Molecular Cloning and Characterization of a Highly Selective Chemokine-binding Protein from the Tick Rhipicephalus sanguineus*

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Ticks are blood-feeding parasites that secrete a number of immuno-modulatory factors to evade the host immune response. Saliva isolated from different species of ticks has recently been shown to contain chemokine neutralizing activity. To characterize this activity, we constructed a cDNA library from the salivary glands of the common brown dog tick, Rhipicephalus sanguineus. Pools of cDNA clones from the library were transfected into HEK293 cells, and the conditioned media from the transfected cells were tested for chemokine binding activity by chemical cross-linking to radiolabeled CCL3 followed by SDS-PAGE. By de-convolution of a single positive pool of 270 clones, we identified a full-length cDNA encoding a protein of 114 amino acids, which after signal peptide cleavage was predicted to yield a mature protein of 94 amino acids that we termed Evasin-1. Recombinant Evasin-1 was produced in HEK293 cells and in insect cells. Using surface plasmon resonance, we were able to show that Evasin-1 was selectively selective for CCR1, CCL3 and CCL4 and the closely related chemokine CCL18, with Kd values of 0.16, 0.81, and 3.21 nM, respectively. The affinities for CCL3 and CCL4 were confirmed in competition receptor binding assays. Analysis by size exclusion chromatography demonstrated that Evasin-1 was monomeric and formed a 1:1 complex with CCL3. Thus, unlike the other chemokine-binding proteins identified to date from viruses and from the parasitic worm Schistosoma mansoni, Evasin-1 is highly specific for a subgroup of CC chemokines, which may reflect a specific role for these chemokines in host defense against parasites.

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†‡ The abbreviations used are: CKBP, chemokine-binding protein; PBS, phosphate-buffered protein; RANTES, regulated on activation normal T cell expressed and secreted; BS3, bis(sulfosuccinimidyl) suberate; Ni-NTA, nickel-nitrilotriacetic acid; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; SPA, scintillation proximity assay; CHO, Chinese hamster ovary; HEK, human embryonic kidney; SPR, surface plasmon resonance.

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Hajnicka et al. (11, 12) demonstrated the presence of anti-CXCL8 (interleukin-8) activity in salivary gland extracts from several ixodid tick species and have recently shown that tick saliva contains a variety of inhibitory activities directed against pro-inflammatory cytokines such as interleukin-2 and the chemokines CCL2/MCP-1, CCL3/MIP-1α, CCL5/RANTES, and CCL11/eotaxin (13). We have, therefore, used an expression cloning approach to identify chemokine-binding proteins secreted in tick saliva. A cDNA library was prepared from salivary glands, dissected from the ixodid tick, *Rhipicephalus sanguineus* (common brown dog tick), during feeding. A chemokine cross-linking assay was used to detect chemokine binding activity in conditioned medium harvested from HEK293 cells which had been transfected with the tick salivary gland cDNA library. Using this method we identified a family of chemokine-binding proteins that we have termed Evasins. In this paper we describe the cloning and characterization of Evasin-1, a CC chemokine-binding protein highly specific for CCL3, CCL4, and CCL18 (Pulmonary and activation-regulated chemokine (PARC)).

**EXPERIMENTAL PROCEDURES**

*Reagents and Recombinant Chemokines—*Unless otherwise stated all reagents and chemicals were purchased from Sigma. Enzymes were obtained from New England Biolabs (Beverly, MA). 125I-Radiolabeled chemokines and chromatographic materials were obtained from GE Healthcare. Chemokines were purified as described previously (14). The cDNA encoding the ectromelia virus chemokine-binding protein (GenBank™/EBI Data Bank accession number AJ277111) was kindly provided by Dr. A. Alcami (University of Cambridge). The cowpox virus CKBP p35 protein was a gift from Dr. D. B. Wigley (Howard Hughes Medical Institute, Boston, MA).

