JOURNAL OF VIROLOGY, May 1999, p. 4272–4278 Vol. 73, No. 5
0022-538X/99/$04.00+0

The Cholesterol Requirement for Sindbis Virus Entry and Exit and Characterization of a Spike Protein Region Involved in Cholesterol Dependence

YANPING E. LU, TODD CASSESE,† AND MARGARET KIELIAN*

Department of Cell Biology, Albert Einstein College of Medicine, Bronx, New York 10461

Received 28 December 1998/Accepted 16 February 1999

Semliki Forest virus (SFV) and Sindbis virus (SIN) are enveloped alphaviruses that enter cells via low-pH-triggered fusion in the endocytic pathway and exit by budding from the plasma membrane. Previous studies with cholesterol-depleted insect cells have shown that SFV requires cholesterol in the cell membrane for both virus fusion and efficient exit of progeny virus. An SFV mutant, *srf-3*, shows efficient fusion and exit in the absence of cholesterol due to a single point mutation in the E1 spike subunit, proline 226 to serine. We have here characterized the role of cholesterol in the entry and exit of SIN, an alphavirus quite distantly related to SFV. Growth, primary infection, fusion, and exit of SIN were all dramatically inhibited in cholesterol-depleted cells compared to control cells. Based on sequence differences within the E1 226 region between SFV, *srf-3*, and SIN, we constructed six SIN mutants with alterations within this region and characterized their cholesterol dependence. A SIN mutant, SGM, that had the *srf-3* amino acid sequence from E1 position 224 to 235 showed increases of ~100-fold in infection and ~250-fold in fusion with cholesterol-depleted cells compared with infection and fusion of wild-type SIN. Pulse-chase analysis demonstrated that SGM exit from cholesterol-depleted cells was markedly more efficient than that of wild-type SIN. Thus, similar to SFV, SIN was cholesterol dependent for both virus entry and exit, and the cholesterol dependence of both steps could be modulated by sequences within the E1 226 region.

Alphaviruses such as Semliki Forest virus (SFV) and Sindbis virus (SIN) are simple enveloped viruses that have been particularly useful in studies of virus entry, membrane fusion, and virus biosynthesis and assembly. Alphaviruses are comprised of a nonsegmented positive-strand RNA genome associated with a capsid protein, a lipid bilayer derived from the plasma membrane during budding, and a spike protein containing two transmembrane polypeptides, E1 and E2, each about 50 kDa (14, 27). Each virus particle contains 240 copies of E1 and E2 arranged as 80 trimers of E1/E2 heterodimers.

Alphaviruses enter cells by endocytic uptake in clathrin-coated vesicles (5, 14). The acid pH in endosomes triggers the fusion of the virus membrane with that of the endosome and releases the viral nucleocapsid into the cytoplasm to initiate infection (9, 10, 14). The virus RNA is then translated and replicated, and new capsid proteins are synthesized in the cytoplasm and assembled with viral RNA into nucleocapsids (14, 27). The spike polypeptides are translocated into the lumen of the endoplasmic reticulum and assembled into a dimer of E1 with the E2 precursor, which is termed p62 in SFV and PE2 in SIN. The p62/E1 dimer is transported via the cellular secretory machinery to the plasma membrane. In a late stage of the secretory pathway, the p62 (PE2) precursor is cleaved by furin-like proteases into E2 and E3. The latter is a small soluble protein that is secreted in SIN but that associates with the E1/E2 dimer as a peripheral protein in SFV (27). At the plasma membrane, efficient budding is driven by both lateral interactions between viral spike proteins and an interaction between the E2 cytoplasmic tail and nucleocapsid (8, 9, 14, 27).

A variety of evidence indicates that E1 is the fusogenic spike subunit and contains the virus fusion peptide (14, 16). Studies of low-pH-dependent conformational changes in the SFV spike protein suggest an overall scheme for the E1-mediated fusion reaction (reviewed in references 9 and 14). Upon exposure to low pH, the normally stable E1/E2 dimer dissociates. The conformation of the E1 subunit then changes, exposing previously hidden sites for monoclonal antibody binding and forming a stable E1 homotrimer believed to be a key fusion intermediate. E1 associates with the target membrane (19) and mediates the mixing of the viral and target membranes.

