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# Antiinflammatory and Immunosuppressive Activity of Sialostatin L, a Salivary Cystatin from the Tick *Ixodes scapularis*\*

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Here we report the ability of the tick *Ixodes scapularis*, the main vector of Lyme disease in the United States, to actively and specifically affect the host proteolytic activity in the sites of infestation through the release of a cystatin constituent of its saliva. The cystatin presence in the saliva was verified both biochemically and immunologically. We named the protein sialostatin L because of its inhibitory action against cathepsin L. We also show that the proteases it targets, although limited in number, have a prominent role in the proteolytic cascades that take place in the extracellular and intracellular environment. As a result, sialostatin L displays an antiinflammatory role and inhibits proliferation of cytotoxic T lymphocytes. Beyond unraveling another component accounting for the properties of tick saliva, contributing to feeding success and pathogen transmission, we describe a novel tool for studying the role of papain-like proteases in diverse biologic phenomena and a protein with numerous potential pharmaceutical applications.

Access to a nutritious blood meal has been gained independently numerous times and in various families and orders during invertebrate evolution (1). In contrast to other successful arthropod bloodsuckers, which feed rapidly, hard ticks feed on their hosts for several days (2). As a consequence, they have evolved a series of strategies to circumvent host defenses during their prolonged meal. Among them, the shedding of saliva in the sites of bite(s) possesses a critical role (3). Tick saliva contains a broad repertoire of potent pharmacologic molecules with vasoactive, antihemostatic, antiinflammatory, and immunomodulatory action (4). A serious side effect of this saliva action in sites of infestation is facilitation of the transmission of tick-borne pathogens (5).

Previous sialotranscriptome work shed light on the salivary complexity of the hard tick *Ixodes scapularis* (6). Large numbers of protease inhibitors were found to be expressed in the tick salivary glands, including a potentially secreted peptide

with mature molecular mass of 12.5 kDa containing the conserved cystatin domain. Cystatins are natural tight binding reversible inhibitors of papain-like cysteine proteases that, in turn, have traditionally been considered as mediators of the terminal bulk proteolysis inside the lysosome. As a result, the vertebrate cystatins have been the focus of extensive research as the guardians or regulators that ensure protection of cells and tissues against the undesirable scission of peptide bonds and damage that could be caused when cysteine proteases are released outside their normal compartment.

Within the last decade, a series of elegant studies changed the limited housekeeping view about cysteine proteases and showed their much more expanded and specific role in certain aspects of vertebrate biology (7). Besides their implication in antigen presentation (8) and immune system development (9), they are also involved in epidermal homeostasis (10), neovascularization (11), extracellular matrix degradation and neutrophil chemotaxis during inflammation (12, 13), apoptosis (14), and (last but not least) proliferation of malignant cells and their subsequent invasion into healthy tissues during metastasis (15, 16).

Since the first description of chicken egg white cystatin in the late 1960s (17), a body of information has been accumulated for this superfamily of proteins present in vertebrates, invertebrates, plants, and protozoa. Cystatins are further subdivided into three individual families, namely 1, 2, and 3. Family 1 members (also known as stefins) are cytosolic molecules with neither disulfide bonds nor carbohydrates. Family 2 contains all of the secreted cystatins that are mainly found in biologic fluids; they form two disulfide bridges, and they do not bear sugars. In contrast to the members of the previous two families, which possess a single cystatin-like domain and display low molecular mass (11–14 kDa), each family 3 cystatin (also known as kinnogens) is made of several cystatin modules, thus being relatively larger molecules (60–120 kDa) (18).

Secreted cystatins have been found in various nonvertebrate organisms, with those expressed by nematode parasites already shown to play a role of cardinal importance in evasion of the host defense system and in modulation of the immune response (19). Although members of the cystatin family are present in ectoparasites, too, including ticks, little is known of their specificity or function. A recent study showed that a cystatin is important for the feeding success of the tick *Amblyomma*

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*americanum*, but the target enzymes and the mechanism of action remain still unknown (20).

Here we describe for the first time expression of an active family 2 cystatin found in the saliva of *I. scapularis*. The sequence divergence of the protein, compared with that of other members of the same family, results in unique and novel target specificity directed among the others against cathepsin L, so that we named the protein sialostatin L. We further show that tick saliva displays an anti-cathepsin L activity that can be attributed to its cystatin component. Finally, we demonstrate that sialostatin L has an antiinflammatory action and reduces the proliferation of CTL. Because this novel inhibitor can be expressed in relatively high amounts in bacteria, many applications of medical importance based on its relatively stringent specificity can be tested in the future.

## EXPERIMENTAL PROCEDURES

Unless otherwise indicated, the protocols followed standard procedures (21), and all of the experiments were performed at room temperature ( $25 \pm 1^\circ\text{C}$ ). All water used was of 18-megaohm quality, produced by a MilliQ apparatus (Millipore Corp., Bedford, MA).

**Bioinformatic Tools**—BLAST searches (22) were used to identify family 2 cystatin genes in different organisms based on their sequence similarity with previously characterized family members. Multiple sequence alignments were done using the ClustalW (23) of the ClustalW Service at the European Bioinformatics Institute (available on the World Wide Web at [www2.ebi.ac.uk/clustalw](http://www2.ebi.ac.uk/clustalw)), and the phylogenetic dendrograms were visualized with the TreeView software (24). To obtain a list of cystatin sequences, we used three iterations of PSIBLAST with an *E* value cut-off of 0.01, retrieved all matches from the nonredundant protein data base, and selected only those sequences that started with a Met and had fewer than 220 amino acids (aa)<sup>2</sup> and more than 2 cysteines. Next, this subset was submitted to the SignalP server (25), and, when existent, the signal peptide was removed. This final set was used as input to the ClustalX analysis.

**Expression, Purification, and Sequence Verification of Sialostatin L**—PCR was performed on the sialostatin L cDNA (NCBI accession number gi|22164282) originating from a  $\lambda$ TriplEx2 cDNA clone, described in our previous work (6). Primers (forward, 5'-GCCCATATGACTGGTGTTCGGTGGCTACAGCGAGAGG-3'; reverse, 5'-GCCCTCGAGCTATGCGGCTTCACACTCGAAGGGGCTGAC-3') were designed to remove the signal sequence (see above), insert an ATG codon directly upstream of the first codon of the mature peptide sequence, and insert NdeI and XhoI restriction sites that allowed cloning into the pET17b bacterial expression vector. The expression vector was moved into *Escherichia coli* strain BL21(DE3)pLysS for expression. Cultures (1 liter) were inoculated and grown to an OD of 0.6–1.0 and induced by adding isopropyl 1-thio- $\beta$ -D-galactopyranoside to a concentration of 1 mM. After 3 h, the cultures were harvested, and inclusion bodies

were prepared using previously described methods (26). The inclusion bodies were dissolved in 6 M guanidine hydrochloride, 20 mM Tris-HCl, pH 8.0, and reduced with 10 mM dithiothreitol. Sialostatin L was refolded in a large volume of 20 mM Tris HCl, pH 8.0, 300 mM NaCl, and stirred overnight at 4 °C. The refolded protein was concentrated with a tangential flow filtration device and purified by gel filtration chromatography on Sephacryl S-100 followed by anion exchange chromatography on Q-Sepharose. Dialysis followed against 20 mM Tris-HCl, pH 7.4, 150 mM NaCl. Potential lipopolysaccharide contamination was removed using the END-X®B15 endotoxin affinity resin (Associates of Cape Cod Inc., East Falmouth, MA). Final protein concentration was determined by measuring protein absorbance at 280 nm. Before use, protein integrity was verified by mass spectrometry.

