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The alternative pathway of complement is an important defense against pathogens and in tick rejection reactions. The tick *Ixodes scapularis* is able to feed repeatedly on its natural host and has a salivary anticomplement activity that presumably facilitates feeding. In this study, we purified and then obtained the amino-terminal sequence of the *I. scapularis* salivary anticomplement (Isac). We found a full-length clone coding for Isac by random screening of a salivary gland cDNA library. Expressing Isac cDNA in COS cells reproduced the activity found in tick saliva, namely, inhibition of rabbit erythrocyte lysis by human serum in the presence of Mg^{2+} and EGTA, inhibition of C3b binding to agarose in the presence of Mg^{2+} and EGTA, and acceleration of factor Bb uncoupling from the C3 convertase generated by the alternative pathway. Recombinant Isac had no effect on the recalcification time of human platelet-poor plasma or in the classical complement pathway, indicating that it is a specific inhibitor similar to the regulators of complement activation of the alternative pathway such as factor H. Isac, however, has no similarity to any protein in the GenBank™ data base, indicating that it is a novel and relatively small (18.5 kDa) anticomplement molecule.

The alternative pathway of complement is an evolutionarily old first line of defense against pathogens (1). Complement activation leads to the production of inflammatory anaphylatoxins (2, 3) and to the formation of a membrane attack complex leading to the lysis of the invading organism (1). Endogenous regulators exist to prevent pathology associated with unconfined or inadvertent complement activation (4, 5). Successful pathogens have developed several mechanisms to evade the host complement system (1, 6, 7). In several of these evasion mechanisms, pathogens may recruit host complement regulatory molecules to their own surface or produce inhibitors of complement activation, which are either secreted or remain associated with their surfaces (1, 6–8).

Ticks are ectoparasites that may feed for several days or even weeks with their mouth parts embedded into their vertebrate hosts. Although unnatural hosts can mount an effective immune response against ticks (9, 10), only minor rejection reactions are observed when ticks feed on their natural hosts (9, 11). The alternative pathway of complement was implicated in rejection reactions of guinea pigs against the tick *Dermacentor andersoni* (12, 13). The tick vector of Lyme disease in Eastern North America, *Ixodes scapularis,* can successfully feed repeatedly on its natural host, the white-footed mouse, *Peromyscus leucopus* (9, 14), perhaps because it has salivary compounds that deactivate anaphylatoxins (15) and inhibit the alternative pathway of complement (16). Indeed, the host range of *Ixodes* correlates with their ability to counteract the alternative complement pathway of their most common hosts (17).

I. scapularis saliva has an inhibitor of the alternative pathway of complement activation (16), as shown by inhibition of the lysis of rabbit erythrocytes by human sera in the presence of EGTA and Mg^{2+} ions. This saliva also prevents deposition of C3[1] to agarose-coated plates and inhibits release of C3a by the C3 convertase formed by the alternative pathway of complement activation (16). Because the molecular nature of this inhibitor remained unknown, we purified, obtained an amino-terminal sequence, cloned, and expressed Isac, which is a novel molecule behaving as a regulator of complement activation. Isac is one of the smallest proteins known to have regulator of complement activation activity and may serve as a tool to understand C3 convertase regulation.

MATERIALS AND METHODS

Ticks—Tick saliva was obtained by inducing partially engorged adult female *I. scapularis* to salivate into capillary tubes using the pilocarpine induction method (18). Briefly, ticks engorging for 4–5 days on the ears of New Zealand White rabbits were harvested, rinsed in distilled water, and fixed to glass microscope slides with double-sided tape. A sterile glass micropipette was placed around the tick hypostome to collect saliva. Salivation was induced by applying 2 μl of pilocarpine (50 mg/ml in 95% ethanol) to the scutum of the tick. Ticks were incubated at 35 °C in a humid chamber until salivation ceased (2–3 h). Volumes ranged from 2.5 to 10 μl/tick. Tick salivary gland extracts were prepared by collecting glands from partially engorged female *I. scapularis* as described. Glands were dissected by first bisecting the tick and then teasing the salivary glands away from the other internal organs and the tick exoskeleton. Glands were rinsed by immersion in HEPES-saline buffer (10 mM HEPES, pH 7.0, 150 mM NaCl) and stored frozen at −75 °C until needed.

Reagents—Water was purified with a Milli-Q system from Millipore (Bedford, MA). Organic reagents were obtained from Sigma. Goat anti-human C3 antibody (catalog no. C-7761) was from Sigma. Goat anti-human factor B (catalog no. 31-785) was from Nordic Immunology

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[1] The abbreviations used are: C3, native complement component 3 possessing an intact thioester and capable of participating in complement activation; C3b, active fragment of C3; Isac, *Ixodes* salivary anticomplement; fB, factor B; HPLC, high pressure liquid chromatography; PCR, polymerase chain reaction; r-Isac, recombinant Isac; c-Isac, control Isac (reverse orientation).

(Tiburg, Netherlands). Rabbit anti-goat IgG, peroxidase conjugated and adsorbed against other human serum proteins (catalog no. A-4174, lot 78H9200), and anti-sheep erythrocyte stroma sera (part no. S 1389) were from Sigma. Agarose (ultraPure; Life Technologies, Inc.) was used to coat Immulon I microplates (Dynatech Laboratories, Inc., Alexandria, VA).

