Glycoprotein G isoforms from some alpha-herpesviruses function as broad-spectrum chemokine binding proteins

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Mimicry of host chemokines and chemokine receptors to modulate chemokine activity is a strategy encoded by beta- and gamma-herpesviruses, but very limited information is available on the anti-chemokine strategies encoded by alpha-herpesviruses. The secretion of chemokine binding proteins (vCKBP’s) has hitherto been considered a unique strategy encoded by poxviruses and gammaherpesviruses. We describe a family of novel vCKBP’s in equine herpesvirus 1, bovine herpesvirus 1 and 5, and related alpha-herpesviruses with no sequence similarity to chemokine receptors or other vCKBP’s. We show that glycoprotein G (gG) is secreted from infected cells, binds a broad range of chemokines with high affinity and blocks chemokine activity by preventing their interaction with specific receptors. Moreover, gG also blocks chemokine binding to glycosaminoglycans, an interaction required for the correct presentation and function of chemokines in vivo. In contrast to other vCKBP’s, gG may also be membrane anchored and, consistently, we show chemokine binding activity at the surface of cells expressing full-length protein. These alpha-herpesvirus vCKBP’s represent a novel family of proteins that bind chemokines both at the membrane and in solution.

Keywords: chemokine/glycosaminoglycan/herpesvirus/immune evasion/inflammation

Introduction

Chemokines are chemoattractant cytokines that regulate trafficking and effector functions of leukocytes, and play an important role in host defence against invading microbes and in the pathogenesis of inflammatory diseases (Baggiolini, 1998). Chemokines are basic 8–10 kDa proteins classified into four subfamilies: C, CC, CXC and CX3C. Seven-transmembrane-domain chemokine receptors are expressed in different cell subsets, thus determining the leukocyte subtype that predominates in different types of inflammation. The interaction of chemokines with glycosaminoglycans (GAGs) is required for endothelial transcytosis and correct presentation of chemokines to leukocytes and function in vivo (Middleton et al., 1997; Cinamon et al., 2001).

Viruses interact with crucial immune molecules to modulate their activity (Alcamí and Koszinowski, 2000; Tortorella et al., 2000). The critical role of chemokines in antiviral defence has been highlighted by the discovery that poxviruses and herpesviruses encode proteins that mimic chemokines or chemokine receptors and secreted chemokine binding proteins (vCKBP) (Lalani et al., 2000; Murphy, 2001). Myxoma virus (MV) MT-7 or vCKBP-1 is an interferon-γ (IFN-γ) receptor that was later shown to interact with a broad range of C, CC and CXC chemokines (Lalani et al., 1997). MT-7 binds CXCL8 (interleukin 8) (Zlotnik and Yoshie, 2000) through the C-terminal heparin binding domain and has been proposed to prevent chemokine–GAG interactions. The 35 kDa/M-T1 protein in vaccinia virus (VV), MV, cowpox virus and variola virus, designated vCKBP-2, binds CC chemokines with high affinity and inhibits their interaction with chemokine receptors and activity (Graham et al., 1997; Smith et al., 1997; Alcamí et al., 1998). VCKBP-3 is encoded by the murine gammaherpesvirus 68 (MHV-68) M3 protein and has broad binding specificity for C, CC, CXC and CX3C chemokines (Parry et al., 2000; van Berkel et al., 2000). Similar to vCKBP-2, M3 inhibits chemokine binding to cellular receptors and activation of biological effects. Neither M3, identified as the first and only vCKBP from a herpesvirus, nor the poxvirus-encoded vCKBP-2 show sequence similarity to human or mouse proteins.

Genes encoding homologues of chemokines or chemokine receptors have been identified in beta- and gamma-herpesviruses (Lalani et al., 2000; Murphy, 2001). In contrast, none of these genes has been found in alphaherpesviruses, with the exception of a biologically active CXCL8 homologue encoded by Marek’s disease virus (MDV), which infects domestic chickens (Parcell et al., 2001).

Members of the alphaherpesvirus subfamily have variable host range and the capacity to establish latent infections primarily, but not exclusively, in sensory ganglia (Roizman and Pellet, 2001). Alphaherpesviruses include human pathogens such as herpes simplex virus 1 (HSV-1) and HSV-2, which replicate in oral or genital mucosal tissue, and varicella–zoster virus (VZV), the causative agent of chickenpox and herpes zoster. The alphaherpesviruses equine herpesviruses 1, 3 and 4 (EHV-1, EHV-3 and EHV-4) are important pathogens of domestic horses that cause immunosuppression, rhinitis, bronchiolitis, abortions and neurological disorders. Bovine herpesvirus 1 (BHV-1) is a major pathogen of cattle associated with abortions and with respiratory and genital infections, and is related to alphaherpesviruses that cause disorders in ruminants: BHV-5, caprine herpesvirus 1
(CaHV-1), cervine herpesvirus 1 (CeHV-1) and rangiferine herpesvirus 1 (RanHV-1).

