

Specific Association of Glycoprotein B with Lipid Rafts during Herpes Simplex Virus Entry

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Herpes simplex virus (HSV) entry requires the interaction of glycoprotein D (gD) with a cellular receptor such as herpesvirus entry mediator (HVEM or HveA) or nectin-1 (HveC). However, the fusion mechanism is still not understood. Since cholesterol-enriched cell membrane lipid rafts are involved in the entry of other enveloped viruses such as human immunodeficiency virus and Ebola virus, we tested whether HSV entry proceeds similarly. Vero cells and cells expressing either HVEM or nectin-1 were treated with cholesterol-sequestering drugs such as methyl- β -cyclodextrin or nystatin and then exposed to virus. In all cases, virus entry was inhibited in a dose-dependent manner, and the inhibitory effect was fully reversible by replenishment of cholesterol. To examine the association of HVEM and nectin-1 with lipid rafts, we analyzed whether they partitioned into nonionic detergent-insoluble glycolipid-enriched membranes (DIG). There was no constitutive association of either receptor with DIG. Binding of soluble gD or virus to cells did not result in association of nectin-1 with the raft-containing fractions. However, during infection, a fraction of gB but not gC, gD, or gH associated with DIG. Similarly, when cells were incubated with truncated soluble glycoproteins, soluble gB but not gC was found associated with DIG. Together, these data favor a model in which HSV uses gB to rapidly mobilize lipid rafts that may serve as a platform for entry and cell signaling. It also suggests that gB may interact with a cellular molecule associated with lipid rafts.

Herpes simplex virus (HSV) is typically responsible for mucosal lesions of the mouth and genital organs in humans, from where it spreads and establishes lifelong latent infections in sensory neurons. Periodically, the virus reactivates, multiplies, and is transported through the axon back to a portal of entry (83). Binding to host cell surfaces and entry is a complex process involving the essential viral glycoproteins B (gB), gD, gH, and gL and multiple cellular molecules, each with various levels of affinity and avidity (reviewed in references 11 and 72). In current models, gC and/or gB binds cell surface heparan sulfate proteoglycans, bringing the viral envelope and plasma membrane close enough for fusion to occur (69). As part of this process, gD must bind to a specific receptor, which can be either herpesvirus entry mediator A (HVEM or HveA), a member of the tumor necrosis factor receptor family, nectin-1 (HveC) or nectin-2 (HveB), two members of the Immunoglobulin superfamily, or a particular type of modified heparan sulfate proteoglycans (HSPG) 3-OST-3 (23, 46, 68). These interactions may then recruit the other essential viral glycoproteins into a functional fusion unit. In addition, entry may involve plasma membrane rearrangement, signaling events, and/or recruitment of additional cellular molecules.

Accumulated evidence indicates that plasma membrane microdomains, or lipid rafts, that are highly enriched in cholesterol and sphingolipids play a crucial role in the lateral organization of the plasma membrane (9, 27, 71). It has been

proposed that constitutive or transient enrichment of a variety of signaling molecules in these defined microdomains plays a major role in the organization of signal transduction. These domains retain substantial lateral mobility and are viewed as highly ordered moving platforms that carry specific proteins. Several viruses have taken advantage of lipid rafts for one or more aspects of their replication cycle (reviewed in references 12, 48, 67, and 76). Such mechanisms include viral particle assembly (4, 28, 38, 43, 63, 66, 86), budding from the plasma membrane (38, 49, 65), signaling (13, 18, 29), fusion (1), and virus entry (3, 5, 39, 42, 55, 74). It was proposed that human immunodeficiency virus (HIV) entry is inhibited by the presence of drugs that remove cholesterol (42, 59). The inhibitory effect was reversed by addition of exogenous cholesterol, indicating that cholesterol-enriched lipid rafts play an important role in HIV entry. Since HIV and HSV enter cells by direct fusion, an intriguing possibility is that rafts also play a role in HSV entry (reviewed in reference 12).

Receptors for HIV, including CD4 (85) and CCR5 (42), and for murine leukemia virus (39) are found in lipid rafts. Binding of gp120 to cells further recruits a larger number of HIV receptors into lipid rafts (42, 59). One evident question is whether the receptors for HSV associate with lipid rafts. It has recently been shown that some members of the tumor necrosis factor receptor superfamily, including CD120a, CD40, and the p75 neurotrophin receptor, are localized in rafts (7, 16, 30, 33). Cross-linking of CD40 with antibodies results in stable association with lipid rafts, leading to activation of tyrosine kinases and mobilization of tumor necrosis factor receptor-associated factors (TRAFs) (78). Such events are essential for downstream events, such as NF- κ B activation and interleukin expression.

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Epstein-Barr virus has exploited this signaling route by virtue of the localization of the latent membrane protein-1 (LMP1) in rafts. Association of LMP1 with rafts is responsible for signaling events that mimic those of a constitutively active CD40 receptor (10). An essential role for rafts has been described for the initiation of Fas-mediated cell death signaling (32). As a tumor necrosis factor receptor-like protein, HVEM contains TRAF-binding motifs in its cytosolic tail (46), and several reports demonstrated activation of NF- κ B during early events of HSV infection (2, 54). In addition, several proteins are rapidly phosphorylated during HSV entry (60). Hence, it is of interest to know whether HVEM is constitutively distributed in raft-like structures on cell surfaces or redistributes there as a consequence of virus binding. If so, is such an association important for virus entry? Similar questions may be asked about a second HSV receptor, nectin-1 (HveC). Strong evidence for membrane raft-dependent scaffolding of signaling complexes has come from studies on the T- and B-cell immunoreceptors and the Fc ϵ receptor, all members of the same protein family as nectin-1 (14, 15, 47, 73).

Here we present data investigating the importance of intact lipid rafts at the cell membrane for efficient virus replication. Second, we analyzed whether two HSV receptors, HVEM and nectin-1, as well as HSV glycoproteins showed an association with lipid rafts during virus entry. We found that HVEM and nectin-1 were not associated with rafts in uninfected cells and that this distribution did not change during infection. Similarly, gD, gC, and gH were not found associated with these microdomains during infection. By contrast, a fraction of gB was associated with rafts after virus attachment and during entry. This last observation points to a unique function for gB during HSV entry that involves lipid rafts and suggests the existence of a gB receptor(s) that is enriched in the cholesterol-rich microdomains.

MATERIALS AND METHODS

Cells and viruses. African green monkey kidney (Vero) and human embryonic kidney (HEK) 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% and 10% fetal calf serum (FCS), respectively. B78-H1 mouse melanoma cells expressing gD receptor HVEM (A10) or nectin-1 (C10) (45) were grown in DMEM supplemented with 5% FCS and 500 μ g of G418 per ml. B78-H1 cells (3E5) expressing a green fluorescent protein (GFP)-tagged form of HVEM (enhanced green fluorescent protein [EGFP] at the C terminus of HVEM) were cultured similarly. These cells were shown to be permissive for infection by HSV (C. Whitbeck, unpublished results). HSV-1 (KOS) and an HSV-1 strain (KOS/tk12) that carries the *lacZ* gene under the control of the ICP4 promoter (80) were both purified on sucrose gradients as described elsewhere (26). HSV-1 (KOS/tk12) and vesicular stomatitis virus were kindly provided by P. G. Spear and R. N. Harty, respectively.

