Characterization of Entry Mechanisms of Human Herpesvirus 8 by Using an Rta-Dependent Reporter Cell Line

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To analyze the mechanisms of entry of human herpesvirus 8 (HHV-8), we established a reporter cell line T1H6 that contains the lacZ gene under the control of the polyadenylated nuclear RNA promoter, known to be strongly activated by a viral transactivator, Rta. We found that infection with cell-free virus, as well as cocultivation with HHV-8-positive primary effusion lymphoma cell lines, activated the lacZ gene of T1H6 in a sensitive and dose-dependent manner. Addition of Polybrene and centrifugation enhanced, but polysulfonate compounds inhibited, the HHV-8 infectivity. RGD-motif-containing polypeptides and integrins did not decrease the infectivity, suggesting the presence of an additional cellular receptor other than the reported one. The entry was dependent on pH acidification but not on the clathrin pathway. Although conditioned media obtained from human immunodeficiency virus (HIV)-infected cells did not have any effect on the early steps of HHV-8 infection, intracellular expression of a proviral HIV type 1, but not of Tat alone, increased the HHV-8-dependent reporter activation slightly, suggesting a potential of HIV-mediated enhancement of an early step of HHV-8 infection.

Attachment and entry represent the first essential steps of viral replication. Enveloped viruses have evolved two main pathways to mediate their entry into the cells after attachment to cell surface moieties (reviewed in reference 25). The first one, low-pH-dependent pathway, involves endocytosis of viral particles followed by viral-cell membrane fusion in endosomes or lysosomes. This fusion is triggered by an acidic-pH-dependent conformational change of viral glycoprotein(s) and allows release of capsid into the cytoplasm. Entry of vesicular stomatitis virus (VSV), the prototype rhabdovirus, exemplifies this pathway. In contrast, in the second one, pH-independent pathway, viral-cell membrane fusion takes place on the plasma membrane at neutral pH. Most retroviruses and paramyxoviruses use this pathway. The pH-independent entry was also demonstrated for herpesviruses, herpes simplex virus (55), and cytomegalovirus (CMV) (16). However, Epstein-Barr virus (EBV) uses membrane fusion both in endosomes and on the plasma membrane differentially (40).

Human herpesvirus 8 (HHV-8), also known as Kaposi’s sarcoma-associated herpesvirus (KSHV), is a member of the gammaherpesvirus subfamily and is etiologically associated with Kaposi’s sarcoma, primary effusion lymphoma (PEL), and multicentric Castleman’s disease (reviewed in references 1 and 11). It interacts with target cells by binding of glycoprotein B (gB) and K8.1 with glycosaminoglycans, such as heparan sulfate, on the cell surface (5, 10, 54). Recent studies found that gB also bound to α3β1 integrin through its RGD motif and induced ERK signaling pathway, implicating that α3β1 integrin functions as a cellular receptor for HHV-8 entry (6, 42). One of the major constraints to study the entry mechanisms of HHV-8 is a lack of fully permissive cell lines to conduct traditional virological assays by measuring virus titers, although a few cell lines, including 293 cells, some carcinoma and human papillomavirus-transformed cell lines, and immortalized endothelial cells, support permissive infection at a low level (32, 41, 45). The currently available assays that allow titration of HHV-8 include (i) enumeration of cells expressing immediate-early antigen ORF73 after infection, (ii) a plaque assay using primary dermal microvascular endothelial cells (15), (iii) quantitative PCR assays to measure encapsidated viral DNA (43, 50), and (iv) an enzyme-linked immunosorbent assay (ELISA) based on virion capture (30). However, some of these assays are laborious or time consuming. In addition, it is not guaranteed that the genome and particle numbers measured by PCR and the virion-capture ELISA reflect infectious particle numbers.

Establishment of the HHV-8 reporter cell line. In this study, we developed a new assay for HHV-8 titration by establishing a reporter cell line and characterized the factors that affect HHV-8 infectivity. For this purpose, we used Rta-dependent activation of the polyadenylated nuclear (PAN) RNA promoter. Rta encoded by open reading frame (ORF) 50 is a key HHV-8 regulator of the switch from a latent to lytic program, and it induces the expression of a number of HHV-8 and cellular promoters and is sufficient to trigger the entire lytic infection process (17, 33, 47, 51). PAN RNA is the most abundant transcript whose expression is activated by Rta through the responsive element RRE (49). We constructed a reporter plasmid, pβgal-T1.1, in which the PAN promoter region −122 to +14 was cloned between XhoI and HindIII sites of βgal-basic (BD Bioscience Clontech, Palo Alto, Calif.). 293T cells were transfected with pβgal-T1.1 and a plasmid encoding hygromycin B phosphotransferase at a 50:1 ratio. The clone T1H6 was selected from 46 hygromycin B-resistant clones.
based on β-galactosidase activities induced by transient transfection of each clone with pCMV-ORF50 expressing Rta (47). β-Galactosidase activities were obtained by a chemiluminescent assay reaction (Luminescent β-galactosidase Detection Kit II, BD Bioscience Clontech) followed by measurement of relative light units with a luminometer (TD-20/20; Turner Designs, Sunnyvale, Calif.) using a sensitivity setting that allows a linear reading over a 3-log range. Transfection of pCMV-
ORF50 activated the reporter gene of T1H6 cells in a dose-dependent manner (Fig. 1A). Treatment with 20 ng of 12-O-tetradecanoylphorbol-13-acetate (TPA)/ml did not activate the PAN promoter in 293T cells transiently transfected with βgal-T1.1 (data not shown) or in T1H6 cells (Fig. 1A). The stability of T1H6 cells for the Rta-dependent activation was confirmed by another transient transfection assay after culturing for more than 1 month.

