VIROLOGY 229, 12–24 (1997)
ARTICLE NO. VY968423

The T1/35kDa Family of Poxvirus-Secreted Proteins Bind Chemokines and Modulate Leukocyte Influx into Virus-Infected Tissues

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Received October 10, 1996; returned to author for revision November 6, 1996; accepted December 18, 1996

Immunomodulatory proteins encoded by the larger DNA viruses interact with a wide spectrum of immune effector molecules that regulate the antiviral response in the infected host. Here we show that certain poxviruses, including myxoma virus, Shope fibroma virus, rabbitpox virus, vaccinia virus (strain Lister), cowpox virus, and raccoonpox virus, express a new family of secreted proteins which interact with members of both the CC and CXC superfamilies of chemokines. However, swinepox virus and vaccinia virus (strain WR) do not express this activity. Using a recombinant poxviruses, the myxoma M-T1 and rabbitpox virus 35kDa secreted proteins were identified as prototypic members of this family of chemokine binding proteins. Members of this T1/35kDa family of poxvirus-secreted proteins share multiple stretches of identical sequence motifs, including eight conserved cysteine residues, but are otherwise unrelated to any cellular genes in the database. The affinity of the CC chemokine RANTES interaction with M-T1 was assessed by Scatchard analysis and yielded a K_d of approximately 73 n*M.* In rabbits infected with a mutant rabbitpox virus, in which the 35kDa gene is deleted, there was an increased number of extravasating leukocytes in the deep dermis during the early phases of infection. These observations suggest that members of the T1/35kDa class of secreted viral proteins bind multiple members of the chemokine superfamily *in vitro* and modulate the influx of inflammatory cells into virus-infected tissues *in vivo.*

INTRODUCTION

One of the key features of the early inflammatory response to an initial virus challenge is the influx and activation of leukocytes which help initiate the earliest phases of antiviral immune activation (Nathanson and McFadden, 1997; Zinkernagel, 1996). Neutrophils, monocytes/macrophages, and NK cells in particular participate in the first wave of cellular infiltration but require directional signals in order to migrate to the injured tissues bearing infected cells (Ben-Baruch *et al.,* 1995; Springer, 1994). The activities of multiple chemotactic cytokines, collectively known as chemokines, are believed to be critical in this process (Ben-Baruch *et al.,* 1995; Furie and Randolph, 1995; Schall and Bacon, 1994). Chemokines may be divided into subfamilies, designated CXC, CC, and C, based upon their cysteine residue distribution within the polypeptide, which has been loosely correlated with their function (Baggiolini, 1993; Kelner *et al.,* 1994; Schall, 1994). Initially it was believed that the CC chemokines, like RANTES (Regulated on Activation Normal T-cell Expressed and Secreted), induce monocyte but not neutrophil chemotaxis and activation, whereas the CXC chemokines, such as IL-8 (interleukin-8), induce chemotaxis and activation of neutrophils but not monocytes. It has now become clear that members of the different subfamilies induce multifunctional biological responses of overlapping sets of leukocytes including eosinophils, basophils, and lymphocytes in addition to monocytes and neutrophils (Baggiolini, 1993; Ben-Baruch *et al.,* 1995; Furie and Randolph, 1995; Kelner *et al.,* 1994; Schall, 1994; Schall and Bacon, 1994). Chemokines signal through a family of serpentine receptors which span the cell surface seven times, are coupled to G proteins, and are generally class restricted, binding to either CC or CXC chemokines, although multiple chemokines from within a family can bind and signal through common receptors (Kelvin *et al.,* 1993; Murphy, 1996). Chemokines have been implicated in a variety of inflammatory disease states, including allergic inflammation (Baggiolini and Dahinden, 1994), rheumatoid arthritis (Hosaka *et al.,* 1994), glomerulonephritis (Brown *et al.,* 1996), and fibrocytic lung disease (Smith *et al.,* 1995). Here we report a novel class of secreted poxvirus proteins that interact with a broad spectrum of chemokines *in vitro* and retard

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0042-6822/97 $25.00

the extent of leukocyte influx into virus infected lesions *in vivo.*

Poxviruses are large DNA viruses that replicate and direct viral gene expression from the cytoplasm of infected cells (Moss, 1996). Myxoma virus, a member of the *Leporipoxvirus* genus, is a well-characterized rabbit pathogen which induces a benign disease in its natural host, the South American rabbit (*Sylvilagus brasiliensis*) (Fenner and Ratcliffe, 1965; McFadden, 1988, 1994). However, in the European rabbit (*Oryctolagus cuniculus*) myxoma virus infection results in myxomatosis, a condition that is usually lethal. Myxomatosis is characterized by swelling and necrosis at the dermal site of infection, rapid dissemination of infected cells throughout the lymphoreticular system, severe immune dysregulation resulting in susceptibility to gram-negative infection, and death within 10–14 days. In contrast, rabbitpox virus is a member of the *Orthopoxvirus* genus and induces a disease syndrome in European rabbits that is also generally lethal but has a distinct pathogenicity spectrum (Martinez-Pomares *et al.,* 1995). Both the rabbitpox virus and myxoma virus systems provide unique insights into the fundamental mechanisms by which poxviruses cause immune dysfunction (McFadden, 1988; McFadden *et al.,* 1995a).

Like many viruses, poxviruses have evolved a number of strategies to counteract the host's immune system, including the inhibition of antigen presentation, apoptosis blockade, and cytokine modulation (Barry and McFadden, 1997; McFadden, 1995; Pickup, 1994; Smith, 1994; Spriggs, 1996). In particular, several secreted poxvirus proteins have been shown to interfere with the host immune response to viral infection by blocking the function of important extracellular immunoregulatory molecules. Myxoma virus and orthopoxviruses such as vaccinia virus and rabbitpox virus have been shown to encode a variety of secreted proteins which mimic host cytokines or cytokine receptors. These viral factors include homologues of epidermal growth factor (McFadden *et al.,* 1995b), tumor necrosis factor receptor (Hu *et al.,* 1994; McFadden *et al.,* 1995b; Schreiber and McFadden, 1994; Smith *et al.,* 1991; Upton *et al.,* 1991), interferon-γ receptor (Alcamí and Smith, 1995b; Mossman *et al.,* 1995, 1996; Upton *et al.,* 1992), interferon-α/β receptor (Colamonici *et al.,* 1995; Symons *et al.,* 1995), and interleukin-1-β receptor (Alcamí and Smith, 1995b; Spriggs *et al.,* 1992). Many of these cytokine binding proteins have also been shown to act as anti-inflammatory virulence factors since disruption of the relevant open reading frames results in an attenuated pathogenic profile *in vivo* (Alcamí and Smith, 1995a; McFadden, 1995; McFadden and Graham, 1994; Pickup, 1994; Smith, 1994; Spriggs, 1996).

As part of our ongoing search for novel secreted poxvirus inhibitors of the cytokine network, supernatants from poxvirus-infected tissue culture cells were screened for viral proteins which interact with proinflammatory chemokines that induce immune cell chemotaxis into sites of injury or infection (Miller and Krangel, 1992). In this report we demonstrate that many, but not all, poxviruses encode a member of a family of secreted proteins, collectively termed T1/35kDa, that bind a variety of CC and CXC chemokines *in vitro.* Furthermore, *in vivo* infection with a rabbitpox virus mutant that does not express the secreted 35kDa chemokine binding protein reveals pronounced alterations in the influx of extravasating leukocytes into virus-infected rabbit tissues.

MATERIALS AND METHODS

Cells and viruses

The poxviruses myxoma virus (strain Lausanne), Shope fibroma virus (strain Kasza), rabbitpox virus (strain Utrecht), raccoonpox virus, swinepox virus, vaccinia virus (strains Western Reserve and Lister) were all obtained from the American Type Culture Collection (ATCC). Cowpox virus (strain Brighton Red) was a gift from D. Pickup. The myxoma virus used throughout this study is a recombinant, vMyxlac, which has a β-galactosidase cassette inserted in an intragenic location (Opgenorth *et al.,* 1992). Myxoma virus in which the M-T7 open reading frame is disrupted, vMyxlac-T7gpt, was previously described (Mossman *et al.,* 1996). Rabbitpox virus in which the 35kDa open reading frame is deleted, RPVΔ35, was also described previously (Martinez-Pomares *et al.,* 1995). Myxoma virus, Shope fibroma virus, and the vaccinia viruses were routinely passaged in a baby green monkey kidney (BGMK, a gift from S. Dales) cell line and rabbitpox virus, raccoonpox virus, and cowpox virus were routinely passaged in a rabbit kidney cell line (RK13, from the ATCC) in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum (Life Technologies, Gaithersburg, MD). Swinepox virus was routinely passaged in a swine kidney (ESK, from the ATCC) cell line in Ham's F12 medium (Life Technologies, Gaithersburg, MD) with 10% newborn calf serum (Life Technologies).

