Salp25D, an *Ixodes scapularis* Antioxidant, Is 1 of 14 Immunodominant Antigens in Engorged Tick Salivary Glands

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Rabbits or guinea pigs infested with *Ixodes scapularis* acquire resistance to tick bites, a phenomenon, known as tick immunity, that is partially mediated by antibody. To determine the salivary gland antigens that elicit antibodies in the host, an *I. scapularis* salivary gland cDNA expression library was probed with serum from tick-immune rabbits. Sera from sensitized rabbits strongly recognized 47 of 100,000 library clones in an antibody-screening assay. These 47 clones encoded 14 different *I. scapularis* genes, including a glutathione peroxidase homologue. Expression of these 14 genes in engorged tick salivary glands was confirmed by reverse-transcription polymerase chain reaction. The *I. scapularis* glutathione peroxidase homologue, named salp25D, was expressed in both unfed and fed nymphal salivary glands. Recombinant Salp25D was able to catalyze the reduction of hydrogen peroxide in the presence of reduced glutathione and glutathione reductase. These results categorize the prominent salivary gland proteins in *I. scapularis* and demonstrate the presence of a potent antioxidant in tick saliva.

Mammals that have been infested by ticks acquire immunity to subsequent bites by the vector, a phenomenon known as tick immunity. Acquired resistance to ticks was first described by Trager in 1939, who showed that guinea pigs on which *Dermacentor variabilis* (the common dog tick) had fed became resistant to future tick bites [1]. Many authors have extended these findings over the last 50 years [2]. Repeated exposure of rabbits, cattle, dogs, and guinea pigs to ticks has been shown to interfere with vector feeding, molting, and fecundity [1, 3–7]. Even a natural host like *Clethrionomys glareolus*, the bank vole, develops resistance to *Ixodes ricinus* with repeated infestations [8].

Tick immunity can interfere with pathogen transmission. Rabbits pre-exposed to uninfected *D. andersoni* were partially protected when exposed to *Francisella tularensis*-infected nymphs [9]. Transmission of tick-borne *Babesia bovis* was impaired in tick-immune cattle [5]. Immunity to *I. ricinus* in *C. glareolus* reduced the efficiency of *I. ricinus*-mediated *Borrelia burgdorferi* transmission [10]. Similarly, *I. scapularis*-sensitized guinea pigs do not acquire infection when challenged with *B. burgdorferi*-infected ticks [11]. Although laboratory mice, compared with larger animals, do not readily develop immunity to ticks after repeated exposures, a tick infestation–induced partial host resistance to vector-borne *B. burgdorferi* transmission has been reported in BALB/c mice [12]. Therefore, the study of the host immunity to ticks and its possible role in disease prevention has been a focus of extensive investigation.

To begin to explain how the host acquires immunity against vectors, several groups have shown that mammals react to tick antigens and that humoral or cellular responses are directed at future ticks during their blood meal. Tick immunity could be partially transferred to guinea pigs never exposed to ticks (tick naive) by using serum from tick-immune animals [13–15], and antibodies, complement, and cutaneous basophil hypersensitivity have been implicated in resistance [2, 16–21]. Tick feeding induces the generation of homocytotropic antibodies that bind to Fc receptors on basophil and mast cell surfaces. These antibodies complex with tick salivary gland antigens during engorgement, and the release of biologically active molecules during degranulation then affects tick feeding [22–28]. A delayed-type hypersensitivity reaction also has been associated with the rejection of ticks by sensitized hosts [29]. Immunization of guinea pigs with tick salivary gland extract has been sufficient for the development of tick immunity and cutaneous basophil hypersensitivity responses [29–31]. Although tick sensitization is at least partially dependent on antibodies, the specific immunogens responsible for tick immunity in *I. scapularis* remain unknown.

The components of tick saliva have many effects that may aid the vector during engorgement and could be important in evading host responses. Reported activities of tick saliva include anticoagulant and immunosuppressive functions, such as inhibition of the complement cascade, impairment of NK cell activity, reduction of homocytotropic and circulating antibody titers, repression of cytokine production, and inhibition of T lymphocyte
proliferation [28, 32–40]. The specific antigenic and functional components of *I. scapularis* saliva have not yet been well characterized. Herein, we describe the major *I. scapularis* salivary antigens that elicit antibodies in the host and demonstrate the antioxidant activity of one of these abundant proteins.

