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# Human herpesvirus-6 infection induces the reorganization of membrane microdomains in target cells, which are required for virus entry

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ABSTRACT

Cell-membrane raft microdomains are important for successful infection by several viruses. However, their role in the cell-entry process of human herpesvirus-6 (HHV-6) is unknown. Here we tested whether HHV-6 requires cell-membrane rafts for its entry. When cell-membrane rafts were disrupted by cholesterol depletion, target-cell entry by HHV-6 was inhibited, although the virus bound normally to the cells. HHV-6 infectivity was partially rescued by adding exogenous cholesterol. Interestingly, the HHV-6 cellular receptor, CD46, was found in the rafts after virus attachment, but not in the rafts of uninfected cells, indicating that HHV-6 infection induces the re-location of its receptor into the rafts. Furthermore, glycoprotein Q1, part of a viral glycoprotein complex that binds CD46, was also associated with rafts immediately after infection. These data suggest that cellular-membrane lipid rafts are important in viral entry and that HHV-6 may enter the target cells via the rafts.



## Introduction

Human herpesvirus 6 (HHV-6) is a betaherpesvirus related to human herpesvirus 7 (HHV-7) and human cytomegalovirus (HCMV). First isolated from the peripheral blood of patients with AIDS and lymphoproliferative disorders (Salahuddin et al., 1986), it is a human pathogen of emerging clinical significance. HHV-6 isolates can be classified into at least two variants, variant A (HHV-6A) and variant B (HHV-6B) (Ablashi et al., 1991; Chandran et al., 1992), based on their genetic, antigenic, and growth characteristics. HHV-6B is the causative agent of exanthem subitum (Yamanishi et al., 1988).

Approximately 15 to 20% of the plasma membrane surface area is believed to consist of membrane lipid microdomains, called rafts (Parolini et al., 1999; Schutz et al., 2000). The raft hypothesis states that attractive forces between sphingolipids with saturated hydrocarbon chains and cholesterol mediate the formation of these lateral lipid assemblies in the otherwise unsaturated glycerophospolipid environment of the cell membrane (Simons and Ikonen, 1997). Under physiological conditions, the raft domains are small, and they are associated with numerous cell processes, such as membrane trafficking and membrane signaling (Simons and Ikonen, 1997; Simons and Toomre, 2000). The tight packaging of sphingolipids and cholesterol with certain proteins forms microdomains with an increased structural order, which are designed as liquid-ordered domains in model membranes (Brown and Rose, 1992). These microdomains resist solubilization by anionic detergents at 4 °C and are therefore also designated as detergent-resistant membrane microdomains (DRMs). However, the extraction of cholesterol by various chemicals destroys the raft organization and renders their components detergent-soluble.

An essential role for cholesterol in different aspects of the replication cycle of viruses has been reported, especially during viral entry (Manes et al., 2003; Rawat et al., 2003). For enveloped viruses, cellular and viral membranes are involved in the fusion process. Successful virus entry may require cholesterol in either membrane, or in both, or may be cholesterol-independent. Murine leukemia virus entry requires cholesterol in the cellular membrane but not in the viral envelope (Lu, Xiong, and Silver, 2002). However the reverse has been reported for influenza virus (Sieczkarski and Whittaker, 2002; Sun and Whittaker, 2003; Takeda et al., 2003). During the entry of herpes simplex virus type 1 (HSV-1) and Varicella-Zoster virus (VZV), cholesterol in both membranes is required (Bender et al., 2003; Hambleton et al., 2007). On the other hand, receptor-mediated endocytosis by vesicular stomatitis virus G protein-pseudotyped retroviruses is lipid raft independent (Guyader et al., 2002; Katzman and Longnecker, 2003; Popik et al., 2002).

Receptors for human immunodeficiency virus type 1 (HIV-1), including CD4 (Xavier et al., 1998) and CCR5 (Manes et al., 1999), are found in lipid rafts. Binding of the viral envelope glycoprotein gp120 to cells recruits even more HIV-1 receptors to the lipid rafts (Manes et al., 2000; Popik et al., 2002). In contrast, in the case of infection by HSV-1, neither its cellular receptors (HVEM and nectin-1) nor its glycoprotein ligand, glycoprotein D (gD), are associated with lipid rafts (Bender et al., 2003).

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Human CD46 is a cellular receptor for HHV-6 (Santoro et al., 1999), and an HHV-6A envelope glycoprotein complex, consisting of glycoprotein H (gH), glycoprotein L (gL), glycoprotein Q1 (gQ1), and glycoprotein Q2 (gQ2), serves as the viral ligand for human CD46 (Akkapaiboon et al., 2004; Mori et al., 2003b). Although viral membrane cholesterol is required for the entry of HHV-6A (Huang et al., 2006), whether or not cholesterol is required in the cell membrane remains to be elucidated, and it is unknown if human CD46 and its viral ligand associate in the lipid rafts during HHV-6A entry.

