A Single Point Mutation Controls the Cholesterol Dependence of Semliki Forest Virus Entry and Exit

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Abstract. Membrane fusion and budding are key steps in the life cycle of all enveloped viruses. Semliki Forest virus (SFV) is an enveloped alphavirus that requires cellular membrane cholesterol for both membrane fusion and efficient exit of progeny virus from infected cells. We selected an SFV mutant, srf-3, that was strikingly independent of cholesterol for growth. This phenotype was conferred by a single amino acid change in the E1 spike protein subunit, proline 226 to serine, that increased the cholesterol independence of both srf-3 fusion and exit. The srf-3 mutant emphasizes the relationship between the role of cholesterol in membrane fusion and virus exit, and most significantly, identifies a novel spike protein region involved in the virus cholesterol requirement.

MEMBRANE fusion is a critical and ubiquitous cellular process involved in the formation and trafficking of endocytic and exocytic vesicles, and in fusions between cells. It is becoming apparent that membrane lipid composition is strategically involved in the control of membrane budding, protein transport, and membrane fusion (Pfanner et al., 1990; De Camilli et al., 1996; Kearns et al., 1997), but the role of specific lipids and their interactions with cellular proteins are not yet clear. A striking example of a defined lipid requirement in membrane fusion is the Semliki Forest virus (SFV)[1] fusion protein, which mediates the cholesterol and sphingolipid-dependent fusion of the virus membrane with the cellular membrane during virus infection.

SFV is a member of the alphaviruses, enveloped positive–stranded RNA viruses with highly ordered icosahedral structures in which the spike and capsid proteins interact in a one-to-one association (reviewed in Strauss and Strauss, 1994; Kielian, 1995). The spike proteins also form extensive lateral interactions, and constitute a protein layer that almost completely covers the virus lipid bilayer (Cheng et al., 1995; Fuller et al., 1995). This simple virus structure protects the virus RNA genome, mediates virus fusion with the cell membrane to release the nucleocapsid and initiate infection, and is efficiently assembled during the budding of progeny virions from the host cell plasma membrane. SFV is a highly developed system to study virus membrane fusion and budding, two key steps in infection by all enveloped viruses.

SFV infects cells by cell surface receptor binding, uptake via receptor-mediated endocytosis, and low pH-triggered fusion of the virus membrane with that of the endosome. Virus membrane fusion is carried out by the 80 spike proteins on the surface of the virus. The spike proteins are trimers containing a complex of the E1, E2, and E3 glycopolypeptides, $(E1/E2/E3)_3$. E1 and E2 are type 1 transmembrane proteins of ~50 kD, and E3 is a peripheral polypeptide of ~10 kD. E1 is the fusion-active spike protein subunit, and a soluble ectodomain form of E1 has been shown to bind membranes in a low pH-dependent reaction (Klimjack et al., 1994). Upon exposure to low pH, the SFV spike protein undergoes a defined series of conformational changes (Garoff et al., 1994; Kielian, 1995). The normally tight dimeric interaction between the E1 and E2 transmembrane subunits is weakened, E1 exposes new antigenic epitopes, forms a trypsin-resistant homotrimer, and associates with the target membrane. E1 contains the putative virus fusion peptide, a highly conserved hydrophobic domain between amino acids 79–97 (Garoff et al., 1980). The importance of these conformational changes and the E1 fusion peptide is supported by the fact that a mutation within the fusion peptide, glycine 91 to aspartate, blocks both the formation of the E1 homotrimer and membrane fusion (Levy-Mintz and Kielian, 1991; Kielian et al., 1996).

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1. *Abbreviations used in this paper*: RT, reverse-transcriptase; SFV, Semliki Forest virus; wt, wild-type.

In addition to the requirement for low pH, SFV fusion has a striking requirement for specific lipids in the target membrane. Fusion requires cholesterol, with optimal concentrations of one cholesterol molecule per two phospholipids (White and Helenius, 1980). Fusion is also dependent on the presence of small amounts (~2 mol%) of sphingolipid in the target membrane (Nieva et al., 1994; Moesby et al., 1995). The SFV cholesterol requirement does not involve the general membrane fluidizing properties of cholesterol, but seems to be specific for the sterol 3β-hydroxyl group (Kielian and Helenius, 1984). The molecular mechanism by which cholesterol affects fusion is unclear at present, but cholesterol is required for the hydrophobic interaction of the virus with the target membrane before fusion (Kielian and Helenius, 1984; Bron et al., 1993; Nieva et al., 1994). In addition, cholesterol is specifically required for both the membrane binding of the E1 ectodomain, and its acid-dependent conformational changes such as epitope exposure, homotrimerization, and acquisition of trypsin resistance (Kielian and Helenius, 1985; Kielian et al., 1990; Klimjack et al., 1994).

To evaluate the role of cholesterol in vivo, we used the observation that insects are cholesterol auxotrophs (Nes and McKean, 1977), and that insect cells, unlike mammalian or avian cells, can be cholesterol-depleted without deleterious effects (Silberkang et al., 1983). Previously, we depleted the C6/36 mosquito cell line to <2% of the control level of cholesterol, and showed that the depleted cells are unaltered in their endocytic uptake and acidification properties, and are permissive for infection by vesicular stomatitis virus, an unrelated, cholesterol-independent virus (Phalen and Kielian, 1991). However, as predicted from the in vitro studies, depleted cells are blocked in SFV fusion and infection, and the block is specifically reversed by cholesterol addition. The depleted C6/36 cells are reduced about 5,000-fold in SFV infection efficiency compared to controls (Marquardt and Kielian, 1996). Using transfection of viral RNA or very high multiplicity infection, we then evaluated the involvement of cholesterol in the SFV exit pathway (Marquardt et al., 1993). Unexpectedly, our studies demonstrated that cholesterol is also involved in the efficient production of progeny virus. Thus, cholesterol seems to play two critical roles in the SFV life cycle, one involving virus fusion and one involving a late step in the formation or release of virus particles.

