

## Poxvirus Genomes Encode a Secreted, Soluble Protein That Preferentially Inhibits $\beta$ Chemokine Activity yet Lacks Sequence Homology to Known Chemokine Receptors

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Poxvirus genomes encode several proteins which inhibit specific elements of the host immune response. We show the "35K" virulence gene in variola and cowpox viruses, whose vaccinia and Shope fibroma virus equivalents are strongly conserved in sequence, actually encodes a secreted soluble protein with high-affinity binding to virtually all known  $\beta$  chemokines, but only weak or no affinity to the  $\alpha$  and  $\gamma$  classes. The viral protein completely inhibits the biological activity of monocyte chemotactic protein-1 (MCP-1) by competitive inhibition of chemokine binding to cellular receptors. As all  $\beta$  chemokines are also shown to cross-compete with MCP1 binding to the viral protein, we conclude that this viral chemokine inhibitor (vCCI) not only interacts through a common binding site, but is likely a potent general inhibitor of  $\beta$  chemokine activity. Unlike many poxvirus virulence genes to date, which are clearly altered forms of acquired cellular genes of the vertebrate immune system, this viral chemokine inhibitor (vCCI) shares no sequence homology with known proteins, including known cellular chemokine receptors, all of which are multiple membrane-spanning proteins. Thus, vCCI presumably has no cellular analogue and instead may be the product of unrelenting sequence variations which gave rise to a completely new protein with similar binding properties to native chemokine receptors. The proposed function of vCCI is inhibition of the proinflammatory (antiviral) activities of  $\beta$  chemokines. © 1997 Academic Press

### INTRODUCTION

All viruses which productively infect eukaryotic hosts must possess mechanisms to avoid immune surveillance that would otherwise lead rapidly to their elimination (Marack and Kappler, 1994; Smith *et al.*, 1990, 1991). Although this maxim has been long accepted *prima facie*, the mechanisms involved have remained enigmatic until recent years. The discovery that an obscure open reading frame, T2, in Shope fibroma poxvirus encodes a soluble secreted form of an acquired cellular receptor for tumor necrosis factor (TNF) proved illuminating for two reasons (Smith *et al.*, 1990, 1991). First, it clearly demonstrated that viruses target host cytokine (inflammatory) networks for disruption, the presumed function of which is to increase virulence. Direct evidence in support of this proposal came from the subsequent demonstration in myxoma poxvirus that targeted inactivation of the T2 gene reversed virulence by almost 70% (Upton *et al.*, 1991). Second, it helped to establish viral acquisition of host immune genes themselves, subverted to viral function, as a general phenomenon, presaged by the identification of a vaccinia virus gene with homology to complement (Kotwal and Moss, 1988; Kotwal *et al.*, 1990). The subsequent identification of the T2 gene in virtually all poxviruses and other genes encoding soluble interleukin-1 (IL-1) receptors (Spriggs *et al.*, 1992),  $\alpha/\beta$ - and  $\gamma$ -interferon

receptors (Symons *et al.*, 1995; Upton *et al.*, 1992), other TNF receptors (Smith *et al.*, 1996), and inhibitors of IL-1 processing (Ray *et al.*, 1992) underscores the validity of both points. Collectively termed virulence factors and often secreted, these proteins are not required for viral DNA replication and usually show clear homology to known genes of the host immune system (Smith and Goodwin, 1994). One presumed virulence gene, the "35K" gene of vaccinia (Patel *et al.*, 1990) and related poxviruses (Hu *et al.*, 1994; Martinez-Pomares *et al.*, 1995), in contrast, shows no sequence homology with any known proteins and is of unknown function, although its sequence is strongly conserved. The 35K gene product of vaccinia (Lister) has been shown to be secreted from infected cells (Patel *et al.*, 1990), and like T2, is diploid, with one copy in either of the ~10-kb inverted terminal repeats of the large (160 kb) duplex DNA genome. To determine its function, we expressed epitope-tagged soluble forms of the same protein from variola virus (vav), the causative agent of smallpox in human, and cowpox virus (cpv) and then used these as probes to identify the cognate. We show that both secreted proteins, 84% identical in sequence, actually promiscuously bind chemokines of the  $\beta$  class with high affinity, bind at least one  $\alpha$  chemokine (IL8) with low affinity, and appear to completely inhibit their (proinflammatory) biological activity.

### MATERIALS AND METHODS

#### Plasmid construction

A soluble form of the cowpox vCCI protein fused to the Fc region of human IgG1 was constructed in the

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mammalian expression vector pDC406 (McMahan *et al.*, 1991). The entire coding region of the cowpox protein was amplified by PCR using as a template an 11.8-kb *EcoRI* fragment of the cowpox genome, which contains the inverted terminal repeat, subcloned into pBR325 (Pickup *et al.*, 1982). The oligonucleotides used were 5'-ATAGCGGCCGCATCTAGAACAGCAATCATGAAAC-3', which introduces a *NotI* site upstream of the initiator methionine, and 5'-GTTAGATCTGGGCTCGACACACGC-TATAAGTTTTGCTG-3', which inserts the amino acids Glu, Pro, and Arg after the last amino acid of the 35K protein. Glu and Pro are the first two amino acids of the hinge region of human IgG1 and are followed by a *BglII* site that was used to fuse the 35K coding region to the remainder of the Fc domain as previously described (Fanslow *et al.*, 1992).

An alternative soluble form of cowpox 35K was created by PCR amplification which was tagged with the FLAG epitope (Hopp *et al.*, 1988) at the 5' end of the mature protein. The 5' oligo utilized in this PCR was 5'-ATAACT-AGTGACTACAAGGACGACGATGACAAGCAATCATTC-TCATCCTCATCCTC-3', which adds an *SpeI* site and the FLAG coding region and begins with amino acid 22 of the cowpox 35K ORF. The resulting PCR product was inserted behind a CMV open reading frame leader sequence as previously described (Wiley *et al.*, 1995).

A soluble form of the variola 35K protein (G5R, India 1967 strain) (Shchelkunov *et al.*, 1993) was also constructed. The entire coding region of G5R was reconstructed with overlapping oligonucleotides with *SalI* and *NotI* restriction sites appended to the 5' and 3' ends, respectively, and cloned into pBluescript SK(+) (Stratagene). This clone was then used as a template for a PCR which removed the termination codon and inserted a *BglII* site that was used to fuse the 35K coding region with the Fc domain as described above.

Isolation of the fusion proteins was performed by transient expression in CV1/EBNA cells (ATCC CRL 10478) and purification on a protein G-Sepharose column as previously described (Fanslow *et al.*, 1992). The flag-tagged cpv p32 protein was affinity-purified using M2 (anti-flag) monoclonal antibody and acid (pH 3.0) elution. The binding activities of cpv flag-p32, cpv p32/Fc, and vav G5R/Fc were completely stable to acid conditions (not shown).

### Cell lines, antibodies, and recombinant chemokines

The TE71 murine thymic epithelial line was grown to near-confluence in RPMI 1640 medium (GIBCO, Gaithersburg, MD) supplemented with 1% low-endotoxin fetal calf serum, 100  $\mu$ g/ml penicillin, 100  $\mu$ g/ml streptomycin, and 2 mM L-glutamine at 37° in a humidified, 7% CO<sub>2</sub> atmosphere. Conditioned medium were harvested at 4 days, 0.2  $\mu$ m filtered, and stored at -20° in aliquots.