Several herpesvirus glycoproteins are present in the virion envelope and play a role in virus morphogenesis, cell attachment and tropism. Glycoprotein G (gG) encoded by alphaherpesviruses is found in the virus envelope and, in some viruses, is also secreted after proteolytic processing, but its function remains largely unknown. Alphaherpesvirus gG was first characterized in HSV-2 as a secreted 92 kDa glycoprotein (Marsden et al., 1984; Su et al., 1987). Homologues of gG were found in HSV-1 (Richman et al., 1986), EHV-1 (Drummer et al., 1998), EHV-3 (Hartley et al., 1999), EHV-4 (Crabb et al., 1992; Drummer et al., 1998), BHV-1 (Keil et al., 1996) and BHV-5 (Engelhardt and Keil, 1996), but the gG gene is absent in VZV (Gomi et al., 2002).

We report here that gG homologues encoded by EHV-1, EHV-3, BHV-1, BHV-5, RanHV-1, CapHV-1 and CerHV-1 have chemokine binding activity. Recombinant gG from EHV-1, BHV-1 and BHV-5 binds a broad range of chemokines with high affinity and inhibits their biological activity in vitro. We demonstrate that gG blocks the interaction of chemokines with both cellular receptors and GAGs, and show that the gG chemokine binding activity may also be expressed at the cell surface.

**Results**

**Identification of soluble chemokine binding activity encoded by alphaherpesviruses**

The secretion of vCKBPs from cells infected with various herpesviruses was tested by chemokine cross-linking assays with ethylene glycol-bis-succinimidy1 succinate (EGS) (Figure 1A). Complexes of radiolabelled human CCL3 [macrophage inflammatory protein 1, (MIP-1α)] with a soluble vCKBP were observed for all the alphaherpesviruses tested. The gammaherpesvirus BHV-4 did not express chemokine binding activity. Binding specificity was demonstrated with the positive controls of MHV-68 (Parry et al., 2000) and VV strain Lister (Alcamí et al., 1998), and the absence of a signal with mock-infected cells and a VV Lister lacking the 35 kDa vCKBP-2 (VV Lister Δ35K) (Alcamí et al., 1998). The size of the ligand–vCKBP complex (54–80 kDa) suggested a vCKBP size of 46–72 kDa after subtraction of the 8 kDa monomeric CCL3. BHV-1, BHV-5, RanHV-1 and CerHV-1 also showed ligand binding to a
Expression of vCKBP by EHV-1

The chemokine binding activity was conserved throughout a collection of EHV-1 field isolates, as well as the virulent laboratory strains AB4 and Army 183 (Turintien and Allen, 1982; Smith et al., 1992) and the apathogenic vaccine strain RacH (Osterrieder et al., 1994) (Figure 1E). CXCL8 binding activity was detected in lysates of EHV-1-infected cells by cross-linking (Figure 1E). Supernatants and cell extracts from infections performed in the presence of tunicamycin lacked chemokine binding activity, suggesting that the vCKBP is N-glycosylated and that the lack of carbohydrate resulted in aberrant expression of the vCKBP or loss of activity (Figure 1E).

Chemokine binding specificity of naturally produced EHV-1 and BHV-1 vCKBP

The chemokine binding specificity was also investigated in cross-linking assays in the presence of excess unlabelled chemokines. Figure 2A shows that the CXC chemokines of human or mouse origin and human XCL1 (lymphotactin) bound the EHV-1 strain AB4 vCKBP and competitively inhibited binding to [125I]CXCL8. Some inhibition was observed with human CCL3 and CCL5, but not with CCL2 or C3XCL1.

A different binding pattern was observed with BHV-1 supernatants (Figure 2B). Addition of excess CXCL8 did not inhibit binding to [125I]CCL3, whereas a weak [125I]CXCL8→vCKBP complex was observed by direct cross-linking (Figure 1A), suggesting a much higher affinity for CCL3. Different degrees of inhibition, and hence of affinity, were observed with various CXC and CC chemokines. XCL1 partially inhibited CCL3 binding but no inhibition was observed with CX3CL1. The addition of unlabelled CCL3 resulted in the formation of multimers.

Identification of the secreted forms of gG as the vCKBP

Open reading frames (ORFs) from the EHV-1 strain AB4 genome sequence (Telford et al., 1992) were selected based on the presence of a predicted signal peptide and/or a size consistent with that observed by cross-linking. EHV-1 ORF8, ORF26, ORF59, ORF60 and ORF70, which have unknown function, were expressed as full-length polypeptides in the baculovirus system. Figure 3A shows that a [125I]CXCL8→vCKBP complex of 50–55 kDa was detected in supernatants from cultures expressing ORF70 (AcEHV-1gG). The smaller size compared with that observed in natural infections (Figure 1A) is probably due to incomplete glycosylation in insect cells. EHV-1 ORF70 encodes gG, which is presumably anchored through the C-terminal transmembrane domain into the virus particle envelope and is also proteolytically cleaved and released into the medium (Crabb et al., 1992; Telford et al., 1992; Drummer et al., 1998). Insect cells can cleave the membrane form of gG to produce a secreted active protein.

