Am. J. Trop. Med. Hyg., 66(3), 2002, pp. 225–233

ISOLATION OF *IXODES RICINUS* SALIVARY GLAND mRNA ENCODING FACTORS INDUCED DURING BLOOD FEEDING

GÉRARD LEBOULLE, CANDICE ROCHEZ, JAMILA LOUAHED, BERNARD RUTTI, MICHEL BROSSARD, ALEX BOLLEN, AND EDMOND GODFROID

Department of Applied Genetics, Université Libre de Bruxelles, Brussels, Belgium; Ludwig Institute for Cancer Research, Université Catholique de Louvain, Brussels, Belgium; Institut de Zoologie, Université de Neuchâtel, Neuchâtel, Switzerland

Abstract. In tick salivary glands, genes induced during blood feeding result in the expression of new proteins secreted into tick saliva. These proteins are potentially involved in modulation of vertebrate host immune and hemostatic responses. In this study, subtractive and full-length cDNA libraries were constructed by use of mRNA extracted from salivary glands of unfed and 5-day engorged *Ixodes ricinus.* Sequences from these 2 libraries were compared with European Molecular Biology Laboratory (EMBL)/GenBank databases, which led to their classification into 2 major groups. The first group comprises cDNAs that failed to match or showed low homology to genes of known function. The second group includes sequences that showed high homology to genes of known function-for example, anticoagulants, inhibitors of platelet aggregation, and immunomodulatory proteins. Analyses of corresponding proteins suggest that they may be secreted by salivary gland cells. To study the properties of the recombinant proteins, selected cDNAs were expressed in mammalian or bacterial systems.

INTRODUCTION

Ticks are remarkable blood-sucking arthropods. Three hundred million years of evolution has led to the development of strategies that allows them to interact with a wide diversity of hosts such as mammals (including humans), birds, and reptiles.[1] Although ticks feed on blood, which may take several days, vertebrate host defenses are raised against the ectoparasite with variable efficiency.[2] Ticks can ingest as much as 15 mL of blood.[3] Moreover, they are vectors of numerous human or animal pathogens, including viruses (e.g., tick-borne encephalitis virus, Thogoto virus), bacteria (e.g., *Borrelia burgdorferi sensu lato, Rickettsia spp.*), protozoans (e.g., *Babesia* spp., *Theileria* spp., *Trypanosoma* spp.), and helminths.[4] In view of this diversity of transmitted pathogens, ticks are important pathogen vectors.

Most tick-borne pathogens invade salivary glands before being transmitted to the host via saliva. Substantial evidence demonstrates that ticks are more than simple injecting needles.[5] Different saliva-activated transmission factors are secreted from the salivary glands that facilitate the transmission of pathogens to vertebrate hosts.[6] Other bioactive factors also secreted by the salivary glands play a key role in the completion of the bloodmeal. Among these, proteinaceous factors are of particular importance. Several anticoagulant proteins have been described: some inhibit the extrinsic and intrinsic pathway of blood coagulation,[7,8] and others inhibit platelet aggregation.[9–11] Tick salivary gland extracts also modulate both innate and acquired immunity.[12–14] Indeed, the alternative pathway of the complement is inhibited[15,16] and the activity of lymphoid cells is modified.[17–23] For tick infestation, interleukin (IL)-4, IL-5, and IL-10 induction and IL-2 and interferon gamma inhibition[24–26] are observed, indicating a polarized Th2 host immune response.[13] It has also been observed that during the bloodmeal, new mRNAs are induced in the salivary glands, leading to the synthesis of a wide range of new proteins.[27,28] It is hypothesized that among these induced genes, several are essential for the completion of the tick feeding process and for the transmission of pathogens such as *B. burgdorferi.*

The goal of this study was to characterize tick salivary gland proteins that influence pathogen-host-vector relationships. Such an understanding could lead to the establishment of new means of control and prevention against tick bites and pathogen transmission. Our approach was to create a subtractive cDNA library by which cDNAs of 5-day blood-fed *Ixodes ricinus* salivary glands would be enriched for novel messages compared with unfed ticks. This approach led to the identification of several mRNAs specifically induced in 5-day blood-fed *I. ricinus* salivary glands, followed by characterization of these gene products.

MATERIALS AND METHODS

Preparation of *I. ricinus* salivary glands mRNA and protein extracts. Salivary glands of 5-day engorged or unfed pathogen-free *I. ricinus* female adult ticks were removed and then immediately frozen in liquid nitrogen and stored at –80°C. Glands were crushed in liquid nitrogen with a mortar and pestle. mRNAs were purified by oligo-dT chromatography (Fast Track 2.0 kit, Invitrogen, Groningen, The Netherlands). Two micrograms and 1.5 μg of mRNAs were extracted from 200 salivary glands of engorged ticks and from 1,000 salivary glands of unfed ticks, respectively. Five-day-fed and unfed tick salivary glands protein extracts were obtained from 70 and 300 *I. ricinus* salivary glands, respectively. This material was crushed for 10 min with a mortar and a pestle in 400 μL (fed tick salivary glands) or 1,600 μL (unfed tick salivary glands) of extraction buffer (1× phosphate-buffered saline, pH7.4; ethylenediamine-tetraacetic acid 10 mM, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride 1 mM (Sigma-Aldrich, Bornem, Belgium). The samples were centrifuged for 8 min at 10,000 × *g,* and the supernatants were recovered and stored at –20°C.

Construction of a representational difference analysis subtractive library. All procedures were performed as described by Hubank and Schatz.[29] Double-stranded cDNAs were synthesized by means of the Superscript Choice System (Life Technologies, Rockville, MD). The cDNAs were digested with *Dpn*II restriction enzyme, ligated to R-linkers, amplified with R-24 primers, and finally digested again with the same enzyme to generate a "tester" pool consisting of cDNAs from salivary glands of fed ticks and a "driver" pool consisting of cDNAs from salivary glands of unfed ticks. The subtractive

TABLE 1
Oligonucleotide primer sequences used in the RT-PCR assays

Clone	Direct primer (5′-3′)	Reverse primer (5′-3′)
seq24	ATGAGGAGGGCACAGAGG	CCTGAATGGGCGGTCAAC
seq28	CCGTTGGAGACCCAGAAG	GTGCTCGGTCTGTCCATG
seq29	CTCCTGTCGCCACCTATG	CCAAGACCGTGTCCGTAG
seq16	CCGATGTGCCCTTTCACCC	ACACTGGAGGATTTCAACACTC
seq7	TGGTAGACACAGCCAACCACAA	GGCAGTCTTCATTCAAGGCTTG
seq6	AACTTAGTCTCACCAATATACGTTT	TCGTACAAGCTCCGCTTTTCTC
seq26	ACATGCGAAGCTGCCGAC	CGTTTATCCGGTCCACGC
seq17	AGGCTCCATCTAGGCAGC	CCGGTCGTCGAATTGCAC
seq3	GTTTGTGCTGTTTGCGATTTGCA	TGGGTAAGTCGGCTCTATGCC
seq2	TTCAGAACCGTGAATTTGAAAAAGT	TGGAACTTCCACGTCTTCTTCAA
seq1	CTTGTAGCCCTTCCTCATCCG	AACGAGGACATTACGCTAAACCT
seq4	CACTCGAAAATGGAGGCTTTGAA	GCAACGTGGCCAGTAGTAATCA
seq5	ATGTTACCATGTCCAACCCGGT	CTCACGACGGAGAAGAACGC
seq19	GACTTGCGCTTAGGTTTCTTAGT	ATGTCATTTGACAACTCATTCGGA

hybridization process consisted of 3 rounds of selection by use of a tester/driver ratio of 1:100, 1:400 and 1:200,000, respectively. The *Dpn*II-digested differential products were subdivided according to their size into 4 different fractions and subcloned into the *Bam*HIsite of the pTZ19r cloning vector. The ligated product was used to transform TOP-10 *Escherichia coli* competent cells (Invitrogen). From this subtractive library, 9,600 clones were randomly selected and stored at –80°C.