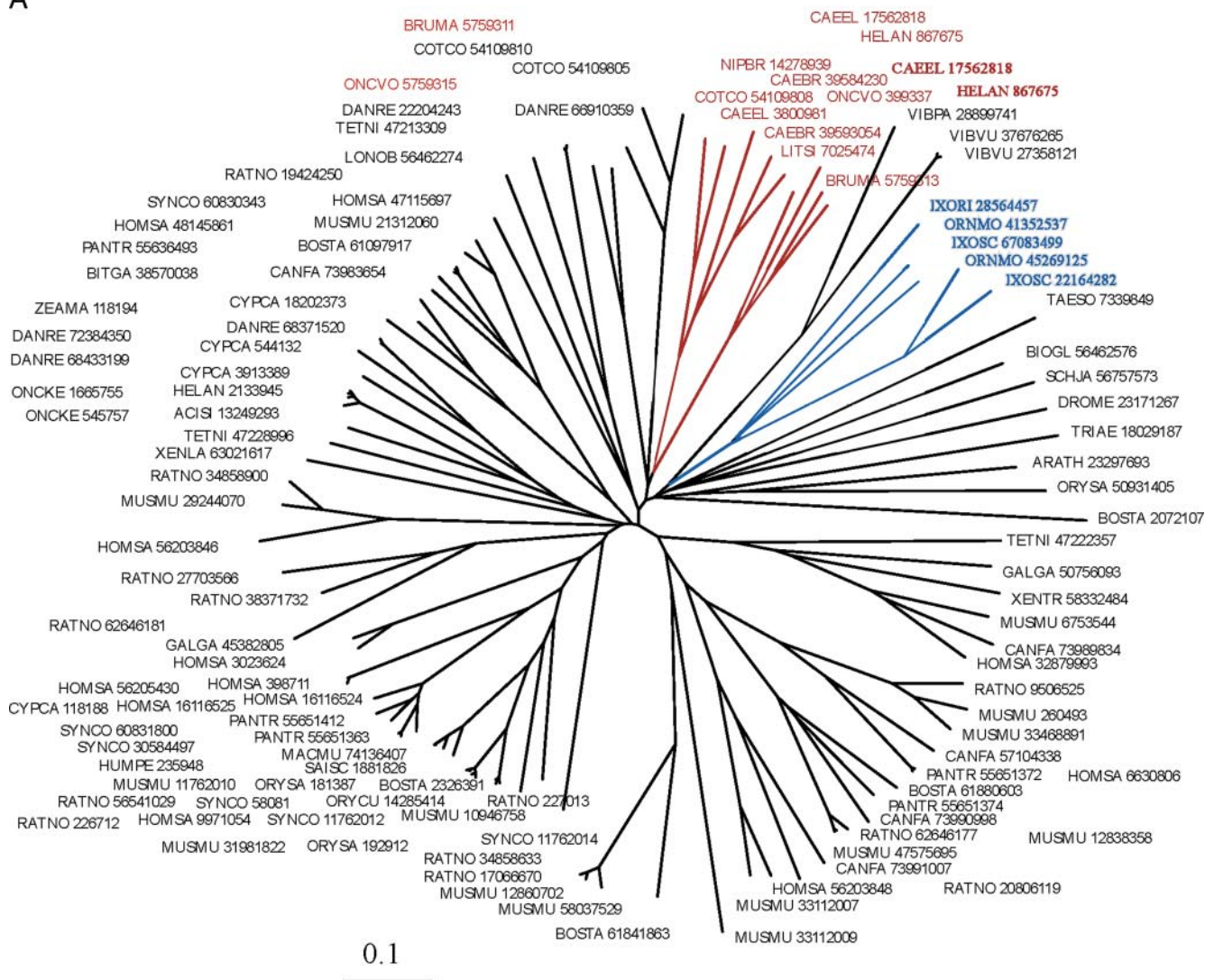
**Enzymatic Assays**—Apparent inhibition constants of sialostatin L for various proteases were obtained as described earlier (27, 28) by measuring the loss of enzymatic activity at increasing concentrations of inhibitor in the presence of a fluorogenic or chromogenic enzyme substrate in large excess. The purified enzymes tested against sialostatin L were as follows: human cathepsin H, S, and L; calpain; recombinant human cathepsin V, X/Z/P, and E; legumain; recombinant mouse cathepsin C (all purchased from Calbiochem); papain; human cathepsin D; G, elastase; bovine cathepsin B; recombinant human caspase 3 (all purchased from Sigma); and bovine trypsin (Roche Applied Science). Calculation of the final molar concentration of the enzymes used during the assays was done as described previously (29).

Assay buffers used were as follows: 100 mM NaAc, pH 5.5, 100 mM NaCl, 1 mM EDTA, 1 mg/ml cysteine, and 0.005% Triton X-100 for cathepsin B, C, H, L, and S and papain; 25 mM NaAc, pH 5.5, 100 mM NaCl, and 5 mM dithiothreitol for cathepsin V; 25 mM NaAc, pH 4.0, 1 mg/ml cysteine, and 0.01% Triton X-100 for cathepsin X/Z/P; 50 mM NaAc, pH 4.0, 100 mM NaCl, and 0.01% Triton X-100 for cathepsin D and E; 20 mM Tris, pH 7.5, 100 mM NaCl, 0.4 mM CaCl<sub>2</sub>, 1 mg/ml cysteine, and 0.01% Triton X-100 for calpain; 100 mM Hepes, pH 7.5, for cathepsin G; 50 mM NaAc, pH 5.0, and 100 mM NaCl for human legumain; 50 mM Hepes, pH 7.4, 100 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, and 0.1% CHAPS for caspase 3; and 100 mM Tris, pH 8.0, and 0.01% Triton X-100 for elastase and trypsin.