Therefore, we investigated the role of lipid rafts in HHV-6 entry. Here we showed that intact cholesterol on the cell membrane, as well as the viral membrane, is required for the successful entry of HHV-6. We also found that the receptor for HHV-6 is distributed in the lipid rafts during viral entry. These observations suggest that lipid rafts in the cellular membrane play an important role in the viral entry process and that HHV-6 may enter target cells via the lipid rafts.

## Results

### *Cholesterol depletion of target cells affects HHV-6 entry*

One way to examine whether the lipid rafts on the target-cell surface are important for virus entry is to deplete the cholesterol from the cell surface with cholesterol-sequestering agents, such as MβCD. We used this method to determine whether cholesterol on target cell surfaces is required for HHV-6 entry. HSB-2 cells were treated with various concentrations of MβCD for 30 min at 37 °C, and the drug was then washed out. The pre-treated cells were infected with HHV-6, washed to remove unbound virus, and cultured. Sixteen hours post-infection, the expression levels of IE1 protein, an immediate early protein, were determined by western blots probed with anti-IE1 (AIE1). As shown in Fig. 1A, the cholesterol depletion of HSB-2 had a strong inhibitory effect on the infection that was noticeable even at 1 mM MβCD. In fact, densitometry showed that the level of IE1 in the cells treated with 1 mM MβCD was approximately 50% of that in untreated cells, and 40% and 20% of the control when 1.5 mM or 2 mM MβCD was used, respectively (Fig. 1B). IE1 was detected faintly in cells treated with 2.5 mM or 3 mM MβCD.

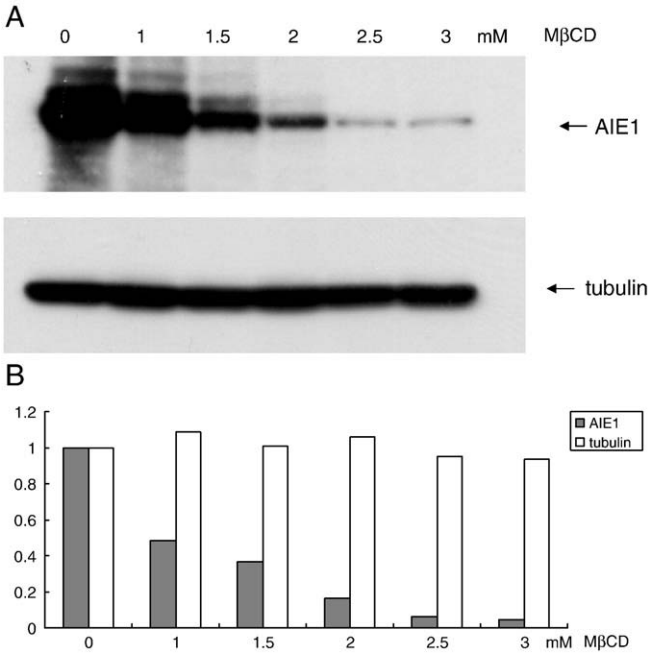


**Fig. 1.** Effect of depleting cholesterol from HSB-2 cells on HHV-6 entry. HSB-2 cells were incubated with increasing concentrations of MβCD, infected with HHV-6A, strain GS, and lysed 16 h post-infection as described in materials and methods. (A) Labeling with an anti-IE1 antibody (AIE1) is shown on Western blot. Anti-tubulin antibody was used as an internal control for the lysed proteins. One of three independent experiments is shown. (B) Quantitative analysis of Western blot by KODAK MI software shows the intensity of the band relative to that obtained with 0 mM MβCD.