Specificity and sensitivity of the reporter cell line. PEL cell lines, including BCBL-1 (46), JSC-1 (12), and BC-1 (13), and other EBV-positive and -negative B-cell lines were cultured in the presence or absence of TPA for 3 days, washed with TPA-free medium, and then cocultured with T1H6 cells for 3 days. Cocultivation with TPA-treated PEL cell lines activated the lacZ gene of T1H6 cells (Fig. 1B and data not shown). On the other hand, untreated PEL cell lines and HHV-8-negative cells did not activate the reporter gene (Fig. 1B), demonstrating the specificity of this assay. The detection limit for the lytically infected BCBL-1 cells was ~100 cells, because ~1,000 cells of TPA-induced BCBL-1 cells (Fig. 1B) with ~10% of lytic infection (the percentage was based on immunofluorescence assay with anti-K8.1 antibody) was enough to provide the measurable signal. The signal was detectable after cocultivation for 2 days. Addition of TPA during cocultivation of untreated BCBL-1 cells with T1H6 cells for 2 days did not enhance the reporter gene activation (Fig. 1C), indicating that the Rta-dependent late phase of lytic infection, probably virion production, is required to activate the reporter gene. Next, the reporter gene activation was shown by infection of T1H6 cells with cell-free virus stocks prepared from culture supernatant of TPA-treated BCBL-1 cells. Polybrene during attachment increased the efficiency of cell-free virus infection (Fig. 1D), which is consistent with an earlier study (32). Additively, a low-speed centrifugation during attachment enhanced HHV-8 infectivity (Fig. 1D), similar to that with CMV (27). The detection limit of cell-free virus was ~1,000 infectious units (IU), because the virus stock used here had ~2 × 10^6 IU/μl, as determined by counting ORF73-positive cells in an immunofluorescence assay after infection of 293T cells, as described previously (28). β-Galactosidase activities were saturated with a multiplicity of infection of more than 1 (5 μl of the stock for 8 × 10^6 cells per well). IU measured on endothelial cells also correlated well with β-galactosidase activities in the reporter cell assay (L. Krug, N. Inoue, and M. K. Offermann, unpublished data). β-Galactosidase activities were detectable at 24 h postinfection (p.i.) and gradually reached a plateau by 72 h p.i. (Fig. 1E). Infection at a higher multiplicity of infection decreased cell viability after 24 h p.i. Treatment of cell-free virus with rabbit antiserum against purified HHV-8 virions, but not with preimmune serum, gifts from K. G. Kousoulas (Louisiana State University), decreased β-galactosidase activities significantly, confirming that the assay is HHV-8 specific (Fig. 1F).

Polysulfonate compounds, but not RGD-containing molecules, inhibit HHV-8 infectivity. By using the reporter cell line, we first examined the initial interaction of HHV-8 with cell surface moieties. Heparan sulfate inhibited HHV-8 infectivity in T1H6 cells (Fig. 2A), confirming the previous studies (5, 10). Suramin, a symmetrical polysulfate naphthylamine derivative of urea, has anti-herpes simplex virus and anti-CMV properties (3) and inhibited HHV-8 infectivity also (Fig. 2A). Thus, the reporter cell assay is useful for screening of HHV-8 antiviral drugs. Next, we examined the inhibitory effects of RGD peptide, fibronectin (FN)-like polymer that contains 13 RGD motifs; RGD, RGD peptide). (A) T1H6 cells were incubated in the presence of inhibitors with HHV-8 inoculums for 2 h. (B and C) The cells were also incubated in the presence of the indicated amount of FN-like polymer and RGD peptide at 4°C (B) or in the presence of soluble α3β1 and α5β1 integrins at 37°C (C) for 90 min, washed with the inhibitor-free medium, and then incubated with the HHV-8 inoculums for 2 h (B, C). After removal of inoculums, the cells were incubated for 52 h (A to C). Means and standard deviations from triplicate experiments are shown. The standard curve was obtained by infection of serial twofold dilutions of the virus stock, and RLU units were converted into % infection by using infection of 1 μl of the virus stock in the absence of inhibitors as a 100% control. Strong cell toxicity was observed after treatment with >4 μg of the peptide/ml. (Fig. 2).
infection, confirming that cell toxicity rather than inhibition of HHV-8 infectivity decreased the β-galactosidase activities (data not shown). We also examined whether soluble α3β1 or α5β1 integrins (Chemicon International, Temecula, Calif.) inhibit HHV-8 infectivity. Although it was previously reported that 5 μg of soluble α3β1, but not α5β1, integrins/ml inhibited HHV-8 infectivity on human foreskin fibroblast cells by >70% (6), there was no significant inhibition on T1H6 cells (Fig. 2C). Therefore, it is possible that T1H6 cells derived from 293T cells express an additional cellular receptor for HHV-8 infection other than α3β1 integrin.

