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The Purified Myxoma Virus Gamma Interferon Receptor Homolog M-T7 Interacts with the Heparin-Binding Domains of Chemokines

ALSHAD S. LALANI,[1] KATHRYN GRAHAM,[1] KAREN MOSSMAN,[1] KRISHNA RAJARATHNAM,[1] IAN CLARK-LEWIS,[2] DAVID KELVIN,[3] AND GRANT MCFADDEN[1]*

Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7,[1] Biomedical Research Centre, University of British Columbia, Vancouver, British Columbia V6T 1Z3,[2] and John P. Robarts Research Institute, London, Ontario N6G 2V4,[3] Canada

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The myxoma virus T7 protein M-T7 is a functional soluble gamma interferon receptor homolog that has previously been shown to bind gamma interferon and inhibit its antiviral activities in a species-specific manner, but gene knockout analysis has suggested a further role for M-T7 in blocking leukocyte influx into infected lesions. We purified M-T7 to apparent homogeneity and showed that M-T7 is an N-linked glycoprotein that appears to be a stable homotrimer with a molecular mass of approximately 113 kDa in solution. M-T7, in addition to forming inhibitory complexes with rabbit gamma interferon, was also shown to bind to human interleukin-8, a prototypic member of the chemokine superfamily. Moreover, M-T7 was able to interact promiscuously with all members of the CXC, CC, and C chemokine subfamilies tested. Binding of human RANTES to M-T7 can be competed by rabbit gamma interferon and also by cold RANTES competitor with a 50% inhibitory concentration of 900 nM. Although M-T7 retains binding to a number of interleukin-8 N-terminal (ELR) deletion mutants, binding to mutants containing deletions in the C-terminal heparin-binding domain of interleukin-8 is abrogated. Furthermore, heparin effectively competes the interaction of M-T7 with the chemokine RANTES but not with rabbit gamma interferon. We propose that this novel M-T7 interaction with members of the chemokine superfamily may be facilitated through the conserved heparin-binding domains found in a wide spectrum of chemokines and that M-T7 may function by modulating chemokine-glycosaminoglycan interactions in virus-infected tissues.

Myxoma virus is a poxvirus that induces a virulent systemic lethal disease called myxomatosis in European rabbits (17). Myxomatosis is characterized by generalized immunosuppression, as evidenced by a notable dysfunction of the host acquired cellular immune response (30, 55). Like all poxviruses, myxoma virus contains a large double-stranded DNA genome with terminal inverted repeats and covalently closed hairpin ends (34). While many genes that map to the central portion of the genome are necessary for virus propagation, those that are clustered near the termini tend to encode factors which are nonessential for replication in tissue culture but confer viral virulence in infected animals (7, 59).

It is becoming increasingly evident that poxviruses have evolved a multitude of strategies to subvert the development of the host inflammatory response, thereby fostering virus survival in the wild (31, 51, 52). Examples of these viral defense mechanisms include interference with major histocompatibility complex antigen presentation (33), inhibition of apoptosis (28, 44, 58), disruption of complement cascade (25), and inhibition of cytokines (3, 6, 31, 43). In particular, poxviruses encode specific gene products that have significant homology to cellular cytokine receptors and may function by sequestering the host cytokine before it can interact with the cognate receptor. To date, secreted poxvirus proteins which have homologies to receptors of tumor necrosis factor (TNF) (60), alpha/beta interferon (IFN-α/β) (13, 56), interleukin-1β (IL-1β) (4, 53), and IFN-γ (61) have been identified. Additionally, two poxvirus genes with sequence similarities to the genes of the serpentine receptors for IL-8 have also been described (8, 29). The myxoma virus T7 protein M-T7 bears significant homology to the ligand-binding domain of cellular IFN-γ receptors (61). M-T7 is produced early in infection and is the most abundantly secreted protein from myxoma virus-infected cells. Previous studies have demonstrated that M-T7 can bind to and inhibit the biological activities of rabbit IFN-γ (rIFN-γ) with an affinity that is comparable to that of its cellular counterpart (36).

We have recently shown that M-T7 is a crucial virulence factor in progression of myxomatosis in European rabbits (35). Rabbits infected with a myxoma virus in which both copies of the M-T7 open reading frame had been disrupted recovered fully and displayed a vigorous and effective cellular immune response to infection. In particular, the absence of M-T7 expression resulted in an elevation of reactive-leukocyte levels in secondary lymphoid organs, contributing to efficient virus clearance. Furthermore, dermal tissue surrounding sites of wild-type virus infection showed a striking disruption of inflammatory cell migration, while tissues from a rabbit with an M-T7 deletion virus infection showed this apparent blockade of leukocyte infiltration to be relieved (35). Since it has yet to be demonstrated that IFN-γ blockading alone influences inflammatory cell trafficking, we speculated that M-T7 may perform additional functions apart from binding and inhibiting rIFN-γ.

The activation and recruitment of specialized leukocyte populations to sites of viral infection and inflammation is largely orchestrated by a growing superfamily of chemoattractant cy-

* Corresponding author. Present address: J. P. Robarts Research Institute and Dept. of Microbiology and Immunology, University of Western Ontario, 1400 Western Rd., London, Ontario, Canada N6G 2V4. Phone: (519) 663-3184. Fax: (519) 663-3847. E-mail: mcfadden@rri.on.ca.

tokines called chemokines (5, 46, 54). This superfamily of proinflammatory molecules is divided into three subfamilies, CXC, CC, and C, based on the number and spatial arrangement of conserved cysteine residues. These designations also loosely correlate with the target cell population to which they impart their biological activity. For example, CXC chemokines such as IL-8 are generally potent activators and chemoattractants of neutrophils but not monocytes. In contrast, CC chemokines such as RANTES (for "regulated on activation normal T-cell expressed and secreted") have been shown to attract and activate monocytes, basophils, eosinophils, and certain T lymphocytes. Lymphotactin (Ltn), the sole member of the C subfamily to date, appears to be a T-lymphocyte-specific chemoattractant (23). Chemokines signal through cell surface receptors, all of which belong to the serpentine family of seven transmembrane G-protein-coupled receptors and display various degrees of ligand and chemokine subfamily binding selectivity (24, 37, 38).

