Kaposi’s sarcoma-associated herpesvirus (KSHV; also known as human herpesvirus 8) is the etiologic agent of Kaposi’s sarcoma, an endothelial neoplasm. This γ-herpesvirus encodes for several unique proteins that alter target cell function, including the virion envelope-associated glycoprotein B (gB). Glycoprotein B has an RGD (Arg-Gly-Asp) motif at the extracellular amino terminus region and binds to the αβ3 surface integrin, which enhances virus entry. We now report that gB can activate the vascular endothelial growth factor receptor 3 (VEGFR-3) on the surface of microvascular endothelial cells and trigger receptor signaling, which can modulate endothelial migration and proliferation. Furthermore, we observed that VEGFR-3 expression and activation enhance KSHV infection and participate in KSHV-mediated transformation. These functional changes in the endothelium may contribute to the pathogenesis of Kaposi’s sarcoma and suggest that interventions that inhibit gB activation of VEGFR-3 could be useful in the treatment of this neoplasm.

Kaposi’s sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), is the primary etiologic agent of Kaposi’s sarcoma (KS), a neoplasm arising from endothelial cells. Viral DNA has been found in KS lesions from patients with all forms of the disease, namely AIDS-KS, classic KS, African endemic KS, and transplantation-associated KS. KS is most commonly found in the dermis but also occurs in viscera including lungs, liver, and intestines (1, 2). The KSHV genome encodes over 85 genes, of which <10% are expressed during latent infection (3, 4). In KS tissues, KSHV infection is localized to KS spindle cells, the majority of which are latently infected. A small subpopulation, however, is lytically infected. Recent evidence suggests that both latency and lytic infection contribute importantly to KS pathogenesis (5–7).

KSHV encodes for several biologically active proteins, including the virion envelope-associated glycoprotein B (gB or ORF-8) (4). Among the herpesvirus gB genes characterized to date, only the KSHV gB has the RGD (Arg-Gly-Asp) motif (amino acids 27–29), which is at the extracellular amino-terminal region after the putative signal sequence. This RGD sequence appears to be highly conserved among the KSHV strains analyzed from different geographical regions (8). The RGD amino acids constitute the minimal region of several extracellular matrix proteins required for interaction with certain cell surface integrins. The RGD motif is the most common integrin recognition motif, but other RGD-independent integrin recognition motifs have also been reported (9, 10). Recently, a gB disintegrin-like domain has been identified in the envelope glycoprotein B of the human cytomegalovirus by analysis of its sequence (11). This gB disintegrin-like domain is highly conserved in most herpesviruses including KSHV, which suggests that other possible interactions may be recruited in KSHV entry and KSHV-mediated signaling pathways beyond the RGD motif. Furthermore, unlike the gB protein of Epstein-Barr virus, KSHV gB is expressed on the surface of the infected cell membrane and on the virion envelope (12–14). Envelope-associated KSHV gB is composed of 75- and 54-kDa polypeptides forming disulfide-linked heterodimers and multimers and binds to heparan sulfate-like molecules on the cell surface (12). In this way, ubiquitous host cell surface heparan sulfate-like molecules may serve as attachment receptors (15). KSHV envelope gB not only binds heparan sulfate through a conserved region found in most of the herpesvirus gBs (16) but also specifically binds integrin αβ3 through its unique RGD motif (17). The integrin αβ3, a receptor for laminin 5 and possibly for fibronectin, is expressed abundantly in endothelial cells (18, 19). It is localized in the cell-cell junctions of the endothelium and regulates cell motility in wound repair assays (20). Recently, it has been reported that KSHV also uses clathrin-mediated endocytosis and creates a low pH intracellular environment to facilitate infection (21).

KS spindle cells are believed to be of lymphatic endothelial origin. Infection by KSHV and virus-induced transformation of the endothelium are key steps in the development of KS. VEGFR-3 (also known as Flt-4) is robustly expressed on the lymphatic endothelium. This receptor mediates signal trans-
duction after engagement of its cognate ligands, VEGF-C and VEGF-D, through a cascade of phosphorylation events. Like VEGFR-1/Flt-1 and VEGFR-2/Flik-1, VEGFR-3 is a member of a subfamily of class III receptor tyrosine kinases characterized by seven extracellular Ig-like domains and a split intracellular domain containing kinase activity. These three receptors have ~31–36% amino acid identity in their extracellular ligand binding domains (22–25). Two isoforms of VEGFR-3 have been described, a short form and long form. The difference between the isoforms arises by alternative splicing in the carboxyl terminus. The long form is the predominant form in most tissues in humans (24). VEGFR-3 is a highly glycosylated protein and migrates as bands with different molecular masses, specifically an ~175-kDa precursor, an ~195-kDa mature form, and an 140-kDa non-glycosylated backbone, and a form that appears to be partially cleaved proteolytically in the extracellular domain to produce an ~125-kDa species (24, 26).

