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Integrin α3β1 (CD 49c/29) Is a Cellular Receptor for Kaposi's Sarcoma-Associated Herpesvirus (KSHV/HHV-8) Entry into the Target Cells

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Summary

Human herpesvirus-8 (HHV-8) is implicated in the pathogenesis of Kaposi's sarcoma. HHV-8 envelope glycoprotein B possesses the RGD motif known to interact with integrin molecules, and HHV-8 infectivity was inhibited by RGD peptides, antibodies against RGD-dependent α3 and β1 integrins, and by soluble α3β1 integrin. Expression of human α3 integrin increased the infectivity of virus for Chinese hamster ovary cells. Anti-gB antibodies immunoprecipitated the virus-α3 and -β1 complexes, and virus binding studies suggest a role for α3β1 in HHV-8 entry. Further, HHV-8 infection induced the integrin-mediated activation of focal adhesion kinase (FAK). These findings implicate a role for α3β1 integrin and the associated signaling pathways in HHV-8 entry into the target cells.

Introduction

Kaposi's sarcoma (KS) is associated with HIV-1 infection, and in the absence of HIV-1, KS occurs in three epidemiologically distinct forms. Kaposi's sarcoma-associated herpesvirus (KSHV) or HHV-8 DNA was detected in all epidemiological forms of KS, and several studies suggest a potential role of HHV-8 in KS pathogenesis (Antman and Chang, 2000). Cell lines with B cell characteristics established from the body cavity-based B cell lymphomas (BCBL) carry HHV-8 in a latent form, and a lytic cycle can be induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) (Schulz et al., 1998).

HHV-8 DNA and transcripts have been identified in vivo in human B cells, endothelial cells, epithelial cells, keratinocytes, and macrophages (Antman and Chang, 2000). HHV-8 has been shown to infect human B, epithelial, endothelial, and foreskin fibroblast (HFF) cells and keratinocytes (Renne et al., 1998; Cerimele et al., 2001; Vieira et al., 2001). However, analysis of HHV-8 infection is hampered by the absence of a lytic replication cycle and a reliable plaque assay. In addition, infection is often latent, as evidenced by the presence of circular latent HHV-8 DNA and by the expression of HHV-8 latency-associated nuclear antigen encoded by the open reading frame (ORF) 73 (Vieira et al., 2001). The broad in vitro cellular tropism of HHV-8 may be in part due to its interaction with the ubiquitous cell surface heparin sulfate (HS)-like molecule (Akula et al., 2001b). HHV-8 encodes for several glycoproteins (Russo et al., 1996), and we have demonstrated the interaction of virion envelope-associated HHV-8 glycoprotein gB(ORF8) and gpK8.1A with HS molecules (Akula et al., 2001a; Wang et al., 2001).

HHV-8 gB ORF consists of 845 amino acids, and among the α, β, and γ herpesvirus gB sequenced to date, only HHV-8 gB possesses the RGD (Arg-Gly-Asp) amino acids (aa 27–29) at the extracellular amino terminus coil region after the putative signal sequence (Russo et al., 1996). Sequence analyses of 63 HHV-8 strains from different geographical locations show that the gB-RGD sequence is highly conserved (Meng et al., 1999). The RGD amino acids are the minimal region of many extracellular matrix (ECM) proteins required for the interactions with a subset of host cell surface integrins (Plow et al., 2000). Integrins are a large family of heterodimeric receptors containing noncovalently-associated transmembrane α and β glycoprotein subunits (Giancotti and Ruoslahti, 1999). There are 17 α and 8 β subunits, and each αβ combination has its own binding specificity and signaling properties (Giancotti and Ruoslahti, 1999). Nonenveloped viruses like adenoviruses (αVβ3, αVβ5, and αMβ2; Nemerow, 2000; Li et al., 2001), rotaviruses (α2β1, α4β1, and αVβ3; Hewish et al., 2000; Guerrero et al., 2000), human parechovirus 1 (αVβ1 and αVβ3; Triantafilou et al., 2000), and enveloped hantavirus (β3; Gavrilovskaya et al., 1998) have been reported to use one or more integrin molecules during the infectious process. To date, members of herpesviruses have not been shown to interact with integrins.

Since HHV-8 gB possesses the RGD sequence, the role of integrins in the infectious process of HHV-8 was investigated. In this study, we provide several lines of evidence that implicate the RGD binding α3β1 (CD49c/29) integrin molecule as one of the in vitro cellular receptors involved in HHV-8's entry into the target cells.

Results

Peptides with RGD Amino Acids Inhibit HHV-8 Infection

To monitor the HHV-8 binding and entry process, HFF, adult human dermal microvascular endothelial cells (HMVEC-d), and a recombinant GFP-HHV-8 expressing GFP under the elongation factor-1-α-promoter control were used (Vieira et al., 2001). Since only a latent HHV-8 infection was observed in these cells, GFP expression represents the input virus entry and infection and not a reinfection from lytic replication. The entry and infection of cells were monitored by counting the number of green fluorescent cells (Figures 1A–1C) and confirmed by the detection of ORF73 protein (Figures 1D–1F).

Since HHV-8 gB possesses the RGD sequence, synthetic peptides with and without RGD sequences were incubated with HFF cells for 1 hr at 4°C before GFP-HHV-8 infection. HHV-8 infectivity was significantly ($p < 0.05$; chi-square test) inhibited by the RGD peptides (RGD, GRGDTP) and by the RGD-containing gB peptide (RGDgBN-1; RGDTFQTSSSPTPPGSSS) at 1.5 mM and 15 mM concentrations and not by the GRGESP peptide

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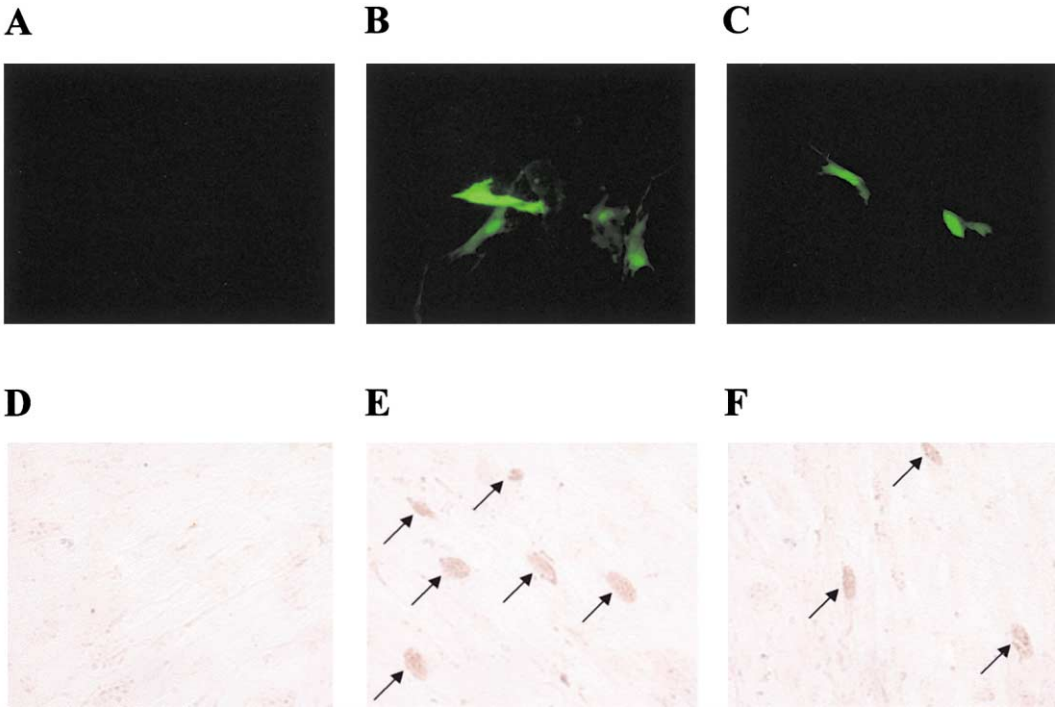


Figure 1. Measurement of GFP-HHV-8 Infectivity

HFF cells treated with DMEM (A, B, D and E) or with DMEM containing 1.5 mM of GRGDTP peptide (C and F) for 1 hr at 4°C were either mock infected (A and D) or infected with GFP-HHV-8 (B, C, E, and F) at 37°C for 2 hr, washed, and incubated at 37°C for 3 days with growth medium. Green fluorescent cells indicative of GFP-HHV-8 entry and infection were counted (A, B, and C). Approximately 190 green fluorescent cells per well were detected after infection with 100 μl of GFP-HHV-8. Cells were fixed with acetone and reacted with MAb against HHV-8 ORF73 protein, biotinylated anti-mouse antibodies, and substrate (D, E, and F). Arrows indicate nuclei of cells expressing the ORF 73 protein (magnification 400×).

