Cellular Cholesterol Facilitates the Postentry Replication Cycle of Herpes Simplex Virus 1

George A. Wudiri, Anthony V. Nicola

Department of Veterinary Microbiology and Pathology and Paul G. Allen School for Global Animal Health, College of Veterinary Medicine, Washington State University, Pullman, Washington, USA

ABSTRACT Cholesterol is an essential component of cell membranes and is required for herpes simplex virus 1 (HSV-1) entry (1–3). Treatment of HSV-1-infected Vero cells with methyl beta-cyclodextrin from 2 to 9 h postentry reduced plaque numbers. Transport of incoming viral capsids to the nuclear periphery was unaffected by the cholesterol reduction, suggesting that cell cholesterol is important for the HSV-1 replicative cycle at a stage(s) beyond entry, after the arrival of capsids at the nucleus. The synthesis and release of infectious HSV-1 and cell-to-cell spread of infection were all impaired in cholesterol-reduced cells. Propagation of HSV-1 on DHCR24^{−/−} fibroblasts, which lack the desmosterol-to-cholesterol conversion enzyme, resulted in the generation of infectious extracellular virions (HSV^{des}) that lack cholesterol and likely contain desmosterol. The specific infectivities (PFU per viral genome) of HSV^{chol} and HSV^{des} were similar, suggesting cholesterol and desmosterol in the HSV envelope support similar levels of infectivity. However, infected DHCR24^{−/−} fibroblasts released ~1 log less infectious HSV^{des} and ~1.5 log fewer particles than release of cholesterol-containing particles (HSV^{chol}) from parental fibroblasts, suggesting that the hydrocarbon tail of cholesterol facilitates viral synthesis. Together, the results suggest multiple roles for cholesterol in the HSV-1 replicative cycle.

IMPORTANCE HSV-1 infections are associated with a wide range of clinical manifestations that are of public health importance. Cholesterol is a key player in the complex interaction between viral and cellular factors that allows HSV-1 to enter host cells and establish infection. Previous reports have demonstrated a role for cellular cholesterol in the entry of HSV-1 into target cells. Here, we employed both chemical treatment and cells that were genetically defined to synthesize only desmosterol to demonstrate that cholesterol is important at stages following the initial entry and transport of viral capsids to the nucleus. Viral protein expression, encapsidation of the viral genome, and the release of mature virions were impacted by the reduction of cellular cholesterol. Cholesterol was also critical for cell-to-cell spread of infection. These findings provide new insights into the cholesterol dependence of HSV-1 replication.

KEYWORDS cholesterol, herpes simplex virus, herpesviruses, sterols

The entry of several herpesviruses into target cells requires cell membrane cholesterol (1–6). Membrane cholesterol is essential for maintaining membrane order, reducing permeability, and a host of other cellular functions, including cell signaling (7–10). Entry of the alphaherpesviruses herpes simplex virus 1 (HSV-1) and pseudorabies virus requires target membrane cholesterol at the fusion step (1, 3), but not for virus attachment to cells (2, 6). Herpesviruses utilize low-pH endosomal entry pathways and pH-independent routes, both of which require cholesterol (11–13). Membrane cholesterol plays a general role in viral entry in that other sterols, such as the

Received 19 March 2017 Accepted 19 April 2017

Accepted manuscript posted online 26 April 2017

Citation Wudiri GA, Nicola AV. 2017. Cellular cholesterol facilitates the postentry replication cycle of herpes simplex virus 1. *J Virol* 91:e00445-17. <https://doi.org/10.1128/JVI.00445-17>.

Editor Richard M. Longnecker, Northwestern University

Copyright © 2017 American Society for Microbiology. All Rights Reserved.

Address correspondence to Anthony V. Nicola, nicola@vetmed.wsu.edu.

cholesterol precursor desmosterol, can partially support HSV-1 entry in place of cholesterol (1). Desmosterol and cholesterol are structurally similar. Both have a hydroxyl head, a steroid ring, and a hydrocarbon tail. The only difference is a C-24=C-25 double bond in the hydrocarbon tail of desmosterol. Desmosterol can also mediate virus-induced membrane fusion (1). Beyond entry, cell membrane cholesterol is also required for the synthesis of several viruses. Cholesterol-enriched lipid rafts in cell membranes play a role in the formation of replication compartments and in the assembly and budding of several enveloped viruses (14–21). Beyond its role in West Nile virus (WNV) entry, the postentry replication of the WNV infectious strain NY385-99 is independent of cell cholesterol (22). Herpesviruses acquire their envelopes from internal membranes of infected cells (23, 24). How cholesterol contributes to the synthesis and assembly of progeny enveloped virions is of great interest.

There are many reports on the role of cellular cholesterol in virus-cell interactions. However, limited attention has been given to the molecular features of cholesterol and their functions in viral replication and infectivity. We recently reported that the hydrocarbon tail of cholesterol facilitates HSV-1 entry (1). This was accomplished with mouse embryonic fibroblasts (MEFs) that lack cholesterol and contain only desmosterol (DHCR24^{-/-} cells) and the parental DHCR24^{+/+} cells, which contain only cholesterol. The DHCR24^{-/-} and DHCR24^{+/+} cell system allows evaluation of the hydrocarbon tail of cholesterol in HSV entry and in the replicative cycle using a drug-free system.

We hypothesized that cell cholesterol is essential for the synthesis and efficient release of progeny virus from infected cells. The cholesterol knockout DHCR24^{-/-} cell system was utilized to assess the contribution of the cholesterol tail. Methyl-beta cyclodextrin (M β CD) treatment was used to ascertain sterol requirements. We identified postentry roles for cholesterol in the HSV-1 replicative cycle. Cholesterol impacted steps after incoming capsid arrival at the nucleus, including viral protein expression, synthesis, and release of mature progeny virus and cell-to-cell spread. The hydrocarbon tail of cholesterol affected the synthesis, but not the release or specific infectivity, of mature viral particles.

RESULTS

Cholesterol plays a role in the HSV-1 replicative cycle beyond initial entry. HSV-1 requires target cell cholesterol for entry into cells. To begin to probe the postentry effects of cellular cholesterol on the HSV-1 replication cycle, we quantitated the yield of infectious HSV-1 progeny from cholesterol-reduced cells relative to untreated cells. Infected cells that were treated with M β CD synthesized up to 1 log unit less total infectious HSV-1 at 19 h postinfection (p.i.) than virus from untreated cells (Fig. 1A). Furthermore, cholesterol-reduced cells released up to 2 log units less extracellular infectious virus than untreated cells (Fig. 1A). To assess further whether cell cholesterol plays a postentry role in the HSV-1 replicative cycle, Vero cells were infected with HSV-1 KOS (multiplicity of infection [MOI] = 0.002) for 4 h at 37°C to allow viral entry. The inoculum was removed, and noninternalized virions were inactivated by sodium citrate (pH 3.0) treatment of the cells. The infected cells were treated with M β CD for 45 min and cultured for an additional 18 h. At the range of M β CD concentrations tested, there was modest if any reduction of HSV-1 plaque formation (Fig. 1B). Replenishment with water-soluble cholesterol restored HSV-1 plaque numbers to levels similar to those in mock-treated cells (Fig. 1B). Treatment of Vero cells with increasing concentrations of M β CD reduced cellular cholesterol levels in a dose-dependent manner by as much as 47% at 50 mM (Fig. 1C). Higher concentrations were cytotoxic; thus, for this experiment, only ~50% of the cholesterol was depleted to maintain cell viability. At the concentrations tested, the viability of the cells treated with M β CD was similar to that of mock-treated cells (Fig. 1D). Altogether, the results suggest that cell cholesterol is important beyond initial entry for the HSV-1 lytic replication cycle and synthesis of progeny.