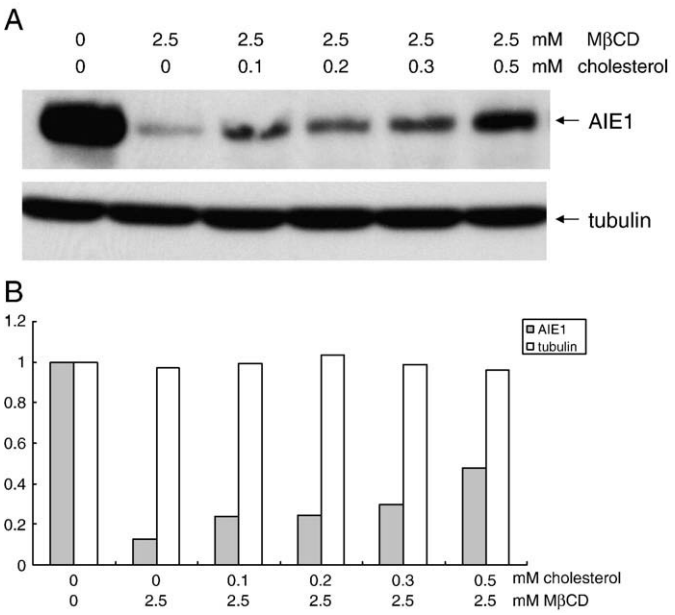


**Fig. 2.** The inhibitory effect of MβCD was partially reversed by exogenous cholesterol. HSB-2 cells, untreated or treated with 2.5 mM MβCD, were then incubated with increasing amounts of cholesterol as described in Materials and Methods. (A) The cells were subsequently infected with HHV-6A, and the expression of IE1 (immunolabeled with AIE1) and α-tubulin was detected in each sample 16 h later by Western blot. One of three independent experiments is shown. (B) Quantitative analysis of Western blot by KODAK MI software shows the intensity of the band relative to that obtained without MβCD and cholesterol.

The viability of the MβCD-treated cells was examined by trypan blue staining. Even with 3 mM MβCD, there were only a few dead cells compared with the untreated cells (data not shown), indicating that the concentrations of MβCD used here had no significant effect on HSB-2 cell viability. Thus, the HHV-6 entry into HSB-2 cells was inhibited in a dose-dependent fashion by MβCD.

### *Cholesterol replenishment restores the HHV-6 entry*

To examine whether the effect of MβCD was permanent or reversible and to confirm that the effects of MβCD were solely due to cholesterol depletion, exogenous cholesterol was used to replenish the cell surface of MβCD-treated cells. After cholesterol was removed from the cell surface by MβCD, various concentrations of exogenous cholesterol were added to the cells. As shown in Fig. 2, the addition of 0.1 mM cholesterol to cells treated with 2.5 mM MβCD restored the expression of IE1. However, the IE1 levels were lower than in cells that were not treated with MβCD, indicating that the exogenous cholesterol was able to restore virus entry only partially.

### *Cholesterol depletion does not prevent the cell-surface expression of the CD46 receptor on HSB-2 cells*

One possible explanation for the decreased IE1 expression following cholesterol depletion (Fig. 1) is that CD46 expression on the cell surface was downregulated by the treatment. To address this question, we examined CD46 expression on the cell surface before and after MβCD treatment. HSB-2 cells were incubated with or without 2.5 mM MβCD, stained with an anti-CD46 antibody, and the CD46 expression was analyzed by FACS. As shown in Figure 3, there was no

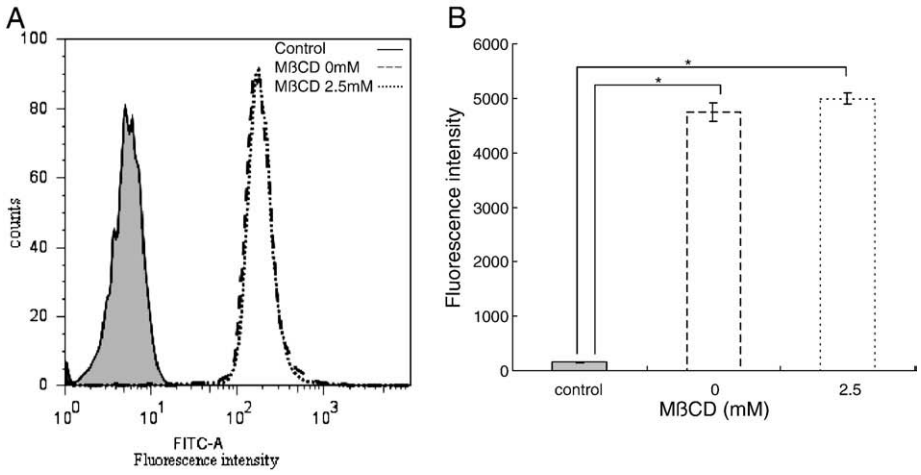


**Fig. 3.** Cholesterol depletion did not affect the expression of CD46 on the surface of HSB-2 cells. HSB-2 cells were incubated with or without 2.5 mM MβCD for 30 min. The cells were fixed with 4% parafolmaldehyde for 15 min at room temperature and stained with the mouse monoclonal antibody to CD46 followed by FITC-conjugated anti-mouse IgG. After the incubation, the cells were washed twice with ice-cold PBS. Finally, the samples were suspended in ice-cold PBS (3% FCS added) and CD46 expression was analyzed on BD FACS Canto (A). Image is one of three independent experiments. As a negative control, cells were incubated with FITC-conjugated anti-mouse (gray area). Quantification of the results obtained by flow cytometry (B). Results are means from three independent experiments, with standard deviations. Asterisks indicate significant ($P<0.01$) values by the *t* test.

