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KSHV attachment and entry are dependent on αVβ3 integrin localized to specific cell surface microdomains and do not correlate with the presence of heparan sulfate

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ABSTRACT

Cellular receptors for KSHV attachment and entry were characterized using tyramide signal amplification (TSA)-enhanced confocal microscopy. Integrins αVβ3, αVβ5 and α3β1 were detected on essentially all the actin-based cell surface microdomains that initially bind KSHV, while the presence of CD98 and heparan sulfate (HS), the putative attachment receptor, was more variable. KSHV bound to the same cell surface microdomains with and without HS indicating that initial attachment of KSHV is not dependent on HS and that receptors other than HS can mediate attachment. A human salivary gland (HSG) epithelial line was identified, which lacks αVβ3 but expresses high levels of HS, α3β1 and other putative KSHV receptors. These cells were resistant to KSHV binding and infection. Reconstitution of cell surface αVβ3 rendered HSG cells highly susceptible to KSHV infection, demonstrating a critical role for αVβ3 in the binding and entry of KSHV that is not shared with other proposed receptors.

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Introduction

The binding of viruses to specific plasma membrane domains stimulates particle movements, receptor clustering, and protein signaling and activation, all of which ultimately triggers uptake of virus by endocytosis or direct transmission of virus to neighboring cells (Burckhardt and Greber, 2009). Plasma membrane domains containing viral receptors are often specialized actin-based structures generated by distinct filament assemblies, including sheet-like protrusions (lamellipodia and ruffled membranes) with branched actin filaments and finger-like protrusions (filopodia and microvilli) that contain parallel filament bundles (Chhabra and Higgs, 2007). Lamellipodia and filopodia adhere cells to the substratum and make contact with adjacent cells, while ruffled membranes and microvilli are non-adherent "free" structures on the apical cell surface.

Particle tracking studies have shown that viruses, such as murine leukemia virus, human immunodeficiency virus, avian leukemia virus (Lehmann et al., 2005; Sherer et al., 2007), human adenovirus (Helmuth et al., 2007; Burckhardt et al., 2011), herpes simplex virus type-1 (HSV-1) (Oh et al., 2010), and human papillomavirus type 16 (Schelhaas et al., 2008) move on filopodia and utilize actin/myosin II driven retrograde flow as a means of virion delivery to sites of particle uptake at connecting lamellipodial edges. Similarly, the spread of viruses from infected to non-infected cells involves virus transport along filopodial bridges contacting adjacent target cells (Lehmann et al., 2005; Sowinski et al., 2008; Gerdes and Carvalho, 2008; Sattentau, 2008). In some cases, virus attachment to filopodia is mediated by plasma membrane heparan sulfate (HS) glycosaminoglycans (Oh et al., 2010; Schelhaas et al., 2008; Liu and Thorp, 2002).

HSV-1 has been shown to bind preferentially to filopodia and not the cell body as a result of restricted distribution of HS on filopodial microdomains (Oh et al., 2010). In this case, HSV-1 attachment involved the interaction of envelope glycoprotein B with HS, while cell infection required the transport of HSV-1 along filopodia toward the cell body where viral glycoprotein D was recognized by a second cell surface entry receptor (Oh et al., 2010). Other herpesviruses, such as cytomegalovirus (Compton et al., 1993), varicella zoster (Jacquet et al., 1998), and Kaposi's sarcoma-associated herpesvirus (KSHV) (Akula et al., 2001b) have envelope glycoproteins with HS recognition motifs, however, it is not known

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if HS binding targets the viruses to specific membrane microdomains to initiate cell infection.

KSHV is the causative agent of Kaposi's sarcoma, pleural effusion lymphoma and multicentric Castleman's disease (Ganem, 2006). Several KSHV virion proteins bind HS, including glycoprotein B (Akula et al., 2001b), K8.1 (Birkmann et al., 2001; Wang et al., 2001), glycoprotein H (Hahn et al., 2009) and complement control protein (KCP) (Mark et al., 2006). Similar to HSV-1, KSHV virions may utilize actin retrograde flow to move to membrane domains containing secondary entry receptors, since perturbation of the actin cytoskeleton causes a reduction in KSHV infection (Greene and Gao, 2009; Sharma-Walia et al., 2004; Raghu et al., 2009). Several cell surface proteins have been implicated as KSHV entry receptors, including DC-SIGN (Rappocciolo et al., 2006, 2008), the cystine transporter (xCT) light chain (Kaleeba and Berger, 2006) – CD98 heavy chain heterodimer (Veettil et al., 2008), the ephrin receptor tyrosine kinase A2 (EphA2) (Hahn et al., 2012), syndecan (Hahn et al., 2009, 2012), and the integrins α3β1, αVβ3 and αVβ5 (Veettil et al., 2008; Akula et al., 2002; Garrigues et al., 2008).

We have shown that the αVβ3 integrin binds specifically to an "RGD" motif at the N-terminus of the KSHV envelope glycoprotein B (Garrigues et al., 2008). The HS binding domain of KSHV glycoprotein B is positioned downstream of the RGD motif (Akula et al., 2001a), suggesting that these motifs may cooperate in virus attachment and entry through cell surface domains containing both HS and αVβ3 integrin. However the distribution of HS and αVβ3 integrin relative to cell surface domains binding KSHV has not been established. Although KSHV infection is inhibited by enzymatic removal of cell surface HS, the inhibition is incomplete as nearly 40% of the cells still become infected at the highest enzyme concentrations tested (Akula et al., 2001a). This raises the possibility that KSHV attachment may not be dependent on HS interactions alone.