To investigate a possible relationship between M-T7 and other proinflammatory cytokines such as the chemokine family, we purified M-T7 to homogeneity and characterized the protein in vitro. We showed that, in addition to binding rIFN-γ, purified M-T7 interacts with multiple members of the chemokine superfamily. Experiments using various NH_2- and COOH-terminal chemokine mutants suggest that this novel activity may be facilitated through interactions between M-T7 and the conserved heparin-binding domains found in a wide spectrum of chemokines.

MATERIALS AND METHODS

Cells and viruses. v-Myxlac, a derivative of myxoma virus (strain Lausanne) containing a β-galactosidase marker gene, has been described elsewhere (42). v-Myxlac was routinely propagated in baby green monkey kidney (BGMK) cells (a gift from S. Dales, University of Western Ontario). BGMK cells were cultured in Dulbecco's minimal essential medium supplemented with 10% newborn calf serum (Life Technologies, Gaithersburg, Md.).

Cytokines and chemokines. Recombinant murine IL-1β (mIL-1β), human IL-2 (hIL-2), hIL-3, mIL-4, mIL-6, mIL-7, and mIFN-α were kindly donated by Hsaing-fu Kung (Biological Response Modifiers Program), and recombinant rIFN-γ was a gift from Genentech Inc. (San Francisco, Calif.). Synthetic chemokines—hRANTES, full-length hIL-8[1–72], human monocyte chemoattractant protein-1 (hMCP-1), hMCP-3, human platelet factor 4, neutrophil-activating peptide 2, human melanoma growth-stimulatory activity, human IFN-γ-inducible protein, mLtn, and full-length rIL-8[1–79]—and chemokine truncation mutant analogs—hIL-8[1–66], rIL-8[6–79], rIL-8[9–79], rIL-8[11–79], and rIL-8[12–79]—were synthesized with *tert*-butoxycarbonyl chemistry on an Applied Biosystems 430A peptide synthesizer as described elsewhere (11).

Purification of M-T7. v-Myxlac was adsorbed onto roller bottles containing 3×10^8 BGMK cells at a multiplicity of infection of 2.5 PFU for 3 h, washed with phosphate-buffered saline (PBS), and incubated with serum-free Dulbecco's minimal essential medium at 37°C. After 18 h, the medium was harvested, centrifuged at 10,000 × *g* for 1 h to remove virus and cellular debris, concentrated 10-fold with a stirred ultrafiltration cell (Amicon, Beverly, Mass.), and dialyzed against 20 mM bis-Tris (Sigma Chemical Co., Mississauga, Ontario, Canada) buffer, pH 6.0. M-T7 was purified from concentrated myxoma virus-infected cellular supernatants by fast-performance liquid chromatography (FPLC). Approximately 5 mg of concentrated supernatant was loaded onto a Mono-Q HR5/5 (Pharmacia, Piscataway, N.J.) column that had been preequilibrated with low-ionic-strength 20 mM bis-Tris buffer, pH 6.0. Proteins were eluted from the anion-exchange column by increasing the salt concentration of the elution buffer to 500 mM sodium chloride in a step gradient. Protein fractions eluting between 120 and 175 mM sodium chloride were subjected to sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS–12% PAGE) and visualized by silver staining (18). Mono-Q fractions containing M-T7 were pooled, dialyzed against PBS overnight, and loaded onto a PBS-equilibrated Superdex 200 (Pharmacia) gel filtration column that had been precalibrated with known molecular weight standards (Pharmacia). Eluted proteins were analyzed by SDS-PAGE as above. Fractions containing purified M-T7 were pooled, concentrated fivefold with a Centriprep (Amicon) concentrator, and stored at 4°C. Approximately 80 μg of M-T7 per mg of crude myxoma virus-infected cellular supernatants was routinely fractionated.

Deglycosylation of M-T7. One microgram of M-T7 was resuspended in 100 mM sodium phosphate buffer, pH 7.0, and boiled for 10 min in the presence of 1% SDS. The denatured protein was then diluted by the addition of 100 mM sodium phosphate buffer (pH 7.0), 20 mM EDTA, 5% Triton X-100, and 1.1 mM dithiothreitol and incubated with 0.2 U of *N*-glycosidase F (Boehringer Mannheim, Laval, Quebec, Canada) at 37°C. After overnight incubation, the reaction products were dialyzed against sodium phosphate buffer, pH 7.0, concentrated to the original volume with an Ultrafree microconcentrator (Millipore, Bedford, Mass.), and analyzed by immunoblotting with an M-T7 antibody.