All recombinant chemokines and cytokines were purchased from commercial vendors: hu and muMCP1,

huMCP3, huRANTES, muMIP1 $\alpha$ ,  $\beta$ , muC10, huGRO $\alpha$ ,  $\beta$ ,  $\gamma$ , huL-8, huENA-78, huSDF-1 $\beta$ , huMCP-2, muMIP2, muMIP1, muKC, huMIDKINE (R&D Systems, Minneapolis, MN); huMCP4, human and mouse Eotaxin, human and rat MIP1 $\alpha$ , muMCP-5, rat GRO  $\beta$ , rat MCP-1, huMIG human lymphotactin (PeproTech, Rocky Hill, NJ); human platelet factor-4 (PF-4), hupP-10, huNAP-2, rat GRO $\alpha$  (Biosource, Camarillo, CA); huL-8 (Genzyme, Boston, MA and R&D Systems); C5a (Fluka); huL-16 (AMS Biotechnology). Human and murine MCP1 and MIP1 $\alpha$  were shown to be biologically active at subnanomolar levels with in-house assays. For all others, particularly those weak or nonbinding  $\alpha$  chemokines, every attempt was made to ensure the validity of *in vitro* bioactivity profiles offered by the vendors.

Flag octapeptide (Hopp *et al.*, 1988) was synthesized by conventional solid-phase synthesis (Applied Biosystems, Foster City, CA) and purified by reverse-phase HPLC on C4 columns. M2 anti-flag monoclonal antibody was produced at Immunex (Hopp *et al.*, 1988). Polyclonal goat anti-human Fc was purchased from Jackson ImmunoResearch (West Grove, PA). Purified human IgG1 was produced at Immunex.

### Radiolabeling, immunoprecipitations, and SDS gel electrophoresis

TE71 cells were cultured in 10-cm petri dishes to near-confluency for 3 days, as described above, washed once in PBS, and recultured at 37° for 30 min in cysteine/methionine-depleted RPMI medium. Cells were washed once in the same medium and then recultured for 5 hr at 37°, 10% CO<sub>2</sub> in 2 ml of the same medium supplemented with 300  $\mu$ Ci of <sup>35</sup>S Translabel (Amersham). The labeled supernatant was precleared by overnight incubation at 4° with 1 ml PBS/0.5% NP-40 containing 5  $\mu$ g/ml hulgG1 and 0.2 ml of a 20% protein G suspension (Pharmacia, Piscataway, NJ) in the same buffer. Protein G was removed by centrifugation at 3000 rpm for 2 min, and the supernatant was incubated for 7 hr at 4° with 5  $\mu$ g/ml of cpv p32/Fc (or IgG1 as control) with 1/10 vol of 20% protein G suspension again. Immunoprecipitates were collected by centrifugation, washed three times with RPPA buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 0.5% deoxycholate, 0.5% Nonidet-P40, 0.1% SDS) and resuspended in Laemmli buffer (Laemmli, 1970), electrophoresed through an 8–16% SDS gel, and the dried gel was PhosphorImaged (Molecular Devices, Palo Alto, CA).

### Affinity chromatography and protein sequencing

Purification of cpv p32 cognates from TE71 conditioned medium was effected by affinity chromatography on cpv p32/Fc-coupled Affigel 10 (Bio-Rad, Richmond, CA). p32/Fc was coupled to Affigel 10, according to the manufacturer's instructions, at 5 mg cpv p32/Fc per 1 ml gel. A preclearing column of huTNFRII/Fc was similarly constructed. TE71 CM (2.1 liters) was twice passed

through a 1-ml p32/Fc column and the flow-through was subsequently passed through the p32/Fc column. The column was then washed with a cocktail of protease inhibitors (1 mM PMSF, 1  $\mu$ M pepstatin A, 10  $\mu$ M leupeptin, 2 mM *O*-phenanthroline) in PBS, 150 ml PBS, and bound protein was eluted with 1.5-ml fractions of 50 mM citrate buffer, pH 3.0. Fractions were assayed for protein on SDS gels, and individual bands were blotted onto PVDF and sequenced on an Applied BioSystems 477A sequencer. The 55-kDa band was determined to be contaminating albumin, but the diffuse 30-kDa and sharp 8- to 10-kDa bands were N-terminally blocked. CNBR digestion was then performed *in situ* (March *et al.*, 1985) and the resulting fragments were sequenced without further modification. Multiple amino acids were detected in each cycle. The major sequence for cycles 10–24 was BLAST searched against the nonredundant protein sequence database maintained at the National Center for Biotechnology Information. An identical match to residues 82–96 of mouse mature MCP1 was obtained. With this information, the sequence data were further analyzed and sequences corresponding to residues 20–21, 23–50, and 73–99 of mouse mature MCP1 were identified. The CNBr fragment corresponding to residues 1–19 of mature MCP1 was not identified, but residue 1 is predicted to be pyroglutamate, which is refractive to Edmann N-terminal sequencing. Previous characterization of recombinant muMCP1 transiently expressed in mammalian cells shows that both diffuse (glycosylated) 30-kDa protein and sharp (unglycosylated) 8- to 10-kDa protein are produced, which also differ in the extent of proteolytic processing (Rollins *et al.*, 1988).

### Chemotaxis assays

Human peripheral blood mononuclear cells (PBMC) were isolated from human donors as described (Smith *et al.*, 1993). The chemotaxis assay (Martinet *et al.*, 1994) was performed in modified Boyden chambers (24-well transwell plates, pore size 5; Costar). Top chambers contained  $5 \times 10^6$  PBMC, and bottom chambers contained 50 ng/ml recombinant huMCP1 with either cpv p32/Fc (166, 500 or 1500 ng/ml) or hulgG at corresponding dilutions as controls. All samples were assayed in triplicate. After incubations for 1 hr at 37°, transwell inserts were removed and the cells (monocytes) that migrated to the bottom well were counted. Migrations were determined to be chemotactic (directional) not chemokinetic (random) by examining migration patterns when both cell number and chemokine concentrations were systematically varied by standard methods. Migration occurred in the direction of increasing MCP1 concentrations.

### Fluorescence assays

THP1 cells were incubated at 37° for 30 min in culture medium (DMEM, 1% FCS, 20 mM HEPES, pH 7.4) containing 5  $\mu$ M Fluo-3 acetoxymethyl ester (Molecular

Probes, Eugene, OR), washed 2× with medium and then seeded at  $5 \times 10^5$  cells/ml in 8-well glass slides (Nunc Inc., Naperville, IL) precoated with poly-L-lysine (Sigma, St. Louis, MO). Cells were then allowed to settle and loosely attach to the substrate for 30 min at 37°. Slides were examined in a Molecular Dynamics (Sunnyvale, CA) confocal laser scanning microscope employing a krypton/argon laser (488 nm excitation; 526 nm emission) maintained at 37° throughout the experiment. Prior to addition of agonists, multiple scans were averaged to establish baseline fluorescence; following addition, scans were recorded at 5-sec intervals. Images were processed to assess mean fluorescence intensity (MFI) using the IMAGE program (Wayne Rasband, NIH), publicly available through the Internet (zippy.nimh.nih.gov).

### Binding and inhibition assays

Murine MCP1 was radiolabeled with  $^{125}$ I (Amersham) using a solid-phase chloramine T analogue (Iodogen, Pierce, St. Louis, MO) to a specific activity of  $3 \times 10^{16}$  cpm/mmol, with a minor loss of specific activity (assessed by inhibition with unlabeled MCP1). Equilibrium binding assays on THP1 cells were performed in 96-well microtiter trays (Corning) as described (Smith *et al.*, 1990). Briefly, serial dilutions of  $^{125}$ I-muMCP1 in binding medium (RPMI 1640, 2.5% BSA, 20 mM HEPES, 0.02% sodium azide, pH 7.2), supplemented with 0.5 mg/ml hulgG1 and 5% human serum, were incubated with cells ( $2.5 \times 10^6$ /well) for 2 hr at 4° in a total volume of 150  $\mu$ l. Free and bound probes were separated by microfugation through a phthalate-oil separation mixture and counted in a gamma counter. Inhibition assays used  $^{125}$ I-MCP1 at a constant concentration of 0.5 nM in the presence or in the absence of serial dilutions of potential inhibitors. Nonspecific binding was determined in the presence of a 100-fold excess of unlabeled MCP1. Theoretical curves based on single-site competitive inhibition model were fitted to the data as described (Dower *et al.*, 1984). Percentage inhibition was calculated according to the equation

$$I(\%) = [100 K_i(I)]/[1 + K_a(L) + K_i(I)],$$

where  $I$  is the molar concentration of inhibitor,  $L$  is the molar concentration of radiolabeled ligand, and  $K_i$  and  $K_a$  are the affinity constants of inhibitor and ligand, respectively.

