Human herpesvirus 6 envelope cholesterol is required for virus entry

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In this study, the role of cholesterol in the envelope of human herpesvirus 6 (HHV-6) was examined by using methyl-β-cyclodextrin (MβCD) depletion. When cholesterol was removed from HHV-6 virions with MβCD, infectivity was abolished, but it could be rescued by the addition of exogenous cholesterol. HHV-6 binding was affected slightly by MβCD treatment. In contrast, envelope cholesterol depletion markedly affected HHV-6 infectivity and HHV-6-induced cell fusion. These results suggest that the cholesterol present in the HHV-6 envelope plays a prominent role in the fusion process and is a key component in viral entry.

INTRODUCTION

Entry of enveloped viruses into host cells relies on fusion of the viral envelope with either the endosomal or plasma membrane of the cell. The lipid composition of both the viral envelope and the host-cell membrane plays an important role in virus infection. Semliki Forest virus fusion is absolutely dependent on the presence of cholesterol in the target (endosomal) membrane (Phalen & Kielian, 1991; Waarts et al., 2002). Human immunodeficiency virus type 1 (HIV-1) and herpes simplex virus entry also requires cholesterol in both the target and the viral membranes (Bender et al., 2003; Campbell et al., 2001, 2002; Graham et al., 2003; Guyader et al., 2002; Viard et al., 2002). Envelope cholesterol is also a crucial factor in the fusion process of influenza virus (Sun & Whittaker, 2003).

Human herpesvirus 6 (HHV-6) is a betaherpesvirus and a human pathogen of emerging clinical significance. HHV-6 was first isolated from the peripheral blood lymphocytes of patients with lymphoproliferative disorders and AIDS (Salahuddin et al., 1986). HHV-6 isolates can be categorized into two variants, A (HHV-6A) and B (HHV-6B); HHV-6B is the causative agent of exanthem subitum (Yamanishi et al., 1988). Therefore, HHV-6 was called roseola virus. Roseola occurs in a minority of infected patients and febrile seizures are associated infrequently with primary HHV-6 infection (Zerr et al., 2005b). There is increasing evidence of HHV-6-associated disease in organ-transplant recipients. HHV-6 reactivation is common after allogeneic haematopoietic stem-cell transplantation (Zerr et al., 2005a). Human CD46 is reported to be a cellular receptor for HHV-6 (Santoro et al., 1999) and the cell–cell fusion induced by HHV-6A requires human CD46 in the target cells (Mori et al., 2002). Recently, we found that the HHV-6A glycoprotein H–glycoprotein L (gH–gL) complex interacts with the glycoprotein Q1–glycoprotein Q2 (gQ1–gQ2) complex and identified the gH–gL–gQ1–gQ2 complex as the viral ligand for human CD46 (Akkapaiboon et al., 2004; Mori et al., 2003a, b). Santoro et al. (2003) have also reported that HHV-6 gH associates with CD46 by co-immunoprecipitation.

Here, we examined the role of cholesterol in the HHV-6 envelope by using methyl-β-cyclodextrin (MβCD) depletion. MβCD efficiently depleted the envelope cholesterol and significantly reduced HHV-6 entry. Virus binding was affected only slightly, whereas depleting the envelope of cholesterol markedly affected virus fusion. Our findings suggest that cholesterol in the viral envelope plays an important role in the viral-entry process.

METHODS

Cells and viruses. T-cell lines (HSB-2 cells and Jurkat cells) were cultured in RPMI 1640 medium with 10% fetal calf serum (FCS). Umbilical cord blood mononuclear cells (CBMCs) were prepared as described previously (Dhepakson et al., 2002). HHV-6A strain GS was propagated in CBMCs and the viral titres were estimated by the TCID50 method using HSB-2 cells. HHV-6 cell-free virus was prepared as described previously (Dhepakson et al., 2002). When HHV-6-infected CBMCs showed evidence of >80% infection by immunofluorescence assay (IFA), the cells were spun at 2500 g for 15 min and the supernatant was used as cell-free virus. Partially purified virions were prepared as follows (Mori et al., 2004). HSB-2 cells were infected with HHV-6 and, at 72–96 h post-infection (p.i.), the cells

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were spun at 2500 g for 15 min at 4 °C. The supernatant was then subjected to centrifugation at 70 000 g for 2 h at 4 °C through a 20% sucrose cushion. Virions were collected from the bottom. The collected virions were then passed through a 0.22 μm filter (Millipore). Sucrose gradient-purified virions were obtained as follows. Supernatant containing the virus from infected cells was collected and virus was precipitated with 10% polyethylene glycol (molecular mass 20 kDa) in the presence of NaCl. Virus was resuspended, layered over a gradient of 15–60% sucrose and spun for 1 h at 70 000 g. The virus was collected from the band in the gradient and concentrated by sedimentation at 70 000 g for 2 h. The pellet was suspended in RPMI 1640 medium containing 10% FCS and passed through a 0.22 μm filter.