In vitro fusion studies with liposomes have demonstrated that SFV fusion requires cholesterol (17, 29) and sphingolipid (23, 30) in the target membrane. The fusion-supporting activity of both lipids showed striking stereospecificity, suggesting specific roles in the fusion reaction. The role of cholesterol in vivo in SFV fusion and infection was investigated by depleting the C6/36 mosquito cell line of both free and esterified cholesterol to a level less than 2% of that of control cells (22, 24). Studies of these cholesterol-depleted cells demonstrated that cholesterol is required not only for SFV fusion and infection but also for efficient SFV exit, while the unrelated virus vesicular stomatitis virus shows no cholesterol dependence for either fusion or exit. The cholesterol-depleted cells were used to isolate a cholesterol-independent SFV mutant termed *srf-3* (sterol requirement in function). *srf-3* is significantly increased in its ability to both fuse with and exit from cholesterol-depleted cells (22, 28). A single point mutation in E1, proline 226 to serine, was shown to be responsible for the *srf-3* phenotype in both fusion and exit (28).

To date, SFV is the only virus that has been demonstrated to require cholesterol for membrane fusion and exit. To examine whether a cholesterol requirement is a general property of the

* Corresponding author. Mailing address: Department of Cell Biology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461. Phone: (718) 430-3638. Fax: (718) 430-8574. E-mail: kielian@aecom.yu.edu.

† Present address: Department of Molecular and Cellular Biology, Harvard University, Cambridge, Mass.

```
          215          225          235
SFV       ANTALKLARP SPGMVHVPYT QTPSGFKYWL
srf-3     ---------- -S-------- ----------
SIN       -S-DIR-LK- -AKN------ -AS---EM-K

SKN       -S-DIR-LK- -SKN------ -AS---EM-K
SGM       -S-DIR-LK- -SGM------ -AS---EM-K

SGN       -S-DIR-LK- -SGN------ -AS---EM-K
SKM       -S-DIR-LK- -SKM------ -AS---EM-K
AGM       -S-DIR-LK- -AGM------ -AS---EM-K
PGM       -S-DIR-LK- -PGM------ -AS---EM-K
```

FIG. 1. Sequence comparison of E1 226 region of SFV, *srf-3*, SIN, and SIN mutants. The sequence begins at amino acid 215 of the E1 subunit. Dashed lines indicate sequence identity with wt SFV. Mutations from the parental sequence are shown in bold.

alphavirus life cycle, we characterized the role of cholesterol in the entry and exit pathway of SIN, an alphavirus distantly related to SFV (27). Our results demonstrated that despite the sequence differences between SFV and SIN, SIN was also highly dependent on cellular cholesterol for infection, fusion, and exit. Specific mutations in the 226 region of SIN E1 decreased the cholesterol dependence of SIN fusion and exit, suggesting that, similar to the situation with SFV, this region of E1 is involved in the virus cholesterol requirement.

(This research was conducted by Yanping E. Lu in partial fulfillment of the requirements for a Ph.D. degree from the Sue Golding Graduate Division of Medical Sciences, Albert Einstein College of Medicine, Yeshiva University, Bronx, N.Y.)

MATERIALS AND METHODS

Cells and viruses. C6/36, a clonal mosquito cell line derived from *Aedes albopictus*, was cultured at 28°C in Dulbecco's modified Eagle's medium with 100 U of penicillin/ml and 100 μg of streptomycin/ml (DME) and containing 10% heat-inactivated fetal calf serum (HIFCS) (22). Cholesterol-depleted C6/36 cells were prepared by at least four passages in DME containing 10% Cab-O-Sil-delipidated HIFCS as previously described (22, 24, 28) and used between passage 5 and passage 15 to avoid potential adaptation (21). C6/36 cells depleted by this method had both free and esterified cholesterol levels of less than 2% of those of control cells (22, 24). BHK-21 cells were used for plaque titration and preparation of virus stocks and were cultured at 37°C in DME containing 5% FCS and 10% tryptose phosphate broth (24).

Wild-type SFV was derived from a plaque-purified isolate (18). *srf-3* is an SFV mutant selected for growth on cholesterol-depleted C6/36 cells and shown to be much less dependent on cellular cholesterol for infection, fusion, and exit (22, 28). Wild-type (wt) SIN and all SIN mutants were derived from the infectious clone Toto 1101 (25). All virus stocks were stored at −80°C in medium containing 10 mM HEPES (pH 7.4).

