Glycosaminoglycan Binding Properties of the Myxoma Virus CC-chemokine Inhibitor, M-T1*

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Poxviruses encode a number of secreted virulence factors that function to mitigate or modulate the host immune response. M-T1 is a secreted 43-kDa glycoprotein produced by the myxoma virus, a poxvirus pathogen of rabbits, that binds CC-chemokines with high affinity, blocks binding to their cognate G-protein coupled receptors, and thereby inhibits chemokine-induced leukocyte chemotaxis. The present study indicates that M-T1, but not the related vaccinia virus 35-kDa CC-chemokine-binding protein, can localize to cell surfaces through an interaction with glycosaminoglycan molecules. In addition to biochemically characterizing the nature of this interaction, we demonstrate that M-T1 can also simultaneously interact with CC-chemokines while bound to heparin, suggesting that the binding sites on M-T1 for chemokines and heparin are distinct. Furthermore, using recombinant baculovirus-expressed M-T1 truncation and internal deletion mutants, we localize the heparin-binding region of M-T1 to the C terminus of the protein, a region that contains a high abundance of basic residues and includes two clusters of basic amino acid residues that resemble Cardin and Weintrab heparin-binding consensus sequences. The ability of M-T1 to simultaneously interact with chemokines and glycosaminoglycans may enable M-T1 to tether to endothelial surfaces or extracellular matrix and capture host chemokines that are expressed close to sites of virus infection.

One of the integral components of the host immune response is the directed infiltration of leukocytes to the site of infection or injury; this component depends on the coordinated action of the chemotactic cytokines known as chemokines (1–3). Chemokines are secreted from a variety of cells in response to a diverse array of stimuli and alert different subsets of leukocytes through high affinity interactions with cell surface G-protein-coupled receptors. Chemokines have been divided into several groups (CXC, CC, C, and CX3C) based on the number and arrangement of conserved cysteines located at the N-terminal region of these proteins. Chemokine presentation is also thought to be influenced by their low affinity association with glycosaminoglycans (GAGs)1 that are found as the posttranslational modification of proteoglycans on the surface of cells and within the extracellular matrix (4–7).

GAGs, such as heparan sulfate and chondroitin sulfate, participate in a variety of important biological processes, such as cell attachment, growth factor and cytokine binding, cell proliferation, migration, morphogenesis, and viral pathogenicity (8, 9). They function by binding to and modulating the biological function of a large and diverse group of proteins generically termed “heparin-binding proteins.” In the case of chemokines, interactions with cell surface GAGs during the process of inflammation or infection are thought to enable the formation of solid-phase gradients that lure leukocytes into the local areas of inflammation (6, 10–14). Given the importance of chemokines for host immune surveillance, their high affinity and low affinity binding sites offer attractive targets for disruption by viruses in order to benefit virus propagation (15–17).

Myxoma virus is a poxvirus that causes a highly virulent systemic disease in European rabbits known as myxomatosis (18). This results from the ability of myxoma virus to cause severe immune dysfunction by producing multiple modulators that disrupt key components of the host innate and acquired immune responses (19). This virus has therefore served as a useful model to examine the methods that poxviruses, such as variola virus (smallpox virus), or viruses in general use to disarm the immune system and contribute to disease pathogenesis. Two distinct myxoma virus virulence factors that have been identified that interact with chemokines are the M-T7 and the M-T1 proteins (20, 21). Originally identified as an interferon-γ receptor homolog (22), the M-T7 protein has an additional ability to interact with low affinity with the heparin-binding domain of CXC, CC, and C chemokines (20). In contrast, M-T1 binds with high affinity and specificity to the CC family of chemokines and can antagonize CC-chemokine binding to their cognate G-protein-coupled receptors, thus inhibiting chemokine-induced chemotaxis (23). M-T1 is also a member of a larger family of related poxvirus chemokine-binding proteins collectively known as the T1/35-kDa family (also called viral CC-chemokine inhibitors) of poxvirus CC-chemokine inhibitors (21, 24–26). Although the crystal structure of the cowpox virus CC-chemokine-binding protein p35 (CPV-p35) has been determined (27), the biophysical mechanism by which this family of

1 The abbreviations used are: GAG, glycosaminoglycan; CPV, cowpox virus; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; Kd, equilibrium dissociation constant; koff, association rate constant; kcat, dissociation rate constant; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; RU, response units; SPR, surface plasmon resonance; VV, vaccinia virus; SA, streptavidin; CSA, chondroitin sulfate A; CSB, chondroitin sulfate B.
viral proteins inhibits the activity of so many CC-chemokines is currently unknown. Nonetheless, characterization of M-T1 has shown that it is a 43-kDa N-linked secreted glycoprotein that not only can inhibit the biological function of CC-chemokines in vitro but also disrupts normal leukocyte trafficking in vivo during virus infection (21, 23). In this study, we show that in addition to binding CC-chemokines, M-T1, under physiological conditions, interacts specifically with cell surface GAGs. In an effort to characterize the nature and significance of this interaction, we demonstrate that M-T1 can bind CC-chemokines while simultaneously bound to GAGs, and we also provide evidence that the CC-chemokine-binding domain of M-T1 is distinct from its heparin-binding domain. As discussed in this report, this property has the potential to augment the ability of M-T1 to inhibit the local activity of CC-chemokines in situ and may represent a general mechanism for maintaining viral cytokine-binding proteins at sites of infection.

