Integrin $\alpha_V\beta_3$ Binds to the RGD Motif of Glycoprotein B of Kaposi’s Sarcoma-Associated Herpesvirus and Functions as an RGD-Dependent Entry Receptor*\v

H. Jacques Garrigues,1 Yelena E. Rubinchikova,1 C. Michael DiPersio,2 and Timothy M. Rose1*

Department of Pathobiology, School of Public Health and Community Medicine, University of Washington, Seattle, Washington 98195,1 and Center for Cell Biology and Cancer Research, Albany Medical College, Albany, New York 12208-34792

Received 1 August 2007/Accepted 13 November 2007

Kaposi’s sarcoma-associated herpesvirus (KSHV) envelope-associated glycoprotein B (gB) is involved in the initial steps of binding to host cells during KSHV infection. gB contains an RGD motif reported to bind the integrin $\alpha_V\beta_3$ during virus entry. Although the ligand specificity of $\alpha_V\beta_3$ has been controversial, current literature indicates that $\alpha_V\beta_3$ ligand recognition is independent of RGD. We compared $\alpha_V\beta_3$ to the RGD-binding integrin, $\alpha_\gamma\beta_1$, for binding to envelope-associated gB and a gB(RGD) peptide. Adhesion assays demonstrated that $\beta_3$-CHO cells overexpressing $\alpha_V\beta_3$ specifically bound gB(RGD), whereas $\alpha_\gamma$-CHO cells overexpressing $\alpha_\gamma\beta_1$ did not. Function-blocking antibodies to $\alpha_\gamma$ inhibited the adhesion of HT1080 fibrosarcoma cells to gB(RGD), while antibodies to $\alpha_V\beta_3$ did not. Using affinity-purified integrins and confocal microscopy, $\alpha_\gamma\beta_1$ bound to gB(RGD) and KSHV virions, demonstrating direct receptor-ligand interactions. Specific $\alpha_\gamma\beta_1$ antagonists, including cyclic and dicyclic RGD peptides and $\alpha_\gamma\beta_1$ function-blocking antibodies, inhibited KSHV infection by 70 to 80%. Additional inhibitory effects of $\alpha_\gamma\beta_1$ on the cell surface expression of $\alpha_V\beta_3$ and on $\alpha_\gamma\beta_1$-mediated adhesion of $\alpha_\gamma$-CHO cells overexpressing $\alpha_\gamma\beta_1$ were detected, consistent with previous reports of transdominant inhibition of $\alpha_\gamma$ function by $\alpha_\gamma$. These observations may explain previous reports of an inhibition of KSHV infection by soluble $\alpha_\gamma$. Our studies demonstrate that $\alpha_V\beta_3$, a cellular receptor mediating both the cell adhesion and entry of KSHV target cells through binding the virion-associated gB(RGD).

Kaposi’s sarcoma-associated herpesvirus (KSHV)/human herpesvirus 8 was originally detected in KS lesions (12) and has been implicated in the etiology of KS and AIDS-associated pleural effusion lymphoma and multicentric Castleman’s disease (52). KS is a multifocal lesion characterized by intense angiogenesis, proliferation, and infiltration by inflammatory cells (18). KSHV is detected in a number of cell types present in KS lesions, including the characteristic KS spindle cells, infiltrating B cells, and neovascular endothelial cells (4). KSHV infection is latent in these cells, with only a small percentage of cells undergoing active virus replication. In vitro, KSHV has a broad tropism and infects endothelial, epithelial, and mesenchymal cells (5, 47).

Similar to other herpesviruses, KSHV’s infection of susceptible cells occurs through at least two separate binding events. The initial binding to heparan sulfate on cell-surface proteoglycans is mediated by the virion envelope-associated glycoproteins, K8.1 (63) and glycoprotein B (gB) (3). Although heparan-sulfate binding is reversible and not essential for virus entry, it is thought to localize virus on the cell surface, enhancing opportunities for direct interactions between virion glycoproteins and specific cellular entry receptors (3, 59). A number of entry receptor candidates have been reported for KSHV.

The cystine transporter, xCT, was identified as a KSHV fusion-entry receptor by functional cDNA selection (27). DC-SIGN was shown to function as a receptor for KSHV on myeloid dendritic cells and macrophages (46). The $\alpha_\gamma$ integrin has also been identified as a potential entry receptor for KSHV (2), and integrin signaling has been implicated in the KSHV infection process and facilitates uptake into the host cell, intracellular trafficking of the viral capsid, and establishment of the latent infection (36, 39, 40, 55). These findings are of particular interest since the integrin receptor family is exploited by a number of other viruses as an entry pathway for infection.

While the KSHV virion proteins that interact with the xCT and DC-SIGN receptors have not yet been identified, the KSHV envelope-associated gB was reported to be the viral ligand recognized by the $\alpha_\gamma\beta_1$ integrin receptor (2). gB is highly conserved within members of the herpesvirus family and plays an important role in the infection of susceptible host cells by different herpesviruses (42). Sequence analysis of KSHV gB identified an N-terminal Arg-Gly-Asp (RGD) tripeptide motif, also found in extracellular matrix proteins that interact with members of the integrin receptor family (2, 49, 50). Integrin receptors consist of noncovalently associated $\alpha\beta$ heterodimers, and ligand specificity is determined by different combinations of $\alpha$ and $\beta$ monomers. The diverse integrin family is divided based on ligand specificity into subfamilies which include integrin receptors specific for RGD (group I), laminin (group II), collagen (group III), and leukocytes (group IV) (24).
presence of the RGD motif in the KSHV gB suggested that it might be a ligand of a group I RGD-binding integrin, such as αvβ5, αvβ3, or α5β1; however, gB was reported to interact with the laminin-binding integrin α5β1 (2). Although α5β1 was once regarded as a promiscuous receptor with weak affinity for RGD and other ligands, more-recent studies have shown that it binds with high affinity to laminin 5, laminin 10/11, and bacterial invasin in an RGD-independent manner (11, 14, 29, 30, 37, 41, 69). These findings raise questions regarding the direct involvement of α5β1 in RGD-mediated binding to KSHV gB and the role of α5β1 in RGD-mediated entry of KSHV into target cells.