In vitro mutagenesis and generation of virus stocks. Mutations were introduced into the Toto 1101 infectious clone by PCR mutagenesis using *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.) and the overlap extension method (28). Mutagenic primer pairs were designed not only to carry the desired mutations (Fig. 1) but also to insert a new restriction enzyme digestion site used as an initial screen of mutant infectious clones. Unique *Bsi*WI and *Xho*I sites at positions 10382 and 11748 were used for subcloning the mutations into the Toto 1101 infectious clone, and the sequence of this region from each mutant infectious clone was determined by the automated sequencing facility of the Albert Einstein College of Medicine to confirm the presence of the desired mutations and the absence of other mutations due to PCR error. To generate wild-type and mutant virus stocks, infectious clones were linearized by *Xho*I digestion, infectious RNAs were synthesized by in vitro transcription, 10 to 20 μg of RNA was introduced into BHK cells by electroporation, and the cells were cultured for ~18 h at 37°C (6, 28).

Assays of virus infection, fusion, and growth. In general, infection of cells by SFV and SIN was carried out by prebinding viruses to control or cholesterol-depleted C6/36 cells on ice for 60 to 90 min in RPMI medium without bicarbonate but supplemented with 10 mM HEPES and 0.2% bovine serum albumin (BSA) (21, 22). Similar levels of infection were obtained without prebinding (data not shown). For analysis of virus growth kinetics, viruses were prebound to cells at a multiplicity of infection of 1 PFU/cell and incubated in Opti-MEM (Gibco/BRL, Gaithersburg, Md.) supplemented with 0.2% BSA (O/B) for 2 h at 28°C to initiate infection. Input virus was removed by washing, cells were incubated in O/B at 28°C for various periods of time, and virus in the medium was quantitated by plaque assay on BHK cells. For comparisons of virus infection efficiency, serial dilutions of virus were bound to control and cholesterol-depleted C6/36 cells grown on coverslips, incubated in O/B medium at 28°C for 2 h to allow primary virus infection, and then cultured in O/B containing 15 mM NH_4Cl to prevent secondary infection for 12 to 18 h for SFV and *srf-3*, or for 24 h for all SIN viruses (21). Infected cells were quantitated by indirect immunofluorescence using polyclonal antibodies against either SFV spikes (22) or SIN spikes (from Ellen G. Strauss, California Institute of Technology). Comparisons of infectivity on control C6/36 cells and BHK cells were performed for SIN and all mutants and demonstrated that the mutations did not produce significant alterations in the virus host range (data not shown). Fusion was assayed by treatment of cells with prebound viruses at pH 7 or pH 5.5 to 5.0 for 1 min at 28°C to trigger virus-plasma membrane fusion. Cells were incubated for 12 to 24 h in O/B plus 20 mM NH_4Cl, and quantitation of infected cells by indirect immunofluorescence was performed as described above (28). The infection resulting from pH 7 incubation was <0.1% of that resulting from acid pH treatment.

Assays of virus exit. Pulse-chase analysis of C6/36 cells in 35-mm plates was used to monitor the release of newly synthesized SIN viruses, using methods similar to those previously published for SFV exit (22). Previous studies have demonstrated that this assay accurately reflects the release of virus particles (22, 28). Briefly, control cells were infected with wt SIN or SGM at 10 PFU/cell for ~22 h at 28°C. Cholesterol-depleted cells were infected with wt SIN or SGM by transfecting with in vitro-transcribed RNA to bypass the cholesterol-dependent infection block. Two and a half micrograms of infectious RNA was mixed with 10 μg of Cellfectin (Gibco/BRL) in 1 ml of Opti-MEM without antibiotics, incubated at room temperature for 30 to 45 min, and then incubated with cholesterol-depleted cells for 4 h, followed by cell culture in fresh O/B with antibiotics for 24 h. Infected cells were pulse-labeled for 15 min with 50 μCi of [^{35}S]methionine-cysteine/ml (control cells) or for 30 min with 200 μCi of [^{35}S]methionine-cysteine/ml (depleted cells) and chased in minimal essential medium containing a 10-fold excess of methionine and cysteine. At each time point, the chase medium was harvested in the absence of detergent, and the cells were lysed in a buffer containing Triton X-100 and protease inhibitors (22). Cell lysates were precipitated with rabbit polyclonal antibodies raised against SIN spike proteins (from Milton Schlesinger, Washington University School of Medicine). The medium samples were precipitated in the absence of detergent with a monoclonal antibody against E2 (SV209 [26], from Richard Kuhn, Purdue University) that efficiently precipitates the virus particle (data not shown). As indicated in the figure legends, different amounts of cell lysate and medium samples were used for immunoprecipitation in order to better visualize protein expression and virus release. Antibody concentrations were adjusted to completely precipitate the virus spike proteins present in each sample (data not shown). Samples were reduced and alkylated (15) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10.8% acrylamide gels followed by fluorography and phosphorimaging (ImageQuant v. 1.2; Molecular Dynamics, Inc., Sunnyvale, Calif.).

