

# Cholesterol Is Required in the Exit Pathway of Semliki Forest Virus

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**Abstract.** The enveloped alphavirus Semliki Forest virus (SFV) infects cells via a membrane fusion reaction triggered by low pH. For fusion to occur cholesterol is required in the target membrane, as demonstrated both in *in vitro* fusion assays and *in vivo* for virus infection of a host cell. In this paper we examine the role of cholesterol in postfusion events in the SFV life cycle. Cholesterol-depleted insect cells were transfected with SFV RNA or infected at very high multiplicities to circumvent the fusion block caused by the absence of cholesterol. Under these conditions, the viral spike proteins were synthesized and transported to the site of p62 cleavage with normal kinetics. Surprisingly, the subsequent exit of virus particles was

dramatically slowed compared to cholesterol-containing cells. The inhibition of virus production could be reversed by the addition of cholesterol to depleted cells. In contrast to results with SFV, no cholesterol requirement for virus exit was observed for the production of either the unrelated vesicular stomatitis virus or a cholesterol-independent SFV fusion mutant. Thus, cholesterol was only critical in the exit pathway of viruses that also require cholesterol for fusion. These results demonstrate a specific and unexpected lipid requirement in virus exit, and suggest that in addition to its role in fusion, cholesterol is involved in the assembly or budding of SFV.

THE entry of viruses into their host cells is a key step in the virus infection pathway. The enveloped alphavirus, Semliki Forest virus (SFV)<sup>1</sup>, infects cells via receptor-mediated endocytosis, followed by a membrane fusion reaction triggered by the acid pH in endosomes (15). This potent low pH-induced membrane fusion activity has been extensively characterized both in infected cells and by *in vitro* fusion assays. Fusion is mediated by the viral spike protein, which contains two transmembrane glycopolypeptides, E1 (50,786 D) and E2 (51,855 D), and a peripheral glycopolypeptide, E3 (11,369 D) (reviewed in references 39 and 41). Low pH has been shown to cause a number of conformational changes in the viral spike protein (1, 15–17, 22, 24, 30, 47–49). In addition to the low pH requirement for fusion, cholesterol has been shown to play a key role in the fusion reaction. Analysis of SFV–liposome interactions showed that fusion requires cholesterol in the target membrane, with maximal effects occurring at a sterol to phospholipid ratio of 0.5 (1, 50). Other sterols containing a 3 $\beta$ -hydroxyl group can substitute for cholesterol, even if they do not produce the same effects as cholesterol on lipid bilayer fluidity, phospholipid condensation, and hydrocarbon chain ordering (18). Thus, cholesterol does not seem to be acting as a membrane fluidizing agent. Instead, the role of cholesterol in fusion

may involve a direct interaction with the SFV spike protein, since a proteolytically truncated form of the E1 subunit requires both low pH and a 3 $\beta$ -hydroxysterol in order to undergo its conformational change at acidic pH (16, 17).

In previous work, we exploited the observation that insect cells are cholesterol auxotrophs (3, 29), and can be maintained virtually devoid of cholesterol by growth in culture medium containing low-density lipoprotein (LDL)-depleted serum (40). Such cholesterol-deficient insect cells are unchanged in phospholipid composition, do not synthesize any replacement sterol, and unlike mammalian cells show no deleterious effects from sterol depletion (40). Using the C6/36 mosquito cell line as a host, we compared all of the steps of the virus infection pathway under control and sterol-depleted conditions (35). The cholesterol-depleted cells are unaltered in their ability to bind, internalize, and acidify virus, but are blocked in SFV fusion and subsequent virus replication. In contrast, the depleted cells are readily infected by the cholesterol-independent vesicular stomatitis virus (VSV), which also enters cells via endocytosis and low pH-triggered fusion (25). The block in SFV infection is specifically reversed by repletion with a 3 $\beta$ -hydroxysterol.

Although these experiments show that SFV entry into the cell requires cholesterol, there are several important viral replication events after the fusion step that may also have a cholesterol requirement. For example, viral RNA replication is known to occur on membranes (7), and synthesis of virus RNA requires continuous cellular phospholipid syn-

1. *Abbreviations used in this paper:* HIFCS, heat-inactivated FCS; LDL, low-density lipoprotein; SFV, Semliki Forest virus; VSV, vesicular stomatitis virus.

thesis (34). The requirement for sterol in these membranes has not been determined. Sterol could also play a role in the transport and function of the transmembrane spike protein, or in correct virus assembly and budding. Here we have analyzed the cholesterol requirements in these postfusion events in the SFV life cycle, using RNA transfection or high multiplicity infection to express the SFV genome in cholesterol-depleted cells. Our results indicate a striking and unexpected requirement for cholesterol in the exit pathway of SFV.

## Materials and Methods

### Virus and Cells

C6/36 cells, a clonal cell line derived from *Aedes albopictus* (13), were grown at 28°C in DME containing 100 U/ml penicillin and streptomycin and 10% heat-inactivated FCS (HIFCS). Cholesterol-depleted C6/36 cells were maintained at 28°C in DME and 10% delipidated HIFCS as previously described (35), and all incubations of C6/36 cells were at 28°C unless otherwise noted. After an adaptation period without cholesterol, the cells appeared morphologically normal, although they grew somewhat more slowly. The depleted cells have currently been passaged for more than a year under "sterol-free" conditions. These culture conditions reduce both free and esterified cholesterol to levels <2% those of control cells (35, 40).

BHK-21 cells were cultured at 37°C in DME containing 5% FCS, 100 U/ml penicillin and streptomycin, and 10% tryptose phosphate broth.

The virus stocks used in these experiments were a plaque-purified SFV wild type isolate (19), the Indiana strain of VSV, and *srf-1*, an SFV mutant selected to be cholesterol-independent. Stocks of *wt* SFV or VSV were grown and titered on BHK-21 cells. *Srf-1* (for sterol requirement in fusion) was selected by serial passage on C6/36 cells without cholesterol, and isolated by limiting dilution on these cells. It is one of three such mutants whose isolation and characterization will be described in detail elsewhere (Phalen, T., M. T. Marquardt, and M. Kielian, manuscript in preparation). The titer of *srf-1* was determined by infectious center assay on cholesterol-depleted C6/36 cells.

