

Cholesterol Is Required for Infection by Semliki Forest Virus

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Abstract. Semliki Forest virus (SFV) and many other enveloped animal viruses enter cells by a membrane fusion reaction triggered by the low pH within the endocytic pathway. In vitro, SFV fusion requires cholesterol in the target membrane, but the role of cholesterol in vivo is unknown. In this paper, the infection pathway of SFV was studied in mammalian and insect cells substantially depleted of sterol. Cholesterol-depleted cells were unaltered in their ability to bind, internalize, and acidify virus, but were blocked in

SFV fusion and subsequent virus replication. Depleted cells could be infected by the cholesterol-independent vesicular stomatitis virus, which also enters cells via endocytosis and low pH-mediated fusion. The block in SFV infection was specifically reversed by cholesterol but not by cholestenone, which lacks the critical 3β -hydroxyl group. Cholesterol thus is central in the infection pathway of SFV, and may act in vivo to modulate infection by SFV and other pathogens.

NUMEROUS viral and bacterial pathogens must gain access to the host cell cytoplasm in order to replicate. This entry pathway is best understood for enveloped animal viruses such as Semliki Forest virus (SFV),¹ which use endocytosis to infect a cell (reviewed in Kielian and Jungerwirth, 1990; White, 1990; Marsh and Helenius, 1989; Kielian and Helenius, 1986). SFV binds to plasma membrane receptors, is internalized in coated pits and vesicles, and is delivered to the mildly acidic environment of endosomes. The acid pH induces conformational changes in the SFV spike protein, and triggers the fusion of the viral and endosome membranes. This critical membrane fusion step releases the viral RNA into the cytoplasm and initiates infection. The fusion reaction is mediated by the viral spike glycoprotein, a heterotrimer of two transmembrane glycopolypeptides, E1 (50,786 D) and E2 (51,855 D), and a peripheral glycopolypeptide, E3 (11,369 D) (reviewed in Schlesinger and Schlesinger, 1986; Simons and Warren, 1984).

Several lines of evidence argued that cholesterol might be playing an important functional role in SFV entry. The in vitro fusion of SFV with artificial liposomes required cholesterol in the liposome membrane above a threshold level of ~ 1 cholesterol per 4 phospholipid molecules (White and Helenius, 1980). No other specific components except for low pH were necessary. Studies with cholesterol analogues showed that the 3β -hydroxyl group is the critical portion of the sterol molecule, and suggested that cholesterol's action is not at the level of membrane fluidity (Kielian and Helenius, 1984). Lastly, the purified E1 ectodomain, a proteolytically truncated form of E1, was found to require both low pH and a 3β -hydroxysterol in order to trigger its acid conformational

change (Kielian and Helenius, 1985). Taken together, these findings suggested a mechanistic role for cholesterol in SFV fusion, but its relevance to virus infection in vivo was unknown. We have used sterol-depleted cells to examine the role of cholesterol in the infective fusion of SFV with cellular membranes. Our results showed that cholesterol was specifically required for infection of host cells by SFV, but not for infection by vesicular stomatitis virus, a virus which also enters cells by endocytosis and low pH-triggered fusion. Thus, in nature the host specificity and tissue tropism of SFV and other intracellular pathogens may be specifically modulated by cholesterol.

Materials and Methods

Virus and Cells

A plaque-purified stock of SFV was grown, radiolabeled, and purified as previously described, using baby hamster kidney (BHK-21) cells (Kielian et al., 1984).

BHK-21 cells were cultured in Dulbecco's modified Eagle's medium containing 100 U/ml penicillin and streptomycin (DMEM), supplemented with 5% FCS and 10% tryptose phosphate broth. CHO cells were cultured as monolayers in alpha-MEM containing 10% FCS and 100 U/ml penicillin and streptomycin. C6/36 cells, a clonal line from *Aedes albopictus* (Igarashi, 1978), were cultured at 28°C in DMEM supplemented with 10% heat-inactivated FCS.

Depletion of Cellular Cholesterol

Lipoproteins were removed from FCS by adsorption on Cab-O-Sil (Research Products International Corp., Prospect, IL) (Weinstein, 1979), which reduced total serum cholesterol levels from 168 to 5.7 $\mu\text{g/ml}$. Lovastatin (a kind gift of Dr. A. Alberts, Merck Sharp and Dohme, Rahway, NJ) was saponified and dissolved (Langan and Volpe, 1986) and the concentration determined by absorbance at 239 nm (Alberts et al., 1980).

BHK-21 cells were partially depleted of cholesterol by growth for 3 d in DMEM with 3% delipidated FCS and 5% TPB. During the last 24 h of

1. *Abbreviations used in this paper:* SFV, Semliki Forest virus; VSV, vesicular stomatitis virus.

growth, 4.5 μ M lovastatin and 0.25 mM mevalonate were added to this medium (Krieger 1983; Renaud et al., 1986). C6/36 cells were cholesterol depleted by gradually adapting to growth in medium with delipidated serum, followed by culture for at least 2 wk in DMEM with 3% heat-inactivated delipidated FCS (Silberkang et al., 1983).

Steroid Repletion

Cholesterol or cholestenone was complexed with BSA essentially by the procedure of Werb and Cohn (1972). In brief, 2 mg steroid dissolved in 1 ml acetone was injected into 2 ml MEM containing 2% BSA, the acetone evaporated under nitrogen, and the resulting emulsion sonicated on ice for 2 min with a Branson microtip (Branson Sonic Power Co., Danbury, CT) at a setting of 4. To quantitate the level of cell-associated steroid, emulsions were made with trace amounts of [7(*n*)-³H]cholesterol or cholestenone. [³H]cholestenone was prepared by oxidation of [³H]cholesterol with cholesterol oxidase. The product was analyzed by thin layer chromatography on silica gel G in chloroform/methanol (50:1) (Lange and Ramos, 1983). 96% of the radioactivity migrated at the position of cholestenone.

