Human Herpesvirus 8 Envelope Glycoprotein K8.1A Interaction with the Target Cells Involves Heparan Sulfate

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Human herpesvirus 8 (HHV-8) or Kaposi’s sarcoma-associated herpesvirus K8.1 gene encodes for two immunogenic glycoproteins, gpK8.1A and gpK8.1B, originating from spliced messages. The 228-amino-acid (aa) gpK8.1A is the predominant form associated with the virion envelope, consisting of a 167-aa region identical to gpK8.1B and a 61-aa unique region (L. Zhu, V. Puri, and B. Chandran, Virology 262:237–249, 1999). HHV-8 has a broad in vivo and in vitro cellular tropism, and our studies showed that this may be in part due to HHV-8’s interaction with the ubiquitous host cell surface molecule, heparan sulfate (HS). Since HHV-8 K8.1 gene is positionally colinear to the Epstein-Barr virus (EBV) gene encoding the gp350/gp220 protein involved in EBV binding to the target cells, gpK8.1A’s ability to interact with the target cells was examined. The gpK8.1A without the transmembrane and carboxyl domains (ΔTMgpK8.1A) was expressed in a baculovirus system and purified. Radiolabeled purified ΔTMgpK8.1A protein bound to the target cells, which was blocked by unlabeled ΔTMgpK8.1A. Unlabeled ΔTMgpK8.1A blocked the binding of [3H]thymidine-labeled purified HHV-8 to the target cells. Binding of radiolabeled ΔTMgpK8.1A to the target cells was inhibited in a dose-dependent manner by soluble heparin, a glycosaminoglycan (GAG) closely related to HS, but not by other GAGs such as chondroitin sulfate A and C, N-acetyl heparin and de-N-sulfated heparin. Cell surface absorbed ΔTMgpK8.1A was displaced by soluble heparin. Radiolabeled ΔTMgpK8.1A also bound to HS expressing Chinese hamster ovary (CHO-K1) cells, and binding to mutant CHO cell lines deficient in HS was significantly reduced. The ΔTMgpK8.1A specifically bound to heparin-agarose beads, which was inhibited by HS and heparin, but not by other GAGs. Virion envelope-associated gpK8.1A was specifically precipitated by heparinagarose beads. These findings suggest that gpK8.1A interaction with target cells involves cell surface HS-like moieties, and HHV-8 interaction with HS could be in part mediated by virion envelope-associated gpK8.1A.
following findings: (i) HHV-8 infection of HFF cells was inhibited in a dose-dependent manner by soluble heparin, a glycosaminoglycan closely related to HS; (ii) enzymatic removal of HFF cell surface HS with heparinase I and III reduced HHV-8 infection; (iii) soluble heparin inhibited the binding of radiolabeled HHV-8 to human B-cell lines, embryonic kidney epithelial (293) cells, and HFF cells, suggesting interference at the virus attachment stage; (iv) cell surface-adsorbed HHV-8 was displaced by soluble heparin; and (v) radiolabeled HHV-8 also bound to wild-type HS expressing Chinese hamster ovary (CHO-K1) cells. In contrast, binding of virus to mutant CHO cells deficient in HS was significantly reduced. These data suggested that the gamma-2-HHV-8 is adsorbed to cells by binding to cell surface HS-like moieties. In this respect, gamma-2-HHV-8 resembles some members of the alpha (herpes simplex virus type 1 [HSV-1], HSV-2, pseudorabies virus [PRV], bovine herpesvirus 1 [BHV-1]), beta (HMV, HHV-7), and gamma-2 (BHVs-4)-herpesviruses, where the initial virus-cell interaction also involves the binding to the cell surface HS (21, 24, 30, 31, 33, 35, 37, 46, 47, 49, 51, 57).

The identity of HHV-8 envelope glycoprotein(s) involved in the interaction with HS is not known. HHV-8 encodes for more than 80 ORFs, and ORFs 4 to 75 are designated based on the similarity to herpesvirus saimiri (HVS) ORFs (1). HHV-8 unique ORFs are designated with the prefix K (36, 43). HHV-8 has counterparts to other herpesviruses glycoproteins such as gB (ORF 8), gH (ORF 22), gM (ORF 39), and gL (ORF47) (36, 43). In addition, K1 and K8.1 genes encode for glycoproteins unique for HHV-8 (10, 36, 43). We have previously reported the identification of cDNAs originating from the HHV-8 K8.1 gene encoding two ORFs from spliced messages (10). One cDNA encoded for a 228-amino-acid (aa) protein designated gpK8.1A and contains a signal sequence, transmembrane domain, and four N-glycosylation sites. The splicing event has generated the transmembrane domain in the gpK8.1A ORF not seen in the genomic K8.1 ORF. Another cDNA encoded for an ORF of 167 aa, designated gpK8.1B. This protein has three N-glycosylation sites and shares similar amino and carboxy termini with ORF K8.1.A but with an in-frame deletion (10). Our studies with human sera demonstrated the immunogenic nature of gpK8.1A and gpK8.1B (10, 61). Using monoclonal antibodies (MAbs), we have also shown that gpK8.1A and gpK8.1B contain N- and O-linked sugars and that gpK8.1A is the predominant form detected within the infected cells and the virion envelopes (60).

HHV-8 K8.1 gene is positionally colinear to the glycoprotein genes in the members of gammaherpesvirus group such as the Epstein-Barr virus (EBV) gene encoding the major envelope glycoproteins gp350 and gp220 (22), gp150 of murine gammaherpesvirus 68 (MHV-68) (53), HVS ORF 51 gene (1), and the BOREFD1 gene of BHV-4 (36, 43). EBV gp350/gp220 glycoprotein has been studied extensively and shown to be involved in the binding of the virus to the target cells (22). HHV-8 gpK8.1A shows several similarities to the EBV glycoproteins. Like EBV gp350/gp220, HHV-8 gpK8.1A elicits a strong human humoral immune response (61) and is a virion envelope- and infected cell membrane-associated glycoprotein containing both N- and O-linked sugars. Because of these similarities to EBV gp350/gp220, we examined the ability of HHV-8 gpK8.1A to interact with the target cells. We expressed gpK8.1A without the transmembrane and carboxyl domains (ΔTMgpK8.1A) in the baculovirus system and purified the protein. Using radiolabeled purified ΔTMgpK8.1A, we show that gpK8.1A interaction with target cells involves cell surface HS-like moieties. These results suggest that HHV-8 interaction with HS could be in part mediated by virion envelope gpK8.1A.