Materials and Methods

Animals

Pathogen-free 4–6-week-old C3H/HeJ (C3H) mice, 300–400-g Hartley guinea pigs, and 500–600-g New Zealand white rabbits were purchased from Jackson Laboratory. All animals were killed by CO₂ inhalation. To obtain *I. scapularis* for experimental use, tick rearing was performed in the laboratory in an incubator at 26°C with 85% relative humidity and a 12 h:12 h light:dark photoperiod regimen. The egg mass was laid by fed female adult ticks in the laboratory. Hatched larvae were fed on uninfected C3H mice to produce pathogen-free nymphs.

Generation of Tick Immunity and Collection of Tick-Immune Serum Samples

To elicit tick immunity, groups of 3 rabbits or guinea pigs were sensitized to ticks by 3 infestations with 100 nymphs per animal, with a resting period of 21 days between challenges. To collect tick-immune serum, selected animals were killed 2 weeks after the final tick challenge. Blood was collected by cardiac puncture and was left at room temperature for 2 h for clot formation. Blood cells were separated from serum by centrifugation at 1000 *g* for 5 min, and the serum samples were stored at −20°C.

To determine whether antibodies to salivary proteins were present in the tick-immune animals, an IgG ELISA was performed, using adult *I. scapularis* saliva as the source of antigen. Saliva was collected from engorged adult female ticks. Fifty microliters of diluted saliva (5 µL saliva/mL of carbonate coating buffer [pH 9.3]) was used to coat 96-well microtitration plates (Titertek; ICN) for 1 h, washed 3 times with PBST, and then incubated again for 1 h. Wells were blocked with 2% bovine serum albumin in PBS for 1 h, and washed again in PBS with 0.1% Tween-20 (PBST). Diluted serum samples (1:100–1:100,000) from tick-immune rabbits or guinea pigs, in PBS with 2% bovine serum albumin, were added to the wells, incubated for 1 h, washed 3 times with PBST, and then incubated again for 1 h with alkaline phosphatase–conjugated goat anti-rabbit IgG or anti-guinea pig IgG (Sigma Chemical), at a dilution of 1:10,000. After the wells were washed 3 times with PBST, 50 µL of 5-nitrophenyl phosphate (Sigma Chemical) was added, was incubated for 30 min, and was read at 405 nm.

To assay active and passive tick immunity, groups of rabbits and guinea pigs either were actively sensitized by exposure to ticks, as mentioned above, or were made passively immune by intraperitoneal injection of 20 mL of tick-immune serum to tick-naïve animals. Each animal then was exposed to 30 nymphs, and the number of attached ticks was recorded daily. The weights of all unattached ticks and ticks fed to repletion also were noted. Erythema was measured for up to 3 days in a double-blind fashion, using a scale of 0–3 (0 represented the absence of erythema, and 1, 2, and 3 represented mild, moderate, and severe erythema, respectively), depending on the intensity of the reaction and the size of the affected area.

Construction and Screening of the *I. scapularis* cDNA Library

RNA from 1000 pairs of salivary glands from engorged *I. scapularis* nymphs was purified, using an RNA microisolation kit (Stratagene). For cDNA library construction, mRNA was purified from 250 µg of total RNA. cDNA was made, using specific oligo-dT primer; was amplified further, using 5′ rapid amplification of cDNA ends; and was made into a cDNA lambda Zap II expression library at Clontech (Palo Alto, CA) [41]. Approximately 1500 lambda phage plaques were grown on a lawn of *Escherichia coli* XL1-blue in 90-mm culture plates. Recombinant protein synthesis was induced with isopropyl-β-D-thiogalactopyranoside (Sigma Chemical) in a soaked nitrocellulose membrane for 3 h, and then the membrane was probed with tick-sensitized rabbit serum. As a control, duplicates of each plate were probed with normal rabbit serum. The immune serum identified 47 different clones from the salivary gland library. Individual plaques were plated for secondary and tertiary screening with immune serum and were purified to homogeneity. The lambda phages then were cotransfected with R408 helper phage, to excise the cDNA inserts from the clones. All sequencing was performed at the W. Keck DNA sequencing laboratory at the Yale University School of Medicine.