Construction of a full-length cDNA library from salivary gland of engorged *I. ricinus* female adult ticks. This library was set up using mRNAs extracted from salivary glands of engorged female adult ticks. The mRNAs (80 ng) were subjected to reverse transcription by means of a degenerate oligo-dT primer (5′-A$(T)_{30}$VN-3′), the Smart oligonucleotide (Clontech, Palo Alto, CA), and the Superscript II reverse transcriptase (Life Technologies). The single-strand cDNA mixture was used as template in a hot-start polymerase chain reaction (PCR) assay including the LA Taq polymerase (Takara, Shiga, Japan), the modified oligo-dT, primer and a 3′-Smart primer specific to a region located at the 5′ end of the Smart oligonucleotide. The PCR protocol consisted of 25 cycles of 1 min at 95°C, 25 sec at 95°C, and 5 min at 68°C, followed by a 10-min incubation at 72°C. The amplified double-stranded cDNA mixture was purified on a Centricon 30 concentrator (Millipore, Bedford, MA). The cDNAs were divided into 4 fractions, 0.3–0.6 kb, 0.6–1 kb, 1 kb to 2 kb, and 2–4 kb, on a 0.8% high-grade agarose electrophoresis gel. After their recovery by the Qiaex II extraction kit (Qiagen, Hilden, Germany), these fractions were ligated into the pCRII cloning vector (Invitrogen). The ligated fractions were then used to transform XL2-Blue ultracompetent *E. coli* cells (Stratagene, Amsterdam, The Netherlands). The resulting recombinant clones were stored individually in microplates at –80°C.

Screening of full-length cDNA library and rapid amplification of cDNA ends. Recombinant bacterial clones derived from the full-length cDNA library were screened by conventional hybridization procedures. Radiolabeled oligonucleotide probes derived from the selected subtractive sequences were as follows: 5′-TCCGGACGACGGACCATGCA-3′ for *seq7;* 5′-AGTCCATGATTGTAGTGTGCT AC-3′ for *seq16;* and 5′-GCAGCTGCAGCCTCTGTGCC-3′ for *seq24.*

Rapid amplification of cDNA ends (RACE) methodology was performed as described by Frohman.[30] The reverse transcription step was carried out with 10 ng of mRNAs extracted from salivary gland of engorged ticks and Thermoscript Reverse transcriptase (Life Technologies). Gene-specific primers (GSP) used for the reverse transcription step for 5′-RACE were: *seq7* 5′-CCTTCGCATCCGCCATAC-3′; *seq16* 5′-CCCGCCAGTCCATGATTG-3′; and *seq24* 5′-CCTGAATGGGCGGTCAAC-3′. The following GSP-primers were used for the amplification step: 3′-RACE: GSP1: 5′-GCGGCCTGCGAAGTGTAA-3′; GSP2: 5′-GGACCATGCAGAGCACGA-3′; 5′-RACE: GSP1: 5′-TCGTGCTCTGCATGGTCC-3′; GSP2: 5′-TTACACT-TCGCAGGCCGC-3′, in the case of *seq7.* 3′-RACE: GSP1: 5′-GCAACTATTGCCGGAGGG-3′; GSP2: 5′-GGCTACTCGGTGTAGTCG-3′; 5′-RACE: GSP1: 5′-GTATGGCTCGTCTGCTGG-3′; GSP2: 5′-GCCAAC-GATCCTGAGCTG-3′, in the case of *seq16.* 3′-RACE: GSP1: 5′-ATGAGGAGGGCACAGAGG-3′, GSP2: AGCTGCCACTGCCATACC-3′; *seq24* 5′-RACE: GSP1: 5′-GGTATGGCAGTGGCAGCT-3′, GSP2: 5′-CCTCTGTGCCCTCCTCAT-3′, in the case of *seq24.* The purified cDNAs were cloned into the pCRII-TOPO cloning vector (Invitrogen) and sequenced.

Evaluation of the differential expression of the cDNA clones isolated in the subtractive and full-length cDNA libraries by reverse transcriptase (RT)-PCR/oligo-dT or RT-PCR/GSP assays. The RT-PCR assays were carried out on mRNAs extracted from salivary glands either of engorged or of unfed ticks. The reverse transcription step of the RT-PCR/oligo-dT assay was performed by use of an oligo-dT primer and the reverse transcription step of the RT-PCR/GSP assay by use of a GSP and the Superscript II RNase H^- reverse transcriptase (Life Technologies), as described by the manufacturer. Each PCR assay included pair of primers specific to each target subtractive or cDNA full-length sequence, and they were performed by Expand High Fidelity polymerase (Roche, Brussels, Belgium) according to the manufacturer's instructions. Primers are provided in Table 1. Single-stranded DNA samples were amplified for 30 cycles under the following conditions: 95°C for 1 min, 72°C for 30 sec, and 60°C for 1 min, followed by a final elongation step of 72°C for 7 min.

Computational analysis. Sequence analyses were performed by the Genetics Computer Group (GCG) sequence analysis software, version 10.0 (Madison, WI).