Sedimentation equilibrium studies. Analytical ultracentrifugation studies were performed at 8,000 rpm in PBS at 20°C on a Beckman Spinco model E analytical ultracentrifuge using absorbance optics. The molecular weight of M-T7 was calculated as $[2RT/(1 - \nu\rho)]\{d(\ln y/[\omega^2 d(r^2)]\}$ where R is the universal gas constant; T is the temperature in Kelvin; ν is the partial specific volume of the protein; ρ is the solvent density; ω is the angular velocity; and $d(\ln y)/d(r^2)$ is the slope calculated from a plot of $\ln y$ versus r^2, where y is the concentration in absorbance units at 280 nm and r is the distance (in centimeters) from the axis of rotation. A linear slope from the plot of $\ln y$ versus r^2 (data not shown) indicated that M-T7 behaves as a single uniform species in solution. On the basis of amino acid composition alone, ν was calculated to be 0.72. However, as M-T7 is glycosylated, ν for the secreted glycoprotein was calculated to be 0.70 with the formula $(M_1\nu_1 + M_2\nu_2)/(M_1 + M_2)$, where $M_1\nu_1$ and $M_2\nu_2$ are the weights and partial specific volumes of the protein and sugar components, respectively.

Gel mobility shift cross-linking assay. The interaction of M-T7 with various cytokines was discovered with a chemical cross-linking assay as described previously (61). Briefly, purified M-T7 was incubated with the appropriate cytokine or chemokine ligand for 2 h at room temperature. For competition binding studies, increasing amounts of rabbit IFN-γ, unlabeled RANTES, or intestinal porcine heparin (Sigma) competitors were incubated simultaneously with the ligands used in the reaction. After incubation, the protein complexes were cross-linked by the addition of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Sigma) to a final concentration of 40 mM for 30 min at room temperature and the reaction was quenched by the addition of 1/10 volume of 1.0 M Tris, pH 7.5. SDS loading buffer was added to the mixtures, the samples were boiled for 3 min and subjected to SDS–12% PAGE, and the resulting protein complexes were analyzed by immunoblotting. For analysis of ^{125}I-labeled RANTES competition assays, cross-linked complexes were analyzed by autoradiography and quantitated on a Fujix BAS 1000 phosphorimager with the MacBAS program. The relative affinity of M-T7 for RANTES was determined by measuring the 50% inhibitory concentration (IC_{50}) of unlabeled RANTES required for displacement of labeled RANTES in M-T7 cross-linking assays by a modification of a technique previously described (62, 63).

Immunoblotting analysis. M-T7 antiserum was prepared as described previously (35) except that rabbits were injected with FPLC column-purified M-T7 protein in Freund's complete adjuvant. Polyclonal antiserum was further subjected to affinity purification against an immobilized bacterially produced M-T7–glutathione *S*-transferase (GST) fusion protein, as outlined elsewhere (35). Detection of M-T7 was performed by immunoblotting analysis as described previously (48) except that the blots were incubated with a 1:5,000 dilution of affinity-purified anti-M-T7 antibody for 1 h. Detection of RANTES–M-T7 complexes by immunoblotting was performed in a similar manner with a 1:1,000 dilution of anti-human RANTES antibody (R&D Systems, Minneapolis, Minn.) and a 1:5,000 dilution of horseradish peroxidase-conjugated donkey anti-goat immunoglobulin G antibody (Jackson ImmunoResearch Laboratories, West Grove, Pa.).

Solid-phase M-T7 binding assay. hRANTES was radiolabeled with ^{125}I (Dupont NEN, Mississauga, Ontario, Canada) by using Iodobeads (Pierce, Rockford, Ill.) according to the manufacturer's recommendations. Falcon 96-well immunoplates were coated with 100 ng of purified M-T7 in 50 μl of PBS overnight at 4°C. Wells were blocked with 5% skim milk powder in Tris-buffered saline containing 0.2% Tween 20 for 4 h at room temperature and then incubated with ^{125}I-labeled RANTES in 100 μl of blocking buffer for 2 h. Wells were washed three times with Tris-buffered saline–0.2% Tween and removed, and radiobound counts were measured on a Packard 5780 gamma counter. To determine the specific binding of M-T7 to RANTES, nonspecific binding in the presence of 50-fold excess cold RANTES was subtracted from total binding.

RESULTS

Purification and characterization of M-T7. To determine whether M-T7 may have additional activities independent of rIFN-γ binding, we purified M-T7 to homogeneity to further characterize its biochemical properties in vitro. M-T7 protein was purified from proteins secreted from myxoma virus-infected cells by a two-step procedure using FPLC. As shown in Fig. 1A, concentrated myxoma virus-infected-cell supernatants were fractionated by anion-exchange and gel filtration chromatography with Mono-Q and Superdex 200 columns, and protein fractions containing a prominent single species of approximately 37 kDa under denaturing conditions were detected. To verify that the purified 37-kDa protein species is