For steroid enrichment, 0.215 mM steroid-BSA emulsion in C6/36 lipid-depleted media was added to depleted cells for 5 h at 28°C, followed by extensive washing. Enrichment was quantitated by trypsinizing the cells, centrifuging through 5 ml of 5% sucrose to separate nonassociated complexes, and determining the radioactivity in the cell pellet. Final levels of cell-associated steroid were \sim 6 μ g cholesterol or 10 μ g cholestenone per 2×10^6 cells, which was 6–10-fold above the usual amount of cholesterol in this number of cells. Cholesterol repletion was also evaluated by staining with filipin, a cholesterol-specific fluorescent dye, exactly as described by Cadigan et al. (1990).

Protein and Lipid Analysis

100-mm plates of cells were washed and scraped into PBS, sonicated for 1 min on ice at a setting of 3 with a Branson microtip, and extracted by the method of Bligh and Dyer (1959). Media and FCS were extracted with petroleum ether (Werb and Cohn, 1971). Tritiated cholesterol was added to the extractions as an internal standard to quantitate recovery. Samples were dried completely under nitrogen and resuspended in spectral grade isopropanol containing 10% Triton X-100. Free and esterified cholesterol were determined by a fluorometric assay (Heider and Boyett, 1978), using an LS-5 fluorometer (Perkin-Elmer Corp., Pomona, CA). The protein concentration of an aliquot of the sonicated cells was determined by a Lowry assay with 0.115% SDS (Lowry et al., 1951).

Cholesterol Oxidase Treatment of CHO Cells

For maximal oxidation, CHO cells in suspension were prefixed with 2% glutaraldehyde for 15 min on ice, and treated for 1 h at 10°C with 8 U/ml cholesterol oxidase in 310 mM sucrose and 0.5 mM NaPO₄, pH 7.4 (Lange and Ramos, 1983). To quantitate the conversion of cellular cholesterol to cholestenone, cells were metabolically labeled with [³H]mevalonolactone. Cells were first grown overnight in lipid-depleted CHO growth media to induce cholesterol synthesis. They were then pulsed 5 h at 37°C with [³H]mevalonolactone and chased for 12 h in lipid depleted growth media. Cells were then oxidase treated, extracted, and cholesterol conversion was analyzed by thin layer chromatography as described above.

Binding, Fusion, Endocytosis, Penetration, and Degradation Assays

Assays for these steps in the virus infection pathway have been previously described. For all assays, radiolabeled SFV was prebound to cells on ice with continuous shaking. To measure binding, cells were scraped and washed twice with ice-cold medium, followed by quantitation of cell-associated radioactivity (Marsh and Helenius, 1980). Cells were treated at 37°C with medium of the indicated pH to trigger virus-plasma membrane fusion; nonfused virus removed by either proteinase K or subtilisin digestion, and cell-associated radioactivity determined (Schmid et al., 1989; White et al., 1980). Cells were warmed to 37°C to initiate endocytosis, nonendocytosed virus removed by proteinase K digestion, and internalized virus quantitated (Marsh and Helenius, 1980). The lysosomal degradation of SFV was followed by determining the release of TCA-soluble [³⁵S]methionine into the medium (Marsh and Helenius, 1980). Penetration assays monitored the accessibility of [³H]uridine-labeled SFV RNA to degradation by ribonuclease added to cell homogenates (Helenius et al., 1982). The

figures shown are representative experiments with duplicates plates at each time point. All experiments were performed at least twice.

Antibodies and Acid Specific Immunoprecipitation

The kinetics of endosome acidification in control and depleted C6/36 cells were analyzed by following the acid-induced conformational change in E1 (Kielian et al., 1990; Kielian et al., 1986). [³⁵S]Methionine-labeled SFV was bound to cells as above, the cells warmed to 28°C to permit endocytosis, the cells lysed at various times, and acid-converted E1 protein reacted with Ela-1, a conformation-specific monoclonal antibody (Kielian et al., 1990). Immune complexes were precipitated with rabbit anti-mouse antibody bound to fixed *Staphylococcus aureus* (Zysorbin; Zymed Laboratories, San Francisco, CA). Samples were analyzed by electrophoresis on nonreducing SDS gels, followed by fluorography (Kielian and Helenius, 1985).

Infection by SFV and VSV

Infection by SFV and vesicular stomatitis virus (VSV) was assayed by quantitating viral protein production in C6/36 cells that were pretreated under depleting or enrichment conditions. Cells were infected with either virus at 100 pfu/cell for 1 h at 28°C in DMEM containing 0.2% BSA and 10 mM Hepes. The inoculum was removed and the infection allowed to continue in C6/36 delipidated growth medium for a total of 3 h for VSV and 8 h for SFV. For labeling, the cells were incubated in methionine-deficient MEM for 15 min, pulsed for 30 min with 50 μ Ci/ml [³⁵S]methionine, and chased for 15 min in DMEM with a 10-fold excess of methionine. Cells were lysed and immunoprecipitated with either a polyclonal serum against SFV spike proteins, or a polyclonal serum raised against VSV. Immunoprecipitates were analyzed by SDS-PAGE. Fluorograms were quantitated by densitometry using a Quantimet Image Analysis System (Cambridge Instruments, Cambridge, England).

SFV infection was also quantitated by measuring the incorporation of [³H]uridine into viral RNA, using quadruplicate cultures on coverslips. Depleted cells were preincubated with steroid-BSA complexes for 5 h as described above. All cells were then infected with SFV in DMEM containing 0.2% BSA and 10 mM Hepes at a multiplicity of 75–90 pfu/cell for 5 h, preincubated for 1 h with 2 μ g/ml actinomycin D to block host cell RNA synthesis, and labeled for 6 h with 25 μ Ci/ml [³H]uridine in actinomycin D. The incorporation of uridine into TCA-precipitable RNA was evaluated as previously described (Helenius et al., 1982).