Given that SFV growth is strongly inhibited in cholesterol-depleted cells, we have now exploited this cell culture system to select for virus mutations that would permit growth in the absence of cholesterol. Such mutants were termed srf mutants, for sterol requirement in function. We previously showed that the srf phenotype allows more efficient virus exit from sterol-depleted cells (Marquardt et al., 1993). Characterization and molecular analysis of a srf mutant here reveals that the mutant's infection efficiency, membrane fusion, growth rate, and exit were all markedly less cholesterol-dependent than those of the parental virus. We demonstrate that the mutant has a single amino acid substitution on the E1 spike protein subunit, proline 226 to serine, which conferred these cholesterol-independent properties. This mutant thus identifies a novel region of E1, separate from the fusion peptide, that is involved in the cholesterol requirement for SFV fusion and exit.

Materials and Methods

Cells

BHK-21 cells were cultured at 37°C in DME containing 100 U/ml penicillin and 100 μg/ml streptomycin, supplemented with 5% FCS and 10% tryptose phosphate broth. C6/36 cells, a clonal cell line from *Aedes albopictus*, were cultured at 28°C in DME supplemented with 10% heat-inactivated FCS (Phalen and Kielian, 1991). Cholesterol-depleted C6/36 cells were prepared by four or more passages in DME containing 10% heat-inactivated FCS previously treated with colloidal silica to remove lipoproteins (Phalen and Kielian, 1991; Marquardt et al., 1993; Marquardt and Kielian, 1996). Depleted cells were repleted with either cholesterol or chlorocholestene (5-cholestene-3β-chloro) (Steraloids Inc., Wilton, NH) by adding either a sterol-BSA emulsion or a stock solution of sterol in ethanol to the depleted cell medium, and incubating the cells overnight (Phalen and Kielian, 1991; Marquardt et al., 1993; Marquardt and Kielian, 1996). Identical results were obtained using control or cholesterol-repleted C6/36 cells.

Isolation of SFV Mutants with Altered Sterol Requirements

A plaque-purified wild-type (wt) stock of SFV (Kielian et al., 1984) was mutagenized to ~20% viability by treatment with 1-methyl-3-nitro-1-nitrosoguanidine (Kielian et al., 1984). The mutagenized stock was grown for 6 h at 37°C on BHK cells at a multiplicity of 0.5 pfu/cell to allow expression of mutant phenotypes. A starting titer of 10^7 pfu of this virus stock was then selected by growth on one plate of chlorocholestene-repleted cells at 0.5 pfu/cell for 48 h in MEM containing 0.2% BSA and 10 mM Hepes, pH 7.0. The selection was repeated by two more passages on chlorocholestene-repleted cells at a starting multiplicity of ≤0.1 pfu/cell. At this point, the selected virus stock was found to have ~100-fold greater infectivity on chlorocholestene-repleted cells than the parental wt virus. Potential mutants were then isolated by limiting dilution on chlorocholestene-repleted cells grown in 96-well tissue culture trays. Cells were infected with 10-fold serial dilutions of virus, and cultured for 2 d at 28°C in serum-free medium (OptiMEM; GIBCO BRL, Gaithersburg, MD) supplemented with 0.2% BSA and 100 U/ml penicillin and 100 μg/ml streptomycin. Medium from the wells was harvested and stored on ice. Wells containing virus able to replicate efficiently in the absence of cholesterol were identified by staining cells with a polyclonal antibody to the SFV spike protein (Kielian et al., 1990), peroxidase-conjugated second antibody, and 3-amino-9-ethylcarbozole substrate, and visualizing by light microscopy. Wells with strongly positive-infected cells were identified as containing potential virus mutants, and the medium from such positive wells at viral dilutions yielding ≤60% positive wells was then used for two additional rounds of limiting dilution. Five potential mutants were isolated, three of which grew to sufficient titers to permit further expansion by three to four successive low multiplicity passages on chlorocholestene-repleted cells. These isolates were termed srf-1, -2 and -3, for sterol requirement in function. Other selections based on nonmutagenized virus or low pH-induced virus–plasma membrane fusion did not result in isolation of mutants.

Preparation of Radiolabeled Virus

Wt SFV and srf mutants were radiolabeled and purified by infecting control, depleted, or chlorocholestene-repleted C6/36 cells for 6 h at multiplicities of 150 pfu/cell, labeling for 18 h in methionine/cysteine free DME containing 150 mCi/ml [^{35}S]methionine/cysteine, followed by pelleting and purification on discontinuous 10–20%/25–50% sucrose gradients containing a 600 μl 50% sucrose cushion (Kielian et al., 1984).

Assays of Virus–Cell Interactions

Binding of radiolabeled wt SFV or srf mutants to C6/36 or BHK cells was measured as previously described (Phalen and Kielian, 1991). Endocytosis of radiolabeled virus was followed by measuring lysosomal degradation and the release of acid-soluble radioactivity (Phalen and Kielian, 1991). Growth curves of wt and mutants on control or depleted C6/36 cells were performed at multiplicities of 1 pfu/cell, and titered on BHK cells (Marquardt and Kielian, 1996). Primary infection of C6/36 cells was quantitated by an infectious center assay using immunofluorescence with a polyclonal antibody to the SFV spike protein to detect infected cells (Mar-

quardt and Kielian, 1996). Fusion of prebound virus with control or sterol-modified cells was induced by treatment for 1 min, pH 5.5, at 28°C. Cells were then incubated overnight in medium containing 20 mM NH_4Cl to prevent secondary infection, and infected cells quantitated by immunofluorescence (Marquardt and Kielian, 1996). Exit of newly synthesized virus from wt and mutant-infected C6/36 cells was followed by pulse–chase analysis as previously described, using increased multiplicity and radiolabel to assay wt virus in cholesterol-depleted cells (Marquardt et al., 1993; Marquardt and Kielian, 1996). Fluorograms were quantitated using a PhosphorImager and ImageQuant software from Molecular Dynamics, Inc. (Sunnyvale, CA).

