Glycoprotein G from pseudorabies virus binds to chemokines with high affinity and inhibits their function

Abel Viejo-Borbolla,1 Ana Muñoz,2 Enrique Tabarés2 and Antonio Alcamí1,3

1Centro de Biología Molecular Severo Ochoa (Consejo Superior de Investigaciones Científicas-Universidad Autónoma de Madrid), Nicolás Cabrera 1, Campus de Cantoblanco, 28049 Madrid, Spain
2Departamento de Medicina Preventiva, Salud Pública y Microbiología, Facultad de Medicina, Universidad Autónoma de Madrid, Arzobispo Morcillo 4, E-28029 Madrid, Spain
3Department of Medicine, University of Cambridge, Addenbrooke’s Hospital, Hills Road, Cambridge CB2 2QQ, UK

Received 17 March 2009
Accepted 22 September 2009

Pseudorabies virus (PRV), also known as suid herpesvirus, is the aetiological agent of Aujeszky’s disease in swine. In other animals, except higher-order primates, PRV infection is often fatal. The mechanisms of PRV pathogenesis and immune modulation are largely unknown. PRV codes for 11 glycoproteins. Among them, glycoprotein G (gG) is the most abundant PRV protein found in the supernatant of PRV-infected cell cultures. PRV-gG has low amino acid sequence similarity with gG from other animal alphaherpesviruses and its function is unknown. gG from other animal alphaherpesviruses, with the exception of at least equine herpesvirus 4, binds to chemokines. We show here that PRV-gG binds to the human chemokine CL1 and several CC and CXC human chemokines with high affinity. Chemokine-binding activity can be detected in the supernatants of PRV-infected cell cultures, and insertional inactivation of the gene encoding gG from the PRV genome results in loss of chemokine-binding activity. Binding of PRV-gG to chemokines inhibits chemokine-mediated cell migration, suggesting a role for PRV-gG in immune evasion.

INTRODUCTION

Pseudorabies virus (PRV) belongs to the family Herpesvirinae, subfamily Alphaherpesvirinae and genus Varicellovirus. PRV is the aetiological agent of Aujeszky’s disease in swine, causing huge economical losses worldwide. Wild-type PRV infection causes primarily respiratory and reproductive disease with low mortality in its natural host, the adult pig, where the virus can invade sensory ganglia and establish a latent infection (Nauwynck et al., 2007). Evidence that infection with PRV elicits both humoral- and cell-mediated immune responses is widely recognized. The relevance of cell-mediated immune responses, in particular of cytotoxic T-lymphocytes, in the protection against disease caused by other herpesviruses suggests that cell-mediated immunity may play a similar role in the control of PRV infections. The best-protected pigs stood out by maintaining strong T cell-mediated immune responses after challenge (van Rooij et al., 2004).

PRV can infect most mammals, with the exception of higher-order primates and humans, causing lethal central nervous disorders (Enquist, 1999; Wittmann & Rziha, 1989). PRV is a particularly aggressive virus in young animals where infection is frequently fatal (Nauwynck et al., 2007). The mechanisms by which PRV evades the innate immune response and causes disease in a variety of animals are not fully understood. One of them involves the inhibition of interferon responsive gene expression by hindering signal transducers and activators of transcription (STAT1) phosphorylation in a species-specific manner (Brukman & Enquist, 2006).

Chemokines are chemotactic cytokines that orchestrate the migration of immune cells to sites of injury and infection, playing a critical role in innate immunity. There are four subfamilies of chemokines classified according to the relative positioning of the N-terminal cysteine residues into C, CC, CXC and CX3C chemokines. Most chemokines are secreted with the exception of CXCL16 and CX3CL1, which are transmembrane proteins. Chemokines have been classified according to their function as inflammatory, homeostatic and dual-function chemokines (Moser et al., 2004). Alterations in the chemokine network are beneath many inflammatory and autoimmune diseases.