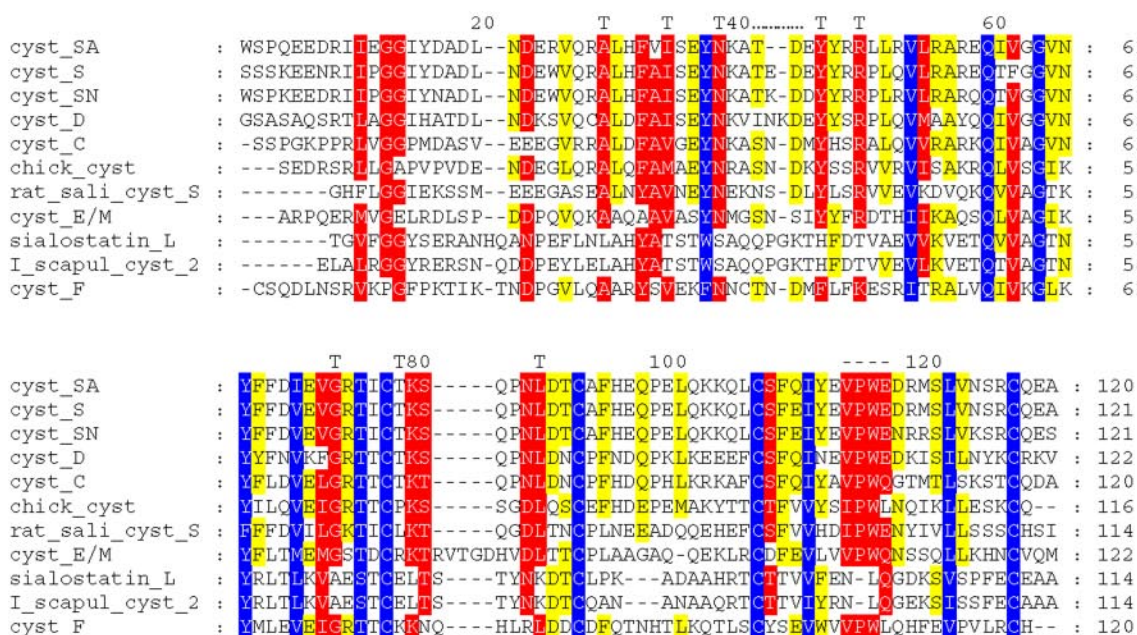
Substrates, used in a 0.25 mM final concentration, were purchased from Calbiochem: *N*-carbobenzoyloxy-Arg-Arg-7-amino-4-methylcoumarin for cathepsin B, L, and papain; H-Arg-7-amino-4-methylcoumarin for cathepsin H; and *N*-succinyl-Leu-Tyr-7-amino-4-methylcoumarin for calpain. The following were from R & D Systems (Minneapolis, MN): (7-methoxycoumarinyl)acetyl-Arg-Pro-Lys-Pro-Val-Glu-norvaline-Trp-Arg-Lys(2,4-dinitro-phenyl)-NH<sub>2</sub> for cathepsin S; *N*-carbobenzoyloxy-Leu-Arg-7-amino-4-methylcoumarin for cathepsin V, L, and C; (7-methoxycoumarinyl)acetyl-Arg-Pro-Pro-Gly-Phe-Ser-Ala-Phe-Lys(2,4-dinitrophenyl)-OH for cathepsin X/Z/P; and (7-methoxycoumarinyl)acetyl-Pro-Leu-Gly-Leu-*N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl-Ala-Arg-NH<sub>2</sub> for cathepsin D and E. The following were from Bachem Bioscience (King of Prussia, PA): *N*-carbobenzoyloxy-Val-Val-Arg-7-amino-4-methylcoumarin for cathepsin S; *N*-acetyl-Ile-Glu-Thr-Asp-7-amino-4-methylcou-

<sup>2</sup> The abbreviations used are: aa, amino acid(s); CTL, cytotoxic T lymphocytes; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

A



B



marin for caspase 3, and *N*-carbobenzyloxy-Ala-Ala-Asn-7-amino-4-methylcoumarin for human legumain. The following were from Sigma: *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide for cathepsin G; *N*-succinyl-Ala-Ala-7-amino-4-methylcoumarin for elastase; and *N*<sub>α</sub>-benzoyl-DL-arginine-4-nitroanilide for trypsin.

**Production of Polyclonal Sera**—DNA vaccination technology was employed for the sialostatin L immunization of Swiss Webster mice as described by Oliveira *et al.* (30).

**Tick Saliva Fractionation**—Tick saliva was obtained by inducing partially engorged adult female *I. scapularis* to salivate into capillary tubes using the modified pilocarpine induction method (31). Microfractionation of tick saliva by reverse-phase high pressure chromatography was achieved with a 0.3 × 150-mm C8 Magic column (Michrom Bioresources, Inc., Auburn, CA) perfused at 2 or 5 μl/min using an ABI 140D pump and 785A UV detector from Applied Biosystems (Foster City, CA). Solution A contained water and 0.1% formic acid, and solution B contained 25% acetonitrile in methanol and 0.1% formic acid. After injecting the sample (previously equilibrated with 10% methanol and containing 0.2% formic acid) into the column, a gradient from 10 to 95% B was imposed for 20 min at a flow rate of 5 μl/min. After this time, the flow rate was decreased to 2 μl/min. Fractions were collected using the Probot machine from Dionex (Sunnyvale, CA) at 1-min intervals on 96-microwell plates containing various solutions as described under "Results."

**Cell Cultures**—Mouse cell line CTLL-2 (CTL) was obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured at 37 °C in 5% CO<sub>2</sub> in a humidified incubator. The culture medium was RPMI 1640 with Glutamax I and 25 mM Hepes buffer (Invitrogen) supplemented with 10% fetal bovine serum (Cambrex BioScience Walkersville, Inc., Walkersville, MD), 4.5 g/liter filter-sterile glucose (Mallinckrodt Chemical, Paris, KY), and 10% rat T-STIM<sup>TM</sup> with concanavalin A (rat IL-2 culture supplement; BD Biosciences). Initial inoculation densities were 1–2 × 10<sup>4</sup> viable cells/ml, and cells were subcultured before reaching 2 × 10<sup>5</sup> cells/ml.

**Cell Proliferation Assays**—The whole procedure was performed at 37 °C. CTLL-2 cells (2.5 × 10<sup>3</sup>/well) were seeded in 96-well polystyrene tissue culture-treated plates (Corning Glass). Sialostatin L was added to the cultures in various final molar concentrations ranging from 1 to 10 μM. As a negative control, cells were cultured in parallel in the presence of 10 μM *Anopheles gambiae* annexin B9, also overexpressed in bacteria (32, 33). Prior to their addition to cell cultures, the proteins were preincubated for 3 h with 100 μg/ml polymyxin B (Sigma) to eliminate the possibility that lipopolysaccharide, potentially present in the recombinant protein preparations, could inter-

fere with the final results of the assay, and polymyxin B was added (in the above mentioned concentration) in the cell culture medium too. Cell proliferation assays were performed 3–4 days post-culture inoculation using the CellTiter 96 aqueous nonradioactive cell proliferation assay kit (Promega, Madison, WI). Briefly, 40 μl of MTS solution was added to each well. MTS solution contains 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium and phenazine methosulfate. It is bioreduced by the cells into a formazan product soluble in tissue culture medium that absorbs at 490 nm. After a 4-h incubation of the cells with MTS, the 490-nm absorbance of the plate was determined using a Versamax microplate reader linked to SOFTmax Pro 3.0 software (Molecular Devices, Sunnyvale, CA). The absorbance displayed by the cells at the time of seeding in the plate (culture inoculation) was considered as zero absorbance. The difference in cell proliferation was additionally monitored by counting their population under the microscope using a Neubauer hemocytometer.