TABLE 2
Comparison of the 27 subtraction cDNA sequences against EMBL/GenBank databases

Ac. Number	Gene	Similar sequences in databases[a,b]	Score	Rate(%)	Alg.[c]
AJ269635	*seq1*	N.S.		5,6	
AJ269636	*seq2*	N.S.		12,3	
AJ269637	*seq3*	N.S.		5,6	
AJ269638	*seq4*	N.S.		2,2	
AJ269639	*seq5*	N.S.		3,4	
AJ269640	*seq6*	*R. melioti* Nitrogen fixation (fixF) [M18272]	0,00089	16,8	T
AJ269641	***seq7***	***H. sapiens* Tissue factor Pathway Inhibitor PI-2 [P48307]**	**4.10^{-12}**	4,5	T
AJ269642	*seq8*	N.S.		3,4	
AJ269643	*seq9*	Probable integrase/recombinase [P42540]	3,3	3,4	T
AJ269644	*seq10*	N.S.		2,2	
AJ269645	*seq11*	N.S.		2,2	
AJ269646	*seq12*	N.S.		3,4	
AJ269647	*seq13*	*C. gloeosporioides* cutinase gene [M21443]	0,082	2,2	F
AJ269648	*seq14*	N.S.		10,1	
AJ269649	*seq15*	mRNA for secretory prot cont. thrombospondine motifs [D67076]	0,014	5,6	T
AJ269650	***seq16***	***Bothrops jararaca*, Jararhagin metalloprotease mRNA [X68251]**	**$2,3.10^{-6}$**	4,5	T
AJ269651	*seq17*	*O. aries* gene for ovine INF-alpha [X59068]	0,7	1,1	T
		Interferon-omega 45 [S69000]	0,88		
		RCPT PGE2 [E06016]	0,85		
AJ269652	*seq18*	N.S.		1,1	
AJ269653	*seq19*	IgG1 L chain directed against human IL2 rcpt Tac prot [S72847]	0,19	1,1	T
AJ269654	*seq20*	N.S.		1,1	
AJ269655	*seq21*	N.S.		1,1	
AJ269656	*seq22*	*Mus Musculus* neuroactin [AF010586]	0,42	1,1	F
AJ269657	*seq23*	N.S.		1,1	
AJ269658	***seq24***	***H. sapiens* thrombin inhibitor [Z22658]**	**$2,1.10^{-12}$**	1,1	T
		Human monocyte/neutrophil elastase inhibitor mRNA [P80229]	**3.10^{-5}**		
AJ269659	*seq25*	N.S.		1,1	
AJ269660	*seq26*	N.S.		1,1	
AJ269661	*seq27*	N.S.		1,1	

The different cDNA clones were compared with sequences in the EMBL/GenBank databases by tFasta or Fasta algorithms (Genetics Computer Group, Madison, WI). Scores and homologous database sequences are presented here. The ratio of each cDNA clone in the subtractive library is shown in the column frequency.
[a] N.S. = No significant identity.
[b] EMBL/GenBank accession numbers are in brackets.
[c] tFasta (T) and Fasta (F).

Synthesis of recombinant proteins in bacterial and mammalian expression systems. Unique restriction sites were added by PCR in order to subclone the different open reading frames (ORFs) into *E. coli* and mammalian expression vectors. Denaturing, annealing, and extension temperatures used for the PCR were 95°C for 15 sec, 58°C for 30 sec, and 68°C for 2 min, respectively, for 25 cycles, except for the amplification of *seq7* ORF, where the annealing temperature was 62°C. The cDNA, obtained from reverse transcription of 5-day fed tick salivary glands mRNA with the 3′ primer, was used as template to amplify the different ORFs. pMALC2-E vector containing maltose binding protein (MBP) fusion partner and pCDNA3.1/V5-His A containing the His tag fusion partner were digested with either Asp718 and XbaI (for *seq7* and *seq16* cloning in pMALC2-E vector) or Asp718 and EcoRI (for *seq24* cloning in pMALC2-E vector) or Asp718 and ApaI (for pCDNA3.1/V5-His A) and ligated to *seq7*, *seq16*, and *seq24* cDNAs. The TG1 *E. coli* cells were transformed with the each pMALC2-E recombinant vector and the expression of the fusion proteins induced by addition of 0.3 mM isopropyl-β-D-1-thiogalactopyranoside. *Escherichia coli* extracts containing Seq16/MBP and Seq24/MBP were solubilized with 6 M urea and purified by amylose chromatography (NEB, Hitchin, UK) according to manufacturer's recommendations. The *seq16*- and *seq24*-pCDNA3.1/V5-His A constructs were transfected into CHOK I cells by means of Fugene 6 (Roche) according to manufacturer's recommendations, and G418 selection was used to generate stable transfectants. Target proteins were purified by Ni-chelate chromatography (Ni-NTA superflow resin; Qiagen) according to the manufacturer's recommendations.

Immunodetection of Seq7, Seq16, and Seq24. Ten-week-old female BALB/c mice were immunized with 5 μg of Seq7/MBP, Seq16/MBP, and Seq24/MBP in Freund complete adjuvant. Three booster immunizations were provided with the same amounts of antigen in Freund incomplete adjuvant at 15-day intervals. We performed DNA immunization by injecting 50 μg of pCDNA3.1/V5-His A vector ligated to *seq16*

TABLE 3
Comparison to EMBL/GenBank databases of 9 clones of the full-length cDNA library

Ac. Number	Clone	Seq.[a]	Length (bp)	Similar sequences in databases[b,c]	Alg.[d]	Score
AJ300190	*412.b7*	C	2378	N.S.		N
AJ300191	*410.e7*	P	~4000	N.S.		N
AJ300192	*416.a12*	C	1311	*A. gambiae* uncharacterised mRNA [U50476]	T/B	8,2.10-12 / N
AJ300193	*419.c3*	P	~800	*A. aegypti* chitinase protein [AF026492]	T/B	2,5.10-5 / 2.10-4
AJ300194	*419.d3*	P	~2400	N.S.		N
AJ269663	*seq29*	C	935	*R. norvegicus* leukocyte common antigen-related protein [X83546]	T/B	4,8.10-9 / N
AJ300195	*419.e5*	P	~2000	*Haloferax volcanii* aminotransferase [U95372]	F	2.10-7
AJ269662	*seq28*	C	2388	Human interferon-related protein [SKMc15] [U09585]	T/B	1,8.10-36/1,7.10-71

The different cDNA clones were compared with EMBL/GenBank databases by tFasta or Blastp algorithms (Genetics Computer Group, Madison, WI). Scores and homologous database sequences are presented here.
[a] Completely sequenced (C) or partially sequenced (P).
[b] No significant homology (N.S.).
[c] EMBL/GenBank accession numbers are in brackets.
[d] tFasta (T) and Blast (B).

ORF into 3-week-old female BALB/c mice. Three booster DNA injections were provided with the same amounts of DNA at 3-week intervals, and serum anti-Seq16/His was recovered.

To examine the expression of native proteins in salivary glands and to detect purified Seq16/His and Seq24/His, the same quantities of fed and unfed tick salivary glands and 50 ng of Seq16/His and Seq24/His were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. The membranes were probed with diluted sera directed against Seq7/MBP (1:1,000) or Seq16/MBP (1:500) or Seq24/MBP (1:1,000) or Seq16/His (1:200) and developed with nitro-blue tetrazolium chloride-5-bromo-4-chloro-3-indolylphosphate toluidine.