To determine whether gG homologues from alphaherpesviruses encode chemokine binding activity, full-length and/or truncated versions, lacking the transmembrane and cytoplasmic domains and in the case of EHV-1, BHV-1 and BHV-5 fused to a C-terminal His6 tag, were expressed in the baculovirus system. Supernatants from infected cultures were tested in cross-linking assays using [125I]-labelled
CCL19 and CXCL8 (Figure 3B). A truncated version of EHV-1 gG was secreted and bound CXCL8 and, with lower affinity, CCL19. Full-length and truncated versions of gG encoded by both BHV-1 and BHV-5 were secreted and bound both CCL19 and CXCL8, confirming that gG from these viruses encodes the vCKBP. BHV-1 gG consists of a glycosylated form (gG; 65 kDa) and a complex glycoproteoglycan of higher molecular mass (gppG; 90 to >240 kDa) which is modified by the addition of chondroitin sulfate (Keil et al., 1996), and this was consistent with chemokines cross-linking to a diffusely migrating species of proteins between 60 and >175 kDa (Figure 1A). These high molecular size forms were also observed after chemokine cross-linking with BHV-5, RanHV-1 and CerHV-1 samples, but not with CapHV-1. However, similar high molecular size forms were only weakly observed after cross-linking of recombinant forms of BHV-1 and BHV-5 gG to CXCL8, suggesting that the glycoproteoglycan form may be produced in very low amounts by insect cells. Expression of secreted versions of gG from HSV-1 (gG-1) and HSV-2 (gG-2) in the baculovirus system confirmed the absence of chemokine binding activity to these chemokines, consistent with the lack of activity in HSV-1- and HSV-2-infected cultures (not shown).

**Chemokine binding affinity and specificity of purified recombinant gG encoded by EHV-1, BHV-1 and BHV-5**

Recombinant secreted forms of gG fused to a C-terminal His$_6$ tag and expressed in the baculovirus system were purified in nickel chelate columns (gG.His), and chemokine binding activity with human [125I]CXCL1 was demonstrated by cross-linking (Figure 4A). Chemokine binding affinity was determined with human [125I]CXCL1 using a scintillation proximity assay. Nickel chelate FlashPlates (Perkin-Elmer Life Sciences), containing a layer of scintillant in the interior of each well, were coated with purified gG.His. Increasing doses of [125I]CXCL1 were added and bound chemokine was determined in a scintillation counter. The affinity constants were determined by Scatchard analysis of the saturation curves (Figure 4B and C). Affinity constants for other chemokines were determined in competition assays of [125I]CXCL1 binding in the presence of increasing doses of unlabelled chemokines (Figure 4C). In all cases, the interaction of gG with chemokines was of high affinity.

Binding of [125I]chemokines to purified gG.His in the presence of a 500-fold excess of unlabelled chemokines was determined in the FlashPlate assay. This provided a broad spectrum of binding specificity for 30 chemokines of human, mouse or viral origin (Figure 5). However, we cannot rule out the possibility that those chemokines that do not competitively inhibit the binding of [125I]chemokines may bind to a different site in gG. Purified gG.His from BHV-1 and BHV-5 bound to most human and mouse CC chemokines, and to some human and mouse CXC chemokines. In contrast, EHV-1 gG.His showed a narrower binding specificity for both human and mouse CC and CXC chemokines. EHV-1 gG.His bound to human and mouse XCL1, whereas BHV-1 gG.His bound only to human XCL1 and BHV-5 gG.His did not bind to the C chemokine. None of the recombinant proteins bound to CX3CL1. BHV-5 gG.His, and to a lesser extent BHV-1 gG.His, interacted with vMIP-2 encoded by Kaposi’s sarcoma-associated herpesvirus.

The binding specificity of recombinant gG.His in the FlashPlate assay (Figure 5) was generally consistent with that obtained by cross-linking to gG secreted from EHV-1- and BHV-1-infected cells (Figure 2). The only exception was with human CXCL8, CXCL12 and CXCL13, which inhibited [125I]CXCL1 binding to recombinant BHV-1 gG.His but did not block the cross-linking of [125I]CCL3 to BHV-1-produced gG. An apparent higher affinity of BHV-1 for CC chemokines than for CXC chemokines.