**Paw Edema Assay**—Female C57BL/6 mice, 6–8 weeks old, were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in the NIAID Animal Care Facility (Twinbrook 3 Building) under pathogen-free conditions in temperature-controlled rooms and receiving water and food *ad libitum*. All treatments were performed in accordance with Ref. 64. The carrageenan-induced hind paw inflammation model was used to investigate the potential antiinflammatory role of sialostatin L. Prior to each injection, the basal footpad thickness of each mouse was recorded using a caliper (Mitutoyo America Corp., Aurora, IL). Subsequently, 50 μl of carrageenan (2% in saline) was administered by intraplantar injection in each footpad in the absence or presence of different concentrations of polymyxin B-treated sialostatin L (see above). Control groups of mice received the same volume of saline (vehicle) in the presence or absence of sialostatin L. As an index of edema formation, paw thickness was then measured at 1, 4, and 24 h postinjection.

**Myeloperoxidase Activity Assay**—Tissue myeloperoxidase activity assay was performed as an index of granulocyte recruitment, as previously described (34). Briefly, the injected paws were cut at 4 h post injection (the time point at which edema peaks) and weighed prior to their homogenization in 2 ml of 0.5% hexadecyl-trimethylammonium bromide phosphate-buffered solution (pH 6.0). The homogenates were then centrifuged at 13,000 × *g* for 3 min. Three aliquots of each supernatant were then transferred into 96-well plates before the addition of a solution containing 3,3'-dimethoxybenzidine and 1% hydrogen peroxide. In parallel, dilutions of pure myeloperoxidase (Sigma) were used for the construction of a standard curve (OD as a function of units of enzyme activity). OD read-

**FIGURE 1. Comparison of sialostatin L sequence with those of other known family 2 cystatins.** A, phylogenetic dendrogram of the family 2 cystatins from vertebrates, invertebrates, and plants. *Blue*, members described to date from ticks; *red*, all members described in nematodes. The names of the sequences are composed of five letters (obtained from the first three letters of the genus name and the first two letters of the species) followed by the NCBI accession number. B, multiple-sequence alignment of the two putative secreted cystatins expressed in the salivary glands of *I. scapularis* (sialostatin L and *I. scapularis*\_cyst\_2, NCBI accession numbers 22164282 and 67083499, respectively) with secreted cystatins of vertebrate origin (*cyst\_SA*, *cyst\_S*, *cyst\_SN*, *cyst\_D*, *cyst\_C*, *chick\_cyst*, *rat\_salivary\_cyst\_S*, *cyst\_E/M*, *cyst\_F*, NCBI accession numbers NP\_001313, NP\_001890, NP\_001889, NP\_001891, NP\_000090, P01038, P19313, NP\_001314, and NP\_003641, respectively). Amino acids denoted with a *blue color* are conserved in 100% of the proteins presented in the alignment, those shown with a *red color* are conserved in 80% of them, and those with a *yellow color* are conserved in 60% of them. *T*, aa substitutions revealed specifically in tick cystatins; *dashes*, the PW dipeptide found in most cystatins studied to date (positions 114–115); *dots*, a tripeptide with the sequence SND (or substitutions with chemically similar aa) (positions 41–44) that is conserved in all cystatins that inhibit legumain (cystatins C, E/M, and F).

## A Novel Tick Salivary Cystatin

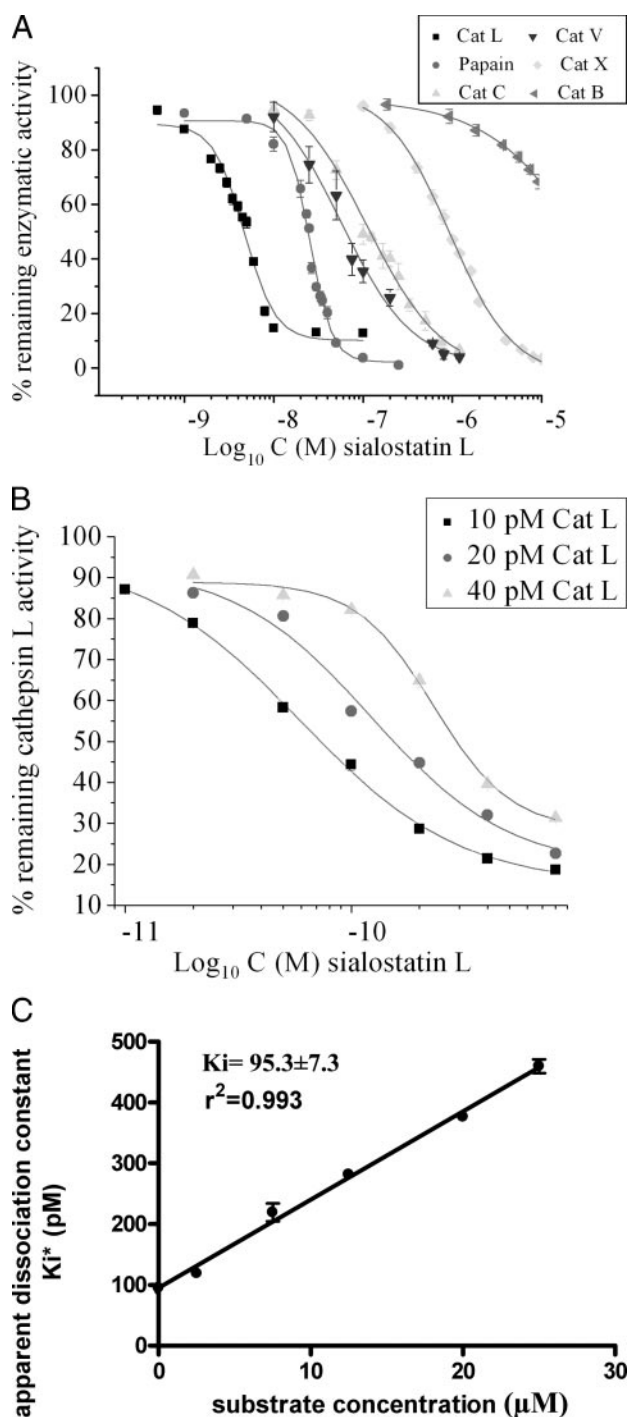
ings at 450 nm were taken at 1 min (time point corresponding to the linear portion of the enzymatic reaction) using a spectrofluorimeter linked to the SOFTmax Pro 3.0 software (Molecular Devices, Sunnyvale, CA). The myeloperoxidase activity detected in the paws was expressed as units of enzyme/g of tissue. A unit of myeloperoxidase activity was defined as that converting 1  $\mu\text{M}$  of hydrogen peroxide to water in 1 min at 22 °C.

**Statistical Analysis**—The statistical differences among the experimental groups in the quantitative experiments were determined by analysis of variance using the Tukey test for multiple comparisons. A *p* value of 0.05 or less was considered to be statistically significant.

