The Tetraspanin Protein CD9 Modulates Infection with Human Herpesvirus 6A and 6B in a CD46-Dependent Manner

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ABSTRACT Tetraspanins are four-span transmembrane proteins that organize the membrane by forming tetraspanin-enriched microdomains. These have been shown to be important for virus entry. The human herpesvirus-6A (HHV-6A) receptor CD46 is known to form complexes with the tetraspanin CD9 and β 1-integrins; however, the significance of this for HHV-6A infection remains unexplored. Using a genetic approach, we demonstrate that knockout of CD46 abolishes binding to and infection of SupT1 cells by both HHV-6A and HHV-6B, establishing CD46 as a necessary receptor for productive infection of these cells. Knockout of CD9 in SupT1 cells had no effect on binding of either virus to the cell surface, but it reduced expression of immediate early transcripts to between 25 and 60%, compared with wild-type cells. Although HHV-6B required CD46 for infection of SupT1, infection of Molt3 cells was independent of CD46 expression. Conversely, the absence of CD9 expression promoted infection of Molt3 cells with HHV-6B, indicating a negative role of CD9 for CD46-independent infection. Taken together, these data demonstrate that CD9 modulates infection with HHV-6A/B by promoting CD46-dependent infection and impairing CD46-independent infection. This also suggests that HHV-6A is strictly dependent on CD46 for entry, although other proteins, like CD9, may enhance the infection, whereas HHV-6B is more promiscuous and may use CD134, as demonstrated by others, CD46 in SupT1 cells, and a novel unidentified receptor in Molt3 cells.

IMPORTANCE The mechanisms of entry of HHV-6A and HHV-6B into host cells are of significance in order to develop novel drugs that may inhibit infection. To elucidate the contributions of the membrane proteins CD9 and CD46, we employed a genetic approach that eliminated these molecules from the host cell. This demonstrated that CD46 is critical for infection by HHV-6A, whereas infection by HHV-6B appeared to be more promiscuous. The infection of a T-cell line in the absence of CD46 and CD134 strongly suggests that an additional receptor for HHV-6B entry exists. Moreover, elimination of CD9 and subsequent reconstitution experiments demonstrated that CD9 promoted infection with HHV-6A and HHV-6B mediated by CD46 but inhibited infection with HHV-6B that occurred independent of CD46. Together, this demonstrated a CD46-dependent role of CD9 during infection with HHV-6A and HHV-6B and emphasized that HHV-6B may employ different entry mechanisms in various cells.

KEYWORDS human herpesvirus 6A, human herpesvirus 6B, CD9, CD46, tetraspanin, HHV-6A, HHV-6B

Human herpesvirus-6A (HHV-6A) and HHV-6B are classified as separate species belonging to the *Roseolovirus* genus within the *Betaherpesvirinae* subfamily (1). They have nucleotide similarities of approximately 90% suggesting a close evolutionary relationship. The primary *in vivo* tropism appears to be T cells for both viruses (2),

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although other cell types may also be infected. Whereas more than 90% of individuals in the Western world have had the childhood disease exanthema subitum and are seropositive for HHV-6B prior to their second year of life, less is known about the epidemiology of HHV-6A infections, mainly due to a lack of serological tests that reliably discriminate between the two viruses.

Santoro and colleagues identified CD46 as the receptor mediating entry of both HHV-6A and HHV-6B (3). CD46 is a complement-inactivating protein in the innate immune system and has more recently also been implicated in the adaptive immune system as a costimulatory protein important for the generation of anti-inflammatory interleukin 10 (IL-10)-secreting T cells (4). A number of pathogens have been shown to use CD46 as a receptor for entry into the host cell. Although HHV-6A and HHV-6B were found to make use of CD46, certain T-cell lines were found to be nonpermissive to HHV-6B infection despite surface expression of CD46 (3), which suggested that CD46 itself might not be sufficient to mediate entry of HHV-6B. In subsequent seminal work, HHV-6A was shown to bind to short consensus repeat 2 (SCR2) and SCR3 of CD46 (5, 6), through expression of a heterotetrameric complex of gH, gL, gQ1, and gQ2 (7, 8), which established a physical interaction between HHV-6A and CD46. A similar complex from HHV-6B was unable to pull down CD46 *in vitro* (9), and later CD134, a membrane protein from the tumor necrosis factor receptor superfamily (TNFRSF), was described as the main receptor for HHV-6B strains KYO, HST, and Z29 (10). CD134 is not found on resting T cells or resting memory T cells but is induced upon activation (reviewed in reference 11), potentially explaining the fact that activation of T cells favors infection. Nevertheless, certain cell types may be infected by HHV-6B even in the absence of, or at least with extremely low levels of, CD134 (12), suggesting that HHV-6B might be more promiscuous in selection of an entry receptor than HHV-6A. Several isoforms can be expressed from the CD46 gene, but whether these are important for HHV-6A and HHV-6B infection remains to be clarified, since different CD46 isoform patterns in T-cell lines were found to correlate with susceptibility to infection by HHV-6B strain PL1 (12).

The viral entry machinery not only depends on the presence of the proper entry receptor but also involves additional cellular membrane components. Tetraspanins are a family of small four-span transmembrane proteins that are involved in anchoring multiple proteins to the cell membranes, associating with one another or different interaction partners (integrins, immunoglobulins, proteases, and cell-specific receptors) in complex networks, so-called tetraspanin-enriched microdomains (13, 14). Tetraspanins are thought to function as plasma membrane master organizers, playing functional roles in cell migration, proliferation, signal transduction, intracellular trafficking, and virus entry and exit (14, 15). At least in epithelial cells, CD46 has been described to assemble in a molecular complex with β 1-integrins and the tetraspanin protein CD9 (16). Although more direct evidence is still lacking, pulldown experiments following cross-linking of surface molecules indicate that CD46 interacts directly with β 1-integrins and indirectly with the tetraspanin protein CD9. In T cells, CD9 may work with the β 1-integrin to mediate signaling in the immunological synapse (17). Although CD9 may exert different functions depending on the cell type and the associated molecular complexes, it is known to be involved in the process of fusion. Mice that were deficient in CD9 had impaired fertilization due to deficient sperm-egg fusion (18–20). Also, CD9 is involved in canine distemper virus uptake, syncytium formation, and production of progeny virus (21), in the entry mechanism of coronavirus (22, 23), and in the susceptibility to measles virus in differentiated human macrophages (24). Despite their expression of CD46, monocytes did not support the replication of measles virus, but maturation to macrophages and the concomitant formation of a complex between CD46, β 1-integrins, CD9, and the tyrosine phosphatase SHP-1 allowed the infection/replication of the virus (24). In contrast, CD9 has also been shown to prevent the syncytia formed by fusion of mononuclear phagocytes (25).