MATERIALS AND METHODS

Cells. HFF cells, 293 cells, CHO-K1 cells (ATCC CCL-61), HS-deficient CHO derivative pgS-D677 cells (ATCC CRL-2244), HS- and chondroitin sulfate-deficient CHO derivative pgS-A745 cells (ATCC CRL-2242) (18, 32), BCB1 cells (HHV-8- human B cells) (40, 50), and BJAB cells (HHV-8- human B cells) were used in these studies. HFF and 293 monolayer cells were grown in Dulbecco modified Eagle medium (DMEM; Gibco-BRL, Grand Island, N.Y.) supplemented with 2 mM glutamine, 10% fetal bovine serum (FBS), and antibiotics. Suspension cultures of 293 cells were grown in 293SF medium (Gibco-BRL). Adult human dermal microvascular endothelial cells (HMEVEC-d Ad; CC-2543; Clonetics, San Diego, Calif.) were grown in endothelial growth medium (EGM; CC-3202; Clonetics). Monolayers of CHO-K1, pgS-D677, and pgS-A745 cells were grown in Ham's F-12K medium (Gibco-BRL) supplemented with 10% FBS and antibiotics. Suspension cultures of BCB1 and BJAB cells were grown in RPMI 1640 medium with glutamax 1 (Gibco-BRL) supplemented with 10% FBS and antibiotics. Spodoptera frugiperda ovarian cells (Sf9) were grown in TNM-FH insect medium (PharMingen, San Diego, Calif.).

Antibodies. The production and characterization of MAbs against gpK8.1A and ORF 59 have been described previously (11, 60). High-titer-antibody-containing ascitic fluids were made by injecting hybridoma cells intraperitoneally into pristane-primed BALB/c mice. Immunoglobulin G (IgG) antibodies from the ascitic fluid and normal mouse sera were purified on protein A-Sepharose columns (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Protein concentrations were adjusted to 1 mg/ml with phosphate-buffered saline (PBS; pH 7.4), and aliquots were stored at −20°C. Rabbit polyclonal antibodies raised against the baculovirus-expressed purified glutathione S-transferase–HHV-8 latency-associated ORF 73 protein (34, 58, 61) were used as a control.

Construction, expression, and purification of recombinant HHV-8 ΔTMgpK8.1A. The 576-bp ΔTMgpK8.1A gene region encoding aa 1 to 192 lacking the transmembrane and the carboxyl domains was amplified from the full-length HHV-8 gpK8.1A cDNA (10) using the primers ΔTMgpK8.1A forward (5′-TTC CGC GTC AGC TCA TGA GTT CAC ACA AGA-3′ with a SacI site) and ΔTMgpK8.1A reverse (5′-GAT GGG TGC GTA CCT CGT CAT TGT AGT-3′ with a KpnI site) (Advantage cDNA PCR Kit; Clontech, Palo Alto, Calif.). The ΔTMgpK8.1A PCR product was cloned into the pAcHLT-A baculovirus transfer vector (PharMingen) and verified by sequencing. To generate the recombinant baculovirus, ΔTMgpK8.1A-pAcHLT-A plasmid was cotransfected with Baculogold DNA (PharMingen) into Sf9 insect cells. Recombinant viruses were purified three times before use by ultracentrifugation.

His-tagged ΔTMgpK8.1A was expressed in Sf9 cells and purified using nickel columns (PharMingen) according to the manufacturer's recommendations. Briefly, Sf9 cells were infected with ΔTMgpK8.1A-baculovirus and, at 2 days postinfection, the cells were labeled with [35S]methionine for 20 h. Cell pellets were lysed with lysis buffer (10 mM Tris, pH 7.5; 130 mM NaCl; 1% Triton X-100; 10 mM NaF; 10 mM sodium orthophosphate; 10 mM sodium pyrophosphate), and centrifuged at 40,000 × g for 45 min at 4°C, and the clear supernatant was passed through an Ni-nitrilotriacetic acid-agarose column. The column was washed extensively with lysis buffer, followed by lysis buffer with 20 and 30 mM imidazole. Washes were monitored by measuring the optical density at 280 nm. When the A280 reached a value of 0.01, column-bound protein was eluted with 0.1 to 0.5 M imidazole and collected in 0.5-ml fractions. The purity of the eluted protein was analyzed by silver staining of sodium dodecyl sulfate (SDS)–12% polyacrylamide gel electrophoresis (PAGE) gels, by Western blots with anti-gpK8.1A MAb, and by autoradiography (60). Fractions containing the purified protein were pooled, dialyzed against PBS, concentrated, and renatured as described above. His-tagged HHV-8 latency-associated ORF 73 protein (61) used as control was purified from Sf9 cells infected with ORF 75-pAcHLT-A baculovirus as described above.

Western blot assays. Samples were boiled in sample buffer with 2-mercaptoethanol (2-ME), subjected to SDS-PAGE, and electrophoretically transferred onto nitrocellulose membranes. Standard prestained molecular weight markers

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(gib) were in parallel lanes. The membranes were soaked in blocking solution (10 mM Tris-HCl, pH 7.2; 150 mM NaCl; 5% skim milk or 5% bovine serum albumin [BSA]; 0.02% NaN₃) at 4°C overnight and then reacted with antibodies for 3 h at room temperature. The membranes were washed five times with washing buffer (10 mM Tris-HCl, pH 7.2; 150 mM NaCl; 0.3% Tween 20) and incubated for 1 h with alkaline phosphatase (AP)-conjugated secondary antibodies (KPL, Gaithersburg, Md.). Bound enzyme-labeled antibodies were detected by evaluating the color reaction of AP with nitroblue tetrazolium and BCIP (5-bromo-4-chloro-3-indolyphosphate) substrates (Sigma). The reactions were stopped by washing the membranes in distilled water.