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**Fig. 3.** Identification of gG as the vCKBP encoded by alpha-herpesvirus. (A) Cross-linking with EGS of [125I]CXCL8 to supernatants from insect cell cultures infected with recombinant baculoviruses expressing the indicated ORF from EHV-1 strain AB4. The baculovirus clone number is shown in parenthesis. (B) Cross-linking with EGS of [125I]CXCL8 or [125I]CCL19 to supernatants from insect cell cultures infected with the indicated recombinant baculoviruses expressing full-length or secreted versions (S) of gG encoded by EHV-1, BHV-1, BHV-5, HSV-1 and HSV-2. Samples were analysed by SDS-PAGE and autoradiography. Molecular masses are in kilodaltons and the positions of 125I-labelled chemokine (CK) and vCKBP-chemokine complexes (asterisks) are indicated.
Inhibition of chemokine binding to cells by gG

Full-length naturally cleaved recombinant gG from EHV-1 and BHV-1 expressed in the baculovirus system inhibited the specific binding of [125I]CXCL8 or [125I]CCL3 to U937 cells in a dose-dependent manner (Figure 6). This suggested that untagged versions of gG have a high affinity for chemokines, sufficient to block the binding of chemokines to high affinity cellular receptors. Purified recombinant EHV-1 gG. His also blocked the binding of CXCL8 to U937 cells (not shown). Recombinant secreted forms of HSV-1 and HSV-2 gG did not inhibit CCL3 binding to cells, confirming the absence of activity (not shown).

Inhibition of the biological activity of chemokines by gG

Figure 7A shows that purified BHV-1 gG. His inhibited, in a dose-dependent manner, calcium mobilization induced by human CXCL1 in U937 cells. Similarly, purified BHV-5 gG. His inhibited calcium mobilization in HeLa cells expressing CCR5 and CXCR4 in response to human CCL3 (Figure 7A).

Cell migration in response to chemokine gradients in vitro, a measure of their ability to induce cell infiltration into tissues, was also inhibited by gG (Figure 7B). Neutrophil migration in response to CXCL8 or CXCL1 was specifically inhibited in a dose-dependent manner by supernatants containing recombinant EHV-1 gG or by purified BHV-1 gG. His, respectively. Similarly, purified BHV-5 gG. His specifically inhibited CCL3-induced chemotaxis in differentiated U937 cells pretreated with IFN-α.

gG can alter the heparin binding characteristics of chemokines

Figure 8B shows that pre-incubating purified gG. His from EHV-1, BHV-1 and BHV-5 with [125I]CXCL8 prior to addition to FlashPlates precoated with heparin–bovine serum albumin (BSA) greatly reduced binding of the chemokine to heparin. No effect was observed with several control proteins, some of which expressed a His tag (Figure 8A). Moreover, gG also disrupted pre-established chemokine–GAG interactions. FlashPlates coated with heparin–BSA were pre-incubated with [125I]CXCL1,
Fig. 5. Binding specificity of gG encoded by EHV-1, BHV-1 and BHV-5. Nickel chelate FlashPlates were coated with gG.His from EHV-1, BHV-1 or BHV-5. Three hundred picomolar iodinated human CXCL8 (EHV-1) or CXCL1 (BHV-1 and BHV-5) was added in the absence or presence of a 500-fold excess of unlabelled competitor CC, CXC, C or CX3C chemokines of human (h) or mouse (m) origin, or vMIP-2. Bars represent the mean percentage inhibition of binding ± SD of the triplicate sample.

and addition of EHV-1 gG.His efficiently detached \([^{125}I]CCL1\) from heparin-BSA over time (Figure 8C). In contrast, no effect was observed on the binding of \([^{125}I]CCL2\), a chemokine not recognized by EHV-1 gG.His, to heparin-BSA (Figure 8D).

**gG encodes chemokine binding activity at the cell surface**

To determine whether the membrane form of gG also binds chemokines, insect cells infected with baculoviruses expressing the full-length gG (AcEHV-1gG) or a truncated form lacking the C-terminal transmembrane domain of EHV-1 gG (AcEHV-1gGs) were tested. The ability of the surfaces of cells infected with AcEHV-1gG to bind human CXCL1 and CXCL8 was clearly increased compared with cells infected with AcEHV-1gGs (Figure 9). No difference was observed in the binding to CCL2, a chemokine not recognized by EHV-1 gG. Chemokine binding activity was observed in media from cells infected with either recombinant (Figure 3B; data not shown).
Discussion

This paper describes the identification and characterization of a family of novel soluble vCKBPs (Table I). We demonstrate that secreted forms of gG encoded by several alphaherpesviruses bind chemokines with high affinity. Baculovirus-expressed gGs from EHV-1, BHV-1 and BHV-5 bind some of the CC, CXC and C chemokines, but not CX3CL1. Biological assays confirmed that gG inhibits signal transduction and migration induced by both CC and CXC chemokines. Remarkably, the alphaherpesvirus vCKBP blocks the interaction of chemokines with both specific receptors and GAGs. We also identified gG as the first vCKBP that interacts with chemokines both at the cell surface and in solution.