Antibodies and reagents. Rabbit R47 and R137 sera were raised against HSV gC-1 and gH-1/gL-1, respectively (20, 56). Monoclonal antibody (MAb) DL6 recognizes a linear epitope on gD (19); MAb SS-10 was generated by immunizing mice with gB purified from an extract of HSV-1-infected BHK cells (unpublished data); MAb CW10 was raised against a recombinant form of HVEM (200t) (82) expressed in a baculovirus expression system (C. Whitbeck, unpublished data); MAbs CK6 and CK41 were raised against a recombinant form of nectin-1 (36). CK41 was also labeled with phycoerythrin at Molecular Probes. MAb to flotillin-2/ESA was obtained from BD Transduction Laboratories. Anti-mouse and anti-rabbit immunoglobulin secondary antibodies coupled to horseradish peroxidase (HRP) were purchased from Kirkegaard and Perry Laboratories. Cholera toxin B subunit peroxidase conjugate (CTB-HRP) was from Sigma and used at 40 ng/ml. 7-Amino-actinomycin D (BD Pharmingen) was used for the exclusion of nonviable cells in flow cytometric assays following the manufacturer's instructions.

Production and purification of HSV-1 glycoproteins. gC(457t) contains the full ectodomain of gC1 truncated at residue 457 (61). gD(285t), a form of the ectodomain of gD truncated at residue 285 (62), binds the receptor molecules HVEM and nectin-1 with higher affinity than gD(306t) (52, 84). The ectodomain comprising the first 730 amino acids of gB was expressed by recombinant baculovirus-infected insect cells. Based on the nucleotide sequence of the HSV-1 gB open reading frame, we synthesized two PCR primers in order to amplify and modify the gB ectodomain coding region for cloning and expression in a recombinant baculovirus. The first primer, 5'-CGGGATCCGGCGGCTCCGACTTC-3', hybridized to the noncoding strand of the gB ORF immediately beyond the region coding for the predicted signal sequence and incorporated a *Bam*HI restriction enzyme cleavage site (bold letters). The second primer, 5'-GCGTGATCAGGCGGCGTGGCGTCCGCGTGGATGAC-3', hybridized to the coding strand of the cloned gB open reading frame immediately prior to the transmembrane region coding sequence (residue 730) and incorporated a *Bcl*I restriction enzyme cleavage site (bold letters).

After cloning into the *Bam*HI site of the pVT-Bac transfer vector, the gB coding region was positioned downstream of and in frame with the mellitin signal sequence coding region. The resulting plasmid construct was cotransfected with baculovirus DNA (Baculogold; Pharmingen) into Sf9 cells grown in monolayer culture. After 4 days, the culture supernatant (containing recombinant progeny virus) was plated onto Sf9 cell monolayers under Grace's insect cell medium containing 1% agarose. Recombinant virus plaques were picked and amplified, and infected cell cultures were screened for the expression of gB by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis with R69 antiserum. The truncated protein called gB(730t) was purified from cells with a DL16 immunosorbent column. This MAb recognizes a dimer-specific epitope on gB, and hence all of the purified protein is dimeric. Conditions for elution and concentration were essentially the same as described for gD1(306t) (70).

Cholesterol sequestration and virus entry assay. Vero, A10, or C10 cells were seeded in 96-well plates and grown overnight to reach about 4×10^4 cells per well. Cells were then incubated for 30 min at room temperature with serial dilutions of methyl- β -cyclodextrin (M β CD) or nystatin (both reagents from Sigma) in cell culture medium containing 30 mM HEPES. After three washes with medium, cells were infected with HSV-1 (KOS/tk12) at a multiplicity of infection of 10 and allowed to attach to the cells at 4°C for 1 h. The temperature was then shifted to 37°C to allow infection to proceed synchronously. Five hours later, cells were lysed by adding an equal volume of DMEM containing 1% NP-40. β -Galactosidase activity was determined by adding substrate (chlorophenol red- β -D-galactopyranoside; Roche) and measuring the absorbance at 570 nm in a microtiter plate reader.

In replenishment experiments, Vero cells were first treated with 7.5 mM M β CD for 30 min. M β CD was washed out as before, and various amounts of water-soluble cholesterol balanced with M β CD (Sigma) in DMEM without serum were added. After 30 min, the cholesterol-containing medium was washed out, cells were infected with HSV-1 (KOS/tk12), and the process of entry was assayed as before. To deplete cholesterol from purified virions, 2×10^6 PFU was incubated for 30 min at room temperature with serial dilutions of M β CD and then diluted 60-fold with cell culture medium containing HEPES. Vero cells seeded into 96-well plates as above were infected with the treated virus at a multiplicity of infection of 10, and entry was assayed as before.

Plaque formation assay. Vero cell monolayers in 48-well plates were incubated for 30 min at room temperature with serial dilutions of M β CD in cell culture medium containing 30 mM HEPES. After three washes with medium, cells were infected with 100 PFU of HSV-1 (KOS) or vesicular stomatitis virus at 37°C. One hour postinfection, the medium was removed, and the cells were overlaid with DMEM containing 1% carboxymethylcellulose and 5% FCS and incubated for an additional 24 h. The cells were then fixed with methanol-acetone (2:1 ratio) for 20 min at -20°C and air dried. Virus titers were determined by an immunoperoxidase assay (81) with a mixture of anti-gB, -gC, -gD, and -gH/gL polyclonal antisera (HSV) or stained with crystal violet (vesicular stomatitis virus).

Flow cytometry. C10 or A10 seeded in six-well plates was incubated for 30 min at room temperature with serial dilutions of M β CD in cell culture medium containing 30 mM HEPES. After three washes with medium, cells were detached with 0.02% EDTA in phosphate-buffered saline (PBS) (Versene; Gibco-BRL), pelleted, and resuspended in 100 μ l of PBS containing 3% FCS. Cells were stained on ice for 1 h by the addition of 5 μ g of CK41 per ml directly labeled with phycoerythrin to detect nectin-1. After a PBS wash, cells were fixed with 3% paraformaldehyde in PBS and analyzed by fluorescence-activated cell sorting (FACS).

Transient transfection of 293T cells. HEK-293T cells (80% confluent) grown in a T75 flask were transfected with 5 μ g of endotoxin-free (Qiagen) purified

plasmid pBEC14, encoding HVEM (46), or pBG38, encoding nectin-1 (23), with GenePorter, as recommended by the manufacturer (Gene Therapy Systems). Twenty-four hours later, cells were fractionated on sucrose gradients as described below. Plasmids pBEC14 and pBG38 were kindly provided by P. G. Spear.

Isolation of low-density detergent-insoluble membrane fractions on sucrose gradients. Low-density detergent-insoluble membrane microdomains were isolated essentially as described by others with some modifications (64). Briefly, confluent cell monolayers in a T75 flask were washed twice with PBS and then scraped into 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) and a cocktail of protease inhibitors (Roche). Cells were further homogenized with 10 strokes in a Dounce homogenizer, and then 800 μ l of the homogenate was adjusted to 45% sucrose (prepared in MNE) and placed at the bottom of an ultracentrifuge tube. A discontinuous gradient was formed by overlaying the homogenate sequentially with 1.6 ml of 35% and of 5% sucrose (both prepared in MNE). These mixtures were centrifuged at 200,000 \times *g* and 4°C for 16 h in an SW50 swinging-bucket rotor. The gradient was fractionated from the top, and 12 fractions of 400 μ l each were collected. A 13th fraction was obtained by extracting the pellet at room temperature with 400 μ l of 4% SDS in 125 mM Tris, pH 6.8.