Vaccinia virus expressing the myxoma virus M-T1 gene, VV-T1, was generated by subcloning a 1.2-kb *Kpn*I–*Bam*HI fragment (containing the M-T1 gene) from the myxoma virus *Bam*HI S fragment into the *Kpn*I–*Bam*HI sites of (Davison and Moss, 1990), creating pMJ-T1 in which M-T1 is targeted for insertion in the vaccinia thymidine kinase gene. The plasmid pMJ-T1 was then used to generate VV-T1 by homologous recombination into vaccinia virus (WR).

In vitro chemokine binding assays and immunoblots

Serum-free, concentrated supernatants were prepared from poxvirus-infected cells as previously described (Upton *et al.,* 1992). Briefly, 2×10^7 cells were infected at a multiplicity of infection of 10, washed extensively with

phosphate-buffered saline (PBS) after the adsorption period, and incubated in serum-free medium (10 ml) for 12–16 hr at 37°. The medium, or supernatant, was then collected and concentrated 15-fold using Amicon centriprep 10 concentrators (Beverly, MA). The human chemokines RANTES and IL-8 were labeled with ^{125}I using Iodobeads (Pierce, Rockford IL). Five micrograms of chemokine was reacted in 175 μl PBS with one Iodobead, and 0.2 mCi Na^{125}I (Dupont NEN, Missasauga, Ontario, Canada) for 7 min. The mixture was then passed over a KwikSep column (Pierce) and eluted with PBS to separate the labeled protein from unbound Na^{125}I.

Chemokine binding assays were performed by both cross-linking and direct solid-phase binding protocols. Cross-linking of chemokines with viral proteins was measured by mixing concentrated secreted viral proteins from virus-infected cells (5 μl) with ^{125}I-labeled chemokine (25 ng) and 10 m*M* sodium phosphate buffer (pH 7.0) in a total volume of 15 μl, and the mixture was then incubated at room temperature for 2 hr. The protein complexes were cross-linked by the addition of 2 μl of 200m*M* 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDC) (Sigma Chemical Co., Missasauga, Ontario, Canada) in 0.1 *M* potassium phosphate buffer, (pH 7.5) for 15 min. Another 2-μl aliquot of 200 m*M* EDC was added and incubated for 15 min, followed by the addition of 2 μl 1 *M* Tris–Cl, pH 7.5, (Sigma Chemical Co.) to quench the reaction (Upton *et al.,* 1992). The cross-linked products were then subjected to 12.5% denaturing SDS–PAGE and autoradiography. Competition assays using unlabeled ligand were performed as previously described (Mossman *et al.,* 1995). In addition to chemokines, the competitors used were human interleukin-3 (IL-3), murine IL-4, murine IL-6, murine IL-7, murine IL-9, human IL-10, and human IL-11, and were kindly supplied by Dr. H. Kung (NIH, Biological Response Modifiers Program).

To confirm that the higher order cross-linked complexes contain both IL-8 and viral protein, cross-linked complexes were prepared as described for the gel shift assay. Cross-linked complexes were separated on denaturing SDS–PAGE and immunoblotted as previously described (Schreiber and McFadden, 1994). Immunoblots were probed with anti-human-IL-8 (1/1000 dilution, R&D Systems, Minneapolis, MN) and detected using horseradish peroxidase–donkey anti-human IgG (1/2000 dilution, Jackson ImmunoResearch Laboratories, West Grove, PA) and a chemiluminescence detection system (Amersham, Oakville, Ontario, Canada) according to the manufacturer's recommendations.

The antibody A18691 (Patel *et al.,* 1990) was also used to detect the 35kDa protein on immunoblots. This polyclonal antiserum, a generous gift of Arvind Patel (MRC Virology Unit Glasgow, Scotland), was previously prepared using the purified 35kDa protein from vaccinia virus (Lister), encoded by the C23L open reading frame, as the immunogen. The antiserum was used at a dilution of 1/10,000, detected with horseradish peroxidase–goat anti rabbit IgG (1/5000 dilution, Jackson ImmunoResearch Laboratories), and visualized using chemiluminescence.

Solid-phase binding of M-T1 to RANTES

M-T1 was fractionated from tissue culture supernatants of myxoma virus-infected cells by column chromatography (Lalani *et al.,* manuscript in preparation). Purified M-T1 protein (50 ng) was immobilized on Falcon 96-well immunoplates in 50 μl of PBS and incubated overnight at 4°C. Wells were blocked with 5% skim milk powder in Tris-buffered saline (TBS) containing 0.2% Tween 20 for 4 hr at room temperature, and then incubated with ^{125}I-labeled RANTES in 100 μl blocking buffer for an additional 4 hr. Wells were washed extensively with TBS–0.2% Tween buffer and removed and radiobound counts were measured on a Packard 5780 gamma counter. To determine the specific binding of M-T1 to RANTES, nonspecific binding in the presence of 100-fold excess cold RANTES was routinely subtracted from total binding. All assays were performed in triplicate and the affinity of M-T1 for RANTES was determined by the method of Scatchard.

DNA sequence analysis

To facilitate sequence analysis, DNA was subcloned, or amplified by PCR, from the myxoma or rabbitpox virus genomes. A 1.2-kb *Kpn*I–*Bam*HI fragment which contains the M-T1 gene was subcloned from the myxoma virus *Bam*HI S fragment into pBluescript (SK$^+$), generating the plasmid pBS-M-T1. The gene that encodes the rabbitpox virus 35kDa protein was amplified from rabbitpox virus DNA by PCR using VENT polymerase (Life Technologies) and the primers 5′-GCGCTCGAGATGAAACAATATATCGTCCTG and 5′-GCGAAGCTTTCAGACACACGCTTTGAG. The PCR product was digested with *Xho*I and *Hin*dIII, and cloned into *Xho*I/*Hin*dIII sites in pBluescript (SK$^+$) (Stratagene, La Jolla, CA), yielding pBS-RPV-35k.

With pBS-M-T1 and pBS-RPV-35k as templates both strands of the myxoma virus M-T1 and rabbitpox virus 35kDa genes were sequenced, using nested oligonucleotides, on an ABI 373 DNA sequencer with *Taq* cycle sequencing to a redundancy of greater than fivefold. The sequences were analyzed using Genetics Computer Group (Wisconsin, MI) programs.

Infection of rabbits with rabbitpox viruses

Female New Zealand White rabbits were given intradermal infections on both flanks with 50 or 5 $\times$ 10^4 PFU of rabbitpox virus or RPVΔ35. A total of 12 rabbits were infected and tissue samples of the primary skin lesions were collected at 1, 2, and 3 days postinfection from 2 rabbits per dose for both viruses. Two lesions from each

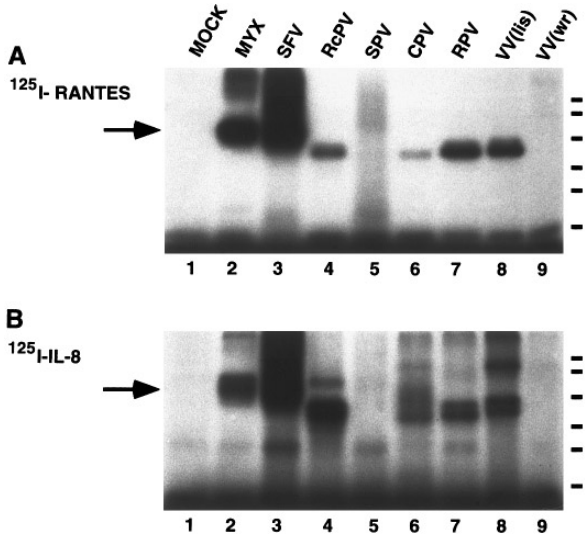


FIG. 1. Secretion of chemokine binding proteins from poxvirus-infected cells. Supernatants from cells which were uninfected (mock, lane 1) or infected with myxoma virus (MYX, lane 2), Shope fibroma virus (SFV, lane 3), raccoonpox virus (RcPV, lane 4), swinepox virus (SPV, lane 5), cowpox virus (CPV, lane 6), rabbitpox virus (RPV, lane 7), vaccinia virus (strain Lister) (VV(lis), lane 8), or vaccinia virus (strain Western Reserve) (VV(wr), lane 9) were cross-linked with ^{125}I-labeled RANTES (A) or ^{125}I-labeled IL-8 (B). The arrow points to a 53-kDa cross-linked species in MYX (lane 1) and SFV (lane 2); a smaller complex of 49 kDa can be observed in RcPV (lane 4), CPV (lane 5), RPV (lane 7), and VV(lis) (lane 8). Molecular weight standards from top to bottom are 101, 83, 50.6, 35.5, 29.1, and 20.9 kDa.

rabbit were assessed. Fixed tissues were paraffin-embedded, sectioned and immunostained with anti-rabbit-CD43 antibody (Spring Valley Laboratories, Woodbine, MD) to stain for infiltrating rabbit leukocytes, particularly T cells and monocytes/macrophages, as previously described (Mossman *et al.,* 1996) except that hematoxylin was used as a counterstain.