Separation of *I. scapularis* Salivary Gland Antigens by 2-Dimensional (2-D) Gel Electrophoresis

Fifty micrograms of total salivary gland proteins, dissolved in 6 M urea buffer, was run on an isoelectric focusing rod gel. The protein gel, separated according to charge in the first dimension, was placed on top of a 12% SDS-polyacrylamide gel and was electrophoresed for separation, according to size in the second dimension. The 2-D gels either were silver-stained (BioRad) or were transferred to nitrocellulose membranes for probing with tick-immune or normal rabbit serum samples. A large protein spot, recognized by the immune serum samples but not by the normal serum samples, was excised from the gel and perfused with trypsin. The resulting peptides were separated by reverse-phase high-performance liquid chromatography into 5 major peaks. One of these peaks was almost 100% pure and therefore was sequenced directly by N-terminal Edman degradation at the W. Keck protein chemistry laboratory at the Yale University School of Medicine.

Identification of Various mRNAs in Tick Salivary Gland

Expression of the genes identified by the screening of the salivary gland library was confirmed by reverse-transcription polymerase chain reaction (RT-PCR). Total RNA was isolated from the salivary glands of 20 partially engorged nymphs (after 66 h of feeding). cDNA was made from 1 µg of each RNA sample, using reverse transcriptase and oligo-dT primer. A negative control, in which reverse transcriptase was not added in the cDNA synthesis reaction for each sample, was always performed, to exclude the possibility
of DNA contamination. Specific primers (5′, bases 1–23, and 3′, last 23 bases) were used to amplify each of these genes from the cDNA made from engorged salivary glands. 3′ rapid amplification of DNA ends (RACE) was performed, according to the manufacturer’s manual (Clontech), with freshly isolated RNA from engorged nymphal salivary glands. These experiments determined whether the full-length clones had been identified.

Subcloning and Expression of Salp25D in pBAD/TOPO Thiofusion Expression System

PCR product synthesis and TA cloning. Specific 5′ and 3′ primers were made for the salp25D gene. pBluescript-inserts, excised from the lambda ZAP II expression library, were used as the DNA template for these reactions. A 50-μL reaction mixture containing 10 ng of DNA template, 10× PCR buffer (5 μL), 50 mM dNTPs (total, 0.5 μL), primers (1 μM each), sterile water, and Taq polymerase (1 unit) was placed in a thermal cycler for 30 cycles, with a denaturation temperature of 94°C (1 min), annealing temperature of 55°C (1 min), and extension temperature of 72°C (3 min). The PCR product was examined by agarose gel electrophoresis, and the DNA band was purified with a gel-purification kit (Qiagen). Ten nanograms of PCR product in 4 μL of sterile water was mixed with 1 μL of pBAD/Thio-TOPO vector (Invitrogen) and was incubated for 5 min at room temperature. Two microliters of the TOPO cloning reaction mixture was added into a vial containing 50 μL of One Shot competent cells (Invitrogen) and then was transformed by heat shock for 30 s at 42°C. One microliter of SOC medium (Invitrogen) was added to the transformed cells and incubated for 1 h in a shaker at 37°C, and then 50 μL of this culture was spread on a prewarmed plate and incubated overnight at 37°C.

Protein expression and purification. To analyze the expression of the recombinant Salp25D, a few colonies from the plate were grown in cultures at 37°C, with vigorous shaking, to an OD600 of 0.5. Arabinoase, at a final concentration of 0.02%, was added to the culture and was grown for 4 h. One-milliliter aliquots of the initial and the induced samples were pelleted at 10,000 g spin for 1 min and were boiled for 5 min in 100 μL of SDS-PAGE sample buffer. Five microliters of each sample was separated by SDS-PAGE. An extra band at 43 kDa, the expected size of the Salp25D fusion protein, appeared in the induced sample. Nickel affinity chromatography was used to purify the recombinant Salp25D. The protein was isolated under native conditions, in which cells first were lysed for 15 min on ice in 20 mM phosphate buffer (pH 7.8) containing 500 mM NaCl and hen egg lysozyme (100 μg/mL). Cells were sonicated and then were flash frozen in an ethanol-dry ice slurry and were thawed at 37°C for 3 cycles. The cell lysates then were treated with RNase (5 μg/mL) and with DNase (5 μg/mL) for 15 min at 30°C. Insoluble debris was removed by centrifugation at 3000 g for 15 min. The clear supernatant was passed over the nickel column, and the recombinant protein was eluted with the same buffer, at pH 6.0, containing 300 mM imidazole.