Isolation of detergent-resistant membranes by sequential centrifugation. Nonionic detergent-insoluble glycolipid-enriched membranes (DIG), which are soluble in octylglucoside, were prepared as described by others (17) with minor modifications. Briefly, confluent cells (T25 flask) were scraped into 1 ml of ice-cold MNE buffer containing 1% Brij-96 (Fluka) and a cocktail of protease inhibitors (Roche). After 10 strokes in a Dounce homogenizer, the nuclei were pelleted by centrifugation at 500 \times *g* for 5 min. Supernatants were sequentially centrifuged at 10,000 \times *g* for 10 min and then at 200,000 \times *g* for 1 h with an SW50 rotor. Supernatants containing detergent-soluble membranes as well as cytosolic proteins are referred to as DSM. The insoluble pellet was extracted with 200 μ l of octylglucoside solution (50 mM β -octylglucopyranoside in 20 mM Tris [pH 8], 200 mM NaCl, and protease inhibitors). Insoluble membranes still attached to the nuclei and the pellet from the 10,000 \times *g* centrifugation run were also both reextracted with 200 μ l of octylglucoside solution. The octylglucoside-soluble fractions were pooled and referred to as DIG.

Measuring binding of virus or soluble glycoprotein to the cell surface. Confluent C10 cells (T75 flask) were washed once with cold culture medium containing 30 mM HEPES and then incubated for 1 h at 4°C with HSV-1 (KOS) (multiplicity of infection, 10 to 20). Cells were then either left on ice or shifted to 37°C for various times before being washed with cold PBS. Cells were then scraped into 1 ml of ice-cold MNE buffer containing 1% Triton X-100, homogenized, and fractionated as described earlier in the section on isolation of low-density detergent-insoluble membrane fractions on sucrose gradients. Alternatively, cells were grown to confluency (T25 flask), washed, and incubated as before but now in the presence of 0.1 μ M purified soluble gB(730t), gC(457t), or gD(285t). Cells were then either left on ice or shifted to 37°C for various times before being washed with cold PBS. The DIG were then isolated as described in the section on isolation of detergent-resistant membranes by sequential centrifugation.

Western and dot blot analyses. To analyze the distribution of proteins in the fractions obtained from centrifugation experiments, an equal volume of each fraction was mixed with SDS-PAGE sample buffer (Pierce), and the proteins were resolved by SDS-PAGE (Novex) under denaturing conditions, followed by Western blotting. In some experiments, the low-density fractions (3 to 6) from a sucrose gradient were pooled, precipitated with methanol-chloroform, and solubilized in gel sample buffer. After transfer, the membranes were reacted with specific antibodies, washed, and then incubated with secondary antibodies coupled to HRP. Bound antibodies were revealed by enhanced chemiluminescence (ECL; Amersham) and exposure to film. For detection of the ganglioside GM1, 50 μ l per fraction was blotted onto a nitrocellulose membrane, blocked with PBS containing 5% nonfat dry milk and 0.2% Tween, and incubated with CTB-HRP diluted in the same buffer. Bound proteins were revealed by ECL as before.

RESULTS

Effect of cholesterol depletion or chelation on HSV entry.

One way to tell if lipid rafts are important for virus entry is to deplete cells of cholesterol with agents that sequester cholesterol, such as cyclodextrins, or agents that chelate cholesterol, such as statins, and then examine the effect of such treatment

on virus entry. We treated Vero and B78-H1 cells with methyl- β -cyclodextrin (M β CD) or nystatin for 30 min, washed out the drug, and then infected them with HSV-1 carrying the *lacZ* (KOS/tk12) gene under the control of the ICP4 promoter (Fig. 1). At 6 h postinfection, the levels of β -galactosidase activity in cell extracts were determined and used as a measure of virus entry (46).

Virus entry into Vero cells was inhibited in a dose-dependent fashion, with 50% inhibition occurring at 7.5 mM M β CD (Fig. 1A) and 80 μ g of nystatin per ml (Fig. 1B). To determine whether this effect was dependent on a particular receptor, we repeated the experiment with cell lines bearing a single HSV receptor. A10 cells, which express HVEM, and C10 cells, which express nectin-1, were both derived by transfection of the mouse melanoma cell line B78-H1 (45). M β CD inhibited HSV entry into A10 and C10 cells in a dose-dependent fashion (Fig. 1C). This indicated that cholesterol was important for HSV entry with either the HVEM or nectin-1 receptor. Based on trypan blue dye exclusion or with actinomycin D in a flow cytometric assay, cells remained viable at drug concentrations as high as 30 mM M β CD or 200 μ g of nystatin per ml (data not shown).

One possible explanation for the inhibition of HSV entry by cholesterol-sequestering drugs is that residual drug present in or on the cells was toxic for the virus. To address this question, HSV-1 (KOS/tk12) virions were incubated with increasing concentrations of M β CD, then diluted 60-fold, and tested for infectivity on Vero cells (Fig. 2). Treatment of virus with 7.5 mM M β CD inhibited its ability to infect cells by 90%. However, virus treated with 1 mM M β CD entered Vero cells almost as efficiently as control untreated virus. Since cells were washed extensively after M β CD treatment and before infection (Fig. 1A), it is unlikely that residual drug left on the cells was as high as 1 mM. So, although a high concentration of M β CD was toxic for HSV, this toxicity was not sufficient to explain the inhibition of entry into cells treated with M β CD.

Another possibility is that M β CD inhibited virus attachment to cells. We treated C10 cells with 30 mM M β CD, washed them extensively, and added HSV-1 (KOS) for 1 h at 4°C. Extracts of untreated and M β CD-treated cells contained the same amount of glycoprotein D (gD), as measured by SDS-PAGE and Western blotting (Fig. 3A). In addition, drug treatment did not alter the amount of nectin-1 detected in the same extracts (Fig. 3A). Surface expression of nectin-1 (Fig. 3B) and HVEM (not shown) was not significantly affected by M β CD, as demonstrated by FACS analysis. Together, these observations rule out the possibility that M β CD inhibits HSV entry by either preventing virus binding to cells or changing the receptor levels present in the cells.

We also evaluated the effect of cholesterol sequestration on HSV replication in a plaque formation assay. Vero cells were treated with M β CD for 30 min, the drug was washed out, and then cells were infected with HSV-1 (KOS). As a control, M β CD-treated cells were also infected with vesicular stomatitis virus, which is known to be raft independent (42, 59). Plaques were stained 24 h later and counted (Fig. 3C). The number of HSV plaques was reduced in a dose-dependent manner, with 50% inhibition occurring at 10 mM M β CD. In contrast up to 30 μ M M β CD did not reduce the number of vesicular stomatitis virus plaques made. These results under-