RESULTS

Establishment of subtractive cDNA library and characterization of select clones. The representational difference analysis technique was performed to identify cDNAs encoding proteins specifically expressed during the blood meal in the salivary glands of *I. ricinus* female ticks. This method consists of an amplification-associated subtractive process that allows the isolation of cDNAs corresponding to mRNAs differentially expressed in 2 distinct physiological stages of the *I. ricinus* salivary glands. The library was set up with mRNAs extracted from salivary glands of unfed and 5-day fed *I. ricinus* female ticks. Ninety-six randomly chosen clones were sequenced. Twenty-seven distinct cDNAs were identified. The frequency of sequences in each pool is indicated in Table 2. We used BLAST searching to compare each cDNA and deduced amino acid sequence against EMBL/GenBank nonredundant databases. Twenty-four sequences showed low or no homology to any known sequences (Table 3). Three sequences had significant hits (E value $< 10^{-4}$): the human tissue factor pathway inhibitor gene (TFPI) (*seq7*); a snake venom zinc-dependent metalloprotease (the *Bothrops jararaca* jararhagin gene [*seq16*]); and the human thrombin inhibitor gene (*seq24*) (Table 4). The deduced amino acid sequences of the 27 DNA fragments were also analyzed by the GCG motifs search algorithm. The peptide sequences of clones *seq7* and *seq16* are predicted to include Kunitz and metallopeptidase motifs, respectively. Clones seq5 and seq8

TABLE 4
Analysis of translated sequences of selected subtracted cDNA clones

Clone	Full-length sequences similarity to databases	tFasta/Blastp Scores[a]	ORF (aa)	Motives	Signal peptide scores[b]
Seq16	Mouse mRNA for secretory protein containing thrombospondin motives [D67076][c]	0,002 / 6.10^{-7}	489	Metallopeptidase	7,9 / S
Seq24	Pig leukocyte elastase inhibitor mRNA. [P80229]	0 / 7.10^{-67}	378	Serpin	8,5 / S
Seq7	Human Tissue Factor Pathway Inhibitor [P48307]	$4,8.10^{-12}$ / 2.10^{-5}	87	Kunitz	6,5 / S

Complete cDNA sequences of 3 selected clones were compared with EMBL/GenBank databases by tFasta or Blastp algorithms (Genetics Computer Group, Madison, WI). Their sequences were analyzed for the presence of either motifs (motifs algorithm) or a specific signal peptide sequence (von Heijne and McGeoch analysis).
[a] N = No score.
[b] Succeeded (S) and failed (F).
[c] EMBL/GenBank accession numbers are in brackets.

encode a predicted prokaryotic membrane lipoprotein attachment site motif (Table 2). On the basis of their high homology to known sequences, the partial *seq7*, *seq16*, and *seq24* cDNA sequences were selected for further characterization of their corresponding complete mRNA sequences. Their full-length cDNA sequences were recovered either by screening of a full-length cDNA library (*seq16*) or by RACE (*seq7* and *seq24*).

Characterization of a full-length cDNA library from blood-fed tick salivary gland mRNA. A full-length cDNA library was constructed with mRNAs from 5-day fed tick salivary glands. To assess the recombinant character of this library, 24 randomly chosen clones were subjected to enzyme restriction analyses. All these clones incorporated cDNA fragments ranging from 0.2 to ~ 3.5 kb. Eight clones were sequenced and similarities sought by BLAST search of public databases. Three cDNAs had no hits; 5 showed significant similarity to known genes. Two cDNAs were homologous to mosquito genes (*Aedes aegypti* and *Anopheles gambiae*), and 2 others showed similarity to proteins with potential immunomodulatory properties. *seq28* resembles a human putative interferon-related gene, whereas the *seq29* shows homology to the *Rattus norvegicus* leukocyte common antigen-related, or *LAR*, gene (Table 3).

Differential expression of cDNA clones isolated from the subtractive and full-length cDNA libraries. The differential expression of 13 subtractive clones (including *seq7*, *seq16*, and *seq24*) and of 2 clones of the full-length library (*seq28* and *seq29*) was assessed via 2 types of RT-PCR (RT-PCR/oligo-dT and RT-PCR/GSP) assays. mRNA expression of the subtractive sequences is upregulated in salivary glands of 5-day engorged ticks (Figure 1). *seq29* mRNA was detected only in salivary glands of blood-fed ticks, whereas *seq28* mRNA was expressed at a similar level both in engorged and unfed ticks' salivary glands. Among upregulated mRNAs, some showed either increased expression (e.g., *seq7*, *seq16*, and *seq24*) in the salivary gland of engorged ticks or no expression (e.g., seq6 and *seq29*) in the salivary gland of unfed ticks.

Sequence analyses of selected cDNAs. The *seq7*, *seq16*, and *seq24* cDNA and deduced amino acid sequences were analyzed by the tFasta, Blastp, and Motifs algorithms of the GCG Wisconsin package software. The size of the deduced amino acid sequences from these coding sequences ranged 87–489 bp (Table 3).

Seq7 is highly homologous ($E = 2.10^{-5}$) (Table 4) to the human TFPI, an anticoagulant protein with 3 tandemly arranged Kunitz-type protease inhibitor (KPI) domains. Seq7 is homologous to the first KPI domain of the human TFPI (Figure 2). Seq16 is homologous to a mouse secretory metalloprotease containing thrombospondin motifs ($E = 3.10^{-7}$) (Figure 2). Seq24 is homologous to the pig leukocyte elastase inhibitor ($E = 7.10^{-67}$) and to human thrombin inhibitor ($E = 5.10^{-55}$), which both belong to the serpin superfamily of protease inhibitor (Table 4) (Figure 2).

As one approach to determining whether these proteins are secreted, the deduced amino acid sequence of the 3 selected cDNAs were analyzed for the presence of a signal peptide sequence by means of both the von Heijne[31] and McGeoch[32] methods (Table 4). Both methods indicated that these cDNAs encode putative secretory signal peptide motifs.

Expression and immune detection of *seq7, seq16*, and *seq24* gene products. Seq7, Seq16, and Seq24 proteins were

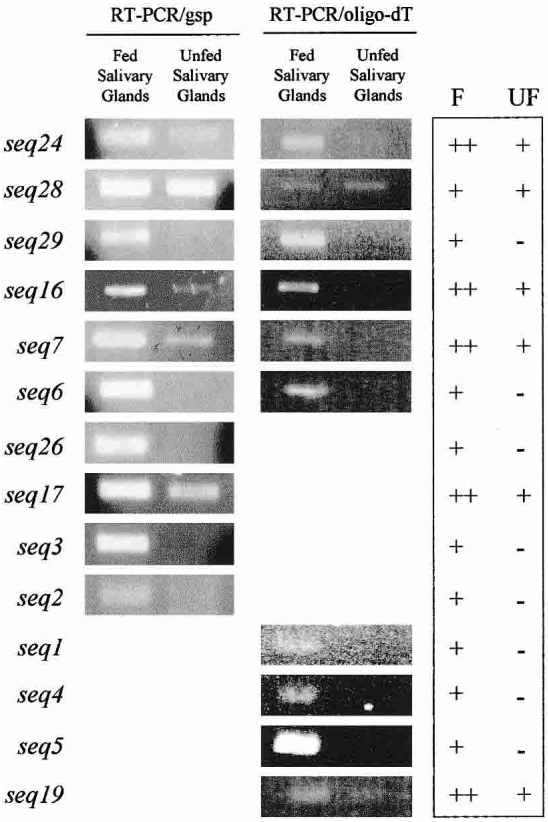

FIGURE 1. Differential expression analysis of 5 full-length cDNAs and 9 cDNA fragments isolated from the subtraction library. Reverse transcriptase-polymerase chain reaction (RT-PCR) assays were carried out on mRNAs extracted from salivary glands either of engorged (F) or of unfed (UF) ticks. The RT step of the RT-PCR/oligo-dT and RT-PCR/GSP assays was performed with an oligo-dT primer and gene-specific primer (GSP), respectively. ++ = strongly positive; + = positive; – = negative.