Glutathione Peroxidase Activity Assay

Glutathione peroxidase reduces H2O2 and oxidizes glutathione in a coupled reaction in the tissue. The oxidized glutathione requires NADPH and an enzyme, glutathione reductase, to return to its reduced form. Glutathione peroxidase activity can be measured in such a coupled reaction by determining the depletion of NADPH. A reaction mixture (total, 2.5 mL) containing 0.5 μmol of glutathione reductase (Sigma Chemical), 0.1 μmol of NADPH, 5 μmol of H2O2, 4.5 μmol of NaN3, 2 μmol of glutathione, and 50 μmol of Tris-HCl (pH 8.0) was used for all assays. Recombinant Salp25D in concentrations of 0.33, 0.66, and 1 mM and equimolar concentrations of thioredoxin were added to separate reaction mixtures at 25°C and were monitored for 5 min in a spectrophotometer at 340 nm, to decrease the NADPH concentration. A positive control of bovine glutathione peroxidase (0.1 mM; Sigma Chemical) and a blank with no proteins added also were used in this enzyme assay.

Accession Number

The sequence of the cDNA encoding all 14 genes was submitted to Genbank with the accession numbers depicted in table 1.

Results

Tick Immunity in Rabbits and Guinea Pigs

Several groups have demonstrated that laboratory animals can acquire resistance to tick bites. We exposed guinea pigs and rabbits to multiple tick infestations to develop tick-immune animals and generated tick-immune serum samples with which to probe an I. scapularis salivary gland cDNA expression library. Rabbits and guinea pigs were each infested with 100 I. scapularis nymphs 3 times over a 9-week period. To accurately assess the development of tick immunity, groups of 3 tick-sensitized animals then were challenged with 50 I. scapularis nymphs per animal, and the duration of tick attachment, the weight of the recovered ticks, and the appearance of erythema at the bite sites were recorded. Ticks did not readily attach to and enorge on these tick-sensitized animals, compared with tick-naïve (control) animals (figure 1). Furthermore, erythema

| Table 1. Antigenic Ixodes scapularis salivary proteins. |
|----------------|----------------|----------------|----------------|
| Protein       | No. of clones | Molecular mass, kDa | pI             | Genbank accession no. |
| Salp9         | 3             | 8.8              | 9.5            | AF278574               |
| Salp10        | 4             | 10.4             | 8.9            | AF278575               |
| Salp13        | 2             | 12.7             | 6.3            | AF209912               |
| Salp14        | 22            | 13.9             | 9.2            | AF209921               |
| Salp15        | 1             | 14.7             | 9.7            | AF209914               |
| Salp16A       | 1             | 16.0             | 8.9            | AF209915               |
| Salp17        | 1             | 17.2             | 9.2            | AF209916               |
| Salp20        | 2             | 20.4             | 4.5            | AF209917               |
| Salp25A       | 5             | 25.4             | 9.5            | AF209922               |
| Salp25B       | 1             | 25.4             | 9.6            | AF209918               |
| Salp25C       | 1             | 25.3             | 8.7            | AF209913               |
| Salp25D       | 2             | 24.6             | 5.7            | AF209911               |
| Salp26A       | 1             | 26.4             | 4.4            | AF209919               |
| Salp26B       | 1             | 25.7             | 9.4            | AF209920               |

NOTE: An I. scapularis salivary gland cDNA expression library was probed with serum samples from tick-immune rabbits, which recognized 47 of the 100,000 plaques screened. These plaques encoded 14 genes, which then were characterized. All the genes, except salp25D, had an N-terminal secretory signal sequence. pI, isoelectric point.
was observed at the tick-bite sites of tick-sensitized, but not control, animals (figure 1).