We have used a variety of approaches to study the binding specificity of the RGD motif of KSHV gB and the role of RGD-binding integrins in KSHV infection. We have analyzed integrin binding to the native KSHV virion envelope-associated gB and to a peptide derived from the N-terminal RGD motif of KSHV gB [gB(RGD)] using cell adhesion and affinity binding studies. We have also used peptide antagonists and function-blocking antibodies to identify the integrin responsible for RGD-mediated binding and entry of KSHV into target cells. Our studies show that the group I integrin, αvβ3, specifically interacts with the RGD motif of KSHV gB and functions as a receptor in cell adhesion and KSHV entry during infection. We were unable to detect any specific interactions between the α5β1 integrin and gB or any of the other envelope-associated glycoproteins present on the KSHV virion. We observed an inhibition of KSHV infectivity and a downregulation of α5β1 expression and function in cells overexpressing α5β1. These effects correlate with the transdominant inhibition of α5β1 function by α5β1, and other integrins containing the β1 subunit seen by others (7, 22, 38) and may explain previous reports of inhibitory effects of soluble α5β1 on KSHV infectivity (2, 39, 55). Our results are consistent with the results of studies showing the importance of integrin-mediated entry and integrin-associated signaling during KSHV infection but suggest that the integrin receptor directly interacting with the RGD motif of KSHV gB is αvβ3 integrin.

MATERIALS AND METHODS

Cell lines. The human HT1080 fibrosarcoma cell line was a gift of W. G. Carter. African green monkey Vero cells were from the American Type Culture Collection. The Chinese hamster ovary (CHO) parental cell line, pB1, and human integrin-expressing transfected cell lines αv-CHO (60) and β1-CHO (61) which overexpress α5β1 and αvβ1 integrins, respectively, were a kind gift from Y. Takada. Cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. The CHO cell medium also contained G418 (700 μg/ml) and 0.01% 2-mercaptoethanol at 37°C. The cell lines include MK-116 (MK-11001), MK-546 (MK-11002), and clone 29A3 [for blotting], anti-K8.1 or antibiotin antibodies using goat anti-IgG–HRP and 488 and 594 anti-IgG–HRP, goat anti-mouse alkaline phosphatase (alk-phos), goat anti-rat IgG–HRP (BioSource), normal goat serum, nonimmune-mouse IgG, and mouse antibiotin–HRP (Jackson). Peptides and purified integrin receptors. Biotinylated peptides corresponding to the 12-amino-acid sequences of the RGD motif within the predicted N terminus of the mature KSHV gB and an RGE motif with a single-amino-acid mutation were synthesized by Res Gen (Huntsville, AL); gB(RGD), AHSGRTDFTQSGGCG, and gB(RGE), AHSGRTDFTQSGGCG. The C-terminal GCG amino acids were added for coupling purposes. The αvβ3 antagonists and peptide controls were cyclo(Args-Asp-D-Phe-Lys) (cRGD), dimeric H-Glu[cyclo(Args-Asp-D-Phe-Lys)] (deRGD), cyclo(Ala-Asp-D-Phe-Lys) (eRAD), and linear GRGDS and GIGGS peptides (Peptides International). Affinity-purified human αvβ3 (cc1020) and α5β1 (cc1092) integrin receptors in an octyl-beta-D-glucopyranoside formulation were purchased from Chemicon.

Flow cytometry. The expression levels of different integrins present on the CHO or HT1080 cells were determined by using fixed-cell suspensions incubated with anti-integrin antibodies and fluorescein isothiocyanate-labeled goat anti-mouse IgG and analyzed on a FACScan instrument. The data are given as the mean fluorescence intensities (MFIs) of the stained cell populations.

Adhesion assay. NeutriAvidin plates were coated with biotinylated gB(RGD) or gB(RGE) peptides at different concentrations. Maleimide plates (Pierce) were coated with laminin (20 μg/ml). The plates were blocked with phosphate-buffered saline–2% bovine serum albumin. HT1080 or CHO cell suspensions (2 × 10^5 cells/well) were incubated with immobilized peptides at 25°C for 1 h to allow cell adhesion. In some experiments, the cells were cooled to 4°C and pretreated for 1 h at 4°C with various function-blocking anti-integrin antibodies prior to being plated on peptide (10 μg/ml). Following cell adhesion at 25°C, the plates were inverted to remove nonadherent cells and then incubated at 37°C for 30 min. Nonadherent cells were fixed with 0.5% toluidine blue, and counted manually. The experiments were performed in triplicate or quadruplicate, and the means and standard errors of the cell numbers were determined.