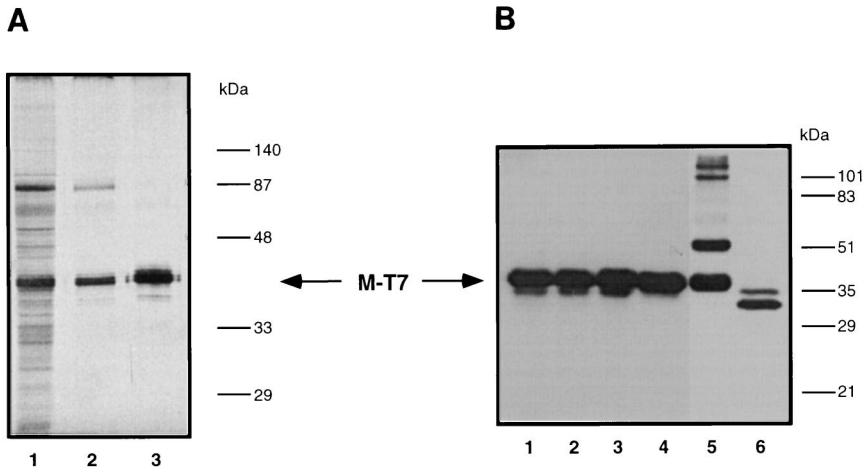

FIG. 1. Analysis of purified M-T7 glycoprotein. M-T7 was purified from crude supernatants of v-Myxlac-infected cells by column chromatography and analyzed by silver staining (A) and immunoblotting (B). (A) Crude supernatants (lane 1) or pooled fractions containing 500 ng of M-T7 following Mono-Q (lane 2) and Mono-Q–Superdex 200 (lane 3) column chromatography were subjected to SDS–12% PAGE and visualized by silver staining. (B) M-T7, as detected by immunoblotting analysis with an affinity anti-M-T7 antibody, from crude myxoma virus supernatants (lane 1) or pooled fractions following Mono-Q (lane 2) and Mono-Q–Superdex 200 (lane 3) column chromatography. One microgram of purified M-T7 was incubated in the absence (lane 4) or presence (lane 5) of 1 μg of rabbit IFN-γ and cross-linked as outlined in Materials and Methods, and complexes were detected by immunoblotting following SDS–12% PAGE. M-T7 was deglycosylated by treatment with *N*-glycosidase F (lane 6) as outlined in Materials and Methods. SDS-PAGE markers are shown on the right of each panel.

indeed the product of the myxoma virus T7 gene, the purified M-T7 sample was subjected to immunoblotting with an anti-M-T7 antibody that had been affinity purified by using a bacterially produced GST-T7 fusion protein. Immunoblotting analysis revealed a major protein species of approximately 37 kDa and a smaller minor variant of approximately 35 kDa from crude myxoma virus supernatants (Fig. 1B, lane 1). Identical M-T7 isoforms were also detected from the pooled fractions collected after both steps of the purification procedure (compare lanes 1 through 3 in Fig. 1B). Similar SDS-PAGE profiles have been detected with a previously described anti-M-T7 antibody (35) prepared exclusively from a bacterially produced T7-GST protein (data not shown). To determine if these isoforms were a result of alternate glycosylation states, purified M-T7 was subjected to treatment with *N*-glycosidase F. Treatment of M-T7 with this enzyme resulted in the deglycosylation of the major 37-kDa M-T7 species to approximately 32 kDa under denaturing conditions, whereas the minor 35-kDa form of M-T7 appeared to be resistant to this treatment (Fig. 1B, lane 6). These data indicate that although the major M-T7 species is N-glycosylated, the minor M-T7 isoform may be posttranslationally modified by a different mechanism. Since M-T7 from crude myxoma virus supernatants has previously been shown to bind and inhibit rIFN-γ (36, 61), we tested whether the purified protein also retained this biological activity. Purified M-T7, when incubated with rIFN-γ and cross-linked, produced a shifted complex of approximately 53 kDa (Fig. 1B, lane 5) as previously observed (35). Thus, the purified protein is indeed M-T7, and it retains the biological property of rIFN-γ binding previously ascribed to this myxoma IFN-γ receptor homolog.

During the purification of M-T7 protein, it was noticed that the native M-T7 species of an approximate molecular mass of 175 kDa migrated as a single species on gel filtration columns, suggesting that M-T7 is an oligomeric species (data not shown). To clarify the discrepancy between the protein's apparent molecular mass observed under denaturing conditions and in nondenaturing gel filtration studies, the mass and oligomeric status of the purified protein were assessed by mass spectrometry and ultracentrifugation studies. According to mass spectrometry, the calculated mass of M-T7 was 33.0 kDa (data not shown). As the theoretical molecular mass of M-T7 is only 29.9 kDa, the difference is most likely due to the extent of N-linked glycosylation. Sedimentation equilibration analysis (see Materials and Methods) revealed that the purified native M-T7 protein behaves as a single uniform species with a calculated molecular mass of approximately 113 kDa (data not shown). Based on this data, we predict that M-T7 likely exists as a stable trimer in solution. This also suggests that the apparent larger mass of M-T7 based on its behavior in Superdex 200 gel filtration chromatography is due to anomalous behavior of the protein likely resulting from glycosylation and/or structural asymmetry of the homotrimer.

Purified M-T7 protein binds to chemokines. Given the pathogenic profiles of rabbits infected with an M-T7 knockout virus, we speculated that M-T7 may be capable of performing additional biological functions apart from the binding and inhibition of rIFN-γ (35). Using a gel mobility shift chemical

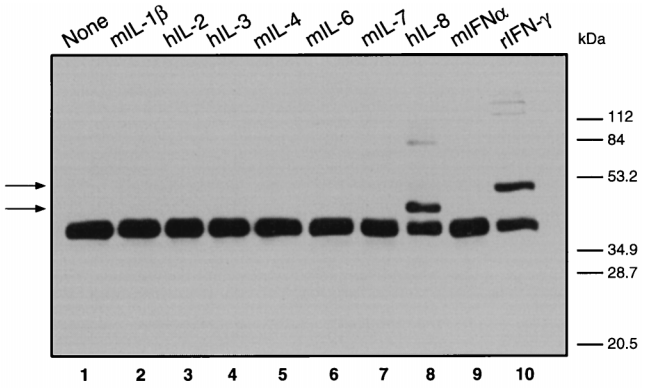

FIG. 2. Purified M-T7 binds to rIFN-γ and hIL-8 but not other cytokines tested. One microgram of purified M-T7 was incubated alone (lane 1) or with 1 μg of the indicated cytokine (lanes 2 through 10) cross-linked as described in Materials and Methods and analyzed by immunoblotting with an affinity-purified anti-M-T7 antibody. SDS-PAGE markers are shown on the right, and arrows indicating novel shifted M-T7–cytokine complexes are on the left.