The humoral response to tick infestation then was examined. Both tick-immune rabbits and guinea pigs developed antibodies to *I. scapularis* antigens, detectable by ELISA at a serum dilution of 1:1000, with tick saliva used as the substrate. To confirm that tick immunity was mediated, at least in part, by antibodies, serum samples from tick-immune guinea pigs or rabbits were passively transferred to tick-naive animals, which then were infected with *I. scapularis* nymphs. As expected, passive transfer of immune serum partially protected guinea pigs or rabbits from tick infestation (figure 1; *P* < .05, Student’s *t* test). This effect, however, was not as striking as that seen with active tick sensitization. Animals passively administered immune serum also developed erythema at the sites of tick attachment, which was similar in intensity to the erythema following active sensitization (figure 1). Twenty-five milliliters of sera from tick-immune rabbits and guinea pigs then was collected for screening of an *I. scapularis* salivary gland expression library, to characterize tick salivary antigens that elicit humoral responses in the host.

**Identification of the Genes Encoding *I. scapularis* Salivary Gland Antigens That Elicit Antibodies in the Host**

An *I. scapularis* salivary gland cDNA expression library was probed with serum samples from tick-immune animals to identify proteins that elicit host antibodies. Screening of 60,000 plaques with tick-immune guinea pig serum yielded only 3 plaques in an earlier study [41]. All 3 plaques encoded salp16, a feeding-inducible gene that has been characterized elsewhere [41]. Further screening of the library with guinea pig serum was complicated by the large degree of background reactivity of tick-immune guinea pig serum with the phage library. Therefore, additional immunoscreening was performed with the serum samples from tick-immune rabbits. Immunoscreening of 100,000 plaques from the cDNA library with tick-immune rabbit serum identified 47 clones that specifically bound to host antibodies. Each clone then was purified to homogeneity and was retested for reactivity, and the rescued plasmids from these clones revealed inserts with a range of 0.5–1.1 kb. On the basis of common DNA sequences in the inserts and cross-hybridization on Southern blot, these 47 plaques were assembled in 14 groups that synthesized gene products that reacted with antibodies in tick-immune rabbit serum. These 14 genes were named according to the calculated molecular mass of the putative proteins (table 1), and a description of each antigen is provided below.

*I. scapularis* salivary protein 9 (salp9). Three of the 47 recovered plasmids contained a 243-bp open-reading frame (ORF) encoding a protein with a molecular mass of 8.8 kDa, an isoelectric point (pI) of 9.5, and an 18-aa signal sequence. Salp9 showed 50% similarity to an anticomplement *I. scapularis* protein, ISAC [42], in a BLAST (Basic Local Alignment Search Tool; National Center for Biotechnology Information) search of the Genbank database.

*I. scapularis* salivary protein 10 (salp10). Four of 47 plaques...
contained a 288-bp gene encoding a 10.4-kDa protein with a 17-aa signal sequence and a pI of 8.9. A BLAST search of the Genbank database revealed homology with several Kunitz-type protease inhibitors and coagulation inhibitors.

I. scapularis salivary protein 13 (salp13). Two plasmids had a 342-bp ORF encoding a 12.7-kDa protein with a 21-aa signal sequence and a pI of 6.3. BLAST search of the Genbank database showed weak similarity with the transforming growth factor (TGF)-β superfamily of proteins.

I. scapularis salivary protein 14 (salp14). Twenty-two clones contained a 375-bp ORF encoding an acidic (pI 5.7) 24.6 kDa protein. BLAST search of the Genbank database did not yield similarities with known proteins.

I. scapularis salivary protein 15 (salp15). One clone contained a 408-bp gene encoding a 14.7-kDa basic protein with a 20-aa signal sequence and a pI of 9.7. Weak homology was found in the active motif regions of inhibin A, a member of the TGF-β superfamily.

I. scapularis salivary protein 16A (salp16A). One clone had a 432-bp ORF encoding a 16.0-kDa basic protein with a 38-aa signal sequence and a pI of 8.9. The protein had 10 cysteine residues. A BLAST search of the Genbank database was unrevealing. This protein is different from Salp16, the first feeding-inducible I. scapularis salivary gland antigen that was described previously [41], and is therefore named Salp16A.