Considerable evidence indicates that VEGFR-3 and its ligands, VEGF-C and VEGF-D, regulate lymphangiogenesis. In the early stages of development, VEGFR-3 is widely expressed in vascular endothelial cells (27, 28). Targeted disruption of the VEGFR-3 gene leads to disorganization in the large vessels, resulting in an embryo that dies of cardiovascular failure (29). However, later in development and in the adult, VEGFR-3 expression becomes restricted mainly to lymphatic vessels (27). VEGFR-3 signaling involves the interaction of several downstream molecules including adaptor proteins (26, 30, 31), the focal adhesion kinases FAK and RAFTK (related adhesion focal tyrosine kinase), transcriptional activators, and certain cytoskeletal proteins such as paxillin (32–34). We also demonstrated that the interaction of VEGFR-3 with integrin β1 can modulate certain endothelial functions (35, 36).

Expression of VEGFR-3 is increased in KS spindle cells, and its ligand, VEGF-C, stimulates the migration and proliferation of KS cells in vitro (37, 38). Histopathological studies indicate that KSHV LANA-1 (latent nuclear antigen 1) and VEGFR-3 co-localize in nodular KS (39).

Akula et al. reported that the integrin αvβ1 and its associated signaling pathways are important in KSHV entry into target cells (17, 21). KSHV gB can mediate cell adhesion via its RGD sequence as well as activate focal adhesion components such as FAK and paxillin (14). Recently, it has been shown that KSHV gB regulates integrin-dependent FAK, Src, PI 3-kinase, and Rho GTPase signaling pathways and modulates cytoskeletal rearrangements, which are critical for KS pathogenesis (40). These observations suggest that KSHV gB-induced signaling pathways may play important roles in the infection of target cells and in KS pathogenesis.

We now report that KSHV gB can activate VEGFR-3 on the microvascular endothelium and modulate endothelial cell migration and proliferation via an interaction between the αvβ1 integrin and the VEGFR-3 receptor. Our studies also show that the activation of VEGFR-3 facilitates KSHV infection and fosters subsequent transformation. This novel mechanism of virus activation of a key lymphatic endothelial receptor could contribute to the pathogenesis of KS.

**Experimental Procedures**

**Cells**—Primary human dermal microvascular endothelial cells (HMVEC; adult) were purchased from Clonetics Inc. (San Diego, CA) and maintained in endothelial basal medium 2 (EBM-2) with EGM-2MV SingleQuots. Recombinant green fluorescent protein (GFP)-KSHV carrying BCBL-1 cells (GFP-BCBL-1) were a gift from Dr. Jeffrey Vieira (Dept. of Laboratory Medicine, University of Washington, Seattle, WA). BJAB is a line of KSHV and Epstein-Barr virus negative human B cells. These cell lines were grown as described previously (17, 41). 293 human embryonic kidney cells and 293 cells stably transfected with VEGFR-3 (293/VEGFR-3) were obtained from Genentech Inc. (San Francisco, CA) and cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum.

**Antibodies**—The anti-VEGFR-3 monoclonal antibody hF4-3C5 was from ImClone Systems (New York, NY). Rabbit anti-human VEGFR-3 polyclonal antibody was from Genentech Inc. Mouse anti-human VEGFR-3 monoclonal antibody was from R&D Systems Inc. (Minneapolis, MN). A neutralizing anti-αv antibody and purified normal mouse IgG were from Chemicon International, Inc. (Temecula, CA). Rabbit anti-Fit-3 (VEGFR-3) polyclonal antibody, mouse polyclonal anti-phospho-tyrosine antibody, rabbit anti-ERK antibody, mouse anti-phospho-ERK antibody, and goat anti-αv antibody were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti-Akt antibody and anti-phospho-Akt (Ser) antibody were from Cell Signaling Technology (Beverly, MA). Rabbit anti-epidermal growth factor receptor (EGFR) antibody and anti-phospho-EGFR antibody were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY).