## RESULTS

The aa sequence of the cystatin (NCBI accession number 22164282) found to be expressed in the salivary glands of *I. scapularis* (6) displays all of the characteristics of the family 2 cystatins. In addition to the secretion signal and the conserved QXVXG module, two disulfide bonds are formed in proximity to the carboxyl terminus, typical for all secreted cystatins (18). On the other hand, multiple-protein sequence alignment with vertebrate and invertebrate family 2 and plant members shows that all cystatins of tick origin cocluster and are divergent from the already known and extensively studied vertebrate, nematode, and plant homorthologs (Fig. 1A). An aa sequence comparison of the tick cystatin with vertebrate-secreted cystatins (most of them found in saliva also) and an additional potentially secreted cystatin from the salivary glands of *I. scapularis* (NCBI accession number 67083499) is shown in Fig. 1B. It uncovers a series of tick-specific aa substitutions (shown with a *T* above the alignment) and a relative short sialostatin L N terminus. Further, substitution of the PW dipeptide (positions 114–115; shown with *dashes* above of the alignment) is impressive, because it is present in most of the known (to date) cystatins. Previous work of Alvarez-Fernandez *et al.* (35) showed that a tripeptide with the sequence SND (or substitutions with chemically similar aa) is conserved in all cystatins that inhibit legumain (cystatins C, E/M, and F). This tripeptide is also absent from sialostatin L (positions 41–44; shown with *dots* above the alignment). In conclusion, certain aa substitutions revealed in the sialostatin L sequence could potentially account for a novel target repertoire.

Since it is known that family 2 cystatins are not subjected to any kind of post-translational modifications, we decided to overexpress sialostatin L in bacteria. Indeed, the recombinant cystatin was active, inhibiting papain, the “archetype” of family C1 of the clan CA cysteine proteases (29). Within the vertebrate members of the family, cathepsins L, V, C, and X were inhibited, whereas only a slight inhibition (30%) of human cathepsin B was achieved at 10  $\mu\text{M}$  final concentration. The effect of sialostatin L presence in the proteolytic activity of the above mentioned enzymes is presented in Fig. 2A. No inhibition could be detected for other members of the papain-like superfamily (cathepsins H and S; see “Note Added in Proof”), although they show high sequence similarity to cathepsin L. The same holds true for cysteine proteases outside the C1 family (human calpain family C2, human legumain family C13, human caspase 3



**FIGURE 2. Proteolytic enzymes targeted by sialostatin L.** A, papain, cathepsin L, cathepsin V, cathepsin C, and cathepsin X are targeted by the inhibitor. In the case of cathepsin B, only 30% inhibition was detected in the presence of 10  $\mu\text{M}$  sialostatin L. The *abscissa* represents sialostatin L concentration (M) in log<sub>10</sub> scale, and the *ordinate* shows the percentage of remaining enzymatic activity in the presence of sialostatin L. B, lower inhibitor concentration is necessary for the same percentage of cathepsin L inhibition to be achieved, since the concentration of the enzyme used in the assays decreases by 2-fold (from 40 pM to 20 and 10 pM, respectively). Each experiment was performed in triplicate. The *abscissa* represents sialostatin L concentration (M) in log<sub>10</sub> scale, and the *ordinate* represents the percentage of remaining cathepsin L activity in the presence of sialostatin L. 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” (cathepsin L, 50 pM; sialostatin L, 0–800 pM; fluorogenic substrate, 2.5–25  $\mu\text{M}$ ). Linear regression of the data yields a  $K_i$  of  $95.26 \pm 7.26$  pM ( $r = 0.9930$ ). Each point in the graph is the mean  $K_i^* \pm$  S.E. of four independent experiments.

TABLE 1

## Effect of sialostatin L in the activity of various proteolytic enzymes

The enzymatic repertoire tested for inhibition by sialostatin L and the concentration of sialostatin L at which 50% inhibition of the activity of the targeted proteolytic enzymes is achieved ( $IC_{50} \pm S.E.$ ) are presented. The enzyme concentration used in the assays is also given for all the targets of sialostatin L. NI, no inhibition (e.g. inhibition of the enzyme was not observed in the presence of  $10 \mu M$  sialostatin L). In the case of cathepsin B, 30% inhibition was detected at the above mentioned concentration of inhibitor.

Enzyme	Class	$IC_{50} \pm S.E.$	Enzyme concentration	Enzyme	Class	$IC_{50}$
Papain	Cysteine protease	$25.4 \pm 0.51 \text{ nM}$	$1.5 \mu M$	Cathepsin D	Aspartic protease	NI
Cathepsin L	Cysteine protease	$4.68 \pm 0.08 \text{ nM}$	0.7	Cathepsin E	Aspartic protease	NI
Cathepsin V	Cysteine protease	$57 \pm 2.39 \text{ nM}$	0.9	Cathepsin G	Serine protease	NI
Cathepsin C	Cysteine protease	$112 \pm 3.58 \text{ nM}$	25	Legumain	Cysteine protease	NI
Cathepsin X	Cysteine protease	$937 \pm 14.06 \text{ nM}$	16	Calpain	Cysteine protease	NI
Cathepsin B	Cysteine protease	$>10 \mu M$	0.8	Caspase 3	Cysteine protease	NI
Cathepsin H	Cysteine protease	NI		Trypsin	Serine protease	NI
Cathepsin S	Cysteine protease	NI <sup>a</sup>		Elastase	Serine protease	NI

<sup>a</sup> See "Note Added in Proof."

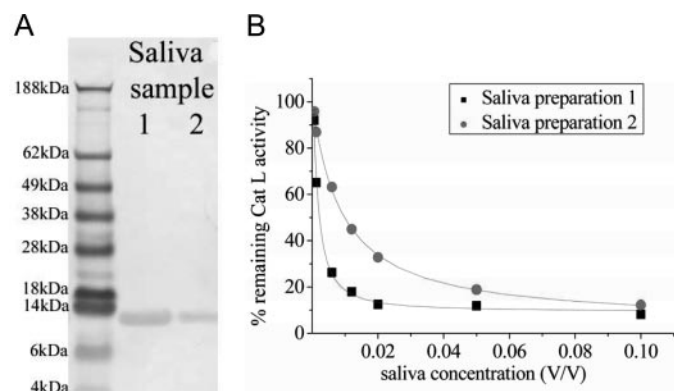


FIGURE 3. Sialostatin L and cathepsin L inhibitory activity is found in two different *I. scapularis* saliva preparations. A, Western blot using anti-sialostatin L mouse polyclonal antibodies against the two saliva preparations. SeeBlue prestained standard (Invitrogen) was used as a molecular weight marker. B, both saliva preparations show anti-cathepsin L activity. The concentration (v/v) of saliva used in the assays is shown in the *abscissa*, whereas the *ordinate* shows the percentage of remaining cathepsin L enzymatic activity in the presence of saliva.

family C14) or cathepsins that belong to the aspartic protease clan AA and the serine protease clan SA (cathepsins D, E, and G). Table 1 presents the complete repertoire of enzymes tested against tick cystatin and their class. For those inhibited by sialostatin L, we further provide the inhibitor concentration necessary to reduce their enzymatic activity by half ( $IC_{50}$ ) as well as the enzyme concentration used in the assays.