Virus purification. KSHV virions were purified from filtered culture supernatant (180 ml) of tetradeoxyglucorolatobolacetate (TAP)-treated BCBL-1 cells (20 ng/ml; 6 days) by centrifugation onto a cushion of 50% Opti-Prep (iodixanol) (density 1.065). The concentrated virus was labeled with 10 μM NHS-PED-biotin (Pierce) and further purified by centrifugation on a 20%, 25%, 30%, and 40% Opti-Prep step gradient. Virus was quantitated by real-time quantitative PCR. A single DNA peak was obtained at the 20%- to 25%-Opti-Prep interface (fractions 11 and 12; see Fig. 6A). The peak fractions were pooled and represented a 450-fold concentration of the original BCBL-1 supernatant. Virions were characterized by immobilization on polys-l-lysine-coated slides and staining with anti-K8.1 or antibiotin antibodies using goat anti-IgG–HRP and 488 and 594 tyramide signal amplification, respectively (Molecular Probes).

Rhesus rhadinovirus (RRV), a close homolog of KSHV (15), was used as a gB(RGD)-negative control virus. RRV, a gift of M. Asthel and S. Wong, was propagated as described previously (9). RRV virions were concentrated, biotinylated, and gradient purified as described above for KSHV. The infectivity of the purified RRV was confirmed by using rhesus primary fetal fibroblasts (data not shown).

KSHV infection. MK cell lines and Vero cells were seeded at 2.5 × 10^5 cells per well 24 h prior to infection. The cell cultures were infected with different dilutions of gradient-purified KSHV virions for 3 h at 37°C, washed, incubated for an additional 24 h, fixed, and stained for KSHV LANA using the LNA3 anti-LANA antibody essentially as described previously (9). The nuclei were stained with TOPRO (Molecular Probes). The percentage of cells expressing nuclear LANA was determined manually using confocal images. In some experiments, the ability of KSHV to infect HT1080 in the presence of specific αvβ3 antagonists was determined. HT1080 cells (3 × 10^4) were chilled on ice and pretreated with peptide (1 mM) or anti-αv,β3 antibody (LM609; 5 μg) for 1 h at 4°C. Gradient-purified KSHV titrated to yield an infection in 75% of the target cells (3 μl, approximately equivalent to 1 ml of BCBL-1 supernatant) was added and incubated for 1 h at 4°C. The cells were washed, cultured for 24 h, and fixed. The percentages of cells expressing LANA and the relative amounts of nuclear fluorescence were determined by using confocal imaging with the LNA3 antibody. The histogram function within the Zeiss LSM software was used to measure the absolute frequency and intensity of fluorescent pixels within the nuclear LANA dots. The fluorescence intensities ranged from 0 (no fluorescence) to 255 (maximum fluorescence), and the lower threshold was set at 100 to eliminate nonspecific fluorescence.
NeutrAvidin beads (50 μl virions/2 mg beads). To monitor coupling, the KSHV-coated beads were stained with anti-K8.1 antibody, while the RRV-coated beads and gB peptide-coated beads were stained with antibody antibody, and immunofluorescence was visualized by confocal microscopy.

(i) Affinity purification of integrins bound to immobilized peptide. β3-, CHO or αv,CHO cells (5 × 10^6) were solubilized with lysis buffer (100 mM octyl-beta-glucoside, 25 mM Tris HCl, 150 mM NaCl, 1.0 mM MgCl2, pH 7.4, containing 2 mM Pefabloc [Roche]) for 1 h at 4°C. The cell lysates were incubated with peptide-coated beads, and bound proteins were eluted with 20 mM EDTA, resolved on 10% NuPAGE gels (Invitrogen), and transferred to polyvinylidene difluoride membranes. The membranes were probed with either anti-β3 (Mab 2032) or anti-αv (Mab 2290) antibody followed by alk-phos-labeled goat anti-λ. Western blue alk-phos substrate (Promega) was used for the final enzymatic development.

(ii) Affinity purification of integrins bound to immobilized virions. KSHV virion-coated beads or dystrobion-coated control beads were incubated with octyl-glucoside lysates of β3, CHO or αv, CHO, or HT1080 cells. The beads were immobilized on poly-L-lysine-coated slides, fixed, and blocked with 10% normal goat serum. Bound integrins were detected by confocal immunofluorescence microscopy using anti-β3 (Mab 2032) or anti-αv (clone P1B5) antibody followed by goat anti-λ-g0-HRP with TSA 488 development. The Zeiss LSM software histogram function was used to determine the sum of the integrin fluorescence pixels (intensity range, 100 to 255) associated with individual beads. The mean sum ± standard deviation (SD) values for 10 beads per group were determined. The fluorescence pixels that were specific for integrin bound to KSHV were quantitated by subtracting the mean control bead values from the mean values for KSHV-bound integrin.

(iii) Binding of purified integrin receptor to immobilized peptides and purified virions. Dynabeads coated with either gB(RGD) or gB(RGE) peptides or KSHV or RRV virions were incubated with 0.3 μg of affinity-purified αv, or β3, integrin receptors in phosphate-buffered saline containing 1 mM MgCl2. The beads were washed and fixed, and bound integrins were detected by confocal immunofluorescence microscopy using the anti-β3 (clone B3A) or anti-αv (clone P1B5) antibodies as described above.