Given the potential association of CD9 with a complex of β 1-integrins and CD46, as well as its potential role during fusion, we asked whether CD9 may modulate CD46-

FIG 1 Susceptibility of T-cell lines to infection with HHV-6B_{Z29} does not correlate with CD46 or CD134 expression. (A and B) The T-cell lines SupT1, Molt3, HSB2, and Peer were incubated with HHV-6B_{Z29} for 24 h, and the level of infection was measured as the relative expression of viral E mRNA transcript U7 (A) and viral L mRNA transcript U23 (B), using real-time PCR. The data represent the $2^{-\Delta CT}$, with PPIB as the reference gene, from at least three independent experiments. (C) Flow cytometry analyses of CD46 and CD134 surface expression were performed. The histograms represent the fluorescence intensity of CD46-PE or CD134-FITC staining, in comparison with an IgG1 isotype control. As a positive control for CD134 staining, we included a CD134-expressing MyLa cell line (dashed line). Data are representative of at least three independent experiments.

dependent infection of T cells with HHV-6A and HHV-6B. To address this, we established CRISPR-Cas9 knockout T-cell lines deficient in CD46 and CD9, respectively. These genetically modified T cells made it possible to investigate the role of CD9 during HHV-6A and HHV-6B binding, entry, and establishment of a productive infection. We propose that CD9 contributes to the CD46-dependent infection mechanism of HHV-6A and HHV-6B, possibly as part of a tetraspanin-enriched microdomain.

RESULTS

T-cell lines differ in susceptibility to infection with HHV-6B_{Z29}. Previous studies demonstrated the involvement of both CD134 and CD46 in the infection with HHV-6B (10, 12). Those studies also indicated that the tropism of HHV-6B is not as straightforward, since the presence of these surface receptors alone was not sufficient to mediate HHV-6B strain PL1 (HHV-6B_{PL1}) entry (12). One explanation for this finding may be the use of different viral strains in the separate studies. In the present study, we used HHV-6B strain Z29 (HHV-6B_{Z29}) to investigate the susceptibility of different T-cell lines.

The level of infection was characterized by analyzing the amount of viral mRNA 24 h postinfection (hpi), assessing the early (E) transcript U7 and the late (L) transcript U23 using real-time quantitative PCR (qPCR) (Fig. 1A and B). SupT1 and Molt3 T-cell lines infected with HHV-6B_{Z29} expressed U7 and U23, in line with previous findings obtained with strain PL1 (HHV-6B_{PL1}) (12), whereas HSB2 and Peer T-cell lines did not express viral transcripts, despite comparable CD46 expression in the four cell lines (Fig. 1C). None of the cell lines expressed detectable levels of CD134, except Molt3, which had a few cells with low levels of CD134, whereas the MyLa T-cell line was highly positive for CD134 expression (Fig. 1C).

CD46 is necessary for infection with HHV-6A. Accumulating evidence suggests that CD46 is an important receptor for infection of human T cells with HHV-6A, but formal testing of this by genetic knockout of CD46 has to our knowledge never been performed. To demonstrate the requirement of CD46 for HHV-6A infection, CD46 was knocked out in SupT1 and HSB2 T-cell lines (SupT1^{ΔCD46} and HSB2^{ΔCD46}) using the CRISPR-Cas9 system.

The functionality of the generated knockout cells was validated by reintroducing CD46 into the SupT1^{ΔCD46} cell line. Using lentiviral transduction followed by puromycin selection, a stable cell line expressing the BC1 isoform of CD46 (SupT1^{BC1}) was obtained. The reconstitution of CD46 on the cell surface was confirmed by flow

FIG 2 Genetic knockout establishes CD46 as necessary for HHV-6A_{GS} infection of SupT1 and HSB2. (A) Flow cytometry analysis of CD46 surface expression on the SupT1 cell line after genetic knockout of CD46 (SupT1^{ΔCD46}) and CD46 reconstitution (SupT1^{BC1}) into SupT1^{ΔCD46}. The isotype control is shown in gray. (B) Flow cytometry analysis of gp60/110 adsorption to the cell surface, determined at 2 hpi with HHV-6A_{GS}. The level of gp60/110 was compared to that of a mock-treated control (shown in gray). For better comparison, the histograms were normalized, i.e., the maximum values were set to 100%. Data are representative of at least three independent experiments. (C) Necessity of CD46 for infection with HHV-6A_{GS}. The T-cell lines were incubated with HHV-6A_{GS} for 24 h. The level of infection was characterized by the relative expression of the viral E transcript U7 and the L transcript U23 using real-time PCR. Data represent the 2^{-ΔCT}, with PPIB as the reference gene, from at least three independent experiments. (D to F) Same as in panels A to C but for HSB2 cell lines.

cytometry analysis, demonstrating a higher expression level, compared with SupT1^{wt} (Fig. 2A).

The role of CD46 in HHV-6A binding was assessed by measuring the adsorption of viral glycoprotein gp60/110 to the cell surface of the generated SupT1^{ΔCD46} and SupT1^{BC1} cells 2 h after addition of HHV-6A_{GS} virus stock (Fig. 2B). Consistent with the known function of CD46 as an HHV-6A receptor, deletion of CD46 from the surface of SupT1 nearly abolished binding of HHV-6A_{GS} to the cell surface. The lost binding capacity was recovered in the reconstituted SupT1^{BC1} variant. In fact, the level of gp60/110 adsorption to the surface of SupT1^{BC1} was elevated, compared to the wild-type SupT1, consistent with the increased expression level of CD46 in the reconstituted SupT1^{BC1} form.

To further confirm the necessity of CD46 for infection by HHV-6A_{GS}, we measured the level of the viral mRNA transcripts U7 and U23 at 24 hpi (Fig. 2C). In the absence of CD46, viral transcripts were not detected following exposure to HHV-6A_{GS}, whereas the presence of viral transcripts was fully recovered upon CD46 reconstitution (SupT1^{BC1}). In fact, the level of transcription was higher than in wild-type cells, in line with the increased expression level of CD46 in the reconstituted SupT1^{BC1} cell line. A similar experimental setup was performed with the generated HSB2^{ΔCD46} cell line, demonstrating the same dependency of CD46 expression for both HHV-6A_{GS} binding and productive infection in the HSB2 cell line (Fig. 2D to F).

