Differential Incorporation of Cholesterol by Sindbis Virus Grown in Mammalian or Insect Cells

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Cholesterol has been shown to be essential for the fusion of alphaviruses with artificial membranes (liposomes). Cholesterol has also been implicated as playing an essential and critical role in the processes of entry and egress of alphaviruses in living cells. Paradoxically, insects, the alternate host for alphaviruses, are cholesterol auxotrophs and contain very low levels of this sterol. To further evaluate the role of cholesterol in the life cycle of alphaviruses, the cholesterol levels of the alphavirus Sindbis produced from three different mosquito (Aedes albopictus) cell lines; one other insect cell line, S21 from Spodoptera frugiperda; and BHK (mammalian) cells were measured. Sindbis virus was grown in insect cells under normal culture conditions and in cells depleted of cholesterol by growth in serum delipidated by using Cab-O-sil, medium treated with methyl-β-cyclodextrin, or serum-free medium. The levels of cholesterol incorporated into the membranes of the cells and into the virus produced from these cells were determined. Virus produced from these treated and untreated cells was compared to virus grown in BHK cells under standard conditions. The ability of insect cells to produce Sindbis virus after delipidiation was found to be highly cell specific and not dependent on the level of cholesterol in the cell membrane. A very low level of cholesterol was required for the generation of wild-type levels of infectious Sindbis virus from delipidated cells. The data show that one role of the virus membrane is structural, providing the stability required for infectivity that may not be provided by the delipidated membranes in some cells. These data show that the amount of cholesterol in the host cell membrane in and of itself has no effect on the process of virus assembly or on the ability of virus to infect cells. Rather, these data suggest that the cholesterol dependence reported for infectivity and assembly of Sindbis virus is a reflection of differences in the insect cell lines used and the methods of delipidation.

Sindbis virus, the prototypic Alphavirus, assembles highly symmetrical particles with an associated membrane of host cell origin. The infectious particle is composed of two nested icosahedral shells of T=4 geometry with an intervening membrane bilayer (41). The three structural proteins which comprise the particle, E1, E2, and capsid, are found in a 1:1:1 stoichiometric ratio. The outer shell, composed of glycoproteins E1 and E2, and the nucleocapsid are associated with the outer protein shell through specific interactions of the E2 endodomain with the capsid protein (28–30, 53). Both E1 and E2 are anchored into the membrane bilayer by transmembrane domains (44). During maturation of the virus, the glycoproteins E1 and E2 are processed and oligomerize into trimers of heterodimers and are delivered to the cell surface by the cellular exocytic pathway (6, 39). In mammalian cells, the glycoproteins are trafficked to the plasma membrane to unite with preformed nucleocapsids (5, 12). The maturation pathway used by this virus in insect cells is also via the exocytic pathway; however, in these cells, the virus particles assemble within cytoplasmic vesicles which release virus directly into the extracellular medium (4, 38). It has been assumed that while the assembly pathway of Sindbis virus differs in certain details during the infection process in these divergent hosts, the molecular details of assembly result in functionally equivalent virus structures (49). Indeed, Sindbis virus is a chimeric structure: the protein and the RNA are specified by the viral genome, while the nature of the lipid in the virus is determined largely by the host cell.

Recent genetic analysis has further demonstrated the chimeric nature of the alphaviruses at the molecular level and has reemphasized the importance of the host-derived membrane as a structural component of the virus particle. Deletions in the protein domain that engages the membrane result in changes in the infectivity of the virus, while having a lesser effect on the process of assembly. The deletions also affect the ability of the virus to assemble in the insect and mammalian hosts (17, 18, 56). These data suggest that the composition of the host cell membrane plays a critical role in the assembly of the virus. The amount of cholesterol in the membrane has been suggested to be critical for both the assembly of virus and the ability of the virus to infect cells (25, 31, 34, 43). To further test this theory, an investigation into the effect of the amount of cholesterol incorporated into the viral membrane on virus infection of and virus production from an insect or mammalian host was warranted.