(Figure 2A). Even though the inhibition observed with 15 mM of RGDgBN-1 peptide was significantly higher than the GRGESP control peptide, it was lower than the inhibition observed with the GRGDTP and RGD peptides. This could be due to aggregation of the 18-aa-long RGDgBN-1 peptide at higher concentrations more than the shorter RGD and GRGDTP peptides. GFP-HHV-8 used here is not clonal and contains both wild-type and recombinant viruses. Nevertheless, when virus-infected cells were monitored for the ORF 73 protein expression (Figures 1D–1F), inhibition comparable to GFP expression inhibition was observed with the RGD-containing peptides (data not shown). Inhibition by the RGD peptides suggests a potential RGD-dependent HHV-8 interaction with the host cell surface and, hence, a role for integrins in the biology of HHV-8.

Antibodies to Peptides Containing RGD Amino Acids Inhibit HHV-8 Infection

Rabbit antibodies directed against the RGD-containing peptide (RGDgB-N1) significantly inhibited the GFP-HHV-8 infection ($p < 0.05$; chi-square test) in a dose-dependent manner (Figure 2B). In contrast, rabbit antibodies against peptides lacking the RGD motif (gB-N2-GVENTFTDRDDVNTTVFLQPVEGLT and gB-C-RGYKP LTQSLDISPETGE) did not show any significant inhibition (Figure 2B). The 50% inhibition by anti-RGDgB-N1 antibodies at 30 μg/ml concentration was comparable to the inhibition by rabbit anti-gB IgG antibodies (Figure 2B), and antibodies against additional immunogenic regions probably have contributed to the higher percentage of inhibition by the anti-gB antibodies. Nevertheless, inhibition by anti-RGDgB-N1 antibodies suggests a role for HHV-8 gB RGD motif in the interaction with the target cells, potentially with integrins.

Inhibition of HHV-8 Infection by Integrin Ligands

To determine the role of integrins in HHV-8 infection, HFF cells were incubated with ECM proteins before GFP-HHV-8 infection. Fibronectin (FN), known to interact with integrins by RGD-dependent and -independent mechanisms, inhibited HHV-8 infectivity by 50% ± 3% at 50 μg/ml concentrations (Figure 2C), and inhibition did not increase significantly at 80 μg/ml concentrations (data not shown). When cells were incubated with 25 and 50 μg/ml of laminin, also known to bind integrins by RGD-dependent and -independent mechanisms, a moderate but significant ($p < 0.05$; chi-square test) increase in GFP-HHV-8 infectivity was observed (Figure 2C). The reason for this increased infection is under further investigation. In contrast, collagen type I, tenascin, vitronectin, BSA, and chondroitin sulfate A and C had no significant effect on GFP-HHV-8 infectivity (Figure 2C). These results also suggested a possible role of integrins in HHV-8 infectivity.

Antibodies to α3 and β1 Integrins Inhibit HHV-8 Infectivity

FN binds to α3β1, α5β1, α8β1, αVβ1, αVβ3, αVβ5, αVβ6, and αIIβ3 integrins via the RGD sequence and to α2β1,

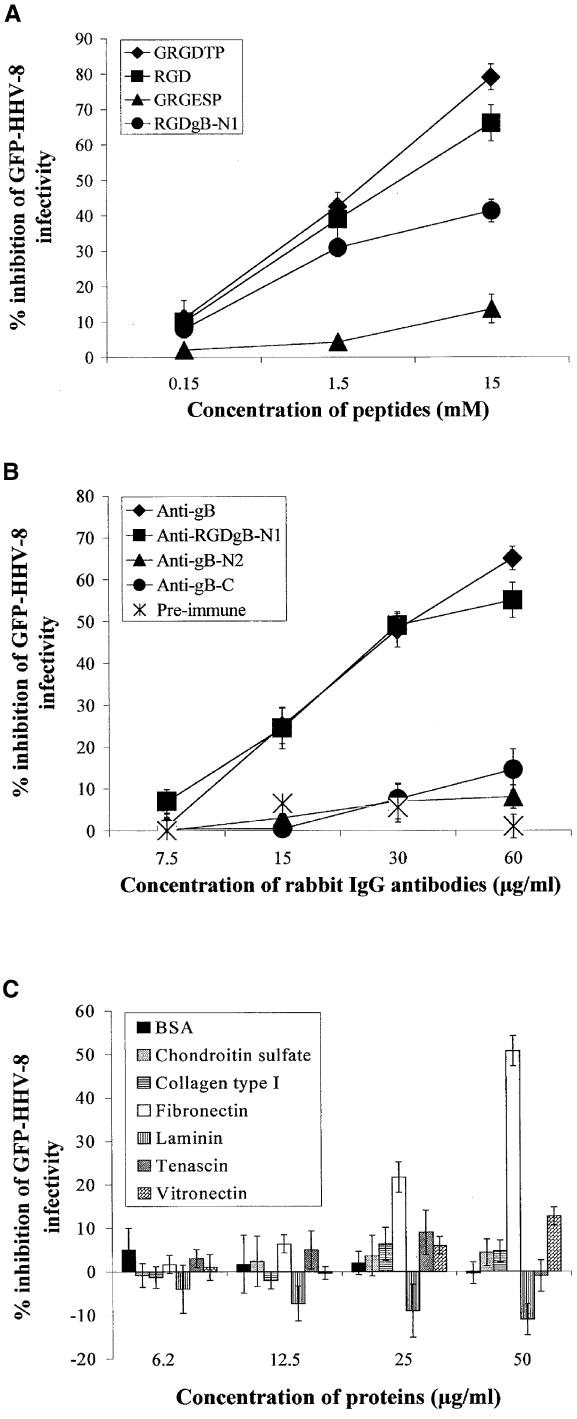


Figure 2. RGD-Dependent HHV-8 Interactions with the Host Cell Surface

(A) Inhibition of GFP-HHV-8 infectivity by peptides with RGD amino acids. HFF cells were incubated with RGD, GRGDTP, GRGESP, and RGDgBN-1 (RGDTFQTSSSPTPPGSSS) peptides in DMEM at 4°C for 1 hr, washed, and incubated with 100 μl of GFP-HHV-8 at 37°C for 2 hr.

(B) Inhibition of GFP-HHV-8 infectivity by antibodies against HHV-8 gB peptide containing the RGD amino acids. GFP-HHV-8 (100 μl) was incubated at 37°C for 1 hr with DMEM alone or with DMEM containing rabbit IgG antibodies against HHV-8 gB (anti-gB) or against HHV-8 gB peptides with (anti-RGDgB-N1) and without RGD amino acids (gB-N2 and gB-C) or preimmune IgG antibodies. HFF cells were incubated with these mixtures at 37°C for 2 hr.

α4β1, α4β7, and αVβ8 integrins in an RGD-independent manner (Plow et al., 2000). Monoclonal antibodies against the RGD binding α3 and β1 integrin subunits inhibited HHV-8 infectivity by 48% ± 4% and 35% ± 5%, respectively, in HFF cells (Figure 3A) and by 46% ± 2% and 36% ± 3%, respectively, in HMVEC-d cells (Figure 3A). The difference between the percentage inhibition of HHV-8 infectivity by MAbs to α3 and β1 integrin subunits was statistically significant ($p < 0.05$). About 30% ± 5% of GFP-HHV-8 infectivity was also inhibited by MAb against the RGD-independent α2β1 integrin, in HFF and HMVEC-d cells (Figure 3A). However, there was no significant difference between the inhibition by β1 and α2β1 MAbs, and the similarities in the inhibition levels suggest that inhibition with anti-α2β1 MAbs could be due to crossreactivity with the β1 integrin subunit. Similar isotype-specific antibodies against other RGD and non-RGD binding (α1, α6, αV, β4, α5β1, αVβ3, αVβ5, and αVβ6) integrins did not show any significant inhibition of HHV-8 infectivity (Figure 3A), thus demonstrating the specificity of inhibition by anti-α3 and anti-β1 antibodies.

MAbs to α3 and β1 integrins inhibited HHV-8 infectivity in a dose-dependent manner with an optimal antibody concentration of about 2 μg/ml (Figure 3B). When antibodies against α3 and β1 subunits or antibodies against α2β1, α3, and β1 integrins were mixed and added to the HFF cells prior to infection, there was no significant additive effect of inhibition (Figure 3B). Incomplete inhibition of HHV-8 infectivity by the anti-α3 and anti-β1 antibodies could be attributed to the nature of antibodies and/or due to additional cell surface molecules recognized by HHV-8. Nevertheless, these results implicate an involvement of α3β1 integrin in the infectious process of HHV-8.

Soluble α3β1 Integrin Blocks HHV-8 Infection

The α3 integrin subunit is expressed on the cell surface only in combination with the β1 integrin subunit (Plow et al., 2000). Since α3β1 integrin binds FN via the RGD sequence and since antibodies against α3 integrin subunit neutralized infectivity better than other integrin antibodies, we focused on the role of RGD-dependent α3β1 integrin in HHV-8 infectivity. Among the RGD binding integrins, only the soluble α3β1 and α5β1 integrins were commercially available in 10 mM octyl-b-D-glucopyranoside formulation. This formulation did not disrupt the cell membranes and was not toxic at less than 20 μg/ml of soluble integrin. Soluble α3β1 blocked infection significantly in a dose-dependent manner with about 75% ± 3% inhibition at 5 μg/ml concentration (Figure 3C). The specificity of this reaction was demonstrated by the absence of inhibition by α5β1 integrin (Figure 3C). These results clearly suggest that α3β1 integrin plays a role in the infectious process of HHV-8.