**Cell Isolation**—HMVEC were incubated with a rabbit or mouse anti-human VEGFR-3 antibody for 1 h. After washing with 1× PBS, VEGFR-3-expressing cells were isolated using MACS colloidal superparamagnetic MicroBeads conjugated with goat anti-rabbit or goat anti-mouse IgG antibodies (Miltenyi Biotec Inc., Auburn, CA) according to the manufacturer’s instructions. Isolated cells were cultured and expanded in the medium described above. For immunofluorescence analysis, cells were fixed, and fluorescein isothiocyanate-labeled cell sorting beads (BD Biosciences) were added. Cells were washed with 5 mM EDTA and 0.5% BSA in PBS and then washed and resuspended in PBS containing 1% normal goat serum. After incubation for 30 min at 4 °C, cells were incubated with anti-VEGFR-3 antibody or isotype control for 60 min on ice. Primary antibodies were detected by incubation with phycoerythrin-conjugated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA). Cellular fluorescence was analyzed on a FACScan® flow cytometer.

**Cell Transfection**—Plasmids containing wild-type VEGFR-3, dominant negative G857R mutant VEGFR-3, or pHK5 vector were used as described (35). Cells were grown to confluence on 60–100% tissue culture dishes. Transfections were done using Super Effectene transfection reagent according to the supplier’s manual (Qiagen). A GFP-labeled pRK5 vector (BD Biosciences) was used as a parallel control to check for transfection efficiency as described (42). At 5 h post-transfection, cells were washed once with 1× PBS, cultured in medium with 10% fetal calf serum, grown for 48 h, and then treated as indicated. The transfection efficiency was determined to be ~30–40% by counting green fluorescent cells under a fluorescent microscope (Nikon Diaphot 300, Tokyo, Japan).

**Virus Preparation and Real Time DNA PCR**—GFP-BCBL-1 cells were grown to a density of 5 × 10^6 cells/ml, induced with 12-O-tetradecanoylphorbol-13-acetate at 20 ng/ml, and grown for 5 days. To harvest the virus, cells were pelleted at 500 × g for 15 min. The supernatant was then removed and centrifuged at 15,000 × g for 4 h. The 15,000 × g pellet was resuspended in one one-hundredth growth volume with complete media and centrifuged at 300 × g for 5 min. The supernatant was then used for virus inoculation and stimulation (17, 41). Real time PCR was performed to quantify the viral DNA. Total viral DNA from purified KSHV samples was prepared using the Qiagen DNeasy tissue kit. The specific RNA-PCR kit (ORF-73) primers, TaqMan probe, and the external KSHV LANA-1 gene standards have been described previously (43). The QuantiTect Probe PCR kit (Qiagen, Valencia, CA) was used to perform real time PCR, and the assays followed a protocol reported previously (44). The cycle threshold (Ct) values were used to plot a standard graph and calculate the relative copy numbers of viral DNA in the samples. Target cells were exposed to KSHV with a multiplicity of infection of 5–6 viral DNA copies per cell for stimulation or infection. The BJAB cells were treated in the same way with 12-O-tetradecanoylphorbol-13-acetate, as were the GFP-BCBL-1 cells, and supernatants were prepared as negative controls.

**Cell Stimulation, Immunoprecipitation, and Western Blotting**—Recombinant wild-type KSHV gB(TM) and a control gB protein, gB(TM-RGA, which is mutated so that it contains a RGA sequence instead of RGD, have been described previously (14, 17). Cells were starved for 4–6 h in serum-free media. To inhibit phosphotyrosine phosphatases, cells were preincubated for 10 min with 0.5 mM Na3VO4 and then stimulated with gB(TM-RGA, or VEGF-C (R&D Systems) as indicated). After stimulation, cell lysates were collected and used for the immunoprecipitation and Western blotting assays as described previously (35).

**Radioiodination and Binding Assays**—Recombinant VEGF-C (carrier-free) was from R&D Systems. This mutant of human VEGF-C containing a C15GS substitution is a selective agonist of VEGFR-3 and does not bind VEGFR-2. 125I-gB(TM/66.4 μCi/μg, 125I-gB(TM-RGA (27.3 kDa).
VEGF-3 Enhances KSHV Infection