### Expression of Virus in Control and Cholesterol-depleted Cells

Two methods were used to express the SFV genome in control and cholesterol-depleted C6/36 cells.

**RNA Transfection.** Viral RNA was isolated by phenol-chloroform and SDS extraction (31) of purified SFV. RNA transfection was performed in Opti-MEM, a serum-free medium (Life Technologies, Grand Island, NY), and used either 6 µg Lipofectin, a cationic liposome mixture (Life Technologies), and 1 µg RNA/35 mm dish of cells, or 1.5 µg lipofectin and 100 ng RNA/well of a 24-well plate. After a 1-h transfection period, cells were incubated in Opti-MEM containing 0.2% BSA (Opti-MEM/BSA) and 100 U/ml penicillin and streptomycin.

**Prebinding and Infection with SFV.** High multiplicity infection was used to express SFV in depleted cells. Virus at multiplicities ranging from 100–2,000 pfu/cell was bound to depleted cells in RPMI 1640 medium containing 0.2% BSA and 10 mM HEPES, pH 6.8, without bicarbonate. Binding was performed on ice with shaking for 60–90 min. The inoculum was aspirated and the cells were cultured in Opti-MEM/BSA at 28°C. This protocol was used for all of the SFV infections of depleted cells. SFV infection of cholesterol-containing cells, or infection of either cell type with *srf-1* followed a similar prebinding procedure, but using the indicated lower multiplicities. Infection of either cell type with VSV was performed at 28°C without prebinding as previously described (35).

### Quantitation of SFV Expression after Infection or Transfection

Virus infection experiments were performed with cells plated on 12-mm round coverslips in 24-well plates. Control or depleted cells were infected with serial dilutions of SFV as described above. 2 h after infection, the medium was replaced with Opti-MEM/BSA plus 15 mM NH<sub>4</sub>Cl to prevent secondary infection, and cells were incubated overnight. For RNA transfection, both control and depleted cells in 35-mm plates containing 22-mm coverslips were transfected as described above, and then incubated for 6 h

in 2 ml Opti-MEM/BSA. All cells were then fixed with methanol and analyzed for spike protein expression by staining with a rabbit anti-spike antibody and a fluorescein-labeled second antibody (17). Cells expressing the spike protein were quantitated by fluorescence microscopy. The 6-h incubation period was chosen as a time when both cell types showed abundant expression of SFV spike protein, but before the infection spread to adjacent cells.

To evaluate delivery of the spike protein to the cell surface, control and depleted cells were transfected as above in 24-well trays containing 12-mm coverslips. After overnight incubation, cells were fixed with paraformaldehyde under non-permeabilizing conditions and stained with previously characterized mAbs against the E1 or E2 spike protein subunits (17).

### Production of Infectious SFV by Transfected Cells

Both control and depleted cells in 35-mm plates containing 22-mm coverslips were transfected as described above, and then incubated for 6 or 8 h in 2 ml Opti-MEM/BSA. Media were collected from the transfected dishes, and cell debris pelleted by centrifugation at 10,000 rpm for 20–30 min in a Sorvall SS34 rotor (DuPont, Newton, CT) at 4°C. The media were then treated with 100 µg/ml RNase A for 30 min at 37°C to remove residual virus RNA. Control experiments showed that this treatment did not reduce infectivity of virus particles, but degraded any SFV RNA remaining from the transfection. The virus in the supernatant was quantitated by plaque assay on BHK-21 cells (19). Transfection efficiency was assessed in each experiment using immunofluorescence as described above.

### Production of Radiolabeled Virus by Infected Cells

Cells were infected as described above by either prebinding on ice or 28°C incubation with the indicated multiplicities of SFV, VSV, or *srf-1*. After infection, cells were incubated for 18–22 h to establish viral expression. Cells were then preincubated for 15–30 min in methionine-free medium, and labeled for 15–30 min with 20–200 µCi/ml [<sup>35</sup>S]methionine (Trans-label; New England Nuclear, Wilmington, DE) as previously described (17). The concentration of label was increased about two- to fourfold for depleted cells infected with *wt* SFV to normalize the level of spike protein expression. Cells were chased for various periods of time in Opti-MEM/BSA plus 10× cold methionine (17). At the time of harvest, the chase medium was removed and the cells were lysed as previously described (17). Protease inhibitors (0.5 mM PMSF, 1 µg/ml pepstatin, and 10 µg/ml leupeptin) were added to the chase medium, and cell debris was pelleted as above. The supernatant was collected and Triton X-100 was added to a final concentration of 1.0–1.4%. Both SFV-infected cell lysates and chase media were analyzed by precipitation with a rabbit antibody against the SFV spike protein, followed by nonreducing SDS-PAGE as previously described (17). For VSV-infected samples, immunoprecipitation was performed using a rabbit antibody against purified VSV, and samples were reduced and alkylated before SDS-PAGE (35). Both SFV and VSV samples were evaluated by reprecipitation to ensure completeness of the immunoprecipitation reaction.

Densitometry was performed on linear exposures of gels using a Molecular Dynamics Class A Computing Densitometer (Sunnyvale, CA) with Quantity One software from PDI Corp. (Huntington Station, NY). Quantitation of SFV and *srf-1* release was expressed as the amount of spike proteins (E1 and E2) in the medium divided by the total amount of spike proteins in the cell lysate plus medium samples. VSV release was quantitated by following the amount of the viral M protein released in the medium divided by the total M in cell lysate plus medium. M protein was quantitated since the amount of G protein expressed in mosquito cells is reduced (10), and a protein migrating close to the position of N protein was secreted from uninfected cells (Marquardt, M., unpublished observations). Similar VSV release was also observed if radiolabeled virus was pelleted through a sucrose cushion and quantitated by SDS-PAGE (data not shown).