The interaction of chemokines with glycosaminoglycans (GAGs) seems to be required for correct presentation of the chemokine and its interaction with specific receptors.
present at the plasma membrane of the target cell (Proudfoot et al., 2003; Rot, 1992). The chemokine receptors belong to the family of seven transmembrane G-protein-coupled receptors. The critical role played by chemokines against infection is emphasized by the fact that several viruses code for proteins that interfere with the chemokine network. Both poxviruses and herpesviruses express secreted proteins that bind to chemokines with high affinity, inhibiting chemokine function (Alcami et al., 1998; Alejo et al., 2006; Bryant et al., 2003; Graham et al., 1997; Parry et al., 2000; van Berkel et al., 2000). These proteins are generally known as viral chemokine-binding proteins (vCKBPs). One of them, gG from some non-human alphaherpesvirus, binds to chemokines with high affinity and inhibits chemokine function (Bryant et al., 2003; Costes et al., 2005). We have shown chemokine-binding activity for the gG of bovine herpesvirus (BHV)-1, BHV-5, equine herpesvirus (EHV)-1 and felid herpesvirus 1 (FeHV-1) (Bryant et al., 2003; Costes et al., 2005). Chemokine-binding activity could not be demonstrated for the gG of EHV-4, or supernatant from cells infected with herpes simplex virus (HSV) or varicella-zoster virus (VZV) (Bryant et al., 2003; Van de Walle et al., 2007). There is no information regarding PRV chemokine-binding activity.

The genome of PRV encodes 11 glycoproteins [gB, gC, gD, gE, gG, gH, gI, gK, gL, gM and gN (Mettenleiter, 2000)]. gG is encoded by the US4 gene, which is present in most members of the subfamily Alphaherpesvirinae with the notable exception of VZV (McGeoch, 1990; Mettenleiter, 2000). PRV-gG has only limited similarity at the amino acid level with gG from HSV-2, in an 81 aa stretch, and even lower similarity with HSV-1-gG (McGeoch et al., 1987; van Zijl et al., 1990). PRV-gG is an early protein expressed as a cell-associated precursor that, following processing, is secreted and accumulates in the medium of infected cells (Bennett et al., 1986; Rea et al., 1985). The function of PRV-gG is unknown. We have investigated the possibility that PRV-gG, despite its low amino acid sequence similarity to gGs from other alphaherpesviruses, could bind to chemokines. We show here that PRV-gG binds to the human (h) chemokine CL1 and with some hCC and hCXC chemokines with high affinity. Chemokine-binding activity is observed in the supernatant of PRV-infected cells and disruption of the PRV-gG gene results in loss of chemokine-binding activity. Binding of PRV-gG to chemokines results in efficient inhibition of chemokine-mediated cell migration, indicating that PRV-gG may play a role in evading the host innate immune response.

**RESULTS**

**Recombinant gG from PRV binds to chemokines**

We have previously reported that gG from other non-human alphaherpesviruses binds to chemokines (Bryant et al., 2003; Costes et al., 2005). PRV is known to encode a gG that is secreted into the extracellular medium but its function is unknown (Bennett et al., 1986; Rea et al., 1985). In order to investigate whether PRV-gG could interact with chemokines we cloned aa 20–417 of PRV-gG downstream of a Histidine tag (His-tag). To increase secretion of His-tagged PRV-gG in insect cells, we substituted the gG signal peptide by that of the honeybee melittin. The final construct was termed PRV-gGs (Fig. 1a). We constructed a recombinant baculovirus for the expression of PRV-gGs in baculovirus-infected Hi-5 insect cells and purified it from culture supernatant by affinity chromatography. The purity of the preparation was assessed by Coomassie staining (Fig. 1b). An antibody raised against bacterially expressed PRV-gG reacted with the purified PRV-gGs but not with purified gG from HSV-1 (A. Viejo-Borbolla & A. Alcamí, unpublished data), whereas an anti-His-tag monoclonal antibody reacted with both gGs (Fig. 1c).

In order to determine whether PRV-gGs interact with chemokines, we screened all 44 commercially available human chemokines (hCCL1, hCCL2, hCCL3, hCCL3L1, hCCL4, hCCL4L1, hCCL5, hCCL7, hCCL8, hCCL11,
Pseudorabies virus gG inhibits chemokine function

The interaction of PRV-gGs and 21 human chemokines (Fig. 2a). Some of these positive interactions and two curves from non-interacting chemokines are depicted in the sensorgram (Fig. 2a). The affinities of the interaction between PRV-gGs and chemokines were determined using the BIAcore X biosensor. As shown in Table 1, the interaction between PRV-gGs and chemokines were determined by multiplex PCR. DNA was isolated and the presence of the gG gene was determined by multiplex PCR.

As an alternative method to demonstrate the interaction of chemokines with PRV-gGs, we also performed a cross-linking assay with purified recombinant PRV-gGs and 125I-labelled hCCL5. As a negative control, a non-relevant protein was used instead of PRV-gGs. As shown in Fig. 2b), cross-linking of PRV-gGs and 125I-labelled hCCL5 resulted in the formation of a slower migrating complex. The apparent molecular size of this complex corresponds approximately to the addition of the molecular masses of PRV-gGs and 125I-labelled hCCL5, indicating an interaction between both proteins.