Erythrocyte Lysis Assays—To test for the alternative pathway of complement activation using rabbit erythrocytes and human serum (19), rabbit erythrocytes in Alsever's solution (Spring Valley Labs, Woodbine, MD) were washed five times with 10 vol of 150 mM NaCl, 10 mM HEPES, pH 7.4 (HEPES saline), followed by centrifugation for 2 s at 10,000 × *g*. Reaction medium, in a 96-well plate, contained 0.4% packed erythrocytes in 40 μl of 6.25 mM EGTA, 2.5 mM $MgCl_2$, 20 mM HEPES, pH 7.4, and 150 mM NaCl. Additionally, 5 μl of the test sample or HEPES saline control was added. In some cases, a larger amount of test sample was added, with corresponding changes in the buffer aliquot to keep the same concentrations of buffers, divalent cations, and chelators. A reaction was started with the addition of 5 μl of human serum. Control reactions were run in which 12.5 mM EDTA was substituted for EGTA and $MgCl_2$. The plate was added to a Thermomax plate reader (Molecular Devices, Menlo Park, CA) set at 37 °C and was monitored at both 405 and 650 nm. The resulting value representing $[A_{405} - (A_{650} \times 1.5)]$ was plotted, and the time to achieve one-half of maximal lysis was determined from the curve. Whereas the absorbance at 405 nm reflects the hemoglobin released by the cells, the absorbance at 650 nm controls for the turbidity change in the microwells. The value of 1.5 corrects for the decreased light scattering at the larger wavelength. In some cases, end point assays were done, and the value representing the absorbance at 405 nm corrected for turbidity as above being taken as a measure of erythrocyte lysis.

Assays to measure classical pathway lysis of sheep erythrocytes using anti-sheep erythrocyte antibody followed manufacturer's instructions (Sigma). Briefly, washed sheep erythrocytes (50 μl of packed erythrocytes/ml) were incubated with 1:100 dilution of the sera for 20 min at 37 °C and stored at 4 °C until needed. The remainder of the assay was identical to that described above to detect alternative pathway-dependent lysis of rabbit erythrocytes as described above, except that 2 mM $CaCl_2$ was used instead of EGTA.

Enzyme-linked Immunosorbent Assays for Measuring C3 and Factor B (fB)—To more specifically identify inhibitory activity within the complement cascade, we measured complement deposition to agarose-coated wells in 96-well plates. Agarose activates the alternative pathway of complement (20). Plates were pretreated with 100 μl of 0.1% agarose in water (melted by boiling and kept at 50 °C) and incubated at 37 °C for 48 h, when dry. Wells were then incubated with 50 μl of 20 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM EGTA, 2 mM $MgCl_2$, and 10% human serum, containing 5 μl of the test solution or HEPES saline. Control wells were run without serum or agarose coating, or 10 mM EDTA was substituted for $MgCl_2$ and EGTA. We chose to use the EDTA method as a routine blank for these assays. To reveal C3 or fB binding to the plate, plates were washed five times (for 3 min each washing, with slow orbital shaking of the plate) with 200 μl of HEPES saline containing 2 mM $MgCl_2$ and 10 mg/ml bovine serum albumin, followed by the addition of 100 μl of a 1:5000 dilution of the anti-C3 or 1:250 dilution of the anti-fB antibody. These were incubated for 1 h at 37 °C, washed five times as before, and the anti-goat peroxidase conjugate was added at 1:5000 dilution. Following incubation for 1 h at 37 °C, the conjugate was washed twice each with HEPES saline containing 10 mg/ml bovine serum albumin, then 0.1% Tween 20, then normal saline. Peroxidase activity was detected by adding a solution of HEPES saline containing 1 mg/ml orthophenylene-diamine (previously diluted in methanol to give 100 mg/ml) and 8.8 mM hydrogen peroxide. The rate of increase in absorption at 405 nm was measured in a Thermomax (Thermo Separation Products, Rivera Beach, FL) plate reader.

Human Plasma Recalcification Assay—To measure recalcification time of human plasma, 30 μl of human citrated plasma (0.38% final citrate concentration) was mixed with equal volumes of either test samples or HEPES saline in a 96-well plate. Reactions were initiated by adding 30 μl of 25 mM $CaCl_2$ to each mixture. The increase in turbidity at 650 nm was monitored in a Thermomax plate reader (Thermo Separation Products). Clotting time was determined as the time to reach 0.025 absorption units.

High Performance Liquid Chromatography (HPLC) Procedures—Thermomax (Thermo Separation Products, Riviera Beach, FL) CM4000 or CM4100 pump was used in conjunction with a dual wavelength UV-visible detector model SM4100 from the same company. Molecular sieving chromatography used a TSK-SW 2000 column (1 x 80 cm for purification purpose or 0.43 × 25 cm for analytical purpose) (TosoHass, Montgomeryville, PA), eluted with 10 mM HEPES, pH 7.0, 150 mM NaCl at the specified flow rates. Reverse-phase chromatography used one or two PRP infinity columns linked in tandem and eluted at 0.5 ml/min with the indicated gradients of acetonitrile in 8 mM HCl or trifluoroacetic acid. HCl, as a modifier in reverse-phase chromatography, protonates and neutralizes the negatively charged protein carboxyl groups, thus increasing their hydrophobicity, but fails to form significant ion pairs of the positively charged amino groups with the chloride anion. Trifluoroacetic acid similarly causes protein protonation, but the anion forms significant ion pairs with the positively charged amino groups, and these ion pairs have increased hydrophobicity and consequent increased retention time. As a result, when a peptide mixture is run in reverse-phase using HCl and trifluoroacetic acid consecutively, the order of peptide elution may change according to the differences in the charge of basic residues. Because trifluoroacetic acid elution gives rise to sharper peaks, it is best that HCl elution is done first followed by trifluoroacetic acid elution, where the peaks are most concentrated. Silica-based octadecyl columns lead to complete loss of anticomplement activity. Fractions of the reverse-phase columns were dried in a Speed-Vac-SC 110 (Savant Instruments, Inc., Holbrook, NY) in the presence of 10 μg of bovine serum albumin (in 10 μl of water) to prevent irreversible protein adsorption to the walls of the plastic tubes. Dried samples were resuspended in buffer.