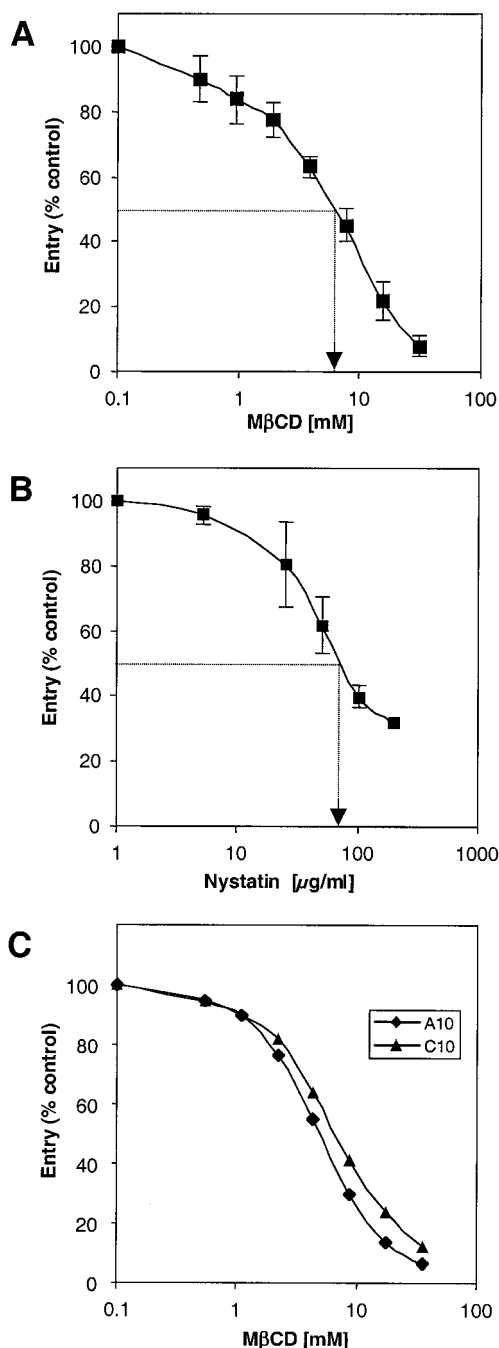


FIG. 1. Effect of cholesterol depletion or chelation on HSV entry. Vero cells (A and B) and B78 A10 and C10 cells (C) were treated with increasing concentrations of MβCD (A and C) or nystatin (B). The drugs were washed out, and the cells were infected with HSV-1 (KOS/tk12) and assayed for β-galactosidase activity at 6 h postinfection. Results presented in A and B are the mean of three independent experiments done in duplicate, with standard deviations. The results shown in panel C are representative of three experiments. A value of 100% entry represents the β-galactosidase activity at 6 h in the absence of MβCD.

score the importance of plasma membrane cholesterol content for efficient viral replication and support the observations made in the entry assays. In addition, we observed that the few plaques that formed in cells treated with MβCD were mark-

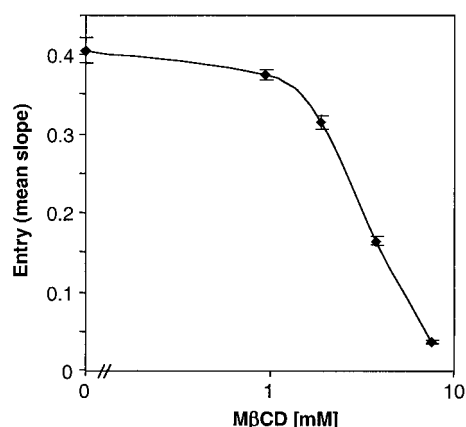


FIG. 2. Effect of cholesterol depletion from virions on HSV entry. HSV-1 (KOS/tk12) particles (2×10^6 PFU) were treated with various amounts of MβCD for 30 min at room temperature. MβCD was then diluted to a final concentration of 0.1 mM or less, a concentration that has no effect on cells, and virus was allowed to infect Vero cells (multiplicity of infection of 10). β-Galactosidase activity at 6 h postinfection was determined as described for Fig. 1. The results presented are representative of two independent experiments done in triplicate, with standard deviations.

edly smaller (not shown). However, plaque sizes were normal after longer periods of infection, and the reduction in plaque number in drug-treated cells was less severe (not shown). These observations suggest that cholesterol depletion imposes a delay in the onset of infection by HSV.

Cholesterol replenishment reverses effect of MβCD. To show that the inhibitory drug effects were specific, we replaced the cholesterol after sequestration and tested whether HSV entry was restored. We treated Vero cells with 7.5 mM MβCD for 30 min, a concentration that inhibited entry by 50% (Fig. 1A, arrow). The drug was washed out, and increasing concentrations of cholesterol were then added to reconstitute plasma membrane cholesterol levels. Excess cholesterol was washed out, and the cells were infected with HSV-1 (KOS/tk12) (Fig. 4). The addition of cholesterol completely reversed the inhibitory effect of MβCD in a dose-dependent fashion. We conclude that plasma membrane cholesterol is very important during HSV entry because sequestration or chelation of cholesterol prior to HSV infection inhibited entry, an effect reversed by the addition of exogenous cholesterol. Since rafts are maintained via cholesterol (reviewed in reference 9), we wondered whether inhibition of HSV entry mediated by cholesterol-sequestering drugs was due to raft dispersion.

Effect of cholesterol depletion after HSV entry. To test whether cholesterol sequestration had an effect after entry but during replication, Vero cells were first infected with HSV-1 (KOS/tk12) and at various times postinfection treated for 30 min with 7.5 mM MβCD (Fig. 5). MβCD markedly inhibited HSV replication when it was added at 30 min postinfection, but the level of inhibition decreased when the drug was added at later times. Normal entry was observed when MβCD was applied to cells between 2 to 3 h after infection. Interestingly, at any time following infection, MβCD-mediated inhibition of entry was not reversible by replenishment of cholesterol. These data suggest that rafts may be important during the early events of HSV replication, like fusion, but play no role later,