Equilibrium binding and competitive inhibition isotherms were also determined in 96-well microtiter plates that had been coated with p32/Fc or variola p35/Fc, captured through goat anti-human Fc polyclonal antibody. Plates were incubated with 5  $\mu$ g/ml goat anti-human Fc in PBS for 4 hr at 4°, washed twice with PBS, and then incubated with 0.1  $\mu$ g/ml Fc chimera in PBS/0.01% Tween 20 for 12 hr at 4° and washed again 2× with PBS. Equilibrium binding isotherms used serial dilutions of  $^{125}$ I-muMCP1 in binding medium, and inhibition assays used

a constant 0.5 nM  $^{125}\text{I}$ -muMCP1 in the presence or in the absence of unlabeled potential competitive inhibitors, as described above. After 2 hr at 4°, plates were washed twice in PBS, and specifically bound ligand was released with 50 mM citrate (pH 3.0) and then gamma counted. Data were processed as described (Dower *et al.*, 1984).

Chemokine binding was also assessed by surface plasmon resonance using a BIAcore biosensor (Biacore AB). Goat anti-human IgG,  $\gamma$  chain-specific (GHFC), was covalently coupled to research grade CM5 sensor chips using a standard amine coupling procedure and reagents, according to the manufacturer's instructions. Cpv p32/Fc (30–50  $\mu\text{g/ml}$ ) was injected over the immobilized GHFC, and concentrated (3.5- to 10-fold) conditioned medium (30  $\mu\text{l}$ ) from cell lines (including TE71) was independently passed over a GHFC-coated chip (negative control) as well as a cpv p32/Fc-coated GHFC chip at a flow rate of 3  $\mu\text{l/min}$ . Similarly, purified chemokines (0.2 and 10  $\mu\text{g/ml}$ ) were passed over both surfaces at 5  $\mu\text{l/min}$ . Regeneration of the chip was accomplished with one 10- $\mu\text{l}$  pulse of 100 mM phosphoric acid at 10  $\mu\text{l/min}$ . All binding was performed in HBS (10 mM HEPES, 0.15 M NaCl, 3.4 mM EDTA, 0.02%  $\text{NaN}_3$ , 0.005% surfactant  $\text{P}_2\text{O}$ , pH 7.4).

## RESULTS

### Construction of viral fusion chimeras and biosensor screening of conditioned medium from diverse cell populations

The complete sequence of the 35K gene from cowpox virus (Brighton Red strain) was expressed both as an N-terminally octapeptide-tagged ("flag") protein and as a C-terminally fused Fc chimera. Similar constructs were made and expressed of the corresponding gene product (G5R) from variola virus (vav; Fig. 1A). The apparent molecular masses of the flag cpv and variola proteins on reducing SDS gels are 32 and 35 kDa, respectively, and those of the corresponding Fc chimeric protomers were 58 and 61 kDa. Most of the initial experiments used the cpv Fc chimera, and we refer to the cpv core protein as p32. Both chimeras were tested for surface binding against a panel of 164 cell lines by FACS analysis, without success (not shown). Since the p32 cognate might be a secreted cellular protein with no surface form, we also tested conditioned medium (CM) from these cell lines using surface plasmon resonance technology (BIAcore, Pharmacia). Figure 1B shows the specific resonance produced by a chip containing immobilized cpv p32/Fc (captured through coupled goat anti-human Fc antibody) when exposed to CM from a murine thymic epithelial line, TE71, suggesting that a specific p32 counter structure(s) is present in the CM.

### Identification and purification of CPV p32 cognate from TE71 cell supernatants

To determine if the putative p32 cognate is a product of TE71 cells, these cells were metabolically labeled with

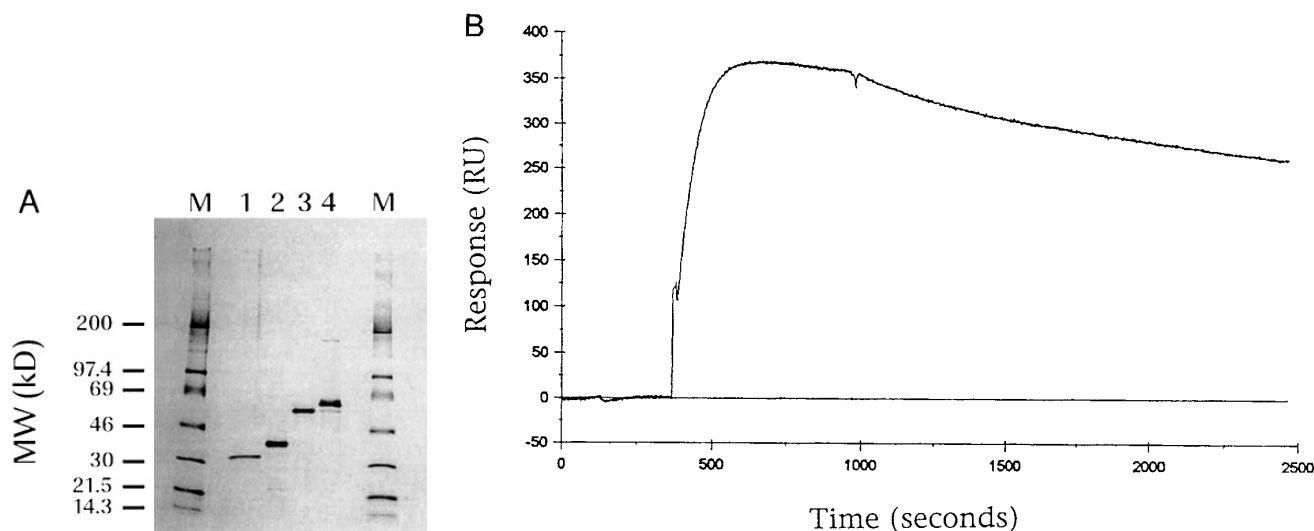
$^{35}\text{S}$  and CM immunoprecipitated with cpv p32/Fc and IgG controls (Fig. 2A). p32/Fc specifically bound two proteins with an apparent molecular mass of 30 kDa (diffuse) as well as an 8- to 10-kDa protein. These cognates were then affinity-purified from 2.1 liters of TE71 CM on a p32/Fc column (Fig. 2B). Twenty micrograms of the purified protein was obtained, and a silver-stained SDS gel showed the same two bands, one diffuse at  $\sim 30$  kDa and a smaller band at 8–10 kDa. Although N-terminally blocked, these proteins were subjected to CNBR digestion *in situ* and a partial sequence was determined (not shown). Both bands were identified as murine monocyte chemotactic protein-1 (MCP1), a chemokine of the  $\beta$  class. The bands differed in the extent of glycosylation and proteolytic processing.