Edman Degradation—Amino-terminal sequencing of the HPLC-purified proteins was performed at the Harvard microsequencing facility under the direction of Dr. William Lane.

Salivary Gland cDNA Library Construction—*I. scapularis* salivary gland mRNA was isolated from 25 pairs of salivary glands dissected from adult female ticks feeding for 3–4 days on a rabbit. We used the Micro-FastTrack mRNA isolation kit (Invitrogen, San Diego, CA), which yielded a total of 200 ng of poly(A)$^+$ mRNA. The polymerase chain reaction (PCR)-based cDNA library was made following the instructions for the SMART cDNA library construction kit (CLONTECH, Palo Alto, CA). The *I. scapularis* salivary gland mRNA (200 ng) was reverse-transcribed to cDNA for 1 h at 42 °C using Superscript II RNase H$^-$ reverse transcriptase (Life Technologies, Inc.) and the CDS/3′-primer (CLONTECH). Second strand synthesis was performed using a PCR-based protocol with the SMART III primer (CLONTECH) as the sense primer and the CDS/3′-primer as antisense primer; these two primers contain, at the ends of the nascent cDNA, *Sfi*I A and B sites, respectively. For double-stranded cDNA synthesis, we used a 9700 Thermalcycler (Perkin-Elmer Corp.) and Advantage Klen-*Taq* DNA polymerase (CLONTECH). PCR conditions were: 94 °C for 2 min; 18 cycles of 94 °C for 10 s, and 68 °C for 6 min. Double-stranded cDNA was immediately treated with proteinase K (0.8 μg/μl) for 20 min at 45 °C and washed three times with water using Amicon filters with a 100-kDa cut-off (Millipore Corp.). The double-stranded cDNA was digested with *Sfi*I for 2 h at 50 °C and then fractionated using columns provided by the manufacturer (CLONTECH). Fractions containing cDNA of more than 400 base pairs were pooled, concentrated, and washed three times with water using an Amicon filter with a 100-kDa cut-off. The cDNA was concentrated to a final volume of 7 μl. The concentrated cDNA was ligated into a Lambda Triplex2 vector (CLONTECH), and the resulting ligation reaction was packed using Gigapack gold III from Stratagene/Biocrest (Cedar Creek, TN) following the manufacturer's specifications. The library obtained was plated by infecting log-phase XL1-blue cells (CLONTECH), and the percentage of recombinants was determined by PCR using vector primers flanking the inserted cDNA visualized on a 1.1% agarose gel with ethidium bromide (1.5 μg/ml).

Sequence of Isac—To isolate and sequence cDNA expressed by the salivary glands of *I. scapularis*, we developed a protocol intended to randomly match the cDNA to the amino terminus of the reverse-phase HPLC-purified Isac. We plated the *I. scapularis* salivary gland cDNA library at a density of ~200 plaques/plate (150-mm Petri dish). The plaques were picked randomly and transferred to a 96-well polypropylene plate containing 100 μl of water/well. The plate was covered and placed on a gyrator shaker for 1 h at room temperature. Five μl of the phage sample was used as a template for a PCR reaction to amplify random cDNA. The primers for this reaction were sequences from the triplEX2 vector, named PT2F1 (5′-AAG TAC TCT AGC AAT TGT GAG C-3′), which is positioned upstream of the cDNA of interest (5′-end), and PT2R1 (5′-CTC TTC GCT ATT ACG CCA GCT G-3′), which is positioned downstream of the cDNA of interest (3′-end). High fidelity platinum *Taq* polymerase (Life Technologies, Inc.) was used for these reactions. Amplification conditions were: 1 hold at 75 °C for 3 min, 1 hold at 94 °C for 3 min, and 34 cycles at 94 °C for 30 s, 49 °C for 30 s, and 72 °C for 1 min 20 s. Amplified products were visualized on a 1.1% agarose gel with ethidium bromide. The concentration of double strand cDNA was

