Analysis of the herpesvirus chemokine-binding glycoprotein G residues essential for chemokine binding and biological activity

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Running title: gG chemokine binding residues

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Summary
Viral chemokine binding proteins (vCKBP) are expressed by large DNA viruses, such as herpes- and poxviruses. vCKBP can bind chemokines with high affinity and efficiently neutralize their ability to induce cell migration. Recently, herpesvirus glycoprotein G (gG) was identified as a member of the vCKBP-4 subfamily. The structural domains of gG important for binding to chemokines and biological activity, however, are unknown. Here, we used equine herpesvirus type 1 (EHV-1) as a model to determine residues in EHV-1 gG that are involved in the processes of chemokine binding and interaction with target cells. First, comprehensive analysis of glycosylation of EHV-1 gG revealed that N-glycosylation is not required for binding of gG to chemokines, but is essential for biological activity of the protein. Second, the epitope responsible for the binding to chemokines was localized to 40 amino acids in the hypervariable region (amino acids 301-340) of the protein. Third, hybrid molecules, designed as loss and gain of function gG proteins were engineered. In these hybrid glycoproteins the hypervariable regions of EHV-1 gG, a vCKBP, and the closely related EHV-4 gG, which does not display any chemokine binding capabilities were exchanged. gG variants containing the EHV-1 hypervariable region were able to bind chemokines and were biologically active, whereas hybrid gG’s containing the corresponding region of EHV-4 gG were not. Taken together, this report is the first to provide insight into the functional residues of an alphaherpesviral vCKBP.

Introduction
Chemokines are chemoattractant molecules that regulate traffic and effector functions of leukocytes, and are key regulators of inflammation and immune surveillance (1). Given the central role chemokines play in antiviral defense, it is not surprising that many viruses have evolved strategies to thwart and/or modulate chemokine function to their benefit. Several virus-encoded proteins with chemokine modulatory properties have been identified, mainly in herpesviruses and poxviruses, based on their sequence similarities with host chemokines or chemokine receptors (2-5). In many cases, viral chemokine modulators are pirated from host genomes. In addition, some viruses have been shown to express viral chemokine binding proteins (vCKBP) which do not share any sequence similarity with known chemokines or chemokine receptors (6;7). vCKBP can bind chemokines with high affinity and efficiently neutralize their ability to establish a gradient along which immune cells migrate. vCKBP are divided in four subfamilies, based on their origin and structures. Proteins from the vCKBP-1 and vCKBP-2 subfamilies are encoded by poxviruses and their chemokine binding profiles have been studied in much detail (7;8).
The crystal structure of the 35kDa vCKBP (also designated viral chemokine inhibitor, vCCI) of cowpox virus and a vCKBP encoded by the vaccinia virus A41L have been resolved, giving better insights in the mechanism of action of one family of vCKBP (9;10). The vCKBP M3 is encoded by the murine gammaherpesvirus-68 (MHV-68) and is the only member of the vCKBP-3 subfamily (11;12). The structure of M3 has also been determined, which is remotely related to that of vCKBP-2 members albeit with a completely different connecting topology (13). The most recently published vCKBP, constituting the vCKBP-4 subfamily, is glycoprotein G (gG) which is encoded by several alphaherpesviruses (6;7). No data on the structure of gG are available to date and the functional domains of this glycosylated vCKBP, involved in chemokine binding and biological activity have thus far remained undetermined.

Members of the Alphaherpesvirinae have a narrow in vivo host range, a short replication cycle and the capacity to establish a lifelong latent infection (14). The length of their linear, double-stranded DNA genome varies between approximately 120 and 180 kbp and generally consists of regions of unique sequences flanked by direct or inverted repeat sequences. This subfamily includes the human pathogens herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2) and varicella zoster virus (VZV), the causative agents of cold sores, genital ulcerous disease, and chickenpox/shingles, respectively. A number of animal viruses related to these human viruses are also allocated to this group, among them equine herpesvirus type 1 (EHV-1) that, genetically and biologically, is most similar to VZV. Significant progress is currently made in demonstrating the potential of alphaherpesviral gG to intercept chemokine networking at different levels (6;7;15). Hereby, EHV-1 has been used most frequently as a model to investigate the pathophysiological importance of gG binding on chemotaxis and cell trafficking, both in vitro and in vivo. EHV-1 initially replicates in the upper respiratory whereupon B and T cells in the tonsils become infected, which ultimately leads to a leukocyte-associated viremia. The viremia enables EHV-1 to reach internal organs and virus infection can result in abortion or disorders of the central nervous system (16). A close relative of EHV-1, EHV-4, generally remains localized to the upper respiratory tract and rarely can escape immune control and cause systemic disease (17;18). Both viruses encode gG as a type I integral membrane protein with their N-terminal part relatively conserved but their extracellular C-terminal part highly divergent (19). The full-length, membrane-anchored form of EHV-1 gG has vCKBP properties as recombinant gG expressed on the surface of insect cells was capable of binding human CXCL1 and CXCL8 (also designated IL-8) (6). Secreted EHV-1 gG (sgG) has also been shown to bind a broad range of chemokines with high affinity and in a species-independent manner: EHV-1 sgG is capable of interfering with chemotaxis of human and equine neutrophils induced by human or equine IL-8, respectively, as well as that of murine neutrophils mediated by the IL-8 relative KC in vitro (6;20). Another study demonstrated a functional interference of EHV-1 gG with chemotaxis of murine neutrophils and macrophages induced by the proinflammatory chemokine CCL3 (21). In addition, EHV-1 sgG was shown to have a significant effect on the migration of immune cells into murine airways in vivo (20;21). Interestingly, whereas EHV-1 gG clearly has vCKBP activities both in vitro and in vivo, no such role was found for its EHV-4 counterpart (6;20).

