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Kaposi's Sarcoma-Associated Herpesvirus (Human Herpesvirus 8) Infection of Human Fibroblast Cells Occurs through Endocytosis

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Kaposi's sarcoma (KS)-associated herpesvirus or human herpesvirus 8 (HHV-8) DNA and transcripts have been detected in the B cells, macrophages, keratinocytes, and endothelial and epithelial cells of KS patients. In vitro, HHV-8 infects human B, endothelial, epithelial, and fibroblast cells, as well as animal cells, and the infection is characterized by (i) absence of lytic replication by the input virus and (ii) latent infection. For its initial binding to target cells, HHV-8 uses ubiquitous heparan sulfate molecules via its envelope-associated glycoproteins gB and gpK8.1A. HHV-8 also interacts with the α3β1 integrin via its glycoprotein gB, and virus binding studies suggest that α3β1 is one of the HHV-8 entry receptors (S. M. Akula, N. P. Pramod, F. Z. Wang, and B. Chandran, Cell 108:407-419, 2002). In this study, morphological and biochemical techniques were used to examine the entry of HHV-8 into human foreskin fibroblasts (HFF). HHV-8 was detected in coated vesicles and in large, smooth-surfaced endocytic vesicles. Fusion of viral envelope with the vesicle wall was also observed. In immune electron microscopy, anti-HHV-8 gB antibodies colocalized with virus-containing endocytic vesicles. In fluorescence microscopic analyses, transferrin was colocalized with HHV-8. HHV-8 infection was significantly inhibited by preincubation of cells with chlorpromazine HCl, which blocks endocytosis via clathrin-coated pits, but not by nystatin and cholera toxin B, which blocks endocytosis via caveolae and induces the dissociation of lipid rafts, respectively. Infection was also inhibited by blocking the acidification of endosomes by NH_4Cl and bafilomycin A. Inhibition of HHV-8 open reading frame 73 gene expression by chlorpromazine HCl, bafilomycin A, and NH_4Cl demonstrated that the virions in the vesicles could proceed to cause an infection. Taken together, these findings suggest that for its infectious entry into HFF, HHV-8 uses clathrin-mediated endocytosis and a low-pH intracellular environment.

Kaposi's sarcoma (KS)-associated herpesvirus or human herpesvirus 8 (HHV-8) is a member of the γ2-herpesvirus family (genus *Rhadinovirus*) (38, 51). HHV-8 DNA has been detected in KS tissues from patients with AIDS-KS, classic KS, African endemic KS, and transplantation-associated KS (6, 17, 21, 56, 57). Numerous studies suggest an etiologic association of HHV-8 with the pathogenesis of KS, body cavity-based B-cell lymphoma (BCBL), and multicentric Castleman's disease (6, 21, 56, 57). Cell lines with B-cell characteristics established from lymphomas carry HHV-8 in a latent form, and a lytic cycle can be induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (6, 21, 48, 56, 57). HHV-8 DNA encodes more than 80 complete open reading frames (ORFs), which are designated ORF4 to ORF75 by their homology to ORFs of herpesvirus saimiri, a simian herpesvirus (38, 51). HHV-8 also encodes more than 20 unique ORFs that are designated with the prefix K (38, 51).

In vivo, HHV-8 DNA and transcripts have been detected in human B cells, macrophages, keratinocytes, endothelial cells, and epithelial cells (6, 21, 56, 57). In vitro, HHV-8 has been shown to infect a variety of human cells, such as B cells, endothelial cells, epithelial cells, and fibroblast cells (4, 6, 21, 37, 48, 57, 70). In addition, HHV-8 also infects a variety of animal cells, such as owl monkey kidney cells, baby hamster kidney fibroblast cells, Chinese hamster ovary (CHO) cells, and primary embryonic mouse fibroblast (Du17) cells (4, 37, 48). If in vitro permissiveness of a cell type is judged by productive lytic replication of HHV-8 after entry into cells, as yet, there is no suitable cell culture system to support lytic replication of input HHV-8. Only latent HHV-8 infection is observed in infected cells (4, 37, 48, 70). If in vitro permissiveness is judged by the establishment of HHV-8 latency and the ability to support HHV-8 lytic replication after activation by agents, cells such as human foreskin fibroblasts (HFF), human carcinoma cells, and endothelial cells are permissive, as evidenced by retention of the viral genome in a latent form, by expression of the HHV-8 latency-associated ORF73 protein, and by the ability to support lytic replication upon activation by TPA and human cytomegalovirus (4, 48, 70). However, in vitro latent HHV-8 infection in primary fibroblast or endothelial cells or in nonadherent B-cell lines is unstable and the viral DNA is not maintained efficiently and is usually lost on subsequent culturing of infected cells (unpublished observations).

The identities of the receptors used by HHV-8 for binding and entry into host cells and the pathways used for infection are critical for understanding the molecular basis of the role of HHV-8 in the pathogenesis of human diseases. Herpesvirus envelope-associated glycoproteins play important roles in binding and entry into target cells (28, 50, 62, 63). Like other herpesviruses, HHV-8 encodes a number of envelope-associated glycoproteins, and HHV-8 glycoproteins gB (ORF8), gH

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(ORF22), gM (ORF39), gL (ORF47), and gN (ORF53) are counterparts to other herpesvirus glycoproteins (1, 36, 38, 51). In addition to these conserved glycoproteins, HHV-8 also encodes K1, gpK8.1A, and gpK8.1B, which are unique to HHV-8 (16, 38, 51). Our previous studies showed that HHV-8 uses cell surface heparan sulfate (HS)-like molecules to bind target cells and suggested that the broad cellular tropism of HHV-8 could be, in part, due to its ability to interact with ubiquitous HS molecules (2). We and others have also demonstrated the interaction of virion envelope-associated HHV-8 glycoprotein gB (ORF8) and gpK8.1A with HS molecules (1, 8, 9, 71).

Among the alpha-, beta-, and gammaherpesvirus gB sequences determined to date, only HHV-8 gB possesses the RGD motif (amino acids 27 to 29) at the extracellular amino terminus coil region after the putative signal sequence (2, 4). The RGD motif is the minimal peptide region of many proteins known to interact with subsets of host cell surface integrins critical for a variety of cell functions, such as regulation of gene expression, activation of focal adhesion kinase (FAK), activation of cytoskeleton elements, endocytosis, attachment, cell cycle progression, cell growth, apoptosis, and differentiation (23). Integrins are a large family of heterodimeric receptors containing noncovalently associated transmembrane α and β glycoprotein subunits (23). There are 17 α and 9 β subunits, generating more than 22 known combinations of αβ cell surface receptors. Each cell expresses several combinations of αβ integrins, and each αβ combination has its own binding specificity and signaling properties (23). We have demonstrated the inhibition of HHV-8 infectivity by RGD peptides, antibodies against α3 and β1 integrins, and soluble α3β1 integrin (4). Anti-HHV-8 gB antibodies immunoprecipitated the virus-α3β1 complex. Radiolabeled virus binding studies suggest that HHV-8 uses the α3β1 integrin as one of the cellular receptors for entry into target cells (4).

Unlike those of the other HHVs, the pathways used by HHV-8 for its entry into various target cells have not been characterized. HHV-8 infection induced the integrin-mediated activation of FAK (4), which implied a role for integrin and the associated signaling pathways in HHV-8 entry into target cells. Since activation of FAK, Src family kinases, and integrin-linked kinases is central to many paradigms of outside-in signaling by integrins, cytoskeleton rearrangement, and endocytosis (13, 23, 55), we examined the mode of entry into adherent HFF by electron microscopy (EM), immunoelectron microscopy (IEM) with anti-gB antibodies, and colocalization of HHV-8 with transferrin. We also examined HHV-8 infection of HFF in the presence of inhibitors of clathrin, caveolae and lipid rafts, and drugs affecting the endosomal pH. Our studies suggest that HHV-8 uses clathrin-mediated endocytosis and the low-pH-dependent intracellular compartments as the predominant pathway for its infectious entry into HFF.

MATERIALS AND METHODS

Cells. HFF (Clonetics, Walkersville, Md.), CV-1 (ATCC CCL-70), BCBL-1 (HHV-8 positive and Epstein-Barr virus [EBV] negative human B cells) (1, 2, 4), and recombinant green fluorescent protein (GFP)–HHV-8-carrying BCBL-1 (GFP–BCBL-1) cells (70) were grown as described before (1, 2, 4).

Virus. To monitor the HHV-8 binding and entry process, GFP–HHV-8 (rKSHV0.152) was used (70). Expression of GFP was under the control of the promiscuous elongation factor 1α promoter. GFP–BCBL-1 cells were induced with 20 ng of TPA (Sigma, St. Louis, Mo.) per ml for 6 days. [^{3}H]thymidine-labeled GFP–HHV-8 was obtained as described before (1, 2, 4). Unlabeled and [^{3}H]thymidine-labeled GFP–HHV-8 in the spent culture medium were centrifuged at 5,000 × *g* and 4°C for 10 min to remove the cells and cell debris. Virus in the clarified supernatant was pelleted by centrifugation at 27,000 × *g* and 4°C for 90 min. Pellets were resuspended in 1/500 of the original volume of RPMI 1640 medium, reclarified by centrifugation at 400 × *g* and 4°C for 10 min four times, and filtered through 0.45-μm-pore-size filters. Concentrated virus was purified with Nycodenz (Sigma) and tested for purity as described previously (37). Herpes simplex virus type 2 (HSV-2; strain 333) grown in CV-1 cells was purified by similar density gradient centrifugation.