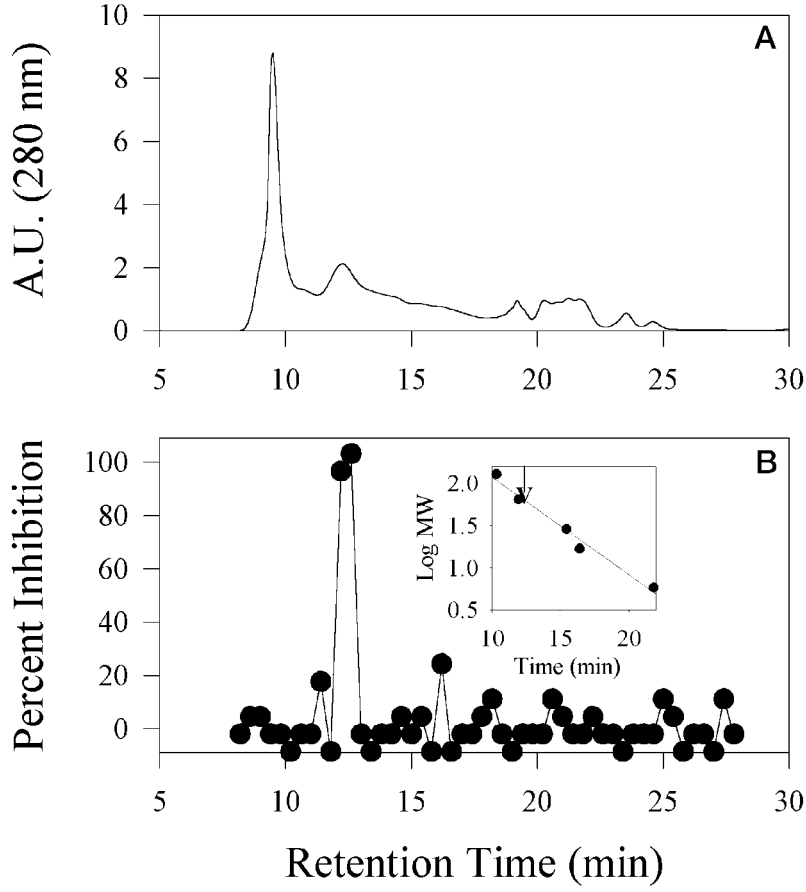

FIG. 1. **Molecular sieving chromatography of homogenized *I. scapularis* tick salivary glands.** *A,* cumulative UV (280 nm) absorbance tracings of 10 chromatograms representing 500 homogenized pairs of glands. *Inset,* log of M_r standards plotted against their retention times. The *arrow* indicates retention time of the salivary anticomplement activity. *B,* anticomplement activity was measured in 12.5-μl aliquots of each fraction by their ability to inhibit lysis of rabbit erythrocytes by human serum in the presence of $MgCl_2$.

measured using Hoechst dye 33258 on a Flurolite 1000 plate fluorometer (Dynatech Laboratories, Chantilly, VA). PCR reaction (3–4 μl) containing between 100 and 200 ng of DNA were treated with exonuclease I (0.5 unit/μl) and shrimp alkaline phosphatase (0.1 unit/μl) for 15 min at 37 °C and 15 min at 80 °C on a 96-well PCR plate. This mixture was used as a template for a cycle sequencing reaction using the DTCS labeling kit from Beckman Coulter Inc. (Fullerton, CA). The primer used for sequencing (PT2F3) is upstream of the inserted cDNA and downstream of the primer PT2F1. The sequencing reaction was performed in a Perkin-Elmer 9700 Thermalcycler. Conditions were 75 °C for 2 min, 94 °C for 4 min, and 30 cycles at 96 °C for 20 s, 50 °C for 20 s, and 60 °C for 4 min. After cycle-sequencing the samples, a cleaning step was done using the multiscreen 96-well plate-cleaning system (Millipore); this plate was prepared by adding a fixed amount (manufacturer's specification) of Sephadex-50 (Amersham Pharmacia Biotech) and 300 μl of deionized water. After 1 h of incubation at room temperature, the water was removed from the multiscreen plate by centrifugation at 750 × *g* for 5 min. After partially drying the Sephadex in the multiscreen plate, the whole cycle sequencing reaction was added to the center of each well and centrifuged at 750 × *g* for 5 min, and the clean sample was collected on a sequencing microtiter plate (Beckman Coulter, Inc.). The plate was dried using a SpeedVac SC 110 with a microtiter plate holder (Savant Instruments Inc.). The dried samples were immediately resuspended with 25 μl of deionized ultraPure formamide (J. T. Baker, Phillipsburg, NJ), and one drop of mineral oil was added to the top of each sample. Samples were either sequenced immediately on a CEQ 2000 DNA sequencing instrument (Beckman Coulter Inc.) or stored at −30 °C.

Isolation of Isac and Construction of Expression Vector—We identified the cDNA with an open reading frame matching the amino terminus of the reverse-phase HPLC-purified Isac. An aliquot (~100 ng) of Isac PCR sample was reamplified using the PT2F1 and PT2R1 primers (conditions as above, but only 25 PCR cycles), and the entire cDNA was fully sequenced as described above on a CEQ 2000 using custom primers. Isac cDNA was cloned into a TOPO TA cloning vector (Invitrogen). For expression of Isac, full-length cDNA was used as a template to amplify only the cDNA fragment that began with the initial methionine and ended at the first stop codon. The single product amplified was immediately cloned into the eukaryotic cloning vector pcDNA3.1/V5/His-TOPO (Invitrogen) following the manufacturer's specifications. The ligation mixture was used to transform TOP10 cells (Invitrogen), and the cells were incubated overnight at 37 °C. Eight colonies were selected and mixed with 10 μl of sterile water. Five μl of each sample were transferred to Luria broth (LB) containing ampicillin (100 μg/ml) and grown at 37 °C. The other 5 μl were used as a template for a PCR reaction using two vector-specific primers from the pcDNA3.1/V5/His-TOPO vector to confirm the presence of the insert and for sequencing analysis. After visualizing the PCR product on a 1.1% agarose gel, we completely sequenced the eight PCR products as described above with a CEQ2000 (Beckman Coulter Inc.). We chose two samples for expression in this vector, one containing the complete sequence (from methionine to stop codon) in the correct orientation of Isac (recombinant Isac (r-Isac)) and a second containing the Isac complete sequence in reverse orientation, which became our control (control Isac (c-Isac)). Cells containing the sample and control were grown overnight at 37 °C on LB with ampicillin (100 μg/ml), and plasmid isolation was performed with the Wizard miniprep kit (Promega Corporation, Madison, WI). After plasmid isolation, the sample and control plasmids were washed three times with ultraPure water using an Amicon-100 (Millipore), measured, and stored before attempting to transform COS-7 cells.