expressed in *E. coli* by the pMALC2-E vector (NEB) fused to the 42-kDa MBP. Seq7, Seq16, and Seq24 recombinant proteins were purified and the predicted 52-kDa (Seq7/MBP), 97-kDa (Seq16/MBP), and 82-kDa (Seq24/MBP) products were visualized by Coomassie blue-stained SDS-PAGE (Figure 3A). In addition, Seq16/His and Seq24/His proteins were produced in a mammalian expression system as V5 epitope/6X His tag fusions by use of the pCDNA3.1/V5-His A as vector (Invitrogen). Purified Seq24/His was detected by Western immunoblot that used anti-V5 antibodies at the predicted size of 46 kDa (Figure 3B). Detection of purified Seq16/His by means of the anti-V5 antibody revealed a major band at 68 kDa and 2 double bands at ~ 53 and 43 kDa.

Immune sera directed against MBP fusion proteins or Seq16/His protein were used to detect recombinant and native proteins by Western immunoblot. By use of an anti-Seq24/MBP sera (Figure 4A), a similar double-band pattern was revealed both in 5-day fed tick salivary gland extract (at 43 and 40 kDa) and purified recombinant Seq24/His protein (at 46 and 40 kDa). Native Seq24 was not detected in unfed tick salivary glands. Anti-Seq16/MBP (data not shown) and anti-Seq16/His (Figure 4B) sera revealed the following: for Seq16/His, the same pattern of bands detected by anti-V5 antibody; and for native Seq16, a 43-kDa band in fed tick

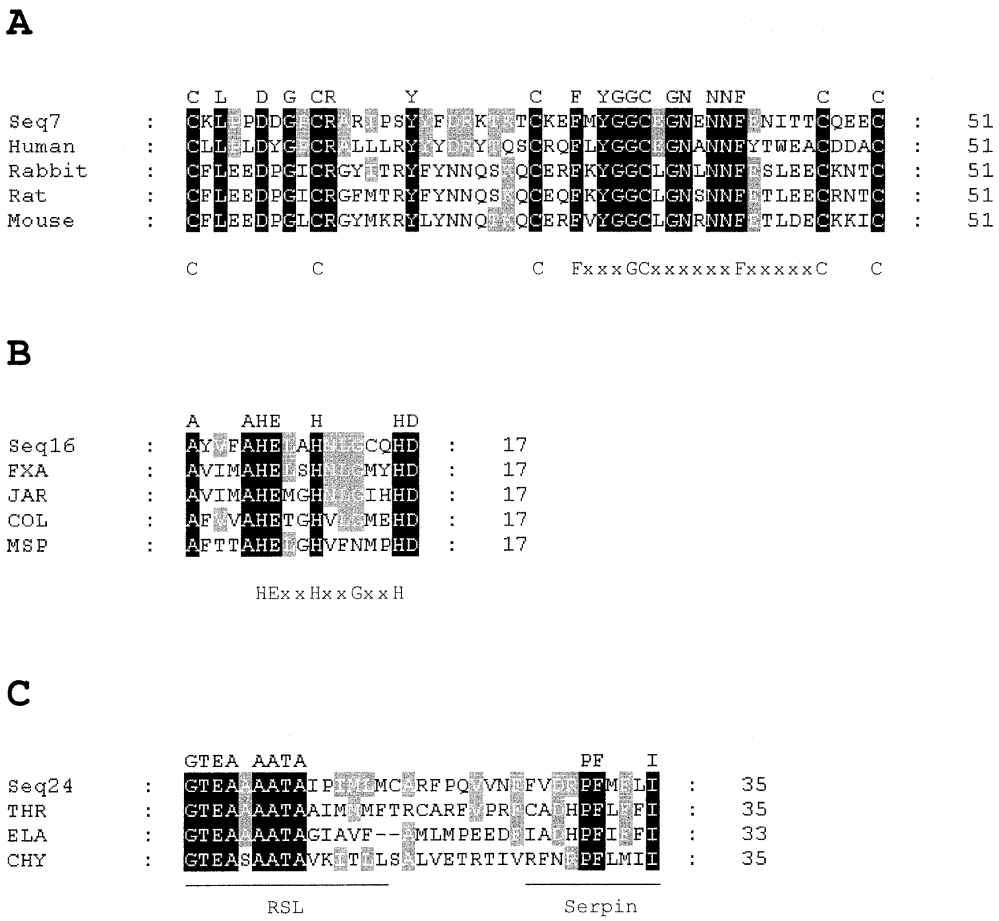

FIGURE 2. Comparison of predicted active sites of *Seq7, Seq16,* and *Seq24* with homologous sequences. **(A)** Alignment of translated *Seq7* and the first Kunitz-type protease inhibitor (KPI) domain of mouse tissue factor pathway inhibitor (TFPI; EMBL/GenBank accession number AF004833), rat TFPI (accession number Q02445), rabbit TFPI (accession number P19761), and human TFPI (accession number P48307) protein sequences. The consensus sequence of KPI is indicated below the alignment. **(B)** Alignment of *Seq16* and different metallopeptidases. Factor X activating enzyme (FXA; accession number A42972), Jararhagin (JAR; accession number P30431), procollagen I-N proteinase (COL; accession number HSAJ3125), and the mouse secretory protein containing thrombospondin motives (MSP; accession number D67076). The consensus sequence of the zinc-binding motif is indicated below the alignment. **(C)** Active site alignment of translated *Seq24,* thrombin inhibitor (THR; accession number Z22658), elastase inhibitor (ELA; accession number P80229), and antichymotrypsin (CHY; accession number AF089747) proteins. Underlined sequences of the active site alignment highlight the reactive site loop (RSL), which interacts with serine proteases, and the specific signature pattern (Serpin) of the serine protease inhibitor family.

salivary glands was weakly detectable in unfed tick salivary glands. Anti Seq7/MBP serum failed to visualize protein in salivary gland extract (data not shown).

DISCUSSION

During bloodmeal ingestion, ticks are thought to modify host defenses by injecting bioactive factors via saliva into the vertebrate host. To identify novel tick salivary factors at the molecular level, 2 cDNA libraries of *I. ricinus* salivary glands were analyzed. The first one, a representational difference analysis subtractive library, was constructed from mRNAs extracted from salivary glands of 5-day engorged and unfed *I. ricinus* ticks. The second library was derived from 5-day fed ticks and contained full-length cDNAs. Analysis of the subtractive library identified 27 distinct sets of sequences (Table 2). Comparisons of sequences from both cDNA libraries to EMBL/GenBank databases led to their classification into 2 groups. The first one comprises all sequences that do not match or show low homology to any sequence found in the databases, and the second group includes all sequences showing significant homology to known genes.