μCi/μg), and 125I-VEGF-C (129 μCi/μg) were radiolabeled by PerkinElmer Life Sciences using the iodogen method. 293/VEGF-3 cells were washed twice in ice-cold binding buffer (Dulbecco's modified Eagle's medium containing 0.15% gelatin and 25 mM HEPES). Duplicate samples (2 × 10^6 cells in 200 μl) were incubated with 0.1 nM 125I-gB(1-23) or 125I-gB(1-23)TM-RGA with or without the indicated concentrations of unlabeled proteins. Unlabeled HIV-1 gp120 protein from the National Institutes of Health AIDS Research and Reagent Program was used as a negative control. Similarly, cells were incubated with 0.1 nM 125I-VEGF-C for 1 h at room temperature as reported previously (45, 46). Unlabeled VEGF-C, KSHV gB(1-23)TM, or KSHV gB(1-23)TM-RGA was added to the cells immediately before the radiolabeled ligand was added. Unlabeled VEGF (R&D Systems) was used as a negative control. Following three washes with ice-cold PBS containing 0.1% BSA, cells were lysed with PBS containing 1% Triton X-100 (Sigma), and the counts per minute of the released 125I-gB(1-23)TM, 125I-gB(1-23)TM-RGA, or 125I-VEGF-C were measured in a γ counter (Beckman Gamma 5500). The percent binding of the 125I-labeled proteins was then calculated.

Migration Assay—Migration assays were performed in triplicate using 24-well transwell plates with 5-μm pore filters (Costar, Boston, MA). The lower side of each filter was coated with 25 μg/ml fibronectin (Invitrogen) for 2 h at room temperature. The filters were blocked with PBS-permeable blocking reagent for 1 h at 22° C and then rinsed with migration medium (RPMI 1640 with 0.5% BSA) and the supernatant was aspirated immediately before loading the cells. Cells (~80% confluent) were harvested by releasing them from flasks with 5 mM EDTA. Cells were washed twice with 1× PBS and one time with migration medium, pre-incubated with anti-α3 and anti-VEGF-3 (hF4-3C5) or with their isotype controls for 30 min at 4 °C, and then loaded into the upper chambers of the inserts (0.1 ml, 2 × 10^5 cells/ml). The inserts were transferred to another well containing 650 μl of migration medium in the absence or presence of gB(1-23)TM, gB(1-23)TM-RGA, or VEGF-C. The plates were incubated at 37 °C in 5% CO₂ and 100% humidity for 3 h. Non-migrated cells were removed from the upper chambers by wiping the upper surface with a cotton tip applicator, and the migrated cells on the membrane were fixed with 4% paraformaldehyde solution, and immunoperoxidase staining was performed with anti-LANA-1 (ORF-73; Advanced Biotechnology Inc., Columbia, MD) antibody by using the VECTASTAIN® ABC system and the DAB substrate kit (Vector Laboratories). Brown staining appeared formed with anti-LANA-1 (ORF-73; Advanced Biotechnology Inc., Columbia, MD) antibody by using the VECTASTAIN® ABC system and the DAB substrate kit (Vector Laboratories). Brown staining appeared.

Viral Infection, Flow Cytometry Analysis, and Immunoperoxidase Staining—Sixty percent confluent cells were either mock-infected or infected with GFP-KSHV in 24-well plates at 37 °C for 3 h and then washed and incubated at 37 °C for 3 days with growth medium. KSHV infections were performed at a multiplicity of infection of 5–6 KSHV copies per cell. Green fluorescent cells were counted under a fluorescent microscope (Nikon Diaphot 300; 200× magnification) or detected by 5 mM EDTA in PBS and analyzed on a FACScan™ flow cytometer. After green fluorescent cells were counted, cells were then fixed with 4% paraformaldehyde solution, and immunoperoxidase staining was performed with anti-LANA-1 (ORF-73; Advanced Biotechnology Inc., Columbia, MD) antibody by using the VECTASTAIN® ABC system and the DAB substrate kit (Vector Laboratories). Brown staining appeared in the nuclei of KSHV-infected cells.

Cell Transformation Assay—HMVECs were exposed to KSHV or mock controls and then cultured in the presence of hF4-3C5 (1 μg/ml) or antibody control in soft agar medium for 21 days following the protocol provided by Chemicon International Inc. After this incubation period, colonies were picked and morphologically using cell-staining solution.

Cytokine ELISA—The supernatant was collected and assayed for VEGF-C and VEGF-D production by using the VEGF-C ELISA kit (ImmuNo-Biological Laboratories, Inc., Minneapolis, MN) and the human VEGF-D immunosay kit (R&D Systems) according to the manufacturer’s manual.

Data Presentation and Analysis—Each experiment has been repeated at least three times, and representative plots, images, or graphs are shown in Figs. 1–7. Statistical significance was determined using the analysis of variance test (p < 0.05).