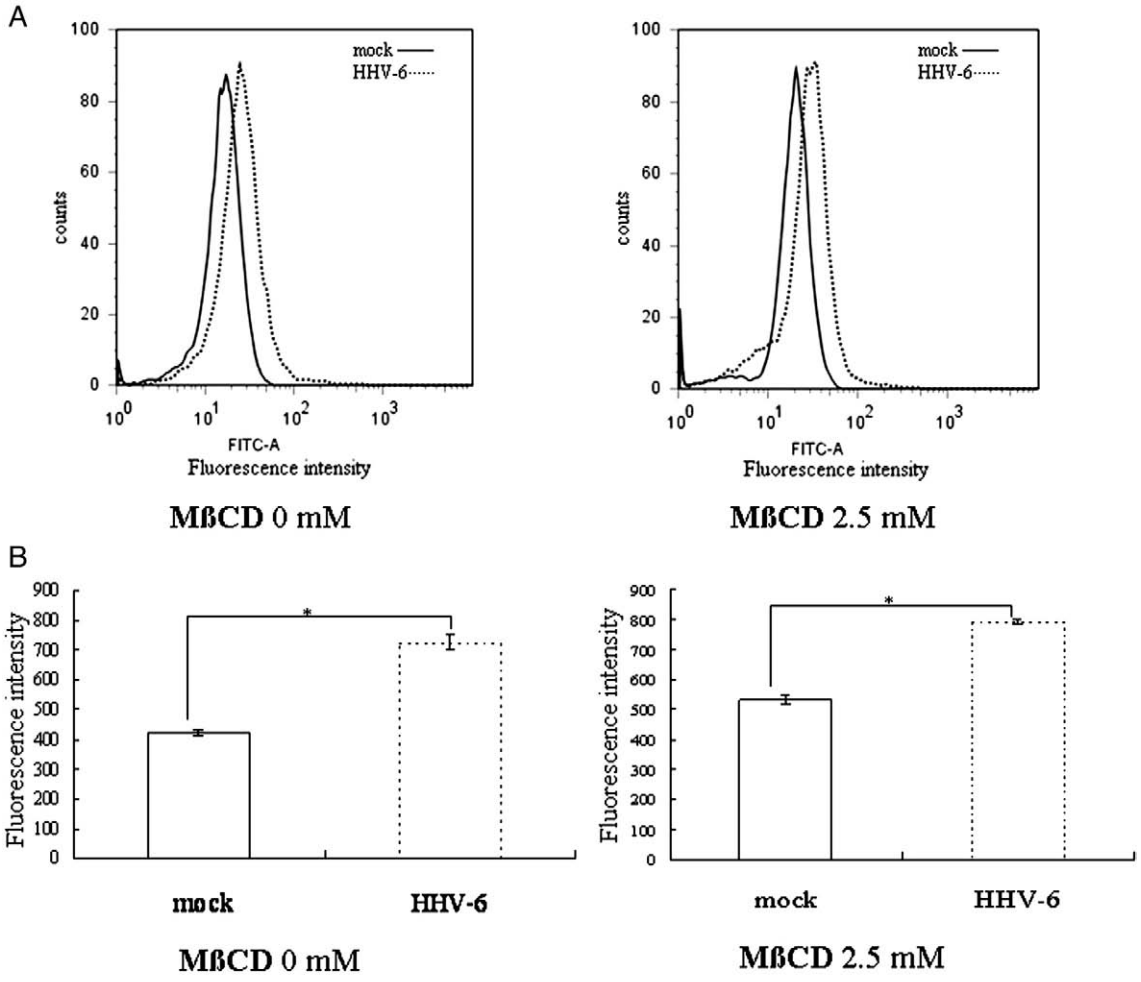


**Fig. 4.** Virus binding was not affected by cholesterol depletion from the target cell membrane. HSB-2 cells were incubated without or with 2.5 mM MβCD and then infected with HHV-6A or mock infected as described in Materials and Methods. At 1 h later, the cells were fixed with 4% parafolmaldehyde for 15 min at room temperature and stained with the mouse monoclonal antibody to viral envelope glycoprotein, gB followed by FITC-conjugated anti-mouse IgG. After the incubation, the cells were washed twice with ice-cold PBS. Finally, the samples were suspended in ice-cold PBS (3% FCS added), and binding to the target cells was confirmed by the presence of gB, analyzed on BD FACS Canto (A). Mock-infected cells were used as a negative control. Image is one of three independent experiments. Quantification of the results obtained by flow cytometry (B). Results are means from three independent experiments, with standard deviations. Asterisks indicate significant ($P<0.01$) values by the *t* test.

difference between the cells treated with MβCD and the untreated cells, indicating that cholesterol depletion did not affect the CD46 cell–surface expression.