I. scapularis salivary protein 17 (salp17). One clone contained a 450-bp ORF, encoding a 17.2-kDa basic protein with a 20-aa signal sequence and a pI of 9.2. Genbank database searches did not show homology with other proteins.

I. scapularis salivary protein 20 (salp20). Two clones contained the same 549-bp ORF, encoding a 20.4-kDa acidic (pI 4.5) with a 22-aa signal sequence. BLAST search of the Genbank database revealed 83% homology with an anticomplement protein, ISAC, from I. scapularis [42].

I. scapularis salivary protein 25A (salp25A). Five clones had inserts containing a 666-bp ORF. The basic protein has a molecular mass of 25.4 kDa, a 20-aa signal sequence, and a pI of 9.5. BLAST search of the Genbank database was unrevealing.

I. scapularis salivary protein 25B (salp25B). One clone, containing a 666-bp ORF, encoded a 25.5-kDa protein, with a pI of 9.6, and a 21-aa signal sequence. BLAST search of the Genbank database showed similarities with Rhipicephalus histamine binding proteins [43].

I. scapularis salivary protein 25C (salp25C). One clone contained a 663-bp ORF. The 25.3-kDa protein had a 21-aa signal sequence and a pI of 8.7. The protein sequence had homology with the histamine-binding protein from Rhipicephalus ticks [43]. Profilescan of this protein with the Prosite database confirmed the presence of histamine-binding motifs.

I. scapularis salivary protein 25D (salp25D). One clone contained a 663 bp ORF, encoding an acidic (pI 5.7) 24.6 kDa protein. BLAST search revealed homology with invertebrate and vertebrate glutathione peroxidases.

I. scapularis salivary protein 26A (salp26A). One clone contained a 699 ORF, encoding a 26.4 kDa protein with an 18 aa signal sequence and a pI of 4.4. BLAST search of the Genbank database did not reveal significant similarities with other proteins.

I. scapularis salivary protein 26B (salp26B). One clone contained a 654-bp ORF. The 25.7-kDa protein had an 18-aa signal sequence and a pI of 9.4. No similarities were found in the Genbank database.

Identification of I. scapularis Salivary Gland Antigens by 2-D Gel Electrophoresis

Two-dimensional gel electrophoresis also was used to directly identify antigenic I. scapularis salivary gland components. Fifty pairs of freshly dissected salivary glands from engorged nymphs were boiled in sample buffer and then were separated by molecular mass and pI in 2-D gel electrophoresis (figure 2A). The proteins were transferred to nitrocellulose membranes and were probed with tick-immune or normal (control) rabbit serum (figure 2B and 2C). Compared with control serum, tick-immune serum recognized several protein spots in the range of 26–100 kDa, and a large spot at ~26 kDa was prominent. This antigen was cut out of the denaturing gel and was digested with trypsin, and the resulting fragments were sequenced by Edman’s degradation. One fragment yielded a FFFENGER sequence, which was used to synthesize degenerate primers. 5’ RACE and 3’ RACE reactions then were performed on salivary gland mRNA from engorged nymphs to amplify the mRNA of the specific gene. The ORF of this mRNA was found to be salp14, which has been described in table 1 and accounted for 22 of the 47 identified plaques. Small quantities of salivary gland extract limited the assessment of additional proteins that reacted with tick-immune serum samples and that were present in minute quantities in the 2-D gel electrophoresis.

![Figure 2](image-url)
Identification of Various salp mRNAs in I. scapularis
Salivary Glands

Tick mRNA then was used to examine the expression of the genes encoding salivary antigens, to substantiate the immuno-screening. Direct expression of the genes identified by the library screening was assessed by RT-PCR. Total RNA was isolated from salivary glands of unfed and partially engorged nymphs (after 66 h of feeding) to make cDNA, using reverse transcriptase and oligo-dT primer. Twenty pairs of salivary glands from fed nymphs and 100 pairs of glands from unfed nymphs were used to extract the RNA. Two micrograms of cDNA from unfed and partially engorged samples was used to amplify the genes. A negative control, in which reverse transcriptase was not added in the cDNA synthesis reaction for each sample, was always performed, to determine whether any DNA contamination was present. Three of the 14 genes (salp20, salp25D, and salp26B) were expressed in unengorged salivary glands, and all 14 genes were expressed in engorged glands (figure 3). The gene salp20 was detected by RT-PCR in unfed nymphs, and expression increased in engorged nymphs (figure 3). Expression levels of salp25D and salp26B appeared to be similar in the salivary glands of both unengorged and engorged nymphs (figure 3).