Surface immunofluorescence assay (SIFA). To detect the binding of gkp8.1A and TMgK8.1A to the cell surface, BAA and HFF cells or CHO mutant derivative cells in 96- or 24-well plates were washed and blocked with 100 μl of RPMI 1640 with 10% FBS were added to the cell pellets, mixed, and incubated for 30 min at 37°C. These cells were washed five times with RPMI 1640 with 10% FBS and 0.01% NaN₃ for 90 min at 4°C. After being mixed for 1 h at 4°C, 100 μl of a 50% slurry of heparin-aragose beads (Sigma) were included in parallel lanes. The membranes were soaked in washing buffer (10 mM Tris-HCl, pH 7.2; 150 mM NaCl; 0.3% Tween 20) and incubated for 1 h at 4°C and then mixed with 100 μl of Tris hydrochloride, pH 7.5; 0.15 M NaCl; 1% sodium deoxycholate; 0.1% Triton X-100, 100 U of aprotinin per ml; and 0.1 m phenylmethylsulfonyl fluoride (60) was added and further mixed for 2 h at 4°C. The heparin-aragose beads were washed five times in RIPA buffer. The material was eluted by boiling the beads in sample buffer with 2-ME, resolved by SDS–12% PAGE, Western blotted, and analyzed with anti-gpK8.1A MAb.

The specificity of TMgK8.1A binding to heparin-aragose beads was tested by preincubating purified TMgK8.1A with different concentrations of heparin for 1 h at 4°C and then incubating it with heparin-aragose beads for 2 h at 4°C. These mixtures were washed five times. The bound materials were eluted by boiling the beads in sample buffer and analyzed by Western blotting with anti-gpK8.1A MAb.

Virally envelope-associated gkp8.1A binding with heparin-aragose. HHV-8 from TPA-induced BCB1-1 cells was purified by two cycles on a sucrose density gradient as per method described before (13). Purified virus was labeled with biotin according to the manufacturer’s recommendations (Gibco-BRL), and the free biotin was removed by extensive dialysis against 0.5 M sodium carbonate buffer (pH 9.0) and then against PBS (pH 7.4). The cell-binding activity of biotin-labeled virus was tested by SIFA as described above, and bound virus was detected by use of gkp8.1A MAb or FITC-labeled streptavidin. To test the binding activity with heparin-aragose, biotin-labeled purified HHV-8 was lysed with RIPA buffer, sonicated, and centrifuged at 100,000 × g for 1 h at 4°C. The resulting soluble biotinylated envelope protein supernatant was mixed with 100 μl of 50% slurry of heparin-aragose or agarose beads in RIPA buffer and mixed for 2 h at 4°C. The beads were washed five times in RIPA buffer, boiled in sample buffer with 2-ME, resolved by SDS-10% PAGE, Western blotted, and analyzed with anti-gpK8.1A MAb or with AP-conjugated streptavidin (Dako, Carpinteria, Calif.).

Purification of HHV-8 full-length gkp8.1A. Full-length gkp8.1A was purified using methods previously described (60). Briefly, TPA (Sigma, St. Louis, Mo.)-induced BCBL-1 cells were lysed on ice for 1 h with lysis buffer (10 mM Tris-HCl, pH 8.0; 140 mM NaCl; 0.025% NaN₃; 2% Triton X-100; 1% sodium deoxycholate; 0.2 U of aprotinin per ml; 1 mM phenylmethylsulfonyl fluoride). Cell lysates were passed over a column of Sepharose 4B covalently coupled with gkp8.1A-specific MAb 4D6 at 4°C. The unbound proteins were removed by extensive washing with lysis buffer. The bound gkp8.1A was eluted with low-pH buffer (50 mM glycine-HCl [pH 2.5] in 150 mM NaCl and 0.1% NP-40) and immediately neutralized with a 1:10 volume of 1 M Tris-HCl (pH 8.0). The peak fractions were pooled, dialyzed against PBS (pH 7.0), and stored at −70°C.

RESULTS

HHV-8 envelope glycoprotein gkp8.1A binds to the target cells. The HHV-8 K8.1 gene encodes two ORFs, gkp8.1A and gkp8.1B, which are derived from spliced mRNAs (10). The gkp8.1A ORF is 228 aa long with a signal sequence and a transmembrane domain, consisting of a 167-aa region identical to the 197-aa amino- terminal 142-aa region of gkp8.1A is identical to the 197-aa genomic K8.1 ORF with the splicing event generating the gkp8.1A ORF transmembrane domain absent in the genomic K8.1 ORF (Fig. 1A). The amino- terminal 142-aa region of gkp8.1A is identical to the 197-aa genomic K8.1 ORF with the splicing event generating the gkp8.1A ORF transmembrane domain absent in the genomic K8.1 ORF (Fig. 1A). (10). HHV-8 gkp8.1A is a virion envelope-associated immunogenic glycoprotein containing both N- and O-linked sugars (60). MAb against gkp8.1A recognizes multiple proteins with molecular masses ranging from 34 to 72 kDa from BCBL-1 cells and 68- to 72-kDa proteins from the
virion particles (60). These multiple proteins represent the precursor and glycosylated forms of gpK8.1A (60).