RESULTS

Several members of the poxvirus family express secreted chemokine binding proteins

Since poxviruses are known to encode multiple secreted immunomodulatory proteins, we screened supernatants prepared from poxvirus-infected tissue culture cells for soluble viral proteins that could bind to representative members of the chemokine subfamilies. Iodinated RANTES, a CC chemokine, and IL-8, a CXC chemokine, were incubated with tissue culture supernatants and exposed to a chemical cross-linker (EDC), and the presence of shifted complexes representing novel protein interactions was assessed. The addition of RANTES (Fig. 1A) or IL-8 (Fig. 1B) to supernatants from poxvirus-infected cells resulted in novel shifted cross-linked complexes of 53 kDa for the leporipoxviruses, myxoma virus (lane 2), and Shope fibroma virus (lane 3), and 49 kDa for the orthopoxviruses, raccoonpox virus (lane 4), cowpox virus (lane 6), rabbitpox virus (lane 7), and vaccinia virus (strain Lister) (lane 8). However, supernatants harvested from swinepox virus (lane 5), vaccinia virus (strain WR) (lane 9), and uninfected cells (lane 1) did not contain any detectable shifted protein species. The cross-linked products exhibited comparable mobility when either RANTES or IL-8 were used in the experiment, suggesting that either the same viral protein is responsible for binding both chemokines or that the virus encodes two or more chemokine binding proteins of similar size. If the molecular mass of RANTES or IL-8 is subtracted from the observed mobility of the shifted complexes, the putative viral chemokine binding protein would be predicted to be approximately 45 kDa for the leporipoxviruses, myxoma virus and Shope fibroma virus, and approximately 41 kDa for the orthopoxviruses, vaccinia virus, rabbitpox virus, cowpox virus, and raccoonpox virus. In addition, the CC chemokines MIP-1α and β (macrophage-inflammatory proteins-1α, and β), and MCP1 (monocyte-chemoattractant protein-1) also gave similar results in cross-linking assays (data not shown). These data indicate that several poxviruses encode and secrete chemokine binding protein(s) that interact with both CC and CXC chemokines.

If both RANTES and IL-8 bind to the same site on the same protein, then they should be able to effectively compete with each other, depending on their relative affinities. Therefore, self- and cross-competition studies were performed (Fig. 2) in which unlabeled ligands were used to compete with ^{125}I-labeled chemokine for binding to a protein in supernatants from myxoma virus-infected cells. RANTES binding was self-competed at a concentration of 10-fold molar excess unlabeled chemokine (Fig. 2A). In a similar experiment unlabeled IL-8 self-competes with ^{125}I-labeled IL-8 for binding to the viral protein (data not shown). Cross-competition studies between RANTES and IL-8 showed that unlabeled RANTES effectively competed with labeled IL-8 (Fig. 2B), for binding to the myxoma virus protein. However, a parallel analysis showed that IL-8 concentrations of up to 200-fold excess were not able to compete binding of the ^{125}I-labeled RANTES (Fig. 2C). Control cytokines were also tested for the ability to compete with labeled RANTES and IL-8 binding. At concentrations of 200-fold excess, interleukins-3, -4, -6, -7, -9, -10, and -11 were unable to displace the binding of RANTES (Fig. 2D) or IL-8 (data not shown) to the secreted myxoma virus chemokine binding protein. These competition analyses suggest that both RANTES and IL-8 specifically interact with the same site on the poxviral protein, but that RANTES binds with a higher affinity.

Sequence of candidate chemokine binding proteins

In order to identify the poxvirus open reading frames which encode the chemokine binding proteins we took advantage of the fact that although no detectable chemo-

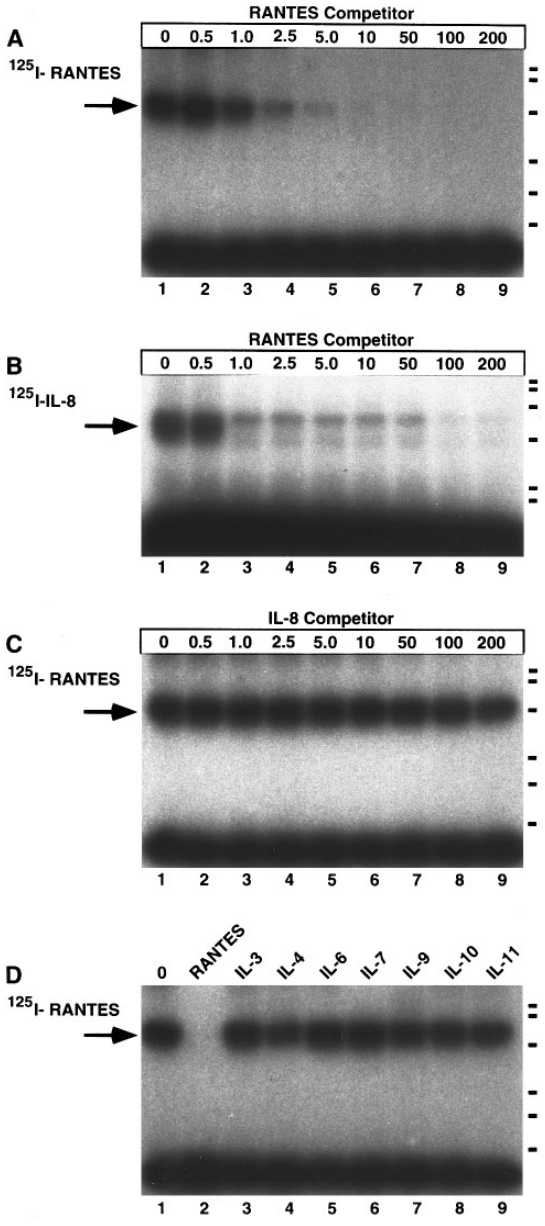
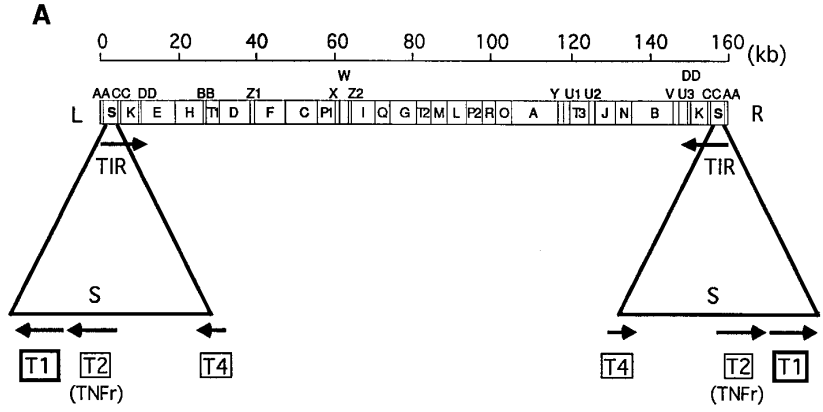


FIG. 2. Competition studies with the myxoma virus chemokine binding protein. Self-competitions for chemokine binding were performed using secreted proteins from cells which were infected with myxoma virus and cross-linked with (A) ^{125}I-labeled RANTES in the presence of increasing concentrations of unlabeled RANTES or with (B) ^{125}I-labeled IL-8 in the presence of increasing concentrations of unlabeled RANTES. Alternatively, (C) a cross-competition was performed in which myxoma virus-derived supernatants were reacted with iodinated RANTES in the presence of increasing concentrations of unlabeled IL-8. In A, B, and C ligand was added in the absence of competitor (lane 1) or in the presence of competitor at concentrations of 0.5× (lane 2), 1.0× (lane 3), 2.5× (lane 4), 5× (lane 5), 10× (lane 6), 50× (lane 7), 100× (lane 8), and 200× (lane 9) fold molar excess. Also, (D) labeled RANTES was reacted with myxoma virus supernatants in absence of competitor (lane 1), in the presence of 200-fold molar excess of unlabeled RANTES (lane 2), or 200-fold molar excess of a variety of nonchemotactic cytokines including huIL-3 (lane 3), muIL-4 (lane 4), muIL-6 (lane 5), muIL-7 (lane 6), muIL-9 (lane 7), huIL-10 (lane 8), and huIL-11 (lane 9). Molecular weight standards are as described in the legend to Fig. 1.