FIG 3 Genetic knockout of CD46 indicates promiscuity in HHV-6B_{Z29} infection of T-cell lines. The cells were exposed to HHV-6B_{Z29} in parallel setups. (A and B) The binding of HHV-6B_{Z29} to the separate cell lines was determined by assessing the cell surface adsorption of gp60/110 by flow cytometry at 2 hpi. The level of gp60/110 was compared with that of cells incubated with medium alone (mock) (shown in gray). For better comparison, the histograms were normalized, i.e., the maximum values were set to 100%. Data are representative of at least three independent experiments. (C and D) The level of infection was characterized at 6 hpi and 24 hpi by relative expression of the viral IE transcripts U86 and U81, the E transcript U7, and the L transcript U23 using real-time PCR. Data represent $2^{-\Delta\Delta CT}$ (fold change relative to the respective wild-type strain).

Knockout of CD46 indicates promiscuity in HHV-6B infection of T cells. To evaluate the role of CD46 for HHV-6B_{Z29} infection, we generated an additional CD46 knockout T-cell line in Molt3, designated Molt3^{ΔCD46}, using the same CRISPR approach as described above. The functional impact of CD46 removal on HHV-6B_{Z29} binding was assessed by measuring the adsorption of gp60/110 to the cell surface of SupT1^{ΔCD46} and Molt3^{ΔCD46} at 2 hpi (Fig. 3A and B). In contrast to the data obtained with HHV-6A_{GS} (Fig. 2), deletion of CD46 did not abolish binding of HHV-6B_{Z29} to the cell surface; indeed, the level was similar to the level of binding in wild-type cells.

The susceptibility of the generated SupT1^{ΔCD46} and Molt3^{ΔCD46} cells to HHV-6B_{Z29} infection was assessed by analyzing the level of immediate early (IE) transcripts U86 and U81 at 6 hpi and E and L transcripts U7 and U23 at 24 hpi. Importantly, the removal of CD46 from the cell surface affected the susceptibility of the SupT1 and Molt3 T-cell lines differently (Fig. 3C and D). Whereas viral transcripts were absent in the SupT1^{ΔCD46} cells, the relative expression level in Molt3^{ΔCD46} was similar to the level measured in Molt3^{wt}. This indicates that CD46 is required for infection of HHV-6B_{Z29} in SupT1, whereas it is dispensable in Molt3. Since CD134 could barely be detected on the Molt3 cells, these data strongly indicate the existence of another receptor involved in HHV-6B_{Z29} entry.

CD9 expression correlates with HHV-6B susceptibility in T-cell lines. CD46 may engage in a molecular complex with β 1-integrin (CD29) and the tetraspanin protein CD9 (16), which has been shown to contribute to infection by measles virus (24). Therefore, we wondered whether the expression levels of CD9 and CD29 differed on the surface of T-cell lines with different susceptibility to HHV-6B_{Z29}.

Flow cytometry analysis demonstrated that only the HHV-6B_{Z29}-permissive T-cell lines SupT1 and Molt3 expressed CD9, whereas the HHV-6B_{Z29}-nonpermissive T-cell lines HSB2 and Peer were negative for CD9 (Fig. 4, upper). Conversely, CD29 was expressed on all four T-cell lines (Fig. 4, lower). This prompted us to examine whether CD9 may represent a hitherto unknown HHV-6B entry cofactor.

FIG 4 Expression of the CD46-associated molecule CD9 correlates with HHV-6B susceptibility in T-cell lines. The T-cell lines SupT1, Molt3, HSB2, and Peer were investigated for surface expression of CD9 and CD29 by flow cytometry. The histograms show fluorescence intensity of CD9-PE or CD29-FITC staining, compared with the respective isotype control (shown in gray). For better comparison, the histograms were normalized, i.e., the maximum values were set to 100%. Data are representative of at least two independent experiments.

CD9 promotes infection of HHV-6A and HHV-6B. To further study the role of CD9 in mediating entry, we generated CD9 knockout SupT1 and Molt3 cell lines, designated SupT1 Δ CD9 and Molt3 Δ CD9, respectively, using the CRISPR system. The CD9 knockout cells were negative for surface expression of CD9, as measured by flow cytometry (Fig. 5A and B). To control for possible off-target effects, we generated two CD9 knockout SupT1 cell lines with different genomic RNAs (gRNAs) targeting separate regions of the CD9 DNA sequence, giving rise to SupT1 Δ CD9.1 and SupT1 Δ CD9.2. We also reconstituted CD9 in the SupT1 Δ CD9.2 cell line, resulting in SupT1 Δ CD9.2^{ex}. Reconstituting the knockout cells brought CD9 expression back to a level slightly above that of the wild-type strain (Fig. 5A). Western blotting confirmed the absence and reconstitution of CD9 protein in the knockout cell lines (Fig. 5C).

The functional impact of CD9 on T-cell susceptibility to HHV-6A_{GS} and HHV-6B_{Z29} infection was assessed by measuring both cell surface binding (gp60/110 analysis) (Fig. 5D and E) and viral mRNA expression (Fig. 5F and G). The gp60/110 binding analyses demonstrated that HHV-6A_{GS} binding to SupT1 was only minimally affected by the absence of CD9, and reconstitution of CD9 in knockout cells (SupT1 Δ CD9.2^{ex}) resulted in binding similar to that of wild-type SupT1 (Fig. 5D). Binding analyses for HHV-6B_{Z29} demonstrated a reduction of approximately 25% in the absence of CD9, which was restored by reconstituting with CD9 (Fig. 5E). Noticeably, a similar dependency on CD9 for binding was not observed in Molt3 (Fig. 5E).

Despite a relatively modest reduction in binding of HHV-6A_{GS} to SupT1 Δ CD9.1 and SupT1 Δ CD9.2, the early infection, as measured by expression of IE viral mRNA, was reduced to only ~30% of the wild-type level, which was somewhat restored (approximately 75% of the wild-type level) by reconstituting CD9 (Fig. 5F). The effect on the level of mRNA was most prominent in the IE transcripts at an early stage of infection, indicating that, in SupT1, CD9 may be involved in the infection with HHV-6A_{GS}.