Glutathione Peroxidase Activity of Recombinant
I. scapularis Salp25D

Because of striking homology (70%) of Salp25D with glutathione peroxidases from various eukaryotic organisms (figure 4A), we determined the activity of Salp25D in a glutathione peroxidase assay. First, Salp25D was expressed in pBad-Topo-Thio expression vector with thioredoxin as the fusion partner and a C-terminal His-tag for affinity purification. The recombinant antigen was soluble, which facilitated the purification of the protein by use of the nickel resin (figure 4B). Glutathione peroxidase activity then was assayed by measuring consumption of NADPH in the presence of reduced glutathione and glutathione reductase. The activities of a positive control of bovine glutathione peroxidase (0.1 mmol) and a negative control of thioredoxin fusion partner (1 mmol) were compared with those of 0.33, 0.66, and 1 mmol/mL of Salp25D fusion protein (figure 4C). Bovine glutathione peroxidase at 0.1 mmol took ~100 s to cleave the substrate (H2O2), compared with ~200 s taken by recombinant Salp25D at a concentration of 1 mmol in the reaction mixture.

Discussion

Although acquired resistance against I. scapularis involves both humoral and cellular responses to the vector, the nature of the antigens that elicit I. scapularis immunity is not known. We now have characterized 14 immunodominant I. scapularis salivary gland proteins that are recognized by tick-immune host serum. These antigens vary in mass from 9 to 26 kDa, and 2 of them, Salp20 and Salp25D, show substantial homology (83% and 70%, respectively) to known proteins. Salp20 is a homologue of an anticomplement protein from I. scapularis [42], and Salp25D has homology to antioxidant proteins across the phyla. Salp25B and Salp25C also have some homology with the histamine-binding proteins from Rhipicephalus ticks [43]. All of these 14 antigens, except Salp25D, contain N-terminal hydrophobic secretory signal sequences, followed by a signal peptidase site. This suggests that these proteins are synthesized in the salivary gland and then are secreted into the saliva. Salp25D does not have a secretory signal sequence; however, since the tick-immune host develops antibody against this protein, Salp25D also must be present in the tick saliva. Salp25D may be synthesized by different types of glandular acinar cells with holocrine or apocrine secretion [44] and may be incorporated into the saliva by cell degradation or leakiness. Alternatively, Salp25D may be secreted via an unknown pathway.

RT-PCR amplification of the 14 genes from salivary gland mRNA of unfed and fed ticks helped to determine which genes...
Glutathione peroxidase activity of recombinant *Ixodes scapularis* Salp25D. Top, Amino acid sequence alignment of Salp25D with selected bovine, human, and nematode glutathione peroxidase homologues. Consensus lines summarize comparisons of the sequence alignments listed above: Asterisks (*) indicate identical or conserved residues in all sequences in the alignment; colons (:), conserved substitutions; periods (.), semiconserved substitutions; and hyphens (-), gaps. Genbank accession nos. for the bovine, human, and nematode antioxidant proteins are O77834, P30041, and AAB83998, respectively. Bottom left, Expression and purification of recombinant Salp25D as a thioredoxin fusion protein. The salp25D gene was inserted in frame with the thioredoxin gene and was induced with arabinose. Cells were sonicated, and the soluble fraction (supernatant) was used to purify the protein by affinity column chromatography. I, Supernatant from lysed induced cells; U, supernatant from lysed uninduced cells; P, purified Salp25D fusion protein. Bottom right, Glutathione peroxidase activity assay. A positive control of bovine glutathione peroxidase (GPX; 0.1 mmol in the reaction mixture) and a negative control of thioredoxin (TR; 1 mmol) also were assayed and were compared with thioredoxin-Salp25D fusion protein (TR-Salp25D; 0.33, 0.66, and 1 mM). One representative assay, of the 4 experiments performed with similar results, is shown.