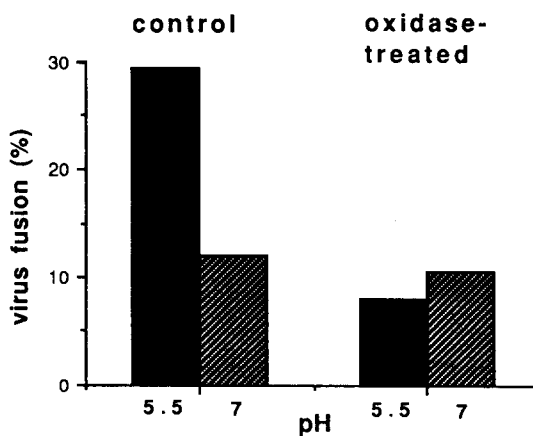


Figure 1. Effect of cholesterol oxidase treatment on SFV fusion with CHO cells. CHO cells in suspension were pretreated with cholesterol oxidase under conditions which oxidized \sim 65% of cellular cholesterol. [³⁵S]Methionine-labeled SFV was bound at 4°C to oxidase or mock-treated cells, and the cells were incubated for 2 min at either pH 7.0 or 5.5 at 37°C to activate virus-plasma membrane fusion. Nonfused virus was removed by digestion with subtilisin, and fusion expressed as the percent of initial bound virus radioactivity which became protease resistant. Control cells bound 23,342 cpm and oxidase-treated cells bound 28,580 cpm.

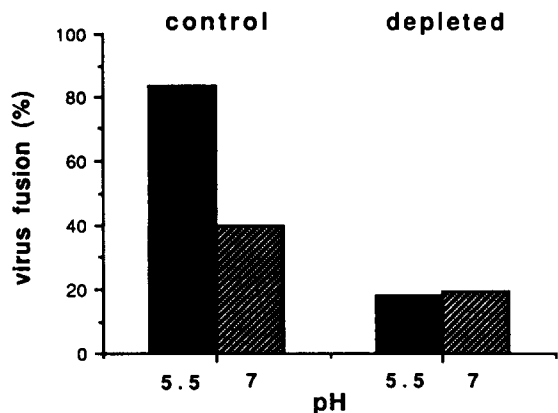


Figure 2. Effect of cholesterol depletion on SFV fusion with BHK cells. BHK cells were depleted of cholesterol by growth in LDL-minus medium containing 4.5 μ M lovastatin and 0.25 mM mevalonate. [35 S]Methionine-labeled SFV was bound at 4°C to control or depleted cells, and treated for 1 min at 37°C with buffers at the indicated pH to activate virus-plasma membrane fusion. Nonfused virus was removed by digestion with proteinase K, and fusion expressed as the percent of initial bound virus radioactivity which became protease resistant. Control cells bound 4,275 cpm and depleted cells bound 4,344 cpm. The radioactivity in the pH 7 sample represents both virus that has been endocytosed and an inherent background of proteinase K-resistant bound virus.

Results

Cholesterol Depletion of Mammalian Cells

We explored several methods to assess the importance of cholesterol in the infectious pathway of SFV. Our first studies used either CHO cells or baby hamster kidney (BHK-21) cells. Both cell lines are productive mammalian hosts for SFV and have been used to develop assays for the stages of endocytic viral entry. Using these well-established assays for the steps of virus entry and fusion, we hoped to be able to define the role of cholesterol in SFV infection.

First, CHO cells in suspension were treated with the enzyme cholesterol oxidase, which specifically oxidizes the 3 β -hydroxyl group on cholesterol to produce cholestenone (Lange and Ramos, 1983). Other components of the membrane remain unaffected. Radiolabeled SFV was bound to oxidase or mock-treated cells in the cold. The bound virus was treated briefly at low pH at 37°C to induce virus fusion with the plasma membrane. Fusion was followed by the re-

Table I. Cholesterol Content and SFV Binding of BHK and C6/36 Cells

Cells	Cholesterol equivalents		SFV binding
	Free	Esterified	
	μ g/mg protein		% control
Control BHK	33.2 (3.6)* [6]‡	22.6 (1.2) [6]	100% [5]
Depleted BHK	11.7 (0.7) [6]	3.7 (3.7) [6]	84.6% (13) [5]
Control C6/36	16.7 (0.6) [5]	29.5 (1.7) [5]	100% [4]
Depleted C6/36	<0.35 [5]	<0.61 [5]	137% (9) [4]

* Standard deviation of the mean.

‡ Number of determinations.