### Electron Microscopy

Control cells were infected as above with *wt* SFV at 10 pfu/cell, and depleted cells with *wt* SFV at 100–2,000 pfu/cell. Control cells were infected with *srf-1* at a multiplicity of 10 infectious centers/cell, and depleted cells with 100 infectious centers/cell. After removal of the inoculum, the medium was changed twice at 1–2-h intervals to wash off non-endocytosed input virus. After a 20-h incubation, cells were fixed in 2.5% glutaraldehyde/0.1 M cacodylate for 30 min, washed in 0.1 M cacodylate buffer, osmicated, dehydrated, and embedded in LX112 (Ladd Research, Burlington, VT). Thin sections were stained with uranyl acetate and lead citrate, and examined in a JEOL 1200EX (Peabody, MA).

## Repletion of Cholesterol-depleted Cells

Cholesterol-depleted cells were infected with 200 pfu wt SFV/cell as described above, and put into repleting medium at 13-h postinfection. Repleting medium consisted of LDL-minus growth medium to which a stock solution of cholesterol in ethanol was added at a final concentration of 50  $\mu\text{g/ml}$  (this method was a personal communication from Dr. Richard Pagano). A parallel plate of depleted cells was put into LDL-minus medium without addition of cholesterol. At various times after repletion, cells were pulse labeled with [ $^{35}\text{S}$ ]methionine, chased for 90 min, and the cells and medium analyzed for SFV expression by immunoprecipitation as described above.

## Results

### Infection and Transfection of C6/36 Cells

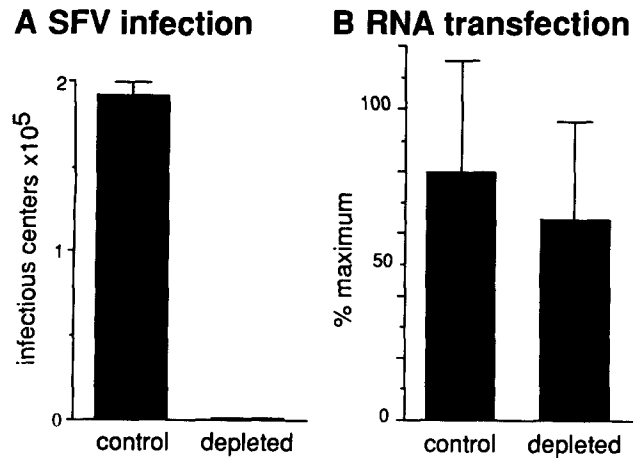
To assess the potential involvement of cholesterol in the post-fusion SFV replication cycle, the SFV genome had to be expressed in cholesterol-depleted cells. SFV is not capable of efficiently infecting cholesterol-depleted cells due to a block in fusion with the endosome membrane (35). The difference in infectability of the two cell types could be quantitated using a sensitive infectious center assay (Fig. 1 A). Cells maintained in the presence of cholesterol were consistently infected  $\sim 2,000$  times more efficiently than cholesterol-depleted cells. If depleted cells were returned to LDL-containing medium, they gradually regained their ability to be infected by SFV (data not shown), suggesting that virus-endosome fusion is dependent on the amount of cholesterol in the host membrane.

Since the cholesterol-depleted C6/36 cells were not efficiently infected by SFV, alternative approaches were required to express the SFV genome. One method to circumvent the fusion block was to transfect the cells using lipofectin and purified SFV RNA. After 1 h, the lipofectin was removed, the cells were cultured 6 h, and virus-expressing cells were quantitated by immunofluorescence with an antibody against the SFV spike. At this time point, the number of primary transfected cells could be quantitated in the absence of secondary infection. In contrast to the  $\sim 2,000$ -fold difference in infection efficiency of the two cell types (Fig. 1 A), RNA transfection was approximately the same in the two cell types (Fig. 1 B). Thus, RNA transfection of depleted cells circumvented the fusion block and delivered SFV RNA into the cytoplasm where it was translated.

Our second approach to infecting cholesterol-depleted cells took into account the large decrease in infectability in the absence of cholesterol. High multiplicities of SFV were prebound to depleted cells on ice, and then warmed to 28°C. The prebinding step appeared to maximize virus uptake by both control and depleted C6/36 cells (Marquardt, M., unpublished results), and in combination with the increased multiplicity allowed a low level of infection to occur under cholesterol-depleted conditions. Although the mechanism of SFV entry into the cell in the absence of cholesterol was not determined, infection was blocked by the addition of  $\text{NH}_4\text{Cl}$  during virus uptake (data not shown). This result suggests that infection of depleted cells occurred by endocytosis and either low pH-triggered fusion or low pH-dependent degradation of virus in lysosomes.

### Kinetics of SFV Exit in Control and Cholesterol-depleted C6/36 Cells

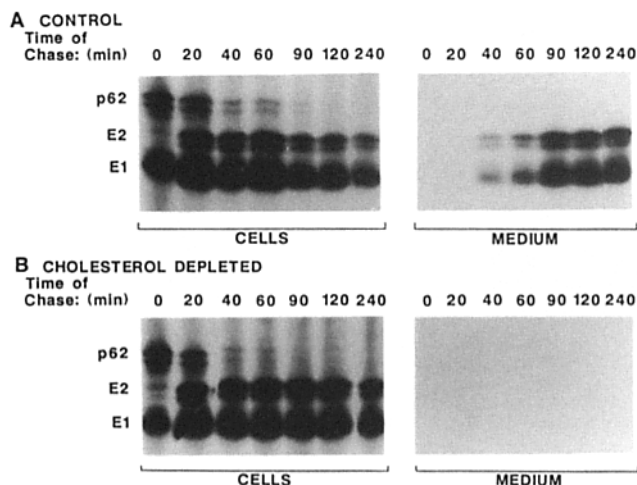
Our results indicated that in the absence of cholesterol the



**Figure 1.** Effect of cholesterol depletion on SFV infection and transfection. (A) Infection. Control and depleted cells were infected by binding with serial dilutions of SFV for 1 h on ice, warming to 28°C for 2 h in Opti-MEM/BSA, followed by overnight incubation in Opti-MEM/BSA containing 15 mM  $\text{NH}_4\text{Cl}$  to block secondary infection. The number of infectious centers was quantitated by immunofluorescence, and normalized to an initial inoculum of one infectious center/cell. Average and standard deviation of eight experiments. (B) Transfection. Control and depleted cells were transfected with 1  $\mu\text{g}$  SFV RNA and 6  $\mu\text{g}$  lipofectin. After incubation for 6 h at 28°C, the infectious centers in 10 random fields of each cell type were quantitated by immunofluorescence. Expression was normalized to the highest expressing cell type in each experiment. Average and standard deviation of three experiments.