Cell cholesterol is important at an early stage of the HSV-1 replicative cycle. To determine further the stage in the HSV-1 replication cycle that is impacted by chole-

FIG 1 Cell cholesterol plays a role in HSV-1 replication beyond entry. (A) Vero cells were infected with HSV-1 KOS (MOI = 5) for 1 h at 37°C. The cultures were mock treated or treated with 50 mM MβCD for 45 min at room temperature and then rinsed and replenished with serum-free medium. At 19 h p.i., the supernatant fraction or cell lysates were collected, and viral titers were determined. The data are means of triplicate determinations with standard deviations and are representative of the results of three independent experiments. Student's *t* test; *, *P* < 0.001. (B) Vero cells infected with HSV-1 (MOI = 0.002) for 4 h at 37°C were treated with sodium citrate buffer (pH 3.0) to inactivate noninternalized virus. The cells were rinsed and treated with MβCD at room temperature for 45 min and then replenished with serum-free medium or medium supplemented with cholesterol and incubated at 37°C for an additional 18 h. Titers were determined at 24 h p.i. The data are the means of at least three replicate samples and representative of the results of three independent experiments. Student's *t* test for MβCD treatment; 0 mM versus 10 mM, *P* = 0.69; 0 mM versus 20 mM, *P* = 0.074; 0 mM versus 50 mM, *P* = 0.063. (C) Vero cells were treated with MβCD at room temperature for 45 min. The cells were rinsed, and cholesterol levels were measured using the Amplex red assay (Invitrogen) according to the manufacturer's instructions. The data are the means of triplicate independent experiments with standard deviations. (D) Viability of mock- or MβCD-treated Vero cells was determined by trypan blue exclusion. The data are the means of quadruplicate determinations with standard deviations.

sterol, we performed a time course of MβCD addition. HSV-1-infected Vero cells were treated with MβCD at different times over the course of a 24-h infection. When cells had their cholesterol reduced at 2, 4, 6, or 9 h postinfection, HSV-1 plaque numbers were decreased by 35 to 50% (Fig. 2A). The reduction of cholesterol in infected cells at 12 or 24 h postinfection did not inhibit HSV-1 plaque formation (Fig. 2A), suggesting that cholesterol influences the first 9 h of the HSV-1 replicative cycle. Following fusion of the viral envelope with the host cell, nucleocapsids are transported to the nucleus via a microtubule-dependent, proteasome-dependent process (25–27). We assessed the effect of cholesterol reduction in already infected Vero cells on incoming capsid transport. At 2.5 h postinfection, capsids were detected at the nuclear periphery of MβCD-treated cells in a manner similar to that in mock-treated cells (Fig. 2B and C). In contrast, when cells were treated with the control proteasome inhibitor MG132, HSV-1 capsids were halted at the cell periphery, as previously reported (25, 28) (Fig. 2D). Thus, capsid transport is not cholesterol dependent, and cell cholesterol impacts HSV-1 replication at a step subsequent to capsid arrival at the nucleus.

HSV-1 protein expression in cholesterol-reduced cells. Cholesterol influences the first 9 h following HSV-1 entry (Fig. 2A), which broadly corresponds to the timing of

FIG 2 Cellular cholesterol is important for HSV-1 replication at a stage following the arrival of viral capsids at the nucleus. (A) HSV-1 KOS-infected Vero cells (MOI = 0.002) were treated with 50 mM MβCD at the indicated times postinfection. The cells were rinsed, replenished with serum-free medium, and fixed at 24 h p.i. Viral titers were determined by immunoperoxidase plaque assay. The data are representative of the results of at least three independent experiments with standard deviations. Student's *t* test: untreated versus MβCD treated, not significant (ns), $P = 0.095$; *, $P = 0.001$; **, $P = 0.004$; ***, $P = 0.013$. (B to D) Vero cells grown on coverslips were infected with HSV-1 K26GFP at an MOI of ~60 for 1 h in the presence of cycloheximide. Infected cells were mock treated (B), treated with 50 mM MβCD (C), or treated with 50 μM MG132 (D). The cells were incubated for an additional 1 h at 37°C and fixed for nuclear staining (blue). The images were captured at $\times 63$ magnification and are representative of the results of five independent experiments.

HSV-1 protein synthesis and viral DNA replication (29). To assess the effect of cholesterol on the expression of HSV-1 structural proteins, mock-infected or MβCD-treated cell lysates were subjected to SDS-PAGE and immunoblotted with an HSV polyclonal antibody. In MβCD-treated cells, there was an apparent decrease in the levels of some viral structural proteins relative to mock-treated cells (Fig. 3A), suggesting that cholesterol influences HSV-1 protein synthesis. Equivalent cell numbers yielded similar tubulin levels, suggesting that cholesterol reduction does not alter cell tubulin levels (Fig. 3B, top row). We examined the effect of cholesterol on the expression of individual HSV-1 proteins representing each of the different kinetic classes. The ratios of a specific protein to tubulin in mock- and MβCD-treated cells were quantitated by densitometry and compared (Fig. 3C to E). ICP4 was expressed in both MβCD-treated and mock-treated cells from 4 h postinfection (Fig. 3B, second row). The ratio of ICP4 to tubulin was decreased at 4 h postinfection by up to 5-fold in MβCD-treated cells relative to mock-treated cells but was similar at 6 h postinfection (Fig. 3B and C). At later time points, the proportion of ICP4 varied; however, in MβCD-treated cells, the greatest decrease in ICP4 levels reproducibly occurred at 4 h p.i. Expression of the HSV early protein ICP8 was detectable in both mock-treated and MβCD-treated cells from 8 h postinfection, suggesting that cholesterol reduction in infected cells does not delay the onset of ICP8 expression (Fig. 3B, third row, and D). However, ICP8 expression was decreased in MβCD-treated cells relative to untreated cells, particularly at 8 to 12 h postinfection. (Fig. 3D). The onset of expression of the HSV late protein VP16 was not delayed by MβCD treatment (Fig. 3E).