Because of the similarity of gpK8.1A to EBV gp350/gp220 involved in target cell recognition, we examined the ability of HHV-8 gpK8.1A to interact with the target cells. The gpK8.1A was affinity purified from TPA-induced BCBL-1 cells (Fig. 1B, lane 1) (60). Paraformaldehyde-fixed BJAB, 293, or HFF cells were incubated with purified gpK8.1A, washed, and reacted with anti-gpK8.1A MAb or MAb against HHV-8 ORF 59 (11) or normal mouse IgG. After incubation and washing, the bound antibody was detected by incubating with FITC-labeled anti-mouse IgG in SIFAs. Cells treated with 0.1% paraformaldehyde were used in the binding assay, since this treatment allows the binding of protein but prevents the entry into cells (25). In addition, binding assays can be performed at 37°C (25). Fluorescence signals representing the cell bound gpK8.1A were detected on the membranes of BJAB or 293 or HFF cells, and the results with BJAB and 293 cells are shown in Fig. 2A and B. No fluorescence signal was detected in cells incubated with anti-gpK8.1A MAbs only (Fig. 2C). Fluorescence signal was also not detected in cells incubated with ORF 59 MAbs or normal mouse IgG or rabbit antibodies against HHV-8 latency-associated ORF 73 protein (data not shown). Fluorescence signal was also not detected in cells incubated with His-tagged ORF 73 protein (data not shown). These results demonstrated the binding of gpK8.1A to the cell surface and suggested a role for gpK8.1A in the interaction between HHV-8 and the target cells.

Expression and purification of HHV-8 ΔTMgpK8.1A without transmembrane and cytoplasmic domains. Since only about 20% of TPA-induced BCBL-1 cells expressed HHV-8 lytic-cycle proteins, the yield of purified gpK8.1A by affinity chromatography was insufficient for binding studies. Hence, a 576-bp ΔTMgpK8.1A gene region encoding aa 1 to 192 lacking the transmembrane and the carboxyl domains was amplified by PCR (Fig. 1A), cloned, and expressed in the baculovirus system. The ΔTMgpK8.1A baculovirus-infected Sf9 cells pellets and the culture supernatant were analyzed in Western blot reactions with gpK8.1A-specific MAbs. The predicted molecular mass of unglycosylated ΔTMgpK8.1A is about 21 kDa. MAbs recognized proteins ranging from 29 to 42 kDa from the ΔTMgpK8.1A baculovirus-infected Sf9 cell pellets and culture supernatant (Fig. 1B, lanes 2 and 3). These proteins represent the different glycosylated forms of ΔTMgpK8.1A. The molecular masses of baculovirus-expressed ΔTMgpK8.1A proteins (Fig. 1B, lanes 2 and 3) were smaller than the gpK8.1A from the BCBL-1 cells (Fig. 1B, lane 1). This could be due to the absence of 36 aa in ΔTMgpK8.1A, as well as to the differences in the efficiency of N and O glycosylation between insect and mammalian cells (60, 61). HHV-8 ORF 59 MAbs or normal mouse IgG did not react with ΔTMgpK8.1A protein in Western blot reactions (data not shown).

Sf9 cells infected with ΔTMgpK8.1A baculovirus were labeled with [35S]methionine and radiolabeled ΔTMgpK8.1A from the cell lysate was purified by use of nickel columns. The purity of the protein was analyzed by silver staining of SDS-PAGE. Western blot reactions with anti-gpK8.1A MAb, and autoradiography. Fractions containing the purified protein were pooled, dialyzed, concentrated, and reanalyzed. Purified radiolabeled ΔTMgpK8.1A proteins of about 32 to 42 kDa were detected in the Western blot reactions and by autoradiography (Fig. 1B, lanes 4 and 5). Contaminating proteins.
were not detected. We used the ΔTMgpK8.1A protein purified
from the Sf9 cell pellets in all subsequent assays, since only a
limited quantity of ΔTMgpK8.1A was detected in the infected
Sf9 cell culture supernatant. This could be due to the weak
cleavage of gpK8.1A signal sequence in the insect cells. A
similar observation was made when HSV gD was expressed
with the native signal sequence (48).

HHV-8 ΔTMgpK8.1A binds to the target cells. To determine
whether ΔTMgpK8.1A binds to the target cells, unlabeled pu-
rified ΔTMgpK8.1A was allowed to bind the paraformalde-
hyde-treated BJAB, 293, HFF, or HMVEC-d cells, which were
washed and tested with anti-gpK8.1A MAbs in SIFAs. Bright-
ring-type fluorescence was observed only on cells incubated
with ΔTMgpK8.1A, and the results with BJAB cells are shown
in Fig. 2D. Binding was not detected when cells were
incubated with [35S]methionine-labeled ΔTMgpK8.1A protein (Fig. 3A).

To determine the specificity of ΔTMgpK8.1A binding, ho-
mologous competition assays were done. HFF cells were pre-
incubated for 15 min at 4°C with different concentrations of
unlabeled ΔTMgpK8.1A and then incubated with a fixed concen-
tration (3.5 and 15 μg for the 96- and 24-well plates, respec-
tively) of purified radiolabeled ΔTMgpK8.1A (7,666 cpm/μg of
protein). Similar results were observed with untreated cells at
4°C or with paraformaldehyde-treated cells at 37°C. The results with untreated cells at 4°C are presented here. Binding was not detected when the cells were
incubated with [35S]methionine-labeled (14,672 cpm/μg of protein)
radiolabeled HHV-8 latency-associated ORF 73 protein (Fig. 3A).