EXPERIMENTAL PROCEDURES

Materials—Heparin-Sepharose and Sepharose beads and 125I-radio-labeled monocyte chemoattractant protein-1 (MCP-1) (2200 Ci/mmol) were purchased from Amersham Pharmacia Biotech; the pAlve vector and Altered Sites Kit II in vitro mutagenesis systems kit were from Promega; porcine intestinal mucosa-derived heparin and chondroitin sulfate B (dermatan sulfate), heparan sulfate from bovine kidney, chondroitin sulfate A from bovine trachea, heparin-albumin-biotin, and albumin-biotin were from Sigma. Oligonucleotides used for site-directed mutagenesis were synthesized by a Beckman Oligo 1000M DNA Directed Mutagenesis kit. Restriction enzymes and T4 DNA ligase from New England Biolabs, and DH5α cells by electroporation. Colonies were screened for the presence of deletion mutant plasmids using polymerase chain reaction. Positive clones containing deletion mutant DNA were isolated and verified by sequencing.

Generation of Recombinant Baculovirus-expressed Vaccinia Virus 35-kDa Protein and M-T1 Mutants—The preparation of the recombinant vaccinia virus-35 kDa (VV-35 kDa) protein-expressing baculovirus is described elsewhere.2 The m001L/R deletion and truncation mutants contained in pAlveT1 were subcloned into pSP6–2 vector (Promega). For the purpose of generating recombinant baculovirus M-T1 protein mutants, we subcloned the m001L/R mutants contained within the pSP6–2 vector into pBacPAK1 (CLONTECH Laboratories Inc.) by PsiI and XhoI restriction enzyme digest of m001L/R deletion fragments. The fragments were isolated and inserted into the PsiI and XhoI restriction-digested sites of the intact m001L/R gene present in pBacPakm001L/R. pBacPAK containing m001L/R deletion mutants (pBacPAKm001) from positive clones were isolated and purified. The expression of M-T1 was accomplished by co-transfecting Spodoptera frugiperda SF-21 insect cells with pBacPAKm001 mutant DNA and Bsu36I linearized Autographa californica nucleopolyhedrovirus (AcMNPV) DNA, BacPAKDNA (CLONTECH Laboratories Inc.). SF-21 cells were seeded in a six-well tissue culture plate at 9 × 105 cells/well in SF9001 medium (Life Technologies, Inc.) and allowed to attach for at least 1 h. As per the manufacturer’s instructions, pBacPAKm001 mutant DNA was transfected into SF-21 cells. After transfection, cells were placed at 27 °C and assayed for protein expression 48 h, 72 h, and 8 days posttransfection by immunoblot. Baculovirus-expressed wild-type M-T1 protein and mutant M-T1 protein (m001) contained in supernatants of virally infected SF-21 cells were detected by immunoblot (30) using polyclonal antiserum against M-T1.

Purification of VV-35 kDa Protein, M-T1, and M-T7—The M-T1 and M-T7 proteins were purified from myxoma virus supernatants by fast protein liquid chromatography as previously described (20, 23). Baculovirus-expressed VV-35 kDa protein was purified from supernatants of baculovirus-infected cells by fast protein liquid chromatography using a three-step purification procedure. Viral supernatants were collected 72 h postinfection, subjected to anion exchange chromatography using a 5-ml HitrapQ column (Amersham Pharmacia Biotech), and eluted with a linear salt gradient (0–600 mM NaCl in 25 mM Tris, pH 6.0). Fractions containing VV-35 kDa were detected by immunoblot using anti-VV-35 kDa antiserum A18691. Fractions containing the protein were collected, pooled, concentrated, and dialyzed against 25 mM Tris, pH 8.0. Samples were subjected to anion exchange chromatography using a MonoQ column and eluted with a linear salt gradient (0–600 mM NaCl in 25 mM Tris, pH 8.0). Fractions with VV-35 kDa protein were pooled, subjected to size exclusion chromatography using a Superdex 200 16/60 column (Amersham Pharmacia Biotech), and isocratically eluted with PBS. Pooled fractions containing viral protein were visualized by silver staining to evaluate the purity of the recombinant viral proteins and demonstrated >95% homogeneity (not shown).

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M-T1 Is a Heparin-binding Protein

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FIG. 1. M-T1, but not M-T7 or VV-35 kDa protein, binds immobilized heparin. Purified M-T1 (top panel), M-T7 (middle panel), or the VV-35 kDa protein (bottom panel) (50 nM) was incubated with Sepharose (control, lane 2) or heparin-Sepharose beads (Hep-SepH; lanes 3 and 4) in binding buffer (see under “Experimental Procedures”) for 2 h. Lane 1 shows an input control of protein loaded directly onto SDS-PAGE. Excess soluble heparin was used in lane 4 to compete the interaction of viral protein bound to the heparin-Sepharose beads. After incubation periods, beads were spun down by high speed centrifugation and were washed with PBS three times. Bound proteins were eluted, subjected to SDS-PAGE, transferred to nitrocellulose, and blotted using either a rabbit polyclonal M-T1, M-T7, or VV-35 kDa protein antibody.

Binding Assays—Heparin-Sepharose beads were swollen in binding buffer (PBS with 0.2% bovine serum albumin) and washed three times in PBS prior to preparing a slurry of 50% (v/v) heparin-Sepharose beads in binding buffer. M-T1, M-T7, or VV-35 kDa protein (50 nM) was incubated with 10 μl of heparin-Sepharose for 1 h at room temperature and rotated in a volume of 400 μl of binding buffer. The beads were then recovered by a brief centrifugation in a microcentrifuge at 15,000 rpm. The supernatant was discarded, and the beads were washed three times with 400 μl of PBS. Bound viral proteins were eluted from the beads by the addition of 25 μl of SDS-gel loading buffer and boiled for 1 min. The gel loading buffer containing eluted viral protein was then subjected to SDS-PAGE and visualized by immunoblotting. As a negative control, the same procedures were repeated with Sepharose beads. This heparin-Sepharose pull-down assay was also varied to include co-attachments of M-T1 with various salt concentrations in 25 mM Tris-HCl buffer, pH 7.4, with 0.2% bovine serum albumin.