In the latter group, some sequences were found to be homologous to anticoagulants, inhibitors of platelet aggregation, and other proteins that might be involved in anti-inflammatory or immunomodulatory processes (Tables 2 and 3), among them different serine protease inhibitors and a metalloprotease. Two sequences showed homology to genes of *Aedes aegypti* and *Anopheles gambiae.* The remaining subtractive sequences showed no significant homologies to known genes. We hypothesize that this set of genes could potentially encode novel proteins that may be involved in the modulation of vertebrate host defenses. It is not surprising that a great number of the sequenced cDNA clones do not present homologies to known tick mRNAs[33] because few have been reported (e.g. ferritin, EMBL/GenBank accession number AF068224, the only *I. ricinus* reported mRNA sequence).

Analysis of the differential expression level of 12 subtractive cDNA sequences suggested that these genes are induced

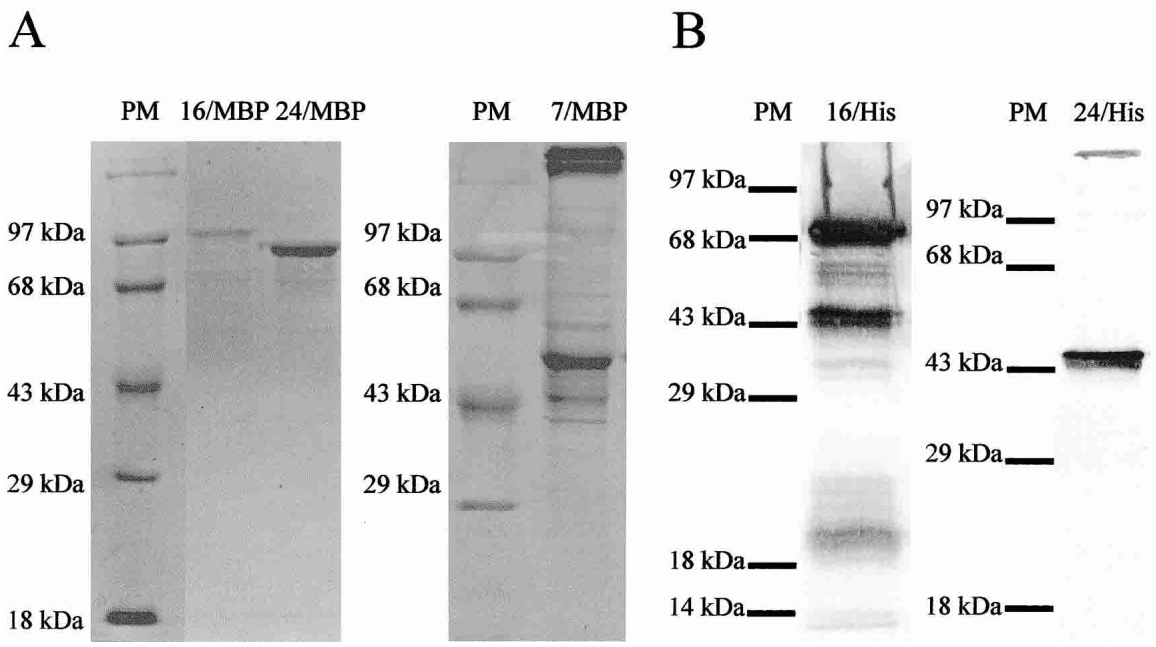

FIGURE 3. Western immunoblot detection of recombinant proteins. **(A)** Purified recombinant Seq7/maltose binding protein (7/MBP), Seq16/MBP (16/MBP), and Seq24/MBP (24/MBP) proteins were visualized on Coomassie blue-stained 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. **(B)** Purified Seq16/His (16/His) and Seq24/His (24/His) were separated on 10% SDS-PAGE gels, and recombinant proteins were detected by an anti-V5 antibody.

during blood meal ingestion. *seq29*, a clone originated from the full-length cDNA library, was detected only in the salivary glands of engorged ticks. In this manner, we have identified 28 partial or complete *I. ricinus* mRNAs differentially expressed between the salivary glands of 5-day fed and unfed ticks. Some show increased expression in the salivary glands of engorged ticks or no expression in the salivary glands of unfed ticks.

seq7, seq16, and *seq24* were selected for further analysis because they were found to be homologous to immunomodu-

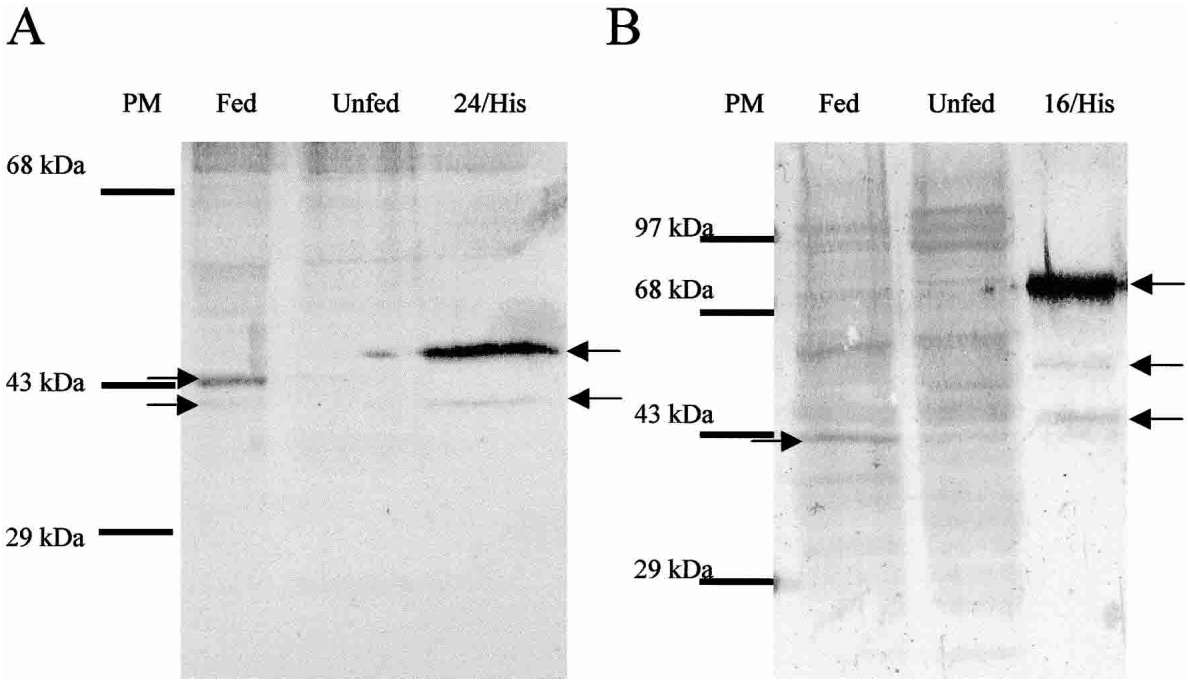

FIGURE 4. Western immunoblot detection of native, Seq16/His, and Seq24/His proteins. **(A)** Detection of a double band pattern both in fed tick salivary glands (fed) and in Seq24/His (24/His), whereas no protein was detected in unfed tick salivary glands (unfed). **(B)** Anti-Seq16/His serum detected Seq16/His (16/His) and native Seq16 in fed tick salivary glands (fed), whereas Seq16 was not detected in unfed tick salivary glands (unfed).