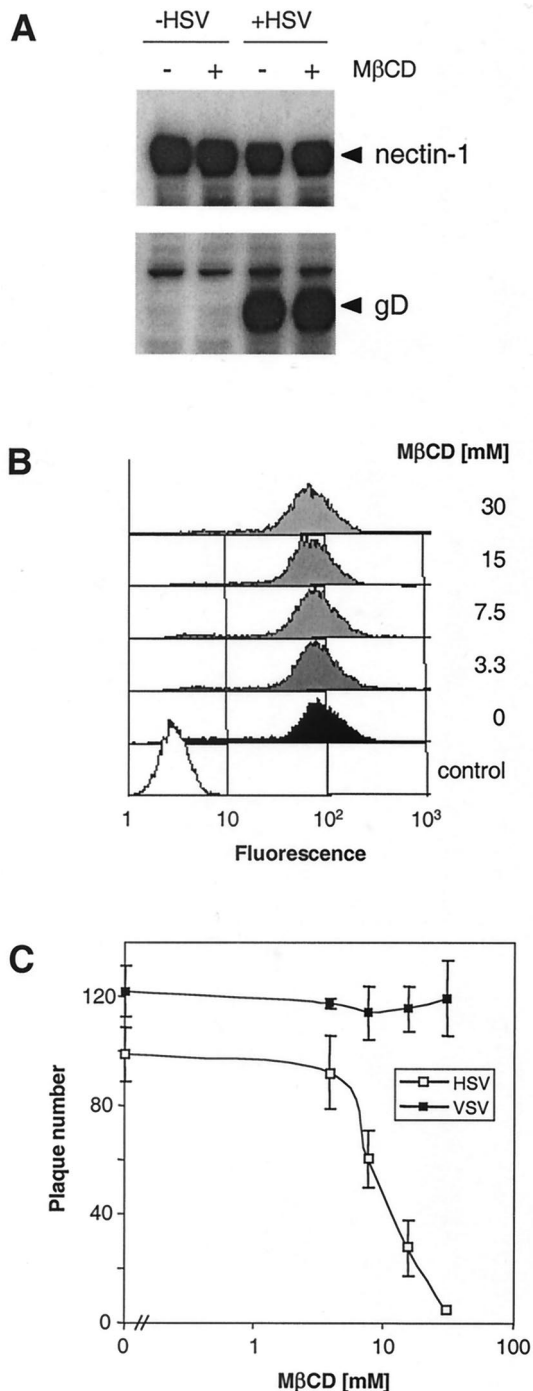


FIG. 3. Effect of cholesterol depletion from cells on expression of nectin-1 and on attachment and replication of HSV. (A) C10 cells were untreated (–) or treated (+) with 30 mM MβCD for 30 min. The drug was washed out, and HSV-1 (KOS) was allowed to bind to the cells for 1 h at 4°C. Total cell proteins were extracted, resolved by SDS-PAGE, and probed with anti-nectin-1 MAb CK6 (upper panel) or anti-gD MAb DL6 (lower panel). (B) C10 cells were treated with increasing concentrations of MβCD (indicated at the right), then stained with anti-nectin-1 MAb CK41 directly labeled with phycoerythrin, and analyzed by FACS. The control (open area) represents the fluorescence of unstained and drug untreated cells. (C) Vero cells were treated with increasing concentrations of MβCD. The drug was washed out, and the cells were infected with HSV1 (KOS) or vesicular stomatitis virus (VSV) for 24 h at 37°C. Then the cells were fixed, and plaques were

once virus entry has occurred. They also provide further evidence that MβCD is not toxic for cells.

Distribution of HSV receptors on the plasma membrane: are the viral receptors HVEM and nectin-1 found in rafts? All these experiments suggested that cholesterol-rich rafts might play a role in HSV entry. One possibility is that HSV receptors are associated with rafts or redistribute into rafts as a consequence of binding to virus glycoproteins. To assess these possibilities, we used sucrose gradients to isolate low-density detergent-insoluble fractions from cells. B78 cells stably expressing HVEM-GFP or nectin-1 were extracted in Triton X-100 and analyzed for receptor distribution into low- and high-density fractions (Fig. 6). HVEM-GFP was used because its larger molecular size allowed us to visualize it more easily than native HVEM by Western blotting. The ganglioside GM1 and the protein flotillin-2 are both associated with rafts and were used as markers.

GM1 was mostly enriched in the low-density fractions (numbers 3 to 7), and a substantial portion of flotillin-2 was recovered in fractions 4 and 5. This is in agreement with other reports (6, 27). Neither HVEM-GFP (Fig. 6A) nor nectin-1 (Fig. 6B) was found in the low-density sucrose fractions (numbers 1 to 6). These proteins were only recovered in the high-density fractions (numbers 7 to 12), where nonraft proteins are found. Similarly, when 293T cells were transiently transfected with plasmids expressing wild-type HVEM (Fig. 6C) or nectin-1 (Fig. 6D), neither receptor was found in the GM1/flotillin-2-rich fractions.

As a second approach, we used a more general procedure for two reasons. First, some proteins, such as prion protein, are enriched in semiordered lipid margins, located at the interface between the highly ordered lipid raft domains and the fluid glycerolipid membrane domain (40). These margin domains are solubilized in Triton X-100 but not in the milder detergent Brij-96. Second, due to strong interactions with the cytoskeleton, some raft-associated molecules, such as TRAF-3, only minimally associate with low-density fractions in sucrose gradients but are mainly recovered in the pellet of the gradient together with the cytoskeleton (29). Nectin-1 associates with afadin, which in turn associates with the cytoskeleton via actin (41, 75). Thus, it was possible that one or both receptors might be minimally associated with rafts.

A less stringent way to examine such interactions is to solubilize the membranes with a milder detergent, such as Brij-96, and then to use centrifugation to separate glycolipid domains (DIG) and the cytoskeleton. Then DIG which also contain rafts are solubilized and separated from the cytoskeleton with octylglycoside (17). Therefore, we lysed HVEM-GFP cells and C10 cells with Triton X-100 or Brij-96 and isolated the DIG fractions (Fig. 7). In no case was HVEM found in fractions containing DIG (Fig. 7A), although a substantial amount of flotillin-2 and all of GM1 were detected in these fractions. These results are consistent with those obtained by sucrose gradient centrifugation. Similarly, nectin-1 was not detected in the DIG recovered from cells lysed with Triton X-100 (Fig. 7B,

visualized by immunoperoxidase staining (HSV) or crystal violet staining (vesicular stomatitis virus).

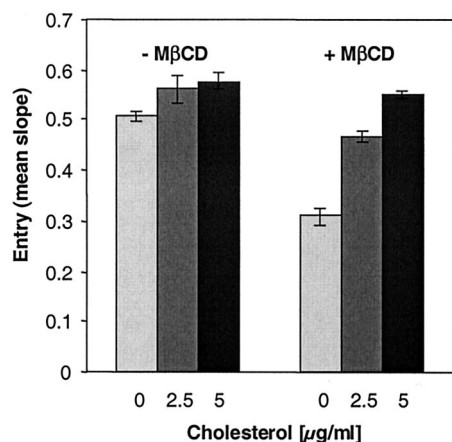


FIG. 4. Effect of cholesterol replenishment on HSV entry. Vero cells were untreated (left, rectangles) or treated (right, rectangles) with 7.5 mM M β CD for 30 min. M β CD was washed out, and various amounts of cholesterol in M β CD were added. After 30 min, the cholesterol was removed, and the cells were infected with HSV-1 (KOS/tk12) and assayed for β -galactosidase activity at 6 h postinfection. The results presented are representative of two independent experiments done in triplicate.

top panel). However, a small but reproducible fraction of nectin-1 was found associated with DIG recovered from cells solubilized with Brij-96, suggesting that a small amount of the protein may associate with rafts (Fig. 7B, bottom). However, the amount of nectin-1 found in the DIG fraction never exceeded the amount of the transferrin receptor found in this fraction (not shown). The transferrin receptor is a classical marker of a nonraft membrane protein (27). This suggests that nectin-1 detected in DIG did not come from a pool of molecules associated with rafts but rather was a contaminant of the fractionation procedure. We therefore conclude that HVEM and nectin-1 are not associated with lipid raft fractions in HVEM cells or nectin-1 cells regardless of the fractionation technique or the detergent used.

Distribution of HSV glycoproteins on plasma membrane during entry. We asked whether HSV receptors redistribute into lipid rafts after initial virus association or during entry. Manes et al. proposed this process for HIV gp120 and its receptors (42). Accordingly, C10 cells were infected with HSV and lysed with Triton X-100 at 0, 15, or 30 min postinfection. Lysates were subjected to sucrose gradient centrifugation (Fig. 8). GM1 was detected with the low-density fractions 3 to 6 at all three times postinfection (Fig. 8A). To facilitate analysis, we chose a representative nonraft fraction (fraction 11) and compared this with pooled fractions 3 to 6 (Fig. 8B and 8C). The raft marker flotillin-2 was found primarily in pooled fractions 3 to 6 (Fig. 8B). However, nectin-1 was excluded from these fractions and instead was located entirely in the nonraft fractions at 0, 15, and 30 min postinfection (Fig. 8B).