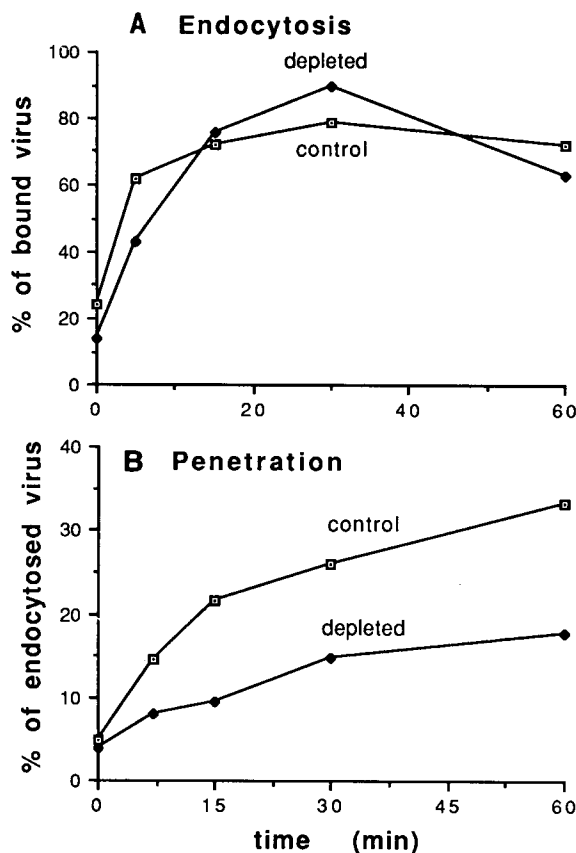


Figure 3. Kinetics of SFV endocytosis and RNA penetration in control and cholesterol-depleted BHK cells. (A) Endocytic uptake. [35 S]Methionine-labeled SFV was prebound for 90 min at 4°C to control or depleted BHK cells. The cells were warmed to 37°C for the indicated period, extracellular viruses stripped off by proteinase K digestion, and internalized virus radioactivity quantitated and expressed as the percent of bound virus radioactivity which became protease resistant. The control cells bound 18,324 cpm and the depleted cells bound 31,067 cpm. The counts at time 0 represent bound virus which was resistant to protease. (B) Penetration of viral RNA into the cytoplasm. [3 H]Uridine-labeled SFV was prebound and internalized as in A. At the indicated time, the cells were homogenized and the accessibility of viral RNA to degradation by exogenous RNase was assayed. Penetration is expressed as the percent of endocytosed viral RNA that was degraded by RNase. The control cells had internalized 82,990 cpm by the 60-min time point, and the depleted cells had internalized 33,873 cpm.

sistance of the fused virus to protease stripping of the plasma membrane (Schmid et al., 1988; White et al., 1980). After oxidation of ~65% of cellular cholesterol, SFV fusion with oxidase-treated cells was completely blocked (Fig. 1). This result suggested that the sterol 3 β -hydroxyl group was required for SFV fusion with a complex biological membrane. However, since in our hands significant oxidation of membrane cholesterol required glutaraldehyde fixation of the cells and treatment in low ionic strength buffers (Lange and Ramos, 1983), the oxidase system was not amenable to further analysis of early virus-cell interactions. Rather surprisingly, the fixed cells did support virus fusion, although it was considerably less efficient than fusion with untreated cells (compare Fig. 1 with Fig. 2).

Mammalian cells can be depleted of cholesterol by using

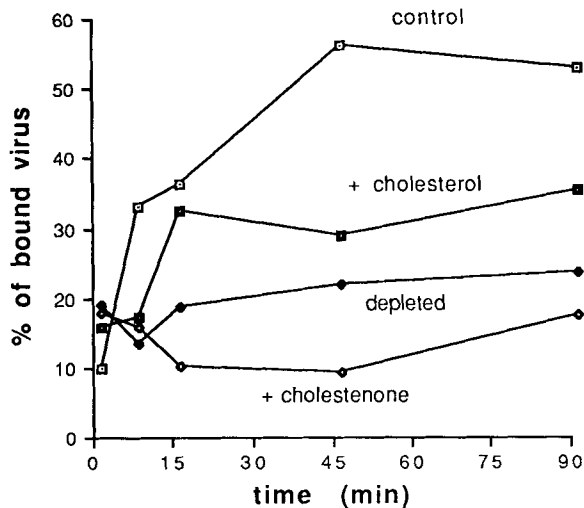


Figure 4. Kinetics of SFV RNA penetration in sterol-modified C6/36 cells. C6/36 cells were depleted of cholesterol by growth in 3% LDL-depleted fetal calf serum. Some depleted cultures were then enriched in cholesterol or cholestenone by the addition of steroid-BSA complexes for 5 h. [^3H]Uridine-labeled SFV was bound to all cells at 4°C, endocytosis initiated by warming to 28°C and the accessibility of viral RNA to degradation by exogenous RNase assayed at the indicated time. Penetration is expressed as the percent of initial bound viral RNA which was degraded by RNase. The counts bound to the four cell types ranged from 36,161 to 39,390 cpm.

drugs such as lovastatin to block the synthesis of mevalonate, the key intermediate of cholesterol biosynthesis (Alberts et al., 1980). Cells are grown in lovastatin-containing, LDL-deficient medium, and given a small amount of mevalonate to support synthesis of essential cellular isoprenoids (Krieger, 1983). In the absence of cholesterol biosynthesis or a source of exogenous cholesterol, cholesterol is diluted out of the plasma membrane as the cells divide (Krieger, 1983). These conditions were modified to deplete BHK-21 cells (see Materials and Methods). After 24 h of culture under depleting conditions, total cholesterol levels were found to have declined to $\sim 28\%$ those of control cells (Table I). Cell surface binding of SFV appeared unaffected by cellular cholesterol depletion (Table I), as predicted by the protein nature of the receptor. However, the low pH-triggered fusion of SFV with plasma membranes of the depleted cells was dramatically decreased (Fig. 2). We then wanted to follow the steps of viral entry into the depleted cells. To assay endocytosis, radiolabeled SFV was bound to control and depleted cells at 4°C, the cultures warmed to 37°C at neutral pH to permit endocytosis, the virus remaining at the surface removed by protease stripping, and the internalized virus radioactivity quantitated (Marsh and Helenius, 1980). Endocytic uptake of virus was comparable in the two cell types, showing that the cells were basically healthy after substantial depletion (Fig. 3 A). Similarly, the kinetics of SFV degradation were equivalent in the two cell types (data not shown). After arrival in the acid environment of the endosome, the fusion of virus with the endosome membrane releases the RNA into the cytoplasm. To measure this RNA penetration step, [^3H]uridine-labeled SFV was bound to cells, the cultures warmed to permit endocytosis, homogenates prepared, and the susceptibility of viral RNA to digestion by RNase

evaluated (Helenius et al., 1982). RNA penetration was significantly decreased in cholesterol-depleted cells, but a low rate of RNA release was still observed (Fig. 3 B). This result argued that sterol depletion decreased intracellular virus fusion, but that the levels of sterol in the depleted cells were still sufficient to permit some fusion. Fusion at the plasma membrane in these cells had appeared to be completely blocked (Fig. 2). This difference in the fusion block may indicate that artificially induced fusion at the plasma membrane is less efficient and requires more cholesterol, or that sterol depletion is more pronounced in the plasma membrane compared to the endosome membrane.