latory and anticoagulant proteins. Computational analysis of the full-length deduced amino acid sequences suggested that Seq7 was homologous to TFPI, a Kunitz-type protein, possessing 3 KPI domains, which is involved in the regulation of blood coagulation by inhibiting FXa and FVIIa.[34] Seq7 contains a domain with homology to the KPI domain 1. Therefore, Seq7 might inhibit factors in the vertebrate coagulation cascade such as FVIIa.[35]

Seq16 was found to have a conserved motif suggesting that it is homologous to different zinc-dependent metallopeptidase proteins of the Metzincin superfamily.[36] Among them, both a mouse secretory protein containing thrombospondin motifs and the *Bothrops jararaca* Jararhagin are known inhibitors of platelet aggregation mediated by different adhesive proteins.[37,38] Thus, we hypothesize that Seq16 encodes a metallopeptidase that may inhibit platelet aggregation during blood feeding.

Seq24 is homologous to several genes from the serine protease inhibitor (Serpin) family, among them the human thrombin inhibitor[39] and the pig monocyte/neutrophil elastase inhibitor proteins.[40] These similarities, and the fact that Seq24 is predicted to encode a Serpin motif, prompt us to suggest that this protein may be a serine protease inhibitor that acts in the modulation of host defenses or interferes with the coagulation cascade.

Recombinant Seq7, Seq16, and Seq24 proteins were produced in bacterial or mammalian expression systems to study expression of the genes in tick salivary glands. Recombinant Seq16 and Seq24 used to immunize mice generated polyclonal antisera that detect the corresponding native proteins in unfed or 5-day fed tick salivary glands extracts or the purified Seq16/His and Seq24/His proteins. Native Seq24 was detected in salivary glands of blood-fed ticks, but not in salivary glands of unfed ticks (Figure 4A). Anti-Seq16/His serum detected recombinant Seq16/His protein expressed in mammalian cells (Figure 4B). The native protein was recognized less specifically in fed tick salivary glands extracts, and no or low expression was detected in unfed tick salivary glands extract (Figure 4B). Recombinant Seq16 was mainly detected at 68 kDa and less abundantly at 43 kDa, whereas the native counterpart was mainly detected at 43 kDa. This could suggest that the 68-kDa form of the Seq16 protein is a propeptide, whereas the 43-kDa form is the active form. The similar patterns of bands observed between the recombinant and the native proteins indicate that *seq16* and *seq24* mRNAs encode proteins that do have their counterpart in *I. ricinus* salivary glands at the fifth day of engorgement. In addition, the lack of detection of the proteins, or their low level of expression, in unfed tick salivary glands confirms the differential expression of Seq16 and Seq24, as was previously showed by RT-PCR.

This work initiates the molecular characterization of a novel set of genes whose expression is induced by the fifth day of tick engorgement. By analyzing 2 cDNA libraries, several mRNAs were identified. Those induced mRNAs may be of particular importance for a better understanding of tick-host-pathogen relationships. Further experiments will evaluate mechanisms by which these genes contribute to the *I. ricinus* blood feeding process and in pathogen transmission.

Acknowledgments: We thank J.-C. Renauld (Ludwig Institute for Cancer Research, Université Catholique de Louvain, Brussels), for critical reading of the article in manuscript and Olivier Rais for providing the *I. ricinus* ticks. This work was supported by an International Brachet Stiftung grant (GR97–1/6). G.L. is supported by a grant from the Fonds de la Recherche Industrielle et Agricole.

Authors' addresses: Gérard Leboulle, Candice Rochez, Edmond Godfroid, and Alex Bollen, Applied Genetics, Université Libre de Bruxelles, Rue des Professeurs Jeener et Brachet, 12, B-6041 Gosselies, Belgium. Jamila Louahed, Ludwig Institute for Cancer Research, Université Catholique de Louvain, Brussels, Belgium. Bernard Rutti, Michel Brossard, Institut de Zoologie, Université de Neuchâtel, CH-2007 Neuchâtel, Switzerland.

Reprint requests: Edmond Godfroid, Applied Genetics, Université Libre de Bruxelles, Rue des Professeurs Jeener et Brachet, 12, B-6041 Gosselies, Belgium, Telephone: 32-2-650-99-35, Fax: 32-2-650-99-00, E-mail: godfroid@sga.ulb.ac.be.

REFERENCES

1. Sauer JR, McSwain JL, Bowman AS, Essenberg RC, 1995. Tick salivary gland physiology. *Annu Rev Entomol 40:* 245–267.
2. Brossard M, Wikel SK, 1997. Immunology of interactions between ticks and hosts. *Med Vet Entomol 11:* 270–276.
3. Nuttall PA, 1998. Displaced tick-parasite interactions at the host interface. *Parasitology 116(Suppl.):* S65-S72.
4. Aeschlimann A, 1991. Ticks and disease: susceptible hosts, reservoirs hosts and vectors. Toft CA, Bolis L, Aeschlimann A, eds. *Parasite-Host Associations: Coexistence or Conflict.* Oxford: Oxford University Press, 148–156.
5. Randolph SE, Gern L, Nuttall PA, 1996. Co-feeding ticks: epidemiological significance for tick-borne pathogen transmission. *Parasitol Today 12:* 472–479.
6. Jones LD, Matthewson M, Nuttall PA, 1992. Saliva-activated transmission (SAT) of Thogoto virus: dynamics of SAT factor activity in the salivary glands of *Rhipicephalus appendiculatus, Amblyomma variegatum,* and *Boophilus microplus ticks. Exp Appl Acarol 13:* 241–248.
7. Limo MK, Voigt WP, Tumbo-Oeri AG, Njogu RM, Ole-MoiYoi OK, 1991. Purification and characterization of an anticoagulant from the salivary glands of the ixodid tick *Rhipicephalus appendiculatus. Exp Parasitol 72:* 418–429.
8. Gordon JR, Allen JR, 1991. Factors V and VII anticoagulant activities in the salivary glands of feeding *Dermacentor andersoni* ticks. *J Parasitol 77:* 167–170.
9. Karczewski J, Waxman L, Endris RG, Connolly TM, 1995. An inhibitor from the argasid tick *Ornithodoros moubata* of cell adhesion to collagen. *Biochem Biophys Res Commun 208:* 532–541.
10. Keller PM, Waxman L, Arnold BA, Schultz LD, Condra C, Connolly TM, 1993. Cloning of the cDNA and expression of moubatin, an inhibitor of platelet aggregation. *J Biol Chem 268:* 5450–5456.
11. Wang X, Coons LB, Taylor DB, Stevens SE Jr, Gartner TK, 1996. Variabilin, a novel RGD-containing antagonist of glycoprotein IIb-IIIa and platelet aggregation inhibitor from the hard tick *Dermacentor variabilis. J Biol Chem 271:* 17785–17790.
12. Kopecky J, Kuthejlova M, 1998. Suppressive effect of *Ixodes ricinus* salivary gland extract on mechanisms of natural immunity in vitro. *Parasite Immunol 20:* 169–174.
13. Wikel SK, 1996. Tick modulation of host cytokines. *Exp Parasitol 84:* 304–309.
14. Wikel SK, 1996. Host immunity to ticks. *Annu Rev Entomol 41:* 1–22.
15. Ribeiro JM, 1987. *Ixodes dammini:* salivary anti-complement activity. *Exp Parasitol 64:* 347–353.
16. Valenzuela JG, Charlab R, Mather TN, Ribeiro JM, 2000. Purification, cloning, and expression of a novel salivary anticomplement protein from the tick, *Ixodes scapularis. J Biol Chem 275:* 18717–18723.
17. Kubes M, Fuchsberger N, Labuda M, Zuffova E, Nuttall PA, 1994. Salivary gland extracts of partially fed *Dermacentor reticulatus* ticks decrease natural killer cell activity in vitro. *Immunology 82:* 113–116.
18. Ushio H, Watanabe N, Kiso Y, Higuchi S, Matsuda H, 1993. Protective immunity and mast cell and eosinophil responses in