Virus Sequence Analysis

Viral RNA was prepared by the method of Ou et al. (1981), using purified wild-type virus propagated on BHK cells, and pelleted srf-3 virus propagated on chlorocholestene-repleted C6/36 cells. 1 μg of viral RNA was reverse transcribed in a 20-μl reaction using oligo dT as a primer and 40 U of AMV reverse transcriptase, as previously described (Kielian et al., 1996). 2–4 μl of the resultant cDNA were then amplified using 500 ng each of primers within E1 and E2 in a 50-μl reaction containing Vent polymerase (New England Biolabs, Inc., Beverly, MA) (Kielian et al., 1996). The amplified DNA was purified using a QIAquick kit (Qiagen Inc., Chatsworth, CA), and both strands of the DNA encoding E1 and 6K were sequenced by the automated sequencing facility at the Albert Einstein College of Medicine (Kielian et al., 1996). Several independent RT-PCR products were sequenced to control for possible errors during transcription and amplification. The E1 sequence from srf-1 was obtained using the virus resulting from infection of one 75-cm^2 flask of cholesterol-depleted C6/36 cells. All of the viral RNA was used for reverse transcription, and 2–4 μl of resulting cDNA were used for amplification and sequencing as above. The E1 sequence from srf-2 was obtained using total cellular RNA from a 100-mm plate of cholesterol-depleted C6/36 cells infected at 1 pfu/cell at 28°C for 24 h. Total cellular RNA was extracted using the RNAzol method and 8 μg RNA was reverse transcribed, as described previously (Kielian et al., 1996), and used for amplification and sequencing as above.

Infectious SFV Clones

The mutation in srf-3 was mapped by transferring a unique 2.1-kb NdeI fragment containing srf-3 nucleotides 8929–11033 into the wt infectious SFV clone. cDNA from srf-3 was prepared as described above, and PCR-amplified using oligos 3′ to the E1 sequence and within E2 to generate an ~2.5-kb product that was then digested with NdeI. The wild-type infectious clone pSP6-SFV4 was digested to completion with NdeI, the large fragment self-ligated and propagated, treated with NdeI and phosphatase, and ligated with the srf-3 NdeI fragment to regenerate the complete infectious clone. Individual clones were transcribed using SP6 polymerase (Duffus et al., 1995), and the RNAs tested for primary and secondary infection on control and cholesterol-depleted C6/36 cells. 50–100 ng of RNA was mixed with 40 μg lipofectin in 250 μl of OptiMEM (GIBCO BRL). Depleted or control C6/36 cells grown on 12 mm coverslips in 24-well trays were incubated with this mixture for 1 h, and then cultured for ~18 h in OptiMEM/0.2%BSA with or without 20 mM NH_4Cl to prevent secondary infection (Marquardt et al., 1993). The cells were then fixed and virus infection assayed by indirect immunofluorescence using an antibody to the SFV spike protein (Marquardt and Kielian, 1996). A clone that produced efficient secondary infection of both control and depleted C6/36 cells was chosen, and used to make a virus stock by lipofection of the transcribed RNA into depleted C6/36 cells. The clone and the recombinant virus stock were referred to as srf-3/ic. The entire NdeI fragment of the srf-3/ic was sequenced on both strands by automated sequencing. Sequences were compared to the original published sequence of SFV spike proteins (Garoff et al., 1980), the sequence of pSP6-SFV-4 in the database, and the sequence of our plasmid isolate of pSP6-SFV-4, termed wt/ic. In addition, we performed RT-PCR and sequence analysis of RNA from the parent virus to srf-3, which is our plaque-purified strain of SFV (Kielian et al., 1984). srf-3 and srf-3/ic contained a unique but silent change at valine 3 of 6K (GUG-GUU), and a change from the published sequence of E1 asparagine 323 to aspartic acid (AAC-GAC). Sequence analysis of both wt/ic and the srf-3 parent strain showed that they also have Asp 323, however, and thus the Asn 323 sequence in the database is probably due to an early sequencing error. The only amino acid change in the NdeI fragment between srf-3/srf-3/ic and the parent virus/published sequences was due to a single base change, from a CCC to UCC in the srf-3 RNA, producing the E1 proline 226 to serine substitution. Mutation of C to U has been previously observed in other alphavirus mutants isolated following nitrosoguanidine treatment (Lindqvist et al., 1986).

The E1 P226S mutation was introduced de novo into the wild-type infectious clone by PCR mutagenesis using the overlap extension method (Levy-Mintz and Kielian, 1991). The sequence of the 5′-3′ mutagenic oligo was GCCCTTCAtCaGGCATGG, and the mutagenized fragment was subcloned into pSP6-SFV4 using the NdeI sites as described above. The P226S mutagenesis also introduced an EcoNI site which was used initially to screen the clones. A clone, termed SFV4-P226S, was selected, and a virus stock was generated by RNA transcription and lipofection as above. The entire NdeI fragment from SFV4 P226S was sequenced and confirmed that no additional changes were introduced during mutagenesis.

Results

Isolation of Semliki Forest Virus Mutants with Decreased Cholesterol Requirements

Given the central role of cholesterol in SFV fusion and exit, we hypothesized that it might be impossible to select sterol-independent SFV mutants. We therefore selected for mutants able to use chlorocholestene, a cholesterol analogue that is normally nonfusogenic and contains chloride at the 3β position. A mutagenized stock of SFV was grown for three successive low multiplicity passages on cholesterol-depleted cells enriched with chlorocholestene. The selected virus stock showed a ~100-fold increase in infectivity on chlorocholestene-enriched cells compared to that of wt virus. Because SFV does not produce plaques on C6/36 cells (Brown and Condreay, 1986), potential mutants in this stock were isolated by limiting dilution on C6/36 cells enriched with chlorocholestene, resulting in three putative mutant isolates. These were termed srf-1, srf-2 and srf-3 for the purposes of phenotypic characterization, although the procedure used to isolate them did not guarantee that they were independent mutants.