Our model for how these truncated proteins retain the ability to assemble into infectious particles in insect membranes invokes membrane thickness as a primary factor in the stability of shortened membrane anchor domains in the lipid bilayer. This hypothesis was put forth because of the evidence that the high cholesterol content found in mammalian cells can increase membrane thickness and alter physical properties, such as ion permeability and viscosity (36). Cholesterol in the eukaryotic membrane enhances acyl chain packing of the phos-
pholipids, increases mechanical strength, and reduces permeability (57). Insects cannot synthesize cholesterol de novo and depend on dietary cholesterol for their physiological requirements (27). As cholesterol auxotrophs, insect cells in culture can withstand a significant level of cholesterol depletion (47); however, in the presence of serum, they will incorporate cholesterol (37). In the present study, we have used wild-type Sindbis virus specifically to investigate the ability of this virus to incorporate cholesterol into the viral membrane when grown under standard conditions and under conditions of lipid depletion. To address this question, Sindbis virus was grown in BHK or mosquito U4.4, C7-10, or C6/36 cells under conditions of high levels of free lipid and various conditions of lipid depletion and in an additional insect cell line, Sf21, grown under serum-free conditions.

MATERIALS AND METHODS

Cell culture, plaque assay, and virus purification. BHK-21 and mosquito cell lines, plaque assay, and virus purification are described in detail in reference 19. The U4.4 cell line, adapted to growth in minimal essential medium containing Earle’s salts (MEM-E) supplemented with 10% fetal bovine serum (FBS) (lot no. 18779, 1.1 mg/ml cholesterol; HyClone, Logan, UT), 5% tryptose phosphate broth, and 5 mM glutamine is a subclone of the U4.4 cell line propagated in M and M medium and described in reference 46. The cell lines U4.4, C6/36, and C7-10 (35) were selected for these experiments because of their different phenotypes and are derived from the original Singh isolate (22). While derived from a common source, these cells respond very differently to Sindbis virus infection (4, 21, 22, 35), and we anticipated that they might respond differently to delipidated serum. These MEM-E-adapted cells were depleted of cholesterol by the addition of methyl-β-cyclodextrin (MβCD) (C-3555; Sigma, St. Louis, MO) (7, 8, 13). MβCD-treated mosquito cells were propagated in MEM-E as described above, with the addition of MβCD to the measured serum cholesterol at a molar ratio of 8:1 (for FBS lot no. 18779, cholesterol was 1.1 mg/ml). The mosquito cells were also adapted to medium containing serum delipidated with Cab-O-sil (COS) (Cabot Corp., Billerica, MA) by using the method described in reference 52. Using COS, the cholesterol concentration of the delipidated serum was between 3 and 6 µg/ml in the range reported previously (43). Additional lipid depletion was conducted by adapting the mosquito cells to SF-900 II SFM (Invitrogen, Carlsbad, CA), a serum-free medium (SF-M) formulation (6.3 µg/ml of cholesterol as determined by an Amplex red cholesterol assay) (36). The cells were adapted to these reduced-serum conditions for a minimum of four passages and are referred to as U4.4-, C6/36-, and C7-10 (MβCD) treated), -COS, or -SF-M, respectively, and were only subjected to limited passage. Sf21 cells (Invitrogen, Carlsbad, CA) are a clonal isolate derived from Spodoptera frugiperda insect cells and are chosen because it has been shown to produce the lowest particle-to-PFU ratios from insect cells in culture under conditions of cholesterol depletion. We grew wild-type Sindbis virus in BHK and mosquito U4.4, C6/36, and C7-10 cells under standard conditions.

Electrospray ionization tandem mass spectrometry lipid profiling. COS was used to delipidate serum as described in reference 52. The COS residue obtained after centrifugation from serum was collected and rinsed five times with PBS-D. Cholesterol and lipids bound to the COS were extracted by using the modified Bligh and Dyer protocol as described above. Lipid extracts were analyzed at the Kansas Lipidomics Center, Kansas State University. An automated electrospray ionization tandem mass spectrometry approach was used, and data acquisition analysis and group identifications were carried out as described previously (1), with modifications.