Disruption of PRV-gG expression results in loss of chemokine-binding activity from the supernatant of PRV-infected cell cultures

The recombinant XGF-N and XGF-G viruses deficient in gG were obtained by substitution of the coding sequences of gG with enhanced green fluorescent protein (EGFP). The gene encoding EGFP was inserted, by homologous recombination in infected cells, into the US4 gene (encoding gG) of the genome of the PRV strains NIA-3 and gIS8 (Gomez-Sebastian & Tabares, 2004), generating the recombinant viruses XGF-N and XGF-G, respectively. To confirm the insertion of the EGFP gene into the US4 gene in the XGF-N and XGF-G recombinant viruses, viral DNA was isolated and the presence of the gG gene was determined by multiplex PCR.

Table 1. Interactions affinities between PRV-gGs and chemokines

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>$K_0$ (M)</th>
<th>$K_a$ (1/Ms)</th>
<th>$K_d$ (1 s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCCL5</td>
<td>$8.4 \times 10^{-9}$</td>
<td>$2.80 \times 10^{-2}$</td>
<td>$2.36 \times 10^{-3}$</td>
</tr>
<tr>
<td>hCCL8</td>
<td>$2.36 \times 10^{-8}$</td>
<td>$5.35 \times 10^{-4}$</td>
<td>$1.27 \times 10^{-3}$</td>
</tr>
<tr>
<td>hCCL13</td>
<td>$3.39 \times 10^{-9}$</td>
<td>$9.96 \times 10^{-2}$</td>
<td>$3.38 \times 10^{-3}$</td>
</tr>
<tr>
<td>hCCL16</td>
<td>$4.69 \times 10^{-8}$</td>
<td>$4.50 \times 10^{-4}$</td>
<td>$2.11 \times 10^{-3}$</td>
</tr>
<tr>
<td>hCCL17</td>
<td>$1.06 \times 10^{-8}$</td>
<td>$8.38 \times 10^{-5}$</td>
<td>$8.86 \times 10^{-3}$</td>
</tr>
<tr>
<td>hCCL18</td>
<td>$3.34 \times 10^{-9}$</td>
<td>$1.97 \times 10^{-2}$</td>
<td>$6.59 \times 10^{-3}$</td>
</tr>
<tr>
<td>hCCL20</td>
<td>$1.16 \times 10^{-7}$</td>
<td>$8.10 \times 10^{-4}$</td>
<td>$9.37 \times 10^{-3}$</td>
</tr>
<tr>
<td>hCCL22</td>
<td>$1.92 \times 10^{-9}$</td>
<td>$7.66 \times 10^{-5}$</td>
<td>$1.47 \times 10^{-3}$</td>
</tr>
<tr>
<td>hCCL25</td>
<td>$9.2 \times 10^{-9}$</td>
<td>$3.72 \times 10^{-3}$</td>
<td>$1.42 \times 10^{-3}$</td>
</tr>
<tr>
<td>hCCL26</td>
<td>$4.0 \times 10^{-9}$</td>
<td>$1.44 \times 10^{-4}$</td>
<td>$5.77 \times 10^{-3}$</td>
</tr>
<tr>
<td>hCCL28</td>
<td>$1.79 \times 10^{-8}$</td>
<td>$4.52 \times 10^{-5}$</td>
<td>$8.07 \times 10^{-3}$</td>
</tr>
<tr>
<td>hCXCL4</td>
<td>$4.4 \times 10^{-7}$</td>
<td>$6.33 \times 10^{-4}$</td>
<td>$2.79 \times 10^{-3}$</td>
</tr>
<tr>
<td>hCXCL6</td>
<td>$3.34 \times 10^{-8}$</td>
<td>$3.28 \times 10^{-4}$</td>
<td>$11.0 \times 10^{-3}$</td>
</tr>
<tr>
<td>hCXCL8</td>
<td>77 aa</td>
<td>$2.55 \times 10^{-8}$</td>
<td>$8.19 \times 10^{-4}$</td>
</tr>
<tr>
<td>hCXCL9</td>
<td>$1.04 \times 10^{-8}$</td>
<td>$1.84 \times 10^{-5}$</td>
<td>$1.91 \times 10^{-3}$</td>
</tr>
<tr>
<td>hCXCL11</td>
<td>$5.82 \times 10^{-9}$</td>
<td>$1.87 \times 10^{-5}$</td>
<td>$1.09 \times 10^{-3}$</td>
</tr>
<tr>
<td>hCXCL12α</td>
<td>$2.89 \times 10^{-9}$</td>
<td>$1.51 \times 10^{-5}$</td>
<td>$4.36 \times 10^{-3}$</td>
</tr>
<tr>
<td>hCXCL12β</td>
<td>$1.08 \times 10^{-8}$</td>
<td>$5.50 \times 10^{-4}$</td>
<td>$6.00 \times 10^{-3}$</td>
</tr>
<tr>
<td>hCXCL13</td>
<td>$2.82 \times 10^{-8}$</td>
<td>$6.70 \times 10^{-4}$</td>
<td>$1.80 \times 10^{-3}$</td>
</tr>
<tr>
<td>hCXCL14</td>
<td>$2.02 \times 10^{-8}$</td>
<td>$2.40 \times 10^{-4}$</td>
<td>$4.85 \times 10^{-3}$</td>
</tr>
<tr>
<td>hCL1</td>
<td>$1.55 \times 10^{-8}$</td>
<td>$1.19 \times 10^{-5}$</td>
<td>$1.86 \times 10^{-3}$</td>
</tr>
</tbody>
</table>
The presence of IE180 and gG was observed in gIS8 and NIA-3 parental viruses (Fig. 3a, lanes 3 and 4, respectively), while IE180 and EGFP in place of gG were detected in XGF-G- and XGF-N-gG deficient recombinant viruses (Fig. 3a, lanes 1 and 2, respectively). In addition, EGFP but not gG was expressed by XGF-N and XGF-G (Fig. 3b, lanes 1 and 2, respectively), while the parental viruses NIA-3 and gIS8 expressed gG but not EGFP as determined by Western blot (Fig. 3b, lanes 3 and 4). PRV-gG was detected in the culture supernatant of Vero cells infected with the NIA-3 or gIS8 virus strains (Fig. 3b, lanes 5 and 6, respectively), but not with the PRV deletion mutants (Fig. 3b, lanes 7 and 8, respectively).

