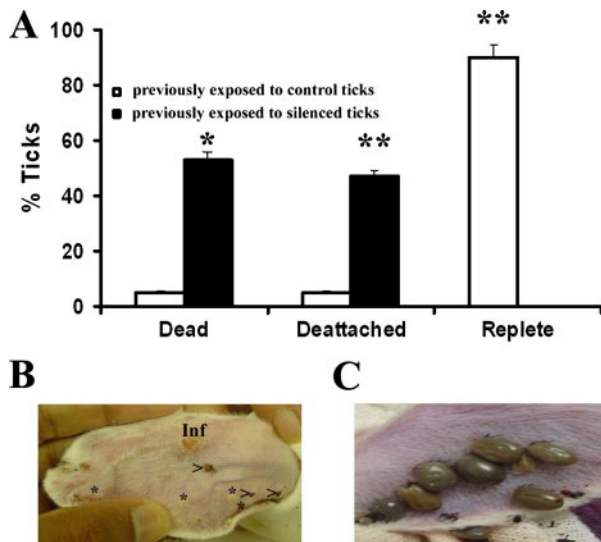


FIGURE 5. **Immunomodulatory role of sialostatin L2 during tick feeding on vertebrate host.** *A*, naïve adult female ticks (*n* = 50) were allowed to feed on rabbits previously exposed to sialostatin L2 RNAi and water-injected control ticks. Each experiment was carried out three times, and the percentage of dead, de-attached, or fed-to-repletion ticks was calculated for experimental and control groups; **, statistically significant difference (*p* < 0.001); *, statistically significant difference (*p* < 0.05). *B*, naïve female adult ticks that attached but died in the first 24 h of infestation in rabbits previously exposed to sialostatin L2 RNAi ticks. In this characteristic photo from the ear of such a rabbit. *Asterisks* indicate swollen skin; *arrowheads* point to dead attached ticks with *Inf* indicating the site of a profound inflammation. *C*, photo of naïve female adult ticks that managed to feed to repletion when attached to rabbits previously infested with water-injected control ticks.

*Sialostatin L2 Is Essential for Tick Blood Feeding Success*—Given this transcriptional induction of sialostatin L2 in tick salivary glands as feeding progresses, we decided to silence the gene using the RNAi technique. Adult unfed female ticks were injected with sialostatin L2 dsRNA and subsequently allowed to recover from the injections and feed on rabbits as described under "Experimental Procedures." Groups of 12 ticks each were pulled from the rabbit after 4 days of feeding, and their salivary glands were dissected and subsequently checked for gene silencing efficiency by RT-PCR. As shown in Fig. 4*A*, ticks injected with sialostatin L2 dsRNA showed an ~80% decrease in sialostatin L2 transcript levels when compared with water-injected controls. Moreover, sialostatin L was completely silenced (data not shown), whereas levels of β-actin and Isac (negative controls) remained unchanged in both experimental and control groups. When attached to a rabbit *in vivo*, ~40% of the silenced ticks were unable to feed and subsequently died (Fig. 4*B*), whereas in most cases apparent inflammatory and swollen skin was revealed in the feeding sites of dead ticks (Fig. 4*C*). For the remaining ~60% of RNAi ticks that fed on the host to repletion, their average weight approximated 60 mg, much lower than the control average weight of 170 mg (Fig. 4, *D* and *E*). Additionally, they showed ~70% egg-laying inhibition and became "stone hard" after detachment from the host (data not shown).

*The Phenotype of Silenced Ticks Can Be Attributed to Enhanced Immune Reaction from the Vertebrate Host*—Rabbits exposed multiple times to ticks eventually develop a strong anti-tick immunity (15). We hypothesized that the signs of inflammation in feeding sites of dead ticks treated with cystatin dsRNA could indicate an accelerated immune response to tick salivary proteins because sialostatins are absent or decreased. Therefore, rabbits exposed to control and silenced ticks were kept and exposed to wild type (normal) adult female ticks 2 weeks after the first infestation. As shown in Fig. 5*A*, when ticks were attached to rabbits previously exposed to RNAi-treated ticks, they fed poorly and were unable to engorge, whereas a severe skin reaction could be seen at the tick attachment site (Fig. 5*B*). In contrast, when adult female ticks were attached to rabbits previously exposed to water-injected control ticks, they managed to feed and engorge (Fig. 5, *A* and *C*), although less efficiently (data not shown) than when attached on naïve rabbits (never exposed to ticks), in agreement with a previous report (15).

## DISCUSSION

In this report we identify two different loci in the tick genome encoding for two *I. scapularis* cystatin transcripts. Given the high similarity of the corresponding transcripts (nucleotide identity in the region 124–388 reaches 86%), it would be almost impossible to show that they are encoded in separate genes in the absence of released genomic sequences. On the other hand, if EST sequences were not available, it would be equally difficult to assemble the corresponding genomic sequence into contigs due to the high number of repetitive sequences in the intronic regions. This is not the first demonstration of the value of a cross-talk between genome and ESTs, but taking into account our difficulties in assembling the intronic regions of the cystatin genes, we propose that all available EST sequences could be valuable additional scaffolds in the assembly of *I. scapularis* genome as long as the repetitive sequences remain at high levels.