Cholesterol influences the maturation and release of infectious HSV-1. The enhanced difference in the yield of extracellular HSV-1 (2 log units) compared to the yield of total synthesized HSV-1 (1 log unit) in mock-treated cells relative to MβCD-

FIG 3 Cholesterol depletion affects the synthesis of viral proteins and the yield and release of infectious HSV-1. (A and B) Vero cells were infected with HSV-1 KOS (MOI = 5) for 1 h at 37°C. The cultures were mock treated (–) or treated with 50 mM MβCD (+) for 45 min at room temperature and then rinsed and replenished with serum-free medium. At 19 h p.i., (A) or the indicated times (B), lysates were separated by SDS-PAGE. The Western blots were probed with anti-HSV polyclonal antibody (A) or monoclonal antibodies to tubulin, ICP4, ICP8, and VP16 (B). (C to E) Densitometry of the HSV-1 proteins in panel B presented as the ratio of viral protein to tubulin. (F) Supernatant fractions or complete whole-cell lysates were collected at 19 h p.i., and viral titers were determined. The data are means of triplicate determinations with standard deviations and are representative of the results of at least three independent experiments. Numerical percentages of extracellular infectious HSV-1 are indicated. (G) DNA was extracted from infected cells that were treated with DNase or left untreated. Cell-associated HSV-1 genome copies were quantitated by qPCR. The data presented are means of the results of three independent experiments. Numerical percentages of copy numbers are indicated to the right of the bars. Student's *t* test; DNase resistance in untreated versus MβCD-treated cells; *P* = 0.032.

treated cells (Fig. 1A) suggested that cholesterol may also have an effect on the release of HSV-1 progeny. To address this, we assessed the effect of cell cholesterol reduction on the percentage of synthesized infectious HSV-1 that was released from infected cells. Extracellular virus (in the supernatant) was expressed as a percentage of the total virus synthesized (combined cell and supernatant fractions). At 19 h postinfection, the percentage of HSV-1 released from infected Vero cells that were treated with MβCD was up to 10-fold less than that of virus released from mock-treated cells (Fig. 3F), suggesting that cellular levels of cholesterol affect viral release. We further investigated whether cholesterol has an effect on the encapsidation of HSV-1. Genome copies of cell-associated HSV-1 were simultaneously quantitated in samples treated with DNase or left untreated. HSV-1 genome that was resistant to DNase represented viral DNA that had been surrounded by capsid. About 17.3% of the HSV-1 genome was resistant to DNase treatment in MβCD-treated cells (Fig. 3G) compared to ~55.2% of the HSV-1 genome that was resistant to DNase treatment in untreated cells. This finding suggests that cell cholesterol influences the packaging of the synthesized HSV-1 genome or a

FIG 4 Cholesterol is required for HSV-1 cell-to-cell spread. (A) Vero cells were infected as described for Fig. 1 and 2. The images were captured with the AMG Evos imaging system. (B and C) Plaque sizes were analyzed using Image J software (NIH). The data are means of at least 130 plaque measurements (AU, arbitrary units) from triplicate wells. The cumulative distributions of plaque sizes between treatment groups are significantly different (Kolmogorov-Smirnov test). *, $P < 0.001$.

step upstream. Taken together, the results suggest that cholesterol affects the genome encapsidation and subsequent release of infectious virions from infected cells.

Cell cholesterol is important for the cell-to-cell spread of HSV-1. The processes of HSV-1 entry and cell-to-cell spread share features in common but are not identical (30). For example, gE and gI are dispensable for HSV-1 entry but critical for cell-to-cell spread (31). To determine the effect of cell cholesterol on HSV-1 cell-to-cell spread, MβCD was added after virus penetration but prior to spread. When Vero cells had their cholesterol reduced after 4 h of infection, plaque sizes were reduced in a concentration-dependent manner (Fig. 4A-B). The reduction in plaque sizes was also observed following MβCD treatment at any time within the first 12 h of infection. Plaque sizes were partially refractory to MβCD treatment at 12 h postinfection and completely refractory to MβCD treatment at 23 h postinfection (Fig. 4C). When infected Vero cells were replenished with cholesterol at 4 h postinfection, plaques were partially restored to sizes similar to those seen in untreated cells (Fig. 4A and B). From these experiments, it is not clear whether cholesterol reduction affects the initially infected cells or the neighboring uninfected cells, which are the target of spread. The data suggest that cell cholesterol facilitates HSV-1 cell-to-cell spread.

Cholesterol is important for HSV-1 synthesis and cell-to-cell spread in a human epithelial cell line. To support our findings in a more relevant cell type, we used HaCaT cells, a nontransformed human keratinocyte line. HaCaT cells treated with MβCD remained viable at levels similar to those of untreated cells (data not shown). MβCD treatment of already infected HaCaT cells at 4 h postentry resulted in a dose-dependent reduction in HSV-1 plaques by as much as ~60% (Fig. 5A). Replenishment with exogenous cholesterol restored HSV-1 plaque numbers to mock-treated cell levels (Fig. 5A). MβCD treatment reduced HSV-1 plaque size in a concentration-dependent manner. When cholesterol was added back, plaque size was partially restored (Fig. 5B),

FIG 5 Cholesterol is important for HSV-1 replication in human epithelial cells. (A and B) Infected HaCaT cells (MOI = 0.0002 to 0.002) were treated under conditions similar to those described for Fig. 1B. (A) Viral titers (means of the results of at least three independent experiments with standard deviations). Student's *t* test; ns, *P* = 0.09; *, *P* = 0.005; **, *P* < 0.0001. (B) Plaque sizes from at least 400 plaque measurements (arbitrary units), representative of the results of at least three independent experiments. The cumulative distributions of plaque sizes between treatment groups are significantly different (Kolmogorov-Smirnov test). *, *P* < 0.005. (C and D) Supernatants or whole-cell lysates were collected at 19 to 24 h p.i., and viral titers were determined. Student's *t* test; *, *P* < 0.001. The data are the means of triplicate determinations with standard deviations and are representative of the results of three independent experiments. (D) The percentage of extracellular virus was determined by dividing the titers of supernatant fractions by the titers of suspension containing cells and supernatant.

consistent with a role for cholesterol in cell-to-cell spread of HSV-1. MβCD-treated, infected HaCaT cells produced >1 log unit less total virus and released ~2 log units less extracellular HSV-1 than mock-treated cells (Fig. 5C). The percentage of HSV-1 released from MβCD-treated, infected HaCaT cells was >10-fold less than the virus released from mock-treated cells (Fig. 5D). The results with human epithelial cells reflected those obtained with Vero cells. HSV-1 plaque sizes were similar in fibroblasts containing desmosterol only and in those containing cholesterol only (data not shown), suggesting that the saturated hydrocarbon tail of cholesterol is not important for cell-to-cell spread.

Cholesterol-free virions derived from DHCR24^{-/-} cells are infectious. Up to this point, we had chemically treated infected cells to assess the impact of cholesterol on the HSV-1 replicative cycle. To circumvent the possible pleiotropic effects of MβCD treatment, we employed cells with a genetic defect at a distinct step in cholesterol metabolism. DHCR24^{-/-} mouse embryonic fibroblasts lack the enzyme that converts desmosterol to cholesterol, allowing assessment of the role of the hydrocarbon tail of cholesterol (1, 32, 33). HSV-1 infects DHCR24^{-/-} fibroblasts, which contain only desmosterol. In fact, HSV-1 enters DHCR24^{-/-} and DHCR24^{+/+} fibroblasts at similar levels (1). We assessed whether the saturated hydrocarbon tail of cholesterol affects the synthesis of infectious extracellular HSV-1. HSV-1 infected DHCR24^{-/-} fibroblasts and produced extracellular virions that were titratable on Vero cells, indicating that these cholesterol-free virions retain infectivity (Fig. 6A). Thus, the native hydrocarbon tail of cholesterol in the viral envelope is not required for virion entry. Released cholesterol-free virions likely contain only desmosterol; they were propagated in cells that contain