mice infested with larval *Haemaphysalis longicornis* ticks. *Parasite Immunol 15:* 209–214.
19. Ganapamo F, Rutti B, Brossard M, 1996. Cytokine production by lymph node cells from mice infested with *Ixodes ricinus* ticks and the effect of tick salivary gland extracts on IL-2 production. *Scand J Immunol 44:* 388–393.
20. Ramachandra RN, Wikel SK, 1992. Modulation of host-immune responses by ticks (Acari: Ixodidae): effect of salivary gland extracts on host macrophages and lymphocyte cytokine production. *J Med Entomol 29:* 818–826.
21. Fivaz BH, 1989. Immune suppression induced by the brown ear tick *Rhipicephalus appendiculatus* Neumann, 1901. *J Parasitol 75:* 946–952.
22. Mbow ML, Rutti B, Brossard M, 1994. IFN-gamma IL-2, and IL-4 mRNA expression in the skin and draining lymph nodes of BALB/c mice repeatedly infested with nymphal *Ixodes ricinus* ticks. *Cell Immunol 156:* 254–261.
23. Urioste S, Hall LR, Telford SR III, Titus RG, 1994. Saliva of the Lyme disease vector, Ixodes dammini, blocks cell activation by a nonprostaglandin E2-dependent mechanism. *J Exp Med 180:* 1077–1085.
24. Ganapamo F, Rutti B, Brossard M, 1996. Immunosuppression and cytokine production in mice infested with *Ixodes ricinus* ticks: a possible role of laminin and interleukin-10 on the in vitro responsiveness of lymphocytes to mitogens. *Immunology 87:* 259–263.
25. Ganapamo F, Rutti B, Brossard M, 1995. In vitro production of interleukin-4 and interferon-gamma by lymph node cells from BALB/c mice infested with nymphal *Ixodes ricinus* ticks. *Immunology 85:* 120–124.
26. Fuchsberger N, Kita M, Hajnicka V, Imanishi J, Labuda M, Nuttall PA, 1995. Ixodid tick salivary gland extracts inhibit production of lipopolysaccharide-induced mRNA of several different human cytokines. *Exp Appl Acarol 19:* 671–676.
27. Wang H, Nuttall PA, 1994. Comparison of the proteins in salivary glands, saliva and haemolymph of *Rhipicephalus appendiculatus* female ticks during feeding [published erratum appears in Parasitology 1995;110(Pt. 3):363]. Parasitology 109(Pt. 4): 517–523.
28. Oaks JF, McSwain JL, Bantle JA, Essenberg RC, Sauer JR, 1991. Putative new expression of genes in ixodid tick salivary gland development during feeding. *J Parasitol 77:* 378–383.
29. Hubank M, Schatz DG, 1994. Identifying differences in mRNA expression by representational difference analysis of cDNA. *Nucleic Acids Res 22:* 5640–5648.
30. Frohman BH, 1995. Rapid amplification of cDNA ends. Dieffenbach CW, Dveksler GS, eds. *PCR Primer: A Laboratory Manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 381–469.
31. Nielsen H, Engelbrecht J, Brunak S, von Heijne G, 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Prot Eng 10:* 1–6.
32. McGeoch DJ, 1985. On the predictive recognition of signal peptide sequences. *Virus Res 3:* 271–286.
33. Das S, Marcantonio N, Deponte K, Telford SR, Anderson JF, Kantor FS, Fikrig E, 2000. SALP16, a gene induced in *Ixodes scapularis* salivary glands during tick feeding. *Am J Trop Med Hyg 62:* 99–105.
34. Baugh RJ, Broze GJ Jr, Krishnaswamy S, 1998. Regulation of extrinsic pathway factor Xa formation by tissue factor pathway inhibitor. *J Biol Chem 273:* 4378–4386.
35. Petersen LC, Bjorn SE, Olsen OH, Nordfang O, Norris F, Norris K, 1996. Inhibitory properties of separate recombinant Kunitz-type-protease-inhibitor domains from tissue-factor-pathway inhibitor. *Eur J Biochem 235:* 310–316.
36. Bode W, Grams F, Reinemer P, Gomis-Ruth FX, Baumann U, McKay DB, Stocker W, 1996. The metzincin-superfamily of zinc-peptidases. *Adv Exp Med Biol 389:* 1–11.
37. Kuno K, Kanada N, Nakashima E, Fujiki F, Ichimura F, Matsushima K, 1997. Molecular cloning of a gene encoding a new type of metalloproteinase-disintegrin family protein with thrombospondin motifs as an inflammation associated gene. *J Biol Chem 272:* 556–562.
38. Kamiguti AS, Moura-da-Silva AM, Laing GD, Knapp T, Zuzel M, Crampton JM, Theakston RD, 1997. Collagen-induced secretion-dependent phase of platelet aggregation is inhibited by the snake venom metalloproteinase jararhagin. *Biochim Biophys Acta 1335:* 209–217.
39. Coughlin P, Sun J, Cerruti L, Salem HH, Bird P, 1993. Cloning and molecular characterization of a human intracellular serine proteinase inhibitor. *Proc Natl Acad Sci U S A 90:* 9417–9421.
40. Remold-O'Donnell E, Chin J, Alberts M, 1992. Sequence and molecular characterization of human monocyte/neutrophil elastase inhibitor. *Proc Natl Acad Sci U S A 89:* 5635–5639.