To determine if the interaction of gpK8.1A with the cell
surfaces is biologically relevant, the ability of purified nonra-

FIG. 2. HHV-8 gpK8.1A binds to the target cells. Binding of purified full-length gpK8.1A and ΔTMgpK8.1A to the target cells was detected
by surface immunofluorescence assay. Parafomaldehyde-treated BJAB, 293, HFF, or HMVEC-d cells were incubated with medium alone
(control) or medium with purified proteins for 30 min at 37°C. After cells were washed, anti-gpK8.1A-specific MAb or anti-HHV-8 ORF 59 MAb
(11) or rabbit anti-HHV-8 ORF 73 antibodies (34) were added, incubated for 30 min at 37°C, washed, and incubated for an additional 30 min at
37°C with FITC-conjugated goat anti-mouse or anti-rabbit IgG antibodies. Cells were washed, mounted, and examined under a fluorescence
microscope. (A and B) BJAB and 293 cells, respectively, incubated with the full-length affinity-purified gpK8.1A and anti-gpK8.1A MAb. (C) BJAB
cells incubated with anti-gpK8.1A MAb alone. (D) BJAB cells incubated with the purified His-tagged ΔTMgpK8.1A and anti-gpK8.1A MAb.
Fluorescence signals detected on the surface of cells indicate the cell-bound gpK8.1A and ΔTMgpK8.1A.

were not detected. We used the ΔTMgpK8.1A protein purified
from the Sf9 cell pellets in all subsequent assays, since only a
limited quantity of ΔTMgpK8.1A was detected in the infected
Sf9 cell culture supernatant. This could be due to the weak
cleavage of gpK8.1A signal sequence in the insect cells. A
similar observation was made when HSV gD was expressed
with the native signal sequence (48).

HHV-8 ΔTMgpK8.1A binds to the target cells. To determine
whether ΔTMgpK8.1A binds to the target cells, unlabeled pu-
rified ΔTMgpK8.1A was allowed to bind the paraformalde-
hyde-treated BJAB, 293, HFF, or HMVEC-d cells, which were
washed and tested with anti-gpK8.1A MAbs in SIFAs. Bright-
ring-type fluorescence was observed only on cells incubated
with ΔTMgpK8.1A, and the results with BJAB cells are shown
in Fig. 2D. Binding was not detected when cells were incubated
with purified His-tagged ORF 73 protein (data not shown).
These data further confirm the interaction of gpK8.1A with the
cell surface and show that the extracellular domains of
gpK8.1A mediate this binding.

To quantitate the target cell bindings, purified [35S]methi-
onine-labeled ΔTMgpK8.1A (7,666 cpm/μg of protein) was
incubated with HFF, BJAB, 293, and HMVEC-d cells. Radiol-
labeled ΔTMgpK8.1A bound to all cells in a dose-dependent
manner, and the results with HFF cells are shown in Fig. 3A.
Similar results were observed when binding assays were per-
diolabeled \( \Delta TMgpK8.1A \) to complete with \([3H]\)thymidine-labeled HHV-8 binding to HFF cells was examined. HFF cells were preincubated with increasing concentrations of purified unlabeled \( \Delta TMgpK8.1A \) for 90 min at 4°C, followed by the addition of a fixed quantity of \([3H]\)thymidine-labeled purified HHV-8 (2,684 cpm) (2). For a control, a fixed quantity of \([3H]\)thymidine-labeled purified HHV-8 (2,684 cpm) was mixed with 10 \( \mu \)g of heparin per ml for 90 min at 4°C and then added to HFF cells. After incubation for 90 min at 4°C with the virus, cells were washed five times and lysed with 1% SDS and 1% Triton X-100, and the radioactivity was precipitated with TCA and counted. The cell-associated virus cpm in the absence or presence of unlabeled \( \Delta TMgpK8.1A \) and heparin and the percentage of inhibition of virus binding were calculated. In the absence of heparin or \( \Delta TMgpK8.1A \), approximately 21% of the input HHV-8 radioactivity became associated with the cells. Approximately 90% of HHV-8 attachment to the cells was blocked by heparin. Each reaction was done in triplicate, and each point represents the average \( \pm \) the SD of three experiments.

Heparin blocks HHV-8 \( \Delta TMgpK8.1A \) binding to the target cells. Heparin is closely related to HS, and inhibition of virus infectivity by heparin treatment has been considered as an

**FIG. 3.** (A) Binding of radiolabeled \( \Delta TMgpK8.1A \) to HFF cells. Different concentrations of \([35S]\)methionine-labeled purified \( \Delta TMgpK8.1A \) (7,666 cpm/\( \mu \)g of protein) or ORF 73 (14,672 cpm/\( \mu \)g of protein) proteins were incubated for 90 min at 4°C with HFF cells in 96- or 24-well plates. After incubation, cells were washed five times and lysed with 1% SDS and 1% Triton X-100, and the cell-bound \( \Delta TMgpK8.1A \) radioactivity was counted. Each reaction was done in triplicate and each point represents the average \( \pm \) the standard deviation (SD) of three experiments. Similar results were seen with cells in 96- and 24-well plates, and the results with the 96-well plates are shown here. (B) Inhibition of labeled \( \Delta TMK8.1A \) binding to cells by unlabeled \( \Delta TMK8.1A \) protein. HFF cells were preincubated with the indicated concentrations of nonlabeled \( \Delta TMgpK8.1A \) for 15 min and then incubated with 3.5 \( \mu \)g (for cells in the 96-well plate) or 15 \( \mu \)g (for cells in the 24-well plate) of \( {35S}\)-labeled \( \Delta TMgpK8.1A \) (7,666 cpm/\( \mu \)g of protein) for 90 min at 4°C. Cells were washed five times and lysed with 1% SDS and 1% Triton X-100, and the cell-bound \( \Delta TMgpK8.1A \) radioactivity was counted. The cell-associated radiolabeled \( \Delta TMgpK8.1A \) cpm in the presence or absence of unlabeled protein was calculated. In the absence of unlabeled \( \Delta TMgpK8.1A \) protein, about 30% of the input labeled \( \Delta TMgpK8.1A \) (1.1 and 4.5 \( \mu \)g for cells in the 96-well and 24-well plates, respectively) became associated with the cells. Each reaction was done in triplicate, and each point represents the average \( \pm \) the SD of three experiments.