We next examined the role of CD9 for infection with HHV-6B_{Z29}. Deletion of CD9 from SupT1 led to a decrease in the level of HHV-6B_{Z29} U86 and U81 at 6 hpi to 25 to 60% of the wild-type cell level, whereas the reduction was found to be less pronounced for the E (U7) and L (U23) transcripts (Fig. 5G). Reconstitution of CD9 (SupT1 Δ CD9.2^{ex}) restored viral transcripts to a level above the wild-type level. This indicated that CD9 had a significant impact on early HHV-6B_{Z29} transcription in SupT1 cells. Deletion of CD9 in Molt3 cells had the opposite effect, giving rise to increased relative expression of viral transcripts, compared with the wild-type Molt3. A comparison of the HHV-6A_{GS} and HHV-6B_{Z29} susceptibility is provided in Table 1.

FIG 5 CD9 enhances infection with HHV-6A and HHV-6B in SupT1. (A and B) Flow cytometry analysis of genetic CD9 knockout (i.e., SupT1^{ΔCD9} and Molt3^{ΔCD9}) (A) and reconstitution (i.e., SupT1^{CD9ex}) (B). The histograms show normalized values, with the isotype control shown in gray. (C) Western blotting of CD9 in the generated cell lines. (D and E) Adsorption of HHV-6A_{GS} (D) and HHV-6B_{Z29} (E) to the cell surface of CD9 SupT1 variants, as determined by flow cytometry of the viral glycoprotein gp60/110 at 2 hpi, compared with a mock control. Data represent the change in median fluorescence intensity (Δ MFI) (i.e., $\text{MFI}_{\text{sample}} - \text{MFI}_{\text{mock}}$), shown as percentage of the respective wild-type level. (F and G) The levels of HHV-6A_{GS} infection (F) and HHV-6B_{Z29} infection (G) characterized at 6 hpi and 24 hpi by analysis of the relative expression of the viral IE transcripts U86 and U81 and the E and L transcripts U7 and U23 using real-time PCR. Data represent the fold change in expression levels relative to the respective wild-type (i.e., $2^{-\Delta\Delta C_T}$). *, $P < 0.05$; **, $P < 0.01$; ns, not significant (Mann-Whitney test, calculated based on the $\Delta\Delta C_T$ values).

CD9 contributes to the entry mechanism of HHV-6B in SupT1. Since our data indicated possible involvement of CD9 in the early entry process of HHV-6B_{Z29} in the SupT1 cells, a kinetic expression analysis of U86 was performed. A marked delay in the production of U86 was observed in the deletion mutant SupT1^{ΔCD9} at all time points

TABLE 1 HHV-6A_{GS} and HHV-6B_{Z29} susceptibility of cell lines

Cell line	HHV-6A _{GS}		HHV-6B _{Z29}	
	Binding ^a	Entry ^b	Binding ^a	Entry ^b
SupT1	Y	++(+)	Y	++
SupT1 ^{ΔCD46}	N	—	Y	—
SupT1 ^{ΔCD9}	Y	+	Y	+
HSB2	Y	++++	Y	—
HSB2 ^{ΔCD46}	N	—	Y	ND
HSB2 ^{CD9ex}	Y	++++(+)	Y	(+)
Molt3	Y ^c	+	Y	+++
Molt3 ^{ΔCD46}	ND	—	Y	+++
Molt3 ^{ΔCD9}	ND	+	Y	++++

^aDetection of gp60/110 on the cell surface (Y, yes; N, no; ND, not determined).

^bThe absence (—) and presence (+) of U86 viral transcripts (6 hpi) is graded from low (+) to high (++++).

^cPreviously published (12).

during the first 10 h (Fig. 6). Importantly, this reduction was reversed to wild-type levels upon reconstitution of CD9 (SupT1^{CD9ex}). In accordance with the results presented above, no viral expression was observed in SupT1^{ΔCD46}.

Ectopic expression of CD9 does not affect HHV-6B_{Z29} susceptibility of HSB2.

Since CD9 appeared to enhance infection in SupT1 and the nonpermissive cell lines HSB2 and Peer do not express CD9, we examined whether ectopic expression of CD9 conferred susceptibility to HHV-6B infection. Ectopic expression of CD9 in HSB2, designated HSB2^{CD9ex}, was achieved by lentiviral transduction. Expression was confirmed by flow cytometry and Western blotting (Fig. 7A and B).

Both HHV-6A_{GS} and HHV-6B_{Z29} were able to bind to HSB2, regardless of ectopic CD9 expression (Fig. 7C and D show comparisons between HSB2^{wt} and HSB2^{ΔCD46}). A productive infection was detected only with HHV-6A_{GS}, whereas HHV-6B_{Z29} infection was barely detectable in HSB2 (Table 1). Nevertheless, while the knockout of CD46 in HSB2 gave rise to a complete absence of HHV-6B_{Z29} transcripts, the ectopic CD9 expression (HSB2^{CD9ex}) appeared to lead to an increase in the level of viral transcripts, compared with HSB2^{wt} (Fig. 7E). This increase, however, still represents an extremely poor infection. Thus, ectopic expression of CD9 is not sufficient to convert HSB2 to a susceptible cell line for HHV-6B_{Z29} infection. However, we also saw a small increase in HHV-6A_{GS} viral transcript levels in the HSB2^{CD9ex} cell line (Table 1) and cannot rule out the possibility that CD9 did improve entry and that the infection with HHV-6B_{Z29} was stalled at an early step prior to transcription of the IE genes.

DISCUSSION

Therapeutics blocking viral entry have been efficient in the treatment of both hepatitis B virus and HIV infections. Thus, detailed understanding of the entry mechanisms of HHV-6A and HHV-6B may also point to new avenues for treatment of these infections. It has been known for decades that CD46 is a receptor for HHV-6A infection of T cells (3). We demonstrate here that, in the absence of CD46 in an otherwise permissive T cell, HHV-6A is virtually unable to establish infection. This strongly supports the idea that CD46 is a necessary receptor for HHV-6A, but whether it is also a sufficient receptor has yet to be determined, since CD46 may be part of a multiprotein complex that may be required for the viral entry process.