All cystatins studied to date form tight equimolar complexes with their preferred targets. A main characteristic of such tightly binding inhibitors is that the  $IC_{50}$  is dependent on the concentration of the enzyme, since the commonly used pseudo-first-order kinetics do not hold under those conditions. This is also true for the inhibition of cathepsin L by sialostatin L. When the concentration of the enzyme used in the reaction reaches picomolar levels, an  $IC_{50}$  of subnanomolar range can be achieved. Fig. 2B shows that as the concentration of the enzyme used in the assay decreases, lower inhibitor concentration is necessary for the same percentage of cathepsin L inhibition to be achieved. A concomitant 2-fold reduction in the calculated  $IC_{50}$  from  $231 \pm 12.29 \text{ pM}$  to  $122.67 \pm 7.77$  and  $59.53 \pm 3.37 \text{ pM}$  is observed, proving that sialostatin L is a tight binding inhibitor that additionally binds stoichiometrically to cathepsin L. We further determined that sialostatin L is a fast binding inhibitor, since the initial velocity of the enzymatic

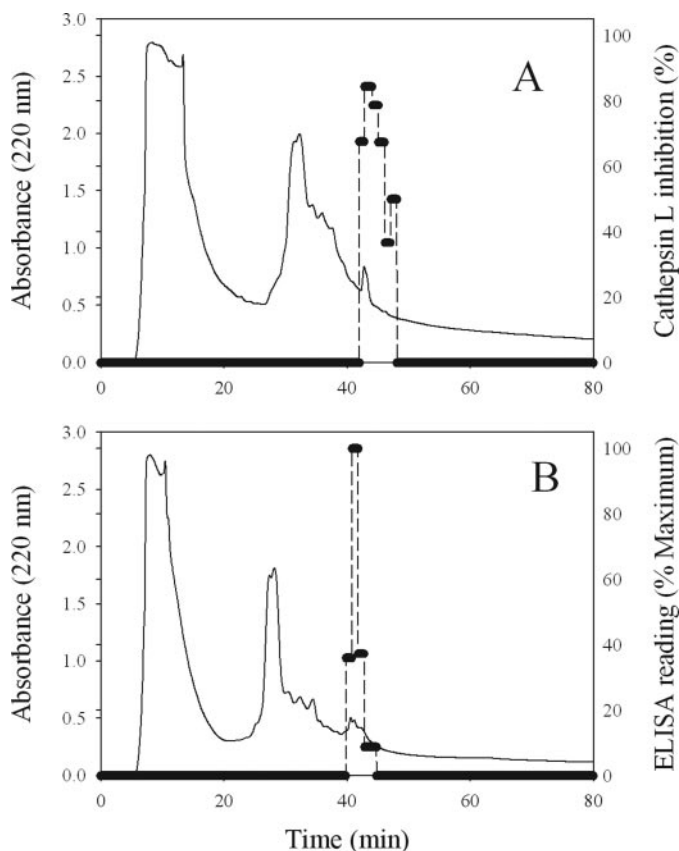
reaction is equally reduced, either when cathepsin L is added to a preincubated mix of inhibitor with substrate or when the substrate is added to a preincubated mix of inhibitor with enzyme (data not shown).

Since conventional Michaelis-Menten kinetics do not apply to the study of tight binding inhibitors, because it assumes that the free inhibitor concentration is equal to the total inhibitor concentration, we applied Morrison's equation for tight binding inhibition (36) to obtain apparent dissociation constants for sialostatin L ( $K_i^*$ ).  $50 \text{ pM}$  of cathepsin L was allowed to interact with increasing sialostatin L concentrations ( $0-800 \text{ pM}$ ) in the presence of varying concentrations of substrate ( $2.5-25 \mu M$ ). When the  $K_i^*$  for several substrate concentrations was plotted against the substrate concentration, a linear regression line ( $r^2 = 0.9930$ ) indicated a *y* intercept of  $95.264 \pm 7.262 \text{ pM}$ , which is the  $K_i$  value for the binding of sialostatin L to cathepsin L (Fig. 2C).

Although it possesses a clear signal for secretion, the presence of sialostatin L in tick saliva was additionally verified by Western blot. The protein content of  $10 \mu l$  of two different saliva preparations was separated by electrophoresis in precast NuPAGE 4–12% bis-Tris gradient gels (Invitrogen) and transferred to a nitrocellulose membrane (Invitrogen). Western blot followed, using mouse anti-sialostatin L polyclonal antibodies. A single band in the expected molecular weight was obtained for both preparations (although fainter for the second preparation) (Fig. 3A). Next, we investigated whether the same saliva preparations bear inhibitory activity against cathepsin L. Fig. 3B presents the effect of tick saliva on cathepsin L activity. A final saliva concentration, ranging from 0.2 to 0.9% (v/v) is sufficient to cause enzymatic activity to drop in half *in vitro*. To clarify whether the anti-cathepsin L activity detected in saliva corresponds partially or fully to the presence of sialostatin L in it, saliva was fractionated by chromatographic means. Fractionation of the same saliva preparation was performed two times. In the first fractionation assay, each saliva fraction was recovered in 96-well plates containing assay buffer to prevent binding of the proteins to the plastic and checked for inhibition of cathepsin L; in the second, each saliva fraction was recovered in 96-well plates containing coating buffer to allow plastic adsorption of the proteins and subsequent sialostatin L detection using mouse anti-sialostatin L polyclonal antibodies in enzyme-linked immunosorbent assays. The polyclonal sera used were verified as non-cross-reactive with the second cystatin found in



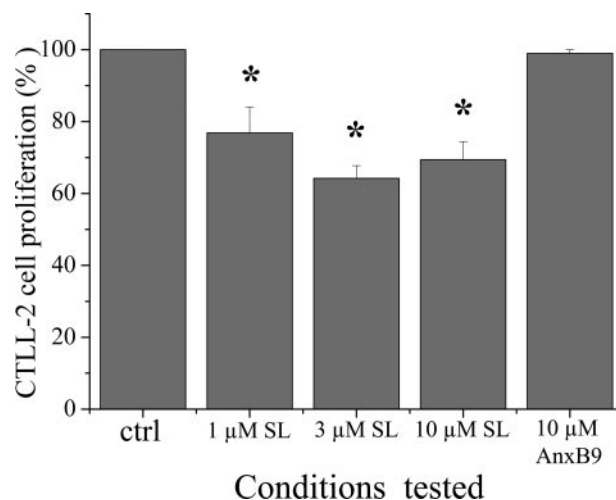
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**FIGURE 4. Fractionation of saliva by chromatography shows that the source of anti-cathepsin L activity and anti-sialostatin L immune reactivity coincide.** *A*, tick saliva (10  $\mu$ l) was fractionated by reverse-phase high pressure liquid chromatography into 1-min fractions collected on 96-well plates containing Triton X-100 to prevent binding of low protein amounts to the plastic, and these fractions were tested for cathepsin L inhibition (percentage of inhibition is shown in the fractionation chromatogram as a *thick black line*). *B*, an additional 10  $\mu$ l of tick saliva was fractionated as above, and each 1-min fraction was collected on 96-well plates containing coating buffer, followed by an enzyme-linked immunosorbent assay using anti-sialostatin L polyclonal antibodies as primary antibodies. The maximum signal detected was considered as 100% reactivity. The percentage of reactivity detected for each saliva fraction is shown in the fractionation chromatogram as a *thick black line*.

the saliva (data not shown). The results are shown in Fig. 4, *A* and *B*, respectively. Both the anti-sialostatin L immune reactivity and the inhibitory activity against cathepsin L describe the same region in the chromatogram, suggesting that sialostatin L contributes to the above mentioned inhibition of cathepsin L by tick saliva.