Cholesterol Independence of srf Mutants

To determine the cholesterol requirements of the srf mutants, we first compared their growth kinetics in control, chlorocholestene-enriched, and sterol-depleted C6/36 cells, measuring the production of progeny virus by plaque assays on BHK cells. Growth of all four viruses on cholesterol-containing cells was rapid and efficient, resulting in titers of ~10^9 pfu/ml by 12 h after infection (Fig. 1 *A*). The *srf* mutants did not exhibit a significant growth advantage, arguing that they were simply not better able to replicate in mosquito cells. As expected, the wt virus grew much less efficiently on chlorocholestene-enriched cells compared to control cells, with titers about four logs lower at 12 h and three logs lower at 48 h (Fig. 1 *B*). In contrast, all three mutants grew more efficiently than wt on chlorocholestene-enriched cells, showing titers four to five logs higher than wt by 24 h. Although the mutants grew more slowly on chlorocholestene-enriched cells than control cells, the final yield of mutant virus was comparable in the two cell types. In cholesterol-depleted, nonenriched cells, growth of wt virus was severely impaired, producing titers of only 10^5 pfu/ml even after a 48-h infection of depleted cells (Fig. 1 *C*). Surprisingly, the mutants were able to replicate on cholesterol-depleted cells that were not enriched with chlorocholestene. Although the growth kinetics were slower than on control cells, final titers of ~10^9 pfu/ml were ob-

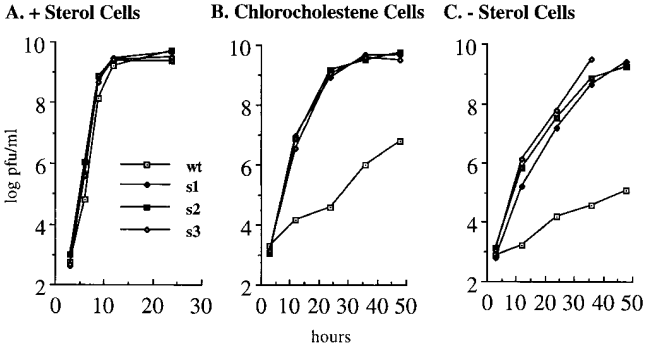

Figure 1. Growth of wt and srf mutants in control and sterol-modified C6/36 cells. Wild-type or mutant viruses were prebound on ice for 1 h to control or sterol-modified C6/36 cells at a multiplicity of 1 pfu/cell. Infection was initiated by warming the cells to 28°C for 1.5 h. The cells were then washed to remove input virus, incubation continued at 28°C, and samples were taken at the indicated times for plaque titration on BHK cells. Representative example of two experiments.

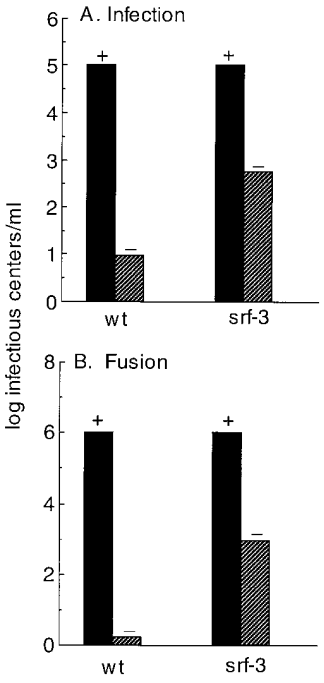

Figure 2. Infectivity and fusion of wt and srf-3 in control and cholesterol-depleted C6/36 cells. (*A*) Infection. Control (+) and sterol-depleted (−) cells were infected with serial dilutions of *wt* and *srf-3*, and primary infection was quantitated by immunofluorescence. Infection was normalized to 10^5 infectious centers/ml on control cells. Data shown are the average of 13 determinations. (*B*) Fusion. Serial dilutions of virus were bound to control (+) or depleted (−) cells in the cold, and the cells treated for 1 min at pH 5.5 to trigger virus fusion with the plasma membrane. Infected cells resulting from virus fusion were quantitated by immunofluorescence, and normalized to 10^6 infectious centers/ml on control cells. Data shown are the average of six determinations for *wt* and seven determinations for *srf-3.*

tained. Thus, the three srf mutants were dramatically altered in their cholesterol requirements for growth. Although the mutants were originally selected for growth on chlorocholestene-enriched cells, in fact they appeared to be relatively sterol independent. This may reflect a lack of chlorocholestene incorporation in the enriched cell membrane, as discussed below. The srf-3 mutant was selected for further study.

The growth properties of SFV are a function of its requirements for cholesterol in fusion (entry) and viral exit from cells. To determine the initial role of cholesterol in srf entry and fusion, we compared the ability of wt and srf-3 to infect control and cholesterol-depleted C6/36 cells using an infectious center assay (Fig. 2 *A*). In agreement with our previous results (Marquardt et al., 1993; Marquardt and Kielian, 1996), there was a four log difference in the ability of wt SFV to infect control cells versus depleted cells. In contrast, although srf-3 most efficiently infected cells with cholesterol, its ability to infect cholesterol-depleted cells was increased about 100-fold compared to that of wt virus. This substantial increase in infection of sterol-depleted cells was not due to a comparable increase in srf-3's infectivity on mosquito cells, such as a host range mutation. This is apparent from the similar growth kinetics of wt and mutants on control C6/36 cells (Fig. 1 *A*). In addition, wt and mutant viruses showed similar ratios of infection on cholesterol-containing BHK cells and mosquito cells (Table I). It is unclear whether the slight increase in infectivity of all four viruses on BHK cells reflects technical differences between plaque assays and infectious center assays, or host cell differences.