FIG 6 Cholesterol-free virions derived from DHCR24^{-/-} cells are infectious. Confluent DHCR24^{-/-} and DHCR24^{+/+} cells were infected with HSV-1 strain KOS (MOI = 5) for 1 h at 37°C. The inoculum was removed, and the cells were rinsed and replenished with serum-free medium. (A) At the indicated times postinfection, the supernatant fraction was collected and the titer was determined on Vero cells. Each data point is the mean of triplicate samples with standard deviation. The data shown are representative of the results of three independent experiments. Student's *t* test; *, *P* < 0.01. (B) HSV-1 titers of supernatant only or whole-cell lysate and supernatant fractions were determined and expressed as the percentage of total infectious virus. (C) Copies of the HSV-1 genome in the supernatant were determined by qPCR. The means of three replicates plus standard deviations are shown. Student's *t* test; *, *P* < 0.001. (D) The specific infectivity of extracellular virus was determined by dividing the genome copies by the viral titers of each of the triplicate samples. Student's *t* test; not significant (ns), *P* = 0.421.

desmosterol only (32). HSV-1 plaques formed on DHCR24^{-/-} and DHCR24^{+/+} fibroblasts were similar in appearance (data not shown), suggesting that desmosterol can also support the cell-to-cell spread of HSV-1. This result further suggests that the synthesis and release of infectious HSV-1 can occur in the absence of cholesterol when desmosterol is present.

To assess the impact of the hydrocarbon tail of cholesterol on the yield of extracellular HSV-1 from infected cells, single-step growth of HSV-1 on DHCR24^{-/-} or DHCR24^{+/+} fibroblasts was analyzed. By 24 h postinfection, DHCR24^{+/+} fibroblasts released up to 1 log unit more infectious HSV-1 (HSV^{chol}) than was released from DHCR24^{-/-} cells (HSV^{des}) (Fig. 6A), suggesting an enhancing effect due to the hydrocarbon tail of cholesterol. The decreased yield of infectious HSV-1 from DHCR24^{-/-} fibroblasts may also suggest a defect in the release of the cholesterol-free HSV^{des}. To assess whether this was the case, extracellular virus was expressed as a percentage of the total virus synthesized. At 19 h postinfection, the percentages of HSV-1 released were similar for DHCR24^{-/-} (HSV^{des}; 9.7%) and DHCR24^{+/+} (HSV^{chol}; 8.9%) fibroblasts (Fig. 6B), suggesting that the double bond in the hydrocarbon tail of desmosterol does not affect viral release. As expected, HSV-1 was highly cell associated in these cells. The results together suggest that the native hydrocarbon tail of cholesterol influences the synthesis, but not the release, of HSV-1.

Infected cholesterol-containing cells yield more HSV-1 particles, yet virions derived from either desmosterol or cholesterol cells have similar specific infectivities. We further quantitated the amount of HSV-1 released and determined the specific infectivity of the viral particles from DHCR24^{-/-} and DHCR24^{+/+} fibroblasts by

FIG 7 Effect of envelope sterol on incorporation of virion proteins and on stability of HSV-1. Proteins from extracellular HSV-1 from infected DHCR24^{-/-} (HSV^{des}) and DHCR24^{+/+} (HSV^{chol}) fibroblasts were separated by SDS-PAGE. (A) Coomassie stain. (B) Western blots were probed with monoclonal antibodies for VP5, VP16, gB, gH, and gD. (C) HSV-1 samples (10^6 PFU) were suspended in 0.05% BSA-PBS. The samples were frozen rapidly and then thawed on ice. After each thaw, the viral titer was determined on Vero cells. This process was repeated three times. The infectivity of each virus at the first thaw was set to 100%. The data are means of the results of quadruplicate independent experiments with standard errors. The infectivity at each freeze-thaw cycle was not significantly (ns) different between HSV^{vero}, HSV^{des}, and HSV^{chol}. One-way analysis of variance (ANOVA); $P > 0.5$ for each.

quantitating viral genomes present in the supernatant of infected cells at 19 h postinfection. Approximately 50-fold more HSV^{chol} genomes were produced than HSV^{des} genomes (Fig. 6C; $P < 0.001$), suggesting that the presence of cholesterol supported enhanced viral synthesis. HSV^{des} and HSV^{chol} had similar specific infectivities (genome copy numbers per PFU) despite more HSV^{chol} particles than HSV^{des} particles being produced (Fig. 6D; $P = 0.421$), suggesting that the saturation in the C-24=C-25 bond in the hydrocarbon tail of cholesterol does not alter the relative infectivity of extracellular HSV-1. Taken together, the data (Fig. 6A to D) suggest that the saturated hydrocarbon tail of cholesterol enhances HSV-1 synthesis but not the release or specific infectivity of extracellular HSV-1.

Protein composition of virions propagated in cells lacking cholesterol. To determine whether the type of envelope sterol impacts virion protein levels, we analyzed the viral protein content of HSV^{des} and HSV^{chol}. Equivalent VP5 amounts of virions were analyzed by SDS-PAGE, followed by Coomassie staining or Western blotting for selected HSV-1 virion proteins. Similar HSV-1 protein ladders were detected by Coomassie stain in HSV^{des} and HSV^{chol} particles by protein staining (Fig. 7A). Similar levels of HSV-1 VP16, gD, gB, and gH were detected in HSV^{des} and HSV^{chol} (Fig. 7B). This was supported by densitometric analysis. Relative to VP5, VP16 and gB levels in HSV^{des} (1.12 and 1.43, respectively) were similar to those in HSV^{chol} (1.14 and 1.45, respectively) (Fig. 7B). gD and gH levels were comparable but slightly reduced in HSV^{des} (1.25 and 0.75, respectively) compared to HSV^{chol} (1.49 and 0.93, respectively) (Fig. 7B). Comparing equivalent PFU also revealed similar levels of viral proteins (data not shown). The data suggest that desmosterol is capable of supporting similar levels of viral protein incorporation into the HSV envelope and that the saturation in the hydrocarbon tail of cholesterol does not alter the protein composition of virions.

FIG 8 Synthesis of viral proteins in the absence of cholesterol. (A and B) DHCR24^{-/-} ("D") or DHCR24^{+/+} ("C") cells were infected with HSV-1 KOS (MOI = 5) as described in the legend to Fig. 6A. At 19 h p.i. (A) or the indicated times (B), lysates containing equivalent amounts of tubulin were separated by SDS-PAGE. The tubulin levels in DHCR24^{-/-} fibroblasts were 0- to 3-fold greater than in DHCR24^{+/+} fibroblasts in three independent experiments (data not shown). Western blots were probed with anti-HSV polyclonal antibody HR50 (A) or monoclonal antibodies against tubulin, ICP4, ICP8, and VP16 (B). The results shown are representative of two independent experiments. (C to E) ICP4 (C), ICP8 (D), and VP16 (E) ratios to tubulin from densitometry in panel B.