PRV-gG is expressed in PRV-infected Vero cells in the presence of phosphonoacetic acid (PAA) (data not shown). Supernatants from mock-, wild-type (wt)- or insertional mutant PRV-infected Vero cell cultures in the presence of PAA to inhibit expression of PRV late proteins, were collected and subjected to a cross-linking assay with 125I-labelled hCCL5. A higher molecular mass band than the one observed with 125I-labelled hCCL5 alone was observed upon incubation of 125I-labelled hCCL5 with the supernatant from both strains of wt PRV-infected cells. However, disruption of the expression of PRV-gG impeded the formation of the higher molecular mass complex, indicating loss of chemokine-binding activity (Fig. 3c).

**PRV-gGs inhibits chemokine-mediated cell migration**

Chemokines are chemotactic cytokines that orchestrate the migration of leukocytes to the site of infection or injury. In order to analyse whether PRV-gGs interferes with chemokine function we carried out an in vitro cell migration assay using the Transwell technology (Neuro Probe). Briefly, chemokine alone or in combination with increasing concentrations of PRV-gGs were placed in the bottom chamber of a 96-transwell plate. MonoMac-1 or m300-19 cells expressing CXCR4 were added to the top chamber, which was separated from the bottom one by a filter. hCXCL12β-induced chemotaxis of MonoMac-1 or m300-19 cells was inhibited with increasing concentrations of PRV-gGs (Fig. 4). As a positive control of inhibition we utilized gG from EHV-1 (EHV-1-gGs) (Bryant et al., 2003). EHV-1-gGs or PRV-gGs reduced hCXCL12β chemotaxis in a similar manner (Fig. 4b).

**Characterization of the chemokine domains involved in the interaction with PRV-gGs**

To trigger migration, chemokines interact with both GAGs and the chemokine receptor. The chemokine domains involved in the interaction with GAGs or chemokine receptor have been characterized for hCXCL12a (Crump et al., 1997). We have tested the ability of PRV-gGs to bind to hCXCL12α mutants defective in GAG or receptor binding using the SPR technology (Fig. 5, Table 2). Deletion of the N-terminal four residues of CXCL12α (mutant 4-67) impairs receptor interaction and activation, whereas it does not affect GAG binding. An hCXCL12α mutant carrying a P2G substitution (mutant P2G) binds to CXCR4 but does not trigger cell migration. Mutation of a cluster of basic residues results in an hCXCL12α variant (mutant 3/6) unable to bind to GAGs. These mutants have been previously used to determine the chemokine domain involved in the interaction with another vCKBP, ectromelia virus E163 protein (Ruiz-Arguello et al., 2008). The number of response units (RU) obtained with the wt and the P2G mutant was similar (Fig. 5). Mutation of the GAG- or the receptor-binding domain resulted in a 40 and 60% decrease in RU, respectively (Fig. 5). The affinities of the interactions between PRV-gGs and the CXCL12α variants correlate with the decrease in RU (Fig. 5). These results indicate that PRV-gGs, in contrast to E163, interacts...
preferentially although not exclusively with the receptor-binding domain of the chemokine.