RESULTS

The RGD motif in the KSHV envelope-associated gB has sequence similarity to extracellular matrix and viral ligands recognized by the αv,β3 integrin. Sequence analysis of KSHV gB identified an Arg-Gly-Asp (RGD) motif near the N terminus of the predicted mature protein (49, 50). RGD motifs are recognized by a number of different integrin receptors, and the amino acids flanking RGD motifs contribute to receptor specificity (44). We compared the KSHV RGD motif and flanking region (Fig. 1A) to the RGD motifs and flanking regions of several integrin (Fig. 1B) and viral proteins (Fig. 1B) for clues to potential KSHV receptor specificity. Visual inspection of the sequences flanking the core RGD motif identified similarities in amino acid composition both upstream and downstream of the RGD motif (Fig. 1A). Alanine (A) residues upstream and a proline (P) residue downstream of the RGD motif were conserved in several RGD-containing ligands. An important conservation of the structurally similar serine (S) and threonine (T) residues flanking both sides of the RGD motif was noted between the KSHV sequence and other ligands. Similar to the sequences of vitronectin and the adenovirus 2 penton base, the KSHV gB contained a phenylalanine (F) residue 2 amino acids downstream of the RGD motif. Overall, the KSHV gB and adenovirus penton base proteins were the most similar, with 6 of 7 identical amino acids within the RGD motif and downstream flanking sequences. The RGD domain of the penton base protein has been shown to interact with the integrin heterodimers αv,β3 and α5,β3 (67), suggesting that the KSHV RGD motif might also bind to these or other integrins containing the αv, integrin subunit.

The glycoprotein B RGD motif is a ligand for cell surface αv,β3 integrin. Previously, the KSHV gB N-terminal domain was shown to promote RGD-dependent fibroblast adhesion; however, the receptor mediating gB binding was not identified (64). We therefore evaluated the ability of the αv,β3 integrin, a proposed KSHV entry receptor (2), to mediate RGD-dependent cell adhesion to KSHV gB and compared it to that of the αv,β3 integrins. One of the integrins predicted to bind the RGD motif of KSHV gB in our sequence alignment study. The αv,β3 CHO cell line, which overexpresses human-hamster chimeric αv,β3 integrin (60), was compared to the β3, CHO cell line, which overexpresses a chimeric αv,β3 integrin (61), for the ability to adhere to gB(RGD), a peptide derived from the N-terminal domain of the KSHV gB (see Fig. 1). Both chimeric integrins have been previously shown to be functional (60, 61). The pBJ-CHO cell line, which lacks an integrin insert, was used as a control. Initial analysis of the CHO cell lines by flow cytometry showed increased surface expression of the appropriate recombiant human integrins (Fig. 2A). To assess adhesion, tissue culture plates were coated with gB(RGD) peptide. Control experiments utilized a gB(RGE) peptide containing a single-amino-acid substitution within the RGD motif. The micrographs in Fig. 2 show that β3, CHO cells (Fig. 2C) adhered significantly more to gB(RGD) than did the αv,β3 CHO cells (Fig. 2D) or the pBJ-CHO control cells (Fig. 2E). Adherent β3, CHO cells showed hallmarks of integrin activation and signaling with a spread morphology and the formation of vinculin-containing focal contacts (data not shown). The adhesion of the β3, CHO cells was specific since no adhesion to the gB(RGE) control peptide was detected (data not shown). Although the αv,β3 CHO cells failed to show significant adhesion to gB(RGD), cell adhesion and spreading was observed when they were plated on a known αv,β3 ligand, such as laminin (Fig. 2F).

Cell adhesion was dependent on the amount of immobilized gB(RGD) peptide. At 0.5 μg/ml, there were >500 adherent β3, CHO cells and no adherent αv,β3 CHO cells.
cells (Fig. 2G). Maximal β3-CHO adhesion was observed at 1 μg/ml of gB(RGD). At the highest concentration of gB(RGD) tested, (2 μg/ml), there were a small number of adherent α3-CHO and pBJ-CHO cells (5% and 13% of the adherent 3-CHO cells, respectively). To determine whether the binding of the α3-CHO and pBJ-CHO cells could be due to endogenous hamster α3 integrins, respectively, using anti-α3β3 (LM609) and anti-α3 (ASC-6) integrin antibodies. (B) Fluorescence-activated cell sorter analysis of α3β3 levels in pBJ-CHO, β3-CHO, and α3-CHO cells. (C to F) Cell adhesion to immobilized gB(RGD) peptide (10 μg/ml) by β3-CHO (C), α3-CHO (D), and the parental pBJ-CHO (E) cells and to immobilized laminin by α3-CHO cells (F) is shown. Bar, 20 μm. (G) Cell adhesion to increasing concentrations of gB(RGD) peptide. The data represent the mean numbers ± standard errors of adherent cells in quadruplicate wells. Control experiments showed no adhesion to the gB(RGE) peptide with a single-amino-acid mutation in the RGD domain (data not shown).

Thus, the levels of endogenous hamster α3β3 expression in the different CHO cell lines correlate with their ability to adhere to gB(RGD) peptide. This suggests that the adhesion of the α3-CHO and pBJ-CHO cells seen at high concentrations of gB(RGD) peptide is mediated by the endogenous hamster α3β3.

To examine the direct receptor-ligand interactions between α3β3 and gB(RGD), gB(RGD) peptide-conjugated magnetic beads were used for affinity binding assays. Biotinylated gB (RGD) or gB(RGE) peptides bound to NeutrAvidin magnetic beads were incubated with octyl-beta-glucoside lysates of β3.
FIG. 3. The RGD motif of KSHV gB specifically binds the α5β3 integrin. Magnetic beads coated with the KSHV gB(RGD) peptide (lanes 2, 5) or gB(RGE) control peptide (lanes 3, 6) were incubated with lysates from either the β3-CHO (lanes 2, 3) or α5-CHO (lanes 5, 6) cells. Bound proteins were eluted with EDTA and characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analysis using anti-β3 antibody (MAb 2023) to detect α5β3 (lanes 1 to 3) and anti-α3 antibody (MAb 2290) to detect α3β1 (lanes 4 to 6). Integrin levels in the initial β3-CHO (lane 1) and α5-CHO (lane 4) cell lysates are shown.