RESULTS

SFV has been shown to require cellular cholesterol for infection, fusion, and exit (22, 24), and the cholesterol dependence of both SFV entry and exit are markedly decreased by the point mutation E1 P226→S (28) (Fig. 1). E1 residue 226 varies among the reported sequences of various alphaviruses, being alanine in a number of alphaviruses, including SIN, the prototype alphavirus, or valine in the case of western equine encephalitis virus, Highlands J virus, and one recently reported SIN strain. Given the availability of SIN infectious clones, useful antibody reagents, background information on SIN infection, and the considerable evolutionary distance from SIN to SFV (27), we chose SIN as a system to evaluate the general significance of cholesterol in alphavirus fusion and exit. Our test SIN strain was derived from the widely used Toto 1101 infectious clone (25) and contains alanine at E1 position 226.

The role of cellular cholesterol in SIN infection, fusion, and growth. The growth rates of SIN, SFV, and *srf-3*, the SFV mutant containing P226→S, were compared on control and cholesterol-depleted C6/36 mosquito cells at a multiplicity of 1 PFU/cell. Production of progeny virus from control cells was very rapid and efficient for all three viruses, although the growth of SIN was somewhat slower than that of SFV and *srf-3*. SFV and *srf-3* titers increased from ~10^3 to ~10^9 PFU/ml during the first 11 h of infection, while SIN titers increased from ~10^4 to ~10^7 PFU/ml during this period and reached a

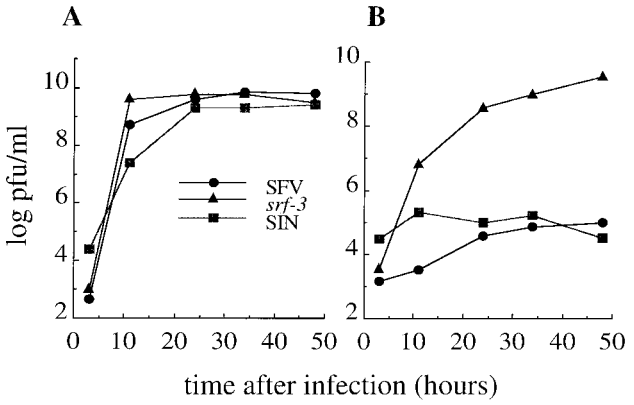

FIG. 2. Growth of wt SFV, *srf-3*, and wt SIN in control and cholesterol-depleted C6/36 cells. Viruses were prebound to either control (A) or cholesterol-depleted (B) C6/36 cells on ice for 1 h at a multiplicity of 1 PFU/cell. Cells were then incubated at 28°C for 2 h to initiate infection, washed to remove input virus, and further incubated at 28°C for the indicated times. Titers of samples of the media were determined by plaque assay on BHK cells. Note that it proved more difficult to remove all input SIN virus inoculum (3-h time point) (A and B) but that no increase in SIN titer was observed in cholesterol-depleted cells (B).

titer of 10^9 PFU/ml after ~24 h of infection (Fig. 2A). The slightly slower growth kinetics of SIN may be due to the fact that a given multiplicity of SIN, as titrated on BHK cells, shows less infectivity on mosquito cells than a comparable amount of SFV (data not shown). In contrast to their efficient growth on control C6/36 cells, both SFV and SIN displayed very inefficient growth in cholesterol-depleted cells, showing little (SFV) or no (SIN) increase in titer even after 48 h of infection (Fig. 2B). Although the cholesterol-independent mutant *srf-3* grew more slowly in the absence of cholesterol, it reached final titers of ~10^9 PFU/ml after ~34 h of infection, similar to previous results (28). Thus, analysis of virus growth properties indicated that SIN, similar to SFV, had a strong requirement for cholesterol in the cell membrane during the virus life cycle, while the *srf-3* mutant was substantially less cholesterol dependent than either SFV or SIN.