kine binding activity was observed from cells infected with vaccinia virus (WR), a complex could be readily detected in supernatants from cells infected with vaccinia virus (Lister) (Fig. 1, lanes 8 and 9). These two vaccinia strains are very similar, but exhibit a few notable differences. One such difference is the expression of a 35kDa secreted protein from vaccinia virus (Lister), designated C23L and B19R, which is truncated in vaccinia virus (WR) to a 7.5-kDa species (Johnson *et al.,* 1993; Patel *et al.,* 1990). Homologous open reading frames have also been sequenced in the genomes of variola virus and cowpox virus, all of which are closely related to the T1 gene of Shope fibroma virus (Martinez-Pomares *et al.,* 1995; Upton *et al.,* 1987). Therefore, members of the orthopoxvirus (rabbitpox virus 35kDa) and leporipoxvirus (myxoma virus M-T1) gene families were chosen for assessment as possible chemokine binding proteins. The myxoma virus M-T1 open reading frame, which is a homologue of the Shope fibroma virus T1, is the first open reading frame from the termini of the virus genome (Fig. 3A). It is located within the *Bam*HI S fragment and is present in two copies within the terminal inverted repeats. Since previously reported myxoma virus DNA sequences (Upton *et al.,* 1991) extend only into the 5′ end of the T1 coding sequence, we have completed the nucleotide sequence extending from the initiating M-T1 codon to the end of the myxoma virus *Bam*HI S fragment (Fig. 3B). The 783-bp myxoma virus M-T1 gene encodes a 260-amino-acid protein with a putative signal sequence and two predicted N-glycosylation sites. The related rabbitpox virus-secreted 35kDa protein, which was previously identified using amino terminal sequencing (Martinez-Pomares *et al.,* 1995), is located within the terminal inverted repeats mapping within the *Hin*dIII B and C fragments of the rabbitpox virus genome, and is therefore also present in two copies. The 777-bp rabbitpox virus 35kDa protein gene encodes a 258-amino-acid protein that contains a signal sequence but only one predicted N-glycosylation site (Fig. 3C). Based on deduced amino acid sequence the M-T1 and the 35kDa proteins are predicted to be 28.3 and 27.7 kDa, which is less than the calculated 41- to 45-kDa mobilities (Fig. 1), but the predicted N-glycosylation of both proteins suggests that the sizes of the fully processed secreted proteins will be in the range of the observed chemokine binding species.

The derived amino acid sequences of M-T1 and rabbitpox virus 35kDa were compared to the sequences of other known members of the T1/35kDa family (Fig. 4). Within the orthopoxvirus 35kDa family there is 81–99% amino acid identity, whereas the leporipoxvirus T1 proteins are about 70% identical; however, between the leporipoxvirus and orthopoxvirus members, there is only about 40% amino acid identity (Table 1). In comparison to the orthopoxvirus 35kDa family members, the leporipoxvirus T1 proteins have insertions of 19 (myxoma virus) and 16 (Shope fibroma virus) amino acids and both share



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1   ATGAAACGCCTGTGTGTATTATTCGCGTGCCTGGCCGCGACCCTCGCGACGAAGGGCATC
    M  K  R  L  C  V  L  F  A  C  L  A  A  T  L  A  T  K  G  I
61  TGCAGACAAGGCGAAGATGTCCGATACATGGGAATAGACGCCGTGGCCAAAATTACAAAG
    C  R  Q  G  E  D  V  R  Y  M  G  I  D  A  V  A  K  I  T  K
121 AGGACTACCGGAAGCGACACGCCGTGTCAGGGTCTGCGTACGACTATTGAATCCGCGTAT
    R  T  T  G  S  D  T  P  C  Q  G  L  R  T  T  I  E  S  A  Y
181 ACAGAAGACGAAAACGAAGACGATGGCGCGACGGGTACGGAGCAGCCCGACGATCTTAGC
    T  E  D  E  N  E  D  D  G  A  T  G  T  E  Q  P  D  D  L  S
241 GAGGAATACGAGTACGACGAAAACGACGAATCGTTTCTAACCGGTTTCGTGATCGGAAGT
    E  E  Y  E  Y  D  E  N  D  E  S  F  L  T  G  F  V  I  G  S
301 ACTTACCACACGATCGTCGGAGGAGGACTCTCCGTCACGTTCGGATTTACGGGATGTCCT
    T  Y  H  T  I  V  G  G  G  L  S  V  T  F  G  F  T  G  C  P
361 ACCGTTAAGGCGATATCCGAACACGTCAAAGGACGCCACGTCTACGTCCGACTGTCCAGC
    T  V  K  A  I  S  E  H  V  K  G  R  H  V  Y  V  R  L  S  S
421 GACGCTCCTTGGAGAGATACGAATCCCGTGTCTATGAACCGTACAGAGGCGCTCGCCCTA
    D  A  P  W  R  D  T  N  P  V  S  M  N  R  T  E  A  L  A  L
481 CTCGACACGTGTGAAGTGTCCGTAGATATCAAATGCAGTCGCGTCAACGTAACCGAAACG
    L  D  T  C  E  V  S  V  D  I  K  C  S  R  V  N  V  T  E  T
541 ACGTACGGAACCGCGGCGCTTGTCCCGCGTATAACTCAAGCGACGAGACGCAGTCATATT
    T  Y  G  T  A  A  L  V  P  R  I  T  Q  A  T  R  R  S  H  I
601 ATCGGATCTACCCTGGTCGACACGGAATGTGTGAAGAGTCTAGACATAACCGTCCAAGTG
    I  G  S  T  L  V  D  T  E  C  V  K  S  L  D  I  T  V  Q  V
661 GGTGAAATGTGTAAGAGAACGTCTGATCTCTCGGCGAGAGACAGTCTTAAGGTAAAGAAC
    G  E  M  C  K  R  T  S  D  L  S  A  R  D  S  L  K  V  K  N
721 GGCAAACTACTCGAGGACGATATCCTTGTCCTTCGTACGCCTACCCTCAAGGCGTGTAAC
    G  K  L  L  E  D  D  I  L  V  L  R  T  P  T  L  K  A  C  N
781 TAATCCTATCTACGATCGATGTCGTATTTTTCTGACCGTTACGCGTCACGTTTTTATACC
    *
841 TATATAAATAGTTAAAACCCATATAGGGAATACCGCTCGCTTTTTTTTCCTTCGTAGTTG
901 TTTACCCGCTCGATAGATCGCGTCGAGGAAGTACCAACCGTGACCACTCCTCCGGCGGGG
961 ATCC
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C

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1   ATGAAACAATATATCGTCCTGGCATGCATGTGCCTGGCGGCAGCTGCTATGCCTGCCAGT
    M  K  Q  Y  I  V  L  A  C  M  C  L  A  A  A  A  M  P  A  S
61  CTTCAGCAATCATCCTCATCCTCATCCTCGTGTACGGAAGAAGAAAACAAACATCATATG
    L  Q  Q  S  S  S  S  S  S  S  C  T  E  E  E  N  K  H  H  M
121 GGAATCGATGTTATTATCAAAGTCACAAAGCAAGACCAAACACCGACCAATGATAAGATT
    G  I  D  V  I  I  K  V  T  K  Q  D  Q  T  P  T  N  D  K  I
181 TGCCAATCCGTAACGGAAATTACAGAGTCCGAGTCAGATCCAGATCCCGAGGTGGAATCA
    C  Q  S  V  T  E  I  T  E  S  E  S  D  P  D  P  E  V  E  S
241 GAAGATGATTCCACATCAGTCGAGGATGTAGATCCTCCTACCACTTATTACTCCATCATC
    E  D  D  S  T  S  V  E  D  V  D  P  P  T  T  Y  Y  S  I  I
301 GGTGGAGGTCTGAGAATGAACTTTGGATTCACCAAATGTCCTCAGATTAAATCCATCTCA
    G  G  G  L  R  M  N  F  G  F  T  K  C  P  Q  I  K  S  I  S
361 GAATCCGCTGATGGAAACACAGTGAATGCTAGATTGTCCAGCGTGTCCCCAGGACAAGGT
    E  S  A  D  G  N  T  V  N  A  R  L  S  S  V  S  P  G  Q  G
421 AAGGACTCTCCCGCGATCACTCATGAAGAAGCTCTTGCTATGATCAAAGACTGTGAGGTG
    K  D  S  P  A  I  T  H  E  E  A  L  A  M  I  K  D  C  E  V
481 TCTATCGACATCAGATGTAGCGAAGAAGAGAAAGACAGCGACATCAAGACCCATCCAGTA
    S  I  D  I  R  C  S  E  E  E  K  D  S  D  I  K  T  H  P  V
541 CTCGGGTCTAACATCTCTCATAAGAAAGTGAGTTACGAAGATATCATCGGTTCAACGATC
    L  G  S  N  I  S  H  K  K  V  S  Y  E  D  I  I  G  S  T  I
601 GTCGATACAAAATGTGTCAAGAATCTAGAGTTTAGCGTTCGTATCGGAGACATGTGCAAG
    V  D  T  K  C  V  K  N  L  E  F  S  V  R  I  G  D  M  C  K
661 GAATCATCTGAACTTGAGGTCAAGGATGGATTCAAGTATGTCGACGGATCGGCATCTAAA
    E  S  S  E  L  E  V  K  D  G  F  K  Y  V  D  G  S  A  S  K
721 GGTGCAACCGATGATACTTCACTCATCGATTCAACAAAACTCAAAGCGTGTGTCTGA
    G  A  T  D  D  T  S  L  I  D  S  T  K  L  K  A  C  V  *
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FIG. 3. Location of the M-T1 gene in the myxoma virus genome and the nucleotide and deduced amino acid sequences of the myxoma virus M-T1 and rabbitpox virus 35kDa open reading frames. (A) A *Bam*HI restriction map of the myxoma genome shows the location of characterized genes in the *Bam*HI S fragment. These genes are present in two copies as they are fully within the terminal inverted repeats (TIR). The myxoma virus M-T1 (B) and rabbitpox virus 35kDa (C) open reading frame sequences. The derived amino acid sequences are shown with the predicted signal sequence cleavage sites (arrow) and putative N-glycosylation sites (underline).