RESULTS

Growth of wild-type Sindbis virus in mammalian or insect cells under standard conditions. Sindbis virus grows efficiently to 10^10 PFU/insect in adult mosquitoes (3), which cannot synthesize cholesterol (26) and contain very low levels of cholesterol (9, 37, 45). These data led us to expect that Sindbis virus would be able to assemble efficiently into infectious virus particles from insect cells in culture under conditions of cholesterol depletion. We grew wild-type Sindbis virus in BHK and mosquito U4.4, C6/36, and C7-10 cells (Fig. 1) under standard tissue culture conditions and in Sf21 cells grown in SFM as described in Materials and Methods. Wild-type Sindbis virus grown under conditions of cholesterol abundance was measured by plaque assay (see Materials and Methods). Sf21 cells were chosen as an alternate insect line because these cells grow in semidefined medium in very low levels of cholesterol; they were used also as a control for the effects of any chemical depletion of lipids in the mosquito cell system. The control (nondelipidated) cells were found to produce infectious wild-type Sindbis virus to approximately the same levels (∼10^10 PFU/ml), with the exception of the U4.4 line, which produces ∼10-fold less virus than the other aedine cells (Fig. 1). These results are similar to those we have published previously (35), and the results presented in Fig. 1 served as control values for the experiments that follow.

Growth of wild-type Sindbis virus in delipidated insect cells. Wild-type Sindbis virus was grown in BHK or mosquito cells depleted of cholesterol by growth in MβCD, COS, or SFM (see Materials and Methods) and in Sf21 insect cells grown in
defined medium without serum. The levels of virus released into the medium of delipidated cells are shown in Fig. 2. After adaptation of the cells to the appropriate growth medium, cells were infected at a multiplicity of infection of 10 to 50 PFU/cell and incubated at 28°C. Virus was harvested at the times indicated on the graphs. For U4.4 (Fig. 2A), C6/36 (Fig. 2B), and Sf21 cells (shown in Fig. 2A with U4.4 cell-grown virus), peak virus growth was seen at 48 h postinfection. C7-10 cells however, showed peak virus production at 72 h postinfection both for delipidated and nondelipidated cells. The most significant finding in this experiment is that only C6/36 cells (Fig. 2B) showed diminished virus production when grown in serum delipidated with COS, CD, or SFM. In U4.4 cells (Fig. 2A), the only delipidation method which affected virus production was growth in SFM. No significant differences in titers from those of the nondelipidated control cells were seen for any of the other treatments. Conversely, for C7-10 cells, delipidation had no effect on virus production under any growth conditions. Thus, of all the cell lines tested, the C6/36 cells are the most sensitive to lipid depletion. To investigate if the loss of virus titer from delipidated cells was from a block in virus assembly or the production of noninfectious particles, the particle-to-PFU ratio of virus from each nondelipidated and delipidated cell line was determined.