Using a genetic approach, we demonstrate that CD46 is indispensable for HHV-6B infection of SupT1 cells. Although a role for CD46 in the entry of HHV-6B was described by Lusso and colleagues (3), molecular work from Mori's group examined the mechanisms by which HHV-6A and HHV-6B might bind CD46. Important work identified a glycoprotein complex of gH, gL, gQ1, and gQ2 of HHV-6A, which coprecipitate CD46 in pulldown assays (7, 8). Although a homologous complex does exist in HHV-6B, it does not coprecipitate CD46 (9). This is not due to gH or gL, which can be switched between

FIG 6 CD9 promotes HHV-6B_{Z29} entry. The T-cell lines were incubated with HHV-6B_{Z29} for the indicated times. The level of infection was characterized by relative expression of the viral IE transcript U86 using real-time PCR. The experiment was performed in two independent setups, with similar results. The data represent the $2^{-\Delta CT}$, with PPIB as the reference gene, from one experiment.

HHV-6A and HHV-6B without losing CD46 binding, but rather is caused by the necessity for HHV-6A gQ1 and gQ2 within the gH-gL-gQ1-gQ2 complex for significant binding to CD46 (26).

Conversely, in agreement with Mori and colleagues, we observe that HHV-6B retains the ability to infect Molt3 cells in the absence of CD46 (Fig. 3), demonstrating that, in Molt3 cells, CD46 is not necessary for HHV-6B infection. Although Mori and colleagues described CD134 as a primary receptor for HHV-6B (10), our SupT1 and Molt3 cells expressed no or barely detectable levels of CD134, orders of magnitudes less than the T-cell line MyLa, which cannot be infected by HHV-6B. In our hands, SupT1 cells expressing CD46 and CD9, but not CD134, are susceptible to HHV-6B, whereas Mori and colleagues needed to express CD134 in SupT1 to allow HHV-6B infection (10).

FIG 7 Ectopic expression of CD9 in HSB2. We used lentiviral transduction to generate a CD9-expressing HSB2 cell line (HSB2^{CD9ex}). (A and B) The ectopic expression of CD9 was verified using flow cytometry analysis (A) and Western blotting (B). (C and D) To assess the binding of HHV-6A_{GS} and HHV-6B_{Z29}, we incubated the cells with the respective virus and investigated the cell surface adsorption of gp60/110 by flow cytometry at 2 hpi. The level of gp60/110 was compared with a mock control (shown in gray). For better comparison, we included HSB2^{ΔCD46} in this analysis. The histograms were normalized, i.e., the maximum values were set to 100%, and the respective wild-type level is included on the graphs as a dashed line. The graphs are representative of two to four independent experiments. (E) HHV-6B_{Z29} infection was assessed by analyzing the relative expression level of viral IE transcripts U86 and U81 at 6 hpi and E and L transcripts U7 and U23 at 24 hpi using real-time PCR. Data represent the fold change in expression levels relative to the wild-type SupT1 (i.e., $2^{-\Delta \Delta CT}$).

Greninger et al. demonstrated that, while both SupT1 and Molt3 could be infected by HHV-6B_{Z29}, more coding sequences were detected in Molt3 than in SupT1 (27). Moreover, different splicing forms of U79 were detected in the two cell types. In addition, they also identified differences among HHV-6B strains. Although the overall diversity between strains of HHV-6B was limited, geographical clustering could be detected. Specifically, differences in HHV-6B_{HST} and HHV-6B_{Z29} were detected in, for example, U12 and U27, which may account for certain differences in results when using these strains.

The incongruence between our data and those from Mori and colleagues may in part be explained by differences in the cells, as mentioned above. Also, the virus production systems differ, as we propagated HHV-6B_{Z29} in SupT1 cells, whereas Mori and colleagues used mitogen-stimulated cord blood mononuclear cells (10). According to the data from Greninger et al., differences in protein expression in the cell lines may also contribute to the different results (27). Importantly, we found a barely detectable level of CD134 on the surface of Molt3 cells, whereas Mori and colleagues found a comparably high CD134 expression level on Molt3 cells (10).

We emphasize that our data do not demonstrate that CD134 is not a receptor for HHV-6B; rather, they suggest that there may be one or more unidentified receptors. This would indicate that the cellular tropism of HHV-6B is much more promiscuous than that of HHV-6A. Besides differences in the cellular systems used to study HHV-6B, we speculate that the virus may exploit different receptors depending on the host cell, the cytokine environment (28), and possibly other factors. Thus, whereas HHV-6B may use CD134 in certain situations, we think that it also has the capacity to exploit other, yet unidentified, surface proteins to gain access to the host cell.

The architecture of the membrane is important for the entry of viruses, and several tetraspanins have been associated with viral entry (14). Studies in epithelial cells suggest a complex of CD46, β 1-integrins, and CD9, possibly by a direct interaction between CD46 and β 1-integrins and an indirect association with CD9 (16). Therefore, we speculated that CD9 might be involved in a complex with CD46 during infection with HHV-6A and HHV-6B. The molecular complexes containing CD46 remain poorly characterized in T cells, but clearly tetraspanins might also be involved in viral infections in these cells.

We examined the expression of CD9 in the four T-cell lines, two of which were efficiently infected by HHV-6B (SupT1 and Molt3) and two of which were uninfected by HHV-6B (HSB2 and Peer). Unexpectedly, the cell lines that were infected expressed high levels of CD9, whereas the cell lines that were not infected expressed no CD9. To further examine the correlation between CD9 and infection by HHV-6B, genetic knock-out of CD9 in SupT1 and Molt3 cells was generated. Although the absence of CD9 had only minor effects on the binding of HHV-6A to SupT1, the early infection by HHV-6A, as measured by expression of IE genes U86 and U81, was significantly impaired but was partially restored by ectopic expression of CD9. This suggests that CD9 may promote the infection with HHV-6A. We cannot rule out the possibility that CD9 is not a receptor *per se* but rather advances the infection by modifying the signal transduction, thereby allowing more efficient transcription of viral genes. Indeed, this seems to be a thematic strategy among viruses and has also been described for cytomegalovirus, a member of the *Betaherpervirinae* subfamily (29).