**DISCUSSION**

A rational approach to the control of Aujeszky’s disease requires an understanding of the molecular mechanisms that mediate a protective immune response against infection and the viral proteins that evade immunity and contribute to pathogenesis. Evidence that infection with PRV results in both humoral- and cell-mediated immune responses in pigs is widely recognized. Vaccination of pigs with attenuated or killed vaccines is practised in many countries. At present, PRV has been officially eradicated from several European countries due to a combination of marker vaccines and discriminating ELISA (Nauwynck et al., 2007). Immunization with PRV mutants lacking gG or gI results in complete protection against PRV challenge (Kimman et al., 1992).

We have investigated the function of gG, the most abundant PRV protein in the supernatant of infected cell cultures (Rea et al., 1985) but of unknown function. We show here that PRV-gG is a vCKBP that binds to a wide range of human chemokines with high affinity. We show binding of PRV-gGs to chemokines both by SPR technology, using the BIAcore X biosensor and by cross-linking assays, and we have determined the affinity of the interaction with chemokines. Chemokine-binding activity was demonstrated using both recombinant purified PRV-gGs and the supernatant from PRV-infected cells containing gG.

PRV-gGs shows a broad binding activity, interacting with hCL1 and members of the hCC and hCXC chemokine subfamilies. Like the gG encoded by EHV-1, BHV-1 and BHV-5, PRV-gGs does not bind to hCX3CL1. Approximately 50% of the chemokines interacting with PRV-gGs are also recognized by EHV-1- and BHV-1-gG, including hCCL5, hCCL13, hCCL17, hCCL18, hCCL20, hCCL22, hCXCL8, hCXCL12a, hCXCL12b and hCL1 (Bryant et al., 2003; this report and N. Bryant & A. Alcami, unpublished data). The binding affinities of the interactions between PRV-gGs and chemokines differ from those of the other gGs (Bryant et al., 2003). Moreover, PRV-gGs also binds to hCCL8, hCCL16, hCCL25, hCCL26,
Table 2. Interaction affinities between PRV-gGs and hCXCL12α analogs

The derived kinetic parameters and the affinity constants for the PRV-gGs/chemokines interactions are shown.

<table>
<thead>
<tr>
<th>CXCL12 variant</th>
<th>$K_0$ (M)</th>
<th>$K_a$ (1/Ms)</th>
<th>$K_d$ (1 s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>$2.2 \times 10^{-8}$</td>
<td>$6.81 \times 10^5$</td>
<td>0.015</td>
</tr>
<tr>
<td>Mutant 3/6</td>
<td>$5.0 \times 10^{-8}$</td>
<td>$1.71 \times 10^{-4}$</td>
<td>$6.55 \times 10^{-7}$</td>
</tr>
<tr>
<td>Mutant 4-67</td>
<td>$1.71 \times 10^{-7}$</td>
<td>$8.11 \times 10^4$</td>
<td>0.0139</td>
</tr>
</tbody>
</table>

hCCL28, hCXCL4, hCXCL6, hCXCL9, hCXCL11, hCXCL13 and hCXCL14. However, PRV-gGs does not interact with hCCL2, hCCL19, hCXCL1, hCXCL2, hCXCL3 and hCXCL5 (bound by both BHV-1- and EHV-1-gG), hCCL1, hCCL7, hCCL11, hCCL12, hCXCL7 and hCXCL10 (bound by BHV-1-gG) or hCCL21, hCCL24 (bound only by EHV-1-gG) (Bryant et al., 2003; N. Bryant & A. Alcami, unpublished data).