Antibodies. HHV-6A monoclonal antibodies (mAbs) anti-gQ1 (AgQ1-119), anti-gL (AgL-3), anti-IE1 (AIE1) and anti-gB (OHV-1) (Mori et al., 2002) have been described previously (Akkapaiboon et al., 2004). The B subunit of cholera toxin conjugated to fluorescein isothiocyanate (FITC) was obtained from List Biological Laboratories. Thiocyanate (FITC) was resuspended in RPMI 1640 medium containing 10% FCS and passed through a 0.22 μm filter. Enzyme-linked secondary conjugates and enhanced buffer containing the mAbs. Reactive bands were visualized by using thiocyanate (FITC) was obtained from List Biological Laboratories. 2004). The B subunit of cholera toxin conjugated to fluorescein isothiocyanate (FITC) was obtained from List Biological Laboratories. 2004). The B subunit of cholera toxin conjugated to fluorescein isothiocyanate (FITC) was obtained from List Biological Laboratories. 2004).

Western blotting. Cells were lysed with sample buffer containing 32 mM Tris/HCl (pH 6–8), 1.5% SDS and 5% glycerol. Lysed proteins were resolved by SDS-PAGE and electrotransferred onto a PVDF membrane for immunoblotting. After blocking with 10 mM Tris/HCl (pH 7–2), 0.15 M NaCl, 3% skimmed milk and 0.75% Tween 20 for 1 h, membranes were incubated for 1 h with blocking buffer containing the mAbs. Reactive bands were visualized by using a horseradish peroxidase-linked secondary conjugate and enhanced chemiluminescence detection reagents (Amersham Biosciences).

Immunohistochemistry. An IFA was performed as described previously (Akkapaiboon et al., 2004).

Cholesterol depletion. MβCD and filipin III were obtained from Sigma. HHV-6 was mixed with PBS or with various concentrations of MβCD or filipin III and incubated for 1 h at 37 °C. Virus treated with MβCD or filipin III was subjected to ultracentrifugation through a 20% sucrose cushion at 70 000 g for 2 h to remove the MβCD or filipin III. Virus was resuspended in 500 μl RPMI 1640 medium containing 10% FCS and passed through a 0.22 μm filter before being used to infect cells.

Cholesterol replenishment of MβCD-treated HHV-6. Dihydrocholesterol was used in this study and obtained from Sigma. The exchange of virion-associated cholesterol with exogenous cholesterol was performed as described previously (Huang et al., 2003a, 2004). 2004). Recombinant baculovirus was prepared according to the manufacturer’s protocol (Invitrogen). H5 cells were infected with the recombinant baculovirus (bac-CD46 or bac-CD4). At 72 h p.i., the supernatant was centrifuged. The supernatant was concentrated 10–30-fold by using a Centricon apparatus (Millipore). The concentrated supernatant was used for co-sedimentation of HHV-6 proteins.

Construction of the soluble form of CD46. The soluble form of the CD46 or CD4 ectodomain was produced from baculovirus-infected cells by using recombinant baculoviruses as described previously (Mori et al., 2003b). Briefly, the CD46 or CD4 ectodomain with six histidine codons added was amplified by PCR. The PCR product was inserted into the plasmid pFastBac-Msp-Fc (Mori et al., 2003b). Recombinant baculovirus was prepared according to the manufacturer’s protocol (Invitrogen). H5 cells were infected with the recombinant baculovirus (bac-CD46 or bac-CD4). At 72 h p.i., the supernatant was clarified by centrifugation. The supernatant was concentrated 10–30-fold by using a Centricon apparatus (Millipore). The concentrated supernatant was used for co-sedimentation of HHV-6 proteins.

Flow cytometry. For flow cytometry, cells were washed twice with PBS, fixed with 4% paraformaldehyde and incubated with primary antibody for 30 min, followed by incubation with secondary antibody for 20 min. Cells were analysed on a FACSCalibur cytometer (Becton Dickinson Immunocytochemistry Systems). At least 10000 cells were analysed for each sample.