RESULTS

The gB-induced Activation of VEGF-3 Is Inhibited by Blocking VEGF-3 or Integrin α₅β₁—Engagement of integrins by extracellular matrix proteins can induce the activation of growth factor receptors in the absence of their cognate ligands (47–49) and thereby modulate a variety of cell functions including migration and growth. We previously observed significant VEGF-3 phosphorylation in model 293 cells transfected with VEGF-3 and in HMVECs after adhesion to fibronectin (35, 36). Three major VEGF-3 isoforms were detected in HMVECs as described (50). These results demonstrated that ligation of the β₁ integrin can induce the tyrosine phosphorylation of VEGF-3. KSHV gB can bind to integrin α₅β₁ through its unique RGD motif (14, 17). We thus asked if KSHV gB could mimic fibronectin and result in the activation of VEGF-3.

The KSHV-gB ORF is 845-amino acids-long with a signal sequence (amino acids 1 to 23), a transmembrane domain (amino acids 710 to 729), and 13 N-glycosylation sites. The gB(1-23)TM encoding amino acids 1 to 702 is a recombinant wild-type gB without the transmembrane and cytoplasmic domains. The gB(1-23)TM-RGA is a recombinant mutant gB with a single amino acid mutation (RGD to RGA) (14, 17, 40). Similarly to the FAK activation described previously (14, 40), we found that an ~15 min treatment with 10 nM of gB(1-23)TM maximally activated VEGF-3. No significant activation was seen after treatment with the mutant gB(1-23)TM-RGA (data not shown).

We addressed the specificity of our observation by using an anti-VEGF-3 monoclonal antibody, hF4-3C5 (ImClone Systems), that antagonizes the activation of VEGF-3 by its cognate ligand. This antibody inhibits VEGF-C-mediated VEGF-3 signaling and its related functions such as angiogenesis (51). Treatment with the hF4-3C5 antibody inhibited receptor activation by KSHV gB and VEGF-C. The KSHV gB protein induced a modest level of VEGF-3 phosphorylation as compared with VEGF-C, the natural ligand of VEGFR-3 (Fig. 1A, top). Similarly, a lower level of VEGF-3 activation by fibronectin as compared with VEGF-C was observed in prior studies (35, 36). Likewise, modest stimulation of receptor tyrosine kinases by other viral proteins, as compared with their natural ligands, has been observed in T cells and endothelial cells (52–54). However, a significantly enhanced association of VEGF-3-RG and α₅β₁ was observed in cells stimulated with gB (Fig. 1A, middle). As expected, VEGF-C (the native ligand) did not cause such an association. These results suggest that the interaction of VEGF-3 and integrin α₅β₁ may be part of a unique KSHV gB-mediated signaling pathway.

Recently, the EGFR has been identified as a potential cellular attachment and signaling co-receptor for the human cytomegalovirus (11). KSHV and human cytomegalovirus both contain similar and conserved disintegrin-like domains in their envelope glycoproteins. Moreover, dividing endothelial cells have been reported to express the EGFR (55–57), and endothelial cells express EGFR when exposed to epidermal growth factor (58). Transactivation of EGFR by integrins has also been reported previously (48, 60). To investigate whether the EGFR signaling pathway is activated in the endothelium, we examined the tyrosine phosphorylation of EGFR upon stimulation with KSHV gB or its cognate ligand, the epidermal growth factor (EGF) (as a control). We did not observe KSHV gB-mediated activation of EGFR (data not shown). These results indicate that EGFR may not be involved in KSHV-mediated signaling.

To further confirm that the gB-stimulated tyrosine phospo-
by blocking of VEGFR-3 or integrin α1. HMVECs were pretreated with the anti-VEGFR-3 antibody hF4-3C5 (1 μg/ml) or its isotype control at 4 °C for 30 min. B, HMVECs were pretreated with an anti-α3 antibody (15 μg/ml) or its isotype control at 4 °C for 30 min. Cells were then stimulated with gB(TM) (10 nM), gB(TM)-RGA (10 nM), or the positive control VEGF-C (250 ng/ml) for 15 min. Total cell lysates were immunoprecipitated (IP) with the anti-VEGFR-3 antibody and developed by Western blotting (WB) with an anti-phosphotyrosine (anti-pTyr) antibody. After stripping, the blot was reprobed with an anti-αβ integrin antibody as indicated. The blot was probed again with anti-VEGFR-3 antibody to assure uniformity in the protein loading. Fold increase was calculated by densitometric scanning of the bands using ImageQuant software (Amersham Biosciences), according to the manufacturer’s instructions.