a common 9-amino-acid deletion in the amino terminal region. However, all 8 cysteines in the predicted mature proteins are strictly conserved among all of the homologues, suggesting that the overall protein folding domains are likely maintained. Database searches have revealed no significant homology between the T1/35kDa poxvirus family and any of the reported chemokine serpentine receptors or indeed to any other proteins in the database. Thus, the closely related family of T1/35kDa proteins are candidate chemokine binding proteins based on their expression pattern in different vaccinia virus isolates, but the sequence information does not provide predictive insights into the basis for any interaction, other than to exclude receptor mimicry.

Identification of myxoma virus M-T1 and rabbitpox virus 35kDa proteins as chemokine binding proteins

Although it is demonstrated elsewhere that the purified myxoma virus interferon-γ receptor homologue, M-T7, binds to chemokines *in vitro* (Lalani *et al.,* submitted for publication), unfractionated secreted proteins obtained

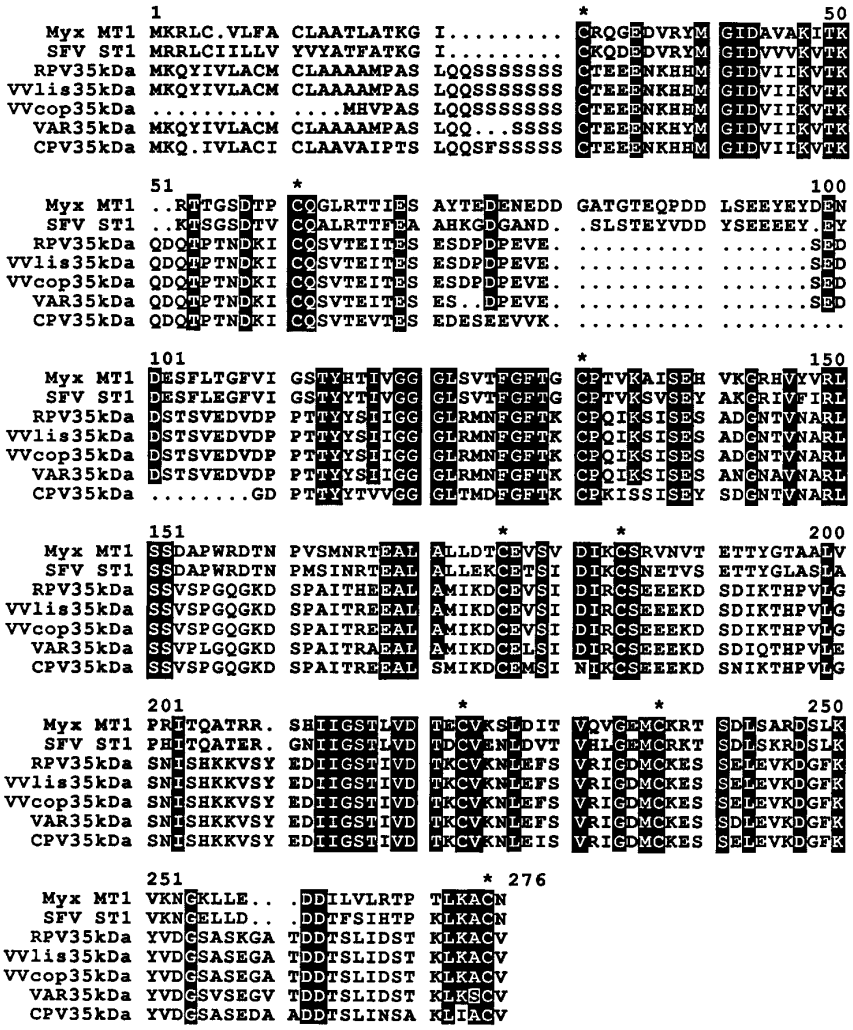

FIG. 4. Alignments of the T1/35kDa family members. The PIR (release 48.0) Swiss-Prot (release 33.0), and GenBank (release 94) databases were searched with the myxoma virus M-T1 and rabbitpox virus 35kDa amino acid sequences and an alignment of homologues was constructed: from top to bottom, myxoma virus M-T1 (U62677), Shope fibroma virus S-T1 (A43692), rabbitpox virus 35kDa (U64724), C23L 35kDa of vaccinia virus (Lister) (P19063), C23L 35kDa of vaccinia virus (Copenhagen) (A42529), G3R 35kDa of variola virus (Somalia) (U18341), and ORF B of cowpox virus (L08906). Amino acids are boxed if they are conserved in both leporipox- and at least four orthopoxviruses. Conserved cysteines are indicated (*).

from cells infected with a myxoma virus construct in which the M-T7 gene has been disrupted (Mossman *et al.,* 1996) still exhibit a chemokine binding species (Fig. 5A, lane 1). This indicates an independent chemokine binding activity in crude supernatants from virus infected cells that is distinct from M-T7. Using recombinant poxviruses, the ability of the myxoma virus M-T1 and rabbitpox virus 35kDa secreted proteins to interact with chemokines was assessed. The M-T1 ORF was inserted, under the control of a synthetic late promoter, into the *tk* locus of a vaccinia virus (WR) background, that itself does not express any chemokine binding protein activity, to create VV-T1. To assess the potential ability of the homologous orthopoxvirus 35kDa protein to bind chemokines, a rabbitpox virus mutant in which the gene encoding the 35kDa secreted protein has been deleted, RPVΔ35, was utilized (Martinez-Pomares *et al.,* 1995). Supernatants from cells infected with the two sets of recombinant viruses were reacted with ^{125}I-labeled RANTES to test for CC chemokine binding (Figs. 5A and 5B). Supernatant from cells infected with VV-T1 (Fig. 5A, lane 4), but not the parent vaccinia virus (WR) (Fig. 5A, lane 3), formed 53-kDa cross-linked complexes with RANTES. Furthermore, the size of the VV-M-T1/RANTES complex (Fig. 5A, lane 4) was comparable to the complex generated from myxoma virus-infected supernatants (Fig. 5A, lane 2), indicating that the myxoma virus M-T1 gene does in fact express a secreted CC chemokine binding protein of the correct size.

Supernatants from cells infected with RPVΔ35 were compared with the parental rabbitpox virus for RANTES binding activity, to confirm that the 35kDa secreted protein of rabbitpox virus is also responsible for CC chemokine binding. As shown in Fig. 5B, rabbitpox virus super-

TABLE 1

Percentage Similarity and Identity between the Amino Acid Sequences of M-T1 and Several Family Members[a]

Virus	Myx T1	SFV T1	RPV 35 kDa	VV (lis) 35 kDa	VV (cop) 35 kDa	VAR 35 kDa	CPV 35 kDa
Myx T1		84.4	56.0	56.0	55.3	57.3	57.0
SFV T1	70.4		58.1	58.5	59.2	60.3	62.3
RPV 35 kDa	37.8	40.2		99.6	99.6	96.0	92.3
VV(lis) 35 kDa	38.2	41.1	99.2		100.0	96.4	92.7
VV(cop) 35 kDa	38.3	41.6	98.8	99.6		96.2	92.3
VAR 35 kDa	37.7	42.6	94.1	94.9	94.1		88.9
CPV 35 kDa	39.6	41.6	85.0	85.8	85.0	85.1	

[a] Similarity is shown at the top right; identity is shown at the bottom left. Accession numbers are given in the legend to Fig. 4.

natants contained a protein which could be cross-linked to RANTES, forming a 49-kDa complex (Fig. 5B, lane 1) but supernatants harvested from cells infected with RPVΔ35 did not exhibit any detectable chemokine binding protein (Fig. 5B, lane 2), verifying that rabbitpox virus 35kDa protein, like M-T1, binds RANTES.