Previous studies of SFV and *srf-3* demonstrated that the virus cholesterol requirement for growth was due to the requirement for cholesterol in two steps in the virus life cycle, fusion and exit (22, 24, 28). To resolve cholesterol-dependent events during SIN entry, we measured infectivity and fusion of SIN on control and cholesterol-depleted cells. Primary infection levels of SIN, SFV, and *srf-3* were assayed in parallel (Fig. 3A), and in keeping with previous results, wt SFV infection of depleted cells was decreased by about 4 logs compared to its infection of control cells (22, 28). In contrast, *srf-3* infection of depleted cells was increased ~100-fold compared to the level for wt SFV. Strikingly, SIN primary infection displayed an even stronger cholesterol requirement than that of wt SFV, showing a decrease of ~5 logs in infection of depleted cells compared to control cells (Fig. 3A). This stronger sterol requirement for SIN infection probably at least partially explains the somewhat stronger SIN growth defect in depleted cells (Fig. 2B).

The decreased infection of depleted cells by SIN could conceivably be due to alterations in virus binding to the cell surface receptor, endocytic uptake, endosome acidification, and/or virus fusion with endosome membranes. Previous studies showed that the decrease in infection by SFV in depleted cells is due to a decrease in virus fusion, while receptor binding, endocytosis, and endosome acidification are unaffected (24). In order to examine whether a similar fusion block explained the decrease in SIN infection, levels of SIN, wt SFV, and *srf-3* fusion were compared on control and depleted cells (Fig. 3B). In agreement with our previous results (28), wt SFV fusion with cholesterol-depleted cells was reduced by ~5 logs compared to fusion with control cells. While the *srf-3* mutant showed maximal fusion on control cells, it fused about 1,000-fold more efficiently with depleted cells than did wt SFV. SIN showed a cholesterol dependence for membrane fusion similar to that of wt SFV, suggesting that the strong block in SIN infection in the absence of cholesterol was due to a block in SIN fusion with the endosome membrane of depleted cells.

Rationale for mutagenesis of the SIN E1 226 region. The single point mutation at position 226 (P→S) makes *srf-3* much less cholesterol dependent than wt SFV for both fusion and exit (28). We compared the sequences of the E1 226 region between SFV and SIN (Fig. 1). The 12 amino acids from position 224 to 235 are identical between the two viruses except for the residues proline^{226}-glycine^{227}-methionine^{228} (PGM) in SFV, which are alanine-lysine-asparagine (AKN) in SIN. Given the similar, highly cholesterol-dependent phenotypes of SFV and SIN, we wished to test whether the SIN E1 226 region plays a role in its cholesterol dependence. We therefore constructed two SIN mutants, containing either the P226→S point mutation on the SIN background or a change of the SIN AKN sequence to SGM to give the *srf-3* sequence from residues 224 to 235 (Fig. 1). The mutants will be referred to as SKN or SGM, with the amino acid sequence from position 226 to 228 listed and changes from the wild-type SIN sequence underlined. To obtain virus stocks, SKN and SGM RNAs were prepared from the corresponding infectious cDNA clones and transfected into BHK cells and infection was allowed to proceed for 18 h. Transfection of RNA containing either mutation resulted in efficient primary and secondary infection in BHK cells and production of high-titered virus stocks (data not shown). This result suggests that the SKN and SGM mutations did not affect SIN spike protein folding, virus exit, or fusion in cells with cholesterol and is in keeping with the fact that the *srf-3* mutation (P226S) does not affect SFV infection on cholesterol-containing BHK or C6/36 cells (28). Electron micros-

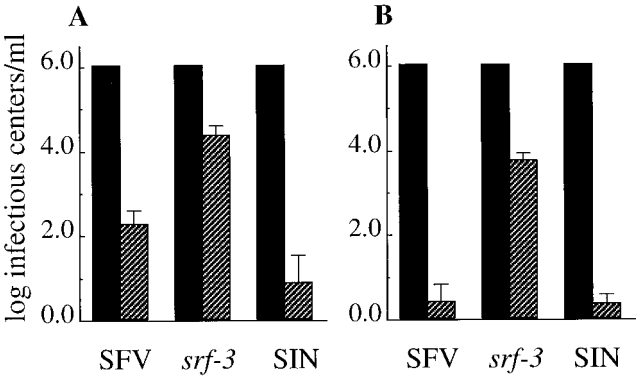

FIG. 3. Infection and fusion of wt SFV, *srf-3*, and wt SIN in control and cholesterol-depleted cells. (A) Infection. Control (solid bars) and cholesterol-depleted (hatched bars) C6/36 cells grown on coverslips were infected with serial dilutions of SFV, *srf-3*, and SIN virus stock, and primary infection was quantitated by immunofluorescence. Infection was normalized to 10^6 infectious centers/ml on control cells. Data are the means of five independent experiments ± standard deviations. (B) Fusion. Serial dilutions of the indicated virus stocks were bound to control (solid bars) and depleted (hatched bars) C6/36 cells on coverslips in the cold for 90 min and then warmed to 28°C in low-pH (either 5.5 or 5.0) medium for 1 min to trigger virus fusion with plasma membrane. The cells infected due to low-pH fusion were quantitated by immunofluorescence, and the titers were normalized to 10^6 infectious centers/ml on control cells. Data are the means of four independent experiments ± standard deviations.