During this time period, gD also was restricted to nonraft fractions (Fig. 8B), suggesting that any association of gD with nectin-1 at the time of or shortly after infection occurred outside lipid rafts. Similarly, gC and gH, two other glycoproteins involved in entry, were restricted to nonraft fractions (Fig. 8B). Interestingly, a substantial fraction of gB associated with rafts at 4°C, and this proportion remained constant through the

early steps of entry (Fig. 8B). However, when purified virions were fractionated on a sucrose gradient in the absence of cells, gB could not be detected in raft fractions (not shown). Similarly, gB was not selectively associated with DIG compared to gD and gH/gL when transfected in 293T cells (not shown). Thus, it is unlikely that gB interacts with rafts during the extraction procedure or during sedimentation. The association of gB with rafts requires the initial binding of the virus to a cell surface.

Distribution of soluble HSV glycoproteins after association with plasma membrane. These studies suggested that gB interacts specifically with lipid rafts during virus entry. Because this association was observed at time zero, it was possible that the ectodomain of virion gB might associate with a raft-associated cell molecule. To test this, we incubated cells with purified ectodomains of gB, gC, or gD and evaluated the association of the different glycoproteins with the DIG fraction. Also, to mimic the situation of virus entry, the proteins were added to the cells at 4°C and the temperature was shifted to 37°C for various times (Fig. 9). At 4°C and time zero, soluble gB was already associated with the DIG fraction (Fig. 9A, 0 min). Interestingly, the apparent amount and proportion of gB in the DIG fraction increased during incubation at 37°C for up to 1 h (Fig. 9A, DIG). During the same period, the proportion of the full-size gB ectodomain found in the DSM was constant, although there was an accumulation of degradation products over time (Fig. 9A, DSM).

By contrast, gC (Fig. 9B) and gD (Fig. 9C, lower panel) were almost excluded from DIG at all time points. The low levels of gD found in DIG are consistent with the low levels of nectin-1 found in this fraction (Fig. 9C, top panel). The fact that gC was also found exclusively in the DSM is important because it, like gB, associates with heparan sulfate on cells (61). Thus, it is unlikely that the distribution of gB into DIG is related to

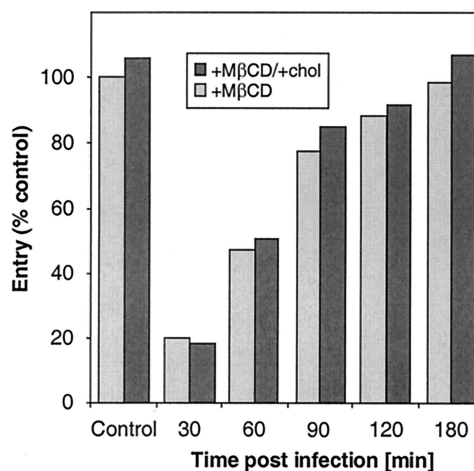


FIG. 5. Effect of cholesterol depletion from cells on the course of HSV entry. Vero cells were infected with HSV-1 (KOS/tk12). At various times following infection (indicated on the abscissa), cells were treated for 30 min with M β CD. The drug was washed out, and medium or cholesterol (chol) was added. After 30 min, the cholesterol was removed, and infection was allowed to proceed. β -Galactosidase activity at 6 h postinfection was determined. The results shown are representative of three independent experiments, and 100% entry represents the β -galactosidase activity at 6 h in the absence of M β CD.

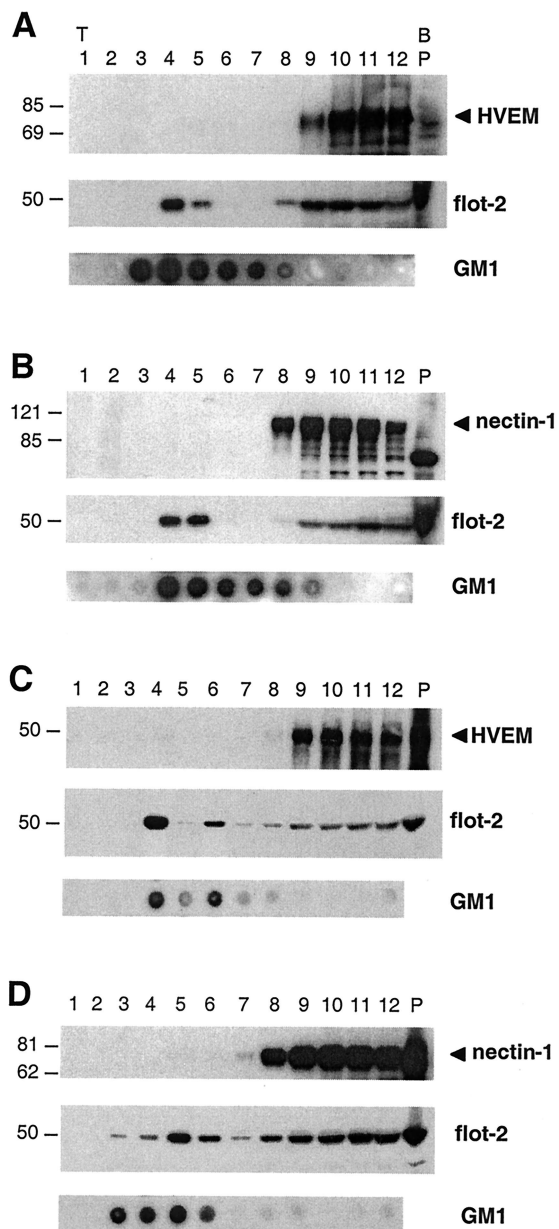


FIG. 6. Association of HSV receptors with detergent-insoluble low-density sucrose fractions containing rafts. 3E5 cells stably expressing HVEM-GFP (A), C10 cells stably expressing nectin-1 (B), and 293T cells transiently transfected with a plasmid expressing the HVEM (C) or nectin-1 (D) gene were homogenized in Triton X-100 and fractionated on sucrose gradients. Proteins (10 μ l of each fraction) were resolved on SDS-PAGE and transferred to nitrocellulose, and the distribution of HSV receptors was analyzed by Western blotting with MAb CW10 to HVEM or MAb CK6 to nectin-1. Fraction numbers are indicated at the top of the figure (P for pellet). T and B refer to the top and bottom of the gradient, respectively. The distribution of the known raft-associated protein flotillin-2 (flot-2) was analyzed with a commercial MAb and served as a positive control for rafts (A, B, C, and D, middle panels). Also shown is the distribution of the raft-specific ganglioside GM1 analyzed by dot blot with CTB-HRP (A, B, C, and D, lower panels). Sizes are shown on the left (in kilodaltons).

heparan sulfate binding. If so, gB but not gC would associate with a specific type of HSPG enriched in DIG, or gB and gC could have differential effects when bound to HSPG. The association of soluble gB with DIG corroborates our observations made with virion gB. Together, the data suggest that the interaction of gB with rafts does not depend on its transmembrane domain or carboxy terminus but is mediated by the ectodomain.