To prove that the same viral proteins can bind to CXC in addition to CC chemokines, the rabbitpox virus 35kDa protein was assessed for its ability to bind IL-8 (Fig. 6A). Similar to the RANTES binding profile, rabbitpox virus (Fig. 6A, lane 1) supernatants exhibit IL-8 binding activity, detectable as a 49-kDa cross-linked protein complex, whereas the RPVΔ35 (Fig. 6A, lane 2) or uninfected (lane 3) supernatants do not form any comparable cross-linked species with IL-8. M-T1 expressed from VV-T1 but not vaccinia virus (WR) also exhibited IL-8 binding activity comparable to that observed in Fig. 5A for RANTES (data not shown). A Coomassie-stained SDS–PAGE gel (Fig. 6B) of untreated supernatants illustrates the 35kDa secreted protein of rabbitpox virus which is absent in RPVΔ35 supernatants; note that in the gel system used here this viral protein migrates at about 41 kDa. To show that both IL-8 and a specific viral protein are detectable within the shifted complexes, immunoblots of IL-8/35kDa cross-linking assays were probed with either an anti-IL-8 antibody or the anti-35kDa antiserum, which was previously generated against the 35kDa, C23L, protein of vaccinia virus (Lister) (Patel *et al.,* 1990). The anti-IL-8 antibody detects the IL-8/35kDa complex derived from rabbitpox virus-infected cell supernatants (Fig. 6C). In addition to the shifted complex containing IL-8 (Fig. 6C, lane 1), monomers and cross-linked dimers of IL-8 can be observed in all cases. The anti-35kDa antiserum detects the 35kDa protein of vaccinia virus (Lister) in supernatants from infected cells (Fig. 6D, lane 1) and also detects the shifted species cross-linked with IL-8 (Fig. 6D, lane 2). Thus, these experiments indicate that the T1/35kDa family of secreted poxvirus proteins are soluble chemokine binding proteins that bind to members of both the CC and CXC classes of chemokines.

Solid-phase binding analysis of purified M-T1

The results obtained thus far depend upon chemical cross-linking to demonstrate the chemokine interactions

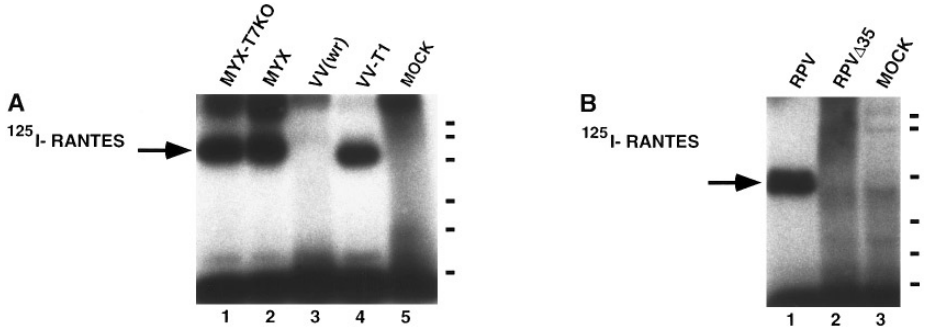

FIG. 5. Identification of myxoma virus M-T1 and rabbitpox virus 35kDa as soluble poxviral CC chemokine binding proteins using chemical cross-linking. (A) ^{125}I-labeled RANTES was cross-linked with supernatants from cells infected with a myxoma virus construct in which the MT-7 ORF was disrupted (Mossman *et al.,* 1996) (MYX-T7KO, lane 1), myxoma virus (MYX, lane 2), vaccinia virus (WR) (VV(wr), lane 3), vaccinia virus (WR) recombinant expressing M-T1 (VV-T1, lane 4), or from uninfected cells (Mock, lane 5). The arrow indicates a chemokine/viral protein complex of identical size in MYX-T7KO (lane 1), MYX (lane 2), and VV-T1 (lane 4) supernatants. (B) ^{125}I-labeled RANTES was cross-linked with supernatants from cells infected with rabbitpox virus (RPV, lane 1), rabbitpox virus in which the 35kDa gene has been disrupted (Martinez-Pomares *et al.,* 1995), (RPVΔ35, lane 2) or from uninfected cells (Mock, lane 3). The arrow indicates the 49-kDa cross-linked complex in supernatants harvested from rabbitpox virus-infected cells which is absent in RPVΔ35 and mock samples. Molecular weight standards are as described in the legend to Fig. 1.

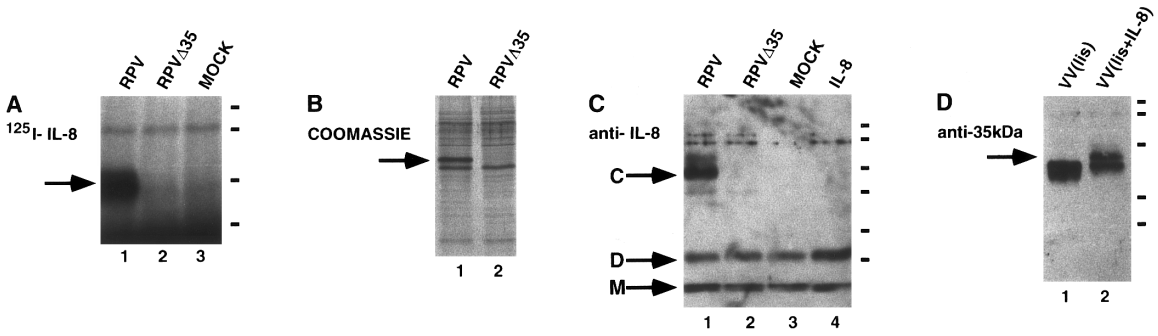

FIG. 6. Identification of rabbitpox virus 35kDa as soluble poxviral CXC chemokine binding protein. (A) ^{125}I-labeled IL-8 was cross-linked with supernatants from cells infected with rabbitpox virus (RPV, lane 1), rabbitpox virus in which the 35kDa gene has been disrupted (Martinez-Pomares *et al.,* 1995), (RPVΔ35, lane 2) or from uninfected cells (Mock, lane 3). The arrow indicates the 49-kDa cross-linked complex in supernatants harvested from rabbitpox virus-infected cells which is absent in RPVΔ35 and mock samples. In a Coomassie-stained gel (B) of the rabbitpox virus (RPV, lane 1) and RPVΔ35 (lane 2) supernatants, the arrow indicates the 35kDa rabbitpox virus secreted protein, which migrates at about 41 kDa in this gel system, and is absent from the RPVΔ35 supernatants. (C) An anti-IL-8 immunoblot was performed to demonstrate that IL-8 is present in the shifted complexes. Unlabeled IL-8 (1 μg) was cross-linked alone in the absence of supernatant (IL-8, lane 4), or with supernatants from uninfected (Mock, lane 3), or from cells infected with rabbitpox virus (RPV, lane 1), or RPVΔ35 (lane 2). The arrows indicate the monomer (M), and dimer (D) of IL-8, and the 49-kDa shifted complex (C) which contains IL-8. To show that the viral 35kDa protein is also present in the shifted complexes, an anti-35kDa immunoblot (D) was performed using the A18691 antiserum which detects the vaccinia virus (Lister) 35kDa protein (Patel *et al.,* 1990). Supernatant harvested from vaccinia virus infected cells was cross-linked in the absence (lane 1) or presence of 1 μg IL-8 (lane 2), the arrow indicated the shifted species. Molecular weight standards are as described in the legend to Fig. 1.

with the T1/35kDa members. To show that this interaction occurs in the absence of cross-linking with a physiologically relevant affinity, solid-phase equilibrium binding studies was performed. Saturable binding of radiolabeled RANTES to immobilized M-T1 was demonstrated under nanomolar concentrations of RANTES (Fig. 7A), demonstrating that the interactions observed earlier between the soluble viral proteins and chemokines occur under physiological conditions and are independent of cross-linking. To quantify the affinity of M-T1 and RANTES interaction, Scatchard analysis of the solid-phase binding data was subsequently performed and yielded a dissociation constant (K_d) of approximately 73 n*M* (Fig. 7B).