CHO or α5-CHO cells in the presence of 1 mM MgCl2. The β3-CHO and α5-CHO cell lysates contained significant amounts of β3 (105 kDa) and α3 (160 kDa) integrins, respectively (Fig. 3, lanes 1 and 4, respectively). The beads were washed, and bound proteins were eluted by using EDTA. RGD interactions are stabilized by divalent cations, and dissociation with metal chelators, such as EDTA, is diagnostic of RGD-mediated binding (56–58). The eluted proteins were analyzed by immunoblotting using antibodies specific for either the α3 or β3 integrin. The α5β3 integrin was detected in the EDTA eluates from the gB(RGD) beads (lane 2), whereas no α5β3 integrin was detected (lane 5). Neither integrin was detected in the eluates from the gB(RGE) control beads (Fig. 3, lanes 3 and 6). These studies demonstrate a specific interaction between α5β3 and the RGD motif of KSHV gB.

KSHV gB(RGD)-mediated cell adhesion is blocked by α5β3 antibodies. To show that the chimeric human/hamster integrin receptors overexpressed in CHO cells did not have an altered affinity for ligand, we also performed adhesion assays using the human fibrosarcoma cell line HT1080, which expresses human integrin receptors. In these studies, we evaluated the ability of integrin function-blocking antibodies to inhibit the adhesion of HT1080 cells to the gB(RGD) peptide. HT1080 cells were chosen due to their wide variety of expressed integrin receptors (65). The surface integrin expression on HT1080 cells was first determined by flow cytometry using a panel of antibodies to different α and β integrin subunits and receptor dimers. In all cases, single-peak histograms were observed, and the percentage of HT1080 cells staining with each antibody was high (87 to 99%) (Fig. 4A). Significant levels of the laminin-binding integrin α5β1 and the RGD-binding integrins αvβ3, αvβ5, and α5β1 were detected; however, the levels of αvβ3 were comparatively low, as has been previously reported (70).

HT1080 cell suspensions were pretreated at 4°C with function-blocking antibodies to different integrin receptors prior to being plated on immobilized gB(RGD), as described in Materials and Methods. Antibodies specific for α5β3 blocked more than 95% of cell adhesion to gB(RGD) at concentrations as low as 0.4 μg/ml (Fig. 4B). These results confirmed the role of α5β3 in cell adhesion to gB(RGD) seen with the transfected β3-CHO cells, described above. In contrast, function-blocking antibodies to the fibronectin receptor α1β1 and the laminin receptor α5β1 were unable to block the adhesion of HT1080 cells to gB(RGD). In fact, these antibody treatments increased cell adhesion to 110% and 125%, respectively, of that observed without antibody pretreatment. Antibodies to the RGD-binding integrin, αvβ3, inhibited 30% of adhesion at the highest antibody concentration. These results indicate that the RGD motif of KSHV gB has a strong specificity for integrins containing the αv integrin subunit, with the strongest reactivity to αvβ3, the vitronectin receptor.

Purified α5β3 receptor binds directly to gB(RGD) and intact KSHV virions. To demonstrate a direct interaction between the α5β3 integrin and KSHV gB, we evaluated the binding of purified soluble α5β3 integrin receptor to both gB(RGD) peptide and KSHV virion-coated beads. The results were compared to the binding of purified α5β3 integrin which
has been previously reported to inhibit KSHV infection of fibroblasts (2). Initial experiments were performed to determine the binding specificities of the commercial preparations of affinity-purified functional $\alpha_v\beta_3$ or $\alpha_v\beta_1$ integrin receptors (R). Bound $\alpha_v\beta_3$ or $\alpha_v\beta_1$ integrins were detected by using anti-31 (A, B) or anti-$\alpha_3$ ($\alpha_3\beta_1$) (C, D) antibodies and confocal microscopy (green fluorescence).

In order to examine the binding of purified integrins to native envelope-associated gB present on intact virions, KSHV virions were purified from TPA-induced BCBL-1 cell cultures by density gradient centrifugation in Opti-Prep (iodixanol) and biotinylated, as described in Materials and Methods. Real-time PCR analysis of KSHV DNA in the gradient fractions showed consistent with the presence of intact, enveloped KSHV virions suitable for receptor binding studies. Infectious KSHV virions were purified from culture medium of TPA-induced BCBL cells by double-step gradient centrifugation in Opti-Prep. (A) Virus-containing fractions were identified by real-time quantitative PCR analysis for KSHV genomic DNA. (B, C) Biotinylated virions showed discrete staining and colocalization (arrows) of antibody reactivity to biotin (B) and KSHV envelope-associated glycoprotein K8.1 (C). Bar, 5 $\mu$m.