Virions propagated in cells containing desmosterol or cholesterol only have similar stability. Cholesterol is crucial for the optimal ordering of the phospholipid bilayers of cell membranes, so it may affect the stability of the HSV-1 envelope. Cellular membranes containing desmosterol only may have less order due to the altered hydrocarbon tail (34). To assess whether desmosterol affects the stability of the HSV-1 envelope, we tested the effect of multiple freeze-thaw treatments on HSV^{des}. The decrease in infectivity observed in HSV^{des} over the course of four freeze-thaw cycles was similar to the decrease observed in HSV^{chol} virions (Fig. 7C). Similar results were obtained for HSV-1 KOS propagated on Vero cells (Fig. 7C). The results suggest that the saturated hydrocarbon tail of cholesterol does not contribute to virion stability.

HSV-1 protein expression in cells lacking cholesterol. We probed further the decreased yield of HSV-1 observed in DHCR24^{-/-} fibroblasts (Fig. 6A and C) and assessed the impact of the hydrocarbon tail of cholesterol on HSV-1 protein synthesis. The levels of some HSV-1 structural proteins in infected DHCR24^{-/-} fibroblasts detected by the polyclonal antibody HR50 were decreased relative to DHCR24^{+/+} fibroblasts (Fig. 8A). This suggests that cholesterol impacts a step prior to or including viral protein synthesis. The two cell types support equivalent levels of HSV-1 entry (5). The expression of proteins representing the immediate-early (ICP4), early (ICP8), and late (VP16) classes of HSV-1 genes was determined by Western blotting (Fig. 8B). The ratio of a specific protein to tubulin was quantitated by densitometry. ICP4 was detectable in both DHCR24^{-/-} and DHCR24^{+/+} fibroblasts from 3 h postinfection (Fig. 8B, row 2). However, the ICP4-to-tubulin ratio was ~5-fold less in DHCR24^{-/-} (0.09) than in DHCR24^{+/+} fibroblasts (0.41) at 3 h postinfection. Expression of ICP8 was delayed in DHCR24^{-/-} fibroblasts relative to DHCR24^{+/+} fibroblasts at 7 h postinfection (Fig. 8B,

row 3), perhaps suggesting a delay in ICP8 activity. However, ICP8 levels became fairly similar in both cell types at later times of infection, suggesting that the initial impact of the hydrocarbon tail on ICP8 levels became less apparent as the infection progressed. VP16 expression in DHCR24^{-/-} fibroblasts was delayed by up to 4 h relative to DHCR24^{+/+} fibroblasts. VP16 levels were decreased in DHCR24^{-/-} fibroblasts by up to 5-fold from 11 to 19 h p.i. (Fig. 8B, row 3). Altogether, we propose that the hydrocarbon tail of cholesterol affects HSV-1 replication by impacting the expression of viral proteins, contributing to the decreased synthesis of mature virions.

DISCUSSION

Cholesterol, the end product of sterol biosynthesis, is a key component of cell membranes and is critical for cell growth and protein synthesis (35, 36). Cholesterol decreases membrane permeability while providing stability and is intimately involved in different replication stages of several viruses. Here, we demonstrate that well beyond the initial entry and arrival of viral capsids at the nucleus, cell cholesterol is important for HSV-1 replication and plaque formation.

M β CD treatment of cells depletes plasma membrane cholesterol but also decreases the cholesterol levels of intracellular membranes (37, 38). Drugs such as lovastatin have been used to lower cell cholesterol levels in virus-infected cells (39–41) by affecting intermediate steps in the cholesterol-biosynthetic pathway but function only after several hours. The HSV-1 replicative cycle is complete within 18 to 24 h, and the rapid action of M β CD in reducing cell cholesterol was better suited to this study.

When cholesterol levels are pharmacologically reduced in HSV-infected cells during the first 9 h p.i., there is a significant decrease in HSV infectivity by several measures. This is a period that coincides with the temporal expression of a subset of HSV-1 genes and the initiation of viral DNA replication (29). At points in this time frame, there was a concomitant decrease in the levels of the representative viral proteins ICP4 and ICP8 (Fig. 3A and 8A). Viruses, including HSV-1, hijack cellular translational processes to promote viral protein production (42, 43). It is possible that the sterol content or the reduced levels of cholesterol in internal membranes impact viral protein translation by membrane-bound polyribosomes used by HSV-1. Cholesterol may also directly or indirectly influence the transcription or function of HSV-1 proteins in manners yet to be determined.

M β CD treatment markedly reduced both plaque numbers and average HSV-1 plaque size in a concentration-dependent manner (Fig. 4B and 5B). Moreover, plaque sizes were partially restored upon addition of exogenous cholesterol, suggesting that cell-to-cell spread of HSV-1 is cholesterol dependent. Cell cholesterol modulation has been proposed to limit the progression of HIV infection in a subset of the infected population (44, 45). In nonprogressors, a reduction in cell cholesterol inhibited the ability of HIV-infected antigen-presenting cells to *trans*-infect susceptible T cells by interfering with the formation of a virological synapse (44, 45).

The yield of HSV-1 progeny was decreased in infected cells (MOI = 5) that contain desmosterol only or infected Vero cells treated with M β CD (Fig. 1A, 5C and 6A). A similar decrease in HSV-1 yield was observed in cells infected at an MOI of <1 (data not shown), consistent with cholesterol's role in viral production. It is unclear how cholesterol might affect nucleocapsid assembly of HSV-1 (Fig. 3G). However, reduced detection of DNase-resistant genomes may stem from the decreased levels of viral proteins expressed when cholesterol is reduced. Alternatively, since viral DNA packaging and capsid maturation are ATP dependent, reduction of cholesterol in infected cells may impact metabolism, which in turn reduces encapsidation. The virions released from cholesterol-free DHCR24^{-/-} fibroblasts were found to be infectious, with specific infectivity similar to that of cholesterol-containing particles, suggesting desmosterol and cholesterol play a shared basic sterol role in the viral envelope. Decreased HSV-1 derived from cells lacking cholesterol (Fig. 6A and C) suggests that the saturated hydrocarbon tail, although not necessary, enhances the sterol function of cholesterol in virus production.

Interestingly, there was a 10-fold decrease in the extracellular release of infectious HSV-1 from cholesterol-reduced Vero or HaCaT cells (Fig. 3F and 5D), but not from DHCR24^{-/-} fibroblasts (Fig. 6B). HSV-1 commandeers the cellular exocytic machinery for the release of mature virus (46–49). The role of cholesterol in the release and spread of HSV-1 might be explained partly by cholesterol-dependent vesicular exocytosis (50, 51). Desmosterol may also function in this context, which would explain the decrease in the release of HSV-1 from cholesterol-reduced cells, but not from DHCR24^{-/-} fibroblasts, which contain physiologically relevant levels of desmosterol. The cholesterol-free virions will be a valuable tool to evaluate the role of viral envelope sterols in HSV-1 entry.

MATERIALS AND METHODS

Cells and viruses. MEFs lacking the enzyme converting desmosterol to cholesterol, DHCR24 (DHCR24^{-/-} cells), were provided by Stacey Gilk, University of Indiana (32). MEFs were propagated in fibroblast basal medium supplemented with essential cell supplements (American Type Culture Collection [ATCC], Manassas, VA) under serum-free conditions. Vero cells (ATCC) and HaCaT cells (provided by Harvey Friedman, University of Pennsylvania) were propagated in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum (Life Technologies). HSV-1 strain KOS was provided by Priscilla Schaffer, Harvard Medical School, and HSV-1 KOSTk12, which contains the *lacZ* gene under the control of the HSV-1 ICP4 promoter, was provided by Patricia Spear, Northwestern University. HSV-1 KOS K26GFP contains green fluorescent protein (GFP) fused to the N terminus of the VP26 capsid protein (provided by Prashant Desai, Johns Hopkins University).