Since the structural features of gG important for binding to chemokines are unknown, the objective of this study was to systematically evaluate and analyze residues of EHV-1 gG to provide insights into the region(s) of alphaherpesviral gG responsible for the binding to chemokines and its bioactivity as a vCKBP.

**Experimental Procedures**

**Cells.** Equine fibroblasts (NBL6) were grown and maintained in growth medium (MEM supplemented with 10% FBS, 100 U/ml penicillin and 0.1mg/ml streptomycin) at 37°C under a 5% CO₂ atmosphere. Equine neutrophils were isolated by density centrifugation of heparinized blood from healthy horses on a discontinuous Percoll gradient as described previously (22). After washing, cells were resuspended in MEM at a concentration of 1x10⁵ cells/ml and used immediately in chemotaxis assays.

**Plasmids.** The His-tagged secreted fragment of EHV-1 gG (pBac11EHV-1sgG) has been
described previously (20). A similar strategy was used to amplify and add a His tag to the secreted region of EHV-4 gG. Briefly, DNA was purified from NBL6 cells infected with the EHV-4 isolate VLS#829 (20) using the Gentra Puregene cell kit (Qiagen, Valencia, CA), following manufacturer’s instructions. This DNA served as a template for PCR amplification of the truncated secreted version of EHV-4 gG. Primers containing CpoI restriction sites within the 5’ overhang, EHV-4 gGFCpoI and gGRCpoI (Table I), allowed insertion of EHV-4 gG into the pBac11 vector (Novagen, Madison, WI). DNA purified from correct clones were subsequently electroporated into DH10Bac cells (Invitrogen) harboring the baculovirus BAC sequence exactly as detailed previously (20).

For bacterial expression of EHV-1 sgG plasmid pBadsG-His was generated. The expression cassette, which includes EHV-1 sgG and a C-terminal 6X His tag, was amplified from pBac11EHV-1sgG. To allow efficient translocation into the periplasm, the OmpA signaling sequence was introduced as an N-terminal fusion protein with EHV-1 sgG. EcoRI and a HindIII restriction sites in the 5’ primer overhangs of gG-OmpA-for and EHV-gG-His-Rev (Table I) facilitated the insertion of secreted EHV-1gG into the pBad24 vector (ATCC, Manassas, VA), which was used for inducible expression of gG upon addition of arabinose. Recombinant plasmids containing EHV-1 gG were isolated and their identity confirmed via restriction digestion and sequencing. For periplasmatic expression of proteins spanning the extracellular part of EHV-1 gG, a standard vector containing an N-terminal OmpA signaling sequence and a C-terminal 6X His-Tag was generated based on pBAD24. Briefly, the OmpA-His cassette was generated by PCR using the overlapping primer 5’GGAAATTCCAGTGAGAGAGA as forward and 5’GCAAGCTTCTCTAGATGCTGTGATGATGGTAGTGAGGCTGG as reverse primer respectively, and was cloned into pBAD24 via the EcoRI and HindIII restriction sites (underlined). A StuI restriction site between OmpA and the 6X His-tag facilitated the insertion of the EHV-1 gG peptides, which were 100 amino acids (aa) in length and overlapped by 50 aa each (primers A-F are given in Table I).

Gene splicing by overlap extension PCR (SOE by PCR). To exchange the hypervariable regions of EHV-1 and EHV-4 gG (Figure 1A) recombinant plasmids pBac11EHV-4sgG and pBac11EHV-1sgG were used as templates to create chimeras using SOE by PCR as described previously (23). Briefly, SOE by PCR involves different sets of primers (Table II) to create three separate PCR products (Figure 1B). With this technique, two sgG chimera PCR products were created: EHV-1sgG259-359, containing the hypervariable region of EHV-4 gG within the EHV-1 gG backbone (loss-of-function chimera) and EHV-4sgG258-325, containing the hypervariable region of EHV-1 gG within the EHV-4 gG backbone (gain-of-function chimera) (Figure 1C). The final PCR products were cloned into the pBac11 for baculovirus expression using CpoI primer pairs as described in Table I (pBac11EHV-1sgG259-359 & pBac11EHV-4sgG258-325).