The rabbitpox virus 35kDa protein influences leukocyte migration in infected tissues

To determine if the T1/35kDa family of chemokine binding proteins influence leukocyte migration *in vivo,* their role in the early inflammatory process during poxvirus infection was assessed using the rabbitpox virus system. Rabbits were infected with rabbitpox virus or RPVΔ35 at a low (50 PFU) or high (5×10^4 PFU) dose of virus and tissue samples were harvested at various times in order to measure leukocyte infiltration into primary sites of infection. Although histological analysis of the tissue sections resulting from the higher virus dose on Days 1 and 2 postinfection revealed only minimal differences in cellular infiltration between the lesions induced by the two viruses, by Day 3 there were distinctive differences. The lower virus inoculation dose exhibited the same trend, but the differences were less evident, possibly because of reduced tissue dosages of secreted viral proteins in general. While the deep dermal layer of lesions infected with wild-type rabbitpox virus (Fig. 8A) still exhibited only a few scattered infiltrating cells at 3 days postinfection, the lesions infected with RPVΔ35 (Fig. 8B) were characterized by a significant leukocyte influx and an accompanying edema typical of an acute inflammatory reaction. To characterize the infiltrating cells, tissue sections were immunostained with an anti-rabbit CD43 antibody which specifically stains rabbit lymphocytes and monocytes/macrophages. In the RPVΔ35 tissue sections about 30% of the infiltrating cells can be immunostained for rabbit CD43, while the remaining 70%, based on nuclear morphology, are presumed to be CD43-negative granulocytes, which are predominantly neutrophils. In the rabbit, granulocyte subclasses have not been as extensively characterized by surface marker expression studies as in the mouse and human systems and the term heterophil is sometimes used to describe such rabbit polymorphonuclear leukocytes. In the lesions caused by the parental rabbitpox virus very few ($<$3%) of the cells in the dermal layer can be stained at all for CD43. Thus, in the absence of the 35kDa chemokine binding protein in rabbitpox virus infection there is an increased infiltration of multiple classes of immune cells, predominantly granulocytes, macrophages, and NK cells, into the infected lesion. This suggests that *in vivo* the expression of the 35kDa secreted chemokine binding protein functions during the early stages of rabbitpox virus infection to reduce the initial influx of extravasating leukocytes into the site of infection.

DISCUSSION

Poxviruses encode an impressive array of proteins which assist in virus evasion of the collective host de-

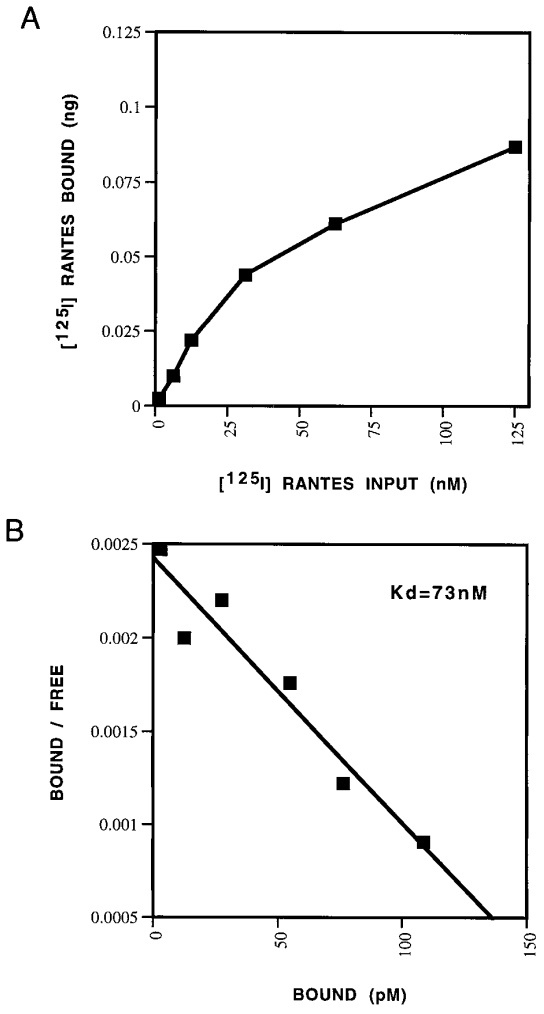

FIG. 7. Solid-phase equilibrium binding analysis of ^{125}I-labeled human RANTES to M-T1. (A) Solid-phase binding analysis of immobilized myxoma M-T1 protein with ^{125}I-labeled RANTES as outlined under Materials and Methods. (B) Scatchard plot analysis of the binding curve of ^{125}I-labeled RANTES and M-T1.

fense systems (Barry and McFadden, 1997; McFadden, 1995; Spriggs, 1996). The relationship between the leporipoxvirus T1 and orthopoxvirus 35kDa genes was previously noted by sequence homology analysis (Martinez-Pomares *et al.,* 1995); however, no function has been previously attributed to this family of proteins. We now report that the T1/35kDa family of secreted proteins bind both CC and CXC chemokines *in vitro* and, in the case of rabbitpox virus infection, modulate early-stage leukocyte migration into virus-infected lesions *in vivo.*

Chemokine binding proteins are secreted from cells infected by several different poxviruses, including myxoma virus, Shope fibroma virus, rabbitpox virus, raccoonpox virus, cowpox virus, and vaccinia virus (Lister), but not vaccinia virus (WR) or swinepox virus. It was unexpected that the cowpox virus-derived supernatants exhibit chemokine binding, as previous analysis of [^{35}S]Met-labeled supernatants from cowpox-infected cells did not reveal a comparable 35kDa protein species (Martinez-Pomares *et al.,* 1995). However, in this study immunoblot analysis using antiserum prepared against the 35kDa homologue of vaccinia virus (Lister) (Patel *et al.,* 1990) in fact revealed a smaller related protein in the cowpox virus supernatant (data not shown). This protein, which is approximately 4 kDa smaller in size than the rabbitpox virus protein, likely corresponds to a smaller secreted protein that was observed in the previous study, but not at that time attributed to the 35kDa protein homologue. The resolution of the gel shifts presented here (Fig. 1) does not illustrate this size difference between the orthopoxvirus 35kDa homologues but these can be observed under different gel conditions (not shown).

Although the secreted T1/35kDa proteins are not related to any known cytokine receptor species, numerous cell surface virus-encoded chemokine receptors with classic seven transmembrane domains have been previously identified. The viral proteins US28, of human cytomegalovirus, and ECRF, of herpesvirus saimiri, are functional serpentine chemokine receptors (Ahuja and Murphy, 1993; Murphy, 1994); however, their role in viral pathogenesis remains unclear. Several other viruses encode similar transmembrane proteins with homology to chemokine receptors, including two herpesviruses (HHV-6, HHV-8) and two poxviruses (capripox and swinepox viruses), but these proteins have not yet been shown to be functional for chemokine binding or signal transduction (Cao *et al.,* 1995; Massung *et al.,* 1993; Murphy, 1994).

The observation that the complexes induced by either RANTES or IL-8 are identical in size for each virus suggests that the same polypeptide is responsible for binding both CC and CXC classes of chemokines. This notion is supported by the following data: (1) unlabeled RANTES can compete for radiolabeled IL-8 binding to the viral protein; (2) the expression of one open reading frame, M-T1, from a recombinant vaccinia virus (WR) construct confers upon the virus the ability to secrete a protein which binds both RANTES and IL-8; and (3) the deletion of the single open reading frame encoding the 35kDa secreted protein from rabbitpox virus results in the loss of all chemokine binding activity from the supernatants of cells infected by this virus. Although the previously characterized viral chemokine receptors are fairly class restricted, binding either CC or CXC chemokines, the T1/35kDa proteins are far less restricted in terms of binding specificity. However, M-T1 clearly binds RANTES more avidly than IL-8 because RANTES can effectively compete for IL-8 binding but IL-8 fails to compete with RANTES. These results indicate that both IL-8 and RANTES bind to the same site on the viral protein but that RANTES binds with a higher affinity.