*Preparation of Tick Saliva—* *R. sanguineus* ticks were laboratory-reared as previously described (15). All ticks used for infestations were 1–3-month-old adults. To obtain engorged ticks for saliva collection, 20 dogs were infested with 70 pairs of adult *R. sanguineus* ticks contained in plastic feeding chambers fixed to their backs. The saliva-collection procedure was performed using engorged female ticks (after 3–5 days of feeding) by inoculating their backs. The saliva-collection procedure was performed using engorged female ticks (after 3–5 days of feeding) by inoculation of 10–15 μl of a 0.2% (v/v) solution of dopamine in phosphate-buffered saline (PBS), pH 7.4, using a 1.27 × 0.33-mm gauge needle (BD Biosciences). Saliva was harvested using a micropipette and placed on ice. Pooled saliva samples were centrifuged through a 0.22-μm pore filter (Costar-Corning, Inc., Cambridge, MA) and stored at −20 °C until further use. Each saliva pool consisted of material harvested from more than 200 female ticks. The saliva protein concentration, determined using a bicinchoninic acid solution (Sigma), ranged from 1000 to 2000 μg/ml.

*Chemical Cross-linking Assay—*Lyophilized, iodinated chemokines were resuspended at 0.23 nm in 50 mM HEPES buffer, pH 7.5, containing 1 mM CaCl₂, 5 mM MgCl₂, and 0.1% bovine serum albumin and incubated with 10 μl of conditioned media from transfected HEK293 cells or with 10 μl of tick saliva in the presence or absence of 25 mM bis(sulfosuccinimidyl) suberate (BS²⁺) in a final volume of 50 μl for 2 h at room temperature with shaking. The cross-linking reaction was quenched by the addition of 5 μl of 10× SDS-PAGE sample buffer. The samples were analyzed by SDS-PAGE and scanned using a Personal FX phosphorimaging system (Bio-Rad) at a resolution of 100 μm.

*Construction of a Tick Salivary Gland cDNA Library—*Tick salivary glands were dissected, rinsed with ice-cold PBS, and stored in RNAlater™ solution (50 mg of tissue/ml) (Ambion, Inc., Canada) at −70 °C until use. Total RNA was extracted from −50 mg of salivary glands using TRIzol™ (Invitrogen) according to the manufacturer’s directions. A tick salivary gland cDNA library was constructed using a SMART® cDNA library construction kit (Clontech, Palo Alto, CA) according to the manufacturer’s directions. An aliquot of the resultant λTriplEx2 phage cDNA library containing 2 × 10⁶ plaque-forming units was converted into a plasmid cDNA library in pTriplEx2 in BM25.8 cells according to the manufacturer’s protocol. The pTriplEx2 cDNA library was stored at −80 °C in LB medium containing 50% glycerol. For subcloning of the cDNA library into the mammalian cell expression vector, pEXP-Lib (Clontech), plasmid DNA was prepared from a 5-ml overnight bacterial culture inoculated with 5 μl of the glycerol stock (containing 1 × 10⁶ colony-forming units) using the Wizard® Plus SV Miniprep DNA purification system (Promega, Madison, WI). The resultant plasmid DNA was digested with SfiI, fractionated on a 1.1% agarose gel and cDNA inserts were excised and purified using the QAQuick gel extraction kit (Qiagen, Basel, Switzerland) according to manufacturer's instructions. The cDNA inserts were ligated into SfiI-digested and dephosphorylated, pEXP-Lib vector (Clontech, Palo Alto, CA, USA) using the Quick Ligation™ Kit (New England Biolabs). Ligation reactions were transformed by heat shock into UltraMAX DH5alpha-FT competent cells (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The pEXP-Lib cDNA library transformation mix was iterated and plated at ~100 colonies per 10 cm diameter LB-agar plate containing 50 μg/ml ampicillin. A total of 120 plates were prepared and grown for ~18 h at 37 °C, to yield large colonies (~2 mm diameter). Bacterial colonies were harvested in 5 ml LB medium by scraping the plates with a sterile, triangular plastic loop. Cells were pelleted by centrifugation at 3500 rpm for 10 min at 4 °C. Plasmid DNA was prepared from each pool using a BioRobot 8000 (Qiagen) and stored at −20 °C in 10 mM Tris-HCl buffer, pH 8.