Many viral molecules that modulate chemokine activity have been described in beta- and gammaherpesviruses (Lalani et al., 2000; Murphy, 2001), but a biologically active CXCL8 homologue encoded by MDV is the only such molecule found in alphaherpesviruses to date (Parcells et al., 2001). We show that expression of soluble vCKBPs is a strategy encoded by many alphaherpesviruses to block chemokine activity. vCKBPs were initially described in the poxvirus family, and the gammaherpesvirus MHV-68 M3 protein was the only one previously identified in the herpesvirus family (Parry et al., 2000; van Berkel et al., 2000).

gG is unique to alphaherpesvirus, with no sequence similarity to known cellular proteins or previously identified vCKBPs (Figure 10), suggesting that gG interacts with chemokines in a different way. Expression of secreted gG provides a mechanism to sequester chemokines and to neutralize their effect by blocking chemokine–receptor interaction and activation of cell migration.

Interestingly, we found that gG blocks the interaction of chemokines with heparin. Recent experiments show that EHV-1 and BHV-1 gG interfere with the interaction of chemokines with complex GAGs at the surface of CHO-K1 cells (N.A. Bryant and A. Alcamì, unpublished data). Chemokines are retained at the surface of endothelial cells by interacting with GAGs, an interaction critical for correct chemokine presentation to the passing leukocyte (Middleton et al., 1997; Cynamon et al., 2001). The chemokine domains that interact with receptors or GAGs may be different and have been well defined for some chemokines (Proudfoot et al., 2001). Thus, the alphaherpesvirus vCKBP may mask both receptor and GAG-binding sites in the chemokine, or alternatively may induce a conformational change in the chemokine. Future studies will identify the chemokine domains involved in the interaction with gG. We have not been able to detect direct binding of purified gG to heparin–Sepharose columns (N.A. Bryant and A. Alcamì, unpublished data). MV M-T7 binds to the GAG binding domain of CXCL8 and has been

Fig. 6. Secreted gG from EHV-1 and BHV-1 expressed in the baculovirus system inhibits chemokine binding to cells in a dose-dependent manner. Binding assay of [125I]CCL3 and [125I]CXCL8 to U937 cells in the absence (solid triangles) or presence of increasing amounts of supernatants from SF21 cells infected with recombinant baculovirus expressing full-length gG from either EHV-1 (B and D) or BHV-1 (A and C) (solid squares), or with control baculovirus (AcNPV) (open squares). The dose of supernatant is expressed as cell equivalents. Binding specificity was determined in the presence of a 500-fold excess of unlabelled CCL3 or CXCL8 (open circles). Purified M3 protein was used as a positive control (open triangles). Binding of chemokines is expressed as the mean ± SD of triplicate assays.
proposed to block chemokine–GAG interactions (Lalani et al., 1997) (Table I). The poxvirus 35 kDa/M-T1 and gammaherpesvirus M3 proteins block the interaction of chemokines with their receptors (Smith et al., 1997; Alcamí et al., 1998; Parry et al., 2000). This novel property of gG of blocking both receptor and GAG binding should enhance its ability to inhibit chemokine function. gG can also displace chemokine once it has bound to heparin, suggesting that it may disrupt established chemokine gradients. Recently, we have found that MHV-68 M3 also disrupts chemokine–GAG interactions (L.M.C. Webb, V.P. Smith, I. Clark-Lewis and A. Alcamí, unpublished data).

The diversity of chemokine binding specificities among gGs from different viruses is probably related to host cell and tissue tropism and the requirement to block different subsets of chemokines in vivo. We have only been able to test human and mouse chemokines in these studies, but these are not the natural hosts of EHV-1, BHV-1 and BHV-5. However, species cross-reactivity exists among chemokines and their receptors, and thus the overall binding specificity of gGs for chemokine from natural hosts is likely to be similar. In contrast to previously identified vCKBP, the finding of a family of vCKBP with different chemokine specificities suggests that gG may provide the molecular scaffolding for designing

Fig. 7. Inhibition of chemokine biological activity by gG. (A) Chemokine-induced calcium flux. The indicated amount of purified BHV-1 gG.His was pre-incubated with 75 ng of CXCL1 before addition to Indo-1-loaded U937 cells. Similarly, purified BHV-5 gG.His was pre-incubated with 75 ng of CCL3 before addition to Indo-1-loaded HeLa cells transfected with CCR5 and CXCR4. Intracellular calcium mobilization was measured by FACS analysis. (B) Chemokine-induced chemotaxis in a Transwell migration assay. The top panel shows the migration of human neutrophils in response to 100 ng/ml CXCL8 in the presence of supernatants, expressed as cell equivalents, from insect cells infected with a recombinant baculovirus expressing full-length EHV-1 gG. The middle panel shows chemotaxis of human neutrophils induced by 100 ng/ml CXCL1 in the presence of increasing doses of purified BHV-1 gG.His. The bottom panel shows migration of U937 cells in response to 100 ng/ml CCL3 in the presence of purified BHV-5 gG.His. Supernatants from AcNPV-infected insect cells or an irrelevant purified His-tagged VV protein (A40R.His) were used as negative controls. Leukocytes migrating in duplicate assays are expressed as the percentage (mean ± SD) of the total cells added or as the number of cells counted (mean ± SD) in four high powered fields.
soluble chemokine inhibitors with different specificities. The broad binding specificity of gG, together with their ability to interfere with the interaction of chemokines with both specific receptors and GAGs, may be advantageous to inhibit chemokine-mediated inflammatory reactions. This family of vCKBP's have the potential of being used, like other virus-encoded chemokine inhibitors (Murphy, 2000), as therapeutic reagents in immune-related diseases.