DISCUSSION

Requirement of cholesterol for HSV entry. The entry of HSV into cells is a complex process that is not completely understood. It involves four viral glycoproteins, gB, gD, and gH/gL, and at least one of the gD receptors (reviewed in references 11 and 72). Lipid rafts represent a privileged microdomain that may facilitate virus entry (reviewed in references 9, 12, 48, 67, 71, and 76). We found that sequestration of cholesterol, an essential constituent of rafts, inhibits entry into cells expressing either HVEM or nectin-1. Entry was restored by replenishing the cells with cholesterol, confirming the requirement for cholesterol. Since cholesterol is an essential

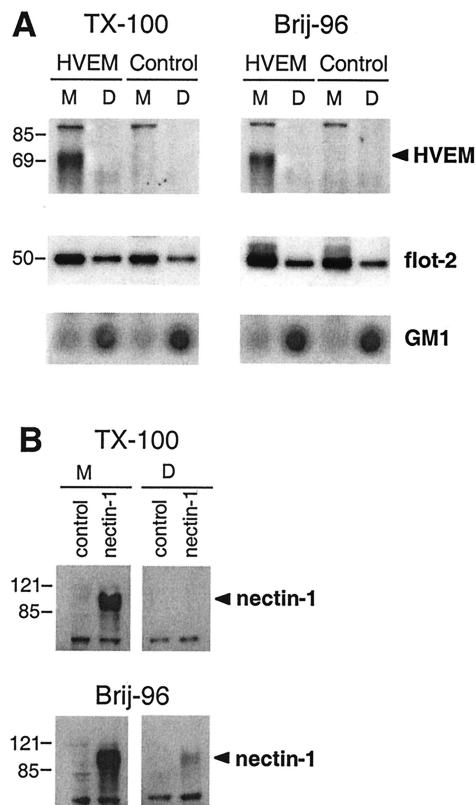


FIG. 7. Association of HSV receptors with detergent-insoluble glycolipid complexes. 3E5 cells (A), C10 cells (B), and control parental B78-H1 cells were fractionated into detergent-soluble (DSM) and insoluble (DIG) membrane fractions with either Triton X-100 (TX-100) or Brij-96. The distribution of the two HSV receptors was analyzed by Western blotting as described for Fig. 6. At the top are indicated the receptor expressed (control, HVEM, or nectin-1), the detergent used (Triton X-100 or Brij-96), and the fraction, DSM (M) or DIG (D). Sizes are shown on the left (in kilodaltons).

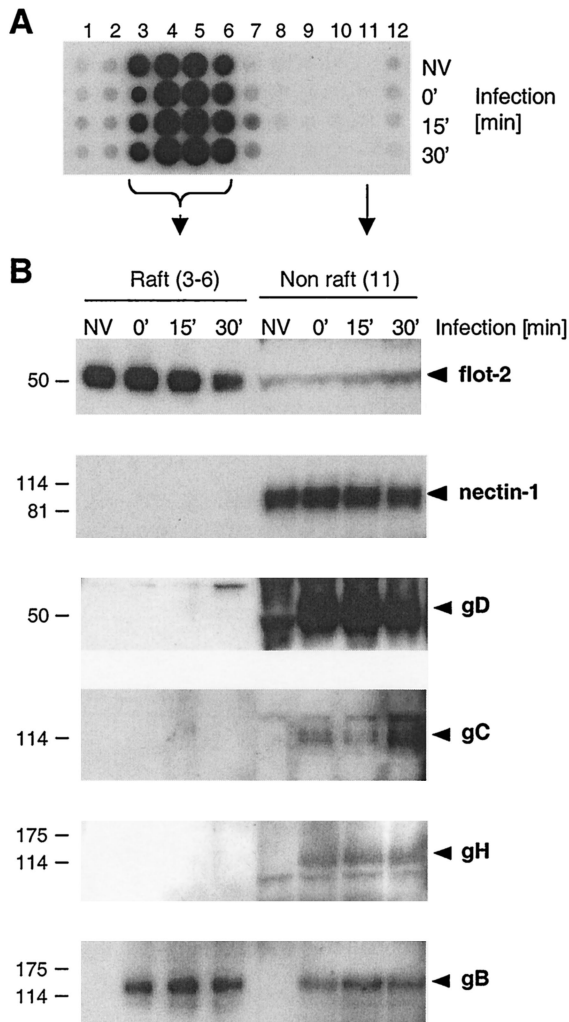


FIG. 8. Association of HSV receptors and glycoproteins with detergent-insoluble low-density sucrose fractions during entry. C10 cells were infected with HSV (KOS), and at various times postinfection, cells were extracted in Triton X-100 and fractionated on a sucrose gradient as in Fig. 6. (A) The distribution of GM1 in the different sucrose gradient fractions was analyzed by dot blot in noninfected cells (NV) and after infection for various times, indicated at the right of the panel. (B) Low-density fractions 3 to 6 containing the raft marker GM1 (raft) were pooled, precipitated with methanol-chloroform, resuspended in gel sample buffer, and resolved by SDS-PAGE. The distribution of flotillin-2 (flot-2) and nectin-1 at various times during infection (indicated at the top of the panel) was compared to that found in 10 μ l of the GM1-negative fraction 11 (non-raft) by Western blotting on separate membranes. The distribution of the glycoproteins (indicated at the right) in raft and nonraft fractions was analyzed with MAb DL6 to gD, polyclonal antibody R47 to gC, polyclonal antibody R137 to gH, and MAb SS-10 to gB. Sizes are shown on the left (in kilodaltons).

structural molecule of rafts (9), we hypothesize that rafts may be directly involved during entry. HSV entry may also require the presence of cholesterol in the plasma membrane independently of rafts. In particular, fusion may require cholesterol regardless of the maintenance of rafts.

Fusion of alphaviruses requires the presence of both cholesterol and sphingolipids in target membrane, but the fusion

activity is not dependent on the presence of DIG (79). Similarly, depleting plasma membrane cholesterol inhibits HIV-1 entry, but the presence of HIV-1 receptors in rafts is not required for infection (57). These observations suggest that cholesterol modulates the HIV-1 entry process independently of its ability to promote raft formation. Cholesterol is, however, essential for the conformation and function of CXCR4 and CCR5 (50, 51). Alternatively, it was proposed that binding of HIV may be favored by the presence of CD4 in rafts, but rafts may then disperse prior to the membrane fusion reaction (35). Since gB was found in lipid rafts during attachment of HSV, this suggests a function for these structures during entry, in particular for fusion.

HVEM and nectin-1 are not associated with lipid rafts. HIV infection induces lateral membrane diffusion following interaction of the viral envelope with cell surface receptors, which are necessary for infection (42). We found that HVEM was not associated with rafts in uninfected cells regardless of which detergent was used for extraction. This was a surprise, because other tumor necrosis factor receptors such as CD40, CD120a, and the p75 neurotrophin receptor are associated with rafts (7, 16, 30, 33). The cytosolic tail of CD120a containing the death domain is necessary and sufficient for both localization of the receptor to lipid rafts and signaling apoptosis. This implies that receptor localization to lipid rafts is important for the function of this receptor (16).