### Affinity measurements of recombinant MCP1 for cpv p32

To accurately determine the affinity and specificity of MCP1 for cpv p32, we used a combination of cellular and solid-phase binding assays with recombinant murine MCP1, radiolabeled without significant loss of biological activity.  $^{125}\text{I}$ -muMCP1 showed saturation binding to cpv p32/Fc immobilized in 96-well plates, with an affinity constant ( $K_d$ ) of 0.083 nM (Fig. 3A). Unlabeled muMCP1 and flag p32, but not purified flag peptide, completely inhibited  $^{125}\text{I}$ -muMCP1 binding to immobilized cpv p32/Fc with inhibitory constants of 0.037 and 0.13 nM (Fig. 3B). To test whether p32 could inhibit MCP1 binding to MCP1 receptors, we used THP1 cells, a human macrophage line known to express MCP1 receptors (CCR2b), as a source of native receptors.  $^{125}\text{I}$ -muMCP1 showed saturation binding isotherms to THP1 cells (Fig. 3C), with 7015 high-affinity sites ( $K_d = 0.82$  nM). Binding was similarly completely and specifically inhibited by unlabeled recombinant muMCP1 ( $K_i = 1.0$  nM) as well as by both forms of cpv p32 (Fig. 3D). As the  $K_i$  of flag p32 and p32/Fc was 100 and 13 pM, respectively, cpv p32 binds (and inhibits) muMCP1 with an affinity at least 10-fold higher than that at which muMCP1 binds its own cellular receptors.

### CPV p32 binds $\beta$ chemokines promiscuously with high affinity but binds $\alpha$ and $\gamma$ chemokines with low affinity or not at all

To test cpv p32 binding to a variety of chemokines, as well as to assess potential cross-competition issues, we examined a total of 29 recombinant chemokines representing all three known classes, as well as C5a, the complement C5 anaphylatoxin cleavage product with strong chemotactic activity, in two assays: competition assays to detect inhibition of MCP1 binding, and BIAcore screening to detect direct binding. Figure 4 depicts typical inhibition assays of  $^{125}\text{I}$ -muMCP1 binding to immobilized cpv p32/Fc with unlabeled muMCP1, human eotaxin, murine MIP1 $\alpha$  ( $\beta$  chemokines), human Gro $\alpha$ , and



**FIG. 1.** Specific binding to cpv p32 detected in TE71 medium by plasmon resonance. (A) SDS gels (reducing conditions) of purified recombinant flag-cpv p32 (lane 1), flag-vav p35 (lane 2), cpv p32/Fc (lane 3), and vav p35/Fc (lane 4). Markers at left. Proteins were affinity-purified as described under Materials and Methods. (B) Cpv p32/Fc was immobilized to the BIAcore chip via a capture antibody (goat anti-human Fc) and exposed to conditioned medium from the TE71 cell line, as described under Materials and Methods. The specific response is plotted as resonance units (RU) vs time (sec) and incorporates subtraction of the nonspecific signal from CM independently passed over a chip containing only the capture antibody. The decay characteristic of the response represents slow dissociation of the CM cognate protein from cpv p32, and the leading shoulder represents its association.

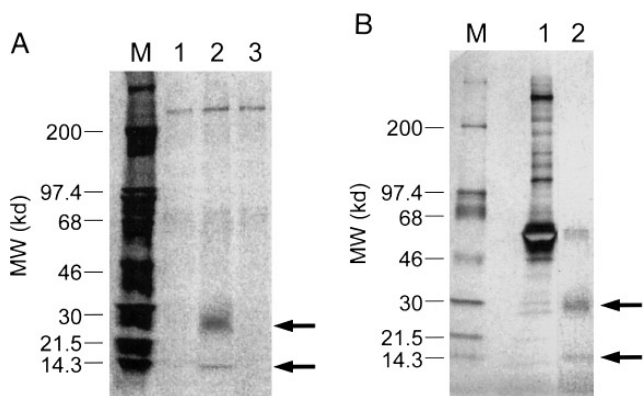
interleukin 8 (both  $\alpha$  chemokines). MCP1, human eotaxin and MIP1 $\alpha$  bound with high affinity [ $K_d$  (muMCP1) = 37 pM;  $K_d$  (huEotaxin) = 91 pM;  $K_d$  (muMIP1 $\alpha$ ) = 83 pM] and showed cross-competition for binding to p32, suggesting a common binding site. Gro $\alpha$ , however, produced no detectable inhibition even at a 5000-fold molar excess

over  $^{125}$ I-muMCP1 (see also Fig. 6B for variola vCCI), and IL8 showed only very-low-affinity binding ( $K_d$  = 0.05  $\mu$ M). Complete cell and plate binding data are summarized in Table 1, along with results from the Biosensor screen.

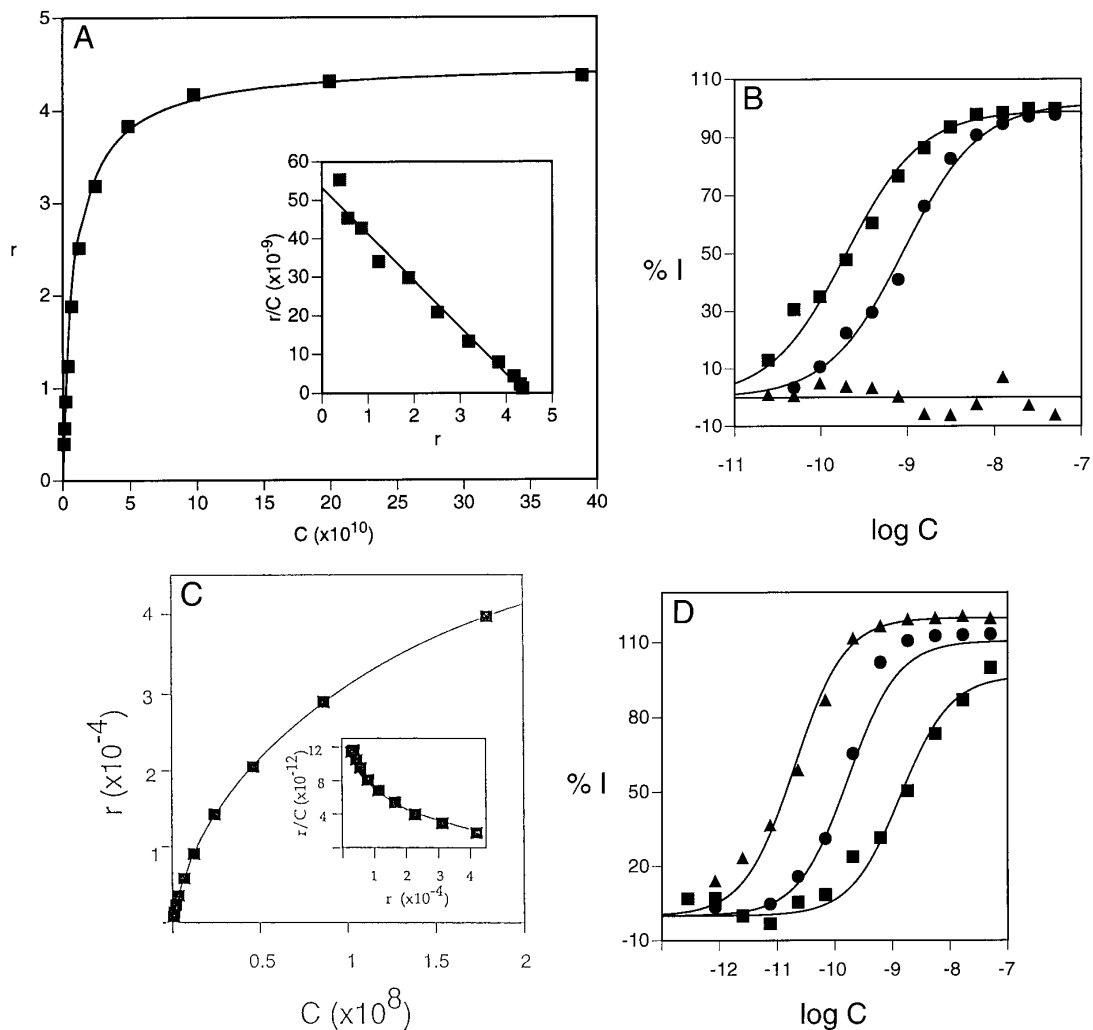
Virtually all 16  $\beta$  chemokines bound p32, clearly detected by induced resonance signals on the BIAcore, and completely inhibited  $^{125}$ I-muMCP1 binding to immobilized p32 in plates. None of the 13 members of the  $\alpha$  or  $\gamma$  classes (or C5a) showed detectable direct binding activity in the Biosensor. These included the following: human platelet factor 4 (PF-4), hULP-10, huGRO $\alpha$ ,  $\beta$  and  $\gamma$ , rat GRO $\alpha$ , huIL-8, huSDF1- $\beta$ , huNAP-2, huMIG, muMIP2, muKC, huENA-78, huIL-16 (cytokine with chemoattractant properties); human lymphotactin ( $\gamma$  chemokine); huC5a (complement), and human midkine, a 15-kDa cytokine that induces neurite outgrowth and displays (chemokine-like) heparin-binding characteristics. The more sensitive plate inhibition assay, however, detected low-affinity IL8 binding ( $K_d$  = 0.05  $\mu$ M), no binding for 11 other  $\alpha$  chemokines, and only limited inhibition with huMIG and lymphotactin even at 5000-fold molar excess, precluding an estimate for their affinities.