*Transfection of HEK293 Cells—*HEK293 cells were maintained in a 5% CO₂, humidified incubator in Dulbecco’s modified Eagle’s medium-F-12 Nut medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine (Invitrogen), and 1% 100X penicillin-streptomycin solution (Invitrogen). The day before transfection a confluent culture of HEK293 cells was harvested by trypsinization and seeded at ~2 × 10⁶ cells/well in a 96-well plate that had been precoated with 10 μg/ml poly-d-lysine hydrobromide. The next day cells were transfected with 100 ng of plasmid DNA using the Geneporter2 transfection kit (Gene Therapy Systems) using the manufacturer’s protocol for low concentration DNA and adherent cells and incubated at 37 °C for 3–4 days. The conditioned medium was then harvested, cell debris was removed by centrifugation, and the supernatants stored frozen at −80 °C until further use.
Selective Tick Chemokine-binding Protein

Expression Cloning—Approximately 80–130 ng of plasmid DNA from each library pool or the positive control plasmid was transfected into HEK293 cells as described above. For the positive controls we used the pEXP-lib vector containing the p35 or vCCL cDNA-coding sequence. Cell culture supernatants (200 μl) were harvested 3 days after transfection and stored frozen. Before use culture supernatants were thawed and concentrated four times using a speed vacuum system. The concentrated culture supernatant (conditioned medium) was tested in a cross-linking assay using 125I-labeled CCL3 as described above. Plasmid DNA from pools that gave a positive signal in the cross-linking assay were retransformed into Escherichia coli, and the transformation mix plates on 10-cm-diameter LB-amp plates to yield ~100 colonies per plate. Plasmid DNA was then prepared from 100 individual colonies and re-transfected into HEK293 cells. The conditioned medium from each transfection was harvested after 3 days and retested in the cross-linking assay using 125I-labeled CCL3 as described above. Plasmid DNA derived from individual colonies which gave a positive signal in the cross-linking assay was sequenced on an Applied Biosystems 3700 DNA sequencer using a T7 and pEXP-Lib-3 reverse primer (Clontech, Palo Alto, CA). The plasmid, which contained the cDNA sequence encoding the CCL3-binding protein (pEXP-Lib-Evasin-1), was subsequently used as a PCR template to generate a six-histidine (His)-tagged version of the cDNA, which was subcloned into the baculovirus vector, pDEST8 (Invitrogen), and into the mammalian cell expression template to generate a six-histidine (His)-tagged version of the template to generate a six-histidine (His)-tagged version of the

Deglycosylation—Evasin-1 produced in TN5 insect cells was subjected to enzymatic deglycosylation with endoglycosidase Hf and peptide-N-glycosidase F (New England Biolabs). A solution of 1 mg/ml Evasin-1 in 50 mM sodium citrate buffer, pH 5.5, was incubated with 25,000 units of endoglycosidase Hf at room temperature for 10 h. A second digestion with peptide-N-glycosidase F (PNGase F) was carried out by incubating 1 mg/ml Evasin-1 in 50 mM sodium phosphate buffer, pH 7.5, containing 10% Nonidet P-40 with 12,500 units of PNGase F at room temperature. The extent of digestion was followed by SDS-PAGE.

Surface Plasmon Resonance—Real-time biomolecular interaction analyses were performed using a Biacore 3000 surface plasmon resonance (SPR) system. Recombinant Evasin-1-His was suspended at 50 μg/ml in 10 mM sodium acetate buffer, pH 4.5, and directly immobilized on the flow cell of a CM4 chip (Biacore) by a standard amine coupling chemistry according to the manufacturer’s instructions using the Biacore 3000 Wizard software. Approximately 750 response units of Evasin-1-His were coupled to the cell using this method. A blank cell was prepared using the chemical coupling as a control in the absence of protein. Experiments were performed at 25 °C with a flow rate of 30 μl/min using HBS-P running buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, and 0.005% surfactant P20) (Biacore). For all binding experiments chemokines were resuspended at 0.1 μg/ml in running buffer and filtered through a 0.22-μm filter. The injection time was 2 min followed by a dissociation time of 2.5 min after injection. The chip was regenerated using 50 mM glycine buffer, pH 2.0, for 30 s. For each experiment chemokines were injected in triplicate in random order.

For the kinetic experiments, serial 2-fold dilutions of CCL3 (ranging from 25 to 1.5 ng/ml), CCL4 (ranging from 50 to 3 ng/ml), CCL18 (ranging from 0.5 μg/ml to 50 ng/ml) were prepared in running buffer, filtered through a 0.22-μm filter, and injected over the experimental blank flow cells. The injection time was 3 min followed by a dissociation time of 15 min. The chip was regenerated using 50 mM glycine buffer, pH 2.0, for 30 s. Each chemokine dilution was injected in triplicate in random order.