The diverse chemokine-binding specificities of the gG encoded by different alphaherpesviruses may reflect an adaptation of the viruses to the immune system of different hosts. Here, we have tested the chemokine-binding potential of PRV-gGs using all commercially available human chemokines. The chemokines targeted by PRV should be tested in its natural host. Similarly, the interaction of PRV-gG with chemokines of other mammals should be analysed considering that the virus infects a variety of mammals. However, these investigations are not feasible due to the very limited availability of such reagents. Analysis of the genomic organization of the chemokines reveals that for some chemokine groups, tandem gene duplication occurred independently in the mouse and human (Zlotnik et al., 2006). Chemokines can be classified as clustered or non-clustered depending on their genomic organization (Yoshie et al., 2001; Zlotnik et al., 2006). In general, chemokines within one cluster are not well conserved between species, whereas the non-clustered chemokines tend to be so (Yoshie et al., 2001). PRV-gGs binds both clustered (i.e. CCL13 and CCL16) and non-clustered (i.e. CCL20 and CCL28) chemokines. It is possible to envisage that PRV-gG could interact with similar, conserved chemokines but also do so with other, not so conserved chemokines in its natural host or in other mammals. Analysis of the interaction of PRV-gGs with human chemokines shows no preference for binding to inflammatory or constitutive chemokines.

Lack of gG expression results in a loss of hCCL5-binding activity in cultures infected with the presence of PAA, indicating that gG is the only PRV-secreted protein expressed at early times post-infection with the ability to interact with hCCL5. A recent report shows that insertion of EGFP within the US4 gene of PRV affects correct expression of the US3 gene and impedes proper cell-to-cell spread of PRV (Demmin et al., 2001). We do not know whether other alphaherpesviruses code for secreted proteins different than gG that interfere with the chemokine network. However, only gG has been shown to have such an activity in EHV-1, -3, BHV-1, -5 and FeHV-1 to date (Bryant et al., 2003; Costes et al., 2005). A chemokine-binding activity that could correspond to gG was also observed in the supernatants of cells infected with rangiferine herpesvirus 1, caprine herpesvirus 1 and cervine herpesvirus 1 (Bryant et al., 2003). No activity was observed in the supernatants of cell cultures infected with HSV-1, HSV-2 and VZV. However, VZV does not code for gG and chemokine-binding activity in VZV-infected cells has not been reported yet. Whether VZV codes for a secreted protein other than gG with the ability to bind to chemokines not tested so far is unknown at present.

The interaction of PRV-gGs with chemokines results in inhibition of chemotaxis. The use of chemokine mutants indicates that PRV-gGs interacts preferentially, although not exclusively, with the receptor-binding domain of the chemokine (Fig. 5). Other vCKBPs also bind to both the receptor- and GAG-binding domains of the chemokines and inhibit chemotaxis in vitro (Bryant et al., 2003; Webb et al., 2003, 2004). However, inhibition of migration in vitro could not be shown for vCKBP that interact solely through the GAG-binding domain of the chemokine (Ruiz-Arguello et al., 2008; Seet & McFadden, 2002). These vCKBPs may impair chemotaxis in vivo but not in vitro through the inhibition of chemokine presentation by GAGs (Seet & McFadden, 2002). These sets of data together with our results suggest that PRV-gGs inhibits chemotaxis in vitro through the impairment of the chemokine–receptor interaction. Chemotaxis of immune cells to sites of infection is one of the first responses to counteract infection. Thus, inhibition of chemotaxis by alphaherpesvirus gG could interfere with the ability of the immune system to respond to viral infection and initiate antiviral protective mechanisms. Deletion of gG from the EHV-1 genome results in increased pathogenicity in mice (von Einem et al., 2007) and correlates with a higher neutrophil migration into the lungs, indicating a role for EHV-1-gG in chemotaxis inhibition in vivo (Van de Walle et al., 2007). Similarly, gG has been shown to play an important role in the pathogenesis of infectious laryngotracheitis virus (ILTV) in vivo (Devlin et al., 2006). Lack of gG expression resulted in an attenuated ILTV and higher inflammation in the trachea of infected birds (Devlin et al., 2006), which would be consistent with ILTV-gG binding to chemokines, although this has not been formally demonstrated. In PRV, deletion of the gG gene does not dramatically affect virulence or immunogenicity in pigs (Kimman et al., 1992; Thomsen et al., 1987). In PRV, strains with deletion of the gG gene showed normal virulence, induced neutralizing antibodies and complete protection in pigs (Kimman et al., 1992; Thomsen et al., 1987). However, there is currently no information regarding leukocyte infiltration induced by the PRV-gG deletion mutant. Further studies on the effect of PRV-gG
in in vivo cell migration and inflammation would provide useful information regarding PRV biology, pathogenesis, immune modulation and vaccine development. Moreover, these studies would be relevant since PRV is also used as a live tracer of neuronal pathways and as a model to study herpessvirial biology.