### CPV p32 specifically inhibits MCP1-mediated chemotaxis and calcium flux in a dose-dependent manner

To examine the ability of cpv p32 to inhibit chemokine-induced biological responses, two assays were used. First, the classic chemokine-mediated monocyte chemotaxis assay was exploited using a mixed population of PBMC and recombinant muMCP1 (with or without varying concentrations of p32/Fc or IgG1 control) in opposite



**FIG. 2.** Metabolic labeling of TE71 cell line and purification of cpv p32 secreted cognate. (A) TE71 cells were  $^{35}$ S-labeled for 30 min at 37 $^{\circ}$ , supernatants were immunoprecipitated with cpv p32/Fc or control reagents, and immunoprecipitates were resolved on an 8–18% SDS gel, as described under Materials and Methods. Lane 1 (control), huIgG1; lane 2, cpv p32/Fc; lane 3 (control), huTNFR1/Fc. Arrows mark the position of diffuse 30-kDa and sharp 8- to 10-kDa proteins specifically immunoprecipitated by cpv p32/Fc. Markers appear at left. (B) Silver-stained SDS gel of purified proteins from TE71 CM. 20  $\mu$ g of p32 cognate protein was affinity-purified from 2.1 liters of CM, as described under Materials and Methods. Lane 1, unfractionated CM from TE71 showing prominent albumin band at 60 kDa; lane 2, proteins affinity-purified with cpv p32/Fc showing diffuse 30-kDa and sharp 8- to 10-kDa bands.



**FIG. 3.** Equilibrium binding isotherms of recombinant cpv p32 and  $^{125}\text{I}$ -muMCP1. (A) Saturation binding of  $^{125}\text{I}$ -muMCP1 to immobilized p32/Fc in plates. Inset shows data replotted in Scatchard format.  $r$ , molecules bound (times  $6 \times 10^8$ );  $C$ , free molar concentration of  $^{125}\text{I}$ -muMCP1.  $K_d = 83$  pM. (B) Inhibition assays using plate-immobilized p32/Fc,  $^{125}\text{I}$ -muMCP1 (0.5 nM) in solution, and flag-p32 (●), muMCP1 (■), or flag peptide (▲) as competitive inhibitors. I, inhibition;  $C$ , molar concentration of inhibitor. Cpv p32 shows complete inhibition while flag peptide control shows none. (C) Saturation binding of  $^{125}\text{I}$ -muMCP1 to THP1 macrophage cells. Inset shows data replotted in Scatchard format.  $r$ , molecules bound/cell;  $C$ , free molar concentration of  $^{125}\text{I}$ -muMCP1. (D) Inhibition assays using THP1 cells,  $^{125}\text{I}$ -muMCP1 (0.5 nM), and unlabeled muMCP1 (■), flag-p32 (●), or cpv p32/Fc (▲) as inhibitors. The amount of  $^{125}\text{I}$ -muMCP1 chosen filled only the high-affinity class of sites.  $r$ , molecules bound/cell;  $C$ , free molar concentration of inhibitor. See Materials and Methods for details.

wells of a modified Boyden chamber (Martinet *et al.*, 1994). After incubation for 1 hr at  $37^\circ$ , cells that migrated to the lower well were counted, and results were plotted as "number of cells migrated" vs inhibitor concentration (Fig. 5A). p32/Fc, but not IgG1, clearly inhibited monocyte chemotaxis in a dose-dependent manner, with the molar equivalence of p32 to MCP1 producing complete inhibition. The IgG1 control measures the number of monocytes present in the absence of MCP1 (background). Conventional checkerboard analysis clearly demonstrated that monocyte migration was chemotactic (not chemokinetic or random), since migration occurred in the presence of a concentration gradient of MCP1 toward the point of highest concentration (not shown).

Second, the ability of p32 to inhibit muMCP1-induced  $\text{Ca}^{2+}$  mobilization in the huTHP1 macrophage line was

assessed by confocal fluorescence scanning microscopy. MCP1 specifically induced a dose- and time-dependent fluorescence burst in THP1 cells loaded with fluo-3 reporter, indicating  $\text{Ca}^{2+}$  flux, and 100 ng/ml concentrations of MCP1 could be completely inhibited by a molar equivalence cpv p32 of either construct (Fig. 5B). Thus, CPV p32 is an efficient and specific inhibitor of chemokine-induced biological responses.

#### Variola p35 also binds $\beta$ chemokines specifically

The variola virus equivalent of cpv p32 is a 253-amino-acid open reading frame, termed G5R in the India 1967 strain, and is 85% identical in sequence, including conserved leader segments, eight cysteines, and the single potential N-linked glycosylation site. It is localized near

the right genome terminus. A recombinant G5R/Fc construct was synthesized, expressed in mammalian cells, purified, and tested for the ability to specifically bind muMCP1 in a plate binding assay (Fig. 1A). Figure 6A shows that G5R/Fc binds <sup>125</sup>I-muMCP1 with saturation isotherms and high affinity ( $K_d = 1.0$  nM). The inhibition assay in Fig. 6B, involving immobilized G5R/Fc and <sup>125</sup>I-muMCP1, documents that both unlabeled muMCP1 and human eotaxin ( $\beta$  chemokines) cross-compete, as was shown for cpv p32/Fc (Table 1), and with similar affinities [ $K_i$  (MCP1) = 1.0 nM;  $K_i$  (eotaxin) = 1.25 nM].