incoming viral RNA was translated to yield a functional membrane-bound RNA polymerase. As qualitatively evaluated by immunofluorescence, cells that became infected had similar levels of viral spike protein expression in the presence or absence of sterol (data not shown). Spike protein transport and assembly into virus was then assayed for possible cholesterol-dependence. Cells were infected at high multiplicity, incubated overnight, and pulsed with [ $^{35}\text{S}$ ]methionine to label virus proteins. The cells were chased for sufficient time to permit transport of the viral spike protein, budding, and release of progeny virions. Cells and media were assayed at the indicated times for the presence of the viral spike polypeptides by immunoprecipitation. Both the virus inoculum and the amount of label were increased for the depleted cell samples to compensate for their decreased infectability (see legend to Fig. 2).

Both control and depleted cells expressed immunoprecipitable spike protein (Fig. 2). The E2 and E3 spike subunits are synthesized as a p62 precursor that is posttranslationally cleaved (5). The kinetics of this cleavage were the same in control and depleted cells. Although these initial steps in virus biosynthesis were unaltered, the release of radiolabeled virus from the cell was drastically affected by the absence of cholesterol. Significant amounts of release from the control C6/36 cells were observed after 90–120 min of chase (Fig. 2 A), and the release at 4 h represented  $\sim 21\%$  of the total E1 and E2 by densitometry. In contrast, virtually no release was observed from depleted cells after 4 h of chase (Fig. 2 B) (undetectable by densitometry). Some virus appeared to be released after 12–24-h chase times, but the proportion of spike proteins released from depleted cells was consistently less than that of control cells, even after a 72-h chase (data not shown).



**Figure 2.** Kinetics of SFV exit in control and cholesterol-depleted C6/36 cells. Control and sterol-depleted cells were infected with SFV at multiplicities of 10 and 100 pfu/cell, respectively. After an overnight incubation cells were pulse labeled for 15 min with [<sup>35</sup>S]methionine at concentrations of 50  $\mu$ Ci/ml for control and 200  $\mu$ Ci/ml for depleted cells. Cells were chased for various periods of time, the chase media were collected, and the cells were lysed. Cells and media were incubated with an antibody against the SFV spike protein, and immunoprecipitates were analyzed using SDS-PAGE and autoradiography. Shown is a representative example of seven experiments.

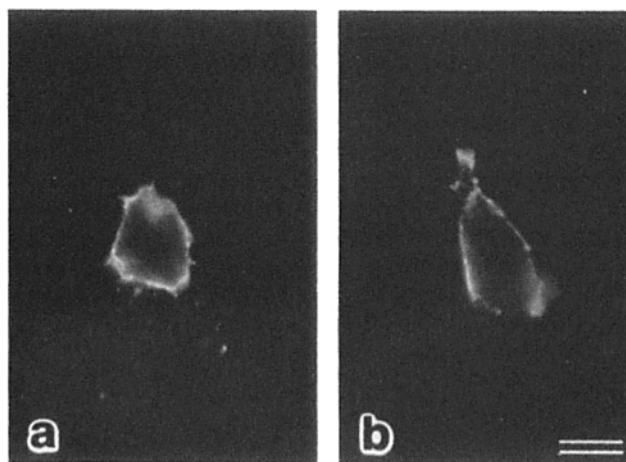
It was possible that virus was released in the absence of cholesterol, but in a form not recognized by our polyclonal antibody. The media from a 4 h chase were therefore assayed by precipitation with TCA, rather than immunoprecipitation. Large amounts of radiolabeled virus were precipitated from control but not depleted cell medium (data not shown). The experiment shown in Fig. 2 used high-multiplicity infection of depleted cells, but similar results were obtained by the RNA transfection method (data not shown).

We also assayed the production of infectious virus by control and depleted cells transfected with virus RNA. Cells were incubated for 6 h after transfection, and the medium assayed by plaque assay. At this time point, primary transfectants were strongly positive but the infection had not spread to adjacent cells. As shown in Table I, the amount of infectious SFV released from cholesterol-depleted cells at 6 h was consistently 3–4% of the amount released from control cells.

**Table I. Production of Infectious SFV by Control and Cholesterol-depleted Cells**

Experiment number	Cell type	pfu/ml	Percent control
1	control	1,640	100
	depleted	64	4
2	control	186	100
	depleted	6	3
3	control	1,100	100
	depleted	38	4

35 mm dishes of C6/36 cells were transfected with 1  $\mu$ g SFV RNA using lipofectin as described. 6-h posttransfection, the media were harvested and treated with 100  $\mu$ g/ml RNase for 30 min at 37°C. Infectious virus was then quantitated by plaque titration on BHK cells.



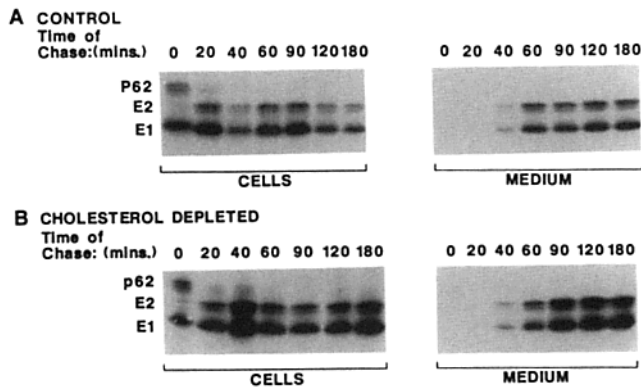
**Figure 3.** Surface expression of spike protein subunits in cholesterol-depleted cells. Cholesterol-depleted C6/36 cells were transfected with SFV RNA, cultured overnight, and fixed with paraformaldehyde under nonpermeabilizing conditions. Cells were stained with either mAb E1-1 against the E1 subunit (A) or mAb E2-1 against the E2 subunit (B), followed by rhodamine-labeled second antibody. Bar, 10  $\mu$ m.