Expression of Isac in COS Cells—COS-7 cells (ATCC CRL 1651) were cultivated in Dulbecco's modified Eagle's medium supplemented with 4 mM glutamine, antibiotics, and 10% heat-inactivated fetal calf serum (complete medium) as described previously (21). Subconfluent COS-7 cells were transfected with Isac cDNA (r-Isac) or with the Isac in the reverse orientation (c-Isac). Transfections were performed with the Bio-Rad electroporation system using 200 V and 1000 millifarads in a 0.4-cm gap cuvette. DNA (200 and 400 μg) was used for each transfection at a cell density of 10^7/ml. After electroporation, the cells were allowed to sit for 10 min at room temperature and then were placed into 10 ml of complete medium in a 75-cm^2 culture flask at 37 °C/5% CO_2. After a 16-h incubation, the supernatants were collected, and 10 ml of serum-free Dulbecco's modified Eagle's medium was added to the cultures. This procedure was repeated daily up to 3 days after transfection. The resultant supernatants were centrifuged at 10,000 × *g* for 5 min, concentrated 20-fold on Centricon Plus-20 (5-kDa cut-off) (Millipore Corp.), and kept at −20 °C for bioassays. Control cells were transfected

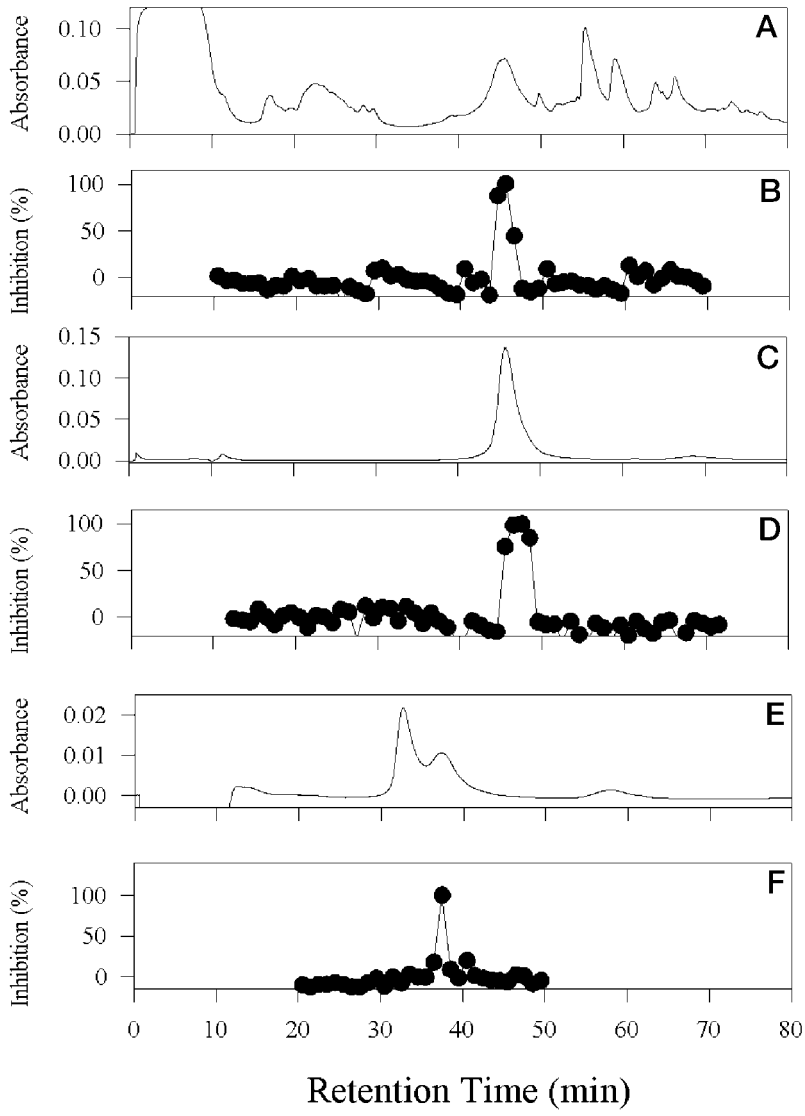

FIG. 2. **Purification of *I. scapularis* salivary anticomplement.** *A*, UV absorbance at 220 nm from reverse-phase chromatography using HCl as a modifier from 1/3 of active fractions obtained from the molecular sieving chromatography of 500 pairs of homogenized salivary glands from *I. scapularis*. *B*, anticomplement activity. *C*, reverse-phase chromatography using HCl as a modifier, of the combined active fractions obtained by reverse-phase chromatography as in *A*. *D*, anticomplement activity. *E*, reverse-phase chromatography using trifluoroacetic acid as a modifier of the active fractions obtained by reverse-phase chromatography using HCl as a modifier. *F*, anticomplement activity.