Protein expression and purification. Recombinant baculovirus bacmid DNA encoding His-tagged secreted gG’s (EHV-4 sgG-His, EHV-1sgG259-359-His or EHV-4sgG258-325-His) were transfected into Sf9 insect cells using Cellfectin (Invitrogen) and recombinant viruses were plaque purified twice. For the expression of His-tagged secreted gG’s, Sf9 cells were infected at a multiplicity of infection (MOI) of 1.0 and supernatants were collected and dialyzed overnight against PBS (2.5mmol NaH2PO4, 7.5 mmol Na2HPO4, 145mmol NaCl, pH 7.2). For bacterial expression of His-tagged EHV-1 sgG (or EHV-1 gG peptides), E. coli strain BL21 (Novagen) was transformed with pBaD24sgG-His DNA and incubated in a shaker (220 rpm) at 32°C in LB medium containing 100µg/ml ampicillin and 34 µg/ml chloramphenicol until an OD650 of 0.5 was reached. To induce gene expression, arabinose (Sigma, 1% final concentration) was added to the cell culture for 3 h. Subsequently, cells were harvested by centrifugation at 4,000Xg for 15 min at 4°C. Following centrifugation, cells were resuspended in buffer P (100 mM Tris-Cl (pH 8.0), 500 mM sucrose, 1 mM Na2-EDTA) and incubated for 30 min on ice. To eliminate spheroplasts, the suspension was centrifuged twice at 4,000Xg for 15 min, followed by 27,000Xg for 15 min, and dialyzed twice against PBS at 4°C.
Protein expression was verified by western blotting with antibodies against EHV-1 gG, EHV-4 gG (24) and/or the polyhistidine tag. For purification of His-tagged proteins, TALON resin (Clontech, La Jolla, CA) was used, exactly as described previously (25). Fractions containing His-tagged proteins were collected and their purity was determined by SDS-PAGE and Coomassie Blue staining. Protein concentrations were determined with a Bradford assay following the manufacturer’s instructions (Biorad).

**Enzymatic deglycosylation of EHV-1 gG.** O-deglycosylation of secreted EHV-1 gG was performed with 10mU neuraminidase (Sigma) for 1h at 37°C, followed by 1mU O-glycosidase (Sigma) for 4h at 37°C. For N-deglycosylation reactions, peptidase N-glycosidase F enzyme (PNGase F) (NEB) was used according to the manufacturer’s instructions. Briefly, EHV-1 sgG was incubated first for 10 min at 100°C in 1X denaturation buffer (NEB) and then in the presence of PNGase F enzyme for 1 h at 37°C in 1X G7 buffer (NEB) containing NP-40.

**Western blotting.** Western blot analyses were performed essentially as described previously (20). To detect gG, polyclonal rabbit anti-gG antibodies (1:500) were used that recognize the hypervariable domain of gG of EHV-1 or EHV-4 (both antisera were kindly provided by Drs. C. Hartley and M. J. Studdert) (24). Penta-His (1:1000; Qiagen) and anti-HA (1:1000; Zymed) antibodies were used to detect His-tagged and HA-flagged proteins respectively. Anti-mouse (1:7,500) or anti-rabbit (1:5,000) immunoglobulin G (IgG) peroxidase conjugates were obtained from Jackson Immunoresearch Laboratories and Amersham Biosciences respectively.

**Peptides.** Small peptides of 20 aa in length spanning the hypervariable region of EHV-1 gG were commercially synthesized by GenScript (Piscataway, NJ) and are shown in Table III.

**Enzyme-Linked Immunosorbent Assay (ELISA).** Wells of a microtiter plate (Nunc, Rochester, NY) were coated with recombinant equine IL-8 (5µg/ml in coating buffer consisting of 15mmol Na2CO3, 35mmol NaHCO3, pH 9.6) overnight at 4°C. Wells were blocked with 3% skim milk powder in PBS for 2h at RT and subsequently washed with PBS containing 0.05% (v/v) Tween 20. To detect the binding of recombinantly expressed His-tagged gG proteins, a dilution series of the protein was added to the wells and incubated for 2h at 37°C, followed by penta-His antibodies (1:500) for 1 h and peroxidase-conjugated anti mouse immunoglobulin (Ig)G (1/5000) for 1h at 37°C, diluted in 0.3% skim milk powder in PBS. To determine the inhibitory effect of the 20 aa-long peptides on the binding of baculovirus-expressed EHV-1 sgG, a dilution series of the small peptides (or a combination of the small peptides) was first added to the coated wells for 2h at 37°C, followed by baculovirus-expressed His-tagged EHV-1 sgG (6µg/ml) for 1h at 37°C. Penta-His antibodies and peroxidase-conjugated anti-mouse IgG were used as described above. Binding of peroxidase-conjugated antibodies was colorimetrically determined at 450nm with a microplate reader (Bio-Tek, Richmond, VA) and BD OptEIA reagent A and B (BD Biosciences, San Jose, CA) as a substrate. In general, wells were washed three times after coating and blocking, and nine times between other steps.