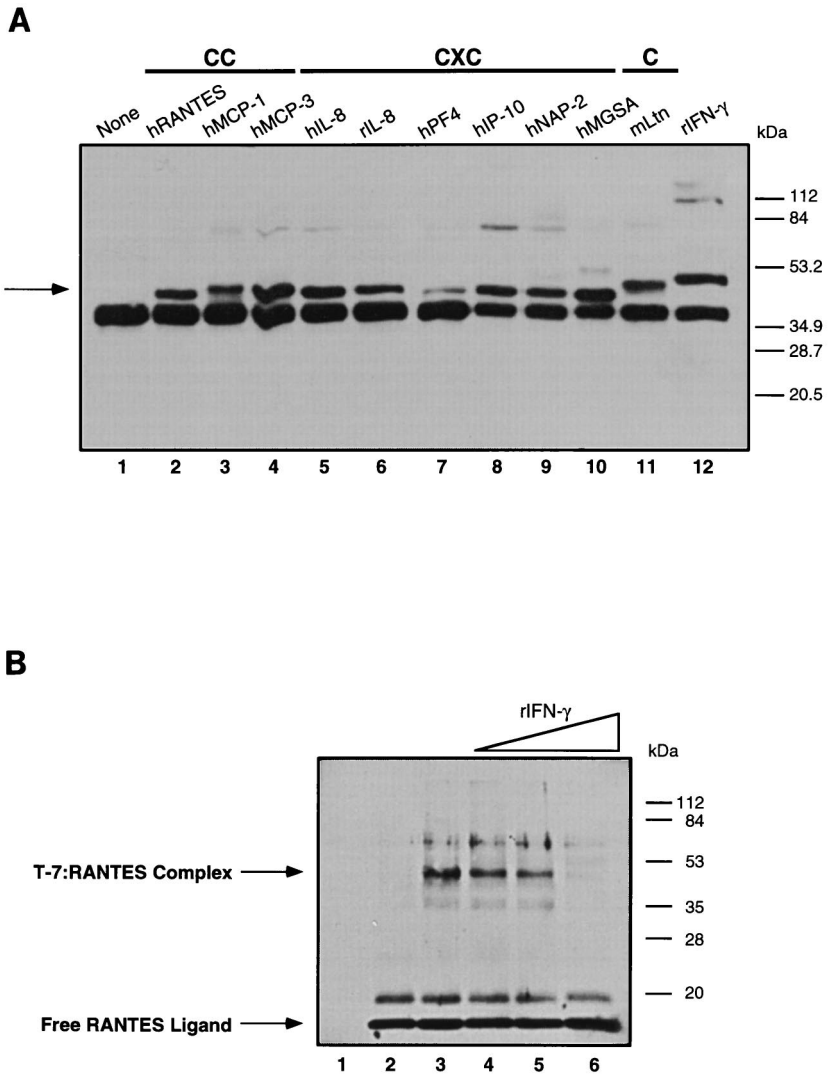

FIG. 3. M-T7 binds members of the CC, CXC, and C subfamilies of chemokines. (A) One microgram of purified M-T7 was incubated alone (lane 1) or with 1 μg of the indicated chemokine (lanes 2 through 12) cross-linked and analyzed by immunoblotting as described in the legend to Fig. 2. The arrow indicates shifted M-T7–chemokine complexes. hPF4, platelet factor 4; hIP-10, IFN-γ-inducible protein; hNAP-2, neutrophil activating peptide 2; hMGSA, melanoma growth-stimulatory activity. (B) Binding of RANTES to M-T7, as detected by anti-human RANTES antibody, can be competed by molar-excess amounts of rIFN-γ. Stoichiometrically equal amounts of M-T7 (lane 1) and hRANTES (lane 2) were incubated together in the absence (lane 3) or presence of 0.01× (lane 4), 0.1× (lane 5), or 1.0× (lane 6) molar-excess amounts of rIFN-γ cross-linked as described in the text and analyzed by immunoblotting with an anti-human RANTES antibody. SDS-PAGE markers are shown on the right of each panel.

cross-linking assay, we investigated whether purified M-T7 could bind to other cytokines, which could help explain the phenotype observed from the pathology of the deletion virus. Although M-T7 demonstrates species specificity for rIFN-γ (36), we were limited to screening with human and murine cytokines in our assays since many rabbit cytokines are unavailable. As shown in the immunoblot in Fig. 2, M-T7 protein did not bind mIL-1β, hIL-2, hIL-3, mIL-4, mIL-6, mIL-7 (lanes 1 through 7), or mIFN-α (lane 9). However, we readily observed a shifted complex between M-T7 and the human chemokine IL-8 when these two proteins were incubated together (lane 8). The retarded complex of approximately 50 kDa, as monitored by SDS-PAGE, corresponds to the predicted size of the M-T7 monomer (37 kDa) binding to IL-8 (8 kDa) in a 1:1 stoichiometric ratio. Thus, this observation indicated that purified M-T7 has the ability to bind to at least one other cytokine in addition to rIFN-γ and prompted us to investigate the specificity of this novel interaction.