A

```
  1 - ACGGTTGCTCACAGCACTGAGGTTTCAGAGCAAGGGTCTACCAGCCATGAGGACTGCGTT
    -                                                 M  R  T  A  F
 61 - TACCTGTGCTCTTTTGGCGATTTCGTTTCTAGGAAGCCCGTGTTCGTCCAGCGAAGACGG
    -  T  C  A  L  L  A  I  S  F  L  G  S  P  C  S  S  S  E  D  G
121 - TCTCGAGCAAGATACCATAGTGGAAACTACTACACAAAATCTCTACGAACGTCATTATAG
    -  L  E  Q  D  T  I  W  E  T  T  T  Q  N  L  Y  E  R  H  Y  R
181 - AAATCATTCTGGATTGTGCGGGGCACAGTATAGGAATTCAAGCCATGCGGAAGCCGTTTA
    -  N  H  S  G  L  C  G  A  Q  Y  R  N  S  S  H  A  E  A  V  Y
241 - CAACTGCACGCTCAATCATTTGCCCCCAGTCGTGAATGCAACCTGGGAAGGAATTAGGCA
    -  N  C  T  L  N  H  L  P  P  V  V  N  A  T  W  E  G  I  R  H
301 - TCGAATTAATAAAACCATACCTCAGTTTGTAAAATTGATTTGCAACTTTACTGTTGCGAT
    -  R  I  N  K  T  I  P  Q  F  V  K  L  I  C  N  F  T  V  A  M
361 - GCCTCAAGAATTCTACTTAGTTTATATGGGGTCAGATGGAAACTCAGACTTTGAAGAGGA
    -  P  Q  E  F  Y  L  V  Y  M  G  S  D  G  N  S  D  F  E  E  D
421 - CAAAGAGAGCACAGGCACTGATGAAGACAGTAACACGGGATCTTCTGCTGCAGCTAAAGT
    -  K  E  S  T  G  T  D  E  D  S  N  T  G  S  S  A  A  A  K  V
481 - TACAGAAGCGCTAATAATAGAAGCAGAGGAAAACTGCACGGCGCATATAACTGGTTGGAC
    -  T  E  A  L  I  I  E  A  E  E  N  C  T  A  H  I  T  G  W  T
541 - CACTGAAACCCCGACCACGCTGGAACCTACGACAGAGTCTCAATTTGAGGCAATTCCCTG
    -  T  E  T  P  T  T  L  E  P  T  T  E  S  Q  F  E  A  I  P  *
601 - AGGCATCGTGTGCCGATCTATCACCCGGTTATTTCACCGCGTGGTTTTGATGCCCGTGCA

661 - AGCACAGTTCTGATACGGGTGGCGAGGTGCTTTGCCGAGATTGATTTAAATAAAATTCGA

721 - TAAAGAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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B (S/G)EDGLEQDSKVEXXXQNLYE

FIG. 3. Nucleotide and predicted translated protein sequences from Isac cDNA (*A*). The amino acids in *bold* were obtained by Edman degradation (*B*). *(S/G)* indicates equal signal of serine and glycine, whereas *X* indicates that no amino acid could be identified. This is GenBank™ accession number AF270496.

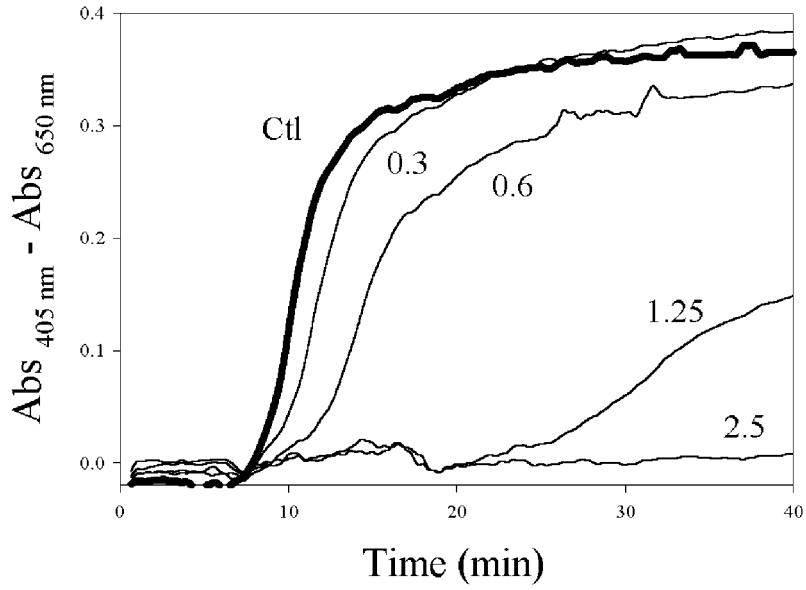

FIG. 4. **Inhibition of rabbit erythrocyte lysis by human serum in the presence of different microliter amounts (*numbers*) of COS cell supernatants transfected with r-Isac or c-Isac (*Ctl*).** The plate reader was blanked with wells containing 10 mM EDTA instead of EGTA and $MgCl_2$.

with the Isac in the reverse orientation construct (c-Isac), and supernatants were obtained as for the r-Isac-transfected cells.

Sequence Analysis—Sequence similarity searches were performed using the Blast (22) program. Cleavage site predictions of the mature proteins used the SignalP program (23).

RESULTS AND DISCUSSION

We initially attempted to purify *I. scapularis* salivary anticomplement from a 550-μl sample of pilocarpine-induced tick saliva. After one step of molecular sieving chromatography followed by reverse-phase HPLC on a polymeric column using a gradient of acetonitrile containing 8 mM HCl and a final step on the same column using trifluoroacetic acid instead of HCl, we obtained small amounts of a product with an amino-terminal sequence (G)EDGLE, where (G) was a doubtful result. Because of difficulties in collecting larger volumes of tick saliva, a second effort was made to purify the protein from tick salivary homogenates, which also possess anticomplement activity as determined by its ability to inhibit lysis of rabbit erythrocytes by human sera in the presence of Mg^{2+}/EGTA (not shown). Accordingly, 500 pairs of salivary glands were homogenized in 50 ml of HEPES saline, and the supernatant (after 10 min at 10,000 $\times$ *g*) was concentrated using a 10-kDa cut-off Centricon (Millipore Corp.) filter. Ten aliquots of the concentrated homogenate were repeatedly injected into a molecular sieving column, and the fractions were collected into the same tubes at 0.4-min intervals. Two consecutive fractions were active eluting with an apparent molecular mass of 62 kDa (Fig. 1).