To examine interactions of M-T1 with heparin and chemokines, the heparin-Sepharose pull-down assay was also combined with a cross-linking electrophoretic mobility shift assay that we have previously used to examine chemokine interactions with M-T1 (21). After incubation of M-T1 with MCP-1 with heparin-Sepharose in binding buffer, all proteins that bound the immobilized heparin were pulled down by centrifugation of the heparin-Sepharose beads. To cross-link the interacting proteins, 15 μl of 10 mM sodium phosphate buffer, pH 7.0, was mixed with 2 μl of 0.2% 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and added to the beads for 10 min. After 10 min, 2 μl of 1 M Tris-Cl, pH 7.5, was added to quench the reaction. The cross-linked products were eluted from the heparin-Sepharose beads with the addition of SDS-gel loading buffer and boiling. The sample was subjected to SDS-PAGE and immunoblotted using an anti-M-T1 antibody. To ensure that MCP-1 had not complexed with M-T1 in the cross-linking solution, the beads were washed with PBS after cross-linking, and proteins were eluted as described and subjected to SDS-PAGE.

Cell binding assays were performed using L cells, gro2C cells, and sog2 cells. Cells were grown to confluency in 48-well dishes and were washed three times with PBS before binding studies were performed. Binding studies with L cells, gro2C cells, and sog2 cells were performed by incubating M-T1 (200 nM) in cell medium. After 2 h incubation at 4 °C, cells were washed three times. Cells were lysed with the addition of SDS gel loading buffer, and samples were subjected to SDS-PAGE. After electrophoretic transfer to nitrocellulose, M-T1 was detected by immunoblot against M-T1 as described. Relative quantities of M-T1 were determined densitometrically and expressed as the percentage bound relative to the specific binding of M-T1 to parental L cells. An identical method was used with L cells preincubated for 3 h at 37 °C with 5 units/ml of heparinase I (Sigma), 2 milliunits/ml chondroitinase ABC (Sigma), or both enzymes together. To ensure equal loading of lysates onto gels, blots were reprobed for β-actin (Santa Cruz Biotechnology).

To examine the effect of M-T1 on MCP-1 binding to cell surface GAGs, L cells grown in 48-well dishes were incubated with constant concentrations of [125I-MCP-1 (250 pCi) and increasing concentrations of M-T1 for 2 h at 4 °C in medium without newborn calf serum. Nonspecific binding was determined using a 1000-fold excess of unlabeled MCP-1. Cells were washed with PBS three times and lysed using 200 μl Tris-HCl buffer, pH 8.0, with 0.2% Triton X-100. Whole lysates were transferred to polystyrene tubes and [125I-MCP-1 was measured using a gamma counter (Wallac).

Biomolecular Interaction Analysis Using Surface Plasmon Resonance—To measure the kinetic and affinity binding parameters between M-T1 and heparin, we performed biomolecular interaction analysis (BIAcore) to measure real-time binding. We developed an artificial proteoglycan surface using a streptavidin (SA) chip (BIAcore, Inc.) and measured binding using a BIAcoreX apparatus (BIAcore, Inc.). The active binding surface was constructed by immobilizing 400 response units (RU) of heparin-albumin-biotin (Sigma) onto flow cell 2 of the SA chip that noncovalently captured the biotinylated heparin-albumin. On the control channel, flow cell 1, biotinylated albumin (Sigma) was injected, and 400 RU of protein was noncovalently immobilized. 50 μl of glycine, pH 2.0, was injected over both surfaces to remove any loosely bound heparin-albumin-biotin.

For kinetic studies, M-T1 was serially diluted into HBS-EP (10 mM Heps, 150 mM NaCl, 0.005% polysorbate (v/v), 3 mM EDTA). 100 μl of each concentration of M-T1 was injected over both flow cells at a rate of 100 μl/min at 25 °C. Following the association phase, HBS-EP was allowed to flow over the two surfaces to monitor the dissociation phase of M-T1 for up to 200 s. Specific binding was determined automatically using BIAcore Control software 2.1 by subtracting the control cell sensogram (nonspecific binding) from the active flow cell sensogram. Flow cells were regenerated by eluting bound protein with three 10-μl pulses of a 1 M NaCl solution. To eliminate sensogram deviations introduced by the BIAcoreX hardware, we subtracted from the initial data a sensogram generated from the injection of buffer alone (31). During preliminary experiments, an observed heterogeneity of binding occurred at higher concentration of M-T1, possibly due to oligomerization at the surface. We therefore confined kinetic analysis to three lower concentrations of M-T1 (4, 12, and 36 nM). Because kinetic analysis allowed the ability to generate accurate affinity measurements using the association and dissociation phases of binding, these curves were adequate for determining the kinetic parameters for M-T1 binding to immobilized heparin conjugated to biotinylated albumin. Curves were globally fit using BIAevaluation software 3.0 using a 1:1 mass transport model. Using the determined association (kₐ) and dissociation (k₉) rate constants, the dissociation equilibrium affinity constant (Kₐ) was calculated (Kₐ = k₉/kₐ).