*Cholesterol depletion does not affect virus binding*

Generally, the initial step of virus entry into a host cell is the attachment of the virus to the cell surface, which is followed by the fusion of the viral envelope with the cell membrane or by the internalization of virus. Therefore, we explored the possibility that MβCD treatment impairs the binding of HHV-6 to the cell surface. Control HSB-2 cells (Fig. 4A) or HSB-2 cells treated with 2.5 mM MβCD (Fig. 4B) were washed and incubated with HHV-6 (dotted lines in Fig. 4) or were mock infected (solid lines in Fig. 4). Virion binding was analyzed by FACS. The expression level of gB was essentially the same in the MβCD-treated and untreated cells (Fig. 4). Therefore, the depletion of cholesterol from the cell surface did not affect HHV-6's binding to the cell.

*The HHV-6 receptor, CD46, associates with detergent-resistant membrane microdomains (DRMs) during HHV-6 entry*

HIV-1 infection triggers lateral membrane diffusion following the engagement between the viral envelope ligand gp120 and the cellular receptor CD4 (Manes et al., 2000). These membrane changes are necessary for infection, because the redistribution and clustering of membrane microdomains enables the subsequent interaction of the gp120-CD4 complex with its co-receptors. Therefore, we examined whether the HHV-6A receptor, CD46, was present in the lipid rafts following the initial virus association with the cell membrane or during viral entry. To avoid confounds caused by other factors, Histodenz gradient-purified virions (see Materials and Methods) were used in this study, and the isolation of purified virions was confirmed by electron microscopy (Fig. 5C). HSB-2 cells were infected with the purified HHV-6 virions (Fig. 5A) or mock infected (Fig. 5B). At 1 h post-infection, the cells were washed to remove the unbound virions and lysed with Triton X-100. The lysates were spun through a sucrose gradient (see Materials and Methods). The fractions containing lipid rafts were identified by the specific markers GM1 (labeled by cholera toxin in Fig. 5) and CD59, which were detected mainly within low-density fractions 4 and 5 from both HHV-6- and mock-infected cell lysates (Fig. 5A and B). EEA1, a cellular marker of the early endosome that does not associate with DRMs, was detected exclusively in high-density fractions (11 and 12). Interestingly, although CD46 was detected only in the high-density fractions in the mock-infected cell lysates, in the HHV-6-infected cell lysates, it was detected in the low-density fractions as well. These results suggest that CD46 was distributed into the DRMs immediately after HHV-6 attachment, and that the lipid rafts may play a role in virus entry.