A similar difference between control and depleted cells was observed 8 h after transfection (data not shown). These results are in agreement with the difference in virus release observed in the pulse–chase experiments, although any noninfectious virus released from either cell type is not quantitated by the plaque-assay analysis.

The delivery of the E1 and E2 spike protein subunits to the plasma membrane of control and depleted cells was assayed by immunofluorescence with previously characterized subunit-specific mAbs (17). After RNA transfection, both spike protein subunits were expressed on the plasma membrane of control or depleted cells, as shown in Fig. 3 for depleted cells. This experiment indicates that the inhibition of virus exit in cholesterol-depleted cells is not due to the failure of the spike protein to be transported to the plasma membrane.

#### **Kinetics of Exit of a Cholesterol-independent SFV Mutant**

Although these results suggested an effect of cholesterol depletion on the exit pathway of SFV, it was important to control for any nonspecific effects of cholesterol depletion on the secretory pathway in C6/36 cells. Pulse–chase studies of endogenous protein secretion from control and depleted cells suggested that the overall kinetics of secretion in the two cell types were similar (data not shown). To address this question more quantitatively, we took advantage of *srf-1*, an SFV mutant recently isolated in our laboratory (Phalen, T., M. T. Marquardt, and M. Kielian, manuscript in preparation). This mutant was isolated by selecting for growth on C6/36 cells without cholesterol, and showed about 50-fold more efficient fusion with cholesterol-depleted cell membranes than that of wild type SFV. We reasoned that if cholesterol was playing a virus-specific role in SFV exit, a virus that does not require cholesterol for entry into the cell by fusion might also be independent of cholesterol during exit. The results in Fig. 4 show that under conditions of identical multiplicity and labeling, *srf-1* produced similar

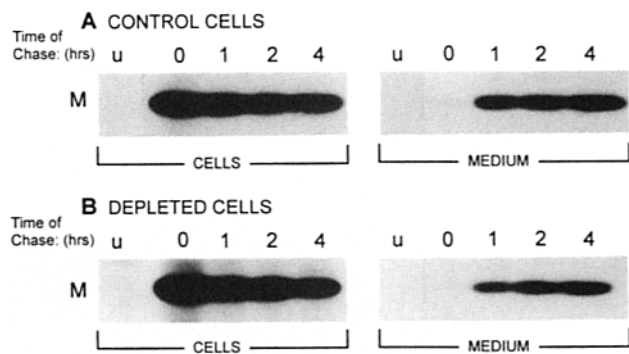


**Figure 4.** Kinetics of *srf-1* exit in control and cholesterol-depleted C6/36 cells. Control and cholesterol-depleted cells were infected with *srf-1* at a multiplicity of 10 infectious centers per cell. Cells were incubated overnight, pulse labeled with [<sup>35</sup>S]methionine at a concentration of 100  $\mu$ Ci/ml, chased for the indicated times, and the cells and media assayed for labeled spike proteins by immunoprecipitation followed by SDS-PAGE and autoradiography. Shown is a representative example of four experiments.

amounts of spike protein in control and depleted cells (Fig. 4). The kinetics of *srf-1* p62 processing were also similar in the two cell types. Finally, and in marked contrast to results with *wt* SFV (Fig. 2), the kinetics of *srf-1* exit from depleted cells were found to be equivalent or faster than those in control cells (Fig. 4). The release of *srf-1* at 3 h was 6% from control cells and 24% from depleted cells by densitometry (average of two experiments). Similar cholesterol-independent release was observed with two other *srf* mutants, and will be described in detail elsewhere (Phalen, T., M. T. Marquardt, and M. Kielian, manuscript in preparation). These results suggest that the secretory pathway per se is unaffected by cholesterol depletion, and that the sterol requirement is specific for a step in the SFV exit pathway.

#### Effect of Cholesterol Depletion on the Assembly and Release of VSV

As a second control for the specificity of the cholesterol effect, we assayed the exit of VSV, an unrelated rhabdovirus. VSV also infects cells by endocytosis and low pH-mediated fusion, but its fusion activity is cholesterol independent (6, 35). VSV-infected control and depleted cells showed similar expression of virus proteins, as quantitated by following synthesis of the VSV M protein (Fig. 5, *cell samples*). In contrast to results with *wt* SFV, release of VSV from control and depleted cells was comparable (Fig. 5, *medium samples*). After a 3-h chase period, M protein release ranged from 13–28% of the total from control cells, and from 13–22% of the total from depleted cells (quantitation from two experiments). These results in C6/36 cells are in contrast to the tenfold reduction in VSV production previously reported in a cholesterol-depleted mammalian cell line (32), which could reflect the deleterious effects of cholesterol depletion in mammalian cells. Taken together, our results with *wt* SFV, *srf-1*, and VSV suggest that the cholesterol requirement for virus exit was specific for viruses requiring cholesterol for entry into the cell by membrane fusion.