Cholesterol Depletion of a Mosquito Cell Line

SFV, an alphavirus, is capable of infecting a wide variety of host cells in culture, and is transmitted in the wild by insect vectors (reviewed in Brown and Condreay, 1986). Insects are cholesterol auxotrophs, obtaining any necessary cholesterol from dietary sources (Nes and McKean, 1977). In contrast to mammalian cells, insect cell lines can be radically depleted of sterol simply by culture in LDL-depleted medium (Silberkang et al., 1983). Depletion produces no compensatory changes in cellular phospholipid or fatty acid composition, and unlike mammalian cells, insect cells appear to suffer no adverse effects from long-term cholesterol depletion (Silberkang et al., 1983). We used a mosquito cell line, C6/36, as a host for SFV, and monitored the cholesterol levels in the cells after extended growth in LDL-deficient medium. As shown in Table I, both free and esterified cholesterol in depleted cells were below the limits of detection, or $<2\%$ of control levels. The cells appeared morphologically normal, but did grow somewhat more slowly. Again, SFV binding to the depleted cells was not inhibited, and was actually somewhat higher than in control cells (Table I).

Since sterol depletion was much more efficient in the insect cells, we continued our analysis of SFV cholesterol re-

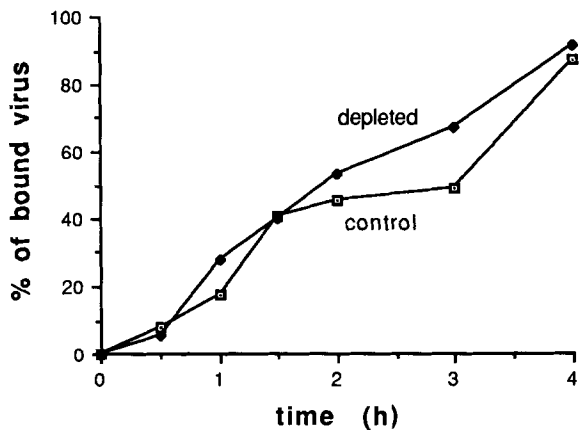


Figure 5. Degradation of internalized SFV in control and depleted C6/36 cells. [^{35}S]Methionine-labeled SFV was prebound for 90 min at 4°C to control or depleted C6/36 cells. The cells were warmed to 28°C for the indicated time, an aliquot of the medium removed and precipitated with 10% TCA, then the TCA-soluble radioactivity was determined and expressed as percent of the initial bound virus counts. Control cells bound 69,602 cpm and depleted cells bound 84,944 cpm. At time 0, approximately 30 cpm were TCA soluble.

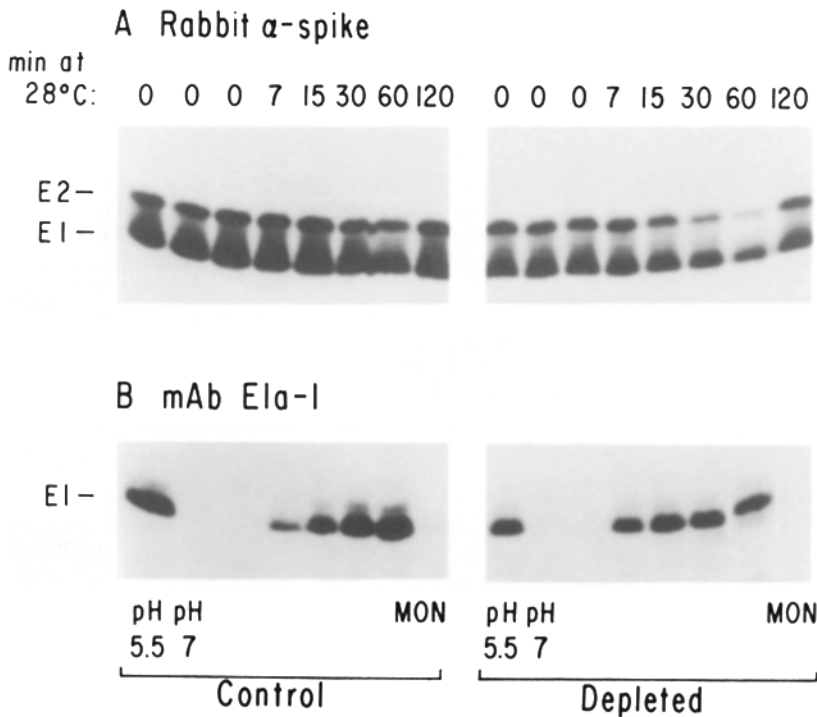


Figure 6. Kinetics of E1 conversion to the acid conformation in control and depleted C6/36 cells. [³⁵S]Methionine-labeled SFV was pre-bound for 90 min at 4°C to control or depleted C6/36 cells. The cells were warmed to 28°C for the indicated time, lysed, and duplicate aliquots analyzed by immunoprecipitation and gel electrophoresis. Controls included samples that were pretreated for 1 min at 28°C at pH 5.5 or 7.0, and samples that were warmed to 28°C for 120 min in the presence of 10 μ M monensin (MON). (A) Samples precipitated with a polyclonal antibody against the SFV spike protein. (B) Samples precipitated with monoclonal antibody Ela-1, which recognizes the acid conformation of E1.