(C) Inhibition of GFP-HHV-8 infectivity by integrin ligands. HFF cells were incubated with ECM proteins and glycoproteins at 4°C for 1 hr, washed, and infected with GFP-HHV-8. HHV-8 infectivity was measured as described in the Figure 1 legend. Data are presented as percentage of inhibition of virus infectivity obtained when the cells were preincubated with DMEM only. Each reaction was done in duplicate, and each point represents the average ± the SD of three experiments.

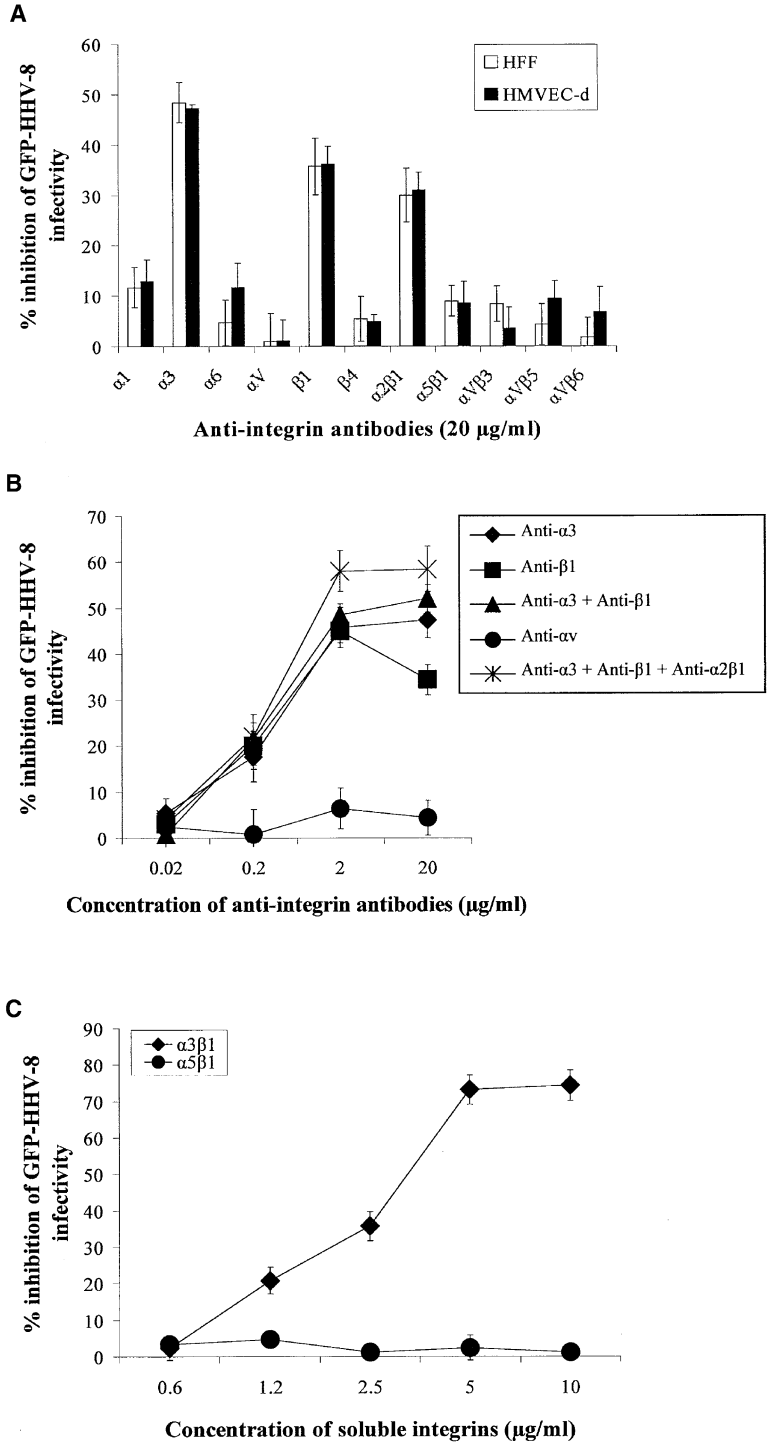


Figure 3. α3β1 Integrin Plays a Role in the Infectious Process of HHV-8

(A) Inhibition of GFP-HHV-8 infectivity by anti-integrin antibodies. Monolayers of HFF and HMVEC-d cells were incubated with 20 μg/ml of MAbs against integrins and integrin subunits for 1 hr at 4°C, washed, and infected with GFP-HHV-8.

(B) Dose-dependent inhibition of GFP-HHV-8 infectivity by antibodies to α3 and β1 subunits of integrins. HFF cells were incubated with MAbs (IgG1 isotype) against α3, β1, αV, and α2β1 integrins, alone or in combinations, for 1 hr at 4°C, washed, and infected with GFP-HHV-8.

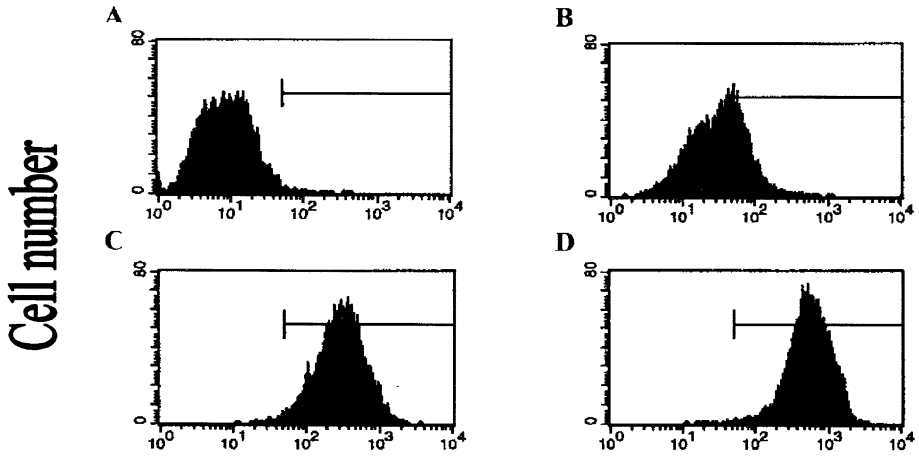
(C) Inhibition of GFP-HHV-8 infectivity by soluble α3β1 integrin. GFP-HHV-8 (100 μl) was incubated with soluble α3β1 and α5β1 integrins at 37°C for 1 hr and added to HFF cells. After 2 hr at 37°C, cells were washed and incubated for 3 days with growth medium. HHV-8 infectivity was measured as described in Figure 1. Data are presented as percentage of inhibition of virus infectivity obtained when the cells were preincubated with DMEM as control. Each reaction was done in duplicate, and each point represents the average ± the SD of three experiments.

Expression of α3β1 Integrin in the In Vitro Target Cells of HHV-8

To determine the relative abundance of α3β1 integrin in the various target cells of HHV-8, flow cytometric analysis with various anti-integrin antibodies was carried out. We analyzed the percentage of fluorescent cells with a fluorescence intensity higher than the negative control, and we analyzed the mean fluorescence intensity (MFI) (Figure 4). The β1 integrin is relatively expressed at a higher level than other integrins and in more than 87% of BCBL-1, BJAB, 293, HFF, and HMVEC-d cells (Figure 4E). The α3 integrin is expressed in more than 90% of BJAB, HFF, and HMVEC-d cells and at a higher level in HFF cells compared to other cells tested (Figure 4E).

HHV-8 Infection of CHO-B2 Cells Is Promoted by the Expression of Human α3 Integrin

HHV-8 binds and enters a variety of human (BCBL-1, BJAB, Raji, 293, HFF, Hela, and endothelial), monkey (Vero and CV-1), hamster (BHK-21 and CHO), and mouse



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Integrins	% distribution and level (MFI) of integrins on target cells				
	BCBL-1	BJAB	293	HFF	HMVEC-d
α1	34 (29)	9 (27)	82 (34)	96 (225)	56 (26)
α3	*42 (29)*	*96 (57)*	39 (26)	*99 (335)*	*90 (29)*
α6	62 (46)	92 (48)	72 (32)	35 (69)	24 (17)
αV	44 (30)	15 (31)	28 (26)	80 (111)	31 (23)
β1	99 (75)	99 (88)	90 (79)	99 (597)	87 (23)
β4	22 (27)	6 (26)	8 (25)	15 (64)	3 (14)
α2β1	12 (25)	65 (29)	87 (43)	90 (145)	65 (27)
α5β1	18 (17)	ND	91 (24)	80 (22)	91 (41)
αVβ3	51 (36)	12 (27)	14 (29)	33 (75)	51 (25)

Figure 4. Flow Cytometric Analysis of α3β1 Integrin Expression

Cells were incubated with MAbs against integrins followed by FITC-anti-mouse antibodies and examined by FACS. HFF cells were incubated with FITC-goat anti-mouse IgG (A), anti-αVβ3-MAb LM609 (B), anti-α3-MAb ASC-6 (C), and anti-β1-MAb 6S6 (D). The percentage of fluorescent cells with a fluorescence intensity higher than the negative control (A) and the MFI as an indication of expression levels were measured. The histograms display relative cell numbers as a function of relative fluorescence intensities. (E) Table showing percentage number of target cells positive for different integrins with MFI values for the gated positive cells within parenthesis. ND indicates not done.