DISCUSSION

We have demonstrated the p32 protein from cpv, whose equivalent in vaccinia is the 35-kDa virulence protein (Patel *et al.*, 1990), is a virtually universal inhibitor of chemokines of the  $\beta$  (but not  $\alpha$  or  $\gamma$ ) class. The variola equivalent (G5R), 85% identical in sequence, appears to have the same properties, and we have therefore termed the gene product vCCI (viral C-C chemokine inhibitor) to reflect this crucial and general characteristic. vCCI specifically binds  $\beta$  chemokines with high affinity and completely inhibits MCP1 biological activity and binding to a known cellular receptor for MCP1 and MCP3 (Charo *et al.*, 1994; Franci *et al.*, 1995) Further, since we show all that 35K-binding chemokines also competitively inhibit MCP1 binding, they presumably share a common binding site on the viral protein, making it likely that vCCI also generally inhibits chemokine binding to cellular receptors, and hence biological activity. The most accurate measure of vCCI affinity for muMCP1 ( $K_d = 13\text{--}100$  pM), based on <sup>125</sup>I-muMCP1 inhibition against cellular receptors, is at least 10-fold higher than the affinity that MCP1 displays for its own native receptors, providing evidence

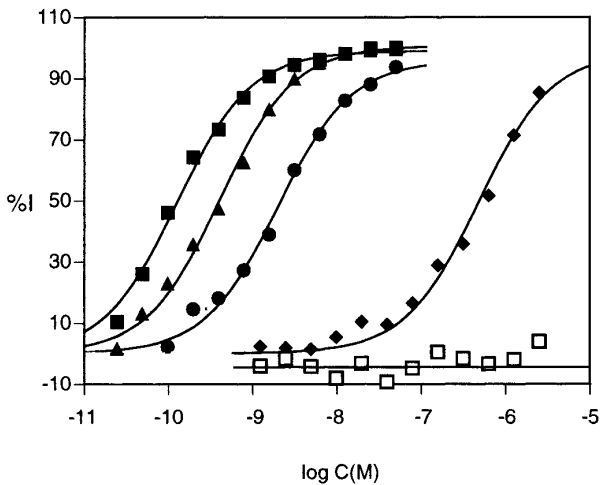


FIG. 4. Representative inhibition assays with immobilized cpv p32/Fc reflecting chemokine class-specific binding. Inhibition assays used immobilized cpv p32/Fc and <sup>125</sup>I-muMCP1 (0.5 nM) in solution with and without unlabeled muMCP1 (■), human eotaxin (▲), or muMIP1 $\alpha$  (●) ( $\beta$  chemokines), huGro $\alpha$  (□), or IL-8 (◆) (both  $\alpha$  chemokines). I, inhibition; C, free molar concentration of inhibitor.

TABLE 1		
Summary of Promiscuous $\beta$ Chemokine Binding to vCCI		
Cell-based assay		
Inhibitor	$K_i$ (nM)	BIACore binding
mu MCP-1	1.000	
CPVp32/Fc	0.013	
flag p32	0.100	
Plate assay		
Mouse		
MCP-1	0.037	+
MCP-5	0.053	+
MIP-1 $\alpha$	0.083	+
MIP-1 $\beta$	0.060	+
C10	0.441	+
Eotaxin	0.347	+
Human		
MCP-1	2.381	+
MCP-2	0.526	—
MCP-3	0.157	+
MCP-4	0.126	+
Rantes	0.096	+
Eotaxin	0.091	+
MIP-1 $\alpha$	0.080	+
Rat		
MCP-1	0.023	+
MIP-1 $\alpha$	0.005	+
Rantes	n.d.	+

*Note.* The top part of the table shows the results for the THP1 cell-based inhibition assay with <sup>125</sup>I-muMCP1. See also Figs. 3 and 4 for details. The bottom part of the table shows results for solid-phase inhibition (plate) or direct binding (BIACore) assays with immobilized cpv p32/Fc. A plus sign denotes a clear resonance signal on BIACore. I, inhibition;  $K_i$ , inhibition constant, n.d., not determined. A total of 13  $\alpha$  or  $\gamma$  chemokines, C5a, IL16, and huMidkine were also tested and showed no binding in the BIACore and poor or no binding in plate assays (see text for list and Methods and Materials for details.)

that vCCI is a potent general inhibitor of  $\beta$  chemokine activity. Rat MIP1 $\alpha$ , for example, shows an even higher affinity ( $K_d = 5$  pM; Table 1). The proposed primary viral function of vCCI is competitive inhibition of an entire class of proinflammatory host ( $\beta$ ) chemokines elicited by viral infection itself. The selective advantage is increased virulence arising from, in effect, partial immunosuppression of the host. Evidence that  $\beta$  chemokines serve a proinflammatory role in normal immune responses is strong. They are induced early and are chemoattractive to mononuclear and antigen-presenting dendritic cells, lymphocytes, and eosinophils, but they also mediate immunoenhancing effects by acting as costimulants of lymphocyte cytotoxicity, proliferation, and cytokine production (Baggiolini *et al.*, 1994; Murphy, 1996). Blocking antibodies to MIP1 $\alpha$ , for example, reduces the severity of experimental autoimmune encephalomyelitis in mice (Karpus *et al.*, 1995). Direct evidence for antiviral effects of  $\beta$  chemokines comes from experiments with MIP1 $\alpha$  knock-out mice challenged with either

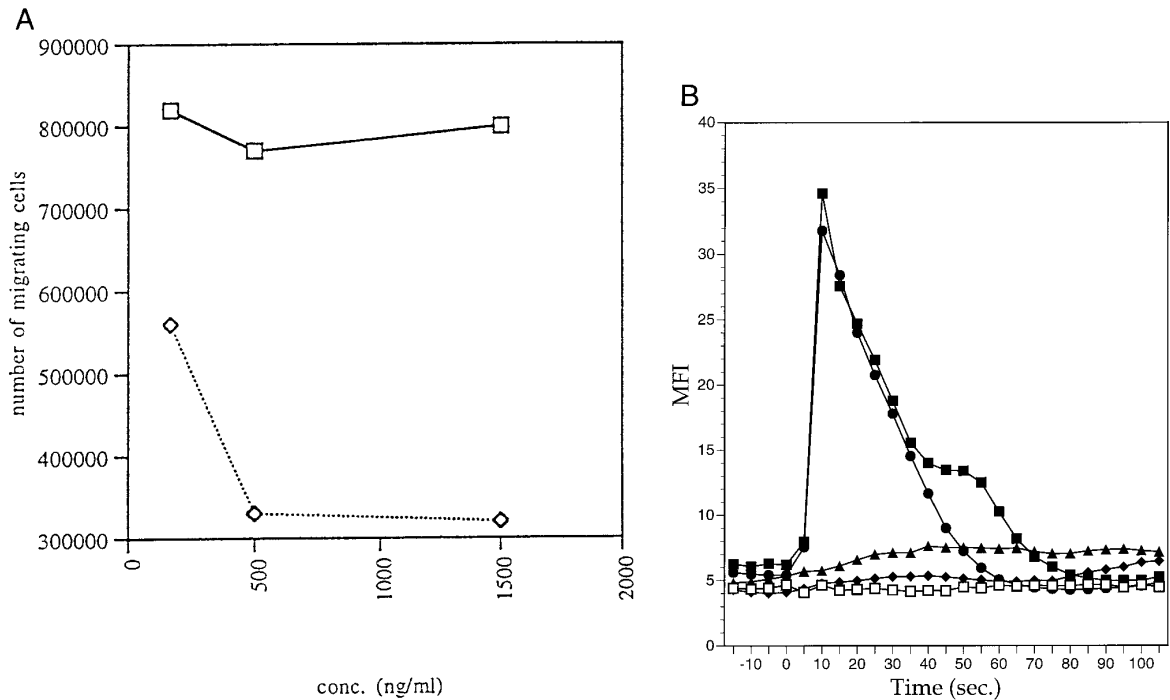


FIG. 5. Inhibition of MCP1-induced cellular responses by cpv p32. (A) HuMCP1-induced macrophage chemotaxis was measured in modified Boyden chambers using unfractionated human PBMC. Upper chambers contained  $5 \times 10^6$  cells, and lower chambers contained 50 ng/ml of recombinant huMCP1 with or without varying concentrations of cpv p32/Fc ( $\diamond$ ) or hulgG1 control ( $\square$ ). The p32 protein completely inhibited all migration at molar equivalence. Migrations were determined to be macrophage-specific and occurred only in the direction of increasing MCP1 concentrations. See Materials and Methods for details. (B) HuMCP1-induced  $\text{Ca}^{2+}$  mobilization in huTHP1 macrophage cells was measured by fluorescence in fluo-3 (reporter)-loaded cells, quantified as mean fluorescence intensity (MFI) using confocal fluorescence microscopy. See Materials and Methods for details. The MFI from 100 ng/ml of MCP1 ( $\blacksquare$ ) was inhibited in a dose-dependent manner by cpv p32/Fc ( $\bullet$ , 100 ng/ml;  $\blacktriangle$ , 1  $\mu\text{g}/\text{ml}$ ;  $\blacklozenge$ , 10  $\mu\text{g}/\text{ml}$ ), while p32/Fc alone induced no detectable change in fluorescence even at 10  $\mu\text{g}/\text{ml}$  ( $\square$ ).