Purified virus titers and particle-to-PFU ratios. The particle-to-PFU ratio is an indicator of the portion of a virus population which is noninfectious. Virus grown in each of the cell lines tested, under standard and delipidated conditions, was gradient purified in potassium tartrate gradients using isopycnic centrifugation (as described in Materials and Methods). The titer of the purified virus was then determined by plaque assay on BHK cells, and the protein content was also measured, using a BCA assay. These results are shown in Fig. 3. These data demonstrate that when virus grown under standard conditions is gradient purified, there is little loss of infectious virus as determined by particle-to-PFU ratios. In contrast, when virus is concentrated after production in delipidated cells, virus titers are greatly reduced, indicating that virus produced from delipidated cells is structurally fragile. Wild-type virus grown in BHK, U4.4, C6/36, and C7-10 cells all gave titers above $10^{12}$ PFU/ml after gradient purification. Assay of the particle-to-PFU ratio of virus purified from the control nondelipidated cells revealed that the virus contained noninfectious virus in a range from 5 to 50 particles/PFU, as previously reported (16, 18). Titers of purified virus grown in delipidated insect cells correlated with the titers from the growth curve experiments (compare titers to those of virus from supernatants shown in Fig. 2) for U4.4CD and -COS and Sf21 cells. The titers of purified virus from the remaining delipidated cells were lower, in the range of $10^{11}$ to $10^{10}$ PFU/ml for C6/36/COS to $10^{9}$ PFU/ml for C7-10/COS and -SFM. With the exception of virus from U4.4CD, however, all virus from the delipidated cells showed an increased particle-to-PFU ratio, ranging from 500 for U4.4/COS to $10^6$ for C7-10/COS (Fig. 3). Because the only difference in the infections was the delipidated state of the respective insect cells, it is reasonable to suggest that the large numbers of noninfectious particles could be ascribed to a change in the membrane composition that resulted in an inability of the virus to survive the purification process without loss of infectivity of many of the particles. These changes affected the production of virus in the seven delipidated cell lines tested and the adapted Sf21 cells (see Fig. 3). Biochemical differences in the membrane composition could induce instability of the metastable virus structure, increasing the particle-to-PFU ratio. Virus from U4.4 and C6/36 cells grown in SFM did not survive the purification process, precluding further analysis. For all the other virus produced from delipidated cells, the total particles/ml were between $10^{13}$ and $10^{14}$ PFU/ml, demonstrating that the cells are assembling approximately the same numbers of total particles as the control cells. This observation makes it reasonable to ascribe the large numbers of noninfectious particles from some cell lines to a defect(s) affecting infectivity. To determine if the increase in noninfectious particles from the delipidated cells was the result of a lack...
FIG. 2. Growth curves of SVHR grown in nondelipidated and delipidated medium. (A) Growth curves of SVHR grown in U4.4, U4.4COS, U4.4CD, U4.4SFM, and Sf21 cells. The virus titers ($10^9$ PFU/ml) were similar for all the mosquito cells, with the exception of those grown in SFM ($10^8$ PFU/ml). The titers for the Sf21 cell line are shown in this graph for comparison. Sf21 virus grew to $10^{10}$ PFU/ml in SFM. All cultures
of cholesterol in the virion, cholesterol/protein levels were determined.

Cholesterol contents of whole cells and purified wild-type Sindbis virus. The largest significant difference in the compositions of mammalian and insect membranes is the amount of cholesterol found in the mammalian host or insect vector. Sindbis virus grown in the cell lines was gradient purified twice, and the cholesterol content was determined by using an Amplex red cholesterol assay (Materials and Methods). As shown in Fig. 4, the amount of cholesterol measured from the control nondelipidated cells roughly reflected the amount found in the purified virus from those respective cells. These values are shown as the percentages of the control BHK level (100%, the largest amount of cholesterol) normalized to the protein content as a weight/weight measurement. The levels of cholesterol content in the control insect cells were found to be U4.4CD > C7-10 > C6/36 > SF21. Comparison of cells delipidated with MβCD to those delipidated with COS shows that cells in MβCD contained more cholesterol than those grown in COS, perhaps reflecting the different mechanisms of delipidation. In the case of MβCD, the sequestered lipids are still in the medium, whereas COS removes the lipids from the serum. The data show that the insect cells treated with serum delipidated with COS had very low levels of cholesterol, as did the viruses that were produced from these cells. If the presence of cholesterol in the virus particle was the only limiting factor in the infectivity of the virus, then it would be expected that the virus with the least cholesterol would have the highest particle-to-PFU ratio. Of all the virus tested, virus grown from C7-10SFM contained the least amount of cholesterol (0.04%, wt/wt), although the titer of this virus did not differ significantly from that of the nondelipidated C7-10 control and this virus was not found to have the highest particle-to-PFU ratio. The observation that the amount of cholesterol in the virus particles did not correlate with the numbers of noninfectious particles suggested that some other component may be limiting and that this component affects virus infectivity, possibly by affecting the structural stability of the glycoproteins in the delipidated membrane. The possibility that these cells were depleted of some component other than cholesterol as a result of the delipidation treatments was considered. While it was not possible to determine the composition of the MβCD-lipid complexes, it was possible to analyze the composition of the lipids adsorbed onto the COS.
Lipid profiles from extracted COS. Given the results shown in Fig. 2, displaying a cell-specific response to the method of delipidation, the content of the lipid bound to the COS was determined by using mass spectrophotometry, performed by the Kansas Lipidomics Center, Kansas State University. This analysis of the lipids extracted from COS revealed nanomolar amounts of phosphatidylcholine, sphingomyelin, phosphatidyl ethanolamine, and phosphatidylinositol (30 nM total), in addition to cholesterol (64 nM total), adsorbed to 50 mg of extracted COS residue. These results confirm that serum extraction using COS extracts not only cholesterol but “>96% of all neutral lipids” as was originally described by Weinstein (52).