These active fractions were combined, and acetonitrile and HCl were added to give 10% organic solvent and 8 mM of the acid. This fraction was further divided into three aliquots, each injected into a reverse-phase polymeric resin eluted with a gradient of acetonitrile in 8 mM HCl (Fig. 2*A* and *B*). Active fractions from these three reverse-phase columns were combined, diluted with water, and concentrated by passing over the same column again with a gradient of acetonitrile HCl to yield a single peak of UV-absorbing material (Fig. 2, *C* and *D*). These fractions containing the UV-absorbing peak were combined and injected into the same column and eluted with a gradient of acetonitrile in 0.1% trifluoroacetic acid instead of HCl. Two UV-absorbing peaks eluted, the second of which had anticomplement activity (Fig. 2, *E* and *F*). Fractions corresponding to the active peak were concentrated by evaporation and then sent for amino-terminal sequencing, which produced primary

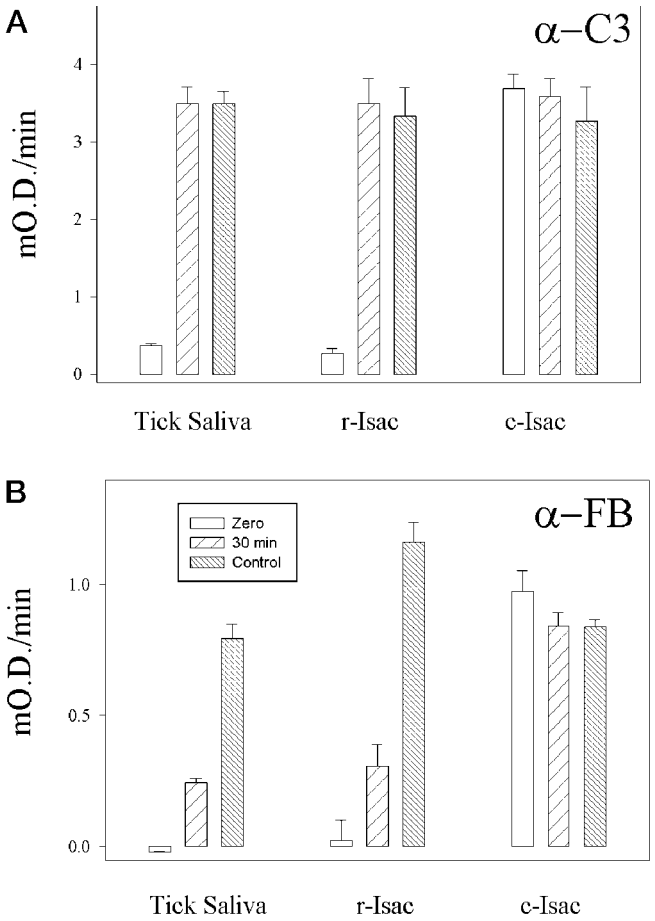

FIG. 5. **Inhibition of C3b (*A*) and factor B (*B*) deposition to agarose-coated plates by *I. scapularis* saliva and r-Isac-transfected COS supernatants.** Controls were run with supernatants of COS cells transfected with the c-Isac cDNA sequence. Blanks were obtained with wells incubated with 10 mM EDTA instead of EGTA and $MgCl_2$ during the complement activation step of the assay.

sequence (S/G)EDGLEQDSKVE*XXX*QNLYE. This sequence contained the shorter sequence obtained from HPLC purification of the anticomplement from tick saliva.

Attempts to obtain a PCR product using primers based on the above sequence failed, perhaps because of the high codon

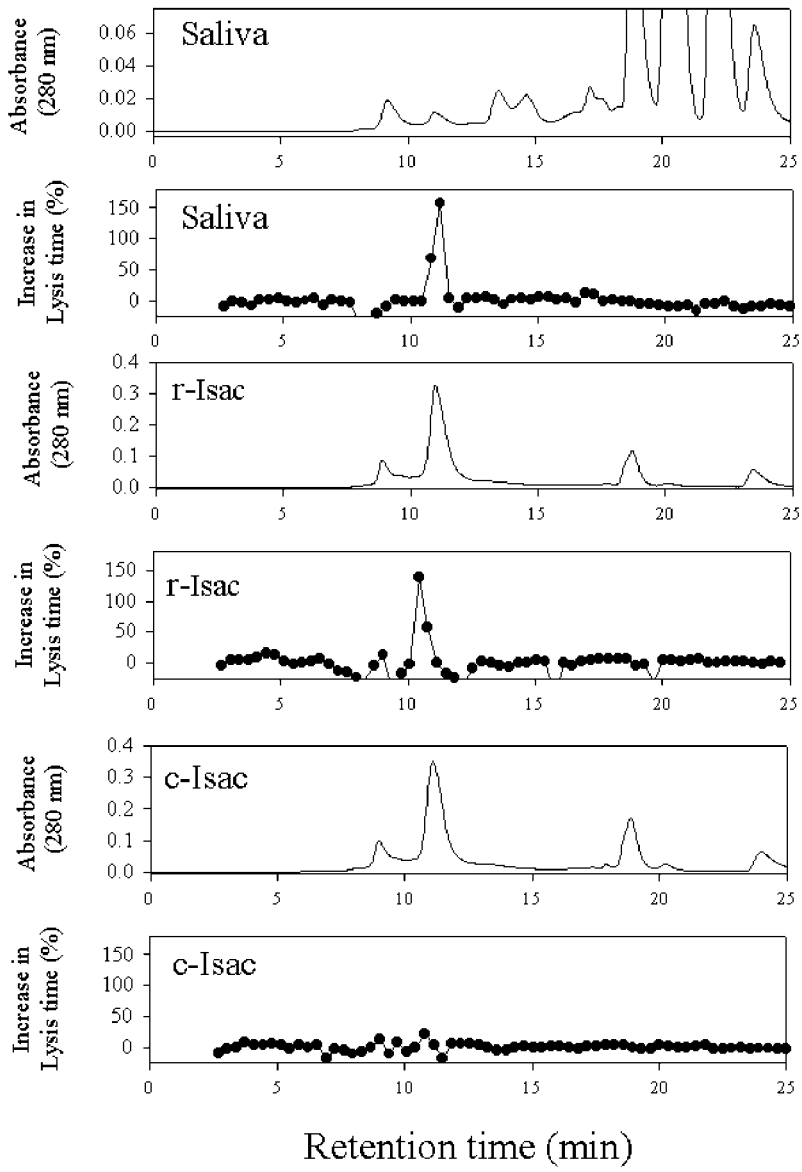

FIG. 6. **Molecular sieving chromatography of 50 μl of *I. scapularis* saliva or 50 μl of concentrated supernatant of COS cells transfected with r-Isac or with c-Isac.** UV absorbance of the chromatogram or inhibition of the lysis of rabbit erythrocytes by human sera in the presence of Mg^{2+}/EGTA is shown.

degeneracy presented by the majority of amino acids found in the sequence. A cDNA library from *I. scapularis* was randomly sequenced, and 709 sequences were obtained. This library is unidirectional with the cDNA being restricted with a rare cutter restriction enzyme (*Sfi*I) before ligation to the vector. The probability of obtaining amino-terminal information from the clones was enhanced by starting the cycle sequencing of the library's clones from the 5′-region of the cDNA. In this manner, a clone was found leading to the predicted Isac protein shown in Fig. 3. The predicted protein has a putative signal peptide of 21 amino acids, the mature peptide having an amino-terminal sequence in agreement with the measured sequence (Fig. 3). The predicted mature peptide has 163 amino acids, a theoretical pI of 4.48, and a M_r of 18,136.77, assuming that no carboxyl-terminal residue beyond the terminal proline is lost. No significant similarities were found between Isac and any other protein in the GenBank™ data base, when the blastx program invoking the BLOSUM 62 matrix was used (22). The predicted Isac sequence has seven Asn glycosylation sites, seven casein kinase 2 phosphorylation sites, and three myristoilation sites, as determined by scanning the Prosite data base.

To confirm that the cDNA sequence corresponded to an anticomplement molecule, we expressed it in COS cells. Supernatants of cells transfected with r-Isac, but not those transfected with c-Isac, had anticomplement activity as measured by their ability to inhibit lysis of rabbit erythrocytes by human serum (Fig. 4).