![Image 1](http://www.jbc.org/content/262/19/26219/F2.large.jpg)

**Fig. 1. VEGF-C stimulation was inhibited by transfection with a dominant negative mutant VEGFR-3, G857R-MT.** These results indicate that KSHV can bind to cell surface heparan sulfate-like molecules and integrins via its envelope-associated gB and gpK8.1A proteins (12, 14, 17). The heparin-binding domain and the RGD motif of gB are believed to be involved in this process. However, how KSHV binds to host cells has not been fully elucidated. To examine whether KSHV gB binds to VEGFR-3, we performed binding studies in 293/VEGFR-3 cells expressing high levels of VEGFR-3. Our results showed a specific binding of KSHV gB(TM) to VEGFR-3, because an excess of unlabeled VEGF-C, but not the control HIV-1 gp120 protein, competed with the 125I-labeled gB(TM) (Fig. 3A). The binding affinities of gB(TM) and gB(TM)-RGA were comparable, as described previously (14). Mutation of the RGD motif had no significant effect on KSHV gB binding to 293/VEGFR-3 cells or HMVECs (data not shown). VEGF-C competed with the 125I-labeled gB(TM) (Fig. 3A) and 125I-labeled gB(TM)-RGA (data not shown) in a similar dose-dependent manner. These results indicate that the natural VEGF-3 ligand, VEGF-C, can inhibit the binding of KSHV gB to VEGFR-3-expressing cells. To further confirm these observations, corollary binding displacement studies were performed. When recombinant KSHV gB(TM) or gB(TM)-RGA proteins were added together with 125I-VEGF-C, we found a dose-dependent inhibition of binding by each viral protein (Fig. 3B). There was no significant difference in inhibition between gB(TM) and gB(TM)-RGA. These results indicate that KSHV gB can compete with the natural ligand of VEGFR-3 to bind to VEGFR-3.

**KSHV Virions Activate VEGFR-3**—To study further whether KSHV gB can stimulate VEGFR-3, we asked whether intact KSHV virions obtained from 12-O-tetradecanoylphorbol-13-ac-
and the extent of specific $^{125}$I-VEGF-C binding to 293/VEGFR-3 cells, open squares, circles, or VEGF-C (open triangles) was determined. Unlabeled KSHV gB, or VEGF-C, VEGF-C (open squares), or open triangles, was added to 293/VEGFR-3 cells, and the extent of specific $^{125}$I-VEGF-C binding to VEGFR-3-expressing cells by KSHV gB. Unlabeled VEGF-C (open squares), KSHV gB-VEGF (open circles), or VEGF (open squares) was added to 293/VEGFR-3 cells, and the extent of specific $^{125}$I-VEGF-C binding to the cell surface was determined. Data shown are the means of three independent experiments, each performed in duplicate.

**FIG. 3.** KSHV gB competes with VEGFR-C to bind to VEGFR-3. A, inhibition of gB$\Delta$TM binding to VEGFR-3-expressing cells by VEGF-C. Unlabeled KSHV gB$\Delta$TM (open triangles), VEGF-C (open circles), or HIV-1 gp120 (open squares) was added to 293/VEGFR-3 cells, and the extent of specific $^{125}$I-gB$\Delta$TM binding to the cell surface was determined. B, inhibition of VEGF-C binding to VEGFR-3-expressing cells by KSHV gB. Unlabeled VEGF-C (open triangles), KSHV gB$\Delta$TM (open circles), KSHV gB$\Delta$TM-RGA (solid circles), or VEGF (open squares) was added to 293/VEGFR-3 cells, and the extent of specific $^{125}$I-VEGF-C binding to the cell surface was determined. Data shown are the means of three independent experiments, each performed in duplicate.

KSHV gB-mediated cell migration as suggested by the lack of effect with the gB$\Delta$TM-RGA mutant, we pretreated cells with anti-VEGFR-3 antibody, or VEGF-C, or VEGF (open squares) was added to 293/VEGFR-3 cells, and the extent of specific $^{125}$I-VEGF-C binding to the cell surface was determined. Data shown are the means of three independent experiments, each performed in duplicate.

**FIG. 4.** KSHV activates VEGFR-3. HMVECs were stimulated with purified GFP-KSHV produced in BCBL-1 cells or with its mock control produced in BJAB cells for the indicated times. Total cell lysates were immunoprecipitated (IP) with anti-VEGFR-3 antibody. The immunoprecipitates were resolved on SDS-PAGE and subjected to immunoblot analysis (WB, Western blot) with anti-phosphotyrosine antibody (top) and anti-VEGFR-3 antibody (bottom). Fold increase was calculated as described above.