Cholesterol reduction and replenishment. Cells were treated with the indicated concentrations of M β CD (Sigma) in serum-free, sodium bicarbonate-free DMEM containing 20 mM HEPES for 45 min at room temperature. The cells were rinsed with warm phosphate-buffered saline (PBS) and were replenished either with serum-free medium or with medium supplemented with 200 μ g/ml water-soluble cholesterol (SyntheChol; Sigma) for 18 h.

Subcellular localization of entering GFP-tagged HSV. Vero cells were grown overnight on glass coverslips in 24-well dishes. The cells were pretreated with 0.5 mM cycloheximide for 15 min at 37°C and then rapidly chilled on ice (25). HSV-1 KOS K26GFP was spinoculated onto the cell surface by centrifugation at 200 $\times g$ for 1 h at 4°C (MOI, $\sim$ 60). Entry was initiated by shifting the cells to 37°C for 1 h in the continued presence of cycloheximide, followed by mock or M β CD treatment for 30 min at room temperature. For the MG132 control experiment, cells were infected in the presence of 25 μ M MG132 as previously described (28). The cells were washed with warm PBS and returned to 37°C for 1 h. The cells were washed three times with PBS and fixed in 3% paraformaldehyde-PBS. Nuclei were counterstained with 5 ng/ml of 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Roche). Coverslips were washed with PBS and mounted with Fluoromount G (Electron Microscopy Sciences, Hatfield, PA). Images were captured at $\times 63$ magnification with a Leica D4000 epifluorescence microscope and processed with Adobe Photoshop CS5.1 and Image J (NIH).

SDS-PAGE. HSV-1 was boiled for 5 min in Laemmli buffer with 200 mM dithiothreitol. Proteins were resolved by SDS-PAGE on 8% or 4 to 20% Tris-glycine gels (Invitrogen, Carlsbad, CA). The gels were fixed and stained with 0.025% Coomassie brilliant blue (Baker, Phillipsburg, NJ), 40% methanol, and 10% glacial acetic acid, followed by destaining with 30% methanol and 7% glacial acetic acid (52). The gel was dried and imaged with a GelDoc XR imager (Bio-Rad).

Immunoblotting. Virions or infected cell lysates were separated by SDS-PAGE on 4 to 20% Tris-glycine gels (Invitrogen). After transfer to nitrocellulose, the membranes were blocked and probed with the following mouse monoclonal antibodies: H1A02210 (1:2,000) for ICP4 (Virusys, Taneytown, MD), 11E2 (1:1,000) for ICP8 (Abcam, Cambridge, MA), H1359 (1:5,000) for gB (Virusys), DL6 (1:5,000) for gD (provided by Gary Cohen and Roselyn Eisenberg), HA108 (1:1,000) for VP5 (Virusys), SC7545 (1:20,000) for VP16 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), BBH1 (1:1,000) for gH (Abcam), and DM1A (1:20,000) for tubulin (Santa Cruz). After incubation with horseradish peroxidase-conjugated goat anti-mouse antibody (Pierce, Rockford, IL), enhanced chemiluminescence substrate (Pierce) was added, and the membranes were exposed to X-ray film (Genesee).

Quantitation of DNase-resistant HSV-1 genomes in infected cells. Cells were infected with HSV-1 KOS at an MOI of 5 for 1 h at 37°C, followed by treatment with 50 mM M β CD for 45 min at room temperature. Mock- or M β CD-treated cells were rinsed, replenished with serum-free medium, and incubated at 37°C. At 19 h p.i., each group of infected cells was collected in serum-free medium and $\sim 1 \times 10^4$ cells were treated with 2 μ g/ml Turbo DNase (Bio-Rad) or mock treated. Viral DNA was extracted using the QIAamp Blood DNA kit (Qiagen), and HSV-1 genome copies were quantitated by quantitative PCR (qPCR) as described below. DNase-resistant and DNase-sensitive fractions were expressed relative to total HSV-1 genomes in a non-DNase-treated sample, which was set to 100%.

Determination of HSV-1 genome copy number by real-time PCR. Samples were treated with 2 μ g/ml DNase (Bio-Rad), and viral genomic DNA was extracted using a QIAamp Blood DNA kit (Qiagen, Valencia, CA). HSV-1 transcripts were quantitated using the CFX96 Real-Time PCR detection system (Bio-Rad). Primers were based on the KOS ICP22 sequence, (forward, 5'-GAG TTT GGG GAG TTT G-3', and reverse, 5'-GGC AGG CGG TGG AGA A-3') (53, 54). A standard curve for the assay was generated using known quantities of a plasmid containing the HSV-1 ICP22 coding region diluted in glycogen.

Single-step growth assay. DHCR24^{-/-} or DHCR24^{+/+} cells were grown in 6-well plates in triplicate wells under serum-free conditions. HSV-1 KOS (MOI = 5) was added for 1 h at 37°C. The virus inoculum was removed, and the cells were rinsed with warm PBS, replenished with fresh medium, and incubated at 37°C (time zero). At the indicated times postinfection, supernatants and infected cells were collected from triplicate wells and the titer was determined in triplicate on Vero cells. The HSV-1 genome copy number was determined by qPCR.

Determination of virion stability. HSV-1 (10⁶ PFU) in culture medium containing 0.05% bovine serum albumin (BSA) was rapidly frozen in a dry ice-ethanol bath and then thawed on ice. The viral titer was immediately determined, or samples were subjected to a total of 2 to 4 freeze-thaw cycles prior to determining the titer. Titers are indicated relative to the first freeze-thaw, which was set to 100%.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AI119159 and AI007025 from the National Institute of Allergy and Infectious Diseases.

We thank Jean Celli, Glen Scoles, and Kathy Mason for the use of microscopes; David Schneider for help with statistical analysis; and Gary Cohen, Prashant Desai, Roselyn Eisenberg, Harvey Friedman, Stacey Gilk, Priscilla Schaffer, and Patricia Spear for the gifts of reagents.