quirements using this system. The penetration of labeled viral RNA into the cytoplasm of control and depleted C6/36 cells was measured by RNase sensitivity (Fig. 4), and compared to the amount of radioactive virus bound to the cells. In control cells, \sim 50% of the bound virus counts became accessible to RNase after 60 min of uptake at 28°C. Penetration of SFV RNA appeared to be totally blocked in the depleted cells, with little or no RNA release after 90 min at 28°C. We attempted to reverse this striking inhibition by the addition of exogenous cholesterol. BSA-steroid complexes were prepared using either cholesterol or the nonfusing analogue cholestenone (Werb and Cohn, 1972). The complexes were added to depleted cells for 5 h at a concentration of 2×10^{-4} M, which represents a steroid excess of \sim 50-fold over the amount of cholesterol found in control cells. The association of the complexes with the cells was followed by adding trace amounts of radiolabeled steroid. Association was maximal by 5 h, and both cholesterol and cholestenone became cell associated to levels above those of cholesterol in control cells (see Materials and Methods). However, the actual incorporation of steroid into the depleted cell membranes is unknown, and other studies have demonstrated that cholesterol is very slowly reincorporated into depleted membranes (Silberkang et al., 1983). Using filipin, a cholesterol-specific fluorescent dye (Cadigan et al., 1990), we found that there was also considerable heterogeneity in the amount of cholesterol uptake within the repleted cell population (data not shown). Our attempts to add back cholesterol more rapidly or efficiently by using sterol-containing liposomes were unsuccessful. We therefore chose 5 h as a time when significant amounts of steroid-BSA complexes were associated with the cells. To assay reversal of the penetration block, depleted cells were incubated with either steroid complex for 5 h, and then assayed for viral RNA entry (Fig. 4). Cholesterol partially reversed the inhibition, resulting in RNA penetration to \sim 50% of the level seen in control cul-

tures. No reversal was seen after incubation with cholestenone complexes. With increasing time of incubation, viral RNA became somewhat less accessible to RNase in cholestenone enriched cells. Since the uptake and degradation of SFV in cholestenone cells was similar to controls, the decrease in RNase susceptibility may be due to a stabilization of the virus membrane by cholestenone.

Localization of the Block in Viral RNA Entry

The inhibition of viral RNA entry could be blocked at any of the steps in the endocytic pathway preceding RNA penetration. Thus, an effect of cholesterol depletion on initial endocytic uptake, transport within the endocytic vacuolar system, acidification of endosomes, or virus-endosome fusion would result in a block in RNA release. We were unable to assay endocytosis directly, since the virus-receptor interaction in C6/36 cells appeared insensitive to any of a number of proteases (data not shown). We therefore followed the degradation of [³⁵S]methionine-labeled virus as a measure of endocytic uptake, transport to lysosomes, and lysosomal function. As shown in Fig. 5, degradation was identical in control and depleted cells. Essentially all virus radioactivity initially bound at the cell surface was released into the medium in TCA-soluble form after 4 h at 28°C.

The unaltered lysosomal degradation suggested that acidification within the lysosomes of depleted cells was not significantly affected by the lack of cholesterol. However, it was critical to demonstrate that acidification within endosomes was fully functional, since this provides the trigger for virus-membrane fusion. For this assay, we took advantage of the low pH-dependent conformational change in the E1 spike polypeptide (Kielian and Helenius, 1985). This conformational change is irreversible, occurs at the pH threshold of fusion, and results in altered protease sensitivity and recognition by acid-specific monoclonal antibodies. We have pre-

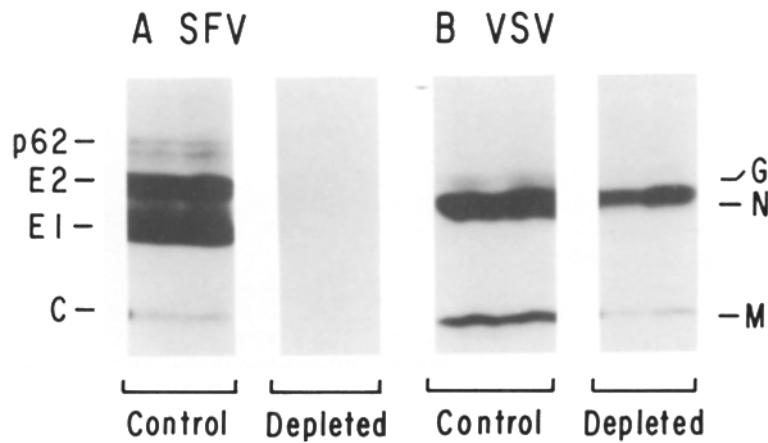


Figure 7. Viral protein production in control and depleted C6/36 cells. Control or depleted C6/36 cells were infected with (A) SFV or (B) VSV at a multiplicity of 100 pfu per cell. After incubation for 8 (SFV) or 3 h (VSV), the cells were pulse-labeled with [³⁵S]-methionine for 30 min, chased for 15 min, and harvested. The cell lysates were immunoprecipitated with a polyclonal antibody against SFV spike proteins (A), or a polyclonal antibody against purified VSV (B), followed by analysis on SDS gels.

viously demonstrated that the conformational change occurs during exposure of virus to endosomal acidity in BHK or CHO cells (Kielian et al., 1986; Schmid et al., 1989). ³⁵S-labeled SFV was bound to control or depleted C6/36 cells and the cells warmed to 28°C for various times to permit endocytosis. The cells were lysed and duplicate aliquots of the lysates were precipitated with a rabbit anti-spike antibody or with Ela-1, an acid conformation-specific monoclonal antibody (Kielian et al., 1990). As controls, bound virus was pretreated at either pH 7 or 5.5, or virus was internalized in the presence of 10 μM monensin to block endosomal acidification (Marsh et al., 1982). Fig. 6 A shows that the polyclonal antibody precipitated comparable amounts of radiolabeled virus polypeptides from control or cholesterol-depleted cell lysates after binding and uptake of SFV. A decreased amount of the E2 spike subunit was seen after 30 and 60 min of endocytosis, especially in the depleted cells. This decrease resulted from lysosomal degradation, since it was blocked in cells that were treated with monensin during endocytosis. When the samples were precipitated by monoclonal antibody Ela-1 (Fig. 6 B), most of the bound E1 protein was in the acid conformation after surface treatment at pH 5.5, but not pH 7. Comparison of the endocytosed samples (7–60 min) showed that E1 was rapidly converted to the acid conformation in both control and depleted cells. The kinetics of acidification in the two cell types were comparable. Conversion was due to endosomal acidification, since it occurred earlier than lysosomal degradation (Fig. 5) and was blocked

by monensin treatment (Fig. 6 B). Thus, both endocytosis and vacuolar acidification were unimpaired in sterol-depleted cells, and the inhibition of RNA entry was due to a block in SFV-endosome fusion.