**Chemotaxis assays.** Chemotaxis assays were performed as previously described (20). Briefly, 50 ng/ml of equine IL-8 (Serotec, Oxford, UK) were pre-incubated for 30min at 37°C with recombinantly expressed gG constructs (0.3 µg/ml) and applied to the lower chambers of 12-well Costar Transwell plates (Corning Costar Co., Cambridge, MA). Wells were covered with a polycarbonate membrane with a 3 µm pore size, 1x10^5 equine neutrophils were added to the top chamber and assay plates were incubated at 37°C for 45min. Equine IL-8 and medium alone were included in each experiment to serve as positive and negative controls, respectively. After incubation, cells in the lower chamber were stained, counted under a light microscope (Zeiss Axiosvert 25) and expressed as percent chemotaxis based on the amount of input cells, unless indicated otherwise.

**Statistical analysis.** Student’s t-test for paired data was used to test for statistically significant differences. Data given are the means and bars show standard deviations.

**Results**

N-glycosylation sites present on EHV-1 gG do not bind chemokines directly, but are essential for biological activity of the vCKBP. In the
original description of alphaherpesviral vCKBP’s it was discussed that glycosylation of gG might be important for proper function as a vCKBP (6). However, since no information was available on the exact glycosylation status of EHV-1 gG, we performed enzymatic deglycosylations of EHV-1 gG to evaluate the extent of post-translational modifications of EHV-1 gG and investigate their importance for chemokine binding. The programs NetNGlyc 1.0 and NetOglyc 3.1 were used to predict possible N- and O-glycosylation sites on gG. First, as EHV-1 gG was predicted to contain three O-linked glycosylation sites, we performed enzymatic O-deglycosylation of EHV-1 gG using supernatants of EHV-1-infected cells. However, western blot analysis with anti-EHV-1 gG antibodies showed that removal of O-linked carbohydrates from EHV-1 gG did not increase the mobility of the protein, suggesting that EHV-1 gG either does not contain O-linked glycans or that there are too few O-glycans added to the protein that would result in a discernable change of mobility as assayed by SDS-PAGE (Figure 2A). We were able to exclude ineffective cleavage of O-glycans and proper action of O-glycosidase and neuraminidase because we detected increased mobility of the HA-flagged secreted gC protein from supernatants of VZV-infected cells that was used as a control (Figure 2A). The gC of the VZV vaccine strain P-Oka is predicted to contain 24 O-linked glycosylation sites, based on the NetOglyc prediction server (version 3.1). As to the importance of O-glycosylation for biological activity of gG, we used enzymatically O-deglycosylated gG in our previously established chemotaxis assays (20). It was observed that gG treated with enzymes removing O-linked sugars was able to interfere with the migration of equine neutrophils in the IL-8 induced chemotaxis assay to the same extent as seen with untreated gG (Figure 2B). Taken together, these results indicated that post-translational modification by addition of O-glycans is not important for proper function of the vCKBP encoded by EHV-1 gG. We also performed N-glycosidase F treatment of EHV-1-infected supernatants, as EHV-1 gG is predicted to contain four N-linked glycosylation sites. This treatment resulted in an increased mobility of gG from 51 to 41 kDa after SDS-PAGE, and this lower apparent molecular weight exactly corresponded to the predicted molecular weight of the unglycosylated form of secreted gG (Figure 2A). In addition, the VZV gC-HA control predicted to contain five N-linked glycosylation sites (26), also exhibited increased mobility after N-glycosydase F treatment (Figure 2A). The N-deglycosylated gG was dialyzed twice against PBS, as it was observed that the buffer used for enzymatic deglycosylation had an inhibitory effect on neutrophil migration by itself (data not shown). Testing the N-deglycosylated gG in chemotaxis assays revealed that removal of N-linked glycans impaired vCKBP activity of EHV-1 gG, as the viral protein was no longer capable of reducing neutrophil migration (Figure 2B). To corroborate this finding, a second experimental approach was employed and secreted EHV-1 gG was recombinantly expressed in E. coli to yield His-tagged EHV-1 gG without glycosylation. As seen in Figure 3A, expression of gG by bacteria resulted in a protein of around 41 kDa, which exactly corresponds to the size of N-glycosidase F treated gG (Figure 2A). As a control, the glycosylated His-tagged gG expressed in insect cells was included and migrated as a glycosylated gG protein of approximately 51 kDa, as shown previously (20) (Figure 3A). First, E. coli-expressed gG-His protein was evaluated with an ELISA for binding to recombinant IL-8. The experiment showed that E. coli-expressed gG was capable of binding to IL-8 with the same affinity as glycosylated gG, indicating that glycosylation sites on gG are not important for direct binding to chemokines (Figure 3B). The His-tagged baculovirus-expressed CCL-2 protein (20) was used as a negative control to exclude non-specific binding of the His-tag to coated IL-8 and did not result in any binding (Figure 3B). Non-glycosylated gG used in chemotaxis assays, however, did not result in a reduction of neutrophil migration, which is in contrast to the situation observed with glycosylated gG (Figure 3C). This result strongly indicated a functional importance of N-glycosylation sites on the vCKBP gG with respect to biological activity and its thwarting of neutrophil migration. Since bacteria are known to produce endotoxins which induce activation of neutrophils and can result in altered locomotory properties (27), chemotaxis assays were repeated in the presence of 10ng/ml lipopolysaccharides (LPS, Sigma). LPS by itself did not result in migration of neutrophils in our chemotaxis assay.
and the observed migration of 2.0±1.0% of neutrophils was not significantly different from the 3±1.2% normally seen with the medium alone control (data not shown).