The increased ability of srf-3 to infect depleted cells could be due to an increase in receptor binding, endocytic uptake, or fusion with the endosome membrane. Virus-receptor binding and endocytosis were measured using purified ^{35}S-labeled wt and srf-3 in control and depleted cells, and were comparable in all cases (data not shown). We then specifically assayed the ability of wt and srf-3 to fuse with the plasma membrane bilayer of control and depleted cells. Virus was bound to cells in the cold and treated briefly at low pH to trigger fusion with the plasma membrane. The cells were then cultured for 12 h in the presence of ammonium chloride to block secondary infection, and infected cells quantitated by immunofluorescence. Under these conditions, infection only results from direct low pH-triggered fusion of bound virus with the plasma membrane (Helenius et al., 1982). As expected from previous liposome fusion assays, wt virus–plasma membrane fusion was strongly cholesterol dependent (Fig. 2 *B*). In contrast, although the srf-3 mutant showed a preference for cholesterol-containing cells, its ability to fuse with the depleted cell membrane was increased about three logs compared to wt virus. Similar cholesterol-independent fusion was observed when srf-1, -2, or -3 were assayed on either depleted cells or chlorocholestene-enriched cells (data not shown). Taken together, these data strongly suggest that the increased infectivity of srf mutants on sterol-depleted cells was caused by an increase in their cholesterol-independent membrane fusion activity.

We compared the cholesterol dependence of wt SFV and srf exit, using pulse–chase analysis in control and cho-

Table I. srf Mutants Do Not Have an Altered Host Range

Virus	Ratio of titer on BHK cells to titer on control C6/36 cells
Wild type	32
srf-1	28
srf-2	37
srf-3	19

Virus stocks were titered on BHK cells by standard plaque assay. The titer on control C6/36 cells was determined by infectious center assay. Representative data from one of two experiments are shown.

Table II. The srf-3 Mutation Is Stable

Virus/passage history	Ratio of titer on control C6/36 cells to titer on sterol-depleted C6/36 cells
Experiment 1	
Wild type/control C6/36 cells	17,860
srf-3/chlorocholestene C6/36 cells	14
srf-3/depleted C6/36 cells	7
srf-3/control C6/36 cells	12
Experiment 2	
Wild type/control C6/36 cells	5,433
srf-3/chlorocholestene C6/36 cells	77
srf-3/BHK cells	55

To test the stability of the srf-3 phenotype, virus stocks were grown for ~ 24 h (BHK) or 48 h (C6/36) at multiplicities of 0.01 pfu/cell (BHK) or 0.05 pfu/cell (C6/36). The resulting virus stocks were then titered by infectious center assay on control and cholesterol-depleted C6/36 cells.

lesterol-depleted cells (Marquardt et al., 1993; Marquardt and Kielian, 1996). wt SFV exit was inhibited in cholesterol-depleted cells compared to control cells, with total radiolabeled spike proteins released in virus particles by 90 min chase averaging 24% in control cells and 2% in depleted cells (n = 7). In contrast, srf-3 exit averaged 32% from control cells and 16% from depleted cells (n = 6). srf-1, -2, and -3 showed similar increases in exit from cholesterol-depleted cells. Electron microscopy showed that srf virions budding from sterol-depleted cells appeared morphologically similar to srf or wt virions released from control cells (data not shown; Marquardt et al., 1993). Thus, the srf mutants are increased in both their ability to fuse with and exit from cholesterol-depleted cells. These data also suggest that the cholesterol requirement for wt virus fusion may be more stringent than that for virus exit (see also Marquardt and Kielian, 1996). To test the genetic stability of the srf mutants, srf-3 was passaged on either depleted C6/36 cells, chlorocholestene-enriched C6/36 cells, control C6/36 cells, or BHK cells. The srf phenotype was then assayed by comparing its infectivity on C6/36 cells with and without cholesterol (Table II). srf-3 showed dramatically increased infectivity on depleted cells irrespective of its passage history, and thus the srf-3 mutation is stable for at least one passage in the absence of selective pressure. In parallel, we assayed wt virus that had been passaged on control C6/36 cells, and found that its infectivity was strongly cholesterol-dependent (Table II), in agreement with the results using BHK-grown virus (Fig. 2 *A*). Thus, the srf-3 phenotype appears to be due to a virus mutation, rather than to differences in lipid and carbohydrate composition between mammalian and mosquito grown virus (Kielian, 1995).

Role of Cholesterol in SFV Structure

The srf mutants grew to comparable final titers in either control or sterol-depleted cells (Fig. 1), and showed similar overall morphology in the absence of cholesterol. These results suggested that once formed, the virus particle was independent of cholesterol until virus fusion was triggered. However, during purification of radiolabeled srf-3 we discovered an important role of cholesterol in virus particle stability. When srf-3 was prepared from depleted cells and

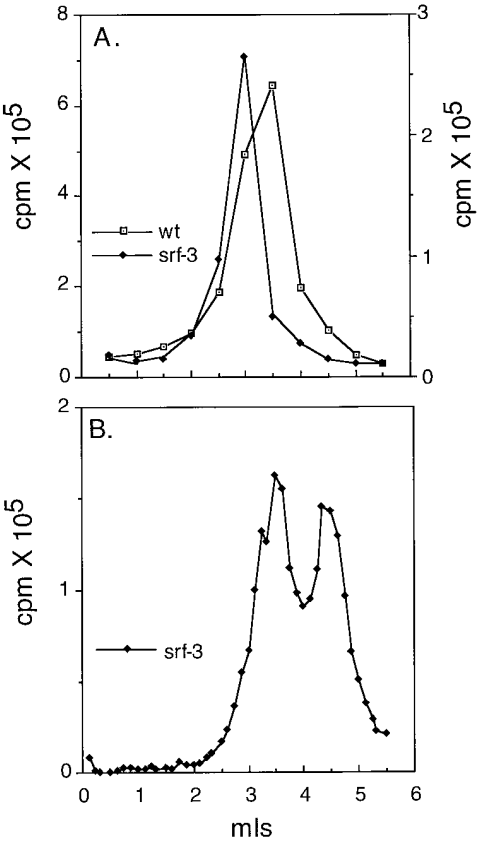

Figure 3. Cholesterol is required for virus membrane stability. (*A*) [^{35}S]methionine-labeled *wt SFV* or srf-3 were prepared by growth in control C6/36 cells. (*B*) [^{35}S]methionine-labeled srf-3 was prepared by growth in cholesterol-depleted C6/36 cells. Radiolabeled viruses were pelleted, centrifuged on sucrose gradients, the gradients fractionated, and radioactivity determined by scintillation counting. Sedimentation is to the left. Representative example of two experiments.

centrifuged on a standard sucrose gradient, the virus was found to be highly unstable to shear force, and dissociated during centrifugation to give a visible, more slowly sedimenting band in the gradient (Fig. 3 *B*). Electron microscopy revealed that this lower density peak consisted of capsid-free virus membranes, and that even the peak at approximate normal density contained highly disorganized virus particles (data not shown). In contrast, srf-3 grown in the presence of cholesterol showed similar sedimentation properties as wt SFV (Fig. 3 *A*). This effect of cholesterol is surprising given the efficient growth properties of the srf mutants on depleted cells, and suggests a critical role of cholesterol in the virus structure. Such a role could explain the requirement for cholesterol in the efficient exit of SFV from the host cell. Enrichment of depleted cells with chlorocholestene did not protect the virus from disruption by shear force (data not shown), suggesting either that this sterol does not partition significantly into the plasma membrane, or that it does not structurally substitute for cholesterol.