For the analysis the sensograms from the blank cell in addition to the sensograms obtained with the running buffer alone were subtracted from the binding to remove the nonspecific background. For the kinetic analyses the association (k_a) and the dissociation (k_d) values were determined simultaneously by globally fitting sensograms for an entire range of chemokine concentrations according to the 1:1 Langmuir fitting model. The apparent equilibrium dissociation constants (K_D) were over the mass range of interest using a standard set of reference proteins provided by the manufacturer. Sinapinic acid was used as the matrix. Size exclusion chromatography was performed by injecting 200 μl of a 1 mg/ml solution onto an analytical Sephadex 75 10/300 GL column (GE Healthcare) previously equilibrated in PBS and eluted at 0.5 ml/min. Elution profiles were monitored by UV absorption at 280 nm. The void volume (V_v) was determined with blue dextran, and the column was calibrated with the following standards purchased from GE Healthcare: albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (20.4 kDa), and ribonuclease A (13.7 kDa).

Physicochemical Characterization—N-terminal sequence analysis of Evasin-1 was performed using an Applied Biosystems 475A protein sequenator with on-line phenylthiohydantoin derivative detection. MALDI-TOF spectra were obtained on a Voyager DE-PRO Biospectrometry Work station (Applied Biosystems, Foster City, CA). The instrument was calibrated...
determined from the mean kinetics values with the equation
\[ K_D = \frac{k_d}{k_a}. \]

**Saturation Binding**—A saturation binding experiment was used to determine the affinity constant of \(^{125}\text{I}\)-labeled CCL3 binding to Evasin-1 using an scintillation proximity assay (SPA). His-tagged Evasin-1 (40 pm) was incubated with increasing concentrations of \(^{125}\text{I}\)-labeled CCL3 with or without a 500-fold excess of unlabeled CCL3 in 75 \(\mu\)l of PBS, pH 7.2 containing 1 mM CaCl\(_2\), 5 mM MgCl\(_2\), and 0.2% bovine serum albumin. Copper chelate-coated polyvinyl toluene-SPA beads containing scintillant (Amersham Biosciences RPNQ0095) (350 \(\mu\)g) were added in 25 \(\mu\)l of PBS and incubated for 72 h at room temperature with shaking. Competition of heparin for the binding of Evasin-1 to CCL3 was determined by the inclusion of heparin ranging from \(1 \times 10^{-3}\) to \(1 \times 10^{-5}\) \(\mu\)g/ml in the assay. The amount of bound \(^{125}\text{I}\)-labeled CCL3 was determined by measurement of the radioactivity using a \(\beta\) counter. Nonspecific binding, determined in the presence of a 500-fold excess of unlabeled CCL3, was subtracted and represented between 2 and 4.1% of the total counts bound per minute. The ability of Evasin-1 to bind to a chemokine receptor was investigated using iodinated Evasin-1 (custom product from GE Healthcare, specific activity 222 Ci/mmol) and CCR1 expressed in CHO membranes. Data were analyzed using GraphPad Prism software. Measurements were performed in duplicate.

**Equilibrium Competition Receptor Binding**—The ability of Evasin-1 to inhibit binding of radiolabeled CCL3 and CCL4 (GE Healthcare) to their cognate receptors was determined using a SPA. Membranes expressing recombinant CCR1 and CCR5 were prepared as described (16). Serial dilutions of Evasin-1 were prepared in binding buffer (50 mM HEPES, pH 7.2 containing 1 mM CaCl\(_2\), 5 mM MgCl\(_2\), 0.15 M NaCl, and 0.5% bovine serum albumin) to cover the concentration ranges shown in Fig. 5. Wheat germ agglutinin SPA beads (Amersham Biosciences) were suspended in binding buffer at 10 mg/ml, and the final concentration in the assay was 0.25 mg/well. CHO cell membranes expressing CCR1 or CCR5 were diluted in binding buffer to 80 \(\mu\)g/ml. Equal volumes of membrane and bead stocks were mixed before performing the assay to reduce background. The final membrane concentration in the assay was 2 \(\mu\)g/ml, and that of \(^{125}\text{I}\)-labeled CCL3 and CCL4 was 0.1 nm. The plates were incubated at room temperature with shaking for 4 h. Measurements were performed in triplicate.