Due to their relevant role in many autoimmune and inflammatory disorders, chemokines and chemokine receptors are the target for the development of new therapeutic strategies (Johnson et al., 2005; Proudfoot, 2002). Therapies aiming at inhibiting a single chemokine have failed due in part to the redundancy present in the chemokine network, where several chemokines bind to one receptor and some receptors interact with more than one chemokine. Thus, the inhibition of several chemokines involved in a particular pathogenic process is required.

vCKBPs are good candidates as therapeutic molecules due to the specificity and high binding affinities of their interactions with chemokines (Fallon & Alcami, 2006). The different chemokine-binding specificities observed among gGs could serve to create chimeric vCKBPs able to bind only the desired chemokine subset that is involved in a particular inflammatory or autoimmune disease.

In summary, we report here for the first time that PRV-gG, the most abundantly secreted protein from PRV-infected cells, binds to a broad range of chemokines with high affinity and inhibits chemotaxis, suggesting that PRV-gG could act as a modulator of the host immune response.

METHODS

Viruses and cells. The PRV strains used were NIA-3 and gIS8 (Fernandez et al., 1999). Vero cells (green monkey kidney cells) were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 5% fetal calf serum (FCS). Macrophage-monoctye 1 cells (MonoMac-1) were grown in RPMI 1640 (Sigma) supplemented with 5% FCS. m300-19 cells (mouse B cells) were a gift from Bernhard Mosser (Cardiff University, UK) and were grown in RPMI 1640 containing 10% FCS, 10 mM HEPES buffer containing 100 mM NaCl and 10% glycerol (v/v) by using a Vivastin500 device (VivaScience). Purified recombinant proteins were analysed by SDS-PAGE and Coomassie blue staining or by SDS-PAGE followed by Western blotting using an anti-His antibody (Qiagen). PRV-gG was also detected by Western blotting using a rabbit polyclonal anti-PRV-gG antibody.

Analysis of gG expression. Early expression of gG was examined in Vero-infected cells, in the presence of 200 μg FAA ml⁻¹, which inhibits expression of PRV late proteins, at 16 h post-infection. NIA-3, gIS8 and PRV recombinant viruses were added to Vero cells in 125 cm² Falcon flasks, at a m.o.i. of about 5 TCID₅₀ per cell. Expression of gG was analysed in the supernatant and cell lysates. The infected cell culture medium supplemented with 1% FCS was harvested and the cell debris was discarded by centrifugation at 10 000 g for 10 min. Proteins in the medium were precipitated with 10% trichloroacetic acid and analysed by SDS-PAGE.

Antibody production and Western blotting. Cell lysates and medium proteins were analysed by SDS-PAGE using 15% acrylamide gels, electro-transferred to a PVDF membrane (Amersham Biosciences) to detect EGFP and gG from infected Vero cells or to a nitrocellulose membrane (Perkin Elmer) to detect gG from baculovirus supernatant. Membranes were probed either with a polyclonal antibody against gG, or a monoclonal anti-His-tag antibody or monospecific anti-GFP antibodies (BD Bioscience). The second antibody was peroxidase-labelled anti-rabbit antibody (Sigma) (to detect gG) or peroxidase-labelled anti-mouse antibody (GE Healthcare). Bands were visualized using the enhanced chemiluminescence detection system (Amersham Pharmacia).

The gG antiserum was obtained by immunization of two rabbits with the internal coding fragment (aa 20–416, included in the pRSETB plasmid, Amersham) and purified by electro elution. The pRSTG-I plasmid was obtained by insertion of the MscI–PmII fragment of the gene, NIA-3 strain, GenBank accession no. EU518619) expressed in Esherichia coli with the plasmid pRSTG-1 and purified by electro elution. The pRSTG-I plasmid was obtained by insertion of the MscI–PmII gG gene fragment into the PsvI site of the pRSETB plasmid (Invitrogen).

Recombinant chemokines. Recombinant human chemokines used in the BIAcore X biosensor and the cell migration assays were obtained from PeproTech with the exception of hCCL25 and hCXCL13, which were from R&D Systems. Recombinant 125I-labelled hCCL5 was purchased from Amersham (GE Healthcare), hCXCL12s wt and mutants used to characterize the chemokine domain involved in the interaction with PRV-gGs were obtained from Fernando Arenzana-Seisdedos (Viral Pathogenesis Laboratory, Institut Pasteur, Paris, France) (Crump et al., 1997).