HHV-8 infectivity assay. The GFP–HHV-8 strain (rKSHV.152) used in our studies is not clonal and contains both wild-type and recombinant viruses (70). Hence, for each batch of stock virus, virus infectivity was first determined by estimating the green fluorescent cells and then by estimating the number of HHV-8 ORF73 protein-expressing cells by immunocytochemistry analysis (4), which estimated the total number of infectious particles. GFP–HHV-8 titers were estimated with HFF monolayers in eight-well chamber slides (Nalge Nunc International, Naperville, Ill.) (1, 2, 4). After the slides were observed for GFP expression, cells were fixed with cold acetone and tested with anti-ORF73 monoclonal antibodies by immunoperoxidase assay (4). Cell nuclei positive for ORF73 staining were counted, and the total number of infectious virus particles per milliliter of virus stock was calculated. The ratio of infectious GFP–HHV-8 (number of GFP infectious units) versus the total infectious virus population (number of ORF73 infectious units) varies from batch to batch and ranged from 1:1 to 1:4. HHV-8 infections were performed at a multiplicity of infection (MOI) of 1 ORF73 infectious unit per cell (37). A mixed population does not deter the conclusions drawn from our experiments.

Antibodies. The production and characterization of rabbit antibodies against the recombinant GST–HHV-8 gB and GST-ORF73 fusion proteins have been described before (1, 37, 71, 75). Immunoglobulin G (IgG) fractions were purified by protein A Sepharose 4B columns (Amersham Pharmacia Biotech, Piscataway, N.J.). Nonspecific antibodies were removed by columns of cyanogen bromide-activated Sepharose 4B covalently coupled with purified GST protein and BJAB cell lysate.

Reagents. Fluorescein isothiocyanate (FITC), tetramethyl rhodamine isothiocyanate (TRITC)-labeled transferrin, heparin, chondroitin sulfate C, chlorpromazine HCl, nystatin, cholera toxin B (CTB), NH_4Cl, bafilomycin A (BFLA1), cytochalasin D, and nocodazole were purchased from Sigma.

FITC–HHV-8. Virus labeling was performed as described earlier (47). Briefly, 50 μl of density gradient-purified HHV-8 (2 mg/ml) was incubated with 50 μl of a solution (5.0 mg/ml) of FITC dissolved in dimethyl sulfoxide (Sigma) at room temperature for 8 h. The FITC–HHV-8 was centrifuged over an 8.5-ml 30% sucrose cushion for 90 min at 4°C in a Beckman SW41Ti rotor at 70,000 × *g* to remove the free dye from the virus preparation. The FITC-labeled virus band was resuspended in phosphate-buffered saline (PBS) and dialyzed against PBS (pH 7.2).

EM and IEM. HFF were washed once with PBS and trypsinized to obtain a single-cell suspension. Approximately 10^6 HFF in 100 μl of Dulbecco modified Eagle medium (DMEM) were kept on ice for 15 min and then mixed with 100 μl of gradient-purified GFP–HHV-8 (MOI, 5 ORF73 infectious units/cell). The cell-virus mixtures were incubated on ice for 60 min. Infection was initiated by shifting the mixture to 37°C. At 0, 5, and 15 min at 37°C, the unadsorbed virus was removed by washing the cells with PBS three times. These cells were fixed in 2% glutaraldehyde, rinsed in PBS, postfixed in 1% osmium tetroxide, dehydrated in a graded ethanol series, and embedded in Embed 812 resin. Thin sections were made and viewed under a JEOL 100CXII transmission electron microscope.

For IEM, HFF were infected as described above, washed, and fixed in 4% paraformaldehyde. The fixed sections on the grids were incubated with a predetermined dilution of rabbit anti-gB or anti-ORF73 or preimmune IgG antibodies at room temperature for 1 h, thoroughly washed in PBS, and incubated at room temperature for 30 min with anti-rabbit antibodies conjugated with 15-nm gold particles (Ted Pella, Inc., Redding, Calif.). The grids were then washed with several changes of PBS and distilled water and counterstained with uranyl acetate and lead citrate before viewing by EM.

Confocal microscopy. HFF were incubated with TRITC-transferrin (35 μg/ml) and a predetermined concentration of FITC-HHV-8 (MOI, 5 ORF73 infectious units per cell) for 5 and 15 min at 37°C. The cells were washed three times in PBS, fixed in 2% paraformaldehyde–PBS at 4°C for 10 min, washed with PBS, and mounted in antifade reagent (Molecular Probes, Eugene, Oreg.). In another set of experiments, HFF were treated at 37°C for 1 h with DMEM or DMEM containing 10 μg of chlorpromazine HCl per ml and then incubated with TRITC-transferrin and FITC–HHV-8 in the presence of 10 μg of chlorpromazine HCl per ml for different lengths of time before monitoring for transferrin and HHV-8

TABLE 1. Primer sequences used for RT-PCR

Transcript and primer	Sequence	Coordinates	Product size (bp)
ORF73			
5′	GAAGTGGATTACCCTGTTGTTAGC	124430–124453	307
3′	TTGGATCTCGTCTTCCATCC	124166–124147	
eGFP			
5′	CACATGAAGCAGCACGACTT		328
3′	TGTTCTGCTGGTAGTGGTCG		
β-actin			
5′	ATCTGGCACCACACCTTCTACAATGAGCTGCG		838
3′	CGTCATACTCCTGCTTGCTGATCCACATCTGC		

internalization. Cells were analyzed with a laser-scanning LSM 510 Carl Zeiss confocal microscope.

Effects of inhibitors on GFP–HHV-8 and HSV-2 infection. Chlorpromazine HCl (5 and 10 μg/ml), nystatin (100 μg/ml), CTB (100 μg/ml), NH_4Cl (0.4, 2, 10, and 50 mM), and BFLA1 (0.003, 0.08, 2, and 50 nM) were used. The stock solutions were prepared in accordance with the manufacturer's recommendations. HFF were incubated at 37°C for 1 h with and without the inhibitors diluted in DMEM, infected with GFP–HHV-8 in the presence or absence of inhibitors at 37°C for 2 h, washed twice with DMEM, and further incubated with growth medium at 37°C. After 3 days, infection was monitored by counting the green fluorescent cells. These cells were subsequently fixed in acetone and examined for ORF73 expression by immunoperoxidase staining (4).

For quantitation of the infectious HSV-2 produced in the presence of inhibitors, HFF in 24-well plates were incubated at 37°C for 1 h with and without the inhibitors diluted in DMEM, infected with HSV-2 at an MOI of 1 in the presence or absence of inhibitors at 37°C for 2 h, washed twice with DMEM, and further incubated with growth medium at 37°C. The cytopathic effect was monitored. After 48 h, cells were collected by being scraped into the medium and frozen and thawed twice, and log dilutions of these samples were used to infect HFF in 24-well plates. The 50% tissue culture infective dose ($TCID_{50}$) of HSV-2 was calculated as described previously (3).

Radiolabeled binding assay. HFF grown in 24-well plates were used for binding assays (2, 4, 71). Cells were treated with DMEM or DMEM containing various inhibitors at 37°C for 1 h and washed three times with DMEM containing 5% fetal bovine serum. Purified, [^{3}H]thymidine-labeled GFP–HHV-8, in the presence or absence of inhibitors, was added to the cells and incubated at 4°C for 1 h. In another set of experiments, a constant quantity of purified [^{3}H]thymidine-labeled virus was mixed with 10 μg of soluble heparin or chondroitin sulfate C per ml and incubated at 37°C for 1 h. These mixtures were then added to HFF and incubated further at 4°C for 1 h. After incubation, cells were washed five times with DMEM and lysed with 1% sodium dodecyl sulfate–1% Triton X-100, and the radioactivity was precipitated with trichloroacetic acid and counted.

RT-PCR. HFF were left untreated or treated with various inhibitors at 37°C for 1 h and then infected with GFP–HHV-8 in the presence or absence of inhibitors at 37°C for 2 h. These cells were washed twice with DMEM and incubated with growth medium at 37°C. After 48 h, total RNA was isolated with an RNeasy RNA isolation kit (Qiagen, Valencia, Calif.) in accordance with the manufacturer's recommendations. Extracted RNA was examined for the presence of viral RNA transcripts with reverse transcriptase PCR (RT-PCR). A 5-μg sample of each RNA was incubated with 2 U of DNase I (Invitrogen, Carlsbad, Calif.) and reverse transcribed in a solution containing 250 ng of random hexadeoxynucleotides and 50 U of Superscript RT (First-Strand cDNA Synthesis System for RT-PCR; Invitrogen) in a final volume of 20 μl. A 1-μl sample of the cDNA was subjected to PCR analysis with different primer combinations to determine the expression of HHV-8 ORF73, GFP, and the human β-actin gene. The primer sequences used in this study and the expected sizes of PCR products are summarized in Table 1. The PCR mixture consisted of each deoxyribonucleotide at 200 μM, 10 U of Advantage cDNA polymerase mix, 10 pmol of each primer, and cDNA in a volume of 25 μl. Aliquots (10 μl) of the PCR-amplified product were subjected to electrophoresis through a 1.2% agarose gel and transferred onto positively charged nylon membranes (Sigma). Nylon membrane was prehybridized for 2 h with DIG Easy Hyb (Roche Applied Science, Indianapolis, Ind.) containing 0.1 mg of poly(A) per ml and 5 μg of poly(dA) per ml and hybridized with internal oligonucleotide probes labeled at the 3′ end with digoxigenin-dUTP (DIG Oligo Tailing kit; Roche Applied Science). The sequences of the probes were 5′-ACAAATTGCCAGTAGCCCACCAGGAGATAATACACCAGACGATG-3′ (ORF73), 5′-ACGGCATCAAGGTGAACTTGAAGATGCGCCACAACATCGAGG-3′ (eGFP), and 5′-GTACCACTGGCATCGTGATGGACTCCGGTGACG-3′ (β-actin). Hybridization and washing were then done in accordance with the manufacturer's protocol (DIG Easy Hyb; Roche Applied Science). The bound probe was detected with anti-digoxigenin–horseradish peroxidase conjugate (Pierce, Rockford, Ill.) and the standard ECL detection system (NEN Perkin-Elmer, Boston, Mass.).