**FIG. 4.** Nonradiolabeled \( \Delta TMgpK8.1A \) blocks HHV-8 attachment. HFF cells were incubated with increasing concentrations of purified unlabeled \( \Delta TMgpK8.1A \) for 90 min at 4°C, followed by the addition of a fixed quantity of \([3H]\)thymidine-labeled purified HHV-8 (2,684 cpm) (2). For a control, a fixed quantity of \([3H]\)thymidine-labeled purified HHV-8 (2,684 cpm) was mixed with 10 \( \mu \)g of heparin per ml for 90 min at 4°C and then added to HFF cells. After incubation for 90 min at 4°C with the virus, cells were washed five times and lysed with 1% SDS and 1% Triton X-100, and the radioactivity was precipitated with TCA and counted. The cell-associated virus cpm in the absence or presence of unlabeled \( \Delta TMgpK8.1A \) and heparin and the percentage of inhibition of virus binding were calculated. In the absence of heparin or \( \Delta TMgpK8.1A \), approximately 21% of the input HHV-8 radioactivity became associated with the cells. Approximately 90% of HHV-8 attachment to the cells was blocked by heparin. Each reaction was done in triplicate, and each point represents the average \( \pm \) the SD of three experiments.
evidence for alpha-, beta-, and gamma-herpesvirus interaction with cell surface HS molecules (21, 24, 30, 31, 33, 35, 37, 46–47, 49, 51, 57). Our recent studies showed that HHV-8 interaction with host cell surface involved HS and soluble heparin prevented HHV-8 infectivity (2). To determine whether heparin inhibits ΔTMgpK8.1A binding, a constant quantity of purified radiolabeled ΔTMgpK8.1A within the linear range of the dose-response curve (3.5 μg for cells in 96-well plates or 15 μg for cells in 24-well plates) (Fig. 3A) was mixed with medium alone or medium with different concentrations of heparin and incubated at 4°C for 90 min. These were then added to the paraformaldehyde-treated target cells and incubated at 37°C for 90 min or to the untreated target cells and incubated at 4°C for 90 min. After incubation, cells were washed five times and cell-associated ΔTMgpK8.1A cpm values were counted. Similar results were observed when binding assays were performed at 4 or at 37°C, and results with untreated cells 4°C are shown in Fig. 5A.

In the absence of heparin, approximately 30% of the input ΔTMgpK8.1A radioactivity became associated with the cells. Soluble heparin significantly inhibited the binding of labeled ΔTMgpK8.1A to all cell lines tested in a dose-dependent manner. The results with HFF cells and HMVEC-d cells are shown in Fig. 5A. The percentage of inhibition plateaued at between 1 and 10 μg of heparin per ml for HFF and HMVEC cells (Fig. 5A) and for 293 cells (data not shown), and the maximum inhibition ranged from 83 to 95%. The specificity of heparin inhibition was shown by the absence of inhibition by CS-A and CS-C, even at a concentration of 100 μg/ml. CS-B also inhibited ΔTMgpK8.1A binding to the cell surface, with about 30 and 70% inhibition at concentrations of 10 and 100 μg/ml, respectively (Fig. 5A). However, these CS-B concentrations required to inhibit 50% of ΔTMgpK8.1A binding to the cell surface were almost 100 times higher than that of the required heparin concentration. The inhibition of ΔTMgpK8.1A binding to the target cells by heparin even at a low concentration suggested that ΔTMgpK8.1A interacts with the cell surface HS. The inability of heparin to completely prevent the protein binding suggests that gpK8.1A also binds to other host cell molecules.

Displacement of cell surface adsorbed HHV-8 ΔTMgpK8.1A by heparin. To determine the specificity of HHV-8 ΔTMgpK8.1A interaction with cell surface HS and the inhibition by heparin, labeled ΔTMgpK8.1A was first allowed to adsorb to the HFF cells and at different times postadsorption, heparin or CS-A, -B, or -C were added to a final concentration of 10 μg/ml. Cells were further incubated for a total period of 90 min, and the cell-associated radioactivity was counted. Similar results were observed when binding assays were performed with paraformaldehyde-treated cells at 37°C or with untreated cells at 4°C. The results with untreated cells at 4°C are shown in Fig. 5. Pretreatment of HFF cells with heparin did not affect ΔTMgpK8.1 binding (data not shown). In the absence of heparin, about 30% of the input labeled ΔTMgpK8.1A (1.1 μg) became associated with the cells. In contrast, when heparin was added to the ΔTMgpK8.1A protein-cell mixture, it was capable of displacing already-adsorbed ΔTMgpK81A even when added 40 min after the protein addition to the cells (Fig. 5B). The partial reversal of binding by the addition of heparin after 50 min of protein-cell interaction (Fig. 5B) could be due to the

FIG. 5. Inhibition of [35S]methionine-labeled purified ΔTMgpK8.1A binding to target cells by heparin. (A) A constant quantity of purified labeled ΔTMgpK8.1A (7,666 cpm/μg of protein) within the linear range of the dose-response curve (3.5 μg for cells in the 96-well plate or 15 μg for cells in the 24-well plate) (Fig. 3A) was mixed with medium alone or with different concentrations of heparin or CS-A, CS-B, or CS-C and then incubated for 90 min at 4°C. These mixtures were then incubated with HFF or adult HMVEC-d (Endo) for 90 min at 4°C and washed five times. Cells were lysed with 1% SDS–1% Triton X-100 and counted. The cell-associated ΔTMgpK8.1A cpm in the presence or absence of heparin and the percentage of inhibition of ΔTMgpK8.1A binding were calculated. In the absence of heparin, approximately 30% of the input ΔTMgpK8.1A radioactivity (1.1 and 4.5 μg for cells in the 96-well and 24-well plates, respectively) became associated with the cells. Each reaction was done in triplicate and each point represents the average ± the SD of three experiments. (B) Displacement of adsorbed ΔTMgpK8.1A from the HFF cell surface by heparin. HFF cell monolayers in 96-well plates were incubated with a constant quantity (3.5 μg) of purified labeled ΔTMgpK8.1A (7,666 cpm/μg of protein). At the indicated time points, cells were incubated with medium (controls) or with medium containing 10 μg of heparin or CS-A, CS-B, or CS-C per ml. Cells were further incubated for a total of 90 min at 4°C, washed five times, and then counted. The cell-associated ΔTMgpK8.1A cpm in the presence or absence of heparin and the percentage of inhibition of ΔTMgpK8.1A binding were calculated. In the absence of heparin, approximately 30% of the input ΔTMgpK8.1A radioactivity (1.1 μg) became associated with the cells. Each reaction was done in triplicate, and each point represents the average ± the SD of three experiments.
onset of interactions between gpK8.1A and cellular receptors other than HS molecules. Reversal of ΔTMgpK8.1A binding to the cells by heparin demonstrated the specificity of HS interaction with HHV-8 gpK8.1A. Specificity was also shown by the absence of any significant inhibition by the same amount (10 μg/ml) of CS-A, -B, and -C (Fig. 5B).