It is very common for stimulation with extracellular matrix proteins to induce a secondary secretion of cytokines. Furthermore, transfection of KSHV GPCR alone or KSHV virus infection has been reported to up-regulate the expression of VEGF receptors and their ligands. To exclude the possibility that VEGFR-3 phosphorylation and related functions induced by KSHV gB were mediated through autocrine stimulation, e.g. that treatment induced its cognate VEGF ligands, the cells were incubated with different concentrations of gB$\Delta$TM (0.1–10 nM) for 24 h. We then assessed the treated cell supernatants by ELISA. The expression of VEGF-D was low and not increased by KSHV gB. VEGF-C was constitutively expressed at higher levels as compared with VEGF-D. Again, however, no difference in VEGF-C levels was observed between the non-treated and treated cells (data not shown). Thus, there was no evidence found that VEGF-C or VEGF-D induction accounted for the VEGFR-3 activation. These results indicate that the KSHV gB-induced phosphorylation of VEGFR-3 and related functions were not mediated by the release of cognate VEGF ligands.

To further investigate whether integrin $\alpha_3$-$\beta_3$ participates in gB-mediated cell migration as suggested by the lack of effect with the gB$\Delta$TM-RGA mutant, we pretreated cells with anti-$\alpha_3$ or its isotype control before conducting the migration assays. We observed that the gB-mediated migration was significantly inhibited after blocking with integrin $\alpha_3$ (Fig. 5B). This observation suggests that the integrin $\alpha_3$-$\beta_3$ can mediate KSHV gB-induced migration. We then studied the effects of KSHV gB$\Delta$TM on HMVEC proliferation using bromodeoxyuridine incorporation assays. We found a marked increase in proliferation upon gB$\Delta$TM treatment but failed to note a similar effect in the gB$\Delta$TM-RGA treatment group (Fig. 5C). Blockade of VEGFR-3 signaling with the hF4-3C5 antibody inhibited gB-mediated cell proliferation as well as VEGF-C-stimulated cell growth. Blocking of integrin $\alpha_3$-$\beta_3$ significantly inhibited cell growth in all groups (data not shown). These results suggest that the RGD motif is critical for both gB-mediated cell growth and migration and that VEGFR-3 is involved in these processes.
VEGFR-3 Enhances KSHV Infection

KSHV gB-induced cell migration and proliferation are inhibited by blocking of VEGFR-3 or integrin αv. A, KSHV gB-induced cell migration is blocked by anti-VEGFR-3 antibody. HMVECs were pretreated with the anti-VEGFR-3 antibody hF4-3C5 (2 μg/ml) or with isotype control for 30 min at 4 °C. Cell migration assays in response to gBΔTM (10 nm), gBΔTM-RGA (10 nm), or VEGF-C (250 ng/ml) were performed for 3 h. The migrated cells were stained and counted. The migration index was calculated by dividing the number of migrated cells in response to stimulus by the number of migrated cells in the control, which was treated by normal IgG and performed without growth factor. B, blockade of integrin αv abolishes KSHV gB-mediated migration. HMVECs were pretreated with anti-αv antibody (15 μg/ml) or its isotype control for 30 min at 4 °C. Cell migration assays in response to gBΔTM (10 nm), gBΔTM-RGA (10 nm), or VEGF-C (250 ng/ml) were performed for 3 h. The migrated cells were stained and counted. The migration index was calculated as described above. C, blockade of VEGFR-3 inhibits KSHV gB-induced endothelial cell proliferation. HMVECs were pretreated with the anti-VEGFR-3 antibody hF4-3C5 (2 μg/ml) or its isotype control for 30 min at 4 °C. Cells were then cultured in 96-well plates in EBM-2 medium with 0.5% BSA in the absence or presence of gBΔTM (10 nm), gBΔTM-RGA (10 nm), or VEGF-C (250 ng/ml) for 48 h. Cell proliferation was quantitated by the BiotrakTM cell proliferation ELISA system. The data represent the mean ± S.D. of three separate experiments performed in triplicate. *, p < 0.05; **, p < 0.01 as compared with the isotype control.

DISCUSSION

KSHV has been called a “molecular pirate” because it encodes several gene products that function like cytokines, chemokines, and G protein-coupled receptors that can co-opt key signaling pathways and cell functions (2, 69). Recent reports indicate that the gB protein of KSHV, which spans the viral membrane, can interact with the specific cell surface integrin αvβ3 that is expressed on target endothelial cells (17), thereby mimicking the extracellular matrix protein fibronectin. Moreover, the αvβ3 integrin appears to be important in virus entry, and ligation of the integrin by virus resulted in the activation of FAK as well as PI 3-kinase and MAPKs (14, 17, 40). Here, we report that KSHV gB can mimic fibronectin by inducing the activation of VEGFR-3 via integrin αvβ3 and by modulating cell proliferation and migration. Furthermore, VEGFR-3 signaling enhanced KSHV infection and participated in transformation of the KSHV-infected endothelium.