Co-sedimentation of HHV-6 proteins with soluble CD46. Soluble CD46 (sCD46) or soluble CD4 (sCD4) was incubated with immobilized cobalt chelate resin (ProFound Pull-Down PolyHis Protein: Protein Interaction kit; Pierce) (Mori et al., 2003b, 2004). After the resin had been washed, it was incubated with lysates of 10 mM MβCD-treated or untreated HHV-6 virions. After extensive washing, proteins were eluted in elution buffer containing 290 mM imidazole and the eluted proteins were detected by Western blotting with anti-gL or anti-gQ1 mAbs.
Virus entry requires HHV-6 envelope cholesterol

(a) M/βCD (0 mM)  M/βCD (1 mM)  M/βCD (2 mM)  M/βCD (3 mM)

(b) Mock  HHV-6A (GS)

(c) Jurkat cells

(d) HSB-2 cells

(e) Filipin III (μg ml⁻¹)

Filipin III

0 μg ml⁻¹  2.5 μg ml⁻¹
RESULTS

Treatment of HHV-6 virions with MβCD to deplete cholesterol reduces virus entry significantly

First, we investigated the role of envelope cholesterol in HHV-6 entry into target cells. HHV-6A strain GS was mixed with PBS or with different concentrations of MβCD and incubated. The treated virus was then subjected to ultracentrifugation through a 20% sucrose cushion to remove MβCD, as described in Methods. Jurkat cells were infected with the treated virus and expression of the HHV-6A IE1 protein (Mori et al., 2002) at 20 h p.i. and fusion from without (FFWO) at 6 h p.i. were examined by indirect IFA and Western blotting and by microscopy, respectively. As shown in Fig. 1(a), exposure to MβCD resulted in decreased HHV-6 IE1 expression and fusion. The percentage of large cells formed by cell–cell fusion was lower when MβCD-treated virus was added, compared with untreated virus (Fig. 1a). Treatment of virus with 2 mM MβCD caused a significant decrease in IE1 expression (Fig. 1a, b). Treatment of HHV-6 with 3 mM MβCD resulted in little detectable IE1 expression in the cells by IFA (Fig. 1a) or Western blotting (Fig. 1b). We used Jurkat cells in these experiments to observe cell–cell fusion. To confirm the block in infection caused by MβCD treatment, we repeated the experiments using HSB-2 cells, which are sensitive to HHV-6A strain GS infection. When HSB-2 cells were infected with MβCD-treated virus, IE1 expression in cells was lower than when untreated virus was used, similar to Jurkat cells (data not shown). Furthermore, similar inhibition of IE1 expression and FFWO was observed when the virus was treated with another cholesterol-depleting drug, filipin III (Fig. 1c). As shown in Fig. 1(c), this band did not indicate the presence of IE1.

To negate the possibility that the infection methods were toxic for the cellular membrane and to confirm the importance of envelope cholesterol, the following experiment was performed. HHV6 incubated with or without 5 mM MβCD was subjected to ultracentrifugation through a 20% sucrose cushion and suspended in RPMI 1640 medium. The purified virions were used to infect HSB-2 or Jurkat cells (data not shown) for 40 min at 37°C. After being washed three times, cells were incubated for 1 h at 37°C. Cells were fixed with 4% paraformaldehyde, stained with FITC-conjugated cholera toxin and analysed by flow cytometry.

MβCD has a slight effect on HHV-6 binding

Next, to investigate HHV-6 binding to target cells, HSB-2 cells were incubated with virus that had been treated with 10 mM MβCD at 4°C or was untreated. Cells were washed, fixed and processed for flow cytometry with the anti-gB and anti-gQ1 mAbs. Virus binding was observed by flow cytometry (Fig. 3b). The staining of gQ1 or gB on the MβCD-treated virions was decreased slightly, indicating that there were slight differences between the ability of MβCD-treated and untreated viruses to bind to cells by flow cytometry. By IFA (Fig. 3a), binding of the MβCD-treated virions also appeared to be different from that of untreated virions, although both could bind to cells. At the same time, HSB-2 cells were incubated with the 10 mM MβCD-treated virus for 40 min at 37°C, as described in Methods. After incubation for 18 h at 37°C, cells were harvested and expression of IE1 was observed by Western blotting (Fig. 3d) and IFA (Fig. 3c). As shown in Fig. 3(d), no IE1 was detected in cells infected with MβCD-treated virus. These results indicated that MβCD-treated virus could bind to the cell surface, even after treatment with 10 mM MβCD, but could not enter cells.