**Stoichiometry of the Evasin-1-CCL3 Complex**—The apparent molecular mass of Evasin-1 in the presence of CCL3 or CXCL8 was analyzed by size exclusion chromatography as described above with a 1 mg/ml solution of Evasin-1 in PBS or a mixture of Evasin-1 and CCL3 or CXCL8, both at 0.5 mg/ml, in PBS. The fractions were analyzed on SDS-PAGE gels stained with Coomassie Blue.

**RESULTS**

**Identification of a CCL3-binding Protein in Tick Saliva**—The existence of a chemokine-binding protein in tick saliva was demonstrated by the detection of a radiolabeled band migrating between 25 and 35 kDa after autoradiography of an SDS-PAGE gel following incubation of tick saliva with \(^{125}\text{I}\)-labeled CCL3 in the presence of the chemical cross-linker, BS\(^3\) (Fig. 1A). This cross-linking assay was subsequently used to screen conditioned media from HEK293 cells transiently transfected with a tick salivary gland cDNA library for the presence of the CCL3-binding protein.

**Construction and Screening of a Tick Salivary Gland cDNA Expression Library**—Total RNA was extracted from salivary glands derived from ~50 female *R. sanguineus* adult ticks that had engorged on dogs for 5 days. Total RNA was used to prepare a directionally cloned cDNA library in the phage vector, \(\lambda\)TriplEx2. The resultant library contained \(~0.575 \times 10^6\) independent clones with an average insert size of between 0.3 and 1.5 kilobases. An aliquot of the phage library was then converted into a \(\lambda\)TriplEx2 plasmid library. cDNA inserts were excised together from the \(\lambda\)TriplEx2 cDNA library using SfiI and ligated into the mammalian cell expression vector, pEXP-lib. Plasmid DNA was prepared from 93 pools of clones, each containing ~85–100 independent cDNAs. Pools were screened by transfecting HEK293/EBNA cells followed by testing of the conditioned media for cross-linking activity to \(^{125}\text{I}\)-labeled...
CCL3. Plasmid DNA from one of the pools which gave a positive signal in the cross-linking assay was retransformed into E. coli, and plasmid DNA isolated from 96 individual colonies was tested by transfection and cross-linking as described above. This process was repeated until a single cDNA (clone 59) giving rise to a positive signal in the cross-linking assay was identified (Fig. 1b, lane 3).

The cDNA insert sequence in clone 59 appeared to be a full-length DNA with an open reading frame of 339 bp encoding a protein of 114 amino acids with a predicted signal peptide of 20 amino acids, which when cleaved gives rise to a mature protein of 94 amino acids, which we called Evasin-1 (Fig. 2). The cDNA contains a single AATTAA polyadenylation site which spans the stop codon. Data base searches indicated that the Evasin-1 cDNA sequence showed no significant homology to any other protein, nucleic acid sequence, or conserved domain by NCBI blast of public databases including available tick genome databases.

The predicted mass of Evasin-1 is 10,466 Da, and the predicted isoelectric point is 4.29. The mature protein contains eight cysteine residues, suggesting the presence of four disulfide bonds. There are also three predicted N-linked glycosylation sites in the mature protein sequence. Given that the 125I-labeled chemokine migrates at 8 kDa, it appears that the binding protein produced by the tick is heavily glycosylated, as it migrates with a mass ranging from ~17–27 kDa, which is considerably larger than the predicted mass.

Protein Purification and Characterization of Evasin-1—The cDNA sequence encoding the predicted open reading frame of Evasin-1 was subcloned with a C-terminal His tag for expression of the recombinant protein in insect (TN5) and mammalian (HEK293/EBNA) cells. Evasin-1 was well secreted in both expression systems. Elution from the Ni-NTA column was accompanied by high Mr-contaminating proteins which were easily removed by size exclusion chromatography as shown for the HEK protein in Fig. 1c. This chromatographic step clearly demonstrated the extent of glycosylation resulting in a mass distribution during size exclusion chromatography, but the identity of the different forms was confirmed by Western blot with an anti-His antibody (Fig. 1d), and activity of all the glycosylated forms was demonstrated by the cross-linking assay (Fig. 1e).