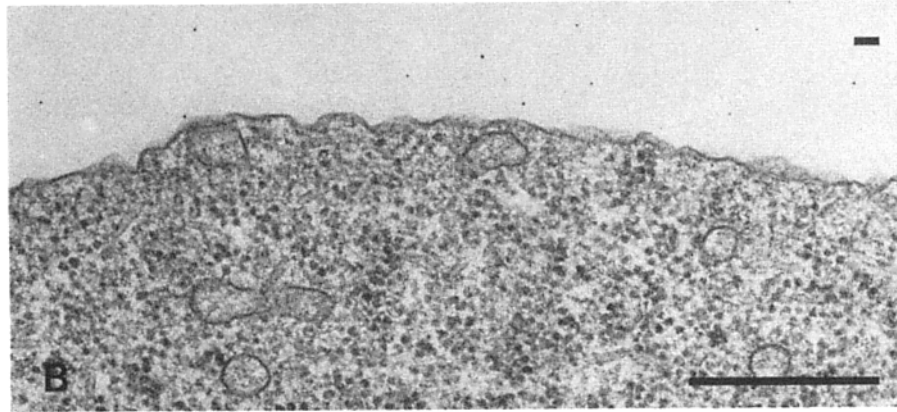
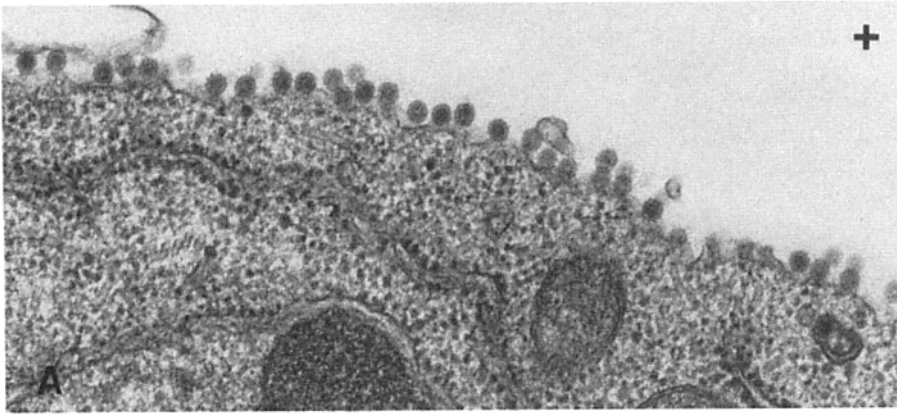
**Figure 5.** Kinetics of VSV exit in control and cholesterol-depleted C6/36 cells. Control and depleted cells were infected with VSV at 200 pfu/cell, incubated overnight, pulse labeled with [<sup>35</sup>S]methionine at a concentration of 50  $\mu$ Ci/ml, chased for the indicated times, and the cells and media assayed for labeled virus proteins by immunoprecipitation with an antibody against purified VSV, followed by SDS-PAGE and autoradiography. The M protein precipitation from a representative example of two experiments is shown.

#### Electron Microscopy of SFV-infected Cells

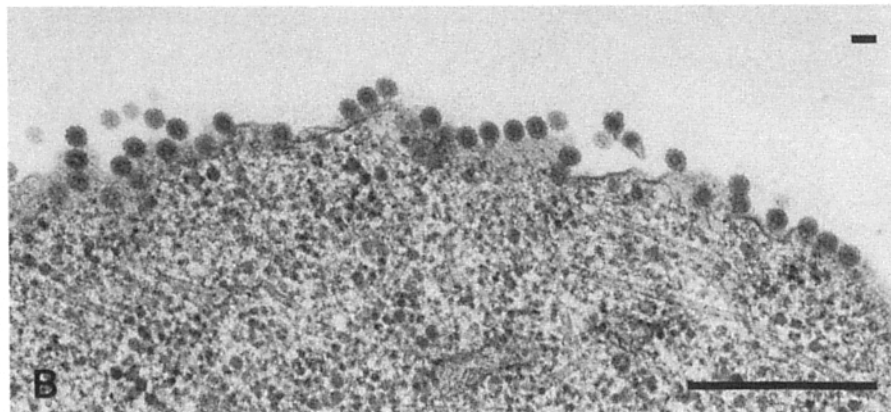
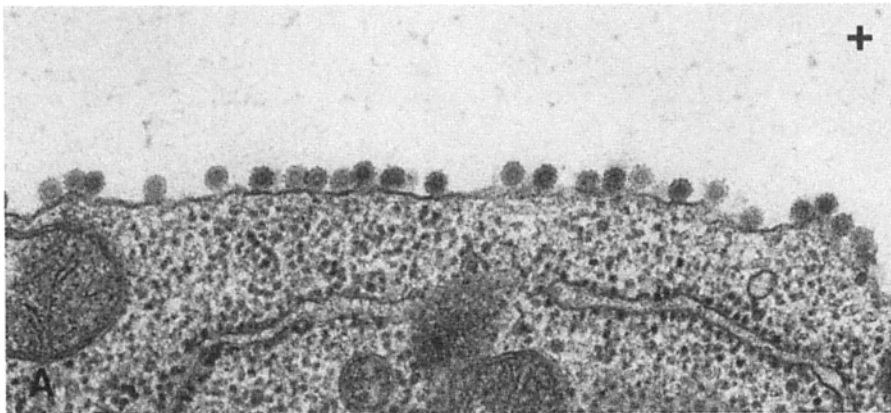
In addition to the biochemical assays for virus production, ultrastructural analysis was used to visualize virus production in infected C6/36 cells. The normal mode of alphavirus release from mammalian cells is by budding from the plasma membrane (39). The mechanism of release from mosquito cells may involve both plasma membrane budding and/or packaging into cytoplasmic vesicles that release virus upon exocytosis (2, 43). Depending on the mosquito cell line used for virus infection, either mode of virus maturation may predominate (26). To examine budding of SFV in control and depleted cells, EM was performed after infection with *wt* or *srf-1* SFV. Parallel cultures were assayed for infection by immunofluorescence. In control cells infected with *wt* virus, abundant virion formation was observed at the plasma membrane, and few cytoplasmic inclusions of virus were seen (Fig. 6 A). In contrast, neither plasma membrane nor intracellular virion formation was observed in cholesterol-depleted cells (Fig. 6 B). We were also unable to detect significant numbers of assembled nucleocapsids or nascent virus buds in the cholesterol-depleted cells. Virus particles were not observed in the depleted cells even after infection with 2,000 pfu/cell. Importantly, EM of cells infected with the *srf-1* mutant revealed large numbers of morphologically normal virions that appeared to be budding from the plasma membrane of both control and depleted cells (Fig. 7, A and B). Thus, the EM experiments corroborated the biochemical assays of both SFV and *srf-1* exit.