(L) cells, as shown by the detection of HHV-8 DNA, limited HHV-8 gene expression, and GFP expression (Renne et al., 1998; Akula et al., 2001b; Vieira et al., 2001). To verify the role of α3β1 in HHV-8 infectivity, CHO-B2 cells expressing low levels of endogenous α3 integrin were transfected with human α3 integrin cDNA (CHO-B2-clones B3 and D5; Wu et al., 1995). The human α3 integrin in transfected cells has been shown to form heterodimeric complexes with hamster β1 and is expressed on the plasma membranes, with B3 cells expressing four times more human α3/hamster β1 integrin than the D5 cells (Wu et al., 1995). FACS analyses showed the expression of human α3 integrin in 23% and 95% of CHO-B2-D5 and B3 cells, respectively (Figure 5A, graphs 1 and 2). The MFI values for human α3 integrin in the D5 and B3 cells were 186 and 116, respectively, and the MFI values for hamster α3 and β1 integrins in CHO cells were 3 and 60, respectively (Weitzman et al., 1995).

CHO cells expressing human α3 integrins were infected with GFP-HHV-8 and GFP (Figure 5B, images 1 and 2), and ORF 73 protein expressions (Figure 5B, images 3 and 4) were measured. The efficiency of infection in CHO-B2 cells was about 25-fold less than the HFF and HMVEC-d cells. However, the expression of human α3 integrin increased the susceptibility to HHV-8 infection, and CHO-B2-B3 and D5 cells were 2–3 times more susceptible to GFP-HHV-8 infectivity than the parental CHO-B2 cells (Figure 5C). The permissiveness of these cells was also shown by the increased expression of ORF 73 protein (Figure 5B). The increase in the level of HHV-8 infectivity was specifically blocked by the preincubation of cells with 2 μg/ml of anti-human α3 antibodies, but not by the isotype-specific antibodies against human β4 integrin (Figure 5C).

The increased infectivity in these cells could be due to the ability of human α3/hamster β1 integrin to mimic a conformation resembling human α3β1 integrin. However, similar to our previous observations (Akula et al., 2001b), we observed that even though the radiolabeled HHV-8 binding to CHO cells mediated by HS-like molecules was comparable to HFF and 293 cells, virus infec-

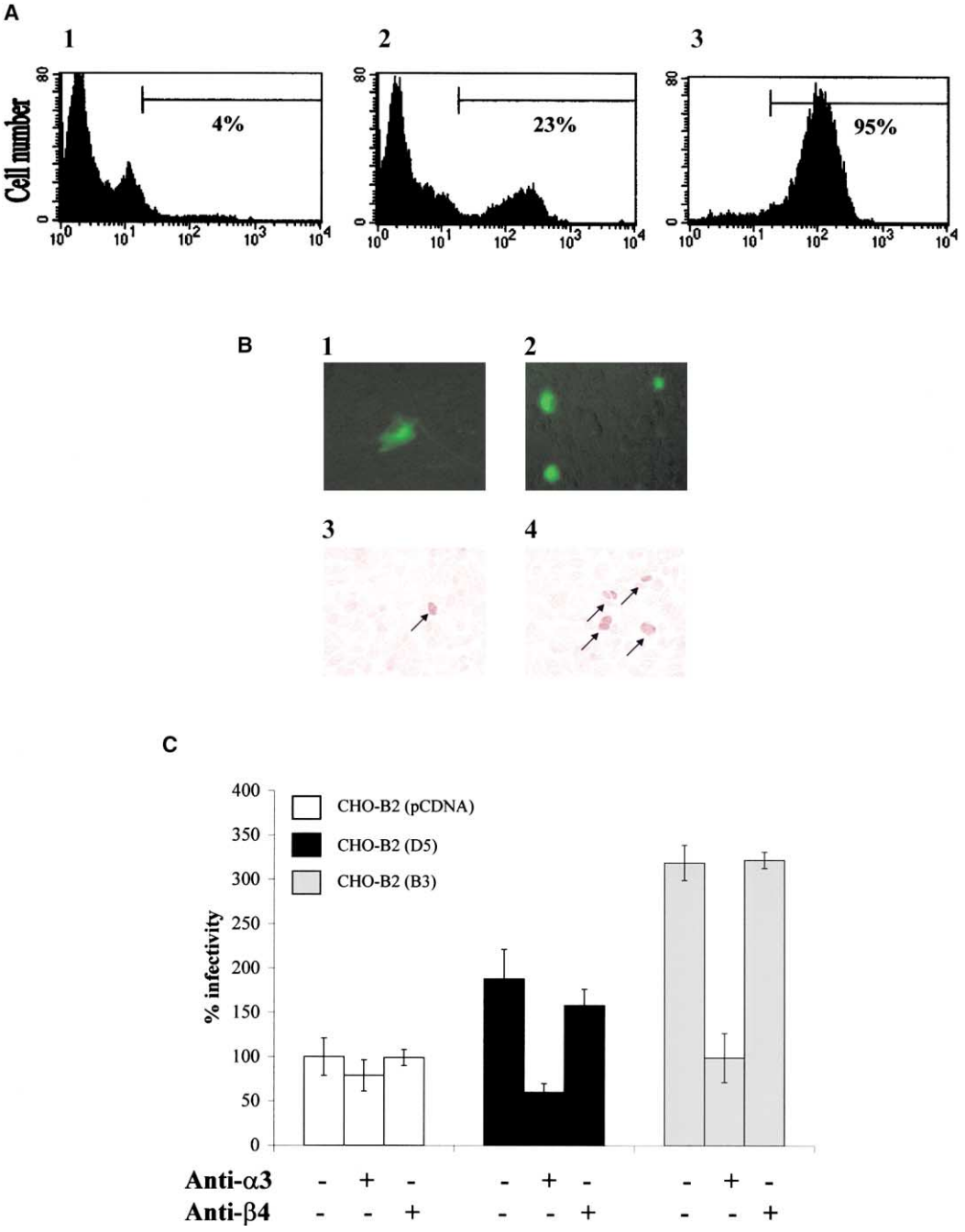


Figure 5. Human α3β1 Integrin Expression Promotes HHV-8 Infection of CHO-B2 Cells

(A) Flow cytometric analysis of human α3 integrin expression in CHO-B2 cells. Cells were incubated with MAbs against human α3 integrin molecules and examined by FACS. (1) CHO-B2 cells transfected with control pCDNA3 plasmid. (2 and 3) CHO-B2 clone D5 and B3 cells, respectively, transfected with pCDNA3-human α3 integrin cDNA. The percentage of cells expressing α3 integrin on the surface is indicated.

(B) Infection of CHO-B2 cells by GFP-HHV-8. CHO-B2 cells transfected with human α3 integrin cDNA (CHO-B2 clone D5 [1 and 3] and CHO-B2 clone B3 [2 and 4]) were infected with 100 μl of GFP-HHV-8 for 3 days at 37°C. (1 and 2) GFP expression in CHO-B2 cells. (3 and 4) ORF 73 protein expression in CHO-B2 cells (arrows) (magnification 400×).

(C) Recombinant human α3 integrin promotes CHO-B2 cell infection of GFP-HHV-8. CHO-B2 cells transfected with pCDNA3 (CHO-B2/pCDNA) and pCDNA3 containing human α3 integrin cDNA (CHO-B2 clone D5 and B3) were infected with GFP-HHV-8 for 2 hr at 37°C. Cells were also infected after preincubation for 1 hr at 4°C with 2 μg/ml of MAbs against human α3 integrin subunit or with similar isotype MAbs (IgG1) against human β4 subunit. Infectivity was measured as described in Figure 1. After infection with 100 μl of GFP-HHV-8, approximately 7–8 green fluorescent CHO-B2/pCDNA cells per well were detected. This was considered as 100% infectivity for comparison with infection of human α3 integrin expressing CHO-B2-D5 and -B3 cells. Each reaction was done in duplicate, and each point represents the average ± the SD of three experiments.

tivity in human α3 integrin-expressing CHO-B2 cells was 8-fold less than the infectivity in human cells. Only very low levels of hamster β1 (MFI value 60) and α3 (MFI value 3) integrins were detected in CHO cells. This suggests that the efficiency of HHV-8 entry in CHO cells may be dependent upon the density of α3β1 integrin, as well as other putative HHV-8 receptor(s). Nevertheless, these results indicate that the augmented HHV-8 infectivity is due to the expression of human α3 integrin and confirm the role of α3β1 integrin in HHV-8 infectivity.

HHV-8 gB Specifically Binds to the α3 and β1 Chains of Integrin

The role of RGD-containing HHV-8 gB in the interaction with α3β1 integrin was next examined. HHV-8 binds to HS on the target cell surface (Akula et al., 2001b). Since it was not clear whether HS would compete with the HHV-8 binding to integrins, heparinase I/III-treated or -untreated HFF cells surface-labeled with biotin were used in immunoprecipitation reactions. Since the cell surfaces were biotinylated, the cell surface locations of the resolved integrin bands were confirmed in parallel experiments by reacting the blots with alkaline phosphatase-labeled streptavidin (data not shown). The α1 integrin was selected as a control since 96%, 99%, and 99% of HFF cells expressed α1, α3, and β1 integrins, respectively, with a relatively similar concentration of expression (Figure 4E). Western-blotted purified α3β1 and α1β1 integrins reacted with anti-α1, -α3, and -β1 integrin antibodies served as markers for identifying the integrin molecules (data not shown).