The gradient-purified biotinylated KSHV virions were coupled to NeutrAvidin-coated beads and fixed. As a binding control, NeutrAvidin beads were coupled to gradient-purified preparations of RRV, a macaque herpesvirus that is closely related to KSHV (15). Sequence analysis of the RRV genome has identified a close homolog of the KSHV gB (53) which lacks a putative integrin-binding RGD motif. The relative amounts and distribution of virions coating the beads were determined by confocal immunofluorescence microscopy using the anti-K8.1A antibody for KSHV and the antibiotin antibody for RRV. Highly concentrated KSHV virions were detected at the bead surface, with some localized enhanced fluorescence concentrations possibly indicating areas of KSHV aggregation (Fig. 7A). The surface density of biotin-
yalated RRV was similar to that of KSHV, but the uniform immunofluorescence pattern suggested fewer surface RRV aggregates (Fig. 7B). The KSHV and RRV virion-coupled beads were incubated with affinity-purified \( \alpha_3\beta_1 \) and \( \alpha_5\beta_1 \) integrin receptors, and bound receptor was detected by confocal immunofluorescence using antibodies to \( \beta_3 \) and \( \alpha_3 \) integrins. The antibodies to \( \beta_3 \) reacted strongly with the KSHV-coated beads, demonstrating the presence of bound \( \alpha_3\beta_1 \) receptor (Fig. 7C). The pattern of fluorescence was similar to that seen with the anti-K8.1 antibody, indicating that the \( \alpha_3\beta_1 \) integrin receptor was bound to the KSHV virions. No binding of the affinity-purified \( \alpha_3\beta_1 \) integrin to the KSHV-coated bead surface was detected (Fig. 7E). The binding of purified \( \alpha_3\beta_1 \) to the KSHV virion was specific since no binding was detected to the control macaque RRV virions which lack the gB RGD motif (Fig. 7D). The purified \( \alpha_3\beta_1 \) integrin receptor was also unable to bind to the RRV virions (Fig. 7F).

**Soluble \( \alpha_3\beta_1 \) integrin in detergent-extracted cell lysates specifically binds KSHV virions.** To determine if \( \alpha_3\beta_1 \) associates with KSHV in the presence of other cellular proteins within whole-cell lysates, gradient-purified biotinylated KSHV virions were coupled to beads, and conjugated virus was detected by confocal immunofluorescence using antibody to the K8.1 envelope glycoprotein (Fig. 8E). The KSHV-coupled beads were incubated with octyl-beta-glucoside lysates of \( \beta_3\)-CHO or \( \alpha_7\)-CHO cells. d-Biotin-conjugated beads were used as a control. Bound integrins were detected by immunofluorescence microscopy. High levels of the \( \beta_3 \) integrin from the \( \beta_3\)-CHO lysate bound to the KSHV beads (Fig. 8A). No binding to the control beads was detected (Fig. 8B). The \( \alpha_3 \) integrin from lysates of the \( \alpha_3\)-CHO cells was also detected on the KSHV beads, but at a lower level than in the \( \beta_3 \) lysates (Fig. 8C). However, similar amounts of \( \alpha_3 \) also bound to the control beads containing no virus, suggesting that the binding was nonspecific. Quantitation of the bead-associated fluorescence revealed a significant and specific reaction between \( \alpha_3\beta_1 \) in cell lysates and the purified KSHV virions (Fig. 8F, G). This binding is similar to that seen with the purified \( \alpha_3\beta_1 \) receptor, described above. No significant association was detected between the KSHV virions and the \( \alpha_5\beta_1 \) integrin even in the context of other cellular proteins in the cell lysate (Fig. 8F, G). These studies were repeated using lysates of human HT1080 cells which express significant levels of functional \( \alpha_5\beta_3 \) and \( \alpha_3\beta_1 \) integrins with the same results (data not shown).

**KSHV cell entry is inhibited by \( \alpha_3\beta_1 \) antagonists.** Since our data showed a strong reactivity between \( \alpha_3\beta_1 \) and KSHV virions, we performed infection-blocking studies to determine whether the \( \alpha_3\beta_1 \) integrin functions as a KSHV entry receptor. Cyclic RGD peptides are selective \( \alpha_3\beta_1 \) antagonists and block \( \alpha_3\beta_1 \) integrin binding at concentrations 80-fold lower than their linear counterparts (21, 43). Furthermore, changing the ligand valency by converting monomeric peptide to dimeric increases the affinity for \( \alpha_3\beta_1 \) (34). Therefore, we tested the ability of the cyclic RGD peptides to block KSHV infection mediated by \( \alpha_3\beta_1 \). cRGD and dcRGD were chosen since these peptides have high affinities for \( \alpha_3\beta_1 \) and low affinities for \( \alpha_5\beta_3 \), \( \alpha_5\beta_1 \), and \( \alpha_3\beta_1 \) integrins (25, 28). The inhibitory activities of the cyclic peptides were compared to the activity of the linear GRGDS peptide derived from the fibronectin sequence. The linear peptide GRGES and the cyclic peptide cRAD, which both contained single-amino-acid alterations in the RGD motif, were used as controls.

Adherent HT1080 cells were trypsinized, chilled (4°C) to prevent receptor internalization, and pretreated with peptide before infection with gradient-purified KSHV, as described in Materials and Methods. Latent infection was assayed at 24 h by confocal immunofluorescence detection of KSHV LANA. In the absence of inhibitor, approximately 75% of the HT1080 cells were infected with KSHV (Fig. 9A). Cells treated with the control peptides, cRAD and GRGES, showed only a small decrease in the number of LANA-expressing cells. In contrast, the \( \alpha_3\beta_1 \)-specific cRGD peptide and the linear GRGDS peptide both inhibited approximately 78% of KSHV infection, while the dcRGD peptide inhibited approximately 64% (Fig. 9A).

Multiple large dots of LANA fluorescence were detected within the nuclei of cells infected in the absence of inhibitor (data not shown) and cells infected in the presence of the control cRAD peptide (Fig. 9B). A noticeable reduction in the number of LANA dots and the level of LANA expression in
cRGD-treated cells were compared. The frequency of fluorescent pixels and the intensity of each pixel (spanning a range of intensities from weakly fluorescent (100) to the brightest pixels (255)) were determined for each dot. As shown in Fig. 9D, LANA expression was inhibited by treatment with the cRGD peptide across the intensity range. More importantly, there was an approximate fivefold reduction in the frequency of the brightest LANA pixels (intensity of 255).