Members of the cystatin superfamily have been isolated from tissues of animals and plants and a variety of microbes. They can be subdivided into three groups (16); family 1 cystatins (also known as stefins) are cytoplasmic and lack disulfide bonds, whereas family 2 cystatins are secreted and bear two disulfide bonds. Members of both groups display low molecular mass (roughly 11–14 kDa) in contrast to the family 3 members (also known as kininogens) that are much larger molecules made of multiple cystatin modules. Structural studies of various cystatins show that they display a wedge-shaped interface that binds to the active site of their target proteases (17). This interface consists of three typical segments (18) (*red* in Fig. 1*A*); they are the amino-terminal domain located around a conserved G (PI segment), a hairpin loop located around the conserved sequence Q*X*V*X*G (PII segment), and a second hairpin loop located around a conserved PW dipeptide (PIII segment). We have previously shown that secreted cystatins from ticks are divergent in their aa sequence from the other family 2 members from animals and lower eukaryotes (9). Now we further show that both *I. scapularis* salivary cystatins lack the two PW residues in the PIII segment that are instead substituted with a conserved NL dipeptide. Single aa substitutions in the PW dipeptide have been shown to reduce cystatin affinity for cathepsins B and H (19). It is possible that sialostatins L and L2 recruited those two aa substitutions for *I. scapularis* to get rid of a potentially undesirable or unnecessary inhibitory activity of their salivary cystatins against vertebrate cathepsins B and H, which can diverge these salivary proteins for their target selectivity.

Of interest, a recent paper (20) describes two secreted cystatins from the soft tick *Ornithodoros moubata.* Soft ticks feed rapidly, so their cystatins are shown in the same paper to play a role in midgut physiology rather than in salivary glands. Both soft tick cystatins display the PW motif in their PIII segment and inhibit cathepsins B and H. The same holds true for a secreted cystatin from the hard tick *Haemaphysalis longicornis* that plays a role in tick midgut physiology/innate immunity (21) but not in salivary glands. Although it is difficult to be conclusive as there are several other aa differences throughout those proteins, another salivary cystatin from the hard tick *Amblyo-*

*mma americanum* has the NL substitution in PIII segment, and RNAi-silenced ticks displayed reduced ability to feed successfully in rabbits (10). Biochemical characterization of this protein and the transcriptional regulation of the gene are both still lacking, but it is tempting to speculate that divergence of the sequence in the PIII segment of salivary hard tick cystatins and the resulting lack of inhibition for cathepsins B and H is a major contributor to the conserved role of those molecules in hard tick feeding success in the vertebrate host.

Identity of the two cystatins at the aa level suggests that the corresponding genes resulted from a relatively recent duplication event. The question arises of why such an event was fixed in the genome. Both inhibitors target the same proteases, namely cathepsins L, V, S, and C, but on the other hand, they differ in their affinity for cathepsins S and C. Additionally, antisera produced against the two proteins were not completely cross-reactive. Furthermore, we uncovered very large differences in their transcriptional regulation; sialostatin L2 transcripts rapidly and constantly accumulate as feeding progresses. Given this transcriptional induction of the sialostatin L2 gene, there is possibly enhancement of the inhibitory activity of saliva against cathepsins L, V, C, and S as feeding to the host continues, assuming that transcript accumulation will result in a corresponding increase of sialostatin L2 secretion from the salivary glands.

Ticks can be considered clever pharmacologists (22) because adaptation to their natural vertebrate hosts has sculptured their saliva composition in such a way that the amount of each salivary constituent is sufficient to counteract any host action that would lead to tick rejection. What could be the reason for salivary cystatin target specificity? Cathepsins V, L, and S are efficient elastinolytic endopeptidases identified as secreted by macrophages during the onset of inflammation (23) and as major contributors to tissue damage under chronic inflammatory conditions (24). Elastic fibers are the key extracellular matrix components conferring elasticity to tissues such as blood vessels and skin. In the absence of salivary cystatins, proteolytic degradation of elastic fibers, resulting from the release of cathepsins in the initial steps of tick infestation, would destroy tissue elasticity and lead to high risk for maintaining the tick feeding cavity. This is the phenotype of the RNAi ticks; immediate rejection or failure to successfully accomplish a blood meal. This phenotype can be further explained from extensive work on the role of cathepsins L and S in antigen presentation/immunity (25, 26). The absence of immunosuppressive action of cystatins during the first infestation (the genes were knocked down by RNAi) led to a much stronger primary immune response from the vertebrate host, as shown by the increase in the number of dead ticks and the signs of inflammation in their attachment sites. Subsequent boost of the same animal with a second tick infestation had detrimental consequences for tick feeding, as shown by almost immediate tick rejection and stronger inflammatory responses in the sites of infestation.

Previous work has shown the importance of anticoagulation in *I. scapularis* feeding success using an RNA interference approach (27). In this study, in addition to confirming the value

of the technique in gene function analysis in this non-model organism, we combine bioinformatics/genomics, biochemistry, and molecular biology to shed light on the mechanism of action of another key mediator in the tick strategy to access the bloodstream for a long time without triggering host reactions; saliva cystatins target a limited number of vertebrate cysteine proteases that possess a pivotal role in vertebrate immunity. Therefore, they should be considered in the process of developing antiparasitic vaccines using a mixture of vector antigens. But before attempting an antibody-mediated inhibition of their detrimental effect to the host, we may first need to resolve their structure and reveal the essential aa for their interaction with the target proteases.

In the era of the human genome, it is now clear that the group of human papain-like cysteine proteases numbers 11 members (26). Our current study presents the most complete (to our knowledge) analysis of cystatins *vis à vis* their target specificity, making them useful tools in the study of their target enzymes in various biologic phenomena. Moreover, extensive work involving transgenic mice that lack the corresponding gene(s) has shown the implication of cathepsins L and S under various pathologic conditions including atherosclerosis and cancer (28, 29). It is the unique and stringent specificity of sialostatins L and L2 that could potentially provide a solid basis for future pharmaceutical applications against those diseases.

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**Selective Cysteine Protease Inhibition Contributes to Blood-feeding Success of the Tick _Ixodes scapularis_**

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