SFV Infection Is Blocked in Cholesterol-depleted C6/36 Cells

Lastly, we wished to assay virus production by the depleted cells. The data up to this point predicted that SFV would be unable to replicate in the depleted cells due to the inability of the virus to fuse with a cholesterol-deficient membrane. Since cholesterol depletion might have general effects on the cells' biosynthetic machinery, infection by VSV, a rhabdovirus, was followed in parallel. This virus also enters cells by endocytosis (Matlin et al., 1982), has a fusion activity with a pH threshold similar to that of SFV (White et al., 1981), and infects insect cells (Gillies and Stoller, 1980). However, VSV fusion with liposomes is independent of cholesterol (Eidelman, et al., 1984). We chose to follow virus protein production in infected cells rather than the release of infectious virus particles, since reduced virus membrane cholesterol can have effects on VSV infectivity (Pal et al., 1988). Control and depleted C6/36 cells were infected with high multiplicities of either SFV for 8 h or VSV for 3 h. The infected cells were then pulse labeled with [³⁵S]methionine, lysed, and immunoprecipitated with antibodies raised against the SFV spike or purified VSV. 3 h after infection with VSV, both control and depleted cells produced large amounts of virus proteins, although the level of VSV protein was somewhat reduced in depleted cells (Fig. 7 B). Densitometry

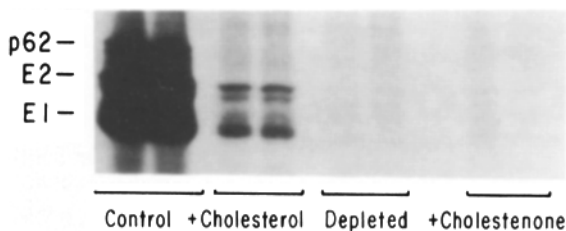


Figure 8. SFV protein production in depleted and steroid-enriched C6/36 cells. Depleted C6/36 cells were incubated with cholesterol- or cholestenone-BSA complexes for 5 h. The cultures were then infected with SFV at a multiplicity of 100 pfu/cell for 8 h. Cells were pulse labeled with [³⁵S]methionine for 30 min, chased for 15 min, and the cell lysates analyzed by immunoprecipitation and gel electrophoresis, using a polyclonal antibody against the SFV spike protein.

Table II. Viral RNA Synthesis In Sterol-modified C6/36 Cells

Cells*	[³ H]Uridine incorporation	
	Uninfected	Infected
	<i>cpm/2 × 10⁵ cells</i>	
Control	5,324	32,393
Depleted	3,696	3,796
Depleted plus cholesterol	5,795	10,055
Depleted plus cholestenone	3,499	3,763

*Depleted cells were preincubated with steroid-BSA complexes for 5 h where indicated. All cells were then infected with SFV, and the incorporation of uridine into RNA measured in the presence of actinomycin D (see Materials and Methods).

showed that the depleted cells made ~47% of the amount of VSV N protein as the control cells. Both cell types were lysed by the VSV infection after 8 h (data not shown).

SFV proteins were synthesized at high levels 8 h after addition of virus to control C6/36 cells. In contrast, SFV proteins were undetectable after infection and labeling of cholesterol-depleted cells (Fig. 7 A). This total block in virus replication thus follows the block in SFV RNA penetration into the depleted cell cytoplasm. The reversibility of this block was assayed by adding cholesterol- or cholestenone-BSA complexes to the depleted cells for 5 h, followed by infection with SFV and assay of either virus protein or RNA production. As shown in Fig. 8, depleted cells enriched with cholesterol supported SFV protein production, although again the reversal was not to the level of the control cells. Densitometry showed that the cholesterol-enriched cells synthesized SFV E1 and E2 proteins at 7–10% the level of control cells. No SFV proteins were produced after cholestenone enrichment (<0.2% by densitometry). Quantitation of virus RNA production by [³H]uridine incorporation showed that depleted cells synthesized no detectable SFV RNA (Table II). Cholesterol-BSA complexes restored the ability of the depleted cells to produce SFV RNA, at levels ~30% those of controls. No viral RNA production was detected in cholestenone-enriched cells.

Discussion

Taken together, these studies demonstrated that cholesterol is required *in vivo* to permit SFV fusion with the endosome membrane of the host cell. The fusion and infection block in cholesterol-depleted cells was at least partially overcome by the addition of a sterol with the critical 3 β -hydroxyl group. Previous studies suggest that this sterol requirement for SFV fusion is an essential feature of the target membrane, rather than the virus membrane. Thus, although purified virus particles contain cholesterol in the virus membrane, they still require cholesterol in the target membrane in order to fuse (White and Helenius, 1980). Conversely, SFV spike proteins reconstituted into phospholipid liposomes without sterol can still fuse with cholesterol-containing membranes (Marsh et al., 1983). Our studies have shown that irreversible conformational changes occur in both the E1 and E2 subunits of the SFV spike protein upon exposure to low pH (Kielian et al., 1990; Kielian and Helenius, 1985). For a proteolytically truncated form of E1, this conformational change required the addition of cholesterol liposomes during low pH treatment. Thus, sterol appears to serve an interactive role with the E1 subunit of the spike protein during fusion, rather than merely affecting target membrane properties such as fluidity. We have not been able to detect a stable association of either E1 or E2 with cholesterol-containing liposomes (Kielian and Helenius, 1985). Any interaction must therefore be either transitory or undetectable by our present assays.