A unique feature not found previously in vCKBP's is that gG is a membrane protein, anchored by a C-terminal transmembrane domain, which may be secreted after proteolytic cleavage. Secretion of gG has been demonstrated during infections with EHV-1, EHV-4, BHV-1, BHV-5 and HSV-2 (Marsden et al., 1984; Su et al., 1987; Crabb et al., 1992; Engelhardt and Keil, 1996; Keil et al., 1996; Drummer et al., 1998). The finding of chemokine binding activity secreted from cells infected with EHV-3, RanHV-1, CapHV-1 and CerHV-1 suggests that gG from these viruses is also proteolytically cleaved and secreted. Inhibition of N-linked glycosylation via tunicamycin blocked EHV-1 expression of chemokine binding activity, consistent with previous evidence that EHV-4 gG expression is greatly reduced and no detectable gG is secreted in the presence of tunicamycin (Drummer et al., 1998).

When expressed naturally, BHV-1 and BHV-5 gG is secreted as a 65 kDa polypeptide and a diffusely migrating species of 90–240 kDa (gpgG), which consists of the 65 kDa gG linked to GAGs (Engelhardt and Keil, 1996; Keil et al., 1996). Chemokines may interact with the GAG present in BHV-1 and BHV-5 gG, but they bind to gG itself since cross-linking to chemokines was observed for the 65 kDa species. The high molecular size species were

![Graph A](image1.png)

**Fig. 8.** Inhibition of chemokine–heparin interactions by purified gG. (A and B) FlashPlates precoated with heparin–BSA were incubated with [125I]CXCL8 in the absence or presence of purified gG from EHV-1 (EHV-1gG.His), BHV-1 (BHV-1gG.His) or BHV-5 (BHV-5gG.His), and the bound radio-activity (mean ± SD of duplicate samples) was determined. FlashPlates coated with BSA alone (BSA) were also tested to determine background binding. Increasing doses (10, 50 and 150 ng per well) of murine TNF (mTNF), human IgG, His-tagged Fas ligand (FasL) or His-tagged glucocorticoid-induced TNFR-superfamily-related protein ligand (GITRL) were also tested. (C and D) FlashPlates precoated with heparin–BSA were pre-incubated with [125I]CXCL1 or [125I]CCL2. Purified recombinant EHV-1 gG (300 ng per well) or medium was added to the wells and the bound radiolabelled chemokine (mean ± SD of triplicate samples) was determined at the indicated times. Background binding to BSA alone was subtracted.
identified by chemokine cross-linking in supernatants from cells infected with RanHV-1 and CerHV-1, but not with CapHV-1. The secretion of EHV-1 and EHV-4 gG as a 120 kDa disulfide-linked homodimer was reported (Drummer et al., 1998), but we have not detected complexes of [125I]chemokines with dimeric EHV-1 gG after cross-linking and SDS–PAGE in the absence of reducing agents (not shown).

Expression of gG on the surface of virus particles has been observed for EHV-1, EHV-4, HSV-1 and HSV-2 (Richman et al., 1986; Su et al., 1987; Drummer et al., 1998). We show chemokine binding activity of recombinant EHV-1 gG when expressed at the surface of insect cells. Membrane-bound EHV-1 gG expressed from infected cells may function as a decoy receptor preventing the interaction of chemokines with cellular chemokine receptors and subsequent signal transduction. It is tempting to speculate that the chemokine binding activity of gG incorporated into the EHV-1 virion envelope may mediate initial virus attachment to cell surfaces presenting chemokines, and play a role in determining cell and tissue tropism in vivo. Further studies will determine whether the chemokine binding activity of EHV-1 gG confers an advantage for virus attachment and replication.

No chemokine binding activity has yet been associated with HSV-1, HSV-2 or EHV-4 gGs, despite the latter having 72.7% amino acid identity with EHV-1 gG (Telford et al., 1998). It is not possible to predict from sequence information whether a particular gG will bind chemokines (Figure 10). We have used laboratory strains of HSV-1 and HSV-2, and gG from clinical isolates may have different properties. However, this is unlikely considering the high degree of conservation of the gG gene (Rekabdar et al., 1999; Liljeqvist et al., 2000). gG from these viruses may bind chemokines different to those included in this study (CCL1, CCL2, CCL3, CCL11, CCL17, CCL20, CXCL1, CXCL8, CXCL10, CXCL12 and CX3CL1). Membrane-bound gGs from HSV-1 and HSV-2 were not tested for chemokine binding, but it is unlikely that the secreted forms tested here will have lost the binding properties of the membrane-bound form. Alternatively, the chemokine binding activity of gG may have been lost from these viruses during evolution. It is notable that HSV-1 gG is significantly shorter that other alphaherpesvirus gGs and is not secreted into the medium of infected cell cultures (Richman et al., 1986). Moreover, the gene encoding gG is not found in VZV or MDV, suggesting that some alphaherpesviruses do not require this function for successful replication in the host. Three VZV strains, including a clinical isolate, lack the gG gene (Gomi et al., 2002) and we have been unable to amplify by PCR the gG gene from two clinical isolates (N.A.Bryant and A.Alcamì, unpublished data). MDV encodes a chemokine homologue and hence subverts the chemokine signalling pathways in an alternative way.