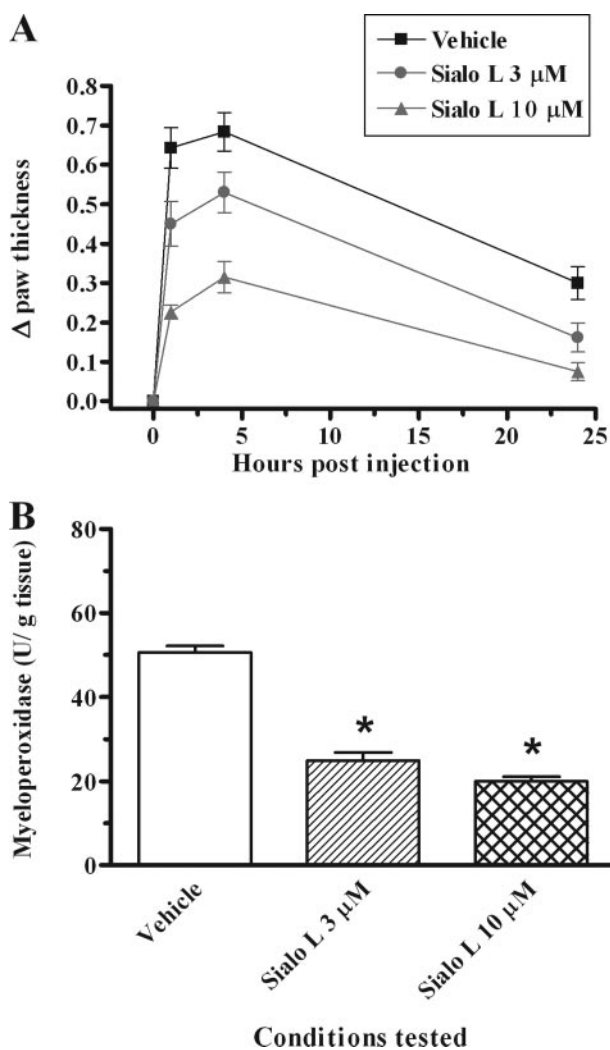
Given the role of papain-like proteases in the function of the immune system (8, 9, 37), we next investigated whether the inhibitory action of sialostatin L can affect the proliferation of CTL. CTLL-2 cells were cultured in the presence of 1, 3, and 10  $\mu$ M concentrations of the inhibitor, and cell proliferation was monitored either by a chromogenic assay or by counting the population of the cells under the microscope. As a negative control, cells were cultured in the presence of recombinant mosquito annexin *ANXB9* (32, 33). All cultures were performed in the presence of polymyxin B to eliminate any potential lipopolysaccharide interference in the final results presented in Fig. 5. A statistically significant ( $p < 0.001$ ) reduction in the proliferation of cells could be detected for



**FIGURE 5. Sialostatin L inhibits the proliferation of a CTL-like cell line.** Cells were cultured in the absence (*bar 1*, numbering *left to right*) or in the presence of 1  $\mu$ M (*bar 2*), 3  $\mu$ M (*bar 3*), or 10  $\mu$ M (*bar 4*) sialostatin L or of 10  $\mu$ M mosquito annexin B9 (negative control, *bar 5*). Cell proliferation in the absence of sialostatin L was considered as 100%; *asterisks* denote a statistically significant decrease in cell proliferation between the cells grown in the presence of sialostatin L ( $p < 0.001$ ) compared with the control (cell proliferation in the absence of sialostatin L). The increase in proliferation between cells grown in the presence of 10  $\mu$ M sialostatin L and those grown in the presence of 3  $\mu$ M sialostatin L is not statistically significant. *ctrl*, control; *SL*, sialostatin L.

all inhibitor concentrations tested, reaching up to 36% in the presence of 3  $\mu$ M sialostatin L in the culture. No effect in cell proliferation could be detected in the presence of recombinant *ANXB9*.

Finally, the role of sialostatin L in acute inflammation was evaluated in a model commonly used to test the antiinflammatory potential of candidate molecules: carrageenan-induced paw edema. As classically described (38), carrageenan induced an edema formation that peaked at 4 h postinjection and slowly decreased but remained significant until 24 h after injection (the end point of the assay). When carrageenan was injected in the presence of sialostatin L, a concentration-dependent inhibition was observed (Fig. 6*A*). In the presence of a 3  $\mu$ M concentration of the inhibitor (final concentration in a total volume of 50  $\mu$ l of injection), the decrease in the edema formation reached 30 and 22% at 1 and 4 h postinjection, respectively, but the above mentioned differences were not statistically significant; however, at 24 h postinjection, carrageenan-induced edema formation was inhibited by 46% ( $p < 0.05$ ). Moreover, in the presence of 10  $\mu$ M sialostatin L, edema formation was inhibited by 65% at 1 h ( $p < 0.001$ ), 54% at 4 h ( $p < 0.001$ ), and 75% at 24 h postinjection ( $p < 0.05$ ). In a further set of experiments, carrageenan-induced recruitment of neutrophils in the footpads was assessed by measuring tissue myeloperoxidase activity (34). Mice received carrageenan injections in the absence or presence of sialostatin L. Myeloperoxidase activity in the tissue was evaluated at 4 h postinjection, the time point at which edema peaks (see above). A statistically significant inhibition ( $p < 0.001$ ) of neutrophil recruitment was observed in the presence of 3 and 10  $\mu$ M sialostatin L (Fig. 6*B*) that reached 51 and 60.6%, respectively. No differences in edema formation or myeloperoxidase activity could be detected when



**FIGURE 6. The antiinflammatory action of sialostatin L.** Mice received carrageenan injections in their footpads either in the absence or in the presence of 3 or 10  $\mu$ M sialostatin L. *A*, edema formation was evaluated at various time points post-injection (*abscissa*) as the increase of paw thickness (in mm). *B*, neutrophil recruitment in the inflamed footpads was evaluated by measuring tissue myeloperoxidase activity, expressed as units of activity/g of tissue (*ordinate*). *Bar 1* (numbering left to right) represents activity detected when carrageenan was administered to the mice, whereas *bars 2 and 3* show the effect of co-administration of 3 and 10  $\mu$ M sialostatin L with carrageenan, respectively. The *asterisks* denote a statistically significant ( $p < 0.001$ ) difference in detected enzymatic activity in the footpads of mice co-injected with carrageenan and sialostatin L compared with the control (mice that received carrageenan injections in the absence of sialostatin L).

bovine serum albumin was co-injected with carrageenan (negative control).