Our current working hypothesis for SFV fusion is that low pH-induced conformational changes in the E2 subunit and possibly in the E1 subunit act in concert to expose a previously hidden site on E1. This domain of E1 then interacts in a stereo-specific manner with cholesterol in the target membrane, allowing further conformational changes and leading

to a disruption in the bilayer that triggers fusion. The role of E2 is hypothesized from its efficient conformational change at the pH of fusion. In addition, E2 is synthesized as a precursor, p62, which must be cleaved in order to make the spike protein fusion-competent (Lobigs and Garoff, 1990; Jain et al., 1991).

What could be the site of cholesterol interaction on the E1 molecule? One possible candidate is a highly conserved 23 amino acid long domain which lies 75 amino acid residues from the E1 NH₂ terminus (Garoff et al., 1980). This region is also strikingly apolar, and for these reasons has been suggested to be involved in fusion (Garoff et al., 1980). Mutation of specific amino acids within this conserved domain causes a dramatic acid shift in the pH threshold required to trigger fusion (Levy-Mintz, P., and M. Kielian, manuscript submitted for publication). This peptide domain could be the region of E1 that interacts with cholesterol. Studies with labeled cholesterol may permit us to localize the interaction site. Perhaps more promising is the possibility that the cholesterol-depleted cells will allow the selection of virus mutants with altered cholesterol requirements. By analyzing the location of such mutations, we hope to obtain insights into the mechanism of cholesterol's action during SFV fusion.

How common is a cholesterol requirement in the entry pathway of viruses and other pathogens? While the alphaviruses that have been examined all appear to need cholesterol for fusion, other enveloped virus families that use endocytosis and low pH to infect cells do not have a similar sterol requirement. Influenza virus, a myxovirus, and VSV, a rhabdovirus, both have cholesterol-independent fusion mechanisms (White et al., 1982; Eidelman et al., 1984). In the case of influenza, fusion appears to be mediated by the low pH-triggered exposure of a hydrophobic fusion peptide, which is then capable of interacting with any hydrophobic substance including target membranes and detergents (reviewed in Wiley and Skehel, 1987). It is interesting that cholesterol has been reported to be required for the fusion of Sendai virus, a paramyxovirus whose fusion protein bears structural and sequence similarities to that of influenza (Kundrot et al., 1983; Hsu et al., 1983; Citovsky et al., 1988). The *in vivo* cholesterol requirements for Sendai virus infection remain to be determined.

Some nonenveloped viruses, such as polio virus, are known to enter cells via endocytosis and a low pH-requiring membrane translocation step (Madhus et al., 1984). Polio virus will bind to membranes after treatment at low pH, but cholesterol is not a required component of the membrane (Lonberg-Holm et al., 1976). An intriguing example is the nonenveloped virus rotavirus, which enters cells by an undefined but apparently pH-independent mechanism (Kalliot et al., 1988; Keljo et al., 1988). There is sequence homology between the 23 amino acid hydrophobic peptide of SFV and amino acids 384–401 of VP4, the rotavirus outer capsid protein (Mackow et al., 1988). Immunoelectron microscopy of rotavirus suggests that this region of VP4 is at the distal tip of the virus spike protein, where it could be involved in virus penetration (Prasad et al., 1990). One speculation on the role of this conserved sequence is that it interacts with cholesterol to mediate the transfer of the rotavirus genome across the host membrane. We are exploring this possibility using a cholesterol-depleted cell system.

Numerous microbial parasites must also gain access to the

host cell cytoplasm in order to replicate, with obvious parallels to viral entry. The infection pathway of the facultative intracellular bacterium *Listeria monocytogenes* is especially provocative. These bacteria secrete a toxin, listeriolysin O, which can lyse cells by interacting with cholesterol in the host membrane (Mengaud et al., 1988; Tweten, 1988). The bacteria are phagocytosed by the host cell, and can only escape from the phagocytic vacuole when expressing the gene for the toxin. The toxin activity has a pH optimum of 5.5, which the bacteria may meet in the phagocytic vacuole of the host cell (Mellman et al., 1986). *L. monocytogenes* infection thus may also depend on the availability of cholesterol in the host cell membrane, in a similar fashion to SFV infection.

Most of the studies described in this paper used a cultured mosquito cell line. It is interesting that the course of SFV infection in an insect host such as the mosquito is different than infection of a vertebrate host. SFV lytically infects vertebrate cells, and can cause a lethal encephalitis upon injection into mice (Griffin, 1986). In contrast, the addition of SFV to mosquito cells in culture leads to a chronic infection without cell lysis, and infection of the mosquito host is not deleterious (Brown and Condreay, 1986). Our results suggest that the localization and concentration of cholesterol in insects may contribute to the protection from lytic infection. The cholesterol in the initial blood meal might allow the primary infection of SFV in mosquitoes, while decreased levels of cholesterol in other tissues could inhibit their subsequent infection by SFV. Other viral and bacterial pathogens with a sterol-dependent entry mechanism might also show effects of cholesterol on their infection or tissue tropism.

One suggestion for the origin of viral fusion proteins has been that they evolved from host cell proteins which carry out cellular fusion reactions (White et al., 1983). If the alphavirus fusion protein evolved from a cellular protein, the cellular homologue would be expected to use cholesterol as an integral part of its function. There are endogenous cellular proteins that have specific interactions and requirements for cholesterol. A well-characterized example is that of HMG-CoA reductase, the rate-limiting enzyme of cholesterol biosynthesis (Goldstein and Brown, 1990). This ER enzyme is down regulated by rapid degradation in the presence of an excess of cellular cholesterol. The increased turnover is due to an interaction of cholesterol with the transmembrane domains of the enzyme (Jingami et al., 1987). Since cholesterol is not present in many insect tissues, an insect cell homologue of the SFV fusion protein would presumably not rely on a sterol-requiring mechanism. In this light, it is interesting that HMG-CoA reductase in insect cells does not down-regulate in response to cholesterol, suggesting that indeed the sterol interaction is missing (Brown et al., 1983). Our findings of a sterol requirement for SFV fusion in vivo may be an example of a common motif between virus proteins and proteins of vertebrate cells.

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