Electron microscopy of negatively stained SVHR from U4.4CD cells. As a qualitative assay of virus structural integrity and to determine if virus particles containing less cholesterol were more susceptible to damage, purified virus was visualized by using electron microscopy. Virus was twice purified on potassium tartrate gradients prior to examination in the electron microscope as described in Materials and Methods. Shown in Fig. 5A are SVHR particles grown in BHK cells and in Fig. 5B, particles from Sf21 cells which contained very low levels of cholesterol. Virus samples from Sf21 cells display a larger number of irregular particles (36%) than the control virus (5%). Virus from U4.4 cells delipidated with MβCD gave results similar to those shown for Sf21 SVHR (data not shown). Virus from other delipidated cells was found to break during electron microscopy preparations, suggesting that the virus membrane composition is a significant determinant of virion stability and that the cholesterol level is only one aspect of the membrane physicochemistry. Although the negative-stain examination of the virus produced from delipidated cells suggests that the virions are structurally unstable, these dramatic changes in morphology cannot alone account for the differences in the particle/PFU ratio, and the electron microscope cannot detect minor changes in structure that might render a virion noninfectious.

We also examined the protein composition of virus produced from delipidated cells and found no obvious difference from virus produced by normal cells (data not shown).

DISCUSSION

The data generated from these studies disclose the relationship between the cholesterol levels contained in the host cells, the virus those cells produce, and the effects of variations in cholesterol concentration on virus production, infectivity, and structure. Virus titers from COS- or MβCD-delipidated cells, with the exception of those from C6/36 cells, showed little to no effect of the treatment, indicating that virus assembly and release was normal. Virus particles from delipidated cells were found to have higher particle-to-PFU ratios than virus from nondelipidated cells, with the exception of virus from U4.4CD. This observation suggests that the total lipid profile of the particles, in addition to the cholesterol content, may determine the infectivity of the virion. This is especially evident in the cells treated with COS, which removes all neutral lipids (52; this study). Removal of any of these components could be responsible for the increased particle/PFU ratios seen in the COS-delipidated cells. The changes in the lipid composition of the virus could directly affect the structure of the glycoproteins by affecting the folding process as protein maturation and assembly take place and thus indirectly affect the structural
stability of the virion. The data also demonstrate that there is no requirement for high levels of cholesterol during virus assembly or infection. The lipid components of the virion, however, do affect structural stability and this conclusion is similar to that of Vashishtha et al. (50).