HHV-8 ΔTMgpK8.1A binds to the HS-expressing CHO-K1 cell line but not to cells lacking HS. To verify the role of HS in the attachment of ΔTMgpK8.1A to the target cells, binding assays were done with wild-type CHO-K1 cell line expressing HS and its two mutant cell lines, pgsD-677 cells (deficient in HS but not in chondroitin sulfate) and pgsA-745 cells (deficient in both HS and chondroitin sulfate). Radiolabeled ΔTMgpK8.1A (3,310 cpm/μg of protein) bound readily to the wild-type CHO-K1 cells to the same extent as HFF cells. About 4 μg or 26% of the input labeled ΔTMgpK8.1A became associated with the CHO-K1 cells (Fig. 6). In contrast, ΔTMgpK8.1A binding to the mutant cells was significantly impaired, and about fivefold-less binding was detected with the pgsD-677 and pgsA-745 cells (Fig. 6). These results confirmed the interaction of HHV-8 ΔTMgpK8.1A with the cell surface HS. The low percentage of ΔTMgpK8.1A binding to the cells lacking HS further supported the notion that gpK8.1A also probably binds other host cell molecules.

HHV-8 ΔTMgpK8.1A specifically binds to heparin. To verify the specificity of gpK8.1A binding to HS, the ability of ΔTMgpK8.1A to bind the heparin-agarose beads was tested. Purified ΔTMgpK8.1A, HHV-8 gL, or HHV-8 ORF 73 (2.5 μg) protein was incubated with heparin-agarose beads. After an extensive washing, the beads were boiled in sample buffer. The eluted proteins were detected by immunoblot using anti-gpK8.1A MAb, rabbit anti-gL antibodies, and rabbit anti-ORF 73 antibodies. Representative results are presented in Fig. 7.

HHV-8 gL and ORF 73 proteins were not precipitated by heparin-agarose beads (data not shown). In contrast, heparin-agarose beads precipitated the various glycosylated forms of ΔTMgpK8.1A (Fig. 7A, lane 1). To determine the specificity of this reaction, various GAGs were tested to compete with the heparin-agarose binding activity of ΔTMgpK8.1A. Heparin-agarose binding activity of ΔTMgpK8.1A was competitively inhibited by preincubating the protein with 350 μg of heparin (Fig. 7A, lane 2) or 350 μg of HS (Fig. 7A, lane 3). In contrast, 350 μg of CS-A, -B, and -C, N-acetyl heparin, and de-N-sulfated heparin did not inhibit the ΔTMgpK8.1A interaction with the heparin-agarose beads (Fig. 7A, lanes 4 to 8). No reactivity was seen with agarose beads alone (Fig. 7A, lane 9), thus demonstrating the specificity of these reactions. These results confirmed the interaction of gpK8.1A with HS and heparin.
The specificity of HHV-8 ΔTMgpK8.1A binding to heparin-agarose beads was also examined by preincubating 2.5 μg of purified ΔTMgpK8.1A with different concentrations of heparin for 1 h at 4°C and then incubating this with heparin-agarose beads for 2 h at 4°C. No reactivity was seen when agarose beads were incubated with purified ΔTMgpK8.1A (Fig. 7B, lane 7). Heparin-agarose beads precipitated the various glycosylated forms of ΔTMgpK8.1A (Fig. 7B, lane 1). This binding was completely inhibited by the preincubation with heparin at a concentration of 300 and 150 μg (Fig. 7B, lanes 2 and 3). Only moderate inhibition was seen with 75 and 38 μg of heparin (Fig. 7B, lanes 4 and 5), and no inhibition was seen with 19 μg of heparin (Fig. 7B, lane 6). These results further verified the interaction of gpK8.1A with HS and heparin.

**Virion envelope-associated gpK8.1A binds heparin-agarose.** To determine whether virion envelope-associated gpK8.1A binds heparin-agarose, density gradient-purified HHV-8 was labeled with biotin. The biotinylated virus bound to the target cells in the surface immunofluorescence assay (data not shown). Similar to our earlier findings (60), gpK8.1A MAbs recognized the 68- to 72-kDa protein in Western blot reactions with purified HHV-8 (Fig. 8, lane 1). Biotinylated purified virus was lysed with RIPA buffer, sonicated, and centrifuged at 100,000 × g. The resulting supernatant containing the soluble biotinylated envelope proteins was mixed with heparin-agarose or agarose beads for 2 h at 4°C and washed. The bound material was eluted by boiling in sample buffer, resolved by SDS-PAGE, Western blotted, and analyzed. Lane 1, purified virus solubilized with sample buffer in Western blot reactions with anti-gpK8.1A MAb; lane 2, biotinylated proteins eluted from the agarose beads reacted with AP-labeled streptavidin and substrate; lane 3, biotinylated proteins eluted from the heparin-agarose beads reacted with AP-labeled streptavidin and substrate; lane 4, biotinylated proteins eluted from the heparin-agarose beads in Western blot reactions with anti-gpK8.1A MAb; lane 5, biotinylated proteins eluted from the heparin-agarose beads in Western blot reactions with rabbit anti-HHV-8 IgG antibodies. The numbers on the left indicate the molecular masses (in kilodaltons) of the standard protein markers run in parallel lanes.