The recombinant protein expressed in mammalian and insect cell systems showed considerable differences in migration behavior on SDS-PAGE. Recombinant Evasin-1 from HEK293 cells migrated as a broad band between 20 and 30 kDa, whereas Evasin-1 produced in insect cells migrated as a broad band between 15 and 25 kDa (Fig. 3a). Because the His tag at the C terminus was present in both proteins, allowing capture on
the Ni-NTA resin, and the N-terminal sequencing showed that the signal sequence was removed in both expression systems according to the in silico prediction (results not shown), the differences in mass were attributed to post-translational modifications, most likely, glycosylation. Treatment of Evasin-1 with a glycosidase (peptide-N-glycosidase F) or Evasin-1 produced by transfected cells cultured in the presence of tunamycin resulted in a band migrating at 12 kDa, confirming that the mass differences could be solely due to differential glycosylation (Fig. 3a). The apparent molecular mass determined by size exclusion chromatography of insect cell expressed Evasin-1 was 22 kDa, and that of the mammalian expressed Evasin-1 was 31 kDa (data not shown), in accordance with their differential migration pattern on SDS-PAGE. Analysis by isoelectric focusing showed that the major difference in glycosylation appears to be in sialylation. The protein expressed in mammalian cells showed a typical ladder pattern on isoelectric focusing characteristic of sialylated proteins, whereas the insect-derived protein showed only three major species (Fig. 3b). The mammalian form displayed a wider and smoother distribution of the mass range, with a bell-shaped spectrum peaking at a mass of around 16 kDa, whereas the insect form resulted in a series of sharp spectra with the center of the mass distribution around 13 kDa (data not shown). When tested in the cross-linking assay to \(^{125}\text{I}\)-labeled CCL3, the extent of glycosylation of the HEK-derived protein appeared to be closer to that of the natural tick protein in saliva (Fig. 3c).

| Chemokine | \(k_a \times 10^5\) | \(k_d \times 10^3\) | \(K_D\) | \(k_a\) | \(k_d\) | \(K_D\) |
|-----------|-----------------|-----------------|-------|----------|----------|-------|
| CCL3      | 24.5 ± 11.5     | 3.56 ± 2.57     | 0.16 ± 0.08 | 0.16 ± 0.08 | 0.16 ± 0.08 |
| CCL4      | 4.27 ± 2.46     | 2.97 ± 0.68     | 0.81 ± 0.30 | 0.81 ± 0.30 | 0.81 ± 0.30 |
| CCL18     | 3.26 ± 0.62     | 11.1 ± 7.61     | 3.21 ± 1.95 | 3.21 ± 1.95 | 3.21 ± 1.95 |

Selectivity and Binding Affinity of Evasin-1—To determine the chemokine selectivity of Evasin-1 we immobilized the recombinant protein on Biacore chips and analyzed the binding profiles of a selection of 23 chemokines using SPR. As shown in Fig. 4, we only observed binding to three chemokines, CCL3, CCL4, and CCL18. Although a binding protein to CCL5 was observed in our initial analyses of tick saliva\(^5\) and has also been reported elsewhere in another tick species (13), we did not observe high affinity binding of CCL5 to purified Evasin-1 by SPR, suggesting that a different protein is probably responsible for this activity in tick saliva. No binding was observed with 9 other CC chemokines or 9 CXC chemokines as well as XCL1 and CX3CL1, demonstrating an exquisite selectivity that has not been described for any other chemokine-binding protein to date.

The affinities for these three chemokines was determined by SPR, giving \(K_D\) values of 0.16 ± 0.08 nM for CCL3, 0.81 ± 0.3 nM for CCL4, and 3.21 ± 1.95 nM for CCL18 (Table 1). The affinity for CCL3 was also determined using a saturation binding experiment using the His tag on Evasin-1 to coat copper chelate SPA beads, resulting in a \(K_D\) of 0.36 ± 0.04 nM, confirming the value obtained using SPR (Fig. 5a). Heparin was unable to compete for the binding of CCL3 to Evasin-1. The \(K_{50}\) values for the inhibition of CCL3 binding to its receptors (see below) demonstrated even greater potency, so we used an SPA assay with CHO membranes expressing one of the receptors, CCR1, to determine whether Evasin-1 could interact with the receptor. As shown in Fig. 5b, whereas \(^{125}\text{I}\)-labeled CCL3 demonstrated saturable binding to CCR1, no binding of \(^{125}\text{I}\)-labeled Evasin-1 was observed.