In the presence of Mg^{2+} and serum, C3 is deposited covalently to agarose via the alternative complement pathway (1, 20). Serum fB binds noncovalently to agarose-bound C3b to form the C3 convertase of the alternative pathway, creating positive feedback for further C3 activation and deposition. This assay is made specific to the alternative complement pathway by the presence of EGTA, a Ca^{2+} chelator, because calcium ions are needed for classical and lectin-mediated complement activation (1). Based on these reactions, we developed a simple test for identification of alternative pathway-dependent C3 deposition to agarose and fB association with C3b using agarose-coated microwells. Using this test, tick saliva and r-Isac cells prevented deposition of C3 to agarose-coated wells if added together with serum to the incubation mixture (Fig. 5); however, if added 30 min after the addition of serum, C3 deposition (which is a covalent phenomenon) cannot be displaced by saliva- or Isac-transformed COS cell supernatants, although fB, which attaches noncovalently to C3b, is significantly displaced by saliva or r-Isac.

To further compare the properties of r-Isac with the native protein, we submitted r-Isac and tick saliva to chromatography

on a TSK-2000 SW column. Both activities eluted with similar retention time, although the recombinant protein eluted one fraction earlier in the chromatogram (Fig. 6). The observed retention times for both activities correspond to a molecular mass of ~65 kDa, larger than the expected 18.5-kDa translation product deduced from Isac cDNA. This discrepancy may reflect the anomalous behavior of some proteins in silica-based molecular sieving columns known to have residual silanol groups that may confer a cation exchange or anion exclusion capability to the column or, alternatively, Isac may be post-translationally modified by glycosylation and/or polymerization. In either case, these results indicate the similarity in chromatographic behavior of r-Isac and the native protein.

Because Isac could be a serine protease inhibitor, we measured its activity on the classical pathway of complement activation using sensitized sheep erythrocytes. We also determined its effect on the recalcification time of citrated human plasma in an assay that measures both the intrinsic and the common pathway of the clotting cascade. Although *I. scapularis* saliva inhibited blood clotting, no effect of saliva was found on the classical pathway (triplicate experiment, using three saliva samples). Purified c-Isac, as well as the supernatant of r-Isac-transfected COS cells, did not have anticlotting or classical anticomplement activity in concentrations that fully inhibited the alternative complement pathway (not shown). These results indicate that Isac is different from the previously identified (but not molecularly characterized) unspecific antiprotease activity found in whole body homogenates of the cattle tick, *Boophilus microplus* (24).

Isac behaves as a regulator of complement activation in a manner similar to decay accelerating factor and factor H, which inhibit complement activation by interacting with the C3 convertase of the classical or alternative pathway. Factor H is a 155-kDa glycoprotein containing 20 repetitive domains termed short consensus repeats (25). In addition to its other effects on the complement cascade, factor H inhibits interaction of factor B with C3b (25), as does Isac. No significant sequence similarity was found, however, between Isac and factor H or with any other protein available in public domain data bases. Isac may be structurally similar to single short consensus repeats, and its four cysteines are consistent with the four cysteines conserved in short consensus repeats of the factor H family (25). It would thus appear that Isac belongs to a new class of complement regulatory molecules. Successful expression of this protein makes it a viable target for anti-tick vaccines.

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Purification, Cloning, and Expression of a Novel Salivary Anticomplement Protein from the Tick, *Ixodes scapularis*

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