were induced following engorgement. The genes *salp20*, *salp25D*, and *salp26B* were expressed in the salivary glands of both unfed and fed ticks, whereas mRNA for all other genes was detected only after feeding. The gene *salp20* was detected at low levels in the salivary gland of unfed ticks, but engorgement induced its expression further. Salp20 may be a homologue of an *I. scapularis* anticomplement protein, ISAC, since they share 83% identity and 90% similarity at the protein level and 91% homology at the DNA level [42]. Salp9 also shows 50% similarity with ISAC [42]. Thus, it is possible that Salp20 and Salp9 also are anticomplement proteins secreted in the saliva in response to the incoming blood meal. In addition, Salp25B and Salp25C, which have histamine-binding motifs, also are induced during engorgement, which suggests their role during the feeding process. We know that Salp25D is an enzyme (figure 4) that may have detoxification function both in vector salivary gland and in the host tissue. The other 9 gene products may be synthesized during feeding to help the processes of feeding and evasion of host immune response, using mechanisms that have yet to be described.

The *I. scapularis* salp25D cDNA contains a single ORF encoding a 222-aa protein with a molecular mass of 25 kDa. A wide variety of antioxidant proteins across the phyla have
strong homology with Salp25D. A representative comparison of Salp25D with antioxidant proteins from cows, humans, and a nematode reveals the conservation of the sequence during evolution (figure 4A). The sequence of Salp25D has 75% similarity with *Drosophila* and bovine glutathione peroxidases [45] and 70% similarity with a human keratinocyte antioxidant protein [46]. The protozoan *Plasmadium falciparum* antioxidant protein (Genbank accession no., AAG14353; 52% identity) and prokaryotic *Pseudomonas putida* antioxidant protein (50% identity; see [47]) also have significant homology with *I. scapularis* Salp25D.

Reactive oxygen species (O$_2^*$, H$_2$O$_2$, and HO·) generated during metabolism can cause cellular damage [45]. Glutathione, with the help of glutathione peroxidase and glutathione reductase, acts as a part of the antioxidant system to protect tissues. During a local tissue injury, such as a wound, reactive oxygen is synthesized in the necrotic tissue by the leukocytes that migrated to the wound site [48]. This, in turn, helps in wound healing. Since *I. scapularis* requires an extended feeding time on a host (4–6 days), one of its strategies to evade host immunity would be to minimize the formation of reactive oxygen species during tick bite and attachment, and thus Salp25D may help in the tick-feeding process. On the other hand, tick-sensitized hosts may mount antibody against Salp25D to influence future tick bites, potentially accounting for tick-immune serum’s recognition of the clones containing salp25D cDNA.

Ticks’ saliva contains anticoagulant, anticomplement, and immunosuppressive activities, which help them during the long engorgement process and help them evade host immune responses [28, 34, 37–40]. Valenzuela et al. have recently described a complement inhibitory protein, named ISAC, from *I. scapularis* [42]. Another notable immunosuppressive pathway working through complement inhibition, a lectin-binding activity of the saliva, has been demonstrated in a related tick species [49]. An immunosuppressant protein from *D. andersoni* salivary gland also has been reported [50]. In this paper, we show the activity of a salivary protein to be antioxidant, which may help the vector to evade host immune responses. Moreover, we have provided the first systematic categorization of the genes encoding *I. scapularis* salivary gland antigens that elicit antibodies in the host. It is likely that salivary gland antigens may play an important role in tick immunity; however, antigens in other tissues also can be important. For example, immunization of cattle with an antigen (Bm86) expressed in the gut of *Boophilus microplus* can interfere with tick feeding [51]. The characterization and determination of the activities of prominent salivary gland antigens may lead to a better understanding of the vector-host interactions and the development of specific antigen vaccines against *I. scapularis*.

**References**

26. Inokuma H, Kemp DH, Willadsen P. Comparison of prostaglandin E2 (PGE2) in salivary gland of *Boophilus microplus*, *Haemaphysalis longi-