Taken together, we concluded from the experiments that EHV-1 gG does not appear to have any apparent O-glycosylation, whereas addition of N-linked carbohydrates were confirmed for EHV-1 gG. Moreover, it was shown that N-glycosylation of EHV-1 gG is dispensable for binding of the vCKBP to chemokines, but does have a functional effect, likely by sterical interference with binding sites on chemokines, which are important for proper chemotactic function.

Identification of the hypervariable region of EHV-1 gG as the region responsible for binding to chemokines. To identify the exact region within EHV-1 gG responsible for binding to chemokines, we created 100 aa His-tagged peptides, with a 50 aa overlap, spanning the whole region of secreted gG (Figure 4A). The peptides were expressed in E. coli and expression was verified by western blot analysis using penta-His antibodies (data not shown). The 100 aa His-tagged peptides were used in ELISA to evaluate their binding to recombinant IL-8. Full-length His-tagged sgG produced in Sf9 cells was used as a positive control and resulted in binding to IL-8 (Figure 4B). E. coli-produced peptides A, B, C and D (spanning aa 21-270) did not bind to IL-8, while peptides E and F (spanning aa 221-358) clearly bound to the chemokine (Figure 4B). These two latter peptides spanned the so-called hypervariable region of EHV-1 gG, which consists of approximately 100 aa of the extracellular part of EHV-1 gG directly adjacent to the transmembrane domain. Unlike other glycoproteins and the remainder of gG, the hypervariable region is highly divergent from the corresponding region of the closely related EHV-4 and contains strong type-specific epitopes which are used for serological differentiation of the viruses (19).

Previous chemotaxis experiments with supernatants of EHV-4 infected cells showed that gG of EHV-4 is unable to inhibit chemotaxis of equine neutrophils, which is in striking contrast to what is observed with EHV-1 gG (20). Here, we expressed secreted His-tagged EHV-4 gG in Sf9 cells using a recombinant baculovirus and were unable to observe any inhibitory effect on neutrophil chemotaxis (Figure 6B). To test our hypothesis that the hypervariable region of EHV-1 gG is responsible for binding to chemokines, we constructed several gG chimeras, where the hypervariable region of EHV-1 gG and EHV-4 gG were exchanged. Therefore, the pBac11EHV-1sgG and pBac11EHV-4sgG plasmids were used as templates to generate gG chimeras, using gene splicing by overlap extension PCR (Figure 1). Western blot analysis using penta-His antibodies showed expression of all His-tagged gG constructs (Figure 5). As expected only EHV-1 sgG and EHV-4sgG258-325, which contains the EHV-1 hypervariable region in an EHV-4 backbone, showed a band of around 55kDa when using anti-EHV-1 gG antibodies (Figure 5). EHV-4 sgG and EHV-1sgG258-359, the chimera containing the variable region of EHV-4 gG in an EHV-1 background, were detected as proteins of 59kDa in size with anti-EHV-4 gG antibodies (Figure 5). Examination of the binding activities of the gG chimeras using the IL-8 ELISA showed that replacing the hypervariable region of EHV-1 with the one of EHV-4 (EHV-1sgG259-329) completely abolished binding to the chemokine (Figure 6A). In contrast, inserting the EHV-1 hypervariable region into the EHV-4 gG(EHV-4sgG258-325), which in its original form is unable to bind chemokines, partially restored binding to coated-IL8 (Figure 6A). Similar results were obtained when evaluating the functionality of the gG chimeras with chemotaxis assays. EHV-1sgG259-359, the loss-of-function EHV-1 gG containing the EHV-4 hypervariable region, could no longer interfere with neutrophil migration, whereas the gain-of-function, EHV-4sgG258-325, was able to inhibit chemotaxis, even though statistical significant differences were not reached (Figure 6B).

In order to further define the exact domain in the hypervariable region of EHV-1 important for chemokine binding, peptides of 20 aa in length, spanning the aa region 261 to 340 of EHV-1 gG, were used in an inhibition ELISA. It was observed that neither P1 (aa 261-280) nor P2 (aa 281-300) were able to competitively inhibit the binding of insect cell produced EHV-1 sgG to coated IL-8 (Figure 7). In contrast, P3 (aa 301-320) showed an intermediate inhibition of around 50% and addition of P4 (aa 321-340) resulted in an approximately 50% inhibition of gG binding.
confirming specific blocking of vCKBP binding to the chemokine was obtained by a combination of P3 and P4 which resulted in an inhibitory effect up to 73% and indicated a synergistic effect of these two peptides with respect to chemokine-vCKBP interaction (Figure 7).