Mutation Responsible for Cholesterol Independence

To define the mutation that confers srf cholesterol inde-

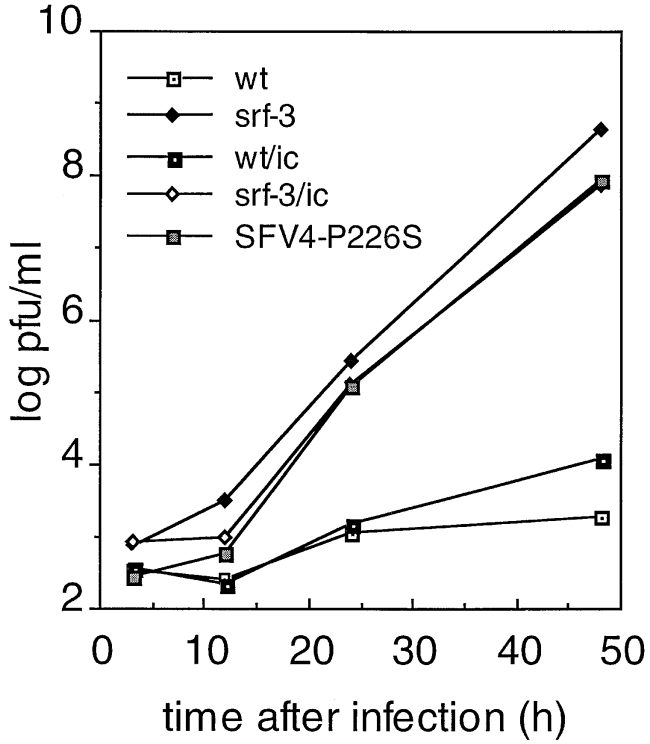

Figure 4. The growth properties of srf-3 are conferred by the E1 P226S mutation. The growth of *wt*, srf-3, and viruses derived from the pSP6-SFV-4 infectious clone (*wt/ic*), pSP6-SFV-4 containing the NdeI fragment of srf-3 (*srf-3/ic*), and pSP6-SFV-4 containing the P226S mutation was determined on cholesterol-depleted C6/36 cells as in Fig. 1. Representative example of two experiments.

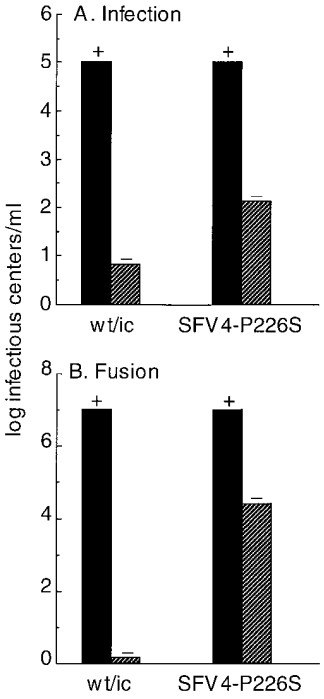

Figure 5. The srf-3 cholesterol-independent infectivity and fusion properties are conferred by the E1 P226S mutation. (*A*) Infection. Primary infection by viruses derived from the pSP6-SFV-4 infectious clone (*wt/ic*) and pSP6-SFV-4 containing the P226S mutation was determined on control (+) or cholesterol-depleted (−) C6/36 cells as in Fig. 2, and normalized to 10^5 infectious centers/ml on control cells. Average of two experiments. (*B*) Fusion. Fusion of viruses derived from the pSP6-SFV-4 infectious clone (*wt/ic*) and pSP6-SFV-4 containing the P226S mutation was determined on control (+) or cholesterol-depleted (−) C6/36 cells as in Fig. 2, and normalized to 10^7 infectious centers/ml on control cells. Average of two experiments.

pendence, we reasoned that the key alteration was probably in the virus spike protein. The central role of E1 in fusion and the cholesterol dependence of its membrane binding and acid-induced conformational changes suggested that it would be the spike subunit most likely to carry the mutation (Klimjack et al., 1994; Kielian, 1995). We prepared RNA from srf-3, and performed RT-PCR amplification. A unique 2.1-kb NdeI fragment encoding most of the srf-3 E1, all of 6K, and the COOH terminal half of the E2 sequence was substituted into the wt infectious SFV clone pSP6-SFV-4 (Liljestrom et al., 1991). This DNA construct, termed srf-3/ic, was used for in vitro transcription of infectious SFV RNA and generation of a virus stock. The NdeI fragment converted the infectious clone-derived virus to cholesterol-independent growth (Fig. 4), and also conferred the srf-3 infection, fusion, and exit properties (data not shown). DNA sequence analysis was performed on this cloned fragment and on several independent RT-PCR products derived from srf-3 RNA. The only amino acid change in the NdeI fragment between srf-3 and the parent virus was due to a single base change, from CCC to UCC in the srf-3 RNA, producing a substitution of proline 226 to serine on the viral E1 (P226S). RT-PCR and sequence analysis of the P226 regions of srf-1 and srf-2 indicated that both contained the P226S mutation, suggesting that the three mutants are separate isolates of the same original mutant that arose during the growth of the virus stock on cholesterol-depleted, chlorocholestene-enriched cells. The results indicated that a single amino acid change could be responsible for the decreased cholesterol dependence of both fusion and exit.