In the case of HHV-6B, the knockout of CD9 in SupT1 had little or no impact on binding, whereas early infection was significantly impaired. In contrast, HHV-6B binding to Molt3 appeared normal in the absence of CD9, and expression of IE genes was normal or even enhanced in these cells. This suggests that CD9 exerted different roles for HHV-6B infection of SupT1 (a CD46-dependent infection) and Molt3 (a CD46-independent infection). A potential negative effect exerted by CD9 on infection of Molt3 with HHV-6B, and consequently a better infection in the CD9 knockout cell, is perhaps mechanistically similar to an effect described for HIV infection of T cells, in which knockdown of CD9 enhanced the infection by HIV, whereas overexpression of CD9

FIG 8 Summary of HHV-6A and HHV-6B infection of HSB2, SupT1, and Molt3 T cells. HHV-6A is dependent on CD46 for infection of HSB2 and SupT1 cells. On the cell surface, a complex between CD46, β 1-integrin, and CD9, a hypothesized "X" molecule, and CD134 are shown. The latter is in parentheses since it was barely detected on the Molt3 cells. The presence of CD9 may positively advance the infection either by mechanisms at the entry level or by promoting early viral gene transcription. It is assumed that CD46 is sufficient for infection, although this has never been formally demonstrated. HHV-6B is dependent on CD46 for infection of SupT1 but not for infection of Molt3. The hypothesized "X" molecule is shown in Molt3 cells since these cells can be infected in the absence of CD46 and with little or no CD134, a molecule previously demonstrated to bind HHV-6B. The question marks next to the entry arrow for "X" and (CD134) indicate that, to date, none of these molecules has been shown to mediate entry of HHV-6B. An arrow from CD9 (or possibly the complex) indicates a positive effect on infection, whereas a blunt-end arrow indicates a negative effect, as shown for Molt3 cells.

inhibited the infection (30). It remains to be determined why CD9 in certain circumstances promotes infections and in other settings inhibits infections. We speculate that this may be related to the complexes that form with the attachment of the virus. Perhaps separate entry mechanisms may be modulated differently by CD9. Also, CD9 may affect postentry stages of infection, such as transcriptional factors or intracellular signaling, and thus modulate steps after entry.

Based on our data, we suggest that CD9 in complex with CD46 and possibly β 1-integrins exerts a positive effect on HHV-6A and HHV-6B entry or early postentry stages of infection (summarized in Fig. 8 and Table 1). Conversely, CD9 may inhibit HHV-6B infection that occurs independent of CD46, possibly by inhibiting a fusion between viral and cell membranes or by an effect on signal transduction. This is also in agreement with our data demonstrating that HHV-6A infection is further enhanced by overexpression of CD9 in HSB2 cells, which normally do not express CD9. It also indicates that expression of CD9 is not required for HHV-6A infection, since the CD9-negative T-cell line HSB2 is easily infected with HHV-6A. In SupT1, HHV-6B infection behaved similarly to HHV-6A infection, as the absence of CD46 abolished infection and the absence of CD9 made infection approximately one-half as efficient as that in wild-type cells. However, we cannot rule out the possibility that other tetraspanin proteins may fulfill the role of CD9 in its absence.

In conclusion, we have shown that CD9 promotes a CD46-dependent infection with HHV-6A and HHV-6B, possibly by participating in tetraspanin-enriched microdomains of importance for infection, whereas it may be inhibitory for CD46-independent infection of T cells by HHV-6B.

MATERIALS AND METHODS

Cell lines. We used the T-cell lines SupT1, Molt3, and HSB2 (kindly provided by the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH) and Peer and MyLa (kindly provided by S. Junker, Denmark). All of these cell lines are human cell lines. SupT1 (catalog number 100, contributed by D. Ablashi) was derived from an 8-year-old male patient with non-Hodgkin's T-cell lymphoma (31, 32). The Molt3 cell line (catalog number 12187, contributed by D. Ablashi) was established from cells isolated from a 19-year-old male patient with acute lymphoblastic leukemia (33). The HSB2 cell line (catalog number 497, contributed by Electro-Nucleonics, Inc.) was derived from the mononuclear cells of a

patient with acute lymphoblastic leukemia (34). The Peer cell line was derived from peripheral blood from a 4-year-old female patient with lymphoblastic leukemia (35). The MyLa cell line was established from a nonmalignant skin-homing T-cell line from a 82-year-old male patient with mycosis fungoides stage II (36). The cell lines were maintained in RPMI 1640 medium (Sigma-Aldrich, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich), 10 mM HEPES (Sigma-Aldrich), 2 mM glutamax (Gibco, USA), 100 U/ml penicillin, and 100 μ g/ml streptomycin. The cells were split 1:3 in fresh medium 2 or 3 times per week. All cells were cultured at 37°C in 5% CO₂.

CRISPR-Cas9-mediated genome editing. For the generation of the SupT1 Δ CD46 and HSB2 Δ CD46 cell lines, we used electroporation for delivery of a Cas9-single guide RNA (sgRNA) complex (RNP complex). We used a chemically modified synthetic sgRNA (3'-CAAUUGUGUCGUGCCAUUCG-5'; Synthego, USA) targeting exon 6 in CD46 (coding for SCR4) in combination with Cas9-3NLS (Integrated DNA Technology, USA). To form the RNP complex, we preincubated the sgRNA and the Cas9 protein in a 1:5 molar ratio prior to electroporation, following the recommendations of the manufacturer (Synthego). Electroporation was performed in medium containing 5 mM KCl, 15 mM MgCl₂, 120 mM Na₂HPO₄, and 50 mM mannitol (pH 7.2), using a 4D-Nucleofector and program CM-138 (Lonza, Switzerland). In general, the electroporation setup was based on the guidelines by Bak and colleagues, using 175,000 cells in a volume of 20 μ l in Lonza 4D Nucleocuvette strips (37). For isolation of the knockout cell population, we used fluorescence-activated cell sorting (FACS). The knockout efficiency was analyzed using flow cytometry and Western blotting.

For the generation of the Molt3 Δ CD46 cell line, and the Δ CD9 cell lines, we used an all-in-one-vector system combining a gRNA sequence targeting CD46 or CD9, respectively, and wild-type SpCas9 in the pLentiCRISPR v2 expression plasmid (purchased from Genscript, USA; gRNA sequences were designed by Zhang's laboratory [38]). The gRNA sequences used were as follows: gRNA-CD46, CAATTGTGTCGCTGCCATCG; gRNA-CD9.1, GAATCGGAGCCATAGTCCAA (Δ CD9.1); gRNA-CD9.2, GCGACATACCGCATAGTGGA (Δ CD9.2). For transfer of the pLentiCRISPR v2 expression plasmid into T-cell lines, we used lentiviral transduction. The transduced cells were selected using 2 μ g/ml puromycin (Gibco) and subjected to limiting dilution to obtain more homogeneous cell populations. To quantify the efficacy and identity of the induced indel mutations, we used TIDE (Tracking of Indels by Decomposition) analysis (39). The knockout efficiency was analyzed using flow cytometry and Western blotting.