#### Reversal of the Exit Block by Sterol Addition

The reversibility of the exit block was assayed by the addition of cholesterol to the culture medium of previously infected depleted cells. The amount of cholesterol added (50  $\mu$ g) represents approximately a 30-fold excess over the amount of cholesterol found in the same number of nondepleted C6/36 cells (35). The release of virus was followed by pulse-chase analysis at various times after cholesterol addition. Reversal of the exit block was observed 9 h after cholesterol repletion (Fig. 8), at which time densitometry showed that  $\sim$ 14% of the total spike proteins were released into the

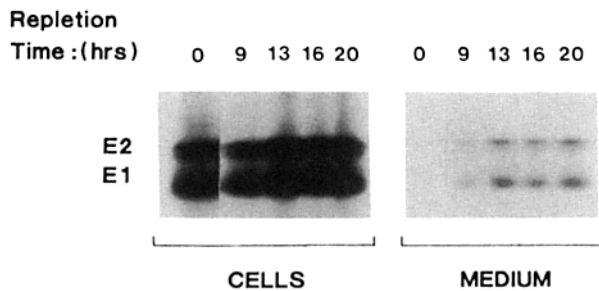


*Figure 6.* Electron micrographs of SFV-infected control and sterol-depleted C6/36 cells. Cells were infected with wt SFV at multiplicities of 10 pfu/control cell (A) or 100 pfu/depleted cell (B). After ~20 h of culture, the cells were fixed and processed for transmission EM. Bar, 0.5  $\mu$ m.



*Figure 7.* Electron micrographs of srf-1-infected control and sterol-depleted C6/36 cells. Control or sterol-depleted cells were infected with srf-1 at a multiplicity of 10 infectious centers/cholesterol-containing cell (A), and 100 infectious centers/depleted cell (B). After ~20 h of culture, the cells were fixed and processed for transmission EM. Bar, 0.5  $\mu$ m.





**Figure 8.** Effect of cholesterol addition on the SFV exit block. Depleted cells were infected with wt SFV at a multiplicity of 200 pfu/cell, and incubated for 13 h at 28°C. The cells were then exposed to 50 µg cholesterol in 1 ml for 9–20 h, and SFV exit assayed by a 30 min label with 20 µCi [<sup>35</sup>S]methionine/ml, followed by a 90-min chase. As a control, depleted cells were incubated without the addition of cholesterol for 20 h (*time 0*). Cells and media were assayed for virus release by immunoprecipitation. Shown is a representative example of four experiments. The time 0 lanes were exposed about fivefold longer to normalize for protein expression levels.

medium after a 90-min chase. The level of release did not change after as much as 20 h of cholesterol repletion. This amount of virus production is comparable or higher than that obtained from control cells after a 90-min chase (Fig. 2). No reversal of the exit block was observed after only 4 h of cholesterol repletion (data not shown).

We also wished to determine if virus structural proteins synthesized in the absence of cholesterol were able to recover their exit competence. Infected depleted cells were pulse labeled, cholesterol was added to the chase medium, and the medium assayed for released radiolabeled virus after various times of chase. Little release was observed even after 12 h of chase (data not shown). This result is difficult to interpret, however, as there was substantial turnover of the labeled spike proteins during the repletion and chase period.

## Discussion

Using cholesterol-depleted mosquito cells, we have identified a requirement for cholesterol in the exit pathway of wt SFV. No cholesterol requirement was observed for the exit of *srf-1*, an SFV cholesterol-independent fusion mutant, or for the rhabdovirus VSV. Cholesterol is thus important both in SFV-endosome fusion (35) and in SFV exit. Since the mosquito is the natural vector for alphaviruses, the availability of cholesterol in the insect would be predicted to affect these two aspects of the SFV infection cycle. Studies have shown that the distribution of cholesterol varies among insect tissues (3), and this distribution might therefore affect their ability to support primary SFV infection or virus production.

Cholesterol was able to reverse the block in the virus exit pathway by 9 h after sterol addition. These kinetics are somewhat slower than reversal of the block in SFV-endosome fusion, which showed reversal 5 h after cholesterol addition (35). It is not clear if this reflects simply the differences in assay sensitivities and experimental protocols, or a true requirement for a higher cholesterol level in exit versus fusion. The slower reversal of the exit block might also reflect the time needed for the *de novo* synthesis of a component, or the

turnover of a virus protein synthesized in the absence of cholesterol.

The intriguing question prompted by the studies in this report is the mechanism of cholesterol's effect on virus exit. To consider possible explanations, we will review the events involved in the SFV life cycle (39, 41, 44). After virus-endosome fusion, the nucleocapsid is released into the cytoplasm and the genomic RNA is translated to give the four subunits of the viral replication complex. This replicase synthesizes a minus-strand copy of the genomic RNA that serves as a template for both full-length plus-strand RNA, and the subgenomic RNA encoding the structural proteins. These reactions occur on membranes that appear to be derived from lysosomes and endosomes (7). The subgenomic RNA is translated to give the cytoplasmic capsid protein, and the p62, 6K, and E1 membrane-bound polypeptides. The capsid protein packages the full-length RNA to form the virus nucleocapsid. The spike proteins are transported as a complex of E1, p62, and 6K through the RER and Golgi compartments (23, 47), and the p62 precursor is cleaved to mature E2 and E3 (5). The mature spike proteins are transported to the plasma membrane, where they form patches that exclude host cell proteins (33). The nucleocapsid then interacts with the 31-residue-long cytoplasmic tail of E2 to drive the budding of virus (14, 45). The virus particle is released by a final pinching off and fission of the plasma membrane to close the virus envelope.

Our studies suggested that depleted cells expressing the SFV genome could translate the SFV genomic RNA, produce a functional RNA polymerase, make subgenomic RNA, synthesize the structural proteins, and transport the spike protein to the plasma membrane. In the absence of cholesterol, however, these spike proteins were not efficiently released in virus particles. The inhibition of virus exit could be due to a block in full-length RNA production, to a decreased rate of spike protein transport to the plasma membrane, or to defects in virus subunit interactions, virus assembly, or the final budding reaction that releases the completed virus particle.

It seems likely that the full-length plus-strand RNA is produced, since analysis of RNA<sup>-</sup> Sindbis ts mutants suggests that if the polymerase can initiate from the subgenomic promoter, it can also produce full-length RNA (12).