RESULTS

EM observation of HHV-8 entry into HFF via endocytic vesicles. Many alpha- and betaherpesviruses deliver their DNA-containing capsids into cells by fusing the virion envelope with the plasma membrane (19, 50, 62, 63). When we examined the entry of HHV-8 into the human B-cell line BJAB, HHV-8 was detected in large endocytic vesicles (2). This is similar to the mode of entry of γ1-EBV into primary B cells (28, 35, 40). EBV infection of primary B cells results in latent infection, immortalization of B cells, and consequently maintenance of latent viral episomes along with host cell division (28). In contrast, infection of primary B cells by HHV-8 does not result in sustained latent infection and immortalization (unpublished observations). Since monitoring of HHV-8 infection in B cells was very difficult, entry of HHV-8 into adherent HFF was analyzed.

To visualize the earliest stages of the internalization process, HFF were incubated with purified GFP–HHV-8 for 60 min at 4°C and warmed to 37°C for 5 and 15 min. After binding at 4°C, enveloped virus particles were observed at or near the plasma membranes; an example of a virus particle near an electron-dense coated pit is shown in Fig. 1A. By transmission EM, HHV-8 particles of about 120 to 170 nm were observed, which is comparable to the reported average size of HHV-8 and other herpesvirus particles (14, 40, 43, 50, 52). As early as 5 min postwarming to 37°C, marked changes were noticed (Fig. 1). Virus particles were observed in thick-walled coated vesicles with the characteristic morphology of clathrin-coated vesicles (Fig. 1B), as well as in noncoated vesicles (data not shown). By 15 min postwarming, most of the virus particles were seen within the noncoated vesicles, which were several times bigger than virus particles (Fig. 1C, D, E, and F). Some of the virus particles were observed in vesicles that were partially coated, and the rest were observed in vesicles with a smooth surface (Fig. 1C). Clathrin-mediated endocytosis is a fast event, and clathrin is recycled back to the cell membrane

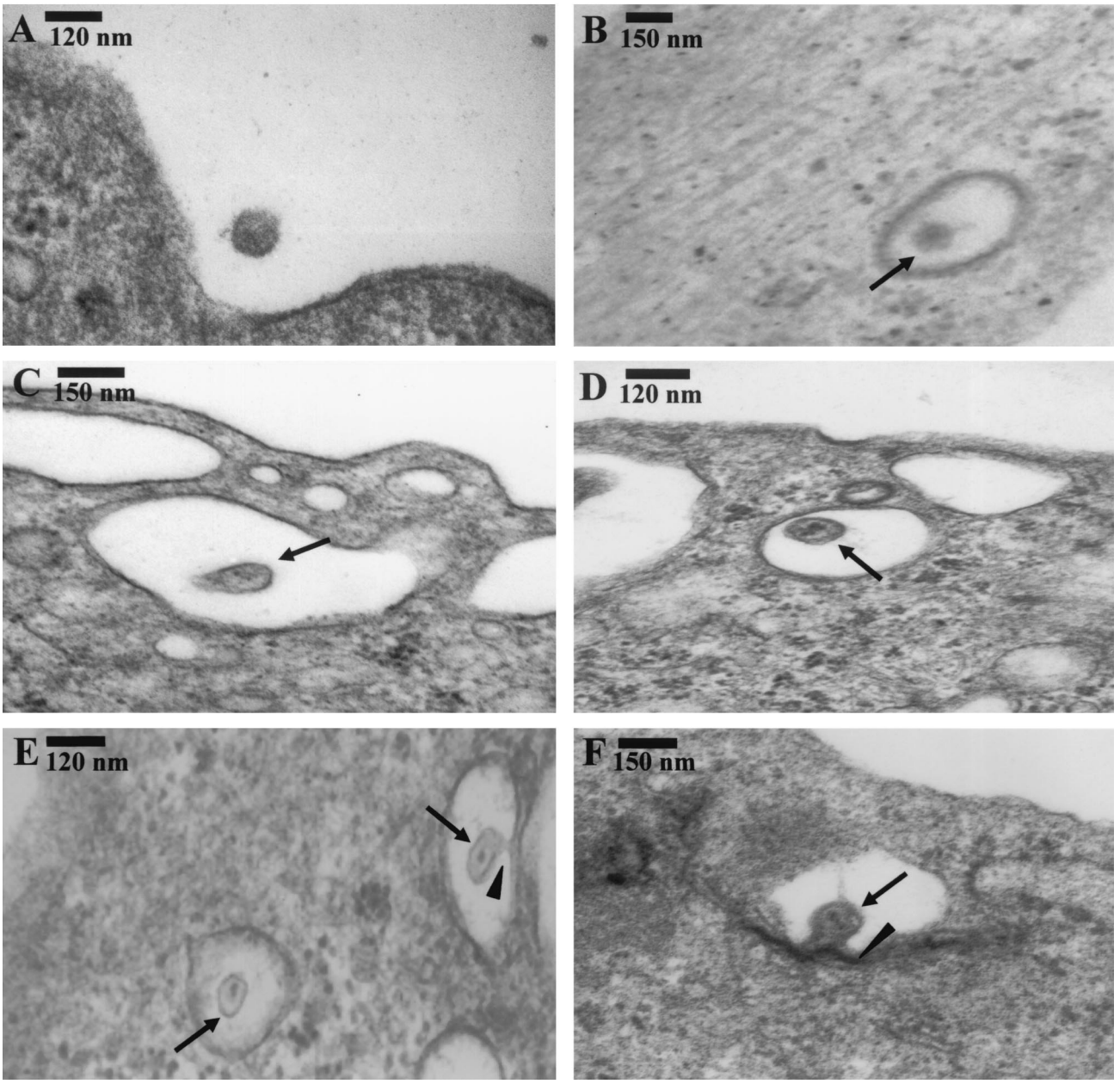

FIG. 1. EM observation of HHV-8 entry into HFF via endocytic vesicles. HFF (10^6) were incubated with GFP–HHV-8 for 60 min at 4°C. Infection was initiated by shifting the temperature to 37°C. After 0 (A), 5 (B), and 15 (C, D, E, and F) min at 37°C, cells were washed in PBS and fixed in 2% glutaraldehyde. Thin sections were made for ultrastructural analysis by transmission EM. Virion particles at various stages of binding and entry and in endocytic vesicles are indicated by arrows. The arrowheads in panels E and F indicate the envelope of a virus particle in contact with the endocytic vesicle membrane and in the process of fusion, respectively.

within minutes of internalization (25, 29, 32, 59). Part of the smooth membrane illustrated in Fig. 1C is in the form of a bleb and appears to be coated. The vesicle may be in the process of losing its coat and perhaps fusing with other thin-walled vesicles. Figure 1E shows a virus particle within a vesicle, and another within a noncoated vesicle, which appears to be in the earlier stage of fusing with the endocytic wall. Figure 1F shows a virus particle within a vesicle in the late stage of fusing its envelope and delivering the capsids into the cytoplasm. We did not observe the fusion of virion envelopes at the cell membranes.

To confirm these results, rabbit anti-HHV-8 gB antibodies were used in IEM. Anti-gB antibodies reacted specifically with HHV-8, as demonstrated by the presence of gold particles on the virion envelopes (Fig. 2A to D). The specificity of the IEM observations was demonstrated by the absence of gold particle binding after reaction with rabbit anti-HHV-8 ORF73 antibodies (data not shown). Gold particles indicating the location of gB were detected on the virion envelope near the contact points with the cell membranes (Fig. 2A and B). Gold particles were also detected on the virion envelopes inside the endocytic