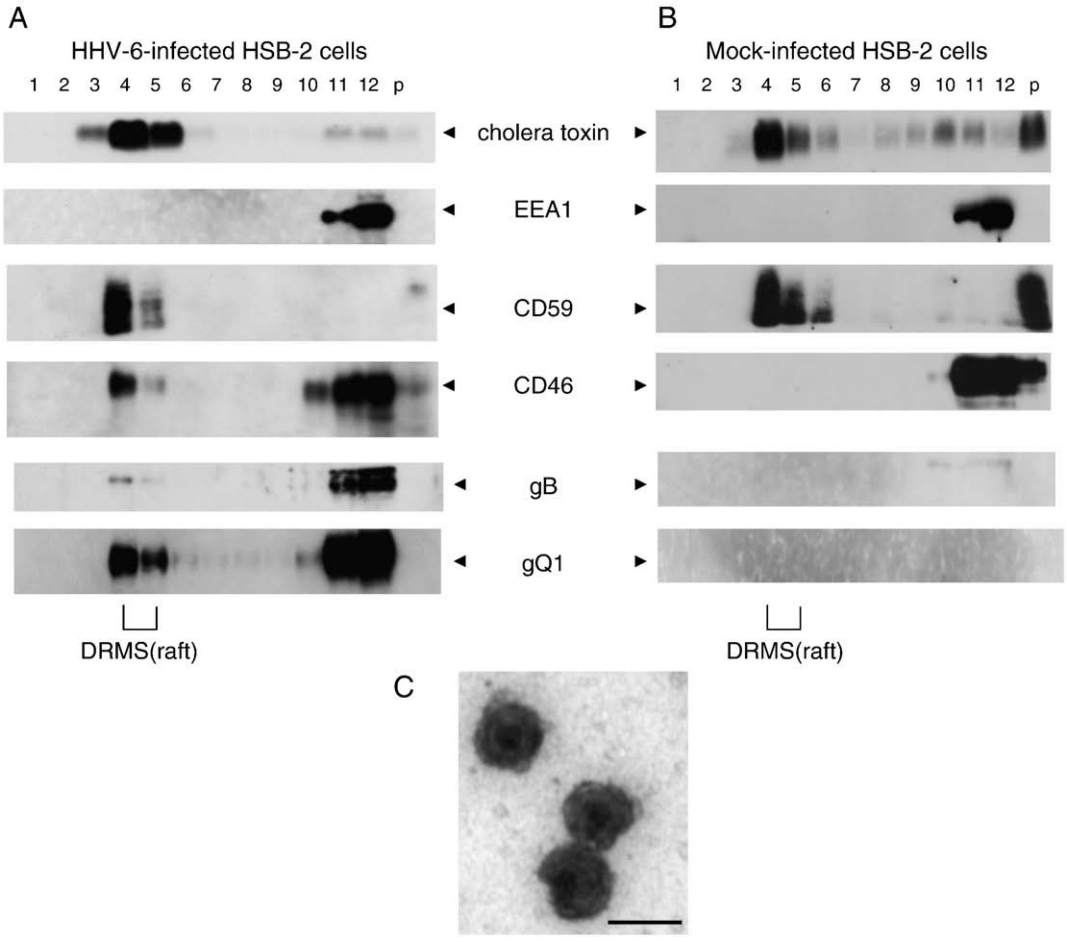


**Fig. 5.** CD46 detected in raft fractions during HHV-6 entry. HSB-2 cells were infected with purified HHV-6A virions (A) or mock infected (B) at 37 °C for 1 h. The raft fractions were obtained by spinning cell lysates through a linear sucrose gradient as described in Materials and Methods. Bottom-loaded sucrose step gradients (fraction 1 represents the top of the gradient) were analyzed on Western blots probed with an anti-EEA-1, anti-CD59, anti-CD46, anti-gB or anti-gQ1 antibody. GM1, which migrated with the dye front, was detected with HRF-coupled cholera toxin. Same amount of protein was used in each case, and EEA1 which is usually detected in nonraft fractions was used as a signal control. "p" indicates pellet. The results of same exposure times in HHV-6- and mock-infected cells were shown in each antibody. One of three independent experiments is shown. DRMs: detergent-resistant membrane microdomains. (C) Electron micrograph of purified virion preparation, demonstrating that intact virions were used. Scale bar: 200 nm.

*HHV-6 envelope proteins associate with cellular detergent-resistant low-density sucrose fractions*

The requirement for cholesterol for HHV-6 entry suggested that the envelope proteins interacted with the lipid rafts. To test this possibility, we looked for viral glycoproteins in the DRM-containing fractions by Western blot analysis. As shown in Fig. 5, gB and gQ1 were detected in the lipid raft fractions of HHV-6A-infected cells as well as in the high-density fractions. Especially, gQ1, part of a viral glycoprotein complex that binds CD46 was detected at higher levels than gB in the lipid raft fractions. The other envelope glycoproteins, gL, gH, gQ2, and gO were assayed, but were not detectable in the fractions. This may be that they are expressed at lower levels or the titter of each antibody used here is lower (data not shown). The results suggest that viral envelope glycoproteins gQ1 and gB associate with cellular lipid rafts during the entry process.

## Discussion

Lipid rafts in the cell-surface membrane represent a privileged microdomain that may facilitate virus entry. Lipid raft microdomains are enriched in sphingolipids and cholesterol (Brown and Lyles, 2003; Schutz et al., 2000; Simons and Ikonen, 1997; Simons and Ikonen, 2000; Takeda et al., 2003). The importance of cholesterol, an essential constituent of rafts, in the viral envelope or the target cell membrane during virus entry has been demonstrated for several viruses (Bender et al., 2003; Hambleton et al., 2007; Huang et al., 2006; Lu et al., 2002; Rawat et al., 2003; Sun and Whittaker, 2003). Depletion of cellular or viral cholesterol can lead to the destruction of the structure of lipid rafts and markedly reduce viral entry into cells. HIV-1, HSV, and VZV require the cellar and viral membrane cholesterol (Bender et al., 2003; Graham et al., 2003; Hambleton et al., 2007; Lee et al., 2003; Liao et al., 2001; Liao et al., 2003; Manes et al., 2000), while the entry of other viruses requires only one of the two membranes to contain cholesterol (Lu et al., 2002; Sieczkarski and Whittaker, 2002; Sun and Whittaker, 2003; Takeda et al., 2003).