coxsackie or influenza virus: post-coxsackie-induced myocarditis and resistance to influenza infection are both severely reduced (Cook *et al.*, 1995). Conversely, the simple existence of the vCCI gene suggests that other  $\beta$  chemokines may also have antiviral activity. In contrast,

a rationale for the general failure of vCCI to effectively inhibit members of the other two known classes of chemokines ( $\alpha$  and  $\gamma$ ) is less clear. Classically,  $\beta$  chemokines are strongly chemoattractive for monocytes/macrophages and eosinophils and activate T cells, but do not

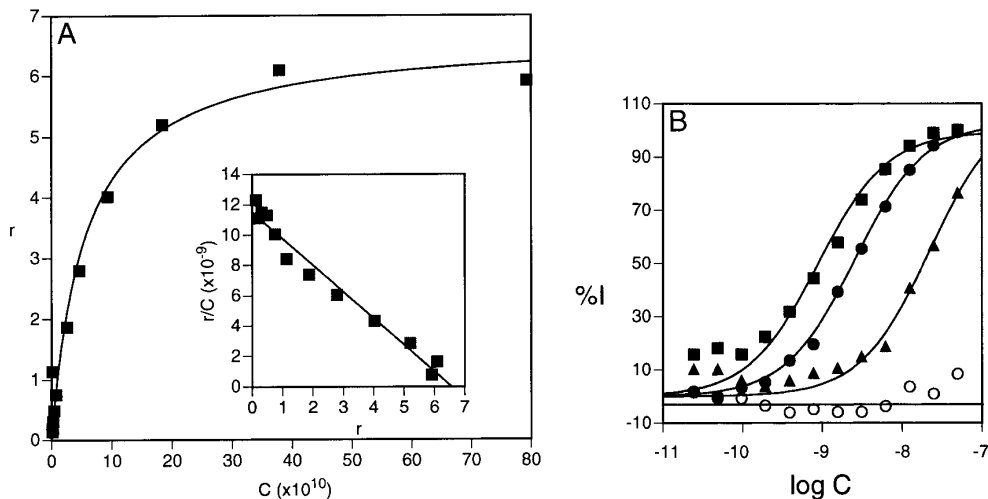


FIG. 6. Variola p35/Fc also binds  $\beta$  chemokines. (A) Equilibrium binding isotherms with plate-immobilized variola virus p35/Fc and  $^{125}\text{I}$ -muMCP1. Inset shows data replotted in Scatchard format.  $r$ , molecules bound;  $C$ , free molar concentration of  $^{125}\text{I}$ -muMCP1. (B) Inhibition of  $^{125}\text{I}$ -muMCP1 (0.5 nM) binding to immobilized variola p35/Fc with unlabeled muMCP1 ( $\blacksquare$ ), human eotaxin ( $\bullet$ ), or mouse eotaxin ( $\blacktriangle$ ) ( $\beta$  chemokines), or huGro $\alpha$  ( $\circ$ ). I, inhibition;  $C$ , molar concentration of inhibitor. See Table 1 and Materials and Methods.



affect neutrophils, while the reverse is true for  $\alpha$  chemokines. Thus, potent viral inhibition of infiltration involving multiple populations is no doubt one selective advantage conferred by vCCI. However, proinflammatory characteristics have been noted for the  $\alpha$  class as well. Chemokines share 25–80% identity in sequence and differ conspicuously in the spacing of the first two of four invariant cysteines ( $\beta$ , C-C;  $\alpha$ , C-x-C). Although formally possible, it seems unlikely that mutations which eliminate binding to those classes would be neutral. More likely is that an unknown selective advantage is accrued, the nature of which awaits a more complete understanding of chemokine action.

The genomic location of vCCI in poxviruses is also consistent with a virulence function. Poxviruses contain large duplex DNA genomes (160–340 kb), covalently closed hairpin termini, and ~10-kb inverted terminal repeats (ITRs) in or near which most virulence genes tend to cluster. In orthopoxviruses, such as cowpox, vaccinia, or rabbit pox, and leporipoxviruses, such as Shope fibroma (Sfv), vCCI is also ITR-localized (diploid), terminus-proximal, extremely conserved in sequence, and expressed at both early and late times, consistent with the proposed virulence function. Although variola lacks protein-encoding ITRs, the analogous gene in both sequenced strains is the right terminus-proximal ORF G5R (India, 1967) and G3R (Bangladesh). These two ORFs purportedly differ by a single amino acid (Massung *et al.*, 1993; Shchelkunov *et al.*, 1993), and are 85% identical to vCCI from the orthopoxviruses cpv and vaccinia (vv). Widely cultured laboratory strains of vv (e.g., WR) have a disrupted vCCI gene and often disruptions of other virulence genes as well, such as T2 (Smith and Goodwin, 1994) and CrmC (both soluble TNF receptors), consistent with their roles independent of viral replication (Smith and Goodwin, 1994). Thus, the clear prediction is that vCCI will be found in most poxviruses, including monkey pox, as yet unsequenced.

While our paper was being submitted, related work by Graham *et al.* (1997) appeared, describing characteristics of presumably the same gene product described here. Our data, however, differ markedly from their results in several respects. First, Graham *et al.* report that this gene product, or a similar gene product, from cpv, vaccinia, and myxoma binds chemokines with broad specificity representing two classes ( $\alpha$ ,  $\beta$ ), although data from only a single member of each class were actually presented (IL8,  $\alpha$  class; RANTES,  $\beta$  class). The authors chiefly relied on chemical cross-linking of  $^{125}\text{I}$ -hu IL8 or  $^{125}\text{I}$ -RANTES to proteins in crude supernatants from poxvirus-infected cells. In contrast, we used purified, well-characterized recombinant vCCI protein expressed in two different forms from each of two different poxviruses, and four different binding assays involving 30 purified recombinant chemokines covering three species (mouse, human, and rat). We found that all 16 recombinant  $\beta$  chemokines tested in multiple assays bound our

recombinant vCCI protein (both cowpox virus and variola) with high affinity, while none of the 13  $\alpha$  chemokines showed detectable binding in the BIAcore system. A low-affinity interaction could, however, be detected in the more sensitive plate binding inhibition assay for IL8 ( $K_d = 50$  nM; Fig. 4) and unmeasurably lower affinities for human MIG and lymphotactin ( $\gamma$  chemokine). All chemokines were previously shown to be biologically active with *in vitro* assays by the vendor. Unlike Graham *et al.* (1997), we conclude that vCCI chemokine binding is preferentially  $\beta$ -specific and the physiologically important class from the viral perspective. Second, we directly demonstrate, by two different assays, that vCCI is a potent competitive inhibitor of MCP1 binding to cellular chemokine receptors and biological activity (chemotaxis and induced calcium flux). As stated above, since we show that all 35K binding chemokines also competitively inhibit MCP1 binding to the viral protein, it seems likely that vCCI also generally inhibits chemokine binding to cellular receptors, and hence biological activity. Graham *et al.* infer this indirectly from the changes in tissue pathology that they report, using a recombinant rabbitpox virus inactivated at the 35K virulence gene locus. However, some of these authors have previously published similar work which reached the opposite conclusion (Martinez-Pomares *et al.*, 1995). Martinez-Pomares and colleagues using the same poxvirus (rabbitpox) inactivated at the same locus (35K) infecting the same host (European rabbit) reported neither gross nor microscopic histopathological host differences between the knockout and the wild-type virus infections. These authors concluded this protein does *not* play a major role in the virulence of virus. Thus, the histopathology data are ambiguous. Third, the vCCI affinity estimate by Graham *et al.* for a  $\beta$  chemokine ( $K_d = 73$  nM) is almost 1000-fold lower than that which we measure ( $K_d = 0.1$  nM) and 50-fold less than published affinities between chemokines and cellular receptors. The large discrepancy in  $\beta$  chemokine affinity is likely accounted for by the quality and sensitivity of the assays: cell-based inhibition assays and plate binding assays using a capture antibody (our data) or plate binding assays lacking a capture antibody (Graham *et al.*, 1997).