Taken together, these data clearly showed that the hypervariable region of EHV-1 harbors the epitope within the alphaherpesvirus gG that mediates binding to chemokines. Replacement of this region also altered the functionality of gG as a vCKBP. Using E. coli-produced peptides and peptides of 22 aa in length, this region was further defined spanning from aa 301 to 340 of EHV-1 gG.

Discussion

Over the past few years, much progress has been made in defining the potential of alphaherpesviral glycoprotein G (gG) to act as a viral chemokine binding protein (vCKBP) (15). Especially gG of equine herpesvirus type 1 (EHV-1) has recently been intensively studied for its potential to interfere with chemotaxis of immune cells in vitro and in vivo (20;21;28). However, no experimental data have been reported so far on the identification of regions and/or residues on gG responsible for the binding to chemokines.

First, the importance of glycosylation sites on EHV-1 gG with regard to its chemokine binding capabilities was investigated. An extensive carbohydrate analysis of gG of the closely related alphaherpesvirus EHV-4, using deglycosylation enzymes and inhibitor of carbohydrate addition had shown that secreted EHV-4 gG is extensively N-glycosylated with complex-type carbohydrate side-chains, but does not undergo O-linked glycosylation (29). Since no such data were available on glycosylation of EHV-1 gG, we used enzymatic deglycosylation to study the glycosylation status of EHV-1 gG in more detail and to evaluate the role of glycosylation for proper function of EHV-1 gG as a vCKBP. It was found that EHV-1 gG does not appear to contain O-linked carbohydrates but does contain N-linked carbohydrates, which mimicks what has been described previously for EHV-4 gG. By using bacterially expressed gG, it was also shown here that N-glycosylation of EHV-1 gG is not important for its binding to chemokines. This is in contrast to an earlier report, where gG glycosylation was shown to be important for binding to chemokines as supernatants collected from tunicamycin lacked chemokine binding activity (6). This discrepancy might be explained by differences in the experimental approach to obtain non-glycosylated gG. Tunicamycin is known to inhibit the en bloc addition of the N-linked oligosaccharide precursor to nascent polypeptides in the endoplasmatic reticulum, and it has been previously described for EHV-4 gG that treatment with tunicamycin resulted in the accumulation and potentially misfolding of gG in infected cells without any detectable secretion of gG in the supernatant (29). Therefore it is highly likely that the lack of chemokine binding activity of supernatants from tunicamycin-treated EHV-1-infected cells is due to the lack of proper secretion of gG and presence of the vCKBP in supernatants. Interestingly, despite the capability of non-glycosylated EHV-1 gG to properly bind chemokines as shown in this study, deglycosylated gG was no longer able to inhibit the migration of immune cells. One possible explanation for this observation is that glycosylation sites, although not important for direct binding to chemokines, are of critical importance for the proper function of EHV-1 gG by providing steric interference. It is conceivable that chemokines are engulfed in a pocket of gG and domains important for binding to chemokine receptors and/or glycosaminoglycans on immune cells might be blocked.

In another set of experiments, we further defined the epitope in EHV-1 gG responsible for the binding to chemokines. Binding experiments using peptides covering parts of gG and chimeric molecules in which regions of EHV-1 and EHV-4 gG were interchanged allowed us to map the chemokine-binding region to 40 aa located in the hypervariable region of EHV-1 gG. In addition, we were able to provide an explanation as to why EHV-1 gG constitutes a vCKBP, whereas gG of the closely related EHV-4 lacks chemokine binding properties. The gG amino acid sequences of both viruses share 72% homology, although approximately 100 amino acids of the ectodomains are highly divergent and harbor type-specific epitopes (19). Interestingly, upon insertion of the highly divergent region of EHV-4 into EHV-1 gG, the vCKBP, we observed a loss-of-function and this chimera was no longer capable of interfering...
with the migration of equine neutrophils. Final proof for this region being important for vCKBP function gave an EHV-4 chimera which harboured the hypervariable region of EHV-1. This molecule exhibited vCKBP properties and a reduction in neutrophil migration was observed with this gain-of-function mutant protein. Such mapping studies and site directed mutagenesis experiments, both on a protein-only level but also in the virus background could be beneficial for determining the importance of alpha herpesvirus vCKBP's with regard to the development of therapeutics and/or the rational design of more safe and efficacious vaccines. In general, vCKBP's do not exhibit sequence similarities with host sequences, and crystal structures of members of the vCKBP-2 and -3 subfamily indicate that vCKBP contain unique amino acid sequences and specific tertiary structures (9;13). This implies that viruses have developed unique ways of chemokine inhibition, and their vCKBP could be used as scaffolds to develop specific chemokine modulators by mutating specific regions. As for vaccine development, it will be of interest to evaluate recombinant EHV-1 in which vCKBP properties of gG are abolished by removing the hypervariable region, critical for binding to chemokines, or replacing it with the hypervariable region of EHV-4 gG. The rationale behind this concept is our recent finding that gG-specific antibodies can help to control its function as a vCKBP and might be important in preventing EHV-1 from evading the immune system (21). These findings put into question the use of gG deletion mutants as modified-live virus marker vaccines for this group of viruses. Our current hypothesis is that an EHV-1 mutant in which gG is expressed and will still be capable of inducing a gG-specific antibody response, but where vCKBP activity is removed, will be more potent with respect to their protective functions against infections. Experiments are in progress to investigate the properties of such mutant viruses in vitro and in vivo.