Previously, we demonstrated the critical importance of viral membrane cholesterol during the entry of HHV-6 (Huang et al., 2006). Here, we showed that HHV-6 infection was markedly decreased when the target (HSB-2) cells were treated with MβCD, an effective cholesterol-depleting drug, indicating that successful HHV-6 infection required cholesterol on both the viral and cellular membranes. The addition of exogenous cholesterol to the target cell membrane restored virus infectivity, although partially, indicating that the decreased infectivity was indeed due to the depletion of cholesterol from the cell membrane. The partial recovery of infectivity is consistent with results reported elsewhere (Imhoff et al., 2007). One possible explanation is that, since only one form of exogenous cholesterol was added, other forms of cholesterol depleted by MβCD, but that are needed for efficient viral entry, were not replenished.

For an enveloped virus, cell entry begins with attachment to the target cell and ends with fusion between the viral envelope and the cellular cytoplasmic or endosomal membrane. To confirm which stage of entry was affected by cholesterol depletion, we first investigated its influence on viral binding with the cellular membrane. No significant effect was detectable, indicating that the depletion of cholesterol from target cells does not perturb the attachment of the virus to the cells, but rather must alter entry events post-attachment, as reported for other viruses (Hambleton et al., 2007).

The sensitivity of the virus to the cholesterol depletion may be explained by the association of virus receptors with lipid rafts. The tightly structured DRMs of the lipid rafts are resistant to ice-cold 1% Triton X-100, whereas other cell membranes are soluble under these conditions. Cellular protein CD46, a receptor for HHV-6, was not detected in the DRMs of HSB-2 cells, except immediately after exposure to HHV-6, when it was easily detected in them, indicating that CD46 is recruited into lipid rafts during HHV-6 entry. The re-distribution of CD46 into the lipid rafts may be important for HHV-6 entry, especially for the post-attachment events.

The HHV-6A glycoprotein gQ1 is part of a heterotetrameric complex, gQ1-gQ2-gH-gL, on the viral envelope (Akkapaiboon et al., 2004; Mori et al., 2003a), that functions as a ligand for CD46 (Mori et al., 2004; Mori et al., 2003b). Although the details of the interaction of the complex with CD46 are still unknown, here we found that gQ1 was associated with the cellular lipid rafts during virus entry. We were unable to detect the other members of the complex, gQ2, gH, and gL, at all. Since gQ1 is generally abundant on the viral envelope, it may have been easier to detect than the other glycoproteins. The association of these viral glycoproteins with lipid rafts will need to be established to clarify the role of the lipid rafts during virus entry. In the case of HSV-1, its cellular receptors, HVEM and nectin-1, are not detected in lipid rafts, nor is its viral ligand, gD (Bender et al., 2003). Therefore, although both viruses use cellular membrane lipid rafts during entry, they may differ in their ligand-receptor interactions and in how they use lipid rafts.

During the entry of HSV-1, gB is associated with lipid rafts (Bender et al., 2003), and this association may contribute to the entry process of the virus. gB is one of five glycoproteins conserved throughout the herpesvirus family (Singh and Compton, 2000; Spear and Longnecker, 2003). Although the function of gB during the entry of HHV-6 still needs to be elucidated, we observed that HHV-6 gB was associated with the DRMs during virus entry. HHV-6 gB may preferentially bind to a raft-associated molecule and play a role following the engagement of the cellular receptor and viral ligand.

The entry of virus into target cells is a complicated process. Here we have elucidated some of the molecular details involved in HHV-6 target-cell entry. However, all of the molecular players may not be accounted for yet, and besides CD46, HHV-6 may elicit the re-distribution of other cellular molecules that are important for its entry into rafts. Future studies will help identify the contributing viral and cellular components and how they interact to effect viral entry.

## Materials and methods

*Cells and viruses*

HSB-2 cells (T-cell line) were cultured in RPMI 1640 medium with 8% fetal calf serum (FCS). HHV-6A strain GS was propagated in HSB-2 cells, and the viral titers were estimated by the $TCID_{50}$ method, using HSB-2 cells. HHV-6 cell-free virus was prepared as described previously (Dhepakson et al., 2002). Briefly, when HHV-6-infected HSB-2 cells showed evidence of >80% infection by immunofluorescence assay (IFA), the cells were frozen and thawed twice, then spun at 2000×g for 10 min. The supernatants were used as cell-free virus. Purified virions were prepared as follows. Supernatants containing the virions from infected cells were collected (spun at 2500×g at 4°C for 15 min) and the viruses were precipitated with 20% polyethylene glycol (molecular mass 20 kDa) in the presence of NaCl (0.9%). The precipitates were resuspended, layered over a 5–50% Histodenz (Sigma) linear gradient and spun for 1 h at 27,000 rpm (Hitachi P40ST-1689 rotor, Hitachi High-Technologies). The fractions were collected from the bottom, and the virus-containing fractions were determined by a PCR analysis of the viral DNA.