To examine the specificity of M-T7 and chemokine interactions, we investigated whether M-T7 could bind to members of other chemokine subfamilies or whether this interaction was restricted to the CXC subfamily, which includes IL-8. We therefore tested the ability of M-T7 to bind to a variety of chemokines using a gel shift assay similar to that described above. As shown in Fig. 3A, M-T7 was found to interact with all representative members of the CC subfamily (hRANTES, MCP-1, and MCP-3) (lanes 2 through 4), the CXC subfamily (hIL-8, platelet factor 4, IFN-γ, inducible protein, neutrophil activating peptide 2, and melanoma growth-stimulatory activity) (lanes 5 through 10), and the C subfamily (mLtn) (lane 11) of chemokines. It appears, therefore, that M-T7 binds multiple chemokines from the three chemokine subfamilies. Neither

purified M-T2 (48) nor SERP-1 (27), both of which are soluble myxoma virus glycoproteins, was able to bind ^{125}I-labeled RANTES (data not shown) under similar conditions, suggesting that chemokine interactions are not a general property exhibited by glycosylated poxvirus proteins. It has previously been demonstrated that M-T7 displays species ligand selectivity by binding to rIFN-γ but not to mIFN-γ or hIFN-γ (36). The M-T7 interaction with chemokines, however, appears not to display this selectivity, because M-T7 was able to bind to rabbit, murine, and human chemotactic ligands (Fig. 3A and data not shown). To examine if the shifted M-T7-containing complexes shown in Fig. 3A reflect an interaction of the viral protein with chemokines, we performed a parallel immunoblot assay probed with an anti-human RANTES antibody. The RANTES antibody did not cross-react with M-T7 when incubated alone (Fig. 3B, lane 1). RANTES was detected as a shifted complex of approximately 50 kDa when incubated with a stoichiometrically equivalent amount of M-T7 but not when incubated alone (compare lanes 2 and 3 in Fig. 3B). This interaction of M-T7 with RANTES could be effectively competed by the addition of molar-excess amounts of rIFN-γ (Fig. 3B, lanes 4 through 6) but not by mIFN-γ or hIFN-γ (data not shown), demonstrating that chemokine and rIFN-γ binding sites may overlap or may be shared.

To demonstrate that the binding of M-T7 with chemokines is independent of cross-linking, we also performed solid-phase binding assays in which the ability of radiolabeled chemokines to bind to immobilized M-T7 was tested under native conditions. Specific binding of radiolabeled RANTES was observed when RANTES was incubated in the presence of M-T7 (Fig. 4A), confirming the interactions between M-T7 and chemokines demonstrated earlier by cross-linking assays. This suggests that M-T7 interacts with the chemokine RANTES in the absence of a cross-linker and under physiological conditions with an apparent submicromolar affinity. To obtain an approximate value for the affinity at which chemokines bind M-T7, we next determined the IC_{50} for displacement of ^{125}I-labeled RANTES binding in M-T7 cross-linking shift assays. Under conditions of saturable binding, we observed that the IC_{50} for displacement of labeled RANTES was approximately 900 nM unlabeled RANTES (Fig. 4B).

Purified M-T7 interacts with the heparin-binding domain of chemokines. Chemokines are basic polypeptides which have been proposed to interact with at least the N-terminal regions of chemokine receptors that are rich in acidic residues (22, 26). It has previously been demonstrated that an amino-terminal domain preceding the first cysteine residue of IL-8, the ELR motif, is essential for binding to IL-8-type receptors (10, 12, 21). ELR deletion mutants of IL-8 are able to neither bind their cognate receptors nor elicit a biological response in target neutrophils. To determine whether the ELR sequence of IL-8 may be a determinant for binding M-T7, we tested the ability of M-T7 to bind to a variety of IL-8 analogs containing successive deletions in the amino terminus or an IL-8 analog that contained a partial deletion in its carboxy terminus (Fig. 5A). All amino-terminal-deletion IL-8 mutants (Fig. 5A, lanes 2 through 6) were able to bind to M-T7, including the IL-8 analog in which the entire ELR motif had been deleted (Fig. 5A, lane 6). Variations in binding intensities observed may be due to the level of cross-linking efficiency in this experimental assay. This data indicates that, although required for binding to CXCR1 and CXCR2 cellular chemokine receptors, the amino-terminal region of IL-8 is not necessary for M-T7 binding.

In addition to binding specifically to their receptors, chemokines interact with glycosaminoglycans such as heparin and heparan sulfate (64) through an α-helix at the carboxy terminus. This interaction is thought to facilitate chemokine localization to the endothelial wall, generating a solid-phase gradient for leukocyte trafficking along the vasculature (45). An hIL-8 partial C-terminal truncation analog, hIL-8[1–66], which displays a markedly lower heparin affinity (64) than does its full-length counterpart, was unable to bind M-T7 (compare lanes 7 and 8 in Fig. 5A). We have also observed that M-T7 is not able to bind to the hIL-8[4–52] analog (data not shown), which lacks the complete COOH-terminal α-he-