Generation of reconstituted versions SupT1^{BC1} and SupT1^{CD9ex} and ectopic HSB2^{CD9}. For reconstitution of CD46^{BC1} and CD9 into the SupT1 Δ CD46 and SupT1 Δ CD9.2 cell lines and ectopic expression of CD9 in HSB2^{wt}, we used lentiviral delivery. Lentiviral vector constructs encoding CD46^{BC1} (pCCL-CD46^{BC1}) or CD9 (pCCL-CD9) were based on the pCCL configuration. pCCL-CD46^{BC1} was generated by combining a CD46^{BC1} PCR amplicon from pcDNA3.1(+)-CD46^{BC1} (GenBank accession number [NM_172351](#); amino acids 56 to 1531; GenScript) with a PCR product containing an internal ribosome entry site (IRES) element and a puromycin resistance gene (Puro), i.e., IRES-Puro, amplified from a pPBT/CMV-IRES-Puro vector (kindly provided by J. Giehm Mikkelsen, Aarhus University, Denmark). We used the NEBuilder HIFI DNA assembly cloning kit (New England Biolabs, USA) to assemble the generated CD46^{BC1} and IRES-Puro PCR products with the BamHI/XhoI-digested pCCL-WPS-PGK-Puro-WHV vector (kindly provided by Jacob Giehm Mikkelsen) (40), generating pCCL-CD46^{BC1}. The assembly of fragments was performed according to the manufacturer's protocol. The primers used for the amplification of PCR products are listed in Table 2. pCCL-CD9 was generated by replacing CD46^{BC1} with CD9 (GenBank accession number [NM_001769](#); GenScript), using BamHI and EcoRV.

Lentiviral production and transduction were performed as described below. Stable SupT1^{BC1}, SupT1^{CD9ex}, and HSB2^{CD9} cell lines were obtained using selection pressure with 2 μ g/ml or 0.5 μ g/ml puromycin (Gibco). Reconstitution and ectopic expression of CD46 and CD9 were confirmed by flow cytometry and Western blotting.

Production of lentivirus and lentiviral transduction. Lentiviral vectors were produced by cotransfecting HEK293T cells with packaging vectors using FuGENE HD transfection reagent (Promega Corp., USA). HEK293T cells were seeded in T150 culture flasks (8×10^6 cells/flask) in Dulbecco's modified Eagle's medium (DMEM), supplemented as described above, 24 h prior to transfection. Transfections were performed using pMD.2G (2 μ g), pRSV-REV (1 μ g), pMDLg/p-RRE (2 μ g), and the respective pCCL transfer vector (3 μ g). The conditioned culture medium was collected 48 h and 72 h posttransfection and filtered through a 450-nm filter. The lentiviral particles were concentrated using 20% sucrose gradient ultracentrifugation at 25,000 $\times$ g at 4°C for 2 h, followed by resuspension in 400 μ l phosphate-buffered saline (PBS) supplemented with 10% FBS (0°C) by applying three cycles of vortex-mixing (15 s) and incubation at 0°C for 2 min.

For transduction, 10^6 cells/well were seeded into 6-well plates 2 h in advance. Transduction was performed in the presence of 25 μ g/ml protamine sulfate (Sigma-Aldrich). The culture medium was replaced 24 h posttransduction. Selection was performed using 0.5 to 2 μ g/ml puromycin (Gibco).

Flow cytometry analysis and FACS. For flow cytometry, approximately 0.5×10^6 cells were washed in PBS supplemented with 2% FBS. The following antibodies were used: anti-CD46-phycoerythrin (PE) (clone MEM-258; Sigma-Aldrich), IgG1-PE isotype control (clone MOPC21; BD Biosciences, USA), anti-CD134-fluorescein isothiocyanate (FITC) (clone ACT35; BD Biosciences), IgG1-FITC isotype control (clone MOPC21; BD Biosciences), mouse monoclonal anti-gp60/110 (Millipore, USA), goat anti-mouse IgG-PE (Invitrogen, USA), anti-CD9-PE (clone SN4 C3-3A2; eBioscience, USA), and anti-CD29-FITC (clone TS2/16; eBioscience). We included the CD134-expressing cell line MyLa as a positive control for CD134 staining (12). For analysis of viability, we used LIVE/DEAD fixable Near-IR (1:100 dilution; Thermo Fisher Scientific, USA). Antibody staining was performed at 4°C for 30 min. The cells were subsequently washed with PBS supplemented with 2% FBS and were resuspended in 0.99% paraformaldehyde. The cells were analyzed

TABLE 2 Primers

Target and oligonucleotide ^a	Sequence (5' to 3')	Amplicon size (bp)	T _m ^b (°C)
Real-time PCR primers			
HHV-6A U81			
Fw	CGTTGTCAGGGGGGAAAAAT	108	64.7
Rev	GCGGCTTGGCCTTCGGTA		62.1
HHV-6A U86			
Fw	ATGGGCGTAGCGGCGTAA	119	63.3
Rev	CAACTACAAAAACAAGGACCGCAAGAA		63.3
HHV-6A U7 ^c			
Fw	CGCAAGCCCGGAGAACTGATT	338/233 ^d	65.1
Rev	AGACTTTTTCGGACTGCGGT		65.0
HHV-6A U23 ^c			
Fw	ATCACCTTGACCGTCGGAA	168	63.3
Rev	GCGAATTCTGAAACGTGGGG		61.8
HHV-6B U81			
Fw	TCAGCATCTACTCTTCGGCA	179	61.2
Rev	CATCGGTGGGTTTGCACAAA		61.2
HHV-6B U86			
Fw	AAAGGACTGGAGTCGAGCTGCA	204	65.6
Rev	ATGTCCAACATACCTTCCCCTCAAACCTT		65.1
HHV-6B U7 ^c			
Fw	ACGACAAACCTGCTGGTAGCG	291/200 ^d	65.0
Rev	GGATGTTGAATGGGGAGTTGCC		65.4
HHV-6B U23 ^c			
Fw	GAATGCCCCGACAAAATAATCCCG	252	65.5
Rev	GATGAGGCGCAGGATATTGAGACGT		65.9
PPIB ^c			
Fw	TGTGGTGTTTGGCAAAGT	278	57.7
Rev	TGGAATGTGAGGGGAGTG		58.4
Cloning primers			
pCCL-BamHI-CD46-IRES-Puro			
Fw	GGGCCTTTCGACCTCTAGCGGGATCCATCTACCATTGTTGCGTCCCATATCTGG		
Rev	TTACTTGACGAAGCCACATTGCAATATTAGCTAAGC		
IRES-Puro-XhoI-pCCL			
Fw	CAATGTGGCTTCGTACAAGTAAAGCATAGCGGCCG		
Rev	AGGTTGATTATCGGAATTCCTCGAGGGGGATCTTCAGGCACCGGGCTTGCG		