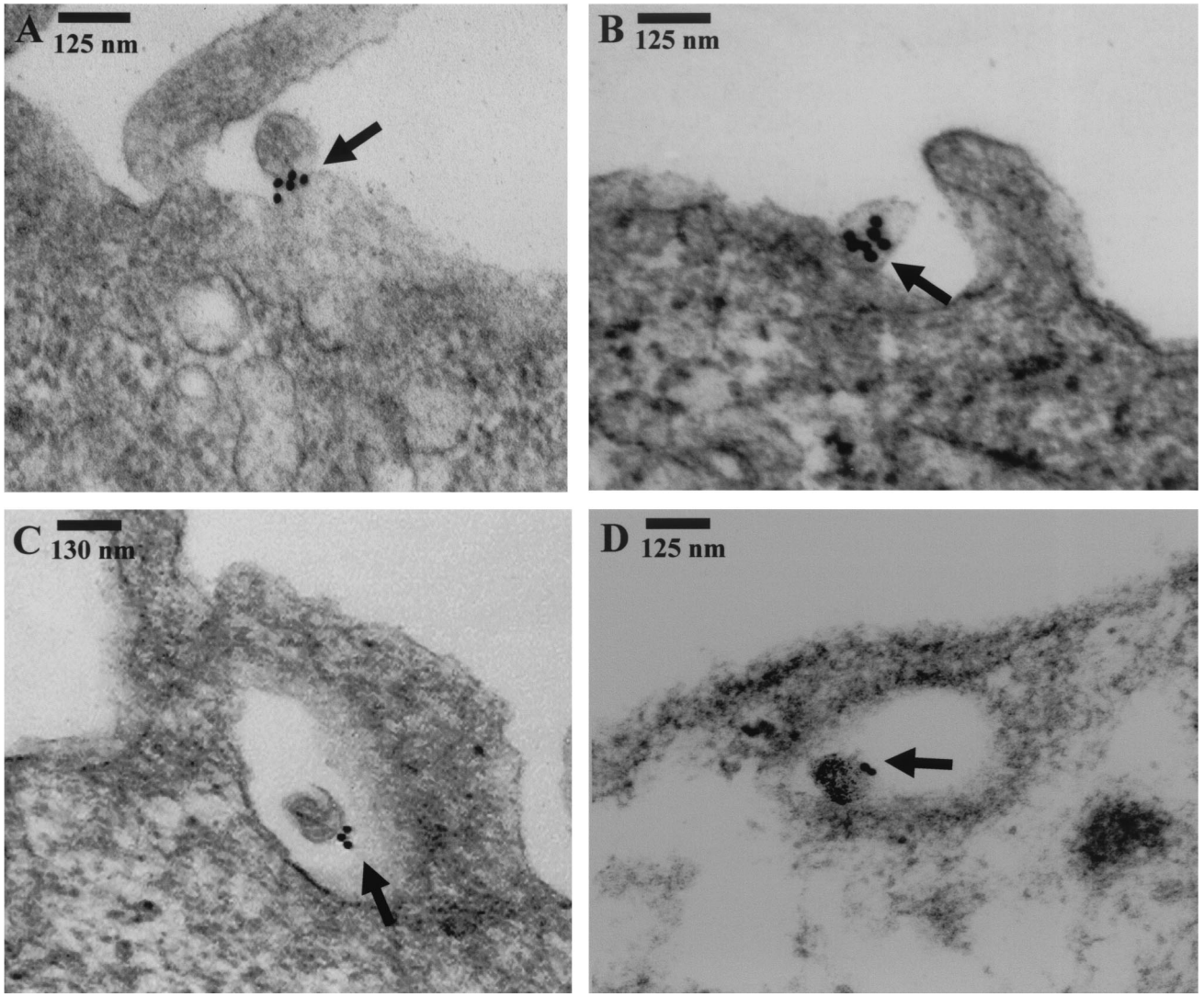

FIG. 2. IEM observation of HHV-8 entry into HFF via endocytic vesicles. HFF were incubated with GFP–HHV-8 for 60 min at 4°C. Infection was initiated by shifting the temperature to 37°C. After 0 (A) and 5 (B, C, and D) min at 37°C, cells were washed in PBS and fixed in 4% paraformaldehyde and thin sections were made. Grids containing the sections were incubated with rabbit anti-HHV-8 envelope glycoprotein gB IgG antibodies for 1 h at room temperature and then incubated at room temperature for 30 min with anti-rabbit antibodies conjugated with 15-nm gold particles. The grids were washed in PBS and distilled H_2O, counterstained, and viewed under an EM. Arrows indicate the gold particles identifying the location of gB near the enveloped virions.

vesicles (Fig. 2B, C, and D). Gold particles representing viral envelopes left over on the cell membranes, representing fusion at plasma membranes, were not observed. Taken together, these results suggest that endocytosis may be the predominant pathway of HHV-8 entry into HFF cells.

Colocalization of transferrin with HHV-8 during entry into HFF. In resting cells, endocytosis occurs via four major routes, i.e., clathrin-coated vesicles, the caveolar pathway, macropinocytosis, and an ill-defined route of non-clathrin-, non-caveola-dependent endocytosis (29, 34, 59). To define the entry pathway of HHV-8, we used FITC-labeled HHV-8 and traced its entry into target cells under a confocal microscope. Since internalization of transferrin occurs by clathrin-dependent receptor-mediated endocytosis (47, 59), to determine the HHV-8 entry pathway, we analyzed the uptake of TRITC-labeled transferrin and FITC-labeled purified HHV-8 by confocal microscopy. FITC–HHV-8 and TRITC-transferrin could both be seen as small punctate clusters within the cytoplasm as early as 5 min postincubation at 37°C (Fig. 3A and B). By about 15 min, FITC–HHV-8 and TRITC-transferrin could be detected in spherical-body clusters (Fig. 3C). Image overlays demonstrated the colocalization of HHV-8 with transferrin, presumably the early and late endosomes. Previous studies have shown that HSV enters target cells via fusion of their virion envelopes with the plasma membrane (50, 62, 63). Similar to our study, in a recent report, entry of HSV-1 was analyzed by fluorescence microscopy (41). Lysosomotropic agents blocked the delivery of virus capsids to the nuclei of HeLa and CHO cells, into which HSV-1 enters via endocytosis, but had no effect on the capsid in Vero cells, in which the virus fuses its envelope with

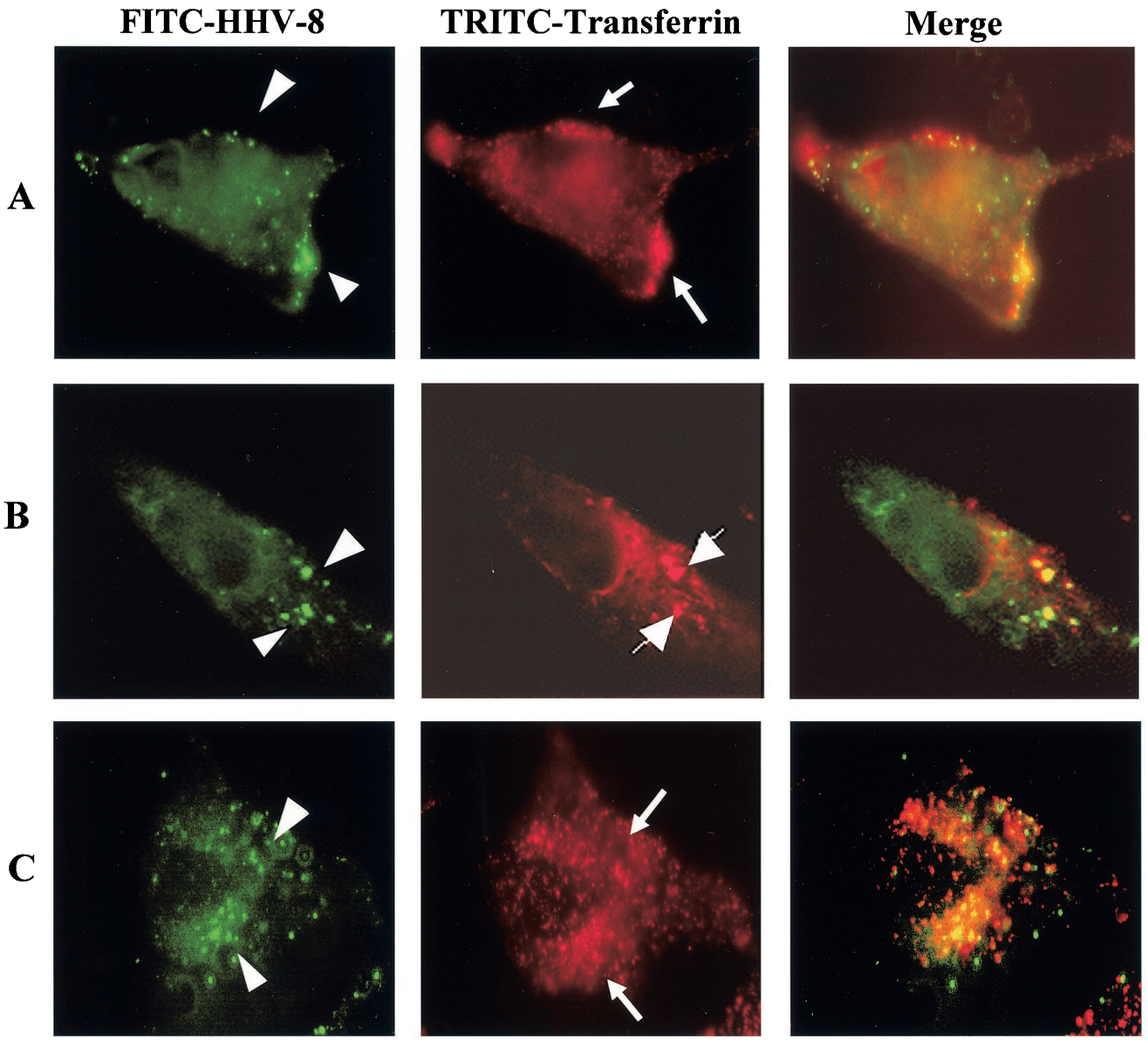

FIG. 3. Colocalization of FITC–HHV-8 and TRITC-transferrin in endosomes. HFF were incubated at 37°C with TRITC-labeled transferrin and FITC-labeled HHV-8 for 5 (A and B) and 15 (C) min, respectively. Cells were washed in PBS, fixed with 2% paraformaldehyde at 4°C for 10 min, washed, mounted in antifade reagent, and analyzed under a confocal microscope with appropriate filters. Magnification, ×62. The arrowheads and arrows indicate internalized HHV-8 and transferrin, respectively.

the plasma membrane (41). Colocalization of transferrin with HHV-8 suggested that HHV-8 probably enters HFF via clathrin-coated vesicles.