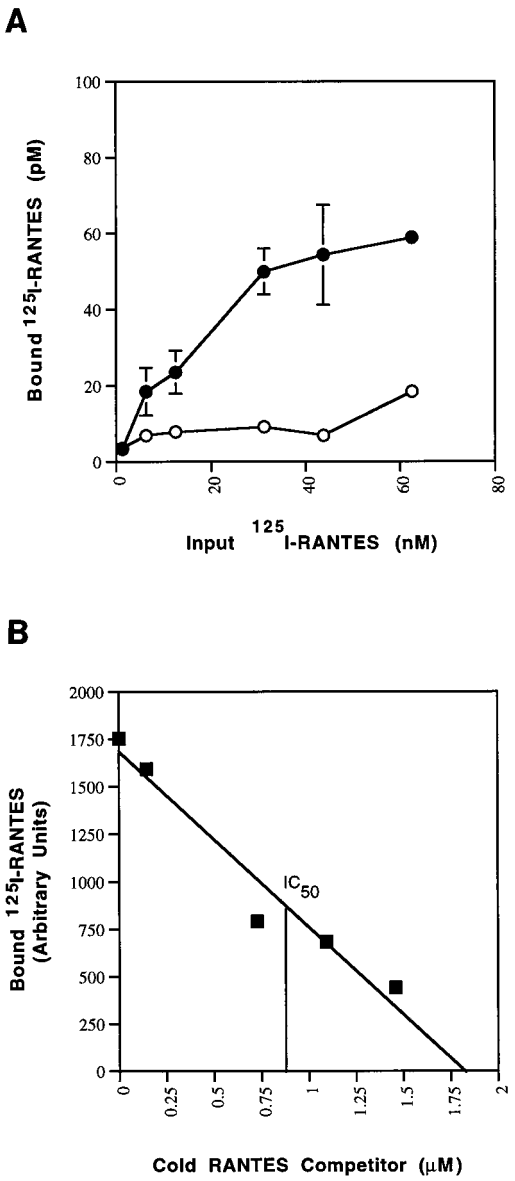

FIG. 4. Solid-phase (A) and self-competition cross-linking (B) binding assays of M-T7 to ^{125}I-labeled RANTES. (A) Results of a representative experiment performed in triplicate of solid-phase binding of ^{125}I-labeled RANTES to M-T7 (solid circles) or control (open circles) as described in Materials and Methods. (B) Determination of the RANTES concentration required to displace 50% binding of ^{125}I-labeled RANTES to M-T7. The amounts of ^{125}I-labeled RANTES bound to M-T7 were quantitated by phosphorimaging after cross-linking assays and plotted against the amounts of increasing cold RANTES competitor. From the graph, the IC_{50} of RANTES for M-T7 was determined to be approximately 0.9 μM.

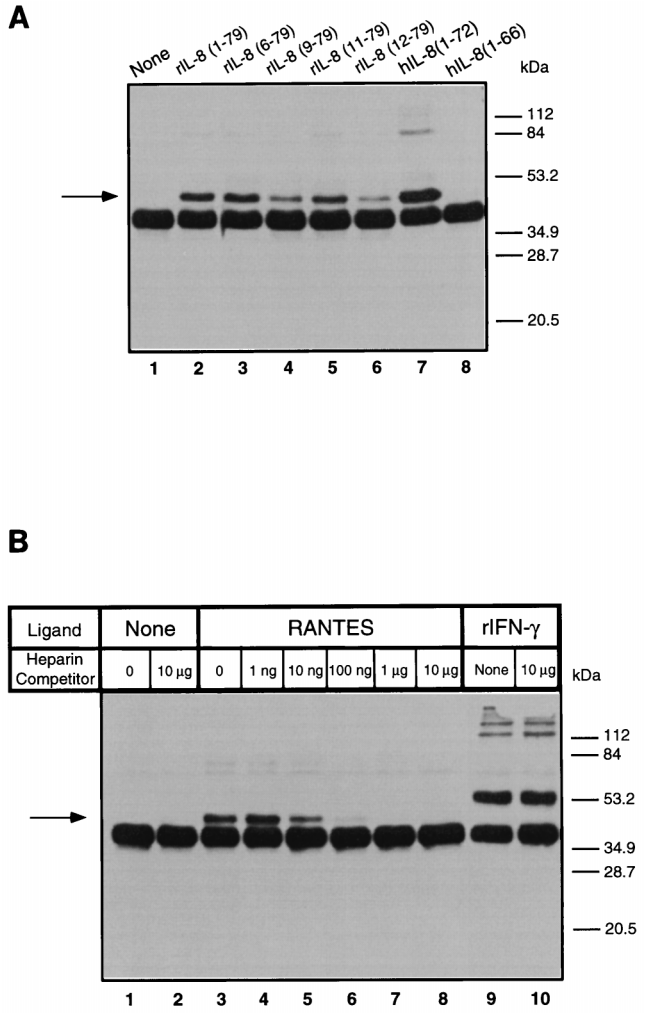

FIG. 5. M-T7 binds to rIL-8 containing N-terminal deletions but is unable to bind to an hIL-8 C-terminal-deletion mutant. (A) One microgram of purified M-T7 was incubated alone (lane 1) or with 1 μg of the indicated mutant (lanes 2 through 8) cross-linked as described in the text; binding complexes were analyzed by immunoblotting with an anti-M-T7 antibody. (B) Heparin competes binding of M-T7 to RANTES but not to rIFN-γ. One microgram of M-T7 was incubated alone (lane 1) or with 1 μg of either hRANTES (lanes 3 through 8) or rIFN-γ (lanes 9 and 10) in the absence (lanes 1, 3, and 9) or presence (lanes 2, 4 through 8, and 10) of increasing amounts of heparin, cross-linked as described in the text and analyzed by immunoblotting with an anti-M-T7 antibody. Arrows to the left of each panel show M-T7–chemokine complexes, and SDS-PAGE markers are indicated on the right.

lix and displays no heparin binding (64). These results indicate that M-T7 binds IL-8, and possibly other chemokines, via the COOH-terminal domain, suggesting that this soluble viral protein may be interacting with these chemotactic ligands in a heparin-like fashion. To confirm this, heparin competition studies were performed. Increasing mass ratios of heparin effectively competed the binding of M-T7 with RANTES (Fig. 5B, lanes 3 through 8), whereas the addition of heparin had no effect on the interaction of M-T7 with rIFN-γ (Fig. 5B, lanes 9 and 10). Addition of heparin to our assays itself did not appear to alter M-T7 mobility (Fig. 5B, lanes 1 and 2). Collectively, this data suggests that M-T7 may interact with the heparin-binding region found in a variety of chemokines of the CXC, CC, and C classes.