Perhaps the most intriguing feature of vCCI is its complete lack of sequence homology with any known chemokine receptor, all of which are G-coupled, seven-membrane-spanning proteins with strong sequence homology (24–80%) among themselves (Charo *et al.*, 1994; Franci *et al.*, 1995; Gerard and Gerard, 1994; Neote *et al.*, 1993; Probst *et al.*, 1992). This contrasts sharply with many other virulence proteins in poxviruses, including soluble, secreted receptors for TNF (T2 and CrmC, encoded at distinct loci), interferons  $\gamma$  and  $\alpha$ , and IL-1. These clearly represent acquired forms of the cellular receptor genes, subverted to viral function as competitive inhibitors, and they show marked sequence homology to their analogs. No such ancestral relation seems possible with vCCI.

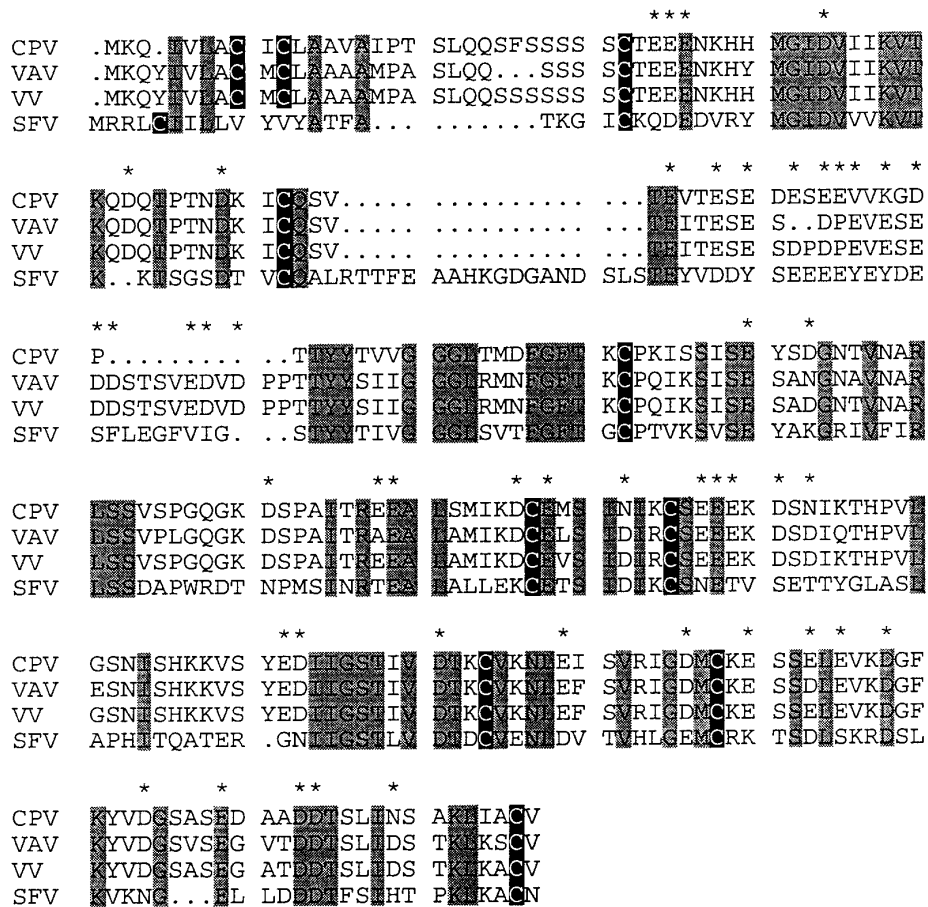


FIG. 7. Sequence alignment of vCCI from cowpox, variola, vaccinia, and Shope fibroma viruses. Cysteines are enclosed in black boxes; residues that are conserved in all four viruses are shaded gray. Asterisks indicate the positions of acidic residues in at least two family members.

Although at least one virulence gene in cytomegalovirus (CMV) encodes a protein (US28) known to bind MIP1 $\alpha$ ,  $\beta$  and RANTES ( $\beta$  chemokines), US28 is structurally a classic chemokine receptor showing dramatic sequence homology and multiple membrane-spanning elements (Neote *et al.*, 1993). Indeed, extensive mutagenesis and deletion studies of known chemokine receptors (Hebert *et al.*, 1993; Horuk, 1994; Lu *et al.*, 1995) imply a ligand binding site composed of discontinuous extracellular loops adopting a membrane-stabilized conformation, suggesting that construction of an effective soluble form is impossible. It seems more likely, therefore, that vCCI may be the product of a kind of "phage display" system in poxviruses, where relentless shuffling of sequences, under inexorable selective pressure, gave rise to a completely new protein with similar binding activity to native chemokine receptors. Alternatively, a formal possibility remains that vCCI represents a new class of cellular chemokine receptors as yet unidentified.

The structural basis for  $\beta$  chemokine binding to vCCI is unclear. The virulence factor shows no sequence homology to any protein in public databases, and at any rate even the nature of chemokine-cellular receptor interactions remains obscure. The strongly conserved fea-

tures of mature vCCI provide modest clues: eight cysteines (presumably four disulfides) and several three- to five-residue motifs are absolutely conserved, one potential N-linked glycosylation site, and a strongly acidic nature, represented as acidic patches throughout the molecule (Fig. 7). Many  $\beta$  chemokines, including MCP1, are quite basic, suggesting that salt links between ligand and vCCI are important. Some acidic clusters are also seen at the (extracellular) N-terminus of many (but not all) chemokine receptors, although mutagenesis studies suggest that they are more relevant to specificity than affinity. The mature leporipoxvirus Sfv vCCI shows an 18-amino-acid apparent insertion element not found in the orthopoxviruses cpv, vav, and vv, but the peptide is only slightly acidic and contains no extra cysteines. Since Sfv vCCI does, however, share only 42% identity with the orthopoxvirus members (85% identical among themselves), it is conceivable that its chemokine binding specificity may vary from cpv or variola. Nonetheless, such divergence is similar to the sequence divergence seen between other virulence factors of different pox species which nevertheless retain almost identical functions and cognate specificities (Hu *et al.*, 1994; Smith and Goodwin, 1994; Spriggs *et al.*, 1992).

The vCCI protein not only adds a new perspective to the genesis of viral immunomodulators, it also proves the existence of soluble, broad spectrum chemokine inhibitors, something previously thought impossible. Crystallographic studies are now possible on chemokine/vCCI complexes and may be relevant to chemokine/cellular receptor interactions. It is interesting to note the recent discovery of a 20-amino-acid peptide erythropoietin mimetic, which, while showing no sequence homology to native erythropoietin, nevertheless displays similar biological activities and binds soluble erythropoietin receptor with contacts reminiscent of the native ligand (Livnah *et al.*, 1996; Wrighton *et al.*, 1996). Finally, it is clearly possible that vCCI, or its derivatives, may prove therapeutically useful in human disease.

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