Our data are consistent with early alphavirus literature which reported that high levels of cholesterol were not required for the assembly or infectivity of Sindbis virus particles (32). The present study confirms these original observations that Sindbis virus infects, grows in, and is produced from insect host cells grown under conditions of cholesterol depletion. Sindbis virus grows efficiently to 10⁹ PFU/mosquito (3), and mosquitoes contain very low levels of cholesterol (9, 37, 45). A study reported by Mitsuhashi et al. (37) found that insect cells grown in SFM were "practically free of sterols" and that when grown in 10% FBS, Aedes albopictus cells incorporated 0.0125 sterol, wt/wt (gm sterol/gm protein), into the cells. Sindbis virus grows to wild-type levels, 10¹⁰ PFU/ml, in insect cells depleted of serum cholesterol (this study). This result is in direct contrast to those of Lu et al. and other studies (31, 34, 43, 50), where the production of infectious Semliki Forest virus (SFV) and Sindbis virus was severely reduced when grown in C6/36 cells with serum depleted of cholesterol (31). It was found that the amino acid sequence of a mutant E1 at the tripeptide E1 226–228 determined the cholesterol requirement. The strain of Sindbis virus employed in our studies has the same sequence at E1 226–228 (AKN) as the strain employed as the wild type in the above-referenced studies; thus, our contrasting results are not due to a genetic alteration in this region. This discrepancy may be explained by the method used to delipidate the serum used for the cell cultures. COS is a fused silica matrix which adsorbs hydrophobic molecules. This treatment removed from the serum not only cholesterol but neutral lipids which may be required by the insect cells in culture. Marquardt and Kielian (33) demonstrated that the centrifugation method of serum delipidation increased the SFV titer in C6/36 cells grown in the serum 100-fold compared to the titer in C6/36 cells delipidated by growth in COS-treated serum, supporting the contention that the delipidation method supports the virus titer. The conclusion in that study was that the C6/36 cells had somehow adapted and were then able to support the growth of SFV. The possibility that the method of delipidation could have made this difference was not considered. In the present study, MβCD was also used to bind cholesterol from the serum component of the medium. While MβCD is qualitatively more selective for cholesterol, it also binds phospholipids at a reduced level (40). Neither of these chemical treatments sequesters cholesterol specifically, and each also removes other serum components which alter the ability of the cells to produce stable virus. Infection of delipidated insect cells with SVHR produced differential amounts of virus, depending on the cell line and the method of delipidation. Weinstein (52) addresses this fact and states that "serum was cleared of >96% of all lipids by treatment with 3% (wt/vol) Cabosil at 4 ° for 24 h," the method employed in the present study. Sf21 cells supported the growth of SVHR to wild-type levels and were not chemically delipidated but biologically adapted. Of the mosquito cell lines, only C7-10 cells adapted well to the SFM, suggesting that this cell line may naturally express a different lipid composition or genetic difference. While these data are in conflict with the prevailing hypothesis that alphaviruses require cholesterol for assembly and infectivity, they do offer an alternate explanation as to the nature of the defect.

The data presented herein have implications reaching far beyond that of the lipid content of SVHR and its effect on virus structural stability. Because the data suggest that neither the process of infection of cells nor the production of virus from cells is dependent on high levels of cholesterol, results from experimental methods requiring high levels of cholesterol should be reexamined. Other published data have shown that the ability of alphaviruses to fuse with artificial liposome requires large mole percentages of cholesterol content (25, 34, 40).
Furthermore, the fusion of liposomes with virus membranes requires only exposure to low pH (24), while the fusion of living cell membranes is a two-step process requiring exposure to low pH followed by a return to neutral pH (11, 42). It has also been shown that at low temperature, the process of cell-cell fusion mediated by Sindbis virus is blocked, while penetration can take place (51). Alkylation of cysteine residues in the virus membrane glycoproteins blocks infection but not low-pH-mediated cell-cell fusion (55). These observations separate the process of membrane fusion from the process of infection and imply that one is not required for the other. These data have significant implications for the use of Sindbis virus functional assays which rely on artificial membranes (liposomes) containing large quantities of cholesterol (10, 14, 23, 25). Those membrane compositions would not reflect the native membrane composition of insect cells in vivo. Alpha- and flavivirus penetration can take place at the cell surface in the absence of membrane fusion (15, 42). While the preponderance of the literature invokes penetration by fusion, other varied biochemical approaches suggesting an alternate mechanism by which alphaviruses infect cells should be considered and models requiring large amounts of cholesterol reexamined.

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