A role for BHV-1 gG in viral infection in vivo has been reported. Deletion of the gene encoding gG from BHV-1 caused viral attenuation in calves and the mutant virus was more immunogenic (Kaashoek et al., 1998). The lack of chemokine binding activity encoded by BHV-1 gG may explain the observed phenotype in vivo. Others have suggested that BHV-1 gG facilitates viral cell-to-cell spread (Nakamichi et al., 2002). In the case of HSV-1, a gG mutant showed no phenotype in vitro and marginal attenuation in the mouse ear model (Weber et al., 1987; Balan et al., 1994), and another study implicated HSV-1 gG in virus entry through apical surfaces of polarized epithelial cells (Tran et al., 2000). Thus, HSV-1 gG may have other functions unrelated to chemokine binding. However, it should be noted that certain functions ascribed to gG from the study of such deletion mutants may need to

![Fig. 9. Chemokine binding activity expressed at the surface of recombinant baculovirus-infected insect cells. SF21 insect cells were infected with AcEHV-1gG or AcEHV-1gGs for 24 h and incubated for 30 min at room temperature with the indicated chemokines in suspension. The bound chemokine was determined. The binding difference between AcEHV-1gG and AcEHV-1gGs was observed three times for CXCL8 and twice for CXCL1.](image)

| Table I. Comparison of vCKBs encoded by different viruses |
|---------------------------------|-----------------|----------------|-----------------|
| **Class** | **Virus and protein** | **Chemokine binding specificity** | **Mechanism of action** |
| vCKBP-1 | MV M-T7 | CC, CCX, C | Secreted, blockade of chemokine–GAG interactions |
| vCKBP-2 | MV M-T1 | CC | Secreted, blockade of chemokine–receptor interactions |
| vCKBP-3 | VV 35 kDa | CC, CCX, C, CCX3C | Secreted, blockade of chemokine–receptor and chemokine–GAG interactions |
| vCKBP-4 | CPV CCI | CC, CCX, C | Secreted and membrane bound, blockade of chemokine–receptor and chemokine–GAG interactions |
| vCKBP-3 | MHV-68 M3 | CC, CCX, C | Secreted, blockade of chemokine–receptor and chemokine–GAG interactions |

CCI, chemokine inhibitor; GAG, glycosaminoglycan; gG, glycoprotein G; MHV-69, murine gammaherpesvirus 68; MV, myxoma virus; vCKBP, viral chemokine binding protein; VV, vaccinia virus.
Fig. 10. Amino acid sequence similarity among members of the gG family. Sequence comparisons (A) among gGs encoded by the equine and bovine herpesviruses and (B) between gGs encoded by HSV-1 and HSV-2. Regions of identity are shown in black boxes and regions of high similarity are shown in shaded boxes. Conserved cysteine residues are marked with an asterisk. DDBJ/EMBL/GenBank Accession Nos are EHV-1 gG (p28967), BHV-1 (o39504), BHV-5 (x99755), EHV-3 (o90421), HSV-1 (p06484) and HSV-2 (p13290).
be re-assessed in the light of evidence that disruption of the upstream gene US3, a protein kinase homologue, can occur with gG deletion mutants of pseudorabies virus, an alphaherpesvirus of swine (Demmin et al., 2001).

In conclusion, we report the identification and characterization of a novel family of viral proteins that interact with chemokines. These findings provide insights into the interaction of viruses with the chemokine network and new strategies of immune modulation.

### Materials and methods

#### Reagents

Radio-iodinated recombinant human CCL1, CCL2, CCL3, CCL11, CCL17, CCL19, CCL20, CXCL1, CXCL8, CXCL10, CXCL12 and CX3CL1 (2200 Ci/mmol) were from Perkin-Elmer Life Sciences (Boston, MA). Recombinant human CCL2, CCL3, CCL4, CXCL8 and CXCL12, and murine CCL11, CCL10, CXCL13 (B-cell-attracting chemokine 1), XCL1, CX3CL1 and TNF were from R&D Systems (Minneapolis, MN). Recombinant human CCL1, CCL5 (regulated upon activation, normal T-cell expressed and secreted), CCL11, CCL17, CCL20, CXCL5 (epithelial cell-derived protein 78), CXCL10, CXCL11 (IFN-inducible T-cell-α chemotactant), CXCL13, XCL1, CX3CL1 and vMIP-2, and murine CCL2, CCL5, CCL20, CCL1, CCL7, CXCL1, CXCL12 (MIP-2), CXCL9 (monokine induced by IFN-γ) and CXCL12 were from PeproTech (Rocky Hill, NJ). Recombinant His-tagged human GITL and FasL were from R&D Systems. Human IgG was from Sigma.