We recently identified cell surface structures initiating attachment of KSHV to target cells using gradient-purified DNP hapten-labeled KSHV virions (Garrigues et al., 2014). An ultra sensitive fluorescent enhancement using tyramide signal amplification (TSA) was developed to visualize DNP-KSHV bound to HT1080 fibrosarcoma cells, which are highly sensitive to KSHV infection (Garrigues et al., 2008). We determined that KSHV binds to specific cellular microdomains, including actin-based filopodia, lamellipodia, ruffled membranes, microvilli and intercellular junctions. KSHV binding domains were also identified on the dorsal cell surface and on a distinct supranuclear domain. Quantitation of bound virus revealed a significant increase on mitotic cells. Importantly, the binding of KSHV to the cell surface domains was inhibited by heparin treatment, confirming previous studies (Akula et al., 2001a). In the current study, we determined that essentially all of the cell surface domains binding KSHV contain the integrins αVβ3, αVβ5 and α3β1, which colocalized with bound DNP-KSHV. We determined that KSHV binds equally well to microdomains with and without cell surface HS, suggesting that receptors other than HS can function in virus attachment. Furthermore, a human salivary gland epithelial cell line that highly expresses all of the putative non-lymphoid KSHV receptor proteins except αVβ3 was resistant to KSHV infection until cell surface αVβ3 was reconstituted by transient expression of β3 integrin. Our findings provide further support for a critical role of αVβ3 integrin in virus binding and entry that is not shared by the other putative KSHV receptors, including HS, integrins α3β1 and αVβ5, EphA2 and xCT/CD98.

Results

KSHV colocalizes with αVβ3 integrin on cell surface binding domains

Using DNP-labeled gradient-purified KSHV virions and ultrasensitive TSA fluorescence amplification, we have previously identified KSHV attachment sites on the surface of highly infectable HT1080 fibrosarcoma cells (Garrigues et al., 2014). The DNP-labeled KSHV was highly infectious, maintained expression of envelope-associated glycoproteins and showed minimal evidence of aggregation. The HT1080 cells were fixed with paraformaldehyde to prevent virus-induced receptor modulation, thus providing a system to capture the initial interactions of the virions with existing cell surface binding sites. These studies revealed that TSA-enhanced fluorescence was 500 fold more sensitive than typical FITC-based techniques, enabling a detailed and accurate localization of DNP-labeled KSHV on the cell surface.

Previously, we also showed that KSHV virions bind specifically to affinity-purified αVβ3 integrin heterodimer in the absence of other cellular proteins (Garrigues et al., 2008). We therefore examined whether the KSHV cell surface binding domains contain αVβ3 using gradient purified DNP-KSHV virions and monoclonal antibodies recognizing the β3 integrin with confocal fluorescence microscopy. Furthermore, we developed a technique for sequential TSA fluorescence enhancement to allow simultaneous detection of antibodies reacting with DNP-KSHV and αVβ3 using tyramides coupled to different Alexa fluors.

Initially, we tested the specificity of sequential TSA enhancement steps using cells incubated with DNP-mock virus control and mouse anti-β3 integrin or cells incubated with DNP-KSHV and non-immune mouse IgG control. First, HT1080 cells were fixed with paraformaldehyde and incubated with a mixture of mouse anti-β3 antibody and DNP-mock virus control, as described in "Materials and methods" section. The cells were washed, fixed again, and incubated with goat anti-mouse IgG G20 gold conjugate followed by HRP-coupled donkey anti-goat IgG and TSA 488. Due to the particle size, the 20 nm gold conjugate is excluded from the intracellular compartment and only cell surface bound anti-β3 antibody is detected via the TSA 488 fluorescence. Residual peroxidase activity was blocked with 1% H_2O_2. Non-specific binding of DNP from the mock virus control was evaluated using monoclonal rat anti-DNP followed by goat anti-rat IgG coupled to HRP and TSA 647. Strong TSA 488 enhanced β3 integrin staining was detected on specific cell surface domains (Fig. 1A). No TSA 647 signal was observed from the mock virus control. The histogram shows the quantitation of detected fluorescence along the dotted arrow.

The converse control experiment was performed using DNP-KSHV and non-immune mouse IgG following the same sequential TSA enhancement protocol described above. Strong staining of DNP-KSHV was detected on distinct cell surface domains, including intercellular junctions, isolated microdomains on the dorsal cell surface and a supranuclear domain (Fig. 1B), as shown previously (Garrigues et al., 2014). No TSA 488 signal was observed from the non-immune mouse IgG control (Fig. 1B). Thus, these control experiments neither showed any evidence of antibody cross-reactions of the species specific HRP-labeled secondary antibodies to the mouse and rat primary antibodies, nor was there non-specific binding of the non-immune mouse IgG or artifactual DNP-labeled material within the mock virus control. Finally, no artifactual enhancement of the reactants in the primary TSA step was induced by the secondary TSA step.

To determine whether DNP-KSHV binds to the cell surface domains containing αVβ3, HT1080 cells were fixed, incubated with both DNP-KSHV and mouse anti-β3 integrin antibody, using the sequential TSA enhancement steps described above. An overview micrograph (Fig. 2A) of the confocal analysis of the β3 integrin (green) and bound DNP-KSHV (red) fluorescence revealed a high level of colocalized pixels (yellow/orange) on specific cellular domains, including actin-based fibrillar structures (double arrow), lamellipodia and ruffled membranes (open arrows). In addition, colocalized pixels were detected in isolated

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