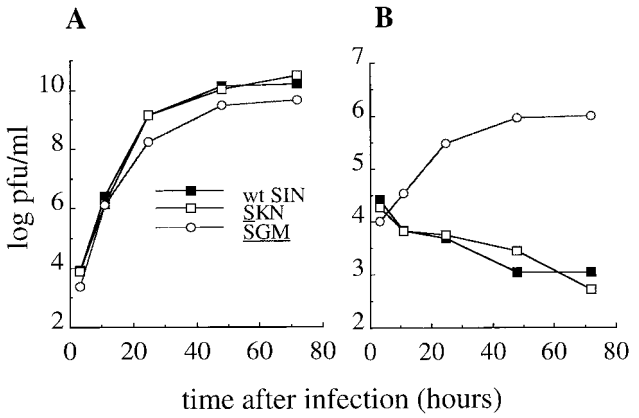

FIG. 4. Growth of wt SIN, SKN, and SGM mutants in control and cholesterol-depleted cells. Control (A) and cholesterol-depleted (B) cells were infected with wt SIN, SKN, and SGM viruses at 1 PFU/cell as described in the legend for Fig. 2, and virus titers were measured after growth for the indicated time. Note that the *y* axes in panels A and B are different.

copy of infected control C6/36 cells also demonstrated that the SGM mutant showed overall morphology, targeting, and budding characteristics similar to those of the wt virus, with one capsid per particle and budding virions observed primarily at the cell plasma membrane (data not shown).

Cholesterol requirements for growth, infection, and fusion of wt and SIN mutants. The growth properties of SIN, SKN, and SGM were compared in control and cholesterol-depleted C6/36 cells. In control cells (Fig. 4A), both mutants produced progeny virus with kinetics similar to those of wt SIN, with titers of $\sim 10^9$ PFU/ml (wt and SKN) and $\sim 2 \times 10^8$ PFU/ml (SGM) 24 h after infection. The titers of SGM at 24 and 48 h were consistently about fivefold lower than those obtained with wt or SKN on control cells. It is not clear if this is due to less efficient plaque production on BHK cells or less efficient growth of SGM on C6/36 cells. Neither wt SIN nor the SKN mutant showed any progeny virus production on depleted C6/36 cells (Fig. 4B). The virus titer in the medium actually decreased during the incubation, presumably due to cellular inactivation of input virus. In contrast, although not as efficient as its growth on control cells or as the growth of *srf-3* on depleted cells, the SGM mutant showed significantly increased progeny virus production in sterol-depleted cells, with a final titer of $\sim 10^6$ PFU/ml after 48 h of infection.

The infectivity of wt and mutant SIN was compared on control and depleted C6/36 cells (Fig. 5A). Both wt SIN and the SKN mutant infected depleted cells very poorly, with an almost 6-log decrease in infectivity on depleted cells compared to control cells. In contrast, although still about 1,000-fold less efficient than its infection of cholesterol-containing cells, the SGM mutant infected depleted cells ~100-fold better than wt SIN. This increase in SGM primary infection was due to an increase of about 250-fold in the fusion of SGM with the cholesterol-depleted cell membrane compared to the level for wt virus (Fig. 5B). Fusion of the SKN mutant with cholesterol-depleted membranes was greatly impaired, similar to the level for wt levels. These data indicate that, in contrast to results with the *srf-3* mutation of SFV, the introduction of 226S in the context of SIN E1 was insufficient for production of a cholesterol-independent fusion phenotype. However, mutations that substituted the *srf-3* sequence from E1 residues 224 to 235 conferred a striking reduction in the cholesterol requirement for SIN fusion and infection.