To confirm the identity of the critical mutation, we introduced the P226S change de novo into the infectious clone using in vitro mutagenesis. The virus derived from this clone (P226S) was then tested for growth on depleted cells, and showed similar efficient growth in the absence of cholesterol as srf-3 or srf-3/ic (Fig. 4). Infectious center assays showed that P226S had significantly increased infectivity on depleted cells (Fig. 5 *A*), and fusion assays showed an increase of greater than four logs in P226S fusion with the depleted cell plasma membrane (Fig. 5 *B*). The exit of P226S was compared to that of srf-3 and wt SFV by pulse-chase analysis of control and cholesterol-depleted cells. Cells were infected with mutant or wt viruses, pulse labeled for 15 min, and chased for 3 h. Radiolabeled virus spike proteins were immunoprecipitated from the cell lysates and chase media, and exit quantitated by gel electrophoresis and phosphorimaging. Increased multiplicity of wt virus infection was used to circumvent the fusion block and express the wt virus in depleted cells (Marquardt et al., 1993). Efficient and comparable exit of all three viruses was observed from control cells (Fig. 6 *A*), with 53, 51, or 46% release of total radiolabeled spike proteins from wt, srf-3, or P226S-infected cells, respectively. As expected, exit from cholesterol-deficient cells was inhibited for wt virus (2.4%), and was significantly more efficient for srf-3 (17%) (Fig. 6 *B*), in agreement with the increased release of virus from depleted cells described for srf-3 above. The P226S mutant also showed increased efficiency of exit in the absence of cholesterol (12%). These data demonstrate that a single amino acid change, P226S, is responsible for the relative cholesterol-independence of both srf-3 fusion and exit.

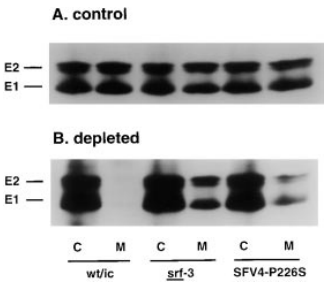

Figure 6. Cholesterol-independent SFV exit is conferred by the E1 P226S mutation. Control or cholesterol-depleted C6/36 cells were infected with the indicated viruses at muliplicities of 50 pfu/cell, except for wt/ic in depleted cells, for which the multiplicity was 1,000 pfu/cell. 18 h postinfection, the release of progeny virus was detemined by pulse-labeling for 15 min with [^{35}S]methionine and cysteine, chasing for 3 h in the presence of excess cold methionine and cysteine, collecting the chase medium, and lysing the cells. The amount of radiolabeled virus spike proteins in the cells (*C*) and media (*M*) was determined by immunoprecipitation and SDS-PAGE. Representative example of two experiments.

```
      215           225           235
SFV   ANTALKLARP SPGMVHVPYT QTPSGFKYWL

RRV   -------S-- ---V------ ----------
ONN   ---Q-I-Q-- AA-AI----S -A---------
CHIK  ---Q-V-Q-- -A-T-----S -A---------
EEE   ---N---Q-- QA-I--T-F- -A----ER-K
VEE   ---N-V-Q-- KA-AI----- -A----EQ-K
AURA  ----IH-Q-- EARNI----- -A----EF-K
WEE   -R-DIR-LK- -VKNI----- -AV--YEM-K
WHATE ---DIR-L-- IARN------ -AA---EM-K
OCK   -S-DIR-LK- -AKN------ -AA---EM-K
SV    -S-DIR-LK- -AKN------ -AS---EM-K
```

Figure 7. Sequence comparison of the P226 E1 region in alphaviruses. Amino acid numbering is given for *SFV E*1, starting with residue *215*, and *P226* is shown in bold. Abbreviations and accession numbers are as follows, other sequence references are listed in (Strauss and Strauss, 1994; Kielian, 1995): *SFV* (Semliki Forest virus); *RRV* (Ross River virus); *ONN* (O'Nyong-nyong virus); *CHIK* (Chikungunya virus) (NCBI 576465); *EEE* (Eastern equine encephalitis virus); *VEE* (Venezuelan equine encephalitis virus); *AURA* (Aura virus); *WEE* (Western equine encephalitis virus); *WHATE* (Whataroa virus) (unpublished data from Drs. Y. Shirako and J. Strauss); *OCK* (Ockelbo virus); *SV* (Sindbis virus).

The amino acid sequence surrounding P226 was compared for the known alphavirus E1 sequences (Fig. 7). This is a conserved area of the E1 polypeptide, with a number of residues that are invariant among 11 different alphaviruses, including the flanking residues leucine 221, histidine 230, and prolines 224 and 232. However, P226 itself is not conserved. At the corresponding position it is a proline residue in the closely related Ross River virus, but alanine in a number of other alphaviruses, and valine in western equine encephalitis virus. Notably, none of the reported alphavirus sequences contain serine at this position. Sequence comparisons showed no significant identity between this region and other, non-alphavirus sequences in the database. Our studies thus have identified a previously uncharacterized region of SFV E1, separate from the fusion peptide, that is involved in the requirement for cholesterol, during virus fusion and exit.

Discussion

In summary, our data indicate that SFV fusion and exit are highly cholesterol dependent, and that this sterol requirement is significantly reduced by the P226S mutation. Interestingly, preliminary data from our lab indicate that two independent mutants selected for cholesterol-independent growth also have the P226S mutation, supporting the importance of this region of the spike protein in the virus cholesterol requirement (Chatterjee, P., and M. Kielian, unpublished results). It is striking that although it is not conserved, the position analogous to SFV E1 P226 is not found as a serine residue in any of the alphavirus sequences in the database, including recent virus isolates from nature (Fig. 7). Other nonconserved proline residues in this and other E1 regions (eg., P237; Fig. 7) are present as a serine in some alphavirus sequences. Preliminary results with Sindbis virus, having alanine at position 226, indicate that both its infection and fusion are highly cholesterol dependent, similar to wt SFV (Lu, Y., and M. Kielian, unpublished results). Although other mechanisms are possible, it therefore appears most likely that the gain of serine 226, rather than the loss of the proline at this position, is responsible for the relative cholesterol independence of srf-3. Although all of the alphaviruses have not been tested, it seems probable that the other members of this genus will require cholesterol for fusion, similar to SFV and Sindbis. By analogy with the SFV studies (Nieva et al., 1994), we also presume that sphingolipids will be necessary for the fusion of other alphaviruses. Taken together, these observations fit a model in which highly cholesterol-dependent infection is an important characteristic of all alphaviruses, and is somehow selected for in vivo. Further in vivo and in vitro studies will be required to determine the lipid dependence of other alphaviruses, and the existence of a possible selection pressure for highly cholesterol-dependent alphavirus fusion.