Determination of gG-chemokine binding specificity and affinity constants using SPR technology. The interactions between human chemokines and gG, and the affinity constants were determined by SPR technology using a BIAcore X biosensor (GE Healthcare). For screening purposes, purified recombinant gG dialysed against acetate buffer pH 4.0 was amine-coupled to CMS chips (GE Healthcare) in
order to reach approximately 5000 response units (RU) (500 pg mm⁻²). Recombinant chemokines were injected at 100 nM in HBS-EP buffer [10 mM HEPES, 150 mM, NaCl, 3 mM EDTA, 0.005% (v/v) surfactant P20, pH 7.4] at a flow rate of 10 μl min⁻¹, and association and dissociation were monitored. The chip surface was regenerated after each chemokine injection by injecting 10 mM glycine–HCl pH 2.0. For kinetic analyses, recombinant gG was immobilized at low densities (Rmax <200 RU). Different concentrations of the corresponding chemokine were injected at a flow rate of 30 μl min⁻¹ over a 2 min period. Following the association period, the chemokine was allowed to dissociate for 5 min. All BIACore sensograms were analysed with the software BIACevaluation 3.2. Bulk refractive index changes were removed by subtracting the reference flow cell responses, and the average response of a blank injection was subtracted from all analyte sensograms to remove systematic artefacts. Kinetic data were globally fitted to a 1:1 Langmuir model.

Chemokine cross-linking experiments. Cross-linking experiments were performed as described previously (Alcamí et al., 1998). Briefly, 125I-labelled hCCL5 (0.4 nM) was incubated with supernatant of uninfected (Mock) or PRV-infected Vero cells, in the presence of PAA, collected 16 h post-infection. In parallel experiments, 125I-labelled hCCCL5 (0.4 nM) was also incubated with purified PRV-gGs. The incubation was done in binding buffer containing 20 mM HEPES, pH 7.5 and 0.1% BSA at room temperature. Then, bis(sulfosuccinimidyl) suberate (BS3) (Pierce) dissolved in 5 mM sodium citrate pH 3 was added to the reaction to a final concentration of 5 mM and incubated for 30 min. The samples were separated by SDS-PAGE, fixed with H₂O containing 20% methanol, 10% acetic acid for 30 min. Then, the gel was dried and exposed to Kodak films for different periods of time.

Recombinant PRV viruses. XGF-N and XGF-G PRV recombinant viruses defective in gG were made by cotransfecting Vero cells with PRV NIA-3 or gIS8 DNA, respectively, and the Xhol–MluI fragment of pSD4-AG plasmid, which contains the EGFP coding gene [Xhol–Ncol fragment of pEGFP-N1 plasmid (Clontech) expressed with both promoter and poly(A) of the gG gene]. The gG promoter was generated by PCR to produce an Ncol restriction site at the gG ATG initiation codon and consists of a −517 nt sequence starting at the ATG initiation codon. The gG poly(A) includes a 357 nt fragment located immediately downstream of the gG TGA terminal codon [120 892–121 249 nt in the viral genome (Klupp et al., 2004)]. This fragment was generated by PCR, flanked by Ncol and MluI restriction sites. The recombinant gG defective progeny was selected in agar medium by fluorescent microscopy using EGFP expression as a marker and characterized by PCR and Western immunoblotting. Recombinant viruses were plaque-purified at least three times in Vero cells. The strain deficient viruses were denominated in base to include the EGFP coding sequence instead of gG (formerly gX) coding sequence of virus infection.

Chemotaxis assays. Different chemokine concentrations were placed in the lower compartment of 96-well ChemoTx System plates (Neuro Probe) with or without recombinant gG in RPMI 1640 containing 1% fetal bovine serum. MonoMac-1 or m300-19 cells (1.25 x 10⁵) were plated on top of the 3 (for MonoMac-1 cells) or 5 μm (for m300-19 cells) pore size filter. The plates were incubated at 37 °C for 2–3 h. Following the incubation period, the presence of migrated cells in the lower chamber was determined by staining the cells with 5 μl CellTiter 96 aqueous one solution cell proliferation assay (Promega) for 1–2 h at 37 °C, followed by measuring absorbance at 492 nm. In parallel, known amounts of cells were incubated with the CellTiter solution.

ACKNOWLEDGEMENTS

We thank Rocio Martin and Soledad Blanco for excellent technical assistance and Nadia Martinez-Martin for her aid with the chemotaxis assay. We thank Fernando Arenzana-Seisdedos for providing the hCXCL12x variants. We are thankful to Bernhard Mosser for providing the m300-19 cell line. This work was funded by The Wellcome Trust (UK), the Spanish Ministry of Science and Innovation, and Comunidad de Madrid. A.V.-B. is supported by a postdoctoral contract programme from the Instituto de Salud Carlos III (Spanish Ministry of Health).

REFERENCES


Glycosaminoglycan binding and oligomerization are essential for the in vivo activity of certain chemokines. Proc Natl Acad Sci U S A 100, 1885–1890.