The present study focused on identifying the regions in gG important for binding to chemokines and biological activity. The structural features of chemokines required to bind gG, however, have not been resolved to date. For proteins of the vCKBP-1 subfamily, it has been shown that they interact with the GAG binding region located at the C-terminus of chemokines (7;30). In contrast, mapping studies of vCKBP-2 and vCKBP-3 have shown that they both interact with the N-loop of chemokines, hereby preventing chemokine binding to chemokine receptors (CR). Prevention of GAG binding is indirect the case of the latter two families of molecules and a consequence of conformational changes rather than a direct interaction (13;31-33). It has been described recently that gG, similar to members of the vCKBP-3 family, can also inhibit chemokine binding to CR and GAG; in addition, it can displace chemokines bound to GAG (6). Based on these observations it is tempting to speculate that gG interacts with the N-loop of chemokines, although structural analyses and site-directed mutagenesis studies are required to further substantiate this hypothesis.

Taken together, we are the first to show that 40 amino acids in the C-terminal region of the extracellular part of the vCKBP gG (aa 301-340 of EHV-1) encoded by alphaherpesviruses are important for its binding to chemokines. In addition, we could demonstrate that N-glycosylation sites on EHV-1 gG are essential for its biological activity. Further studies will explore the impact of mutations in this chemokine-binding region in the virus’ life cycle.

Reference List


Footnotes
Abbreviations: vCKBP: viral chemokine binding protein, gG: glycoprotein G; HSV-1 & 2: herpes simplex virus type 1 & 2; VZV: varicella zoster virus; EHV-1 & 4: equine herpesvirus type 1 & 4; sgG: secreted gG; IL-8: interleukin 8; NBL6: equine fibroblasts; MEM: minimal essential medium; FBS: fetal bovine serum; aa: amino acids; SOE: gene splicing by overlap extension PCR; PBS: phosphate-buffered saline; GAG: glycosaminoglycans; CR: chemokine receptors.

Acknowledgments: The authors are grateful to Drs. Carol Hartley and Michael Studdert for supplying antibodies. We thank Nora Thormann for help with the gG chimera constructs. This work was supported by the Harry M. Zweig Memorial Fund for Equine Research at Cornell University and grant OS143/4-1 from the Deutsche Forschungsgemeinschaft to NO.

Figure Legends
Figure 1. Generation of the gG chimeras EHV-1 sgG259-359 and EHV-4 sgG258-328. (A). The amino acid sequences of the hypervariable region of EHV-1 and EHV-4 is shown. (B). Schematic representation of the ‘gene splicing by overlap extension PCR’ (SOE by PCR). The strategy for generation of EHV-1 sgG259-359 is shown. Starting templates for the generation of EHV-4 sgG258-328 are shown between brackets. Primer b is complementary to b’ and primer c is complementary to c’. Colors used correspond to those given in Table II. (C). Schematic representation of chimeric proteins. The amino acid positions of the hypervariable region of EHV-1 and EHV-4 are shown in bold and color-coded as is depicted in B.

Figure 2. Enzymatic deglycosylations of EHV-1 gG. (A). Western blot analysis. Supernatants of EHV-1 infected cells containing secreted gG (lane 1) or control supernatants of VZV-infected cells, containing HA-flagged gC (lane 1) were subjected to O-glycosylation with 10 mU neuraminidase, followed by 1 mU O-glycosidase (lane 2). N-deglycosylation was performed using PNGase F (lane 3). Each sample was analyzed by SDS-PAGE under reducing conditions. Polyclonal rabbit anti-EHV-1 gG Ab (1/500) and anti-HA Ab (1/10000) were used to detect gG or HA-flagged gC, respectively, followed by anti-rabbit (1:5,000) IgG peroxidase. (B). Chemotaxis assays. Chemotaxis assays with equine neutrophils were performed with 50 ng/ml of equine IL-8, pre-incubated with 300 µl supernatant of EHV-1-infected cells or enzymatically
de-O- or de-N-deglycosylated supernatants of EHV-1-infected cells. Supernatants of mock-infected cells were included as a control. Data are expressed as the mean ± standard deviation of at least three independent experiments. Asterisks indicate a significant difference compared to IL-8 alone (p<0.05).