Cholesterol dependence of SIN and SGM exit. Our previous data indicated that wt SFV, which was cholesterol dependent for fusion, was also cholesterol dependent for exit, while the *srf-3* mutant was less cholesterol dependent for both fusion and exit (22, 28). To correlate the SIN cholesterol requirement for fusion with that for exit, pulse-chase analysis was performed in control and sterol-depleted C6/36 cells. Control cells were infected with either wt SIN or the SGM mutant, pulse-labeled with [^{35}S]methionine-cysteine, and chased for various times. The amount of newly synthesized viral spike proteins in the cells and the amount released as virus particles in the medium were evaluated at each time point (Fig. 6A). Immediately following the 15-min pulse-labeling (0 h), several processed forms of PE2 and E1 from both wt SIN and SGM were observed (e.g., a PE2 doublet). With increasing time of chase, PE2 was cleaved to E2 and E3 and increasing amounts of assembled virus were released into the medium. Phosphorimaging was used to quantitate the percentage of total labeled spike proteins released into the medium (Fig. 6A). Virus release within a 4-h chase was ~17% for wt spike proteins and ~10% for SGM, a difference that appeared to be due to experimental variation. Thus, exit efficiencies of wt SIN and SGM from control cells were similar.

Given the strong block in fusion in the absence of cholesterol, depleted C6/36 cells were infected with SIN or SGM using RNA transfection (see Materials and Methods). Transfected cells were pulse-labeled for 30 min and chased for the indicated times, and the percentage of virus release was determined (Fig. 6B). Both wt SIN- and SGM-infected cells produced abundant viral spike proteins and showed similar kinetics of PE2 cleavage. Similar to the results with SFV, almost no wt SIN virus was released even after 6 h of chase (~1% of total spike proteins released during 6 h). In contrast, the SGM mutant was released from depleted cells with kinetics similar to those of control cells (~14% of total spike proteins released within 6 h). Thus, wt SIN shows a cholesterol requirement for fusion and exit similar to the results previously observed with wt SFV, while the SGM mutant is relatively cholesterol independent for both entry and exit.

Mutations responsible for decreased SIN cholesterol dependence. Our results demonstrated that the SGM mutant was markedly less cholesterol dependent for both virus fusion and

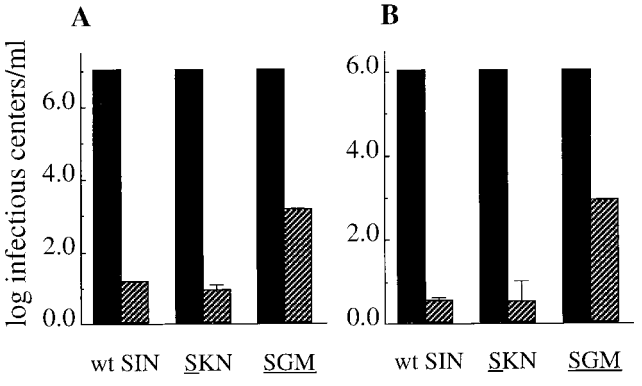

FIG. 5. Infection and fusion of wt SIN, SKN, and SGM mutants in control and cholesterol-depleted cells. (A) Infection. Virus infection of control (solid bars) and depleted (hatched bars) cells was measured as described in the legend for Fig. 3A. Virus titers on control cells were normalized to 10^7 infectious centers/ml. (B) Fusion. Virus fusion with control (solid bars) and depleted (hatched bars) C6/36 cells was quantitated by immunofluorescence as described in the legend for Fig. 3B. Virus titers on control cells were normalized to 10^6 infectious centers/ml. Panels A and B show the averages of two independent experiments, and error bars show the ranges.