^aFw, forward primer; Rev, reverse primer.^bT_m, melting temperature, calculated using CLC Main Workbench 6 and 8.^cPrimer from reference 12.^dLength of amplified DNA or cDNA (from spliced RNA).

on a NovoCyte 3000 (405-, 488-, and 540-nm lasers; Agilent) or Novocyte Quanteon (405-, 488-, 561-, and 637-nm lasers; Agilent) flow cytometer, generally using 50,000 counts for acquisition. Debris and doublets were excluded using forward scatter-side scatter gating. Data processing was performed using FlowJo v10 (BD Biosciences).

For sorting of CRISPR-edited cells, a three-laser BD FACSARIA III instrument (375-, 488-, and 640 nm lasers; BD Biosciences) was used. Cells were stained with anti-CD46-PE antibody (clone MEM-258; Sigma-Aldrich), and the PE-negative cells were sorted from the PE-positive cells. Isolation of the CD46-negative population was verified using flow cytometry and Western blotting.

Cell lysis and Western blotting. For Western blotting, 10⁶ cells were lysed for 30 min in lysis buffer (Pierce IP lysis buffer; Thermo Fisher Scientific) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium fluoride, and complete mini protease inhibitor (Roche Diagnostics, USA), according to the manufacturer's recommendations. Protein concentrations of the lysates were determined by the Bradford method (Bio-Rad, Denmark), and 30 μg protein of each lysate was used for separation on an XT Criterion 12% gel with XT MOPS running buffer (Bio-Rad). The separated proteins were transferred to a Trans-Blot Turbo 0.2-μm polyvinylidene difluoride (PVDF) membrane using the Trans-Blot Turbo transfer system (Bio-Rad). The membrane was blocked with 5% skim milk (Sigma-Aldrich) in Tris-buffered saline (TBS) with 0.1% Tween 20 (Sigma-Aldrich). For detection, the following antibodies were used: mouse anti-CD46 (1:5,000, clone EPR4014; Abcam, UK), mouse anti-CD9 (1:1,000, clone MM2/57; Invitrogen), rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1,000, catalog number ab9485; Abcam), horseradish peroxidase (HRP)-conjugated polyclonal rabbit anti-mouse IgG (1:2,000; Dako, Denmark), and HRP-conjugated polyclonal swine anti-rabbit IgG (1:2,000; Dako). For detection of CD9, samples were run under nonreducing conditions. Membranes were developed with SuperSignal West Femto chemiluminescent substrate (Pierce, Thermo Fisher Scientific), using a ChemiDoc imaging system (Bio-Rad).

RNA purification and cDNA synthesis. Total RNA was isolated using NucleoSpin RNA columns (Macherey-Nagel, Germany) following the manufacturer's instructions. The procedure includes an on-column treatment with DNase for 15 min. The RNA concentration was measured using a NanoDrop 1000 spectrophotometer. For cDNA synthesis, an equal amount of total RNA (standardized to 500 to 1,000 ng) was utilized in a reverse transcription reaction, using the QuantiTect reverse transcription kit (Qiagen, Germany). The cDNA was diluted 1:5 in RNase-free water before use in real-time PCR.

Real-time PCR analysis. Real-time PCR analysis for expression of viral transcripts was performed on a QuantStudio 5 real-time PCR system (Agilent Technologies, USA) using Brilliant II SYBR green qPCR master mix (Agilent Technologies). Peptidylprolyl isomerase B (PPIB) was used as a reference gene. Real-time PCR analysis was performed in technical triplicates. The mRNA expression relative to the reference gene was calculated using $2^{-\Delta CT}$ and is presented as relative units. For normalization to the respective control, we used $2^{-\Delta\Delta CT}$, representing the fold change relative to the respective wild-type strain. Statistical analyses were performed on $\Delta\Delta C_T$ for data sets from more than three independent experiments using GraphPad Prism v8. Primers used are listed in Table 2. Means and standard errors of the means for data sets from more than two independent experiments are shown.

Virus stocks and infection setups. HHV-6B₂₂₉-infected SupT1 cells and HHV-6A_{GS}-infected HSB2 cells were kindly provided by the HHV6 Foundation (34, 41). Virus was propagated in the respective T-cell lines by supplying the cells with uninfected cells and culture medium (RPMI 1640 medium supplemented as described above) every 4 to 6 days, with regular monitoring of enlarged cells and cell viability using light microscopy and trypan blue staining. To harvest the virus, the cells were centrifuged at $300 \times g$ for 8 min. The supernatant was collected and stored on ice. The cell pellet was resuspended in 1 ml of the collected supernatant. To free virus particles from the cells, the cells were subjected to repeated freeze/thaw cycles. The cells were frozen at -80°C for 20 min, followed by incubation at 37°C to thaw the cells. The cells were again centrifuged at $300 \times g$ for 8 min, and the supernatant was collected and stored on ice. The cell pellet was resuspended in 1 ml of the supernatant from the first round of collection, and the cycle was repeated twice. The collected supernatants were pooled, centrifuged at $3,200 \times g$ for 1 h at 4°C , and stored in aliquots at -80°C .

The viral titer was determined in a Reed-Muench assay (42). We used values ranging from 0.001 to 0.04 multiplicities of infection (MOIs). For infection, cells were adjusted to 0.5×10^6 cells/ml on the day before infection. On the day of infection, we mixed 0.35×10^6 cells with HHV-6A or HHV-6B stock at a concentration of 2×10^6 cells/ml. For measurement of gp60/110 binding to the cell surface, we harvested the cells after 2 h by extensive washing with PBS supplemented with 2% FBS. The cells were analyzed by flow cytometry as described above. For measurement of virus entry, we set up the infection as described above and supplemented it with culture medium to a concentration of 10^6 cells/ml at 4 hpi. The cells were harvested at the indicated time points and subjected to RNA isolation and real-time PCR analysis as described above.

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We have no conflicts of interest.

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