From the heparinase I/III-treated cell-virus interaction mixture, anti-gB antibodies specifically immunoprecipitated a 150/110 kDa heterodimer protein, which was identified as α3 and β1, respectively, by the α3 and β1 integrin chain-specific antibodies (Figure 6A, lanes 7 and 8). The α1 chain of the integrin molecule was not immunoprecipitated by the anti-gB antibodies (Figure 6A, lane 6). In the absence of virus, integrin antibodies to α1 (Figure 6A, lane 2), α3 (Figure 6A, lane 3), and β1 (Figure 6A, lane 4) did not react with any immunoprecipitated proteins, thus confirming the specificity of these virus-integrin interactions. HHV-8 also bound to α3 (Figure 6A, lane 10) and β1 (Figure 6A, lane 11), but not to α1 (Figure 6A, lane 9) chain of integrin molecule from the heparinase I/III-untreated HFF cell lysate. The α3 and β1 integrins were immunoprecipitated only by the mouse anti-gB antibodies. MAbs to gpK8.1A (Zhu et al., 1999) did not immunoprecipitate the α1 (Figure 6A, lane 12), α3 (Figure 6A, lane 13), or β1 (Figure 6A, lane 14) chains of integrins. Mouse preimmune antibodies also did not precipitate the β1 integrin subunit (Figure 6A, lane 5). These results demonstrated the loss of enveloped virus integrity in the lysis buffer and confirmed the specificity of HHV-8 gB interactions with α3 and β1 integrins. Taken together, these studies demonstrate a direct role of HHV-8 gB in the interaction with α3 and β1 chains of integrin molecules. Immunoprecipitation of β1 integrin subunit only with α3 subunit, but not with α1 subunit, suggests that HHV-8 gB probably recognizes a specific conformation of α3β1 heterodimers.

HHV-8 Interacts with α3β1 Integrin at a Postcell Attachment Step

HHV-8 interacts with cell surface HS during the initial attachment stage of infection (Akula et al., 2001b). To determine whether HHV-8 interaction with cell surface α3 and β1 integrins occurred during attachment or at a postattachment step, HHV-8 binding inhibition experiments with RGD peptides, antibodies to RGDgB-N1 peptide, antibodies to α3 and β1 integrins, and soluble α3β1 and α5β1 integrins were carried out. Similar to our previous findings (Akula et al., 2001a), heparin at a concentration of 10 μg/ml inhibited more than 90% of [^{3}H]-thymidine labeled HHV-8 binding to the HFF cells (Figure 6B). In contrast, RGD peptides, RGDgB-N1 antibodies, anti-integrin antibodies, and soluble α3β1 integrin at the concentrations used in the virus neutralization assays as well as at higher concentrations did not inhibit HHV-8 binding to the target cells (Figure 6B). These results implicate a role for α3β1 integrin in the entry of HHV-8 into the target cells.

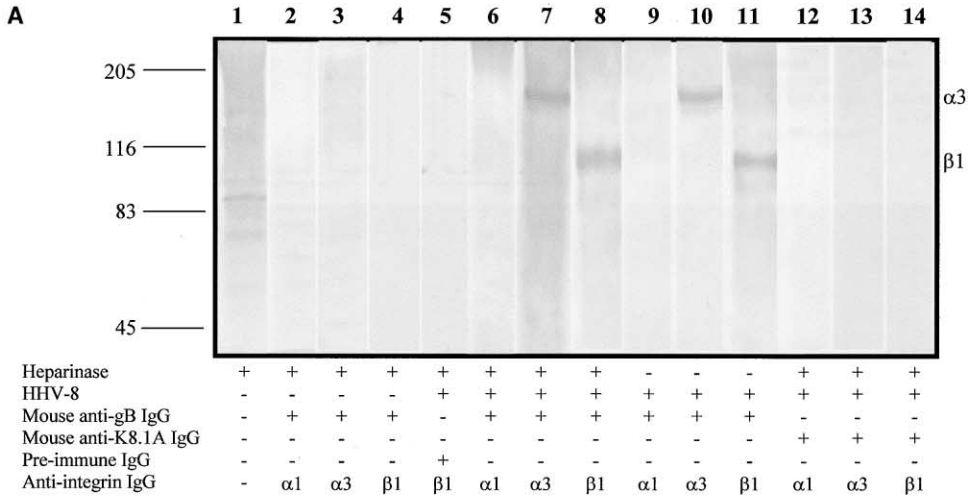
HHV-8 Activates the Integrin-Dependent Focal Adhesion Kinase in the Target Cells

After the interaction with ligands, integrins, numerous signaling molecules including FAK, C-Src, and p130cas, and cytoskeletal proteins like talin, paxillin, vinculin, and α actin assemble into aggregates on each side of the membrane called focal adhesions (FAs) (Giancotti and Ruoslahti, 1999). FAK is a nonreceptor protein-tyrosine kinase that localizes with vinculin at the FAs, and FAK activation is the first step necessary for the outside-in signaling by integrins (Sastry and Burridge, 2000). To examine the potential mechanism of α3β1 integrin's contribution in HHV-8 entry, FAK activation in HFF and HMVEC-d cells immediately after HHV-8 infection was analyzed.

The distributions of FAK and vinculin in serum-starved (data not shown) and mock-infected cells (Figure 7A) were more diffused throughout the cytoplasm and independent of each other. In contrast, distinct patchy patterns of FAK and vinculin distribution was observed in HHV-8-infected cells, and a representative example after 5 min infection is shown in Figure 7A. Image overlays demonstrated the colocalization of FAK with vinculin, indicating the specificity of the altered FAK distribution in the infected cells. These patterns of FAK colocalization with vinculin were readily observed as early as 5 min after infection, and was comparable to the patterns observed in 20 ng/ml of lysophosphatidic acid (LPA)-treated cells (Figure 7A). Even though similar activation and redistribution of FAK were noticed in HMVEC-d cells, the basal level of FAK activity in the quiescent HMVEC-d cells was much higher than the HFF cells (data not shown). Nevertheless, these results demonstrated the activation of FAK during the early stage of HHV-8 infection, presumably by its interaction with integrins.

HHV-8 Induces the Phosphorylation of FAK in the Target Cells

Tyr^{397} of FAK has been shown to specifically undergo autophosphorylation in response to integrin-ligand interactions at the FA sites (McLean et al., 2000). Induction



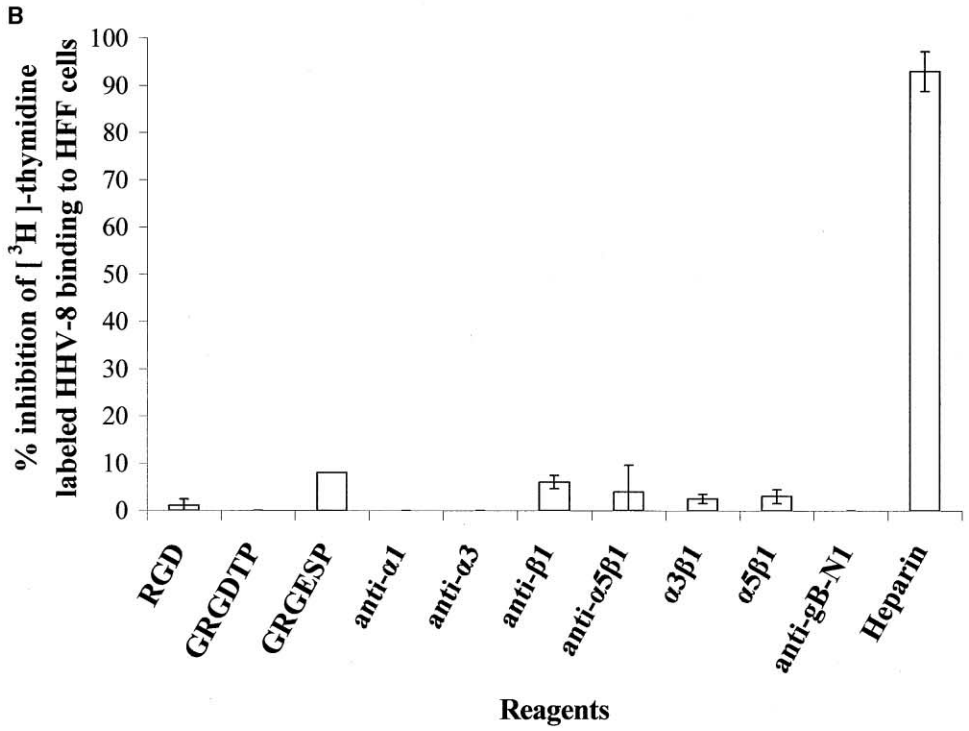


Figure 6. HHV-8 gB Binds to the $\alpha3\beta1$ Integrin

(A) Interaction of HHV-8 gB with $\alpha3\beta1$ integrin molecules. Heparinase I/III-treated and -untreated HFF cell surfaces were biotinylated, solubilized, precleared with preimmune mouse IgG antibodies, and mixed with purified HHV-8 at RT for 1 hr. These were immunoprecipitated with preimmune mouse IgG antibodies (lane 5) or mouse anti-gB IgG antibodies (lanes 2–4 and 6–11) or MAb to gpK8.1A (lanes 12–14). Samples were resolved in SDS-7.5% PAGE and were Western blotted. Membrane strips were probed with rabbit polyclonal antibodies to $\alpha1$ (lanes 2, 6, 9, and 12), $\alpha3$ (lanes 3, 7, 10, and 13), or $\beta1$ (lanes 4, 5, 8, 11, and 14) integrin subunits. Lane 1: Biotinylated HFF lysate probed with AP-conjugated streptavidin and substrate. The numbers on the left indicate the molecular masses (in kDa) of the standard molecular weight markers run in parallel lanes. The $\alpha3$ and $\beta1$ integrins recognized by the antibodies are indicated on the right.