Inhibition of Receptor Binding—We confirmed the affinity of Evasin-1 for CCL3 and CCL4 by equilibrium competition receptor binding to determine the ability of Evasin-1 to inhibit the binding of iodinated CCL3 and CCL4 to their cognate receptors, CCR1 and CCR5, using a scintillation proximity assay. Recombinant Evasin-1 produced in TN5 insect cells was able to inhibit CCL3 and CCL4 binding to their respective receptors (Figs. 6, a–d). Evasin-1 showed remarkable potency in inhibiting CCL3 binding to both CCR1 (Fig. 6a) and CCR5 (Fig. 6b), with IC\(_{50}\) values of 0.05 and 0.015 nM, respectively, and it inhibited CCL4 binding to CCR5 with an IC\(_{50}\) of 3.1 nM. The same potency was observed for recombinant Evasin-1 produced in HEK293 cells, and both proteins showed equivalent activity in this assay after lyophilization (data not shown). Enzy-
matically deglycosylated Evasin-1 or Evasin-1 expressed in insect cells in the presence of tunicamycin had IC50 values almost identical to that of the glycosylated recombinant protein (Fig. 6d), indicating that glycosylation is not required for the inhibitory activity.

**Stoichiometry of the Evasin-1-Chemokine Complex**—To estimate the stoichiometry of Evasin-1 binding to CCL3, equimolar mixtures of Evasin-1 and three chemokines, CCL3, CXCL8, and E66A-RANTES, an obligate dimeric form of CCL5 (17), were applied to a Sephadex 75 10/300 GL column. Confirmation of the selectivity profile described above was demonstrated by the observation of two discrete peaks eluting on size exclusion chromatography for the mixtures of Evasin-1 produced in HEK293 cells with CXCL8 and E66A-RANTES, whereas a single peak was observed for the Evasin-1/CCL3 mixture which eluted with an elution volume corresponding to a mass of 38 kDa (Fig. 7). The analysis of the protein peaks by SDS-PAGE confirmed the formation of a 1:1 complex of Evasin-1 with CCL3, which appears to form a compact structure with an apparent molecular mass only slightly larger than the mass of 34 kDa for the binding protein alone.

**DISCUSSION**

We describe the cloning of a highly selective chemokine-binding protein from a cDNA library constructed from tick salivary glands which we have called Evasin-1. Evasin-1 belongs to a totally novel class of chemokine-binding proteins distinct from the known viral chemokine-binding proteins and EB2 from *S. mansoni* (18). Public database searches indicated that tick cDNA sequence coding for Evasin-1 did not show significant homology to any known sequence. The highest identity against any tick sequence was 30% over 95 amino acids for a *Boophilus microplus* EST (EST768980).

Evasin-1 is a small protein, barely larger than its monomeric chemokine ligands, with a predicted mass of 10.4 kDa, although due to glycosylation it has an apparent mass of 15–30 kDa depending on the system in which the recombinant protein is expressed. Removal of glycosylation results in a protein with an apparent molecular mass close to the predicted mass. Recombinant Evasin-1 is differentially glycosylated when produced in mammalian and insect cell expression systems. The extent of glycosylation of Evasin-1 produced in mammalian cells seems to be closer to that of the natural protein found in tick saliva than the protein produced in insect cells when visualized on SDS-PAGE. Although glycosylation does not appear to play a role in activity, it may be involved in preventing immunogenicity. Hard ticks feed on their hosts for protracted periods of time; therefore, glycosylation of proteins secreted in their saliva may be an important way to disguise their proteins from immune recognition by the host. Specific glycosylation and immunogenicity are important considerations in potential therapeutic applications of Evasin-1.