GAG Competition Assay Using Surface Plasmon Resonance—To examine the oligosaccharide structures that M-T1 may be capable of binding, we used the heparin-albumin chip in a surface plasmon resonance (SPR) competition assay that involved the preincubation of 50 nM of M-T1 with several concentrations of soluble GAG competitors, heparin, heparan sulfate, chondroitin sulfate A, and chondroitin sulfate B for 2 h prior to SPR analysis. For each GAG competitor, M-T1 (50 nM) preincubated with 1000 μg/ml GAG was injected at a flow rate of 5 μl/min. The M-T1 binding curve was allowed to equilibrate (dRUt/dt = 0), and the response at equilibrium was recorded. Subsequently, M-T1 (50 nM) preincubated with 100 μg/ml GAG was then injected over the chip and allowed to equilibrate, and the response was recorded. This was further repeated for M-T1 preincubated with GAG concentrations of 10, 1, and 0 μg/ml.

Structural Modeling of M-T1 and the VV-35 kDa Protein—To identify the location of potential heparin-binding domains within the three-dimensional structure of M-T1, a model of M-T1 was compared with a model of the related VV-35 kDa protein, which does not bind heparin. Both M-T1 and the VV-35 kDa models were constructed using the structure of CPV-p35 (27) (Research Collaboratory for Structural Bioinformatics Protein Data Bank accession number PDB 1CQ3). A multiple sequence alignment containing M-T1, VV-35 kDa, CPV-p35, and four other CC-chemokine-binding proteins from poxviruses was used to match residues from M-T1 and VV-35 kDa to those of CPV-p35 (21). When aligned in this manner, the sequence identity between M-T1 and CPV-p35 is ~38%, with an additional 18% sequence similarity; in
M-T1 binds heparin with submicromolar affinity. A, purified M-T1 was incubated with heparin-Sepharose beads (as described under "Experimental Procedures") in the presence of increasing concentrations of NaCl. At the end of the incubation period, bound protein remaining on beads was eluted, resolved by SDS-PAGE, and immunoblotted for M-T1. B, SPR analysis of M-T1 interacting with an artificial proteoglycan surface. M-T1 was injected at three different concentrations (4, 12, and 36 nM) over flow cells of a BIAcore SA chip having immobilized heparin-albumin-biotin on the active surface and albumin-biotin on the control surface. The amount of M-T1 bound was recorded as RU as a function of time (s). 100 µl of M-T1 was injected over the surfaces at a flow rate of 100 µl/min for the association phase, and HBS-EP only was injected over the surfaces to monitor dissociation phases. The active surface sensorgram was double referenced by subtracting the control surface sensorgram and a blank injection sensorgram from the active sensorgram. Using the global analysis software BIAevaluation 3.0, a 1:1 mass transport model was used to fit theoretical curves (shown as red lines) to the experimental data (black lines) to determine the association and dissociation rate constants (k_{on} and k_{off}).

RESULTS

M-T1 Is a GAG-binding Protein—To examine potential interactions of secreted viral proteins that might interact with GAGs, we employed a heparin-Sepharose bead pull-down assay (35). The purified viral chemokine-binding proteins, M-T1, M-T7, and VV-35 kDa, were mixed with either heparin-Sepharose or unconjugated Sepharose beads; after washing, viral proteins were eluted from the beads and visualized by SDS-PAGE, and visualized by immunoblotting. As shown in Fig. 1, M-T1 uniquely bound to the heparin-Sepharose beads in 140 mM NaCl (Fig. 1, top panel, lane 3), and this interaction was specific for the immobilized heparin because M-T1 failed to bind the control unsubstituted Sepharose beads (top panel, lane 2). In addition, the interaction of M-T1 with the immobilized heparin could be fully competed off by the co-incubation of M-T1 with excess soluble heparin (1 mg/ml) (Fig. 1, top panel, lane 4). M-T7, another secreted myxoma virus chemokine-binding protein, was tested for heparin binding but failed to bind either to control beads or heparin-Sepharose beads (Fig. 1, middle panel, lanes 2 and 3). Because M-T1 belongs to a family of related poxvirus CC-chemokine-binding proteins, we also tested the vaccinia virus (strain Lister) 35 kDa (VV-35 kDa) CC-chemokine-binding protein, but it also failed to bind either the control or heparin-Sepharose beads under these conditions (Fig. 1, bottom panel, lanes 2 and 3).

M-T1 Binds Heparin with Submicromolar Affinity—A large component of heparin-protein interactions is mediated by electrostatic interactions between positively charged amino acids on proteins and anionic sulfate groups present on GAGs (36, 37). The concentration of salt required to elute heparin-binding proteins from heparin has therefore been useful to determine the relative strength of the interaction between proteins and heparin. We used NaCl competition to determine the relative binding affinity of M-T1 for heparin. In a modified heparin-Sepharose pull-down assay, M-T1 was eluted from heparin-Sepharose beads with increasing concentrations of NaCl. M-T1 binding to heparin is completely abrogated at 400 mM NaCl (Fig. 2A). This demonstrates that the M-T1 interaction with heparin is of a low affinity nature. It further demonstrates that M-T1 is able to interact with heparin at ionic strengths above that of physiological levels. To more accurately define the affinity of the M-T1 protein for heparin, we developed a SPR assay to monitor the real-time association and dissociation phases of M-T1 binding to an artificial proteoglycan surface constructed using biotinylated heparin-albumin (Fig. 2B). Using this assay, M-T1 was shown to bind heparin with a K_{D} of 446 nM (k_{on} = 1.64 × 10^{4} M^{-1} s^{-1}, k_{off} = 7.32 × 10^{-3} s^{-1}).