Disruption of clathrin-mediated endocytosis by chemical inhibitors inhibits entry of HHV-8 into HFF. To confirm biochemically the above morphological observations of HHV-8 entry via clathrin-coated vesicles, we tested the abilities of drugs that selectively inhibit the various pathways of endocytosis to modulate the entry of HHV-8 (Table 2). To monitor the HHV-8 binding-and-entry process, recombinant GFP–HHV-8 expressing GFP under control of the elongation factor 1α promoter was used (70). Entry into and infection of cells were monitored by counting green fluorescent cells and confirmed by detection of the ORF73 protein by immunoperoxidase staining (4). As a control for these experiments, we used HSV-2, a virus known to enter target cells by fusion of the virus envelope with the plasma membrane (50, 62, 63).

Chlorpromazine HCl is a cationic amphiphilic drug that prevents clathrin-mediated endocytosis by blocking the assembly and disassembly of a clathrin lattice at the cell surface and endosomes, as well as decreasing clathrin recycling (29, 32, 58, 59). Treatment of cells with nystatin, which blocks the caveola-dependent pathway, and CTB, which causes dissociation of lipid rafts, did not significantly alter the GFP–HHV-8 and HSV-2 infection of HFF (Fig. 4A). At a concentration of 10 μg/ml, chlorpromazine HCl, which blocks clathrin-dependent

TABLE 2. Inhibitors of endocytosis and their modes of action

Drug	Effect	References
Chlorpromazine HCl	Cationic amphiphilic drug that prevents assembly and disassembly of clathrin lattice and decreases recycling of clathrin, thus preventing clathrin-mediated endocytosis	25, 29, 32, 47, 59, 60, 64
Nystatin	Sterol-binding agent acts to remove membrane cholesterol, which is important for both maintenance of caveolae and the ability of caveolae to seal off from the plasma membrane	25, 59
CTB	Ganglioside-binding molecule that causes dissociation of lipid rafts	32, 59
NH_4Cl	Agent most commonly used to study pH-dependent entry of microbes, a lysosomotropic weak base	25, 27, 32, 42, 53, 59
BFLA1	Specific and potent inhibitor of vacuolar H^+-ATPase resulting in inhibition of endosome and lysosome acidification	11, 32, 59

endocytosis, inhibited GFP–HHV-8 infection about 78% ± 5% (Fig. 4A). At a chlorpromazine HCl concentration of 5 μg/ml, HHV-8 infection was inhibited about 46% (data not shown). Similar results were also observed when ORF73 expression was monitored by immunoperoxidase staining (data not shown). In contrast, chlorpromazine HCl did not affect the replication of HSV-2 (Fig. 4A). Chlorpromazine HCl did not alter HHV-8 infection significantly when cells were treated 24 h after infection (data not shown), suggesting that the effect was at an early stage of infection. Treatment of cells with sucrose has also been shown to inhibit clathrin-dependent endocytosis by increasing the hypertonicity of cells (67). GFP–HHV-8 infection, but not HSV-2 infection, was inhibited by more than 50% by pretreatment of HFF with 0.2 M sucrose before infection (data not shown).

To verify that HHV-8 enters cells via clathrin-dependent vesicles, HFF were incubated with DMEM containing chlorpromazine HCl at 37°C for 1 h, incubated with labeled transferrin and HHV-8 in the presence of chlorpromazine HCl, and analyzed by confocal microscopy. Even after 15 min of incubation in the presence of chlorpromazine HCl, HHV-8 and transferrin colocalization did not occur in HFF. Small punctate green or red fluorescence spots representing labeled virus and transferrin, respectively, were observed on the surface of the cells (Fig. 4B). This could be due to a lack of entry and/or a block in the progression into early endosomes due to pretreatment with chlorpromazine HCl. These results provided one line of biochemical evidence for the entry of HHV-8 into HFF via clathrin-coated endocytic vesicles and thus support the morphological evidence shown in Fig. 1 to 3.

Inhibition of acidification of endosomes inhibits HHV-8 entry into HFF. Virions internalized by endocytosis penetrate the cytoplasm by fusing with the membranes of endosomes or lysosomes in a pH-independent or -dependent manner (32, 59). Viruses such as the Semliki Forest virus, influenza virus, and vesicular stomatitis virus (VSV) require exposure to low-pH environments for efficient fusion of the envelope with endosomes (22, 32, 59), while viruses like EBV (in primary B cells) (35, 40) and duck hepatitis virus (32) enter via pH-independent endocytosis. To determine whether internalized HHV-8 requires a change in pH for its entry into HFF, the effects of NH_4Cl and BFLA1 on HHV-8 infection were examined. NH_4Cl is a weak lysosomotropic base that diffuses into acidic endosomes, where it becomes protonated. Once protonated, it is unable to diffuse out, thereby increasing the pH (25, 32, 42, 53, 59). BFLA1 is a potent and specific inhibitor of vacuolar H^+-ATPase, which is the proton pump responsible for acidification of the intracellular compartments of eukaryotic cells (11). These lysosomotropic drugs are known to raise the pH of intracellular organelles and to inhibit low-pH-dependent endosomal fusion within several minutes of cell treatment (32, 59).

Treatment of cells with NH_4Cl (Fig. 5A) and BFLA1 (Fig. 5B) significantly inhibited infection with GFP–HHV-8 in a dose-dependent manner. The percentage of inhibition reached a plateau between NH_4Cl concentrations of 10 and 50 mM, and the maximum inhibition ranged from 82 to 87%. For BFLA1, the percentage of inhibition reached a plateau between concentrations of 25 and 50 nM and the maximum inhibition ranged from 92 to 95%. Similar results were also observed when ORF73 expression was monitored by immunoperoxidase staining (data not shown). In contrast, as expected, NH_4Cl and BFLA1 were unable to block the replication of HSV-2, and an equal quantity of virus was produced in the presence or absence of these drugs (data not shown). This indicated that the inhibition of HHV-8 by these drugs was caused by blocking of endosomal acidification and not by nonspecific cytotoxic effects. Treatment of cells with NH_4Cl and BFLA1 after 24 h of infection at 37°C did not significantly alter HHV-8 infection (data not shown), suggesting that the effects of these lysosomotropic agents occur at an early stage of infection. These results suggested a requirement of intracellular acidic compartments for HHV-8 infection.

Chlorpromazine HCl, NH_4Cl, and BFLA1 do not inhibit binding of HHV-8 to HFF. HHV-8 interacts with cell surface HS during the initial attachment stage of infection (2). To determine whether inhibition of HHV-8 infection by chlorpromazine HCl, NH_4Cl, and BFLA1 affects the virus binding stages, we tested the inhibitors' ability to block the binding of virus to HFF. Similar to our previous findings (2), heparin at a concentration of 10 μg/ml inhibited [^{3}H]thymidine-labeled HHV-8 binding to HFF by more than 90% (Fig. 5C). The specificity of this inhibition was demonstrated by the absence of inhibition by HS-related chondroitin sulfate C (Fig. 6C). In