To determine the physiological relevance of chemokine binding to the M-T1/35kDa proteins solid-phase binding analysis was performed in the absence of cross-

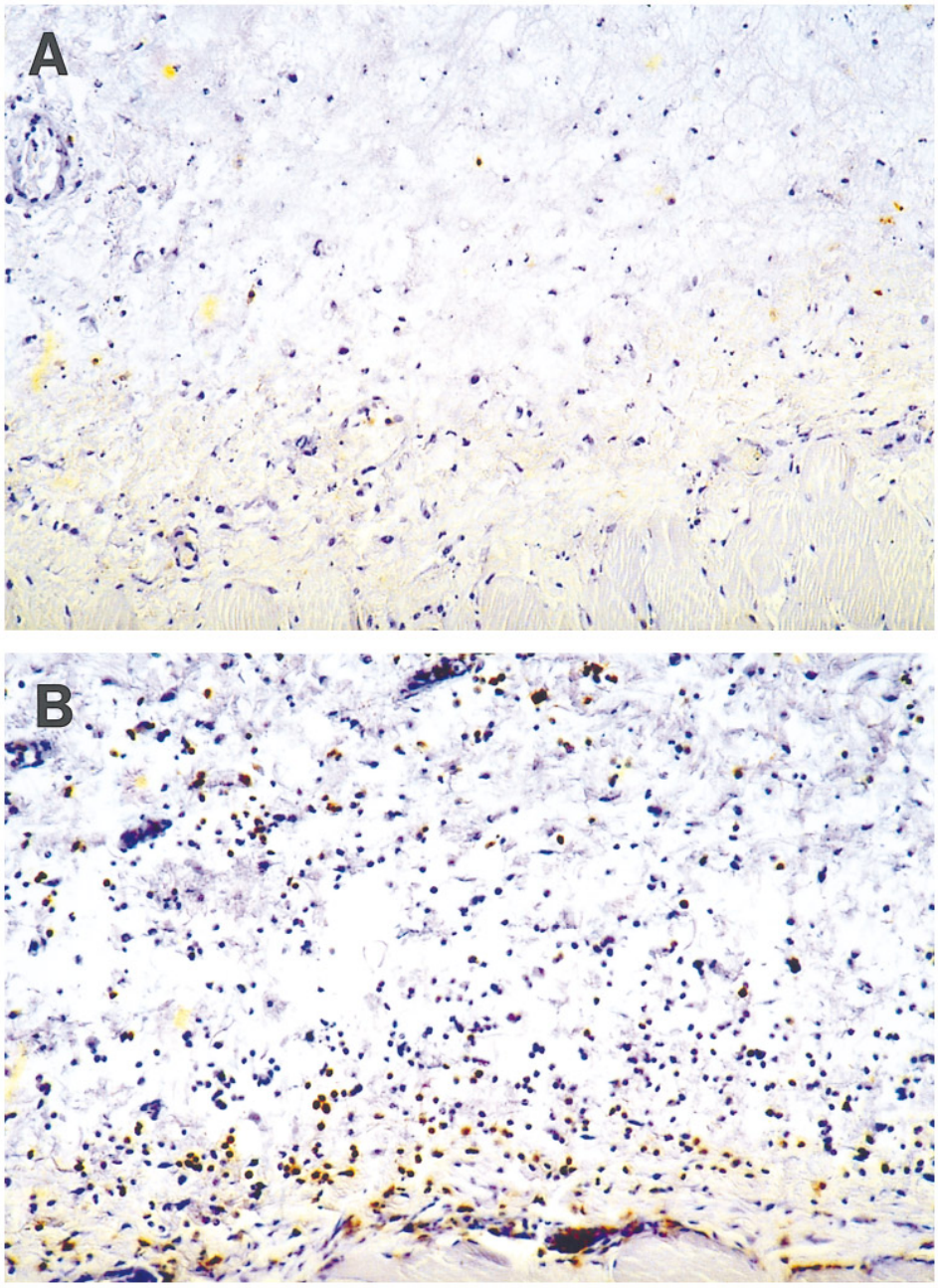

FIG. 8. Leukocyte infiltration into dermal lesions of European rabbits infected for 3 days with 5×10^4 PFU rabbitpox virus or RPVΔ35 viruses. In the deep dermal layer at the primary lesion on Day 3, very little cellular infiltration was induced by rabbitpox virus (A). However, in (B) RPVΔ35-infected tissues there was extensive infiltration of CD43-positive lymphocytes and macrophages (stained brown) and CD43-negative granulocytes (stained blue). Refer to Materials and Methods for experimental details.

linking. As the competition study using unfractionated supernatants suggested that RANTES has apparent greater affinity for the viral protein than IL-8, RANTES was selected for this experiment. The calculated 73 n*M* K_d of RANTES binding with purified M-T1 is comparable with CC chemokine binding to cell-surface receptors (Kelvin *et al.,* 1993) but whether these soluble proteins competitively displace receptor/ligand binding or triggering remains to be determined.

The function of the M-T1/35kDa proteins can also be assessed by infecting susceptible animals with poxvirus mutants in which the M-T1/35kDa gene has been deleted. Unlike myxoma virus, rabbitpox virus expresses only a single chemokine binding protein; therefore, rabbitpox virus was chosen for this study. Previous analysis of rabbitpox virus has shown that virus infection of European rabbits induces an almost uniformly lethal disease even when the secreted 35kDa gene is deleted, and no differences in the pathology were observed between the parental and mutant virus strains. However, when injected intranasally into mice,

the RPVΔ35 virus did induce an inflammatory reaction that developed more rapidly during the initial stages of infection (Martinez-Pomares *et al.,* 1995). There are several important differences between that study and the experiments reported here. Previously, both the parental and mutant viruses were administered to the same rabbit, on opposite sides, at a dose of 500 PFU per site. In this study each rabbit received only one virus at a dose of 50 or 5×10^4 PFU. The results we observed with the lower 50 PFU virus dose is consistent with the previously published experiment. However, when we injected a significantly higher dose of 5×10^4 PFU, the effect of the deletion in the RPVΔ35 virus was evident. Here we show in the RPVΔ35 virus-infected lesions that the influx of at least two classes of infiltrating leukocytes, composed of CD43-positive lymphocytes (possibly NK cells) and monocytes/macrophages as well as CD43-negative granulocytes, occurs at significantly greater levels by Day 3 following infection, compared to the parental virus. Although this increase in leukocyte infiltration did not cause overall attenuation in terms of eventual mortality levels, it should be noted that the relationship between virulence and immunomodulatory proteins can be complex. For example, deletion of the IL-1β receptor in vaccinia virus causes an increase in virulence in mice following an intranasal route of inoculation but a decrease in virulence following intracranial inoculation (Alcamí and Smith, 1992; Spriggs *et al.,* 1992).

Distinct domains of chemokines have been identified that are necessary for binding to their cognate serpentine receptors and to glycosaminoglycans both on cell surfaces and on the extracellular matrix (Webb *et al.,* 1993; Witt and Lander, 1994). The poxviral soluble chemokine binding proteins, alone or in concert with other proteins, could in theory act either by blocking signaling through the chemokine receptors or alternatively by interfering with chemokine gradients mediated by interaction with glycosaminoglycans. Inhibition of either function could have a profound effect on leukocyte chemotaxis in complex tissues (McFadden and Kelvin, 1997). Although the biochemical basis for the T1/35kDa protein activities *in vivo* is not understood, it is known that most chemokines bind tightly to glycosaminoglycans such as heparan sulfate proteoglycan (Webb *et al.,* 1993). Because the T1/35kDa proteins are all very acidic, with predicted p*I* values of 4.3–4.7, it is plausible that they interact with chemokines via the conserved heparin-binding domains; further studies, including mutagenesis analysis of the viral proteins, will be required to address this issue.

There is increasing evidence that chemokines play an important role in the early inflammatory responses to viruses (Cook *et al.,* 1995). Moreover, the recent claim that chemokines can act as the major HIV suppressor factors and the fact that chemokine receptors act as cofactors for HIV entry suggests that chemokines may significantly contribute to the outcome of viral disease progression and viral pathogenesis (Bates, 1996; Bleul *et al.,* 1996; Oberlin *et al.,* 1996). Thus, it would not be unexpected that at least some viruses have evolved counteractive extracellular proteins to alter the ability of chemokines to activate and direct inflammatory cells to the site of virus infection. The mechanism by which the T1/35kDa family of secreted proteins modulate chemokine activities remains to be demonstrated, but this new superfamily may very well provide useful protein probes with which to investigate the complex biological roles of chemokines as regulators of leukocyte trafficking during inflammatory syndromes in general.

ACKNOWLEDGMENTS

We thank Dr. Hsaing-fu Kung (NIH-Biological Response Modifiers Program) for generously supplying samples of chemokines in the early phases of this work, and Robert Maranchuk for excellent technical assistance. This work was funded by the National Cancer Institute of Canada (G.M.) and by the National Institutes of Health, Grant AI 15722 (R.W.M.). G.M., A.S.L., M.B., and A.L. are supported by the Alberta Heritage Foundation for Medical Research and G.M. is a senior medical scientist of the Medical Research Council of Canada.

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