(B) HHV-8 interacts with $\alpha3\beta1$ integrin at a postattachment step of infection. HFF cells were incubated with RGD, GRGDTP, and GRGESP peptides and anti-integrin antibodies at 4°C for 90 min and incubated with [^{3}H]-thymidine labeled, purified HHV-8 (2,684 cpm). Labeled HHV-8 was also mixed with 10 μg/ml of heparin or rabbit anti-RGD-gB-N1 antibodies or soluble integrins for 90 min at 4°C and then added to the cells. After incubation for 90 min at 4°C with the virus, cells were washed, lysed, precipitated with TCA, and counted. The cell-associated virus cpm in the presence of different treatment was calculated as the percentage inhibition of virus binding. Results with 15 mM of RGD, GRGDTP, and GRGESP peptides, 20 μg/ml of anti-integrin antibodies, 1 mg/ml of anti-RGD-gB-N1 antibodies, and 10 μg/ml of soluble integrins are shown here. In the absence of peptides and antibodies, 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 $\pm$ the SD of three experiments.

of cells for 5 min with 100 ng/ml of LPA resulted in about 90% increase in FAK phosphorylation over the control cells (Figure 7B, lanes 1 and 2). HHV-8 infection also rapidly induced the phosphorylation of FAK, with about 20% increase in FAK phosphorylation as early as 5 min postinfection over control cells (Figure 7B, lane 3) and with about 75% increase at 15 and 30 min postinfection (Figure 7B, lanes 4 and 5). The lowering of FAK phosphorylation to undetectable levels by treating infected cell lysates with tyrosine phosphatase 1B (Figure 7B, lane 6) showed the specificity of anti-phospho-FAK antibodies. Infection of HFF cells with herpes simplex virus type-2 (HSV-2), shown to interact with HS and other nonintegrin cell surface molecules (Spear et al., 2000), did not induce any significant levels of FAK phosphorylation (Figure 7B, lane 7). Treatment of cells for 1 hr before infection with a nontoxic dose (100 μM) of genistein, a tyrosine phosphorylation inhibitor, and during the 30 min incubation with virus lowered the HHV-8-induced FAK phosphorylation to levels comparable to the uninduced cells (Figure 7B, lane 8). The levels of actin did not alter in these experiments (Figure 7B, bottom), and dimethylsulfoxide used as a solvent for genistein did not alter the expression of FAK (data not shown). These results further confirmed the activation of FAK during the early stages of HHV-8 interactions with the host cells.

Soluble α3β1 Integrin Blocks the HHV-8-Induced Phosphorylation of FAK

To determine the role of HHV-8-α3β1 integrin interaction in the FAK activation, soluble α3β1 or α5β1 integrins were mixed with HHV-8 and incubated for 1 hr at 37°C before infecting HFF cells. Compared to FAK phosphorylation at 30 min postinfection in the untreated HHV-8-infected cells (Figure 7C, lane 1), 5, 2.5, and 1.25 μg/ml concentrations of soluble α3β1 integrin lowered the HHV-8-mediated FAK phosphorylation by about 72%, 60%, and 20% (Figure 7C, lanes 2–4), respectively. Soluble α5β1 integrin did not significantly alter the HHV-8-mediated FAK phosphorylation (Figure 7C, lane 5). These results demonstrated a direct role of HHV-8-α3β1 integrin interaction in the activation of FAK.

Anti-HHV-8 gB Antibodies Block the HHV-8-Induced Phosphorylation of FAK

Compared to FAK phosphorylation at 30 min postinfection in the untreated HHV-8-infected cells (Figure 7D, lane 1), 100, 50, and 25 μg/ml concentrations of anti-gB antibodies lowered the HHV-8-mediated FAK phosphorylation by about 74%, 39%, and 10% (Figure 7D, lanes 2–4), respectively. HHV-8-mediated FAK phosphorylation was not affected by the rabbit preimmune (Figure 7D, lane 5) and anti-ORF 73 antibodies (data not shown), even at 100 μg/ml concentrations. Together with the data demonstrating HHV-8 gB binding to the cell surface α3β1 integrin (Figure 6A) and the inhibition of FAK activation by soluble α3β1 integrin (Figure 7C), these results demonstrate a direct role of HHV-8 gB-α3β1 integrin interaction in the activation of FAK.

Discussion

Our studies implicate the α3β1 integrin as one of the in vitro cellular receptors involved in the infectious process of HHV-8. Inhibition of more than 75% of HHV-8 infectivity by RGD peptides and by the RGD-dependent soluble α3β1 integrin suggest an RGD-dependent HHV-8 interactions with host cells. Absence of complete inhibition of infection by the anti-α3β1 antibodies and greater but not complete inhibition by the soluble α3β1 suggests that α3β1 integrin is not the only virus entry receptor in the cells under study. The inability of antibodies to other RGD-dependent and -independent integrins with β1 subunit to block HHV-8 infectivity suggests that HHV-8 interacts with the specific combined conformation of α3β1 heterodimers, which is also supported by the immunoprecipitation of β1 integrin subunit along with the α3 subunit, but not with the α1 subunit from the virus-target cell mixture. The ability of integrin antibodies to inhibit HHV-8 infectivity to comparable levels in HFF and HMVEC-d cells suggest that α3β1 integrin is a common receptor for HHV-8 infection of these cells. It is interesting to note that in vivo, α3β1 integrin is expressed abundantly in endothelial cells, epithelial cells, kerationcytes, B cells, and monocytes (Wu et al., 1995; Plow et al., 2000), the same target cells in which HHV-8 DNA and transcripts have been detected (Schulz et al., 1998).

Several viruses utilize multiple receptors for binding and entry into cells. For example, the α herpesviruses utilize HS molecules for cell surface binding and other nonintegrin molecules for entry (Spear et al., 2000). Similarly, HHV-8 utilizes HS molecules for cell surface binding (Akula et al., 2001b), and the present study implicates a role for α3β1 integrin in the entry of HHV-8 into the target cells. Integrin α3β1 is the second of multiple in vitro HHV-8 receptors to be identified, and further studies are required to determine the in vivo roles of these receptors in HHV-8 infection. Even though HHV-8 gB and gpK8.1A interact with the HS molecules (Akula et al., 2001a; Wang et al., 2001), which one of these glycoproteins mediate binding to HS during infection has not been established. HHV-8 gB binds to α3 and β1 integrin subunits to a comparable extent in heparinase I/III-treated or -untreated HFF cells. This could be due to HHV-8 gB interaction with HS via the putative heparin binding domain (HBD 108–117 aa; Akula et al., 2001a), not competing with the virus-α3β1 interaction by the RGD motif (27–29 aa), or it could be due to HHV-8 interaction with HS solely by gpK8.1A.

Many members of α and β herpesviruses deliver their DNA-containing capsids into the cells by fusing the virion envelope with the plasma membrane (Compton et al., 1992; Spear et al., 2000). In contrast, in a similar manner to simian virus-40 (SV-40) (Anderson et al., 1996) and γ1-Epstein-Barr virus (EBV) (Nemerow and Cooper, 1984), the γ2-HHV-8 enters via large endocytic vesicles in the human B cell line, BJAB (Akula et al., 2001b), HFF, 293, and endothelial cells (unpublished data). The ability of HHV-8 gB to interact with α3β1 integrin and the subsequent FAK activation suggests that HHV-8 utilizes integrin and the associated signaling pathways to enter the cells. Since activation of FAK, Src-family kinases, and integrin-linked kinases (ILK) is central to many paradigms of outside-in signaling by integrins, actin assembly, and endocytosis (Calderwood et al., 2000), activation of FAK may have an important role in HHV-8 infection. Preliminary studies with chemical inhibitors suggest that HHV-8 enters cells by endocytosis. Studies