#### Cells and viruses

Human neutrophils were isolated from blood samples in Lymphoprep gradients (Baly et al., 1997). The EHV-1 strains AB4, V592, RaH, Army 183 and those described in Figure 1E and EHV-3 were grown in equine embryonic lung cells. EHV-4 strain MD was grown in equine embryonic kidney cells. Primary equine cells were derived from lung or kidney of an aborted fetus via enzymatic digestion, and recovered cells were passaged in 10% fetal calf serum (FCS) MEM. MDBK were infected with BHV-1 (strain Jara). BHV-4 (strain V. test), BHV-5 (strain N569), RaHV-1 (strain CvHV-2), CapHV-1 (strain E-Ch) and CerHV-1 (strain CvHV-1). H5V-1 strain SC16 and HSV-2 strain HVD were grown in Vero cells. VZV was provided by A.Davison (Institute of Virology, Glasgow, UK) and grown in human MRC-5 cells. VV strain Lister and VV Lister A35K were grown in BSC-1 cells (Alcamì et al., 1998).

#### Construction of recombinant baculoviruses

The gG gene was PCR amplified from infected cell DNA using Pfu polymerase and specific oligonucleotides (Table II), and cloned into pBAC-1 (Novagen). Except for the expression of EHV-1 candidate genes, the nucleotide sequence of the genes was determined. *Aotographa californica* nuclear polyhedrosis virus (AcNPV) recombinants were constructed as described previously (Alcamì et al., 1998). Full-length and secreted forms of gG with and without a C-terminal His tag were produced in *Spodoptera frugiperda* (Sf) 21 insect cells infected with recombinant baculoviruses.

The full-length EHV-1 AB4 gG ORF sequence was identical to the published sequence (Telford et al., 1992), but the truncated version had an amino acid substitution V208A (T203C at the nucleotide level). Full-length and secreted forms of the BHV-1 (strain Jura) (Metzler et al., 1986) gG showed an E331K substitution (G991A) when compared with the Cooper strain (DBB/EMBL/GenBank Accession No. AJ004801). Full-length and secreted BHV-5 (strain N569) (French, 1962) gG had five amino acid substitutions compared with the published sequence: V157L, C560G, G585C, E220D (C660G), P221A (G661C), A266G (C797G), and A392T (G1174A). The sequences of the secreted forms of gG from HSV-1 strain SC16 and HSV-2 strain HVD were identical to the published sequences from HSV-1 strain 17 and HSV-2 strain HGS2, respectively (McCoy et al., 1985; Dolan et al., 1998).

#### Preparation of medium for binding and biological assays

FCS-free supernatants from cultures infected with 5–10 plaque-forming units per cell were harvested 2–3 days post-infection and prepared as described previously (Alcamì et al., 1998). Infected cells were cultured for 24 h with tunicamycin (5 μg/ml; Sigma) when indicated. Infectious virus in the supernatants was inactivated with 4,5,8-trimethylpsoralen (Sigma) and exposure to UV radiation, and concentrated and dialysed against phosphate-buffered saline (PBS) (Alcamì et al., 1998).

#### Protein purification

Recombinant gG.His fusion proteins were purified on nickel–Sepharose columns (Pharmacia) from supernatants of baculovirus-infected H5 insect cells grown in EX-Cell 405 (JR Biosciences). Purified His-tagged proteins were visualized by SDS–PAGE and Coomassie Blue staining, and were quantified by protein assay (Bio-Rad).

#### Chemokine cross-linking assay

The binding medium was RPMI containing 20 mM HEPES pH 7.4 and 0.1% BSA. Cross-linking experiments with BS5 (Pierce), EGS (Sigma) or EDC (Sigma) to [125I]chemokines (0.4 nM) were performed as described previously (Alcamì et al., 1998). The amount of medium used was equivalent to 5–7 × 10⁴ cells. Samples were analysed by 12% SDS–PAGE. Purified His-tagged CmE, a TNFR homologue encoded by cowpox virus (Saraiva and Alcamì, 2001), was provided by M.Saraiva and used as a control in some experiments.

#### Chemokine binding to cells

To determine chemokine binding to U937 cells, samples were pre-incubated with 100 pM [125I]chemokine for 1 h at 4°C, and U937 cells were added and incubated for 2 h at 4°C. Purified M3 protein was provided by C.Bunce and M.Wilson (Xenova Research Ltd, Cambridge, UK). To determine the binding of chemokines to Sf21 cells, 2 × 10⁶ cells were scraped and incubated in suspension with 200 pM [125I]chemokines


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