M-T1 Interacts with Other Sulfated GAGs—Proteoglycans in vivo are modified with a variety of GAG structures that differentially interact with heparin-binding proteins with varying specificity and affinity (8, 36). To examine the GAG binding specificity of M-T1, we developed a SPR GAG competition assay using the heparin-albumin-biotin SA chip described. M-T1 binding to immobilized heparin-albumin-biotin was determined at equilibrium after preincubation with various concentrations of competing GAGs including soluble heparin, heparan sulfate, chondroitin sulfate A (CSA) and chondroitin sulfate B (CSB) (Fig. 3). Whereas soluble heparin was able to competitively inhibit M-T1 interaction with immobilized heparin at concentrations less than 1 µg/ml, the less sulfated heparan sulfate was unable to interfere with M-T1 binding. The concentration of competing GAGs used to determine the association and dissociation rate constants (k_{on} and k_{off}).
sulfate and CSB were less potent by at least an order of magnitude. CSA exhibited a further 10-fold decrease in the ability to inhibit M-T1 binding to the heparin-albumin surface. These results demonstrate that M-T1 is capable of interacting with a variety of sulfated GAG structures but with varying affinities.

Localization of the Heparin-binding Region of M-T1—To examine the region(s) along the polypeptide structure of M-T1 responsible for binding to heparin, N- and C-terminal deletions and internal deletion variants of M-T1 were constructed and expressed in a recombinant baculovirus system (Fig. 4A). Full-
**TABLE II**

Comparison of basic regions of heparin-binding proteins to M-T1

| Consensus I          | M-T1 (site 1)* | Apo B† |
|----------------------|----------------|--------|
|                      | XXBBXBX        | TTS1GRQLHRVSTAF |
| Fn                   | FGTVRPLHETGGYML |
| TS                   | SLRQKKEKTGGTIAL |
| Vn                   | RPSLARKKRFHRNKK |
| THR                  | LVR1KKEKTRYERM1 |
| PCI                  | GLSEKTLRLKLVFKK |

| Consensus II         | M-T1 (Site 2)* | γ-IP-10 | Vn | NCAM | ApoE |
|----------------------|----------------|---------|----|-----|------|
|                      | XXBBXBX        | EIATKKEKKGERCLN |
|                      | SAROSLKVKNKLED |
|                      | QFRRHNRKGR1SQRG |
|                      | DGGSPIRVLKYYKAK |
|                      | SHLKRKERRLLOADD |
|                      | GLSEKTLRLKLVFKK |

* Sequence for M-T1 site 1 corresponds to residues from Ile-191 to Val-206.
† ApoB, apolipoprotein B; ApoE, apolipoprotein E; Fn, fibronectin; γ-IP-10, γ-interferon-inducible protein; Vn, vitronectin; THR, thrombin; NCAM, neural cell adhesion molecule; PCI, protein C inhibitor; TS, thrombospordin.

Hydropathic (X) residues in a database of heparin-binding proteins.

Longer-length bM-T1 and bM-T1 deletion mutants were incubated with Sepharose or heparin-Sepharose beads, and after washing, bound proteins were eluted and detected by immunoblotting (Fig. 4B). We determined from these assays that the N-terminal and central regions of M-T1 are not critical for heparin binding. The lack of binding to heparin by the M-T1 C-terminal deletion mutant (Δ170–260) suggests that the heparin-binding domain of M-T1 is localized within the C terminus (amino acids 170–260) (Fig. 4A). The decreased band intensity of the N-terminal mutant of M-T1 (Δ23–52) is unique to this particular gel. That this region is not involved in heparin binding is also demonstrated by the observation that binding to heparin by the mutant Δ23–102, a mutant that encompasses Δ23–52, is unaffected by the deletion. Nevertheless, inspection of the primary amino acid sequence of the C-terminal region revealed an abundance of basic amino acids (Fig. 4A, asterisks) and at least two clusters of basic residues that resemble the Cardin and Weintraub (38) heparin-binding consensus motifs, XXBBXBX and XXBBXBX, where B represents any basic residue and X represents any nonbasic residue (Table II). Interestingly, the VV-35 kDa protein, which does not bind heparin, does not possess these motifs, nor does it share the abundance of basic charge in its C-terminal region, nor do other orthopoxvirus members of the T1/35-kDa family of proteins.

**M-T1 Is Capable of Interacting with GAGs and Chemokines Simultaneously**—To test whether M-T1 may be able to bind both a CC-chemokine and GAG simultaneously, we combined a heparin pull-down assay with an in vitro cross-linking assay in order to observe whether M-T1 can still interact with a CC-chemokine while bound to immobilized heparin. After M-T1 was co-incubated with MCP-1 and heparin-Sepharose, unbound proteins were washed away with PBS (Fig. 5, lanes 4–6). Using the chemical cross-linking agent EDC, proteins that were associated with each other were covalently cross-linked, and bound proteins were eluted from the heparin-Sepharose bead, subjected to SDS-PAGE, transferred to nitrocellulose, and probed with antisera against M-T1 (Fig. 5, lanes 5 and 6).