*Antibodies*

Monoclonal antibodies (MAbs) against HHV-6A glycoproteins, anti-gQ1 (AgQ1–119), anti-gL (AgL-2), anti-IE1 (AIE1), and anti-gB (OHV-1), were described previously (Akkapaiboon et al., 2004; Huang et al., 2006; Mori et al., 2003a). The rabbit antibody specific for HHV-6 gB was prepared as follows. Rabbits were immunized five times with a purified recombinant protein, named AgB-c. AgB-c was expressed as a

glutathione S-transferase (GST) fusion protein and purified with glutathione sepharose 4B (GE Healthcare). To prepare the fusion protein, primers AgB2232bamF (5′-accggatccacacctagtgttaaggatgttg) and AgBsalR (accgtcgactcacgcttcttctacatttac; underlining indicates restriction enzyme site) were used to amplify the insert from HHV-6A DNA. The expression vector was made by inserting the PCR product into the prokaryotic expression vector, pGEX-4T (GE Healthcare) at the *BamHI* and *SalI* sites. The recombinant protein was expressed in *E. coli* and purified. The B subunit of cholera toxin conjugated with fluorescein isothiocyanate (FITC) was obtained from List Biological Laboratories. Anti-CD46 (J4–48:Immunotech), anti-EEA-1 (clone 14: BD Biosciences) and anti-CD59 (MEM-43: Serotec) MAbs were purchased.

*Western blotting*

Western blotting was performed as described previously (Akkapaiboon et al., 2004).

*Cholesterol sequestration and virus entry assay*

HSB-2 cells were incubated for 30 min at 37 °C with serial dilutions of methyl-β-cyclodextrin (MβCD) in cell culture medium. After being washed with medium twice, the cells were infected with virus by centrifugation at 2000×g at 37 °C for 1 h, then washed with medium again. Sixteen hours later, the cells were harvested and lysed with 2x sample buffer containing 32 mM Tris/HCl (pH 6.8), 1.5% SDS, and 5% glycerol. The proteins from the lysed cells were processed for Western blotting.

*Cholesterol replenishment*

HSB-2 cells were treated with 2.5 mM MβCD for 30 min at 37 °C and washed, and cultured again with various amounts of Dihydrocholesterol (Sigma). After a 1-h incubation, the cholesterol-containing medium was washed out, and the cells were infected with virus. After 16 h, the cells were lysed and processed for Western blotting.

*Virus-binding assay*

HSB-2 cells were incubated with or without 2.5 mM MβCD for 30 min at 37 °C, washed twice with ice-cold medium, and incubated with purified virus solution for 1 h at 4 °C. The cells were fixed with 4% parafolmaldehyde for 15 min at room temperature, and stained with the mouse monoclonal antibody to gB (OHV-1, 1:100) followed by goat anti-mouse IgG-FITC (1:100). After the incubation, the cells were washed twice with ice-cold PBS. Finally, the samples were suspended in ice-cold PBS (3% FCS added) and the binding was analyzed on FACS Canto (BD Biosciences).

*Isolation of low-density detergent-insoluble membrane fractions on sucrose gradients*

Low-density detergent-insoluble membrane microdomains were isolated essentially as described, with some modifications (Bender et al., 2003; Kosugi et al., 2001; Nagafuku et al., 2003). Briefly, $1\times10^8$ HSB-2 cells were infected with GS at 37 °C for 1 h, washed with ice-cold PBS, and lysed with 1 ml of ice-cold MNE buffer (25 mM MES [2-{*N*-morpholino}ethanesulfonic acid, pH 6.5], 150 mM NaCl, 2 mM EDTA) containing 1% Triton X-100 (Fluka) with protease inhibitor cocktail (Sigma). The cell lysates were further homogenized with 20 strokes in a Dounce homogenizer, and then incubated on ice for 20 min. Next, the homogenized cell lysates were mixed with 1 ml 80% sucrose (prepared in MNE) and placed at the bottom of an ultracentrifuge tube. A discontinuous gradient was formed by overlaying the homogenate sequentially with 6.5 ml of 30% and 3.5 ml of 5% sucrose in MNE. The gradient was spun at 39,000 rpm (Hitachi P40ST-1689 rotor, Hitachi High-Technologies) for 16 h at 4 °C, and fractions were collected from the top. The fractions were analyzed on a Western blot.

*Electon microscopy*

The purified virions were spun at 20,000 rpm (Hitachi P40ST-1689 rotor, Hitachi high-Technologies) for 2 h, and fixed with 4 paraformaldehyde plus 2.5 glutaraldehyde at room temperature for 1 h. Processing for electro microscopy (EM) was carried out as described previously (Gabel et al., 1989).

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