To further substantiate the role of the αvβ3 integrin in KSHV entry, the function-blocking anti-αvβ3 MAb LM609 was tested for its ability to inhibit KSHV infection. As shown in Fig. 9A, the LM609 antibody was a potent inhibitor of KSHV infection and inhibited infection levels by 75%, equivalent to the results obtained with the cRGD and GRGD peptides. No inhibition was detected with the normal IgG control antibodies. These studies and those with the αvβ3 peptide antagonists demonstrate that specific blocking of the RGD-mediated interaction with the αvβ3 receptor can inhibit KSHV infection and establishment of latency. The similar levels of inhibition seen with the cyclic and linear RGD peptides strongly implicate αvβ3 as a major KSHV entry receptor.

**KSHV infection of mouse keratinocytes is independent of αvβ3 integrin.** Although our studies failed to confirm the previously reported interaction between KSHV and the αvβ3 integrin, the possibility remained that αvβ3 could facilitate KSHV entry by synergizing with αvβ3 or other receptors, such as the xCT heavy chain which associates with integrins (35). We therefore compared the ability of KSHV to infect mouse keratinocytes derived from (i) wild-type mice (MK+/+), (ii) homozygous αv-null mice which lack functional cell surface αvβ3 integrin receptors (MK−/−), or (iii) αv-null MK cells engineered to produce recombinant αvβ3 (MK−/−αv3) (16). The αv-null and wild-type mouse keratinocytes express equivalent amounts of other integrins, including αv (16). The mouse keratinocytes were infected with different amounts of gradient-purified KSHV, and infection was quantitated 24 h later by immunofluorescence detection of nuclear LANA expression. The Vero cell line, which is highly infectible by KSHV, was used as a positive infection control. The virus infection curves for both the wild-type MK+/+ and the αv-null MK−/− cells were very similar, although at all points the infection of the αv-null MK−/− cells was higher than that of the wild-type cells, attaining 74% at the highest virus dose tested (Fig. 10). By comparison, this virus dose yielded >95% infection of the Vero cells. In contrast, MK−/−αv3 cells which overexpress the αvβ3 integrin showed decreased infection levels, attaining only 55% infection of the cell culture at the highest virus dose tested. These results, which were corroborated in three independent experiments, indicate that KSHV infection of mouse keratinocytes is independent of αvβ3. In fact, the reduced level of infection of the αv-null mouse keratinocytes transfected with recombinant αvA integrin cDNA suggested that the overexpression of αvβ3 negatively affected KSHV entry and establishment of a latent infection.

**DISCUSSION**

This study demonstrates that the αvβ3 integrin is a cellular receptor mediating both the cell adhesion and entry of KSHV into target cells through binding to the RGD motif of the virion-associated gB. This conclusion is supported by multiple lines of evidence. First, analysis of the RGD motif of the
KSHV gB revealed strong sequence similarities to analogous motifs in known ligands of the αvβ3 integrin receptor. Second, the gB(RGD) peptide, derived from the KSHV gB sequence, promoted RGD-dependent cell adhesion that was specifically blocked by an αvβ3 function-blocking MAb. Function-blocking antibodies to αvβ3 or αvβ1 integrins were unable to block adhesion. Antibodies to αvβ3 partially blocked adhesion, supporting the role of the αv integrin subunit in gB(RGD)-integrin interactions. Third, the overexpression of αvβ3 integrin receptors strongly increased the adherence of β3 CHO cells to gB(RGD) over that seen with the parental pBJ-CHO cells or αvβ3 CHO cells overexpressing the αvβ1 integrin. Fourth, immunoblot analysis revealed that the gB(RGD) peptide specifically bound to αvβ3 receptors in octyl-beta-glucoside lysates of β3 CHO cells in a divergent cation-dependent manner. Fifth, confocal microscopy demonstrated a specific binding between affinity-purified αvβ3 receptors and both gB(RGD) peptide and gradient-purified KSHV virions. Finally, KSHV infection of HT1080 fibrosarcoma cells was specifically blocked by αvβ3-specific cyclic RGD peptides and a specific αvβ3 function-blocking antibody.

The αvβ3 integrin is a cell surface receptor that is widely expressed in proliferating endothelial cells, arterial smooth muscle cells, and certain populations of leukocytes and tumor cells (23). Integrin αvβ3 mediates the adhesion of cells to a number of extracellular matrix proteins containing RGD motifs, including vitronectin and fibronectin, and is responsible for mediating cell-cell interactions through binding to cell-associated glycoproteins containing RGD motifs. The αvβ3 integrin has been implicated in cellular processes ranging from wound healing to tumor angiogenesis and tumor progression. In addition, αvβ3 has been coopted by a number of pathogens, including foot-and-mouth disease virus, coxsackievirus, hantavirus, and adenovirus (45), as a receptor for the entry of target cells during the infection process. Our finding that αvβ3 functions as an entry receptor for KSHV further expands the number of viruses that utilize this receptor for entry. In KS tumors, αvβ3 is highly expressed on KS tumor spindle cells and in neovascular endothelial cells and B cells that infiltrate KS tumors (8, 26, 51, 62). These cells are also consistently infected with KSHV in KS lesions, suggesting a central role for αvβ3 in KSHV infection and in the pathogenesis of KS.