In this way, all proteins that interacted with the heparin-Sepharose beads were pulled down, and those proteins that interacted with each other on the surface of the bead were covalently coupled and detected using a gel electrophoretic mobility shift assay. Using this method, a 1:1 interaction between M-T1 and MCP-1 was detected as determined by the appearance of a shifted M-T1 complex, suggesting that M-T1 and MCP-1 could indeed interact with each other while bound to immobilized heparin (Fig. 5, lane 5). To ensure that complexes did not form while in solution with the cross-linking agent, the beads were washed and pelleted again after the reaction was quenched, and heparin-bound proteins were eluted and subjected to SDS-PAGE (Fig. 5, lane 6). Additional control experiments were also performed using radiolabeled MCP-1 to ensure that the apparent shift was a result of M-T1 being cross-linked to the chemokine (data not shown). These results demonstrate that M-T1 is capable of interacting with CC-chemokines even while bound to heparin-Sepharose. This dual interaction with a chemokine and heparin-Sepharose indicates that M-T1 has the potential to bind and neutralize chemokines while affixed to GAGs on cell surface proteoglycans.

M-T1 Localizes to Cell Surfaces via Proteoglycan Interactions—The in vitro binding of M-T1 to immobilized heparin indicates that M-T1 may have the capacity to localize to cell surfaces through interactions with GAGs. To probe the use of cell surface GAGs by M-T1, we employed cell lines that have been well characterized for their defective synthesis of GAGs (39, 40). It has been previously demonstrated that gro2C cells, a cell line derived from parental L cell fibroblasts, are defective in their ability to synthesize heparan sulfate, whereas sog9 cells, derived from gro2C cells, possess an additional defect such that they have lost the capacity to synthesize any sulfated GAG, including chondroitin sulfates (40). M-T1 was incubated with each of the wild-type L cells and gro2C and sog9 cell lines. Cells were washed with PBS, lysed with SDS, and subjected to SDS-PAGE. Cell-associated M-T1 was detected by immunoblotting, and the relative amount was quantified by densitometry (Fig. 6A). Given the cell surface GAG phenotypes of the gro2C and sog9 cells, ~40% of the binding by M-T1 to the surface of L cells can be ascribed to cell surface heparan sulfate, whereas another 40% can be ascribed to cell surface chondroitin sulfate (Fig. 6A).

These cells also possess intact N- and O-linked surface oligosaccharides that could impart a negative charge, depending on levels of sialylation, which could contrib-
ute to a small amount of the observed residual binding to sog9 cell surfaces by M-T1. To confirm that the differences observed for M-T1 binding to each cell line were a result of the deficiencies in GAG expression, we demonstrate, using heparinase I and chondroitinase ABC to specifically digest cell surface GAGs, that the binding of M-T1 to cell surfaces is reduced after L cells were treated with the enzymes in a similar manner as in Fig. 6A (Fig. 6B).

Competition of the Binding of a CC-chemokine to Cell Surface GAG Sites by M-T1—To further understand the interaction of M-T1 with both chemokines and GAGs, we attempted to competitively inhibit the binding of 125I-radiolabeled MCP-1 to cell surface GAGs with increasing concentrations of M-T1 (Fig. 6C). Rather than simple competition curves, we observed an initial increase in the binding of the tracer ligand (human 125I-MCP-1) at M-T1 concentrations between 10 and 100 nM followed by a reduction in binding at 1 and 10 μM. Given that both MCP-1 and M-T1 bind to low affinity cell surface GAG sites, the observed increase in the amount of MCP-1 bound at the surface as a function of M-T1 concentration reflects an increase in cell surface chemokine binding sites bestowed by M-T1. Thus, as M-T1 reaches a critical concentration at which its binding shifts toward the low affinity GAG sites on the cell surface, additional binding sites for MCP-1 are generated. The reduction in cell surface-bound MCP-1 at the highest M-T1 concentrations likely represents the displacement of both 125I-MCP-1 and M-T1 complexed with MCP-1 from the cell surface by excess cold M-T1. These data support the notion that M-T1 not only has the capacity to tether to cell surfaces and bind chemokine out of solution but also may be able to disrupt GAG-bound chemokine gradients.

**Modeled Structure of M-T1 and the VV-35 kDa Protein**—To determine what structural features are present on M-T1 that may accommodate heparin binding and to examine why M-T1, but not the related VV-35 kDa, interacts with GAGs, we constructed three-dimensional models of M-T1 and the VV-35 kDa proteins based on the crystal structure of the cowpox virus CC-chemokine inhibitor, p35 (CPV-p35) (27). The structure of the CPV-p35 molecule forms a β-sandwich of novel topology with the exposed surface of β-sheet II, forming a strongly negative face that is a candidate binding region for CC-chemokines (27). The negative potential of the β-sheet II face is conserved in both the M-T1 and VV-35 kDa models, consistent with their ability to bind positively charged CC-chemokines (Fig. 7C). In fact, the negative potential of the β-sheet II face in M-T1 is expected to be greater than in VV-35 kDa: 11 additional negative charges in M-T1 are provided by an insertion (residues 68–85, which were not modeled in our M-T1 structure) that occurs in a large loop between strands β2 and β4 on the β-sheet II face.

On the opposite side of the putative CC-chemokine-binding region formed by β-sheet II, β-sheet I of M-T1 produces a strong positive electrostatic potential (Fig. 7B). The lack of positive charge on the corresponding region of the VV-35 kDa protein is consistent with its inability to bind heparin (Fig. 7B). The positive potential of this surface in M-T1 is due to the replacement of 5 acidic and 7 neutral residues in VV-35 kDa with basic residues in M-T1, for an overall increase in the surface charge of +17 (Table III). In particular, there are changes in charged residues that produce two heparin-binding consensus motifs in M-T1 (TRRSII, residues 195–200, and LKVKNQKIL, residues 236–243) that are not present in the VV-35 kDa protein. The residues that form these motifs are surface exposed and are accessible as candidate heparin-binding regions.