REFERENCES

- Wudiri GA, Pritchard SM, Li H, Liu J, Aguilar HC, Gilk SD, Nicola AV. 2014. Molecular requirement for sterols in herpes simplex virus entry and infectivity. *J Virol* 88:13918–13922. <https://doi.org/10.1128/JVI.01615-14>.
- Bender FC, Whitbeck JC, Ponce de Leon M, Lou H, Eisenberg RJ, Cohen GH. 2003. Specific association of glycoprotein B with lipid rafts during herpes simplex virus entry. *J Virol* 77:9542–9552. <https://doi.org/10.1128/JVI.77.17.9542-9552.2003>.
- Desplanques AS, Nauwynck HJ, Vercauteren D, Geens T, Favoreel HW. 2008. Plasma membrane cholesterol is required for efficient pseudorabies virus entry. *Virology* 376:339–345. <https://doi.org/10.1016/j.virol.2008.03.039>.
- Favoreel HW, Van den Broeke C, Desplanques A, Deruelle M, Van Minnebruggen G, Nauwynck H, Glorieux S, Van Opdenbosch N, De Regge N. 2010. Alpha herpesvirus use and misuse of cellular actin and cholesterol. *Vet Microbiol* 143:2–7. <https://doi.org/10.1016/j.vetmic.2010.02.007>.
- Rahn E, Petermann P, Hsu MJ, Rixon FJ, Knebel-Morsdorf D. 2011. Entry pathways of herpes simplex virus type 1 into human keratinocytes are dynamin- and cholesterol-dependent. *PLoS One* 6:e25464. <https://doi.org/10.1371/journal.pone.0025464>.
- Hambleton S, Steinberg SP, Gershon MD, Gershon AA. 2007. Cholesterol dependence of varicella-zoster virion entry into target cells. *J Virol* 81:7548–7558. <https://doi.org/10.1128/JVI.00486-07>.
- Yeagle P. 1993. The membranes of cells, 2nd ed. Academic Press, San Diego, CA.
- Goluszko P, Nowicki B. 2005. Membrane cholesterol: a crucial molecule affecting interactions of microbial pathogens with mammalian cells. *Infect Immun* 73:7791–7796. <https://doi.org/10.1128/IAI.73.12.7791-7796.2005>.
- Ikonen E. 2008. Cellular cholesterol trafficking and compartmentalization. *Nat Rev Mol Cell Biol* 9:125–138. <https://doi.org/10.1038/nrm2336>.
- Mukherjee S, Zha X, Tabas I, Maxfield FR. 1998. Cholesterol distribution in living cells: fluorescence imaging using dehydroergosterol as a fluorescent cholesterol analog. *Biophys J* 75:1915–1925. [https://doi.org/10.1016/S0006-3495\(98\)77632-5](https://doi.org/10.1016/S0006-3495(98)77632-5).
- Nicola AV. 2016. Herpesvirus entry into host cells mediated by endosomal low pH. *Traffic* 17:965–975. <https://doi.org/10.1111/tra.12408>.
- Nicola AV, McEvoy AM, Straus SE. 2003. Roles for endocytosis and low pH in herpes simplex virus entry into HeLa and Chinese hamster ovary cells. *J Virol* 77:5324–5332. <https://doi.org/10.1128/JVI.77.9.5324-5332.2003>.
- Barrow E, Nicola AV, Liu J. 2013. Multiscale perspectives of virus entry via endocytosis. *Virol J* 10:177. <https://doi.org/10.1186/1743-422X-10-177>.
- Albulescu L, Wubbolds R, van Kuppeveld FJ, Strating JR. 2015. Cholesterol shuttling is important for RNA replication of coxsackievirus B3 and encephalomyocarditis virus. *Cell Microbiol* 17:1144–1156. <https://doi.org/10.1111/cmi.12425>.
- Chang TH, Segovia J, Sabbah A, Mgbemena V, Bose S. 2012. Cholesterol-rich lipid rafts are required for release of infectious human respiratory syncytial virus particles. *Virology* 422:205–213. <https://doi.org/10.1016/j.virol.2011.10.029>.
- Keller P, Simons K. 1998. Cholesterol is required for surface transport of influenza virus hemagglutinin. *J Cell Biol* 140:1357–1367. <https://doi.org/10.1083/jcb.140.6.1357>.
- Lu YE, Cassese T, Kielian M. 1999. The cholesterol requirement for Sindbis virus entry and exit and characterization of a spike protein region involved in cholesterol dependence. *J Virol* 73:4272–4278.
- Marquardt MT, Phalen T, Kielian M. 1993. Cholesterol is required in the exit pathway of Semliki Forest virus. *J Cell Biol* 123:57–65. <https://doi.org/10.1083/jcb.123.1.57>.
- Ilnytska O, Santiana M, Hsu NY, Du WL, Chen YH, Viktorova EG, Belov G, Brinker A, Storch J, Moore C, Dixon JL, Altan-Bonnet N. 2013. Enteroviruses harness the cellular endocytic machinery to remodel the host cell cholesterol landscape for effective viral replication. *Cell Host Microbe* 14:281–293. <https://doi.org/10.1016/j.chom.2013.08.002>.
- Manes S, del Real G, Lacalle RA, Lucas P, Gomez-Mouton C, Sanchez-Palomino S, Delgado R, Alcamí J, Mira E, Martinez AC. 2000. Membrane raft microdomains mediate lateral assemblies required for HIV-1 infection. *EMBO Rep* 1:190–196. <https://doi.org/10.1093/embo-reports/kvd025>.
- Lorzate M, Krausslich HG. 2011. Role of lipids in virus replication. *Cold Spring Harb Perspect Biol* 3:a004820. <https://doi.org/10.1101/cshperspect.a004820>.
- Medigeshe GR, Hirsch AJ, Streblow DN, Nikolich-Zugich J, Nelson JA. 2008. West Nile virus entry requires cholesterol-rich membrane microdomains and is independent of alphavbeta3 integrin. *J Virol* 82:5212–5219. <https://doi.org/10.1128/JVI.00008-08>.
- Mettenleiter TC, Klupp BG, Granzow H. 2006. Herpesvirus assembly: a tale of two membranes. *Curr Opin Microbiol* 9:423–429. <https://doi.org/10.1016/j.mib.2006.06.013>.
- Mettenleiter TC, Muller F, Granzow H, Klupp BG. 2013. The way out: what we know and do not know about herpesvirus nuclear egress. *Cell Microbiol* 15:170–178. <https://doi.org/10.1111/cmi.12044>.
- Delboy MG, Roller DG, Nicola AV. 2008. Cellular proteasome activity facilitates herpes simplex virus entry at a postpenetration step. *J Virol* 82:3381–3390. <https://doi.org/10.1128/JVI.02296-07>.
- Sodeik B, Ebersold MW, Helenius A. 1997. Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus. *J Cell Biol* 136:1007–1021. <https://doi.org/10.1083/jcb.136.5.1007>.
- Weed DJ, Pritchard SM, Gonzalez F, Aguilar HC, Nicola AV. 2017. Mildly acidic pH triggers an irreversible conformational change in the fusion domain of herpes simplex virus 1 glycoprotein B and inactivation of viral entry. *J Virol* 91:e02123-16. <https://doi.org/10.1128/JVI.02123-16>.
- Delboy MG, Nicola AV. 2011. A pre-immediate-early role for tegument ICP0 in the proteasome-dependent entry of herpes simplex virus. *J Virol* 85:5910–5918. <https://doi.org/10.1128/JVI.00267-11>.
- Lehman IR, Boehmer PE. 1999. Replication of herpes simplex virus DNA. *J Biol Chem* 274:28059–28062. <https://doi.org/10.1074/jbc.274.40.28059>.
- Mettenleiter TC. 1994. Initiation and spread of alpha-herpesvirus infec-