**DISCUSSION**

Proteoglycans are found abundantly in the extracellular matrices or cell surfaces of animal cells and mediate many fundamental cellular processes, including cell-to-cell and cell-to-matrix adhesion, motility, growth, and signaling (28). A proteoglycan is formed by the linkage of glycosaminoglycans such as HS or chondroitin sulfate to a protein core. HS is the initial binding target of many microorganisms, including parasites, bacteria, and viruses (23, 42, 54). Several alphaherpesviruses, such as HSV-1, HSV-2, PRV, and BHV-1 (21, 24, 30, 31, 33, 46, 47, 51, 59), betaherpesviruses, such as HCMV and HHV-7 (35, 37, 45, 49), and gamma-2-herpesviruses, such as BHV-4 (57), interact specifically with HS-like moieties. HS is also recognized by a wide spectrum of other viruses such as human immunodeficiency virus type 1 (38, 41), vaccinia virus (14), Sindbis virus (7), foot-and-mouth-disease virus (26), respiratory syncytial virus (19, 29), and adeno-associated virus (55).

Our recent studies show that the gamma-2-HHV-8, like some members of the alpha-, beta-, and gamma-2-herpesviruses, adsorbs to cells by binding to cell surface HS-like moieties (2). Studies here examined the role of HHV-8 envelope glycoprotein gpK8.1A in the interaction with target cells. Comparison with the human or animal herpesvirus sequences to date show that the gpK8.1A gene is unique for HHV-8. The location of the gpK8.1A gene in the genome clearly suggests an important role of gpK8.1A in the biology of HHV-8. The gpK8.1A gene is positionally colinear to the gamma-1-EBV gp350/gp220 gene (22), the gamma-2-MHV-68 gp150 gene (53), and the gamma-2-HVS ORF 51 gene (1). HHV-8 gpK8.1A shows several similarities with these proteins. Like EBV gp350/gp220 (56) and MHV-68 gp150 (53), HHV-8 gpK8.1A is a virion envelope- and infected cell membrane-associated glycoprotein (60). Antibodies against gp350/gp220 of EBV and gp150 MHV-68 neutralized the respective virus infectivities (53, 56). Binding of gpK8.1A to the target cells and the gpK8.1A blocking the radiolabeled HHV-8 binding shown here suggest that gpK8.1A plays an important role in the initial events of HHV-8 entry into susceptible cells. Our ongoing studies show that anti-gpK8.1A MAbs neutralize HHV-8 infectivity (data not shown). Inhibition of ΔTMgpK8.1A binding...
by heparin, binding of ΔTMgpK8.1A to the HS-expressing CHO-K1 cells, limited binding to the mutant derivatives of CHO cell lines lacking HS, specific binding of ΔTMgpK8.1A to HS but not to other GAGs, and the binding of virion gpK8.1A with heparin clearly demonstrate that gpK8.1A is involved in the interaction with HS. Even though heparin lowered the level of ΔTMgpK8.1A binding, the absence of complete inhibition suggests the interaction with other cell surface molecules. The low percentage of binding of ΔTMgpK8.1A to CHO cells lacking HS also reinforces this suggestion. Our results indicate that ΔTMgpK8.1 interacts with HS is the first important set of ligand-receptor interaction which may lead to the binding of one or more second receptor(s) essential for the subsequent viral entry process (23). The putative second receptor for gpK8.1A needs to be identified.

Inspection of the structure of heparin and/or HS and sequence analysis of the heparin-binding domain (HBD) of several proteins suggested that the negatively charged sulfate or carboxylate groups on heparin could interact via electrostatic interactions to positively charged cationic residues in a protein or peptide (26, 42, 54). HBDs are enriched with positively charged basic amino acids (lysine, arginine, and histidine). Two typical heparin motifs (XBXXBX and XBBxBBX) have been identified, where “B” is a basic residue and “X” can be any other residue but is usually a hydrophobic residue (8). Analysis of amino acid sequence of gpK8.1A revealed two possible, although atypical heparin-binding motifs: gpK8.1A-H1 (150SRTTIRIV537, XBXXBXBBX) and gpK8.1A-H2 (182TRGRDAHY189, XBXXBBXXB). Whether these gpK8.1A putative HBDs play a role in the interaction with HS requires further investigation. It is also possible that several other weak and/or high-affinity HBDs may appear in HHV-8 gpK8.1A in its native quaternary structure, since the basic amino acids separated apart may lie juxtaposed, forming a typical HBD.

Among the eight HHVs, HS has been shown to mediate the attachment of HSV-1, HSV-2, HCMV, HHV-7, and HHV-8 (2, 21, 24, 30, 31, 33, 35, 47–49, 51, 59). In alphaherpesviruses, the glycoproteins gB and gC are known to bind cell surface HS (21, 24, 30, 31, 33, 51). The gC homologue of alphaherpesviruses is absent in the beta- and gammaherpesviruses, HHV-4 (gammaherpesvirus) and HCMV (betaherpesvirus) (35, 45, 57). Predictive analysis of HHV-8 sequence revealed the presence of putative HBD in HHV-8 gB. Ongoing studies show that HHV-8 envelope-associated gB also binds HS and the 75- and 54-kDa proteins precipitated by heparin-agarose from the biotinylated virus (Fig. 8, lane 2) represent the two cleaved-disulfide linked forms of HHV-8 gB (S. M. Akula et al. unpublished results). The presence of two or more heparin-binding glycoproteins within a single virus is not unexpected, since all well-studied human alpha- and beta-herpesviruses contain at least two HS binding glycoproteins, e.g., gC and gB for HSV-1 and HSV-2, gB and gCH for HCMV, and gB and gp63 for HHV-7 (30, 31, 35, 37, 45, 49). The presence of two HS binding proteins within the same virus indicates the importance of cell surface HS as receptors for viral attachment. HSV-1 gC and gB exhibit differences in their relative affinities for distinct cell surface HS proteoglycans (30). Whether HHV-8 gpK8.1A and gB also exhibit such differences needs to be studied.

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