There are many examples of heparin-binding proteins that do not conform to any apparent heparin-binding consensus sequences. This underscores the fact that primary sequence alone cannot define heparin-binding domains and that conformational positioning of basic residues may be sufficient to create energetically favorable arrangements to facilitate interactions with polyanionic sulfates present on GAGs. For instance, human lactoferrin (41) and fibronectin (42) possess a heparin-binding structure termed the “cationic cradle.” This structure is composed of six basic residues, either lysine or arginine, linearly arranged with three on each side of a central cleft. In addition to the proposed heparin-binding consensus...
sequences, the tertiary arrangement of the basic residues within the β-sheet I region of M-T1 appears to adopt a structure that resembles a cationic cradle (Fig. 7B) and may contribute to the ability of M-T1 to bind heparin.

The unique electrostatics of M-T1 compared with the VV-35 kDa protein are dramatic. The shared negative regions of M-T1 and VV-35 kDa proteins support the prediction that the β-sheet II forms the high affinity site for CC-chemokines (Fig. 7, C and D). In contrast, the exposed face of the β-sheet I of M-T1, a region with significant positive charge, forms the candidate heparin-binding domain of M-T1 (Fig. 7, B and D). The discrete surfaces of opposite charge revealed in the M-T1 model not only provide a structural basis for the ability of M-T1 to simultaneously interact with a chemokine and GAG using distinct domains but also illustrate why the VV-35 kDa protein possesses no discernible affinity for heparin.

**DISCUSSION**

The ability of GAGs to modulate the function of proteins that possess heparin-binding domains has yielded new avenues of study within various subdisciplines of biology. It is not entirely surprising that viruses have adopted the ability to exploit the universal presence of GAGs. There are several examples of heparin-binding proteins encoded by poxviruses. Notably, several virion-associated proteins of vaccinia virus have been shown to bind GAGs and facilitate infection of host cells (43–45). Certain secreted viral proteins, such as the human poxvirus *Molluscum contagiosum* MC148 chemokine homolog, have retained the ability to bind heparin, and this property has been exploited during purification (46). Recently, a complement-control protein from vaccinia virus was shown to be a GAG-binding protein with several unique features including the ability to displace chemokines bound to GAGs (47, 48).

In this study, we investigated whether any of the secreted chemokine-binding proteins from poxviruses have a similar propensity to bind GAGs. Using *in vitro* assays, we show that M-T1 uniquely binds heparin ($K_D = 446$ nM), but neither the related VV-35 kDa protein nor the distinct M-T1 protein can interact with GAGs. M-T1 elutes completely from heparin at salt concentrations of 0.4 M. Interestingly, chemokines, such as MIPF-1 (myeloid progenitor inhibitory factor-1), MIP-1α (macrophage inflammatory protein-1α), MIP-1β (macrophage inflammatory protein-1β), RANTES (regulated upon activation, normal T cell expressed and secreted), MCP-3 (monocyte chemoattractant protein-3), HCC-2 (human CC-chemokine 2), and inter leukin-8 elute from heparin-Sepharose columns with 0.3, 0.19, 0.3, 0.7, 0.4, 0.2, and 0.4 M NaCl, respectively (49). The comparable NaCl elution concentration profile of M-T1 suggests that the heparin binding ability alone of M-T1 could displace GAG-bound chemokines.

To characterize the GAG structures that M-T1 interacts with, we demonstrate that M-T1 binds heparan sulfates and chondroitin sulfates with varying affinities: heparin > heparan sulfate > CSA > CSA. The differential ability of M-T1 to bind these GAGs might reflect the constituent units that make up each polysaccharide. Heparin is more sulfated than either heparan sulfate or the chondroitin sulfates. Although heparin, heparan sulfate, and CSB all possess l-iduronic acid, heparin contains the highest proportion of l-iduronic acid. CSA, on the other hand, is devoid of l-iduronic acid residues. Therefore, our observations suggest that the binding affinity of M-T1 to GAGs is dependent on both the level of sulfation and the proportion of l-iduronic acid present in the polysaccharide.

| Residues responsible for the positive electrostatic potential of M-T1 |
|-------------------------|-------------------------|-------------------------|
| VV-35 kDa | M-T1 | ΔCharge |
| Asp-52 | Arg-41 | +2 |
| Asp-124 | Lys-130 | +2 |
| Glu-139 | Arg-145 | +2 |
| Asn-184 | Arg-190 | +1 |
| Glu-193 | Ser-198 | +1 |
| Asp-194 | His-199 | +2 |
| Glu-221 | Arg-226 | +2 |
| Glu-226 | Ser-231 | +1 |
| Val-234 | Lys-239 | +1 |
| Asp-235 | Asn-240 | +1 |
| Ser-237 | Lys-242 | +1 |
| Asp-250 | Arg-252 | +2 |

*a* Calculated by subtracting the charge difference for the residue of M-T1 from the corresponding residue on the VV-35 kDa protein.