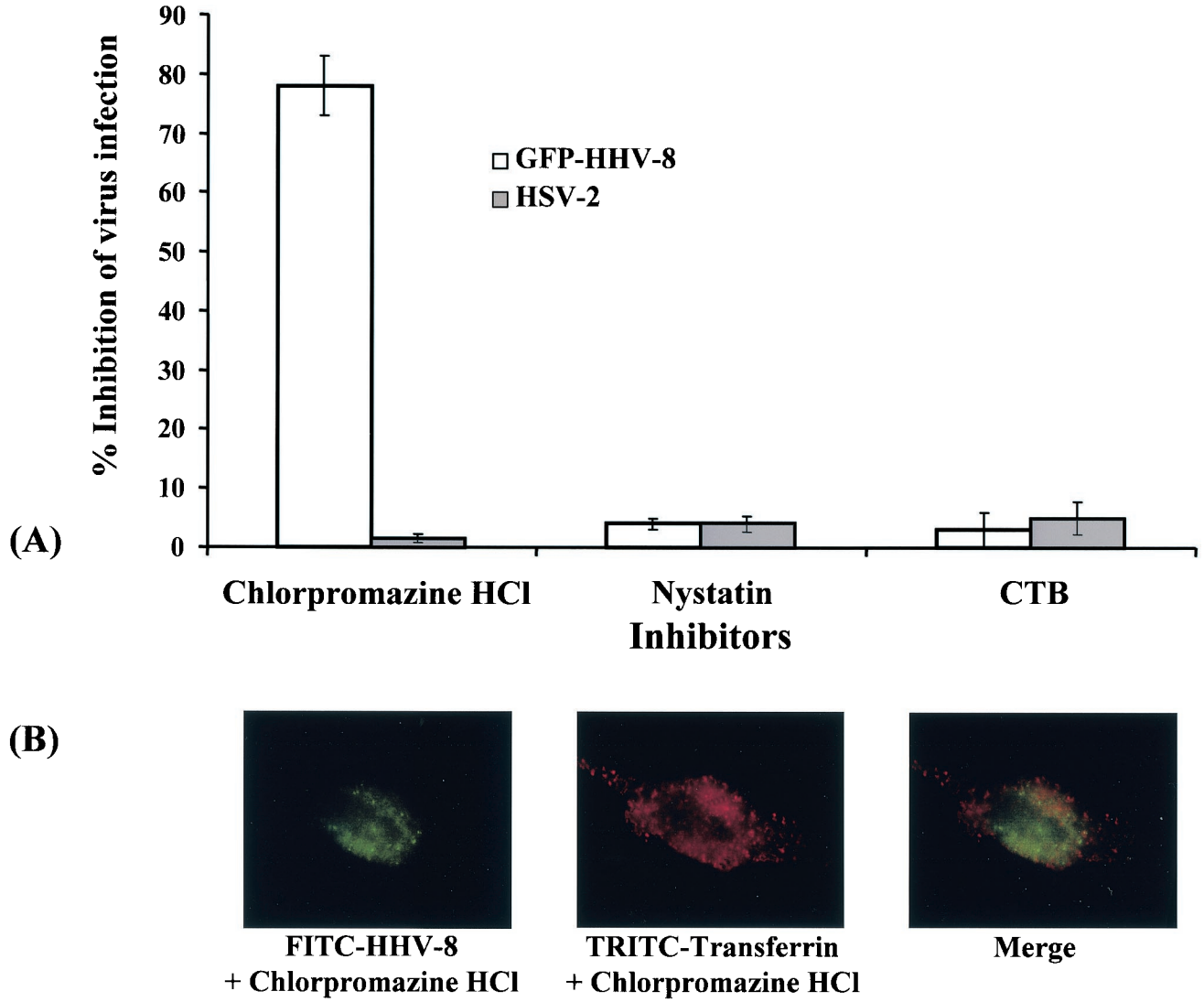

FIG. 4. Inhibition of GFP–HHV-8 infection by chlorpromazine HCl. (A) HFF monolayers in eight-well chamber slides were incubated with DMEM containing no drug or chlorpromazine HCl (10 μg/ml), nystatin (100 μg/ml), or CTB (100 μg/ml) for 1 h at 37°C before infection with GFP–HHV-8. After incubation for 2 h at 37°C with the virus in the presence of inhibitors, cells were washed and further incubated with growth medium for 3 days at 37°C. Green fluorescent cells, indicative of GFP–HHV-8 entry and infection, were counted. In the absence of inhibitors, approximately 300 green fluorescence-expressing HFF per well were detected. A similar procedure was used for infection of HFF in 24-well plates with HSV-2. Cells and supernatant from HSV-2 infection were collected after 2 days, and the HSV-2 $TCID_{50}$ was quantitated by titration in HFF. Approximately $10^{5.8}$ $TCID_{50}$ of HSV-2 was produced in HFF in the absence of inhibitors. Data are presented as the percentage of inhibition of virus infectivity obtained when cells were incubated with the virus without inhibitors. Each reaction was done in triplicate, and each point represents the average ± the standard deviation of three experiments. (B) HFF were incubated with DMEM containing chlorpromazine HCl (10 μg/ml) at 37°C for 1 h and then incubated at 37°C with TRITC-labeled transferrin and FITC-labeled HHV-8 in the presence of chlorpromazine HCl (10 μg/ml) for 15 min. Cells were washed in PBS, fixed with 2% paraformaldehyde at 4°C for 10 min, washed, mounted in antifade reagent, and analyzed under a confocal microscope with appropriate filters. Magnification, ×62.

contrast, chlorpromazine HCl (10 μg/ml), nystatin (100 μg/ml), CTB (100 μg/ml), NH_4Cl (50 mM), and BFLA1 (50 nM) at the concentrations used in the virus neutralization assays, as well as at higher concentrations, did not inhibit the binding of HHV-8 to target cells (Fig. 5C). These results demonstrated that chlorpromazine HCl, NH_4Cl, and BFLA1 inhibit the infectious process of HHV-8 at a postattachment step of infection.

Chlorpromazine HCl, NH_4Cl, and BFLA1 inhibit infectious entry of HHV-8. Since HHV-8 infection of target HFF results only in latent infection with no expression of viral lytic genes (70), we could not examine the expression of viral immediate-early genes. Hence, to confirm the neutralization of GFP–HHV-8 with inhibitors without affecting the binding of radiolabeled virus to target cells, and to provide additional quantitative evidence for the endocytic entry of HHV-8, we examined their effect on indicator GFP mRNA and HHV-8 ORF73 mRNA expression. No detectable signal was observed with reactions performed in the absence of RT or with no template (data not shown), demonstrating the specificity of the

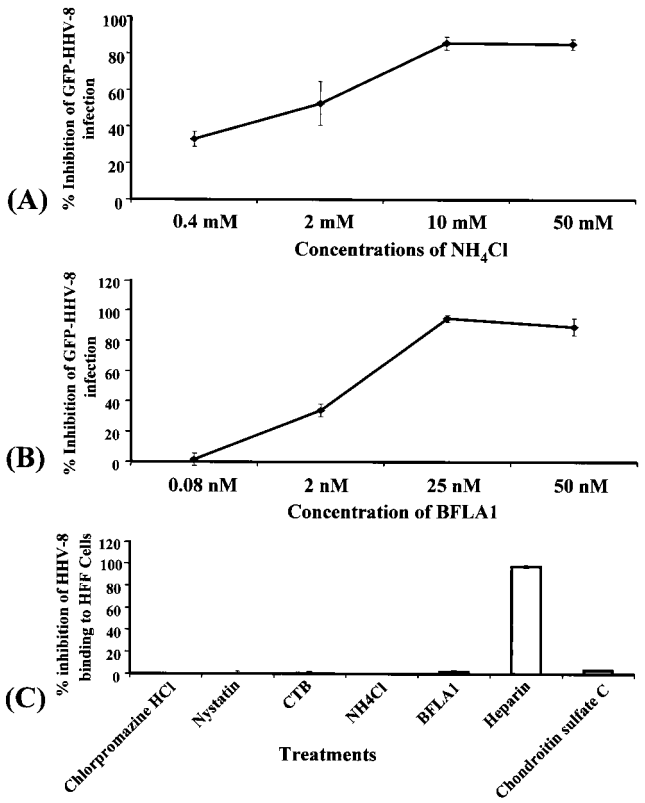

FIG. 5. Effects of NH_4Cl and BFLA1 on HHV-8 binding and infection. HFF monolayers were incubated with DMEM or DMEM containing various concentrations of NH_4Cl (A) and BFLA1 (B) for 1 h at 37°C. These cells were infected with GFP–HHV-8 in the presence or absence of respective inhibitors at 37°C for 2 h, washed twice with DMEM, and further incubated with growth medium at 37°C. After 3 days, infection was monitored by counting of green fluorescent cells. In the absence of inhibitors, approximately 300 green fluorescence-expressing HFF per well were detected. Data are presented as the percentage of inhibition of virus infectivity obtained when the cells were incubated with the virus without inhibitors. Each reaction was done in triplicate, and each point represents the average ± the standard deviation of three experiments. (C) HFF were treated with DMEM or DMEM containing different inhibitors (chlorpromazine HCl at 10 μg/ml, nystatin at 100 μg/ml, CTB at 100 μg/ml, NH_4Cl at 50 mM, or BFLA1 at 50 nM) at 37°C for 1 h. Cells were washed three times with DMEM containing 5% fetal bovine serum. Purified [^{3}H]thymidine-labeled GFP–HHV-8 (2,500 cpm), along with the inhibitors, was added to the cells and incubated at 4°C for 1 h. Inhibition of radiolabeled HHV-8 binding by heparin and chondroitin sulfate C was performed by incubation of a constant quantity of purified [^{3}H]thymidine-labeled virus (2,500 cpm) with 10 μg of soluble heparin or chondroitin sulfate C per ml at 37°C for 1 h. These mixtures were then added to HFF and incubated at 4°C for 1 h. After incubation, cells were washed five times with DMEM and lysed with 1% sodium dodecyl sulfate and 1% Triton X-100, and the radioactivity was precipitated with trichloroacetic acid and counted. In the absence of inhibitors, approximately 21% of the input HHV-8 radioactivity became associated with the cells. Each reaction was done in triplicate, and each point represents the average ± the standard deviation of three experiments.

RT-PCRs. Compared to the gene expression in HHV-8-infected cells in the absence of inhibitors (Fig. 6A, lane 2), chlorpromazine HCl (10 μg/ml), NH_4Cl (50 mM), and BFLA1 (50 nM) significantly inhibited the mRNA expression levels of virus-encoded ORF73 by about 80, 89, and 92%, respectively, and that of the GFP genes by about 84, 91, and 90%, respectively (Fig. 6A, lanes 3, 6, and 7, respectively, and B). These inhibitors did not significantly alter the expression levels of the β-actin gene (Fig. 6A). In contrast, nystatin (100 μg/ml) and CTB (100 μg/ml) did not inhibit the ORF73 and GFP mRNA levels significantly (Fig. 6A, lanes 4 and 5, respectively, and B). Inhibition of HHV-8 ORF73 and GFP gene expression by chlorpromazine HCl, BFLA1, and NH_4Cl demonstrated that they inhibited the infectious entry of HHV-8 via endocytosis. These results correlate with the effect of these inhibitors on HHV-8 infection shown in Fig. 4A and 5A and B and thus indicate that clathrin-mediated endocytosis may be the predominant pathway for the infectious entry of HHV-8 into HFF cells.