- tions. *Trends Microbiol* 2:2–4. [https://doi.org/10.1016/0966-842X\(94\)90335-2](https://doi.org/10.1016/0966-842X(94)90335-2).
31. Dingwell KS, Brunetti CR, Hendricks RL, Tang Q, Tang M, Rainbow AJ, Johnson DC. 1994. Herpes simplex virus glycoproteins E and I facilitate cell-to-cell spread in vivo and across junctions of cultured cells. *J Virol* 68:834–845.
 32. Gilk SD, Cockrell DC, Luterbach C, Hansen B, Knodler LA, Ibarra JA, Steele-Mortimer O, Heinzen RA. 2013. Bacterial colonization of host cells in the absence of cholesterol. *PLoS Pathog* 9:e1003107. <https://doi.org/10.1371/journal.ppat.1003107>.
 33. Wechsler A, Brafman A, Shafir M, Heverin M, Gottlieb H, Damari G, Gozlan-Kelner S, Spivak I, Moshkin O, Fridman E, Becker Y, Skaliter R, Einat P, Faerman A, Bjorkhem I, Feinstein E. 2003. Generation of viable cholesterol-free mice. *Science* 302:2087. <https://doi.org/10.1126/science.1090776>.
 34. Vainio S, Jansen M, Koivusalo M, Rog T, Karttunen M, Vattulainen I, Ikonen E. 2006. Significance of sterol structural specificity. Desmosterol cannot replace cholesterol in lipid rafts. *J Biol Chem* 281:348–355.
 35. Martinez-Botas J, Suarez Y, Ferruelo AJ, Gomez-Coronado D, Lasuncion MA. 1999. Cholesterol starvation decreases p34(cdc2) kinase activity and arrests the cell cycle at G₂. *FASEB J* 13:1359–1370.
 36. Sinensky M, Beck LA, Leonard S, Evans R. 1990. Differential inhibitory effects of lovastatin on protein isoprenylation and sterol synthesis. *J Biol Chem* 265:19937–19941.
 37. Mahammad S, Dinic J, Adler J, Parmryd I. 2010. Limited cholesterol depletion causes aggregation of plasma membrane lipid rafts inducing T cell activation. *Biochim Biophys Acta* 1801:625–634. <https://doi.org/10.1016/j.bbalip.2010.02.003>.
 38. Mahammad S, Parmryd I. 2008. Cholesterol homeostasis in T cells. Methyl-beta-cyclodextrin treatment results in equal loss of cholesterol from Triton X-100 soluble and insoluble fractions. *Biochim Biophys Acta* 1778:1251–1258. <https://doi.org/10.1016/j.bbame.2008.02.010>.
 39. Bryan-Marrugo OL, Arellanos-Soto D, Rojas-Martinez A, Barrera-Saldana H, Ramos-Jimenez J, Vidaltamayo R, Rivas-Estilla AM. 2016. The antitumor virus properties of statins may be associated with alterations in the cellular antiviral profile expression. *Mol Med Rep* 14:2155–2163. <https://doi.org/10.3892/mmr.2016.5519>.
 40. Li D, Li Y, Hernandez JA, Patenia R, Kim TK, Khalili J, Dougherty MC, Hanley PJ, Bollard CM, Komanduri KV, Hwu P, Champlin RE, Radvanyi LG, Molldrem JJ, Ma Q. 2010. Lovastatin inhibits T-cell proliferation while preserving the cytolytic function of EBV, CMV, and MART-1-specific CTLs. *J Immunother* 33:975–982. <https://doi.org/10.1097/CJI.0b013e3181fb0486>.
 41. Ponroy N, Taveira A, Mueller NJ, Millard AL. 2015. Statins demonstrate a broad anti-cytomegalovirus activity in vitro in ganciclovir-susceptible and resistant strains. *J Med Virol* 87:141–153. <https://doi.org/10.1002/jmv.23998>.
 42. Ellison KS, Maranchuk RA, Mottet KL, Smiley JR. 2005. Control of VP16 translation by the herpes simplex virus type 1 immediate-early protein ICP27. *J Virol* 79:4120–4131. <https://doi.org/10.1128/JVI.79.7.4120-4131.2005>.
 43. Larralde O, Smith RW, Wilkie GS, Malik P, Gray NK, Clements JB. 2006. Direct stimulation of translation by the multifunctional herpesvirus ICP27 protein. *J Virol* 80:1588–1591. <https://doi.org/10.1128/JVI.80.3.1588-1591.2006>.
 44. Prasad VR, Bukrinsky MI. 2014. New clues to understanding HIV nonprogressors: low cholesterol blocks HIV trans infection. *mBio* 5:e01396–01314. <https://doi.org/10.1128/mBio.01396-14>.
 45. Rappocciolo G, Jais M, Piazza P, Reinhart TA, Berendam SJ, Garcia-Exposito L, Gupta P, Rinaldo CR. 2014. Alterations in cholesterol metabolism restrict HIV-1 trans infection in nonprogressors. *mBio* 5:e01031–01013. <https://doi.org/10.1128/mBio.01031-13>.
 46. Hogue IB, Scherer J, Enquist LW. 2016. Exocytosis of alphaherpesvirus virions, light particles, and glycoproteins uses constitutive secretory mechanisms. *mBio* 7:e00820–16. <https://doi.org/10.1128/mBio.00820-16>.
 47. Mingo RM, Han J, Newcomb WW, Brown JC. 2012. Replication of herpes simplex virus: egress of progeny virus at specialized cell membrane sites. *J Virol* 86:7084–7097. <https://doi.org/10.1128/JVI.00463-12>.
 48. Johns HL, Gonzalez-Lopez C, Sayers CL, Hollinshead M, Elliott G. 2014. Rab6 dependent post-Golgi trafficking of HSV1 envelope proteins to sites of virus envelopment. *Traffic* 15:157–178. <https://doi.org/10.1111/tra.12134>.
 49. Johnson DC, Spear PG. 1982. Monensin inhibits the processing of herpes simplex virus glycoproteins, their transport to the cell surface, and the egress of virions from infected cells. *J Virol* 43:1102–1112.
 50. Linetti A, Fratangeli A, Taverna E, Valnegri P, Francolini M, Cappello V, Matteoli M, Passafaro M, Rosa P. 2010. Cholesterol reduction impairs exocytosis of synaptic vesicles. *J Cell Sci* 123:595–605. <https://doi.org/10.1242/jcs.060681>.
 51. Wasser CR, Ertunc M, Liu X, Kavalali ET. 2007. Cholesterol-dependent balance between evoked and spontaneous synaptic vesicle recycling. *J Physiol* 579:413–429. <https://doi.org/10.1113/jphysiol.2006.123133>.
 52. Delboy MG, Siekavizza-Robles CR, Nicola AV. 2010. Herpes simplex virus tegument ICP0 is capsid associated, and its E3 ubiquitin ligase domain is important for incorporation into virions. *J Virol* 84:1637–1640. <https://doi.org/10.1128/JVI.02041-09>.
 53. Walker EB, Pritchard SM, Cunha CW, Aguilar HC, Nicola AV. 2015. Polyethylene glycol-mediated fusion of herpes simplex type 1 virions with the plasma membrane of cells that support endocytic entry. *Virol J* 12:190. <https://doi.org/10.1186/s12985-015-0423-0>.
 54. Komala Sari T, Pritchard SM, Cunha CW, Wudiri GA, Laws EI, Aguilar HC, Taus NS, Nicola AV. 2013. Contributions of herpes simplex virus 1 envelope proteins to entry by endocytosis. *J Virol* 87:13922–13926. <https://doi.org/10.1128/JVI.02500-13>.