CD46-binding ability of the gH–gL–gQ1–gQ2 complex on the viral envelope treated with MβCD

Previously, it has been shown that the HHV-6A gH–gL–gQ1–gQ2 complex binds to human CD46 (Akkapaiboon et al., 2004; Mori et al., 2003b, 2004). In this study, we
examined the effect of MβCD on the interaction of this complex and human CD46. To investigate whether the gH–gL–gQ1–gQ2 complex associated with human CD46, sCD46 and sCD4 were prepared and binding experiments were performed as described previously (Akkapai boon et al., 2004; Mori et al., 2003b, 2004). As shown in Fig. 4, the gL and gQ1 proteins were detected in both untreated and MβCD-treated virion lysates. However, of the proteins eluted from the CD46-bound resin, the amount of protein eluted from MβCD-treated virion lysates (Fig. 4a and b, lane 4) was lower than that of proteins eluted from untreated virion lysates (Fig. 4a and b, lane 2), although the amount of gQ1 or gL detected in MβCD-treated virion lysates was nearly equal to that detected in untreated virion lysates (Fig. 4a and b, lanes 6 and 5, respectively). These results indicated that, in spite of 10 mM MβCD treatment of virions, gH–gL–gQ1–gQ2 complexes could bind to CD46, but the CD46-binding ability of the complex was decreased by depletion of cholesterol in the viral envelope.

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Expression of glycoproteins on the viral envelope after incubation with or without MβCD

HHV-6 treated with 10 mM MβCD and untreated virus were lysed with sample buffer and immunoblotted with anti-gQ1 or anti-gL mAb under reducing and non-reducing conditions. The gQ1 and gL proteins were detected in both MβCD-treated and untreated viral lysates at similar levels (Fig. 5). Furthermore, the gH–gL complexes formed by disulfide bonds were also detected in both viral lysates (Fig. 5b), indicating that, even with cholesterol depletion of the viral envelope, the glycoproteins were maintained on the envelope. We also confirmed that there was no detectable IE1 expression in cells infected with MβCD-treated virus (data not shown), indicating that the drug-treated virus did not infect the cells, even though the virions contained envelope glycoproteins.

Measurement of the cholesterol content of MβCD-treated HHV-6

We assayed the relative depletion of envelope cholesterol caused by MβCD treatment. Sucrose gradient-purified virus was treated with various concentrations of MβCD for 30 min at 37°C and the cholesterol content was determined by using an Amplex Red Cholesterol Assay kit (Molecular Probes), according to the manufacturer’s protocol. The virus showed a dose-dependent drop in the level of cholesterol (Fig. 6), indicating that MβCD treatment produced a specific and efficient depletion of envelope cholesterol.

Replenishment of envelope cholesterol restores HHV-6 infectivity

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 dihydroycholesterol was used to replenish the envelopes of MβCD-treated HHV-6. The exchange of virion-associated cholesterol with exogenous cholesterol required the initial removal of cholesterol from the HHV-6 particles by MβCD and subsequent replenishment of the cholesterol-depleted viruses with exogenous cholesterol. This procedure restored the expression of HHV-6A IE1 and HHV-6A-induced FFWO in Jurkat cells (Fig. 7a, b), indicating that it restored HHV-6A infectivity. The addition of 50 μM cholesterol to virus treated with 2-5 mM MβCD restored infectivity. We performed the same experiment using HSB-2 cells and analysed the expression of IE1 in the cells by Western blotting. IE1 expression in HSB-2 cells was restored by adding 50 μM exogenous cholesterol, as in the Jurkat cells (Fig. 7c).

DISCUSSION

In this study, we examined the role of the lipid composition of the viral membrane in HHV-6 infection, focusing on the events occurring during the viral-entry process, in particular binding and fusion. We showed a marked effect of depleting the HHV-6 envelope cholesterol on the expression of IE1 by both IFA and Western blotting, indicating decreased virus entry, and on virus-induced FFWO, which was probably due to the inhibition of virus–cell and cell–cell membrane fusion. Following treatment with the membrane cholesterol-extracting drug MβCD, HHV-6 virions still bound to target cells, but could not enter them. In all of the experiments in this study, after the virus was treated with MβCD, the drug was removed by ultracentrifugation through a 20% sucrose cushion, as reported elsewhere (Sun & Whittaker, 2003); therefore, the inhibition of virus infection by the drug was unlikely to be due to the effects of MβCD itself on the cellular membrane.
Both MβCD-treated and untreated virus bound to cells, as assessed by IFA and flow cytometry, although on visual inspection the level of binding of MβCD-treated virions appeared to be lower than that of untreated virions. However, the addition of 10 mM MβCD to purified virions inhibited the expression of IE1 and cell–cell fusion completely, indicating that, after depletion of cholesterol from the viral envelope, at least some virus could still bind to cells, although no virus could enter the target cells, as the envelope–cell fusion process was inhibited specifically.