The absence of a death domain in HVEM may explain its particular membrane distribution (46). On the other hand, CD40 engagement by MAbs leads to a membrane raft-restricted recruitment of TRAF-3 and TRAF-2 to CD40's cytoplasmic tail. This indicates that the membrane raft structure

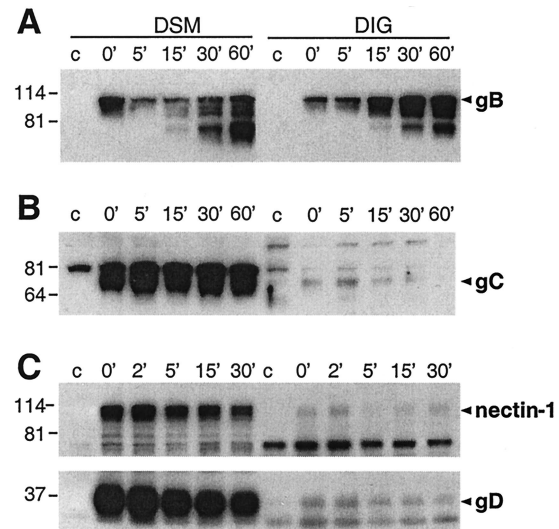


FIG. 9. Association of HSV soluble glycoproteins with detergent-insoluble glycolipid complexes. (A) Soluble gB, (B) gC, or (C) gD (0.1 μ M each) was bound to C10 cells at 4°C, and the temperature was shifted at 37°C for various times (in minutes), as indicated at the top of the panel. Cells were extracted in 1% Brij-96, and the distribution of each glycoprotein into DSM and DIG was analyzed as described for Fig. 8. For each experiment, the control (c) without glycoproteins (A and B) or on parental nectin-1-negative B78-H1 cells (C) is shown. Panel C also shows the distribution of nectin-1. Sizes are shown on the left (in kilodaltons).

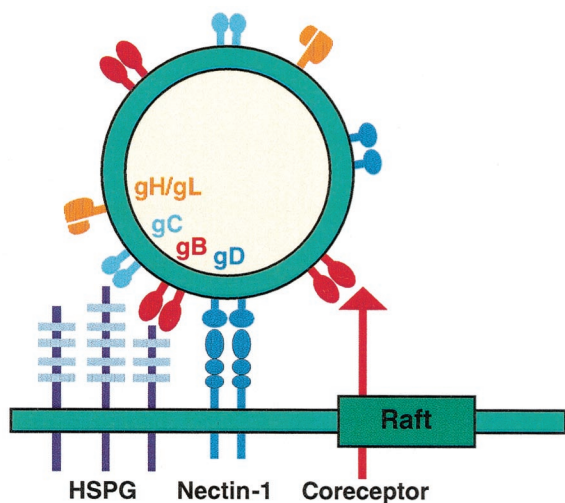


FIG. 10. Model of HSV attachment involving lipid rafts. Initial attachment of HSV is mediated through the interaction of gB and/or gC with HSPG. These interactions are likely to occur in nonraft domains, since gC and a fraction of gB are found in DSM. Then a specific interaction of gD with a cognate receptor (here, nectin-1) is essential for fusion to occur. Both gD and nectin-1 are excluded from rafts during attachment and throughout the entry process. However, a fraction of gB is associated with rafts from the moment of attachment and during entry. Such a distribution can be explained by the interaction of gB with an unknown receptor enriched in rafts. The size of the HSV virion (150 to 200 nm) would allow its envelope to interact with a plasma membrane region that can encompass at least one raft microdomain (70 nm).

plays an integral role in CD40 signaling (78). A similar domain is likely important for HVEM function. However, binding of gD did not result in redistribution of HVEM into rafts. Moreover, deletion of part of the cytosolic tail of HVEM comprising the TRAF-2 and TRAF-5 binding sites required for NF- κ B activation had no effect on its function as an HSV receptor (31, 46).

Based on the model proposed for the T- and B-cell receptors and the Fc ϵ receptor (14, 15, 47, 73), we hypothesized that the immunoglobulin family member nectin-1 would also associate with rafts during entry. Nectin-1 is located at tight junctions (22), and tight junctions are found in membrane microdomains (53). However, we found that nectin-1 was not associated with lipid rafts in uninfected cells and that this distribution did not change during infection. If rafts are involved in HSV entry, it is not because of the localization of the gD receptors in these structures. Localization of nectin-1 in tight junctions and rafts *in vivo* should be confirmed.

gB associates with lipid rafts during entry. The interesting finding that gB but not gD, gC, or gH associated with rafts after virus attachment and during entry may point to a unique function for gB during HSV entry that involves association with lipid rafts. We hypothesize that a cellular gB receptor(s) is enriched in rafts, a situation that would explain the unique association of gB with microdomains during entry (Fig. 10). Localization of this unknown molecule in rafts would also account for the inhibition of virus entry by M β CD and nystatin. Rafts are likely to be less than 70 nm in diameter (21, 77). Thus, the envelope of HSV (which is 150 to 200 nm in diam-

eter) could potentially interact simultaneously with both raft and nonraft domains during attachment. This would explain how only gB is found associated with rafts (Fig. 10).

Among the herpesviruses, gB is the most highly conserved glycoprotein, suggesting that this molecule possesses a central function which is common for the entry of different herpes viruses (58). It is of interest that cytomegalovirus gB activates the interferon response pathway via an interaction with an unidentified cellular receptor different from HSPG (8). Activation of these genes involves the mitogen-activated protein kinase pathway and protein kinase C. Liquid-ordered domains represent the preferential location for the initiation of the mitogen-activated protein kinase as well as other signaling cascades (reviewed in reference 71). Thus, the potential cytomegalovirus gB receptor may also localize in rafts, where it elicits signaling upon cytomegalovirus attachment. The association of HSV gB with lipid rafts during entry may be necessary to elicit rapid signaling events. Transient tyrosine phosphorylation of several proteins occurs within minutes after infection, followed by activation of NF- κ B. Both of these events support the idea that signaling cascades parallel HSV entry (data not shown) (60). The direct involvement of HSV gB in signaling remains to be investigated.

The role of rafts during HSV infection has been investigated by others. Sucrose gradient fractionation suggested that the UL56 gene product is a membrane protein associated with rafts (34). A minor fraction of the tegument protein vhs as well as VP16 and gH localizes to DIG in HSV-infected cells (37). We found that gH as well as gB, gC, and gD was excluded from DIG in the virion envelope (data not shown). Similarly, these glycoproteins were not in DIG from either cells infected with HSV for 8 h or cells transfected with the individual glycoproteins (data not shown). We suspect that the traces detected in rafts are likely a consequence of contamination.

Another interesting observation was that treatment of herpes simplex virions with a cholesterol-sequestering drug inhibited entry. Replenishment with cholesterol partially restored entry. Thus, cholesterol may play an important role in determining the structure of the viral envelope. Whether cholesterol is important for the organization of rafts on the virion is not known. However, rafts are found in the Golgi complex (24), a cellular compartment important for the final envelopment of virus (reviewed in reference 44). Thus, it is possible that some rafts may be incorporated in the viral envelope. Similar experiments with HIV showed that M β CD-treated virus was less infectious (25).

The association of gB with rafts during entry could represent a step in our understanding of fusion. Semliki Forest virus fusion protein E1 inserts preferentially in membrane domains enriched in cholesterol and sphingolipid (1). By analogy, HSV gB may preferentially bind to a raft-associated molecule and play a direct role in fusion through these microdomains. The other glycoproteins would contribute through strong and specific interactions with cognate receptors, bringing the viral envelope and plasma membrane into close apposition. Alternatively, conformational changes in the entry glycoproteins induced by binding to receptors could result in the formation of a fusion complex which includes the association of gB with rafts. Identification of the gB binding molecules as well as characteriza-

tion of the individual involvement of each glycoprotein during this process are essential to answer these questions.

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