The ability of the integrin αvβ3 to bind RGD-containing proteins has been exhaustively reported in the literature. Our finding that αvβ3 binds to the RGD motif of KSHV gB confirms this specificity. Ligand-binding studies have shown that αvβ3 and the closely related αvβ1 interact specifically with peptides containing the consensus sequence RGDXY, where X is S or T (33). Panning studies of phage libraries displaying cyclic peptides demonstrated that both the αvβ3 and αvβ1 integrins selectively bound the peptide sequence SRGDFV, which constitutes the core of the KSHV RGD motif (33). In contrast, the αvβ3 and αvβ1 integrins, which also bind RGD motifs, did not select peptides with sequences similar to that of the KSHV RGD motif. These panning studies correlate with the results of our antibody-blocking studies which showed that antibodies to αvβ3 and αvβ1 could significantly block binding to the KSHV gB(RGD) peptide, whereas antibodies to αvβ1 could not. These data strongly support the hypothesis that the RGD motif of KSHV gB specifically interacts with integrins containing the αv integrin subunit, especially αvβ3.

A previous study has shown that de novo infection of naïve cells with KSHV results in a Poisson distribution of viral episomes within the infected cells (1). Furthermore, the number and summed immunofluorescence of LANA dots in the infected cell nuclei were directly proportional to the amount of intracellular viral DNA. Our results showed that blocking KSHV infection with αvβ3 antagonists not only decreased the number of infected cells but also decreased the amount of LANA fluorescence in those cells that were infected. This suggests that blocking viral entry can reduce the number of virions that enter an infected cell and decrease the number of LANA dots and associated immunofluorescence. Studies are ongoing to confirm this.

A previous study reported that αvβ1 interacts with KSHV gB and functions as a cellular entry receptor for KSHV (2). It was suggested that this interaction may be RGD mediated. However, recent studies have shown that αvβ1 binding to its high-affinity ligands is RGD independent (11, 14, 20, 29, 30, 37, 41, 66, 69). Other studies using purified recombinant αvβ1 detected no binding to RGD-containing proteins, such as fibronectin (17), while transfection studies demonstrated that the αvβ1 integrin is not sufficient for cell attachment to matrices containing RGD motifs (14, 66). Furthermore, the characterized αvβ1 recognition motifs NLR (37) and PPFMLK GSTR (31) are not present in the KSHV gB sequence. Our studies revealed no interaction between αvβ1 and the RGD motif of KSHV gB, and αvβ1 function-blocking antibodies failed to inhibit cell adhesion to the gB(RGD) peptide. Al-
though α3-CHO cells overexpressing αβ3 showed enhanced adhesion to a laminin substrate, no adhesion to the gB(RGD) peptide greater that detected with the parental pBJ-CHO cells was observed. Affinity purification studies revealed no binding between the gB(RGD) peptide and solubilized αβ3 receptors from octyl-beta-glucoside lysates of α3-CHO cells. In addition, the gB(RGD) peptide and the native envelope-associated gB present on gradient-purified KSHV virions were unable to bind to commercial preparations of affinity-purified αβ3 receptors. Infection studies revealed that α3-null mouse keratinocytes lacking αβ3 integrin were competent for infection by KSHV, and the expression of αβ3 in either the parental mouse keratinocytes or α3-null mouse keratinocytes transfected with human α3 decreased infection levels.

While our study failed to detect a direct interaction between αβ3 and the RGD motif of KSHV gB, it did not directly address a potential secondary role for αβ3 in KSHV binding, entry, or downstream signaling. Studies have shown co-localization of αβ3 and αβ1 integrin at cell surface focal adhesions due to shared interactions with other molecules, such as actin, focal adhesion kinase, vinculin, α-actinin, and paxillin which form the adhesion plaque (10, 13, 68). It has been suggested that the αβ3 integrin may mediate secondary responses after other integrins, such as αvβ3, bind their specific ligands (for a review, see reference 6). These secondary responses could include “integrin cross talk” and the modulation of downstream signaling or the recruitment of receptors. Infection studies revealed that α3-null mouse keratinocytes, which are completely lacking in α3-CHO cells, to the gB(RGD) peptide compared to the levels in both α3-null mouse keratinocytes and α3-null mouse keratinocytes transfected with human α3 decreased infection levels.

Several lines of evidence from our study confirm the dominant inhibitory effect of αβ3 and other integrins containing the β3 subunit on the receptor function of αβ3 that has been seen by others (7, 22, 32, 38). First, the antibody blocking of αβ3 function in our cell adhesion assays led to an increased level of αβ3-mediated adhesion of HT1080 cells to the gB(RGD) peptide. Second, the overexpression of α3 integrin in the α3-CHO cells resulted in a 60% decrease in the surface expression of αβ3 and a 62% decrease in αβ3-dependent adhesion of the α3-CHO cells to the gB(RGD) peptide compared to those of the pBJ parental CHO cells. Third, the overexpression of α3 integrin in the rescued α3-null mouse keratinocytes resulted in decreased levels of KSHV infection compared to the levels in both α3-null mouse keratinocytes and wild-type mouse keratinocytes. The inhibitory effect of αβ3 on αβ3 function may explain the ability of soluble αβ3 to inhibit KSHV infection, as reported previously (2, 39, 55).

Several previous studies have implicated integrin binding and the downstream activation of integrin signaling pathways in the KSHV infection process (36, 40, 54, 64). Our data showing that αβ3 functions as a major integrin receptor mediating RGD-dependent interactions with the KSHV gB during viral entry are consistent with the results of these studies.

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