The unique electrostatic potentials (blue, positive; red, negative) as calculated by the program SPOCK, C, opposite side of the viral proteins (relative to the view in B) showing the surface of β-sheet II. This view shows the predicted surface at which the CC-chemokine-binding region is thought to reside because the negatively charged surfaces also contains patches of conserved residues among the poxvirus CC-chemokine-binding proteins (27). D, side view (turned left 90°) showing overall electrostatic potential surrounding the proteins. M-T1 possesses a highly negatively charged face (β-sheet II, left side of M-T1) and on the opposite side displays a highly basic face (β-sheet I, right side of M-T1). The corresponding basic region present in M-T1 is virtually absent in the VV-35 kDa protein.
M-T1 Is a Heparin-binding Protein

The opposite side of M-T1 (the chemokine-binding region for this family of viral proteins) the deduced structure, forming what has been proposed to be a negatively charged surface within the exposed face of M-T1. This region possesses a high occurrence of basic residues, the bulk of which make up the surface region of β-sheet I. This region also contained at least two clusters of basic residues that closely resemble canonical Cardin and Weintraub heparin-binding motifs (38). Inspection of the amino acid sequence of the vaccinia virus counterpart of M-T1, the VV-35 kDa protein, revealed a notable absence of the clustered basic residues present in M-T1 and implies why the VV-35 kDa protein does not have any detectable affinity for heparin.

To examine the structural features of M-T1 that may mediate GAG binding, a model of M-T1 was constructed based on the structure reported for CPV-p35 (27). The M-T1 protein exhibits a negatively charged region on the β-sheet II side of the deduced structure, forming what has been proposed to be the chemokine-binding region for this family of viral proteins (27). The opposite side of M-T1 (β-sheet I) forms what is likely the heparin-binding region of M-T1 because this surface possesses a high degree of positive charge and contains two Cardin and Weintraub heparin-binding consensus sequences. The idea that this region mediates heparin binding is consistent with the observation that the C-terminal deletion mutant, a deletion that would have significant impact on this region of the protein, abolished the interaction of M-T1 with heparin. This region also forms a potential heparin-binding structure found in human lactoferrin and fibronectin known as a cationic cradle (41, 42). Whether the ability of M-T1 to bind heparin is mediated by the Cardin and Weintraub heparin-binding consensus sequences or a cationic cradle structure remains to be determined. Nonetheless, the bipolar charge distribution on the surface of M-T1 is compatible with the idea that the CC-chemokine-binding region on M-T1 is physically distinct from the GAG-binding domain.

Comparison of the models of M-T1 and the VV-35 kDa protein revealed a striking difference in surface electrostatics. Consistent with its inability to bind heparin, the VV-35 kDa protein notably lacks the region of positive charge and the putative heparin-binding consensus sequences observed in β-sheet I of M-T1. In contrast, the two proteins share a negatively charged surface within the exposed face of β-sheet II consistent with their shared ability to bind positively charged chemokines. This common feature of these two proteins lends support to the suggestion by Carfi et al. (27) that the CC-chemokine-binding domain is likely positioned within a region of β-sheet II. Nevertheless, the considerable difference in the surface electrostatics within the β-sheet I regions of M-T1 and the VV-35 kDa protein provides a structural basis for the differential ability of these two proteins to bind GAGs.

We have previously reported that M-T1 and the VV-35 kDa protein exhibit less than an overall 40% amino acid identity yet share similar in vitro effects on inhibiting CC-chemokine activity in Boyden chamber assays (23). Our findings here suggest that the dual ability of M-T1 to simultaneously bind GAGs and chemokines may impart several distinct functions in vivo not shared by the VV-35 kDa protein. One scenario would permit M-T1 to loosely tether to the surface of cells at the site of infection in order to bind CC-chemokines that are induced locally as a result of the infection. In this way, M-T1 could capture and neutralize host CC-chemokine gradients established at sites of infection in addition to its documented ability to antagonize CC-chemokine binding to their cognate receptors. GAG binding by M-T1 within the extracellular matrix may also prevent diffusion effects in vivo and enable the protein to persist in the tissue microenvironment, thereby increasing its local concentration at sites of infection. Furthermore, to prevent clearance, M-T1 may share the ability of other heparin-associated proteins that are protected from protease degradation when bound to GAGs (51). Overall, the ability to bind GAGs may provide a mechanism for enabling M-T1 to better inhibit the activity of CC-chemokines.

Kinetic analysis of the interaction between M-T1 and the VV-35 kDa protein with several CC-chemokines has demonstrated a very rapid association rate ($k_{on} = > 10^5$ M$^{-1}$ s$^{-1}$).3 Comparing these values with the $k_{on}$ for M-T1 with heparin ($k_{on} = 1.46 \times 10^4$ M$^{-1}$ s$^{-1}$) suggests that in vivo, M-T1 would likely associate with CC-chemokines in the immediate microenvironment before interacting with cell surface GAGs. On the other hand, M-T1 is produced early during infection (52) and binding GAGs on cell surfaces or within the matrix would elevate its local concentration, allowing it to remain as a sentinel close to the site of infection, permitting the very rapid neutralizing of any CC-chemokines produced.

The conclusion that M-T1 and the VV-35 kDa protein differ so dramatically by their GAG binding characteristics is likely a reflection of the distinct evolutionary history of the two genera of poxviruses that these viruses represent. The relative efficacies of these two chemokine-binding proteins in inhibiting CC-chemokine functions in tissues remains to be deduced, but the unique ability of M-T1 to bind to cell surfaces suggests that they will exhibit distinctive tissue distribution and clearance profiles. Using model-guided site-directed mutants of M-T1 to identify the precise residues that mediate GAG interactions, these predictions will be amenable to testing not only within the viral model of myxomatosis but also in models of inflammation that are responsive to the application of viral chemokine-binding proteins as anti-inflammatory reagents (53, 54).

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Glycosaminoglycan Binding Properties of the Myxoma Virus CC-chemokine Inhibitor, M-T1
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