Evasin-1 forms a 1:1 complex with CCL3, distinct from the chemokine-binding protein M3, expressed by myxoma virus,
which forms a dimeric complex that binds two chemokine molecules, as established by x-ray crystallographic studies (19). Another feature that distinguishes Evasin-1 from other chemokine-binding proteins is its surprising selectivity profile. Most viral chemokine-binding proteins isolated to date are reported to have broad chemokine binding selectivity. For example, the myxoma virus product M-T7 is the prototype of the type I CKBP family and, as well as binding interferon-γ, also interacts with members of the CXC, CC, and C chemokine families with moderate (submicromolar) affinity (20). Members of the type II CKBP family bind exclusively to CC chemokines with nanomolar affinity (21, 22). The sole member of the type III CKBP family, M3 from murine γ-herpesvirus-68, binds to members of the CX3C, CXC, CC, and C chemokine families with high (nanomolar) affinity (23). A fourth type of CKBP was identified in alphaherpesviruses, which display a broad, yet high affinity binding profile to chemokines of all classes (24). The recently discovered S. mansoni chemokine-binding protein also has broad selectivity, binding to CXCL8, CCL3, and CX3CL1 as well as to CCL2 and CCL5 (18). The binding affinities of Evasin-1 for various members of the chemokine family were determined by SPR. Evasin-1 belongs to a new class of chemokine-binding proteins in that it shows high affinity binding to a very limited set of three highly homologous CC chemokines: CCL3, CCL4, and CCL18. The affinities of Evasin-1 for CCL3 and CCL4 were confirmed by competition in receptor binding assays, where it showed surprising potency for CCL3, with an IC50 value in the picomolar range. We, therefore, investigated whether this potency could be partially due to the binding of Evasin-1 to a CCL3 receptor, but at least for CCR1, no binding could be demonstrated. It has been reported that the binding of chemokines to glycosaminoglycans can aid in sequestration of the chemokine to its receptor (26), but heparin had no inhibitory effect on the binding of Evasin-1 to CCL3. Because the receptor for CCL18 remains unidentified to date, the potency of inhibition of CCL18 receptor binding was not possible.

CCL3, CCL4, CCL5, and CCL18 show high overall sequence identity with each other (>70%), yet CCL5 only shows low, micromolar affinity binding to Evasin-1. Resolution of the three-dimensional structure of the Evasin-1-CCL3 complex has identified two hydrophobic interaction sites, and the residues involved are conserved between CCL3 and CCL5. Thus, the features of Evasin-1, which confer such exquisite binding specificity to these chemokines, remain to be elucidated.

The binding of Evasin-1 to CCL18 is interesting. Sequence analysis of the CCL18 gene indicates that it was probably generated by fusion of two MIP-1α-like genes, with deletion and selective usage of exons (27). The absence of a murine CCL18 ortholog indicates that the generation of the CCL18 gene is likely to have occurred after the diversification of rodents and primates after the tetrapod split. CCL18 has been shown to be one of the most highly up-regulated chemokines in the skin of atopic dermatitis patients, and its expression is associated with an allergy/atopy skin phenotype (25, 28–30). The presence of a chemokine-binding protein in tick saliva, which shows selectivity for CCL18, therefore highlights an important role for this chemokine in the immune response in the skin of primates. Targeting CCL18-mediated immune cell recruitment may, therefore, be of therapeutic use in atopic dermatitis. The human receptor for CCL18 is still unknown, which paves the way for the potential use of Evasin-1 as a biotherapeutic for treatment of this disease. However, the lack of a rodent counterpart for human CCL18 would necessitate the use of primate animal models for target validation.

In conclusion, we have cloned a highly specific CC chemokine-binding protein from tick salivary glands, the first chemokine-binding protein to be identified in an external parasitic organism. Evasin-1 is specific for CCL3, CCL4, and CCL18 and shows no sequence homology to any other known protein in public or patent databases. Using biochemical methods we identified the presence of binding proteins for CCL5 and CXCL8 in tick saliva and have subsequently used the expression cloning method described here to identify other distinct, highly selective CXC and CC chemokine-binding proteins from the tick salivary gland cDNA library, thus confirming results from other investigators that tick saliva contains diverse chemokine-binding proteins. These novel proteins will be useful tools in helping us to understand the role of specific chemokines in inflammatory disease and may have potential use as biotherapeutics in the future.

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