DISCUSSION

Viruses enter target cells by two distinct routes. In the first pathway, a virus fuses its envelope with the plasma membrane

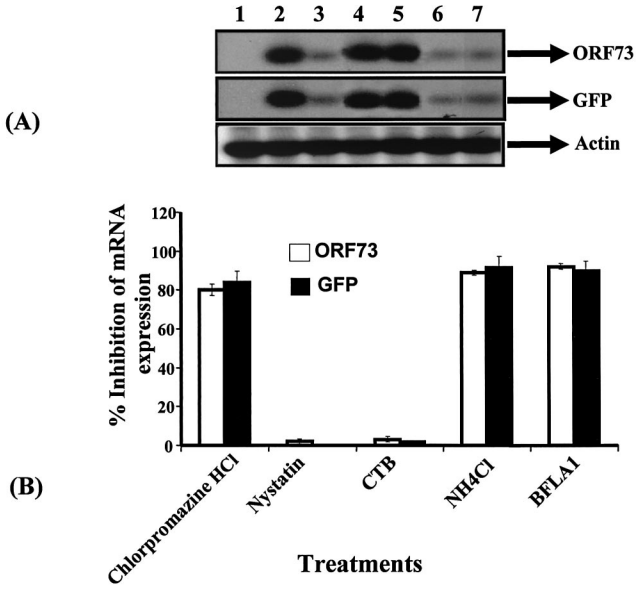

FIG. 6. Chlorpromazine HCl, NH_4Cl, and BFLA1 inhibit infectious entry of HHV-8. (A) HFF were left untreated or treated with various inhibitors at 37°C for 1 h and then infected with GFP–HHV-8 in the presence or absence of inhibitors at 37°C for 2 h. These cells were washed twice with DMEM and incubated with growth medium at 37°C. At 48 h postinfection, total RNA was isolated and reverse transcribed by RT-PCR with random hexamers and the cDNA was subjected to PCR analysis with different primers specific to HHV-8 ORF73 or the GFP and human β-actin genes (Table 1). The PCR-amplified products were resolved in a 1.2% agarose gel and transferred onto nylon membranes, and Southern blot hybridization was performed with different digoxigenin-labeled internal oligonucleotide probes to detect expression of the ORF73 (307 bp), GFP (328 bp), and human β-actin (838 bp) mRNAs. Lanes: 1, uninfected HFF; 2, HFF infected with GFP–HHV-8 in the absence inhibitors; 3 to 7, GFP–HHV-8 infection of HFF incubated with chlorpromazine HCl (10 μg/ml), nystatin (100 μg/ml), CTB (100 μg/ml), NH_4Cl (50 mM), and BFLA1 (50 nM), respectively. (B) The intensities of bands in the absence or presence of the inhibitors in panel A were scanned, the intensities were assessed with the ImageQuant software program (Molecular Dynamics), and HHV-8 ORF73 and GFP gene expression was quantitated. Each point represents the average ± the standard deviation of three experiments.

and releases the genome containing the capsid and other proteins into the cell. This is a pH-independent process and is exemplified by HSV-1 and human immunodeficiency virus type 1 (28, 31, 32). The second pathway is receptor-mediated endocytosis of virus particles into target cells. Nonenveloped DNA viruses (adenoviruses, simian virus 40, JC virus [papovavirus], papillomavirus, adeno-associated virus 2, and parvovirus [5, 32, 39, 44, 47, 54, 58]), nonenveloped RNA viruses (echo virus and rotavirus [26, 33, 39, 64]), and enveloped RNA viruses (influenza virus, Semliki Forest virus, VSV, murine leukemia virus and hantavirus [25, 27, 30, 60]) use the endocytic pathway. Endocytosis is used by many viruses, since endosomes offer a convenient and often rapid system of transit across the plasma membrane and through a crowded cytoplasm, as well as the delivery of viral cargo to the vicinity of the nuclear pore for viruses replicating their genomes in the nucleus (32, 59, 72).

In this report, we have presented several lines of evidence that HHV-8 enters HFF via endocytosis. Some alpha- and betaherpesviruses have been shown to enter target cells via fusion of their virion envelopes with the plasma membrane (19, 50, 61, 62, 63). However, there are exceptions to this dogma, as studies have demonstrated that entry pathways may vary with the herpesvirus and target cell. For example, recent studies have shown that HSV-1 and HSV-2 enter the HeLa and CHO-K1 cell lines via endocytosis and that they depend on exposure to a low pH (41). γ1-EBV infects two human cell types, B lymphocytes and epithelial cells (35, 40). Similar to our observations with the γ2-HHV-8 infection of HFF, EM studies demonstrated the absence of direct fusion of EBV with the outer cell membrane in primary B cells. Instead, EBV entry in large endocytic vesicles was observed (40). Weak bases such as chloroquine, methylamine, and NH_4Cl blocked deenvelopment and viral infectivity (40). In contrast, in Raji cells, a human B-cell line established from Burkitt's lymphoma, EBV entered by fusion of its envelope at the plasma membrane (40). This cell type variation was attributed to probable receptor variations, alterations in membrane fluidity, and cytoskeletal differences (28, 35, 40). These observations were further supported by a fluorescence dequenching assay of fusion with EBV labeled with pH-insensitive and -sensitive probes (35). In these studies also, fusion of the virus with normal B cells was inhibited by chlorpromazine, chloroquine, and sodium azide, but none of these reagents had any effect on fusion with Raji or epithelial cells (35). These studies demonstrated that EBV is incapable of fusing with normal B cells unless it is first endocytosed and suggested a role for clathrin in the uptake of EBV into B cells (35).

IEM analysis of the binding and internalization of HHV-6A strain GS and HHV-6B strain Z29, betaherpesviruses, in a susceptible T-cell line revealed that infection occurs through an endocytic pathway (18). Similar to our observation, when cells with HHV-6 virions bound to the cell surface at 4°C were warmed to 37°C, viral internalization through smooth-surfaced pits and vesicles was observed. Fusion of HHV-6 virions with the cell plasma membrane was never observed (18). Gold immunolabeling before or after viral internalization showed the absence of HHV-6 envelope proteins on the cell plasma membranes at all times of internalization. Treatment of cells with chloroquine resulted in complete inhibition of HHV-6 infectivity, suggesting that the endocytosed virions are responsible for a successful infection (18). Inhibition of HHV-6 infection by chloroquine was effective in two different T-cell lines, as well as in peripheral blood mononuclear cells (18). Similarly, human cytomegalovirus, a betaherpesvirus, has been shown to enter retinal pigment epithelial cells by endocytosis (10) but fuses at the plasma membrane of fibroblasts (19). These studies support the notion that the entry pathways of herpesviruses may vary with the virus and target cells and indicate that entry by fusion at the cell membrane may not be the sole mode of entry for all herpesviruses.

Influenza virus enters cells predominantly via clathrin-mediated endocytosis and has been studied extensively as a model for clathrin-mediated endocytosis (59, 60). However, in a recent work, by combining inhibitory methods to block both clathrin-mediated endocytosis and uptake by caveolae in the same cell, it was demonstrated that influenza virus infects cells by an additional non-clathrin-dependent, non-caveola-dependent endocytic pathway (60). Similarly, human immunodeficiency virus type 1, entering susceptible cells by fusion of its envelope with the plasma membrane after binding to the CD4 molecule and the chemokine coreceptors, has been shown to enter brain microvascular endothelial cells by macropinocytosis, which is dependent on the lipid raft and mitogen-activated protein kinase signaling pathway (31). These studies suggest that viruses may use multiple pathways to enter target cells and some pathways may predominate over others.

Vaccinia virus produces two antigenically distinct infectious virions, intracellular mature virus (IMV) and extracellular enveloped virus (EEV) (69). Structurally, EEV consists of an IMV with an additional outer membrane containing proteins that are absent from IMV. EEV is important for virus dissemination both in vitro and in vivo. Both morphological and biochemical approaches show that IMV enters the cell by fusion in a pH-independent manner at the plasma membrane (69). In contrast, EEV enters cells by endocytosis, followed by low-pH-induced disruption of the EEV outer membrane and fusion of the exposed IMV, with the endosomal membrane releasing the core into the cytosol (69). These studies suggest that entry into target cells may vary even within the different forms of the same virus because of differences in the composition of surface glycoproteins. Hence, it is not surprising to observe different entry pathways for herpesviruses, since, in addition to the glycoproteins that are conserved among the herpesviruses, each of the herpesviruses also possesses envelope glycoproteins that are unique to the particular virus. Even the conserved glycoproteins may have different functions best suited for the particular virus that are probably responsible for their unique biological characters. For example, only HHV-8 gB possesses the integrin-interacting RGD amino acid motif among the alpha-, beta-, and gammaherpesvirus gB sequences determined to date.

The morphological and biochemical evidence presented here suggests that γ2-HHV-8 differs from some alpha- and betaherpesviruses in its entry and that clathrin-coated vesicles may be the predominant pathway of HHV-8 entry into HFF. Inhibition of HHV-8 ORF73 and GFP gene expression by chlorpromazine HCl, BFLA1, and NH_4Cl demonstrated that the virions in the vesicles could proceed to cause an infection. We interpret these results with caution, since the effects of these drugs on HHV-8 infection could also be due to multiple

effects on other cellular functions. However, at concentrations that inhibited HHV-8 infection, these drugs did not inhibit the productive lytic HSV-2 infection of HFF. Additionally, detection of HHV-8 in endocytic vesicles by EM, fusion of the viral envelope with endocytic vesicles, and colocalization of transferrin along with HHV-8 early during infection also strongly suggest the specificity of these drugs for HHV-8 infection and support our conclusion that HHV-8 uses clathrin-coated vesicles as the predominant pathway of entry into HFF cells.