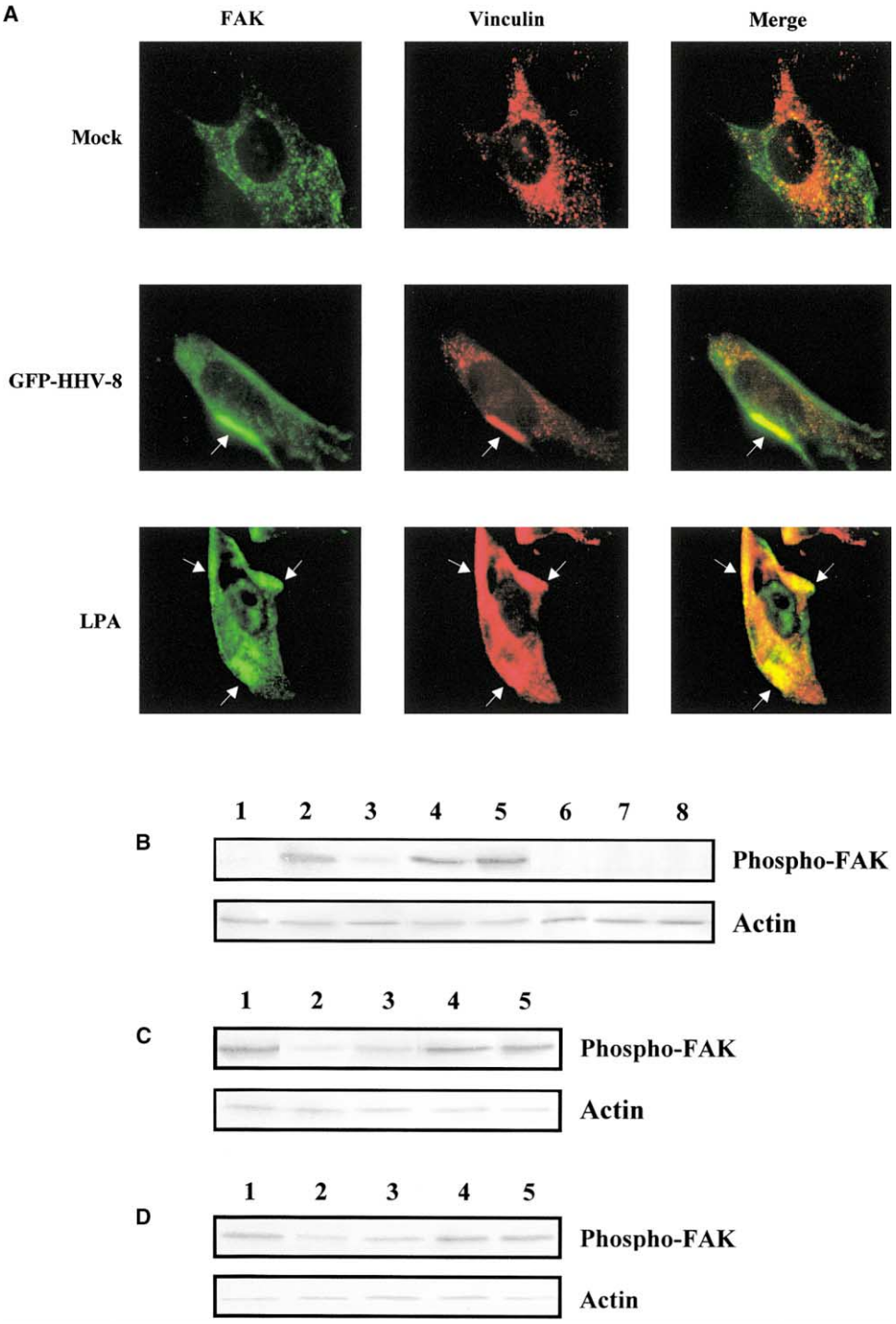


Figure 7. HHV-8 Activates the Integrin-Dependent Focal Adhesion Kinase

(A) Colocalization of $p125^{FAK}$ with vinculin after HHV-8 infection. Serum-starved HFF cells were mock infected, infected with GFP-HHV-8, or stimulated with 20 ng/ml of LPA for 5 min at 37°C. At the end of incubation, cells were fixed, permeabilized, and reacted with rabbit anti-$p125^{FAK}$ antibodies and mouse MAb to vinculin followed by anti-rabbit-FITC and anti-mouse-TRITC antibodies. Stained cells were examined under a fluorescence microscope with appropriate filters. The arrowheads denote representative areas of $p125^{FAK}$-vinculin colocalization. Magnification 1,000×.

(B) HHV-8 induces phosphorylation of FAK. Serum-starved HFF cells were uninduced (lane 1); induced with 100 ng/ml of LPA for 5 min (lane 2); infected with GFP-HHV-8 for 5 min (lane 3), 15 min (lane 4), and 30 min (lane 5); infected with HSV-2 for 30 min (lane 7); or pretreated with 100 μM genistein and then infected with GFP-HHV-8 in the presence of 100 μM genistein for 30 min (lane 8). At different time points at 37°C, cells were lysed and equivalent amounts of lysates were subjected to SDS-PAGE, transferred to PVDF membranes, reacted with anti-phospho-FAK antibodies, and developed using chemiluminescent reagents. After reactions, membranes were stripped and reprobed with MAb to actin. The phosphotyrosine residue in FAK at 30 min post-HHV-8 infection was dephosphorylated by incubating the membranes with 0.5 units of

to further clarify the mechanism by which the FAK activation promotes HHV-8 entry and the role of clathirin and/or caveolin in the formation of endocytic vesicles are in progress.

The discovery that HHV-8 exploits α3β1 integrin for entry into the target cells may have important implications in the unique biology of KS lesions and HHV-8's role in the pathogenesis of KS. HIV-Tat protein is believed to mimic the ECM molecules by interacting with HS and with α5β1 and αVβ3 integrins via its RGD sequence (Barillari et al., 1999). These interactions are believed to displace the basic fibroblast growth factor (BFGF) from the ECM, increase the production of cytokines and BFGF, and thus facilitate KS development. Even though HIV infection accelerates KS development, this may not be the sole inciting event in KS etiology, since KS also occurs in the absence of HIV-1 infection. In contrast, HHV-8 is present in all forms of KS (Antman and Chang, 2000), has the RGD motif in the gB ORF, and binds to HS and the α3β1 integrin molecule. Whether HHV-8 binding to integrin and HS mediates similar biological effects that facilitate KS development, as proposed for HIV-1 Tat, needs to be examined further.

The cells cultured from KS tumors grow for a limited number of passages, contain a mixture of cell types, depend upon growth factors, fail to grow in soft agar, and do not induce tumors in immunodeficient mice (Ganem, 1998). The dominant cultured cell types are fibroblasts and endothelial cells, both of which have a spindle shape. The factors driving the KS tumor spindle cell growth, the inability of KS cells latently infected with HHV-8 to grow in cell culture, and the reason for the loss of HHV-8 DNA within a few passages of KS cells are not known. Evidences such as the reduced risk of KS by ganciclovir treatment that can inhibit HHV-8 replication (Antman and Chang, 2000) and increased antibodies against lytic cycle proteins in KS patients (Zhu et al., 1999; Antman and Chang, 2000) suggest a role for HHV-8 lytic replication in KS. Lytic replication is an obvious necessary precondition to KS lesion formation, as it is required to generate progeny virions that can target endothelial cells for latent infection. In addition to the delivery of viral nucleic acid into the cells, HHV-8 interaction with integrins may also have other important biological consequences. Our studies indicate that HHV-8 interaction with host cell integrins and the associated cell signaling pathways play a critical role in the infectious process. Further studies are needed to examine the consequences of such interactions with latently infected target cells and to discover whether such interactions play a role in the establishment and/or maintenance of latent infection and/or in cellular proliferation of latently infected cells.

Experimental Procedures

Cells

HFF cells, 293 cells, HMVEC-d (CC-2543, Clonetics, Walkersville, MD), BJAB (HHV-8-negative human B cells), BCBL-1 (HHV-8-positive and EBV-negative human B cells), BCBL-1 cells harboring recombinant green fluorescence-HHV-8 (GFP-HHV-8; a gift from Dr. Jeffrey Vieira, Seattle), CHO-B2 cells transfected with pCDNA3 (CHO-B2/pCDNA3), and pCDNA3 containing human α3 integrin cDNA (CHO-B2-clones B3 and D5; gifts from Dr. John A. McDonald, Scottsdale) were grown as per specifications described before (Wu et al., 1995; Akula et al., 2001a).

Virus

BCBL-1 and GFP-BCBL-1 cells were stimulated with 20 ng/ml of TPA (Sigma, St.Louis, MO) for 6 days. Unlabeled wild-type HHV-8, GFP-HHV-8, and [^{3}H]-thymidine labeled HHV-8 in the spent culture medium was concentrated and gradient purified using nycodenz (Sigma), as described previously (Akula et al., 2001b).

Ligands, Peptides, and Integrins

Collagen type I, fibronectin, laminin, tenascin, vitronectin, GRGDTP, and GRGESP peptides were obtained from GIBCO-BRL (Grand Island, NY). Bovine serum albumin (BSA), chondroitin sulfate A and C, and the RGD peptide were obtained from Sigma. Soluble purified human integrins α3β1, α5β1, and α1β1 were obtained from Chemicon International, Temecula, CA.