**HHV-8 entry is pH dependent.** To examine whether HHV-8 entry is dependent on pH acidification, T1H6 cells were treated with 10 to 40 mM ammonium chloride for 24 h beginning at various time points (Fig. 3A). The treatment at the early stage of infection (<2 h p.i.) significantly decreased β-galactosidase activities (Fig. 3B). To ensure that the conditions used were optimal for this type of cell, retrovirus vectors pseudotyped with VSV-G and with amphotropic murine leukemia virus (MLV) envelope were used as positive and negative controls, because it is well known that VSV, but not amphotropic MLV, requires endocytosis and pH acidification for their entry (25), and retrovirus vectors pseudotyped with envelope glycoprotein(s) from heterologous virus display the characteristics of entry of the heterologous viruses (4, 14, 36, 48). The VSV- and MLV-pseudotyped retrovirus vectors were prepared by transient transfection of GP-293Luc (packaging cell line for production of LLRN retrovirus vector; BD Bioscience Clontech) with pVSV-G (BD Bioscience Clontech) and pPAM3 (39), respectively, collection of culture supernatants 2 days later, and passage through 0.45-μm-pore-size filters. Infectivity of the retrovirus vectors on T1H6 cells was measured by transduction of luciferase, as described previously (29). The identical treatment with ammonium chloride inhibited infectivity of HHV-8 and of VSV-pseudotyped, but not of MLV-pseudotyped, retrovirus at the early, but not the late, stage of infection (Fig. 3C), demonstrating that HHV-8 entry is pH dependent. The pH dependence is also confirmed by inhibition with bafilomycin A1 (an inhibitor of vascular H+ ATPase) and monensin (an ionophore that blocks endosomal acidification) (Fig. 3D). Next, we examined whether this pH-dependent infection uses the clathrin-dependent pathway. It is widely accepted that Semliki Forest virus (SFV) requires pH-dependent infection uses the clathrin-dependent pathway. Chlorpromazine, an inhibitor of the pH-dependent infection uses the clathrin-dependent pathway.

**Effects of HIV expression on HHV-8 replication.** Several studies by others proposed potential effects of HIV infection with HHV-8 (24, 26, 37, 38, 52). However, these studies mainly focused on HHV-8 reactivation from latency in PEL cell lines. To characterize the HHV-8 entry mechanisms, here we asked...
pression of HIV had any effect on HHV-8 infection. T1H6 cells were transfected with pSV-Tat72 (22) or with the HIV-1 proviral DNA clone pNL4-3 (2), infected with HHV-8 24 h after transfection, and then harvested 52 h.p.i. We found that HIV-1 gene expression, but not Tat alone, enhanced HHV-8 replication slightly (Fig. 4B). A recent study reported that HIV infection activated the Rta promoter in a transient transfection assay (53) and that this activation was mediated by factors other than Tat. However, because we did not observe any detectable increase of lytic HHV-8 infection in PEL cells, including BCBL-1, JSC-1, and BC-3 cells, by HIV-1 infection (data not shown), further study is required to see whether increase of Rta expression by HIV-1 explains the slight effect that we observed in T1H6 cells. Studies on the factor(s) of HIV-1 that caused the HHV-8 activation in T1H6 cells are under way.

The reporter cell line that we developed in this study will be available for any noncommercial use upon written request.

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REFERENCES


ERRATUM

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Volume 77, no. 14, p. 8147–8152, 2003. Page 8149, legend to Fig. 2, lines 8 to 12: “The cells were also incubated in the presence of the indicated amount of FN-like polymer and RGD peptide at 4°C (B) or in the presence of soluble α3β1 and α5β1 integrins at 37°C (C) for 90 min, washed with the inhibitor-free medium, and then incubated with the HHV-8 inoculums for 2 h (B, C)” should read “The cells were also incubated in the presence of the indicated amount of FN-like polymer and RGD peptide at 4°C for 90 min, washed with the inhibitor-free medium, and then incubated with the HHV-8 inoculums for 2 h (B). The cells were incubated for 2 h with HHV-8 inoculums that had been preincubated in the presence of α3β1 and α5β1 integrins at 37°C for 90 min (C).”