In general, it is not known if members of other virus families have specific lipid requirements for membrane fusion or virus egress. In vitro and/or in vivo studies of influenza virus, a myxovirus, and vesicular stomatitis virus, a rhabdovirus, suggest that both viruses have fusion mechanisms independent of either cholesterol or sphingolipid (White et al., 1982; Eidelman et al., 1984; Phalen and Kielian, 1991; Cleverley et al., 1997). Cholesterol does seem to enhance fusion of Sendai virus, a paramyxovirus whose fusion protein bears structural and sequence similarities to that of influenza (Hsu et al., 1983; Kundrot et al., 1983). Many medically important viruses such as HIV-1 have not yet been tested for lipid requirements in fusion or infection. It is interesting that during budding, HIV-1 appears to select for significantly increased concentrations of both cholesterol and sphingomyelin in the virus membrane compared to the cell plasma membrane (Aloia et al., 1993). It is not known if this selectivity represents an actual lipid requirement for HIV-1 budding.

There are several precedents for protein–cholesterol interactions important in membrane biology. Caveolin, an integral component of specialized membrane domains known as caveolae (Rothberg et al., 1992), binds cholesterol (Murata et al., 1995) and may be involved in trans-

port of sterol from the endoplasmic reticulum to the plasma membrane (Smart et al., 1996). The caveolin residues involved in sterol binding are not yet identified. Several proteins involved in cellular cholesterol metabolism have membrane-spanning domains that may act as cholesterol sensors, although the specific cholesterol–interaction site has yet to be identified (Brown and Goldstein, 1997). The thiol-activated cytolysins are bacterial toxins that act by binding cholesterol in the target cell membrane, inserting into the membrane, and oligomerizing to form a pore (Alouf and Geoffroy, 1991). Similar to the SFV E1 protein, these toxins appear to require the sterol 3β-hydroxyl group for their activity, and at least one, lysteriolysin O, has an acid pH optimum for cholesterol binding and pore formation (Portnoy et al., 1992). The crystal structure of a member of this family, perfringolysin O, was recently determined (Rossjohn et al., 1997). The putative cholesterol-binding site is composed of noncontiguous amino acid residues, and involves interactions with aliphatic side chains and hydrogen bonding between the sterol 3β-hydroxyl and glutamate and arginine residues. Cholesterol oxidase is an enzyme that specifically oxidizes the sterol 3β-hydroxyl group. The crystal structure of the enzyme with a bound sterol substrate shows that the binding site is a solvent-sealed internal cavity in which the sterol rings have extensive contacts and the sterol hydroxyl is hydrogen-bonded to a flovin adenine dinucleotide cofactor and a bound water molecule (Li et al., 1993). Thus, work from other systems suggests that sterol binding is likely to involve noncontiguous residues that interact with the sterol ring structure and hydrogen bond with the sterol hydroxyl. The SFV E1 protein does not show significant regions of sequence identity with these other cholesterol-binding proteins. It remains to be seen if similar binding sites or motifs for cholesterol interaction exist among these molecules.

What might be the mechanism by which the gain of serine 226 confers srf-3 cholesterol independence? One intriguing possibility is that the serine hydroxyl group may act as a substitute for the critical 3β-hydroxyl group of cholesterol. The mutation could act by "filling" a cholesterol-binding site either within the P226 region or in an interacting domain of E1. If this is the case, the mutation could cause a normally cholesterol-requiring step in fusion to be less cholesterol dependent. During low pH-triggered fusion, E1 undergoes at least three separate conformational changes: exposure of new epitopes, formation of a homotrimer, and hydrophobic association with the target membrane bilayer (Kielian, 1995; Kielian et al., 1996). Preliminary data indicate that srf-3 E1 is less cholesterol dependent than wt for both epitope exposure and homotrimer formation (Chatterjee, P., M. Vashishtha, and M. Kielian, unpublished data). Further studies will focus on the role of the serine hydroxyl and on the potential interactions of this region with other spike protein domains.

The most surprising finding of this study is that a single point mutation in E1 substitutes for the specific cholesterol requirement in both SFV fusion and exit. The exact step in the SFV exit pathway that requires cholesterol has not been determined, but our data suggest that the most likely site is at a point after arrival of the spike protein at the cell surface (Marquardt et al., 1993). It is unlikely that E1's fusion activity is directly required for virus exit. During virus budding, the forming virus particle must pinch off in a membrane fission reaction, but unlike virus membrane fusion, this occurs at neutral pH. In addition, virus mutants that are completely blocked in membrane fusion (Duffus et al., 1995) or have a dramatically acid-shifted pH threshold for fusion (Salminen et al., 1992; Duffus et al., 1995) can still assemble into virus particles. Computer reconstructions of alphaviruses indicate that almost all of the surface of the particle is covered by a spike protein shell, with very little of the lipid bilayer exposed (Cheng et al., 1995; Fuller et al., 1995). However, in spite of the fact that the majority of the cholesterol in the virus membrane bilayer appears inaccessible, cholesterol-depleted virus is highly destabilized. The requirement for cholesterol in both efficient wt virus exit and virus particle stability suggests that cholesterol is important in the normal spike protein interactions involved in formation of the alphavirus particle. The phenotype of the srf-3 mutant suggests that these interactions are mediated by spike protein domains that are also required for membrane fusion.

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