Furthermore, the replenishment of envelope cholesterol was partially able to restore HHV-6 infectivity and FFWO, indicating that HHV-6 envelope cholesterol may be important for the virus-induced fusion process, as reported for other enveloped viruses (Guyader et al., 2002; Sun & Whittaker, 2003; Viard et al., 2002). As shown in Fig. 7, the restoration of infectivity after replenishment of cholesterol to the viral envelope did not appear to be perfect. It may be difficult to restore the composition of the envelope glycoproteins completely simply by adding exogenous cholesterol, or it may be that other molecules affected by the cholesterol removal are also required to restore the viral-envelope composition.

Previously, we showed that the HHV-6A gH–gL–gQ1–gQ2 complex binds to human CD46 and that this binding may be important for virus–cell fusion, but not for virus–cell binding (Mori et al., 2002, 2003b). Here, we investigated the
CD46 binding of the gH–gL–gQ1–gQ2 complex itself on virus envelope treated with 10 mM MβCD. As shown in Fig. 4, CD46 binding of the complex was decreased by the addition of MβCD, but the complex still bound to CD46, although the virus could not enter the cells under these conditions. CD46 binding of the complex may require the steric conformation of the complex itself, which may be destroyed by cholesterol depletion of the envelope. However, the result may not reflect virus–cell binding directly, as CD46 binding of the complex may occur after virus attachment to the cell surface. gQ1 and gB staining shown in Fig. 3(a, b) indicated binding of virus itself to the cell surface.

To investigate the role of cholesterol in virus entry, we next examined the expression of HHV-6 envelope glycoproteins gQ1 and gL in MβCD-treated and untreated virions by Western blotting. The levels of gQ1 and gL expression in MβCD-treated and untreated virions appeared similar, indicating that the glycoproteins remained on the envelope even after the depletion of cholesterol. Under non-reducing conditions, the bands of the gL proteins, which form complexes by disulfide bonds with gH and gQ2 or gH and gO, but not with gQ1, shifted to a high molecular mass,

Fig. 7. Replenishment of envelope cholesterol restores HHV-6 cell entry. HHV-6A strain GS was pretreated with 2.5 mM MβCD in the presence of various concentrations of exogenous cholesterol (0, 50, 100 and 200 μM). Treated virus was purified by centrifugation through a 20% sucrose cushion to remove the MβCD. (a) Jurkat cells were infected and analysed by IFA with anti-IE1 mAb (i) or nuclei were stained with Hoechst 33258 (ii) at 18 h p.i. In addition, virus-induced FFWO was examined at 4 h p.i. by microscopy (iii). Cells were also analysed at 18 h p.i. by Western blotting using anti-IE1 mAb (b). (c) HSB-2 cells were infected and analysed at 18 h p.i. by Western blotting using an anti-IE1 mAb. An anti-tubulin mAb was used as an internal control in the Western blots.
indicating that the complexes of glycoproteins joined by disulfide bonds were not destroyed by the depletion of cholesterol in the envelope. In this experiment, we could not use anti-gB and anti-gH mAbs, because they cannot bind to the proteins in Western blots.

For the virus–cell binding experiments shown in Fig. 3, before treatment with MβCD, the viruses were purified over a sucrose gradient; therefore, the finding that the drug-treated virions bound to the cell surface was not due to soluble glycoproteins binding to the cell surface.

Why was the MβCD-treated virus unable to induce the viral envelope–cell fusion required for entry? One possible answer is that the depletion of cholesterol makes the envelope itself less rigid, loosening its support of the glycoproteins. Even though the glycoproteins may still be attached to the envelope by other membrane-organization factors, the conformational change in the glycoproteins required for fusion may not occur because of the looseness of the envelope base. Thus, the virus may not enter the target cells, even though it binds to them. This might also explain why there was a visual difference in the cell-surface binding between the MβCD-treated and untreated virus.

Our results support the idea that cholesterol in the viral envelope plays a role in the conformational changes accompanying the glycoprotein complex-mediated fusion in a manner similar to that reported for HIV-1 (Guyader et al., 2002).

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