DISCUSSION

The proper recruitment and activation of specialized leukocyte subsets to sites of viral infection constitute a pivotal component of the host defense for efficient clearance of a pathogen. Viruses, therefore, have coevolved multiple anti-inflammatory mechanisms in an attempt to circumvent the arsenal of the host's primary defense systems (32, 51, 52). Poxviruses encode a variety of gene products that target the cytokine machinery that regulates the earliest stages of the inflammatory response. These include inhibitors of cytokine synthesis, release, and signal transduction, as well as a variety of cytokine receptor homologs (43). Viral mimics of cytokine receptors have been shown to function by binding to their respective ligands with high affinity and preventing normal cytokine–cellular-receptor interactions. There is also increasing evidence of poxviral virulence factors that may have multiple biological properties that cannot be predicted from conventional sequence analysis. For example, the myxoma virus TNF receptor homolog, M-T2, also blocks apoptosis in myxoma virus-infected rabbit lymphocytes by a mechanism that appears to be independent of its extracellular TNF-α binding property (28, 49). Also, a glycoprotein secreted by tanapox virus has previously been shown capable of binding three distinct cytokines, IFN-γ, IL-2, and IL-5 (16). In the case of M-T7, sequence analysis was used to predict its demonstrated function as a soluble IFN-γ receptor (36, 61). Although, IFN-γ does indeed appear to be a major target for the biological activity of M-T7 in vitro, the pathogenic profiles of rabbits infected with M-T7 deletion viruses indicate that M-T7 may be a multifunctional virulence factor (35). Here we report a new activity for purified M-T7 apart from binding and inhibiting rabbit IFN-γ, namely, promiscuous interaction with members of the chemokine superfamily.

Chemokines appear to be of paramount importance in the early host response to viruses (14, 50). Viral proteins dedicated to subverting these proinflammatory mediators would therefore be expected. Several virus chemokine receptor homologs with sequence similarity to seven-transmembrane-type cellular receptors have been identified (2, 39, 47). These include the gene products encoded by cytomegalovirus US28 (9, 40, 47); herpesvirus saimiri ECRF3 (1, 41); human herpesvirus-6 UL78, U12, and U51 (19); equine herpesvirus-2 74 and E1 (57); and two poxviruses, swinepox virus K2R (29) and capripox virus Q2/3L (8). Although both US28 and ECRF3 have been shown to be functional signaling receptors by binding multiple CC and CXC chemokines, respectively, their roles in viral pathogenesis remain unclear. Sequence analysis of M-T7, however, indicates no homology to any of the extracellular regions of any chemokine receptors identified to date. Considering that M-T7 is a soluble protein with significant amino acid similarity to the extracellular ligand-binding region of cellular IFN-γ receptors, we hypothesize that its interaction with chemokines would thus be distinct from the interaction of chemokines with their cognate cellular receptors.

Given that rIFN-γ specifically competes for RANTES binding to M-T7 (Fig. 3B), it is possible that the two ligands have identical or overlapping M-T7 binding sites. Alternatively, a conformation change in the M-T7–IFN-γ complex could occlude the chemokine-binding domain of M-T7, although this remains to be determined by further studies which map the domains of chemokine and rIFN-γ binding. Note that the ability to bind to chemokines does not appear to be a biochemical property shared by all poxviral IFN-γ homologs. The IFN-γ receptor homolog from vaccinia virus (strain WR) was unable to form cross-linked complexes with radiolabeled che-

mokines in similar binding assays (20). We have also recently identified another family of poxvirus-secreted chemokine binding proteins that are distinct from M-T7. Like M-T7, the M-T1 family of 35-kDa poxvirus proteins binds both CXC and CC chemokines, although the biochemical mechanism of this interaction remains to be demonstrated (20).

Native M-T7 is shown to be a trimer that is N glycosylated, but further investigations are needed to determine whether the carbohydrate moieties of this glycoprotein are important for the interaction. Several lines of evidence suggest that the binding of M-T7 with the chemokines described here may be analogous to the interactions between chemokines and heparin. Firstly, unlike most chemokine receptors that have been identified, M-T7 binds promiscuously to members of all three chemokine subfamilies tested, suggesting that a common conserved structural motif is involved. Secondly, chemokines with a partial deletion in the COOH-terminal α-helix, which displayed decreased affinity for binding heparin in previous work, were also unable to interact with M-T7 in our studies. In contrast, N-terminal determinants that dictate CXC chemokine binding, such as the ELR motif, are not essential for M-T7 binding. Thirdly, heparin acts as a specific competitor for displacing the binding of chemokines to M-T7. We propose, therefore, that this novel M-T7 activity may be facilitated through the α-helix heparin-binding region commonly found within multiple chemokines.

The biochemical characteristics of M-T7–chemokine interactions suggest that the M-T7 protein alone might not effectively occlude chemokine receptor triggering. However, it is still plausible that M-T7 could perturb chemokine localization in the extracellular matrix and/or affect the correct presentation of chemokines to target leukocytes, neither of which can be readily monitored with current in vitro chemotaxis assays. Consistent with this hypothesis is the data demonstrating a submicromolar affinity between RANTES and M-T7, a value reported similarly for chemokine-glycosaminoglycan interactions (65). Given the prodigious quantity of M-T7 secreted from an infected cell, in excess of 10^7 molecules/cell/h (36), we propose that M-T7 is an ideal candidate for interaction with multiple classes of chemokines in virus-infected tissues. A similar role as a chemokine sink has also been proposed for a nonsignaling chemokine receptor found on the surfaces of erythrocytes (15). Nevertheless, the dramatic effect of M-T7 loss on leukocyte migration in myxoma virus-infected tissues suggests that further studies to define the in vivo relationship between extracellular M-T7 protein and the activities of chemokines that mediate leukocyte influx into virus-infected tissues are warranted.

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