A number of spike subunit and capsid interactions are required for viral protein transport, assembly, and budding (39). E1 and p62/E2 dimerize before leaving the RER, and are found as a tightly associated dimer during transport and in virus particles. Our preliminary results suggest that the spike subunits from either control or depleted cells dimerize equivalently (data not shown). There has been some controversy concerning the localization of p62 cleavage in mosquito cells, with the suggestion that cleavage might occur in the RER in this cell type (27). However, we have observed that cleavage is blocked by treating C6/36 cells with either carbonylcyanide *m*-chlorophenylhydrazone or monensin, agents that block protein transit beyond the RER or *medial*-Golgi, respectively (data not shown). It is therefore most likely that p62 cleavage in C6/36 cells occurs in the TGN or immediately thereafter, as has been shown in mammalian cells (5). The apparently normal p62 cleavage kinetics in depleted cells (Fig. 2) therefore implies that spike protein traffic to the TGN was unaffected by sterol. Immunofluores-

cence data indicated that both E1 and E2 were transported to the plasma membrane in cholesterol-depleted cells (Fig. 3). Although the detailed kinetics of plasma membrane transport have not yet been evaluated, it appears unlikely that a markedly slower transport rate is responsible for decreased virus release in the absence of cholesterol.

The spike protein assembles into a trimer (E1-E2-E3)<sub>3</sub> on the surface of virus-infected cells (36) or virus particles (46), and it is possible that this trimeric interaction requires cholesterol. The block could also lie in the formation of nucleocapsids or their interaction with the cytoplasmic tail of E2.

Recent studies have suggested a potential role of the small hydrophobic 6K polypeptide in alphavirus exit. Newly synthesized 6K appears to associate with the E1 and E2 subunits during transport (23), and is incorporated into virus particles at a 10-fold lower stoichiometry than the other structural proteins (9). Using a 6K deletion mutation of an SFV infectious clone, a block in virus release was observed for virus lacking the 6K polypeptide (21). In cells infected with this mutant, spike proteins are synthesized and p62 is cleaved normally, but release of labeled or infectious virus is greatly decreased. 6K is fatty acylated on cysteine residues in or near its putative transmembrane domain (9, 20). Alteration of these cysteines by site-directed mutagenesis of the Sindbis virus infectious clone resulted in underacylated 6K, and also conferred a budding defect on the progeny virions (8). Unlike the block in virus production caused by cholesterol depletion, the acylation mutants produce aberrant particles with multiple nucleocapsids. Taken together, these data suggest that it is important to determine if the exit block in cholesterol-depleted cells involves effects on 6K.

Our EM data to date have not shown a definitive location for the block in SFV exit, but suggest that the block in virus release may lie in a virus assembly step. The limitations in interpreting these results are that immunofluorescence assays show that only about 15% of the depleted cells are expressing SFV, and that EM alone does not differentiate between infected and uninfected cells. Immuno-EM should prove useful to positively identify infected cells and to localize the spike and capsid proteins. Further ultrastructural studies should also reveal if the block is localized to the final pinching off of the virus bud to form a completed particle. This step involves a membrane fission event, which could be mediated by a similar spike protein mechanism as that involved in the cholesterol-requiring fusion activity of SFV. Membrane fusion joins two membrane-bound compartments to form one, while membrane fission divides one membrane compartment into two (42). Although fusion and fission processes appear symmetrical, they may occur via different mechanisms (42). One clear distinction in the SFV system is that fusion in the endosome requires low pH, while membrane fission during virus budding must take place at the neutral pH of the cytoplasm. It is interesting that in the *srf-1* mutant, the cholesterol requirements for fusion and for virus exit were both overcome. Additional SFV mutants are currently being sought in the hope of distinguishing between the cholesterol-requiring events in entry and exit.

The roles of cholesterol in animal cells are largely unexplained. In mammalian cells, cholesterol appears to play a crucial role in membrane structure, affecting both permeability and stability (28, 51). Surprisingly, work with cholesterol-depleted cultured insect cells has demonstrated that

cholesterol is not a required structural component of the insect cell membrane, and thus the dietary sterol required by insects may be needed for hormone production or other factors (40). In mammalian cells, cholesterol is highly compartmentalized among cellular membranes, being low in the ER and high in the plasma membrane (4, 51). Although it is clear that cells expend considerable metabolic energy in establishing and maintaining this cholesterol polarity, its cellular function is completely unknown. Within mammalian cells, there are endogenous proteins that have specific interactions and requirements for cholesterol, but in no case has the molecular basis of such an interaction been defined. For example, HMG-CoA reductase, the rate-limiting enzyme of cholesterol biosynthesis, is an ER enzyme that is down regulated by a cholesterol-dependent degradation event (11). Recent studies have shown that cholesterol is important in the maintenance of receptor clustering for the methyltetrahydrofolate receptor (38), due to a cholesterol requirement for the structure of caveolae, the specialized membrane domains in which the folate receptor is endocytosed (37). Thus, even the limited amount of data currently available suggests that cholesterol plays a variety of important roles in the function of mammalian cells. Perhaps studies in progress in our laboratory on the mechanism of the cholesterol requirement for SFV fusion and exit will also be able to address more general questions about the differences between the mammalian and insect cell cholesterol requirements, and the functions of cholesterol in animal cells. The unexpected finding of an involvement of cholesterol in SFV exit suggests that future studies of the role of lipids in viral and cellular function may uncover additional new roles for these little understood constituents.

M. Kielian dedicates this paper to the memory of Dr. Zanvil Alexander Cohn. We thank Michael Rindler, John Bergman, and John Rose for their gifts of anti-VSV antibodies, and Douglas Lyles for helpful advice on VSV assembly. We thank Frank Macaluso and the members of the Einstein Analytical Ultrastructure Center for their excellent assistance with the EM. Matthew Klimjack and Susan DeCandido provided much appreciated help with tissue culture and SDS-PAGE. We are grateful to Dennis Shields and Pamela Stanley for critical reading of the manuscript.

This work was supported by grants to M. Kielian from the National Institutes of Health (GM-38743), the American Cancer Society (VM-41), the Hirsch Charitable Trust, and the Pew Scholars Program in the Biomedical Sciences, and by Cancer Center Core Support Grant NIH/NCI P30-CA13330. M. T. Marquardt was supported by National Institutes of Health training grant 2T32 CA09173-15, and T. Phalen by National Institutes of Health training grant 15T32 HL-07675.

Received for publication 6 May 1993 and in revised form 6 July 1993.

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