KS is characterized by a multifocal distribution, and its lesions contain multiple cell types including endothelial cells and infiltrating inflammatory cells. In KS tissues, KSHV DNA is present in a latent form in vascular endothelial and spindle cells that express the latency-associated KSHV genes LANA-1, cyclin D (vCYC-ORF-72), vFLIP (ORF-71), and K12. About 1–10% of infiltrating monocytic cells in KS lesions express KSHV lytic cycle proteins (70–73). Several studies demonstrate an increase in KSHV viral load before KS development and during KS clinical manifestations, suggesting reactivation and lytic KSHV replication (74–76). KS is an angiogenic tumor with a high expression level of VEGFRs. VEGFR-3 is increased in KS, and its ligand, VEGF-C, stimulates the migration and proliferation of KS cells in vitro (37, 38). Our studies suggest that KSHV gB may substitute for VEGF-C/D in activating VEGFR-3. We found that KSHV gB can trigger a migratory and
proliferative response in microvascular endothelial cells and that this migration depended on the activation of VEGFR-3. This finding about the viral protein gB mimicked what we observed with the cognate physiological ligand VEGF-C.

The KSHV envelope glycoprotein gB is distinct from Epstein-Barr virus and other herpesviruses as it contains an RGD domain and appears specifically to interact with the cell surface integrin $\alpha_\text{v}\beta_3$. The RGD motif serves as a major determinant in the interaction of a number of proteins with cell surface receptors of the integrin superfamily and mediates cell adhesion (77). It plays an important role in vascular development and remodeling via the interaction of extracellular matrix proteins and endothelial cells (78). The RGD motif has also been exploited by a number of pathogenic organisms besides KSHV in facilitating attachment to target cells (79). Moreover, the HIV-1 Tat protein, a putative contributor to HIV-related KS, also may regulate angiogenesis through its RGD motif (45). Prior studies suggested that KSHV gB binding to integrin $\alpha_\text{v}\beta_3$ through its RGD motif is important in the process of virus entry into target cells (17, 21). KSHV gB can mediate cell adhesion with cell surface integrin molecules via its RGD motif (14, 40). In our studies, the RGD motif was important for the gB-mediated activation of VEGFR-3 and its association with integrin $\alpha_\text{v}\beta_3$. Such interactions between KSHV gB and VEGFR-3 may play critical roles in KSHV infection of the endothelium as well as the KSHV-mediated transformation of infected endothelial cells.

It has been demonstrated that cross-talk between different receptor families occurs in angiogenesis. For example, VEGFR-2 association with the integrin $\alpha_\text{v}\beta_3$ is required for the full activation of VEGFR-2 (80). We found previously that the association of integrin $\beta_3$ with VEGFR-3 is critical for VEGFR-3-mediated survival signaling (35, 36). Here we show that the specific association of integrin $\alpha_\text{v}\beta_3$ with VEGFR-3 followed the KSHV gB treatment of cells. Furthermore, KSHV induces the expression of a series of genes and activates several signaling pathways that modify the status of target cells and may also create an appropriate intracellular environment to facilitate infection (44, 62). Our results indicate that activation of
VEGFR-3 and its downstream signaling pathways by gB enhanced microvascular endothelial cell migration and proliferation as well as colony formation in soft agar, an indicator of transformation. Such functional changes could contribute to the pathogenesis of the neoplasm.

Viruses entry is a complex and multistep process. Many viruses use multiple receptors that can be proteins, carbohydrates, or lipids (79, 81). In particular, KSHV entry and signal transduction. We found that the gB disintegrin-like domain is involved in VEGFR-3-mediated KSHV infection and transformation. Thus, the gB disintegrin-like domain could participate in KSHV entry and KSHV-mediated cell signaling. Thus, the gB disintegrin-like domain is involved in VEGFR-3-mediated KSHV infection and transformation. These data are consistent with previous studies, because a single amino acid substitution of the RGD motif may not alter the conformation of the gB protein (14). Our results and those of others imply that additional integrin functions for VEGFR-3 in KS pathogenesis and suggest that entry of KSHV may be facilitated by its binding to cell surface VEGFR-3. These technical issues require further examination. However, in this study, Polybrene was used during the attachment phase. Polybrene has been used to increase the infectivity of many viruses and to facilitate the delivery of nucleic acids for gene therapy, bypassing native receptors. These findings, along with our observations of changes in endothelial function, provide new insights into KSHV infection and transformation.