By using EM, Dezube et al. (20) have examined primary endothelial cells 1 h after HHV-8 infection. That study revealed naked nucleocapsids in the cytosol and virions in the cytoplasmic vesicles, presumably endosomes. The nature of the endocytic vesicles and the mode of viral entry were not determined in that study. However, although they observed virions in endocytic vesicles, on the basis of the presence of nucleocapsids in the cytoplasm and by analogy with HSV-1, the authors concluded that the virus enters by fusion at the plasma membrane (20). Observation of nucleocapsids in the cytoplasm does not distinguish virus entry by fusion at the plasma membrane or by endocytosis, since nucleocapsids are also observed in the cytoplasm of EBV-infected B cells, which the virus enters by endocytosis (40). Our present study demonstrated the endocytic entry pathway of HHV-8 into HFF, and further studies are in progress to determine the mode of entry into the other target cells of HHV-8. Transport from early to late endosomes involves intermediates known as endosomal carrier vesicles. Entry is a dynamic event, and the virus envelope can fuse at any stage of endosomal trafficking and at different proximities to the nucleus. HHV-8 entry via clathrin-coated vesicles in HFF appears to be a pH-dependent event. Although we have not provided direct evidence in this study to explain why a low pH is critical for HHV-8 infection, it is generally believed that acidification of virus-carrying endosomes may induce conformational changes in the envelope glycoproteins or capsid structures that trigger membrane fusion or penetration reactions (32, 60). In other systems, a reduction in pH vesicles has been found to occur prior to fusion with lysosomes (12, 68). The action of some plasma membrane enzymes has also been suggested to be involved in the pH change (74). Whether HHV-8 capsid release into the cytoplasm requires lysosomal fusion with virion-containing vesicles or some other mechanism that lowers the pH of these vesicles is not clear and needs to be investigated further.

Our studies suggest that the predominant pathway of HHV-8 entry into HFF is low-pH-dependant endocytosis. When the 58-amino-acid HHV-8 gB cytoplasmic tail containing the putative endocytosis signals (YXXΦ) was removed, the truncated form of gB (gBMUT) was expressed efficiently on the CHO cell surface (46). Expression of HHV-8 gBMUT, gH, and gL resulted in the fusion of CHO cells with 293 (human embryonic kidney) cells, implying a pH-independent fusion mechanism (46). The divergence between the study by Pertel (46) and our study could be due to a number of factors. (i) As pointed out by the author (46), fusion by HHV-8 gBMUT, gH, and gL is an inefficient process, since fusion was observed in only a limited number of cells transfected with HHV-8 glycoproteins. Fused cells also exhibited only two to four nuclei. In contrast, the control fusion mediated by HSV-1 gB, gD, and gH-gL was more efficient, with more fused cells and larger polykaryocytes containing more nuclei (46). Cells with more than two nuclei were commonly observed in cultures of normal 293, HFF, COS-1, COS-7, and CHO cells (unpublished observation). (ii) Since the transfected cells were observed after incubation at 37°C for 48 h, a change in the medium pH due to normal cellular metabolism and consequently an acid-dependent alteration in the conformation of proteins leading to the fusion of cells could not be ruled out. (iii) The limited fusion event was observed only when transfected CHO cells were mixed with 293 cells but not when they were mixed with BJAB (human B), CHO, or Vero cells. The reason for this discrepancy was not explained (46). HHV-8 binds and enters a variety of human (BCBL-1, BJAB, Raji, 293, HFF, HeLa, endothelial), monkey (Vero, CV-1), hamster (BHK-21, CHO), and mouse (L, DU17) cells, as shown by the detection of DNA, limited HHV-8 gene expression, and GFP expression (1, 2, 3, 37, 48, 70). Lack of fusion could not be due to lack of the entry receptors in BJAB cells, since we have observed the entry of HHV-8 into BJAB cells via endocytosis (2). Infection of CHO cells was also seen, although the efficiency was about 25-fold less than that of HFF and endothelial cells (1, 2, 3). (iv) HHV-8 gB expressed in CHO cells is not processed and cleaved as its native counterpart in the virion and not expressed on the cell membrane (45). Since removal of the last 58 amino acids of HHV-8 gB was required for expression on the cell membrane and to mediate the fusion of CHO and 293T cells, this suggests the presence of a putative motif in native gB that blocks acid-independent fusion by the native gB molecule. In addition, the function of virion-associated gB, gH, and gL may be tightly regulated because of the coordinated cascade of virus binding and entry processes and by their possible association with other virion proteins, such as the tegument proteins. Such constraints are probably removed when gB is expressed alone and without the last 58 amino acids (46).

The possible cell-to-cell fusion mediated by HHV-8 gB, gH, and gL does not imply that HHV-8 enters cells by fusion at the cell membrane, since studies have shown that when glycoproteins of viruses entering target cells by acid-dependent endocytosis were expressed alone, they could mediate acid-independent fusion. For example, entry of VSV occurs by receptor-mediated endocytosis (49). Subsequently, during traversal through the endosomal compartments, the VSV G protein acquires a low-pH-induced fusion-competent form, allowing fusion of the viral membrane with endosomal and lysosomal membranes. However, when the VSV G protein was expressed in the polarized endometrial HEC cell line, it acquired a pH-independent fusion activity (49). The results showed that VSV can induce cell fusion without exogenous exposure to a low-pH environment. Cell fusion activity is actually a result of a low-pH-mediated conformational change in the viral G protein, acquired as it traverses the exocytic transport pathway (49). Virions released from infected HEC cells were themselves not fusion competent, since viral entry required an active H^+-ATPase and a low-pH-induced conformational change in the viral G protein. These results suggest that in the HEC cell line, the VSV G protein undergoes a conformational change during intracellular transport allowing fusion of virus-infected cells with the surrounding cells in a culture, also referred to as induction of fusion from within (49). It has been suggested that acquisition of a fusion-competent form at

or near the cell surface may allow the virus to spread from cell to cell without the need for virion release and may allow the virus to escape the effects of neutralizing antibodies (49).

It is not unusual for viral glycoproteins to encounter a low-pH environment during exocytotic transport. For example, influenza A virus hemagglutinins (HA) of some strains (fowl plague virus), which are cleaved intracellularly, acquire a low-pH-defective conformation when expressed without the viral M_2 protein (42, 65). Coexpression of the viral M_2 protein neutralizes the vesicular pH during exocytic transport, maintaining the viral HA in an inactive, neutral conformation (42, 65). The limited fusion activity shown for HHV-8 gBMUT, gH, and gL resembles the fusion observed in the influenza A virus HA system (42, 65). Fowl plague virus HA expressed from a simian virus 40 vector exerted fusion activity in CV-1 cells; however, the syncytia observed in cell monolayers were few in number and small in size (42). These studies show that, depending on the cell type and/or other conditions, such as the expression of individual glycoproteins out of the context of other virion proteins, acid-independent cell-to-cell fusion can even be demonstrated for a glycoprotein of a virus entering via a low-pH-dependent pathway. Further work is necessary to determine the biological significance of the limited CHO cell to 293 cell fusion event observed with HHV-8 gBMUT, gH, and gL.

Entry of viruses into cells is a complex, multistep process, and for several viruses, cell attachment and internalization are distinct steps (66). HHV-8 uses its envelope gB and gPK8.1A to make initial contact with the target cell surface HS (1, 2, 9, 71), which is probably the first of a set of ligand-receptor interactions leading to binding with other host cell receptors essential for the subsequent viral entry process. We have demonstrated that HHV-8 uses the α3β1 integrin as one of its entry receptors (4). Ongoing studies have shown that HHV-8 also uses the αvβ3 and αvβ5 integrins as entry receptors (unpublished data). These integrins are expressed in vivo in all HHV-8 target cells, implying that distribution of this integrin matches the permissivity for infection (4). Integrins play a major role in the infectious process of many microbes, including several viruses (39). For example, nonenveloped viruses, like adenoviruses (αVβ3, αVβ5, αVβ1), echovirus (α2β1), foot-and-mouth disease virus (αVβ1, αVβ1, αVβ6), rotaviruses (α2β1, αVβ3), human parechovirus (αVβ3, αVβ1), and papillomaviruses (α6β4, α6β1), as well as enveloped hantavirus (αVβ3), have been reported to use one or more integrin molecules during the infectious process (39). Interestingly, all of these viruses that interact with integrins (adenovirus, echovirus, foot-and-mouth disease virus, parechovirus, parvovirus, rotavirus, and hantavirus) enter target cells via endocytosis (25, 26, 33, 39, 44, 73). This is not surprising since ligand interactions with integrins activating a cascade of outside-in signaling, such as the activation of FAK and the associated kinases, play important roles in endocytosis and cytoskeleton rearrangement (7, 15, 23, 24, 34, 65). To date, HHV-8 is the only herpesvirus that has been shown to interact with integrins (4). Our studies show that, like other viruses interacting with integrins, HHV-8 also uses the endocytic pathway for entry into HFF. Our studies imply a critical role for integrin-associated mitogenic signaling in HHV-8 infection of target cells and suggest that by orchestrating the signal cascade, HHV-8 may create an appropriate intracellular environment to facilitate the infection (37). Further studies are needed to determine the role of integrins and signaling pathways in HHV-8 infection of target cells.

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