## DISCUSSION

Having in hand the set of mRNA and proteins expressed in the salivary glands of the tick *I. scapularis* (6), we focused on a secreted cystatin-like molecule with a potential function in disrupting tick feeding (20). The recombinant protein inhibited papain-like proteases, targeting mainly cathepsin L and cathepsin C. Sialostatin L also inhibits human cathepsin V (also known as cathepsin L2), but this protein seems to be the more closely related homolog of mouse cathepsin L, shows high similarity with human cathepsin L, and is probably a product of a recent gene duplication of a cathepsin L-like gene in the human

genome (39). Finally, the high concentration of inhibitor necessary to inhibit cathepsin X makes it doubtful whether this inhibition takes place *in vivo*.

Certain aa substitutions in sialostatin L could contribute to its unique specificity when compared with other cystatins. It is almost 20 years after the first publication of an attempt to associate certain cystatin residues with the target specificity of those proteins (40), and since then, a series of elegant studies (41, 42) has shown that at least three highly conserved domains mediate inhibition of papain-like proteases: the N-terminal domain located around a conserved Gly (residue 12 in the alignment of Fig. 1*B*), a first hairpin loop located around the conserved sequence QVVAG (residues 59–63 in the alignment of Fig. 1*B*), and a second hairpin loop located around two conserved PW residues (residues 114–115 in the alignment of Fig. 1*B*). The mechanism of cathepsin B inhibition by cystatin C has also received extensive attention, mainly because cathepsin B differs from the other family members in the vicinity of the active site due to the presence of the so-called occluding loop (43). It has been proposed (44) that the flexible N-terminal region of cystatin C binds like an anchor to the proteinase, thus providing the correct orientation of the inhibitor with respect to the enzyme for displacement of the occluding loop and the subsequent inhibitor binding to occur. On the other hand, a substitution of the Trp residue by Pro or Gly in the second hairpin loop PW dipeptide of cystatin C reduced the affinity of the inhibitor not only for cathepsin B but also for cathepsin H (44), suggesting a universal role of this domain for the inhibition of all papain-like proteases: to keep the inhibitor bound to the enzyme once their complex has been formed. Taking into account all of the above information, we can speculate about the possibility that the short N terminus of sialostatin L and the substitution of the PW motif could potentially contribute to its unique specificity, whereas an additional role can be attributed to tick-specific aa substitutions found throughout the protein. Finally, the absence of the SND motif (35) from the sialostatin L sequence appears to be detrimental for its inhibitory activity against legumain.

Cathepsin L is unique among cathepsins by having an important extracellular function. Up to 40% of the cathepsin L proenzyme from fibroblasts is secreted (45) and shows catalytic activity even in the absence of further maturation processing (46). Cathepsin L is more efficient in the degradation of protein substrates than other members of the same family (47) and is more effective in the hydrolysis of extracellular matrix proteins, such as collagen and elastin, even when compared with collagenase and neutrophilic elastase, which are better known for their activity on these substrates (48, 49).

On the other hand, secreted cystatins seem to have access to intracellular compartments (50). As a result, sialostatin L, besides its stringent specificity, could affect the activity of additional enzymes by blocking proteolytic cascades that take place during the maturation of their proenzymes. More specifically, cathepsin L and S are responsible for the removal of the inhibitory pro-region of procathepsin C (51). In the presence of sialostatin L, cathepsin L activity is inhibited, but it is still possible that the noninhibited cathepsin S can partially (or fully) process procathepsin C (see "Note Added in Proof"). Even if this is the

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case, the mature cathepsin C that is produced will be blocked by the inhibitor. Sialostatin L inhibition of cathepsin C should also prevent the activation of granule serine proteases in CTL and natural killer cells (granzymes A and B), mast cells (tryptase, proteinase 3, and chymase), and neutrophils (cathepsin G and elastase), because the N-terminal dipeptides of their proenzymes would not be removed (52–54). Indeed, it could result in prevention of cathepsin B maturation, since the trimming of the N-terminal extensions of cathepsin B propeptide is no longer possible (55). Additionally, cathepsin L inhibition could affect cathepsin D processing (14). From all of the above, it is feasible that sialostatin L targets two fundamental enzymes controlling the activation of proteolytic cascades in both the extracellular and intracellular compartments. It seems that the tick employs a strategy to inhibit a few (but crucial) proteases with its saliva rather than secreting a repertoire of inhibitors with broad target specificity.

Given the implication of the sialostatin L target enzymes in the function of CTL (see above), we next tested whether the presence of sialostatin L affects their proliferation. A statistically significant reduction in proliferation was detected, in agreement with the results of Thiele *et al.* (56), who similarly showed a reduction in cell proliferation when a selective chemical inhibitor for cathepsin C (Gly-Phe-CHN<sub>2</sub>) was administered in cultures of the same cell line we used, further enhancing the idea of a blockade of cathepsin C activity by sialostatin L. Furthermore, the antiinflammatory action of sialostatin L is not surprising, since it is known that upon onset of inflammation, the balance between cystatins and cysteine proteases changes in favor of proteolysis, mediating extracellular matrix remodeling and neutrophil chemotaxis (12, 13, 57–59). Additionally, cathepsin L, one of the target enzymes, is implicated in kinin generation *in vivo* (60). Administration of sialostatin L in the sites of inflammation changed this equilibrium in favor of antiprotease activity and reduced both edema formation and granulocyte recruitment, as shown by the results of the myeloperoxidase assay.

Cysteine proteases have been associated with a number of pathologic events such as cancer, rheumatoid arthritis, osteoarthritis, Alzheimer disease, multiple sclerosis, and muscular dystrophy (29, 61). Our present work, in addition to revealing a saliva constituent that contributes to its known antiinflammatory and immunosuppressive properties (62, 63), describes a novel inhibitor with a very stringent and unique specificity that can shed more light on the role of cysteine proteases in several biologic phenomena, including, among others, immunity and inflammation, and may be the basis for many applications of medical importance.

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*Note Added in Proof*—Although Table 1 reports no inhibition of cathepsin S (purified from human spleen, 62.5 milliunits/mg, Calbiochem) by sialostatin L, inhibition of cathepsin S by sialostatin L (IC<sub>50</sub> = 0.71 nM) could be detected, when using 60 pM of active recombinant human cathepsin S expressed in *E. coli* (80,000 units/mg, Calbiochem).

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**Antiinflammatory and Immunosuppressive Activity of Sialostatin L, a Salivary Cystatin from the Tick *Ixodes scapularis***

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