**Figure 3.** *E. coli*-expressed EHV-1 gG is still capable of binding to chemokines, but no longer inhibits chemotaxis of equine neutrophils. (A). The extracellular domain expressed as a recombinant His-tagged protein in *E. coli* was purified with TALON resin, separated by SDS-10%-PAGE and stained with Coomassie blue (lane 3). As a control, sgG expressed in insect cells using recombinant baculoviruses was included (lane 1). Lane 2 represents crude *E. coli* periplasm containing non-glycosylated sgG. (B). The binding of secreted His-tagged gG recombinantly expressed with baculovirus or *E. coli* to coated equine IL-8 was determined by ELISA. The His-tagged baculovirus-expressed CCL-2 protein was used as a negative control. The Optical Density (OD) of 6 µg/ml (grey bars) and 0.6 µg/ml (white bars) recombinant protein is shown. (C). Chemotaxis assays with equine neutrophils were performed with 50 ng/ml of equine IL-8, pre-incubated with 0.3 µg/ml of secreted gG recombinant expressed with the baculovirus system (glycosylated, black bars) or expressed by *E. coli* (non-glycosylated, white bars). Data are expressed as the mean ± standard deviation of at least three independent experiments. The 100% response equals 47.6 ± 5.5% chemotaxis. Asterisks indicate statistically significant differences (p<0.05).

**Figure 4.** His-tagged peptides, spanning the hypervariable region of EHV-1 gG, are capable of binding to chemokines. (A). Schematic overview of the different His-tagged peptides, which are 100 aa in length and were designed to cover the entire extracellular domain of gG with 50 aa overlap each. The full-length sgG is also shown and the hypervariable region is shown in the dotted line box. (B). ELISA. Binding of the His-tagged peptides (A → F, 100 aa each) to coated equine IL-8 was determined by ELISA. Full-length His-tagged sgG of EHV-1 was used as a positive control.

**Figure 5.** Western blot analysis of baculovirus-expressed His-tagged gG chimeras. Schematic representation of the recombinant His-tagged proteins are given. The hypervariable regions are color-coded as depicted in Figure 1. Recombinant purified His-tagged EHV-1 gG, EHV-1 sgG259-359, EHV-4 sgG and EHV-4 sgG258-325 were analyzed by SDS-10%-PAGE under reducing conditions. Monoclonal Penta-His Ab (1/1,000), polyclonal rabbit anti-EHV-1 gG Ab and anti-EHV-4 gG Ab (1/500), recognizing the hypervariable regions of EHV-1 and EHV-4 gG (24), were used to detect the gG proteins, followed by anti-mouse (1:7,500) or anti-rabbit (1:5,000) IgG peroxidase, respectively.

**Figure 6.** Chimeric EHV-1 sgG259-359 results in a loss-of-function variant, whereas EHV-4 sgG258-325 is a gain-of-function chimera. (A). The binding of secreted His-tagged gG proteins to coated equine IL-8 was determined by ELISA. The Optical Density (OD) of 6 µg/ml (grey bars) and 0.6 µg/ml (white bars) recombinant protein is shown. (B). Chemotaxis assays with equine neutrophils were performed with 50 ng/ml of equine IL-8, pre-incubated with 0.3 µg/ml of secreted His-tagged gG proteins. Data are expressed as the mean ± standard deviation of at least three independent experiments. The 100% response equals 46.3 ± 5.5% chemotaxis. Asterisks indicate statistically significant differences (p < 0.05).

**Figure 7.** Inhibition ELISA with peptides. The relative percentage of inhibition of full-length, insect-cell produced sgG by individual peptides was determined by an inhibition ELISA. The inhibitory effect of 20 µg/ml of P1 (aa 262-280), P2 (aa 281-300), P3 (aa 301-320), P4 (aa 321-340) and a combination of 10 µg/ml of P3 and P4 are shown. Data are expressed as the mean ± standard deviation of at least three independent experiments.
Table I: Primers used for cloning

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* Underlined sequences indicate restriction enzyme sites. Bold indicates the <i>OmpA</i> signaling sequence.
Table II. Primers for designing EHV-1 sgG<sub>259-359</sub> and EHV-4 sgG<sub>258-325</sub> chimera

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EHV-1 sgG<sub>259-359</sub> (loss of function)

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EHV-4 sgG<sub>258-325</sub> (gain of function)

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<sup>a</sup> Directionality of the primer
<sup>b</sup> Colours are in agreement with Figure 1B

Table III. Peptides covering the hypervariable region of EHV-1 gG

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<td>P4</td>
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<sup>a</sup> Sequences are given in the single-letter amino acid code
Figure 1: Van de Walle et al.
Figure 2: Van de Walle et al.
Figure 3: Van de Walle et al.
Figure 4: Van de Walle et al.
Figure 5: Van de Walle et al.
Figure 6: Van de Walle et al.
Figure 7: Van de Walle et al.