Anti-Integrin Antibodies

Function-blocking monoclonal antibodies (MAbs) against integrins FB12 (anti-α1, IgG1), ASC-6 (anti-α3, IgG1), NKI-GoH3 (anti-α6, IgG2a), P3G8 (anti-αV, IgG1), 6S6 (anti-β1, IgG1), 2058Z (anti-β4, IgG1), BHA2.1 (anti-α2β1, IgG1), JBS5 (anti-α5β1, IgG1), LM609 (anti-αVβ3, IgG1), P1F6 (anti-αVβ5, IgG1), 10D5 (anti-αVβ6, IgG2a), and rabbit polyclonal antibodies against integrins α1 (AB1934), α3 (AB1920), and β1 (AB1952) obtained from Chemicon International were used in this study.

Anti-gB Antibodies

The production and characterization of rabbit and mouse polyclonal antibodies against the baculovirus-expressed purified full-length GST-gB fusion protein have been previously described (Akula et al., 2001a). Rabbits were immunized with the gB peptides RGDgB-N1 (aa 27–44; RGDTFQTSSSPTPPGSSS), gB-N2 (aa 167–191; GVEN TFTDRDDVNTTVFLQPVEGLT), and gB-C (aa 828–845; RGYKPLTQ SLDISPETGE) predicted to have antigenic regions with and without the RGD sequence. IgG fractions were purified by Protein-A Sepharose 4B columns (Amersham Pharmacia Biotech, Piscataway, NJ). Nonspecific antibodies were removed by columns of cyanogen bromide-activated Sepharose 4B covalently coupled with purified GST protein and BJAB cell lysate.

Inhibition of GFP-HHV-8 Infectivity

GFP-HHV-8 (100 μl) was incubated at 37°C for 1 hr with 100 μl of DMEM alone or with DMEM containing different concentrations of anti-gB antibodies or with soluble integrins. This mixture was added to cells in 8-well chamber slides, incubated at 37°C for 2 hr, washed, and incubated at 37°C for 3 days. Target cells were also incubated with different concentrations of ECM proteins, RGD peptides, or anti-integrin antibodies at 4°C for 1 hr, washed, incubated with 100 μl of GFP-HHV-8 at 37°C for 2 hr, washed, and incubated at 37°C for 3 days. After 3 days, green fluorescent cells were examined

protein tyrosine phosphatase 1B for 3 hr at 30°C before reacting with anti-FAK antibodies (lane 6). The bands were scanned and the band intensities were assessed using the ImageQuaNT software program (Molecular Dynamics).

(C) Soluble α3β1 integrin inhibits HHV-8-induced FAK phosphorylation. Serum-starved HFF cells were infected with GFP-HHV-8 (lane 1); with GFP-HHV-8 preincubated with 5 μg/ml (lane 2), 2.5 μg/ml (lane 3), or 1.25 μg/ml (lane 4) of α3β1 integrin; or with 5 μg/ml of α5β1 integrin (lane 5) at 37°C for 1 hr before infecting cells. At 30 min postinfection, phosphorylation of FAK was measured as described in (B).

(D) Anti-gB antibodies block the HHV-8-induced FAK phosphorylation. HFF cells were infected with GFP-HHV-8 (lane 1); with GFP-HHV-8 preincubated with 100 μg/ml (lane 2), 50 μg/ml (lane 3), or 25 μg/ml (lane 4) of anti-gB IgG antibodies; or with 100 μg/ml of preimmune IgG antibodies (lane 5) at 37°C for 1 hr before infecting cells. At 30 min postinfection, phosphorylation of FAK was measured as described in (B).

under a fluorescence microscope and were counted using the Nikon Magna Firewire digital imaging system (Akula et al., 2001a).

Immunoperoxidase Staining

After examining for GFP expression, cells were fixed with acetone and incubated with MAb to HHV-8 ORF73 protein (ABI, Rockville, MD) for 30 min at 37°C. Slides were washed and incubated with biotinylated anti-mouse antibodies (Vector Laboratories, Burlingame, CA) for 30 min at 37°C, followed by treatment with Vectastain ABC reagent and 3,3′-diaminobenzidine substrate.

Blocking HHV-8 Binding

Cells were incubated with different concentrations of RGD peptides or anti-integrin antibodies in DMEM at 4°C for 90 min, followed by the addition of a fixed quantity of [^{3}H]-thymidine labeled purified HHV-8 (2,684 cpm) (Akula et al., 2001b). Labeled HHV-8 was also incubated with different concentrations of rabbit anti-RGD-gB-N1 peptide antibodies or soluble $\alpha3\beta1$ and $\alpha5\beta1$ integrins or with 10 μg/ml of heparin for 90 min at 4°C before adding to the cells. After incubation for 90 min at 4°C with the virus, cells were washed five times, lysed with 1% SDS and 1% Triton X-100, radioactivity precipitated with trichloroacetic acid (TCA), and counted.

Flow Cytometric Analysis

Suspension cells (BJAB and BCBL-1) and single-cell suspensions of adherent cells (HFF, 293, HMVEC-d, CHO-B2) were washed, incubated in growth medium at 37°C for 30 min, centrifuged, and resuspended in PBS. Cells (1×10^6) were incubated with different anti-integrin antibodies at 4°C for 30 min, washed, incubated with FITC-conjugated anti-mouse IgG at 4°C for 30 min, washed, and analyzed in a FACScan flow cytometer (Becton Dickinson) with appropriate gating parameters.

Interaction of HHV-8 with Cells, Immunoprecipitation, and Western Blot Assays

HFF cells were either treated or untreated with 4 U/ml of heparinase I/III (Akula et al., 2001b) at 37°C for 2 hr, washed, and labeled with sulfo-NHS-biotin (Pierce, Rockford, IL). Biotin-labeled cells were lysed with lysis buffer (0.1% NP40, 15 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM $CaCl_2$, and 2 mM PMSF) and centrifuged at 100,000 $\times$ g for 90 min at 4°C. Supernatant fluid was incubated with normal mouse serum at 4°C for 1 hr, followed by Protein A-Sepharose beads at 4°C for 1 hr, centrifuged at 15,000 $\times$ g for 2 min, and the supernatant was mixed with purified HHV-8 at room temperature (RT) for 1 hr. The cell lysate-HHV-8 mixture was incubated with 20 μg/ml of mouse anti-gB IgG or preimmune IgG antibodies or anti-gpK8.1A MAb at 4°C for 1 hr, precipitated by Protein A Sepharose beads at 4°C for 1 hr, and washed with lysis buffer. Beads were boiled in sample buffer without 2-mercaptoethanol, and the proteins were resolved by SDS-7.5% PAGE. Proteins were Western blotted onto nitrocellulose membranes, soaked in blocking solution at 4°C overnight, and then reacted with specific rabbit anti-integrin antibodies for 3 hr at RT. Membranes were processed as per methods described previously (Akula et al., 2001b).

Immunofluorescence Examination of FAK Activation

HFF and HMVEC-d cells in chamber slides were incubated for 24 hr with DMEM without serum at 37°C and infected with purified GFP-HHV-8 or mock infected or treated with 20 ng/ml of LPA (Sigma) for 5, 15, and 30 min at 37°C. At different time points, cells were washed in PBS, fixed in 3.7% formaldehyde (in PBS) for 10 min at RT, permeabilized with 0.1% Triton X-100-PBS for 4 min, and blocked with 1% BSA-PBS for 10 min at RT. These cells were washed, reacted with rabbit anti-p125^{FAK} antibodies (Sigma) and MAb to vinculin (Sigma) for 45 min at RT, and incubated with goat anti-rabbit FITC and goat anti-mouse tetramethyl rhodamine isothiocyanate (TRITC, Sigma) for 30 min at RT. Stained cells were washed, counterstained with Evan's blue (1:10,000) for 5 min at RT, washed again, and viewed with appropriate filters.

Immunoblot Examination of FAK Activation

Serum-starved HFF cells were either untreated or treated with 100 ng/ml of LPA for 5 min or were infected with HSV-2 or infected with GFP-HHV-8 for 5, 15, and 30 min at 37°C. HFF cells were also preteated with 100 μM of genistein for 1 hr at 37°C and infected with GFP-HHV-8 for 30 min at 37°C in the presence of 100 μM genistein. At different time points, cells were washed in PBS and lysed in NET lysis buffer, and protein concentrations were determined (Shaw et al., 2000). Equal amounts (4 μg) of protein were resolved by SDS-7.5% PAGE, blotted onto a PVDF membrane (Millipore, Bedford, MA), blocked in blocking buffer overnight at 4°C, reacted with anti-phospho-FAK antibodies (Y397, BD Transduction Laboratories, Los Angeles, CA) for 3 hr at RT followed by HRP-conjugated goat anti-mouse antibodies for 90 min at RT, and developed using chemiluminescent reagent (NEN Life Science, Boston, MA). Membranes were stripped and reprobed with MAb to actin (clone AC-40; Sigma). The phosphotyrosine residue in FAK was dephosphorylated by incubating the cell lysate with 0.5 units of protein tyrosine phosphatase 1B (Calbiochem) for 3 hr at 30°C before testing with anti-FAK antibodies. In other experiments, different concentrations of soluble integrins ($\alpha3\beta1$ or $\alpha5\beta1$) or antibodies (anti-gB or anti-ORF 73 or preimmune IgGs) were mixed with GFP-HHV-8 and incubated for 1 hr at 37°C before infecting HFF cells. After 30 min at 37°C, infected cells were lysed and FAK phosphorylation was measured as described above.

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