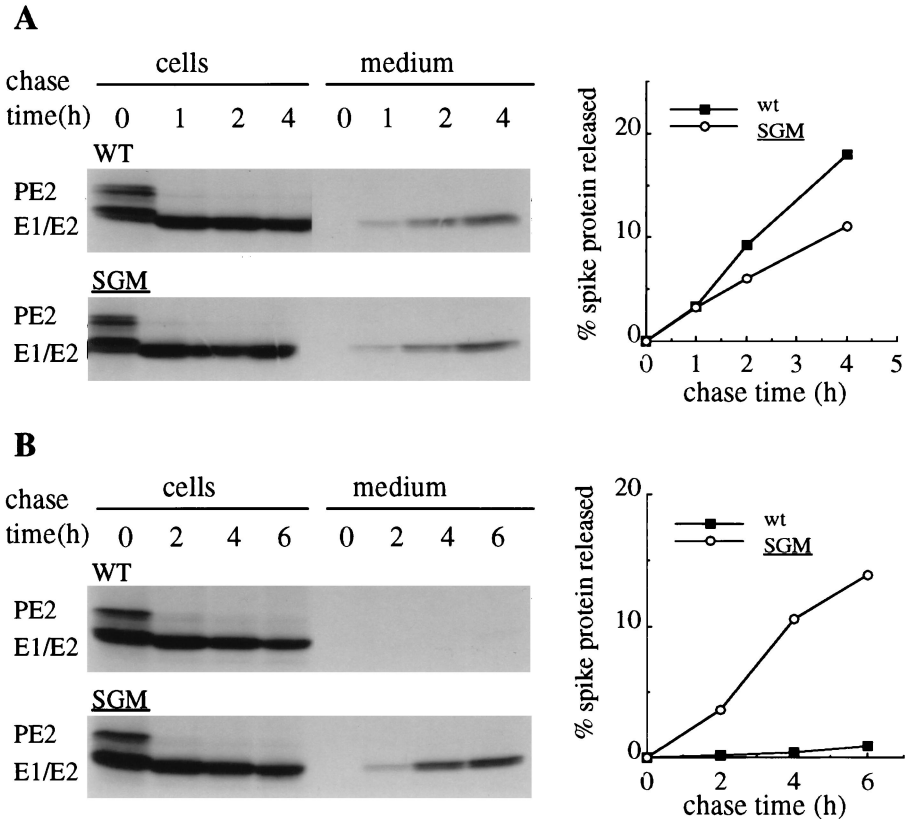

FIG. 6. Kinetics of exit of wt SIN and SGM virus in control and cholesterol-depleted C6/36 cells. (A) Control C6/36 cells were infected with wt SIN or SGM at a multiplicity of 10 PFU/cell for 26 h at 28°C. Cells were pulse-labeled for 15 min with [^{35}S]methionine-cysteine at 50 μCi/ml and chased for the indicated times, and aliquots of the media were immunoprecipitated with a monoclonal antibody against E2 in the absence of detergent. The cells were lysed and immunoprecipitated with a polyclonal antibody against the SIN spike protein. The immunoprecipitates were assayed by SDS-PAGE, fluorography, and phosphorimaging, for which 1/20 of the cell lysates and 1/10 of the medium samples were loaded. E1 and E2 comigrate in this gel system. The graph shows the quantitation of virus exit, determined as the percentage of spike proteins in the medium divided by the total amount of spike proteins in the cell lysate at 0 min of chase. Shown is a representative example of two experiments. (B) Cholesterol-depleted C6/36 cells were transfected for 4 h with 2.5 μg of wt SIN RNA or SGM RNA plus 10 μg of Cellfectin, incubated in fresh medium at 28°C for 20 h, pulse-labeled for 30 min with [^{35}S]methionine-cysteine at 200 μCi/ml, and chased for the time periods indicated. Analysis was performed as described for panel A, except that for the wt virus, 1/10 of the cell lysates and 1/5 of the medium samples were loaded. Shown is a representative example of three experiments.

exit than either wt SIN or the SKN point mutant. Selections for additional cholesterol-independent mutants of SFV have independently yielded the *srf-3* P226→S mutation eight times, suggesting the importance of the serine substitution (3). We therefore predicted that the cholesterol-independent phenotype of the SGM mutant was due to the combination of serine with glycine and/or methionine. Four additional SIN mutants were constructed to evaluate the importance of serine and the adjacent residues (Fig. 1). The SGN and SKM mutants were used to test the effect of serine^{226} in combination with either glycine^{227} or methionine^{228}. The mutant AGM retained the SIN alanine at position 226 but substituted the SFV glycine^{227}-methionine^{228}. We also constructed the mutant PGM, which has the wt SFV sequence from residues 224 to 235 on the SIN background. RNAs containing the mutations were prepared from the cDNA clones and transfected into BHK cells to obtain virus stocks. All four additional mutant constructs resulted in high-titer virus stocks capable of carrying out efficient secondary infection on BHK cells (data not shown), again indicating their lack of deleterious effects on spike protein folding, assembly, and normal fusion function. Efficiencies of infection of control and sterol-depleted C6/36 cells by wt SIN, SGM, and the new mutants were then compared in parallel (Fig. 7). As expected, wt SIN showed a strong cholesterol requirement, with a difference of about 5 logs between infection of depleted versus control cells. The SGM mutant was increased ~100-fold in its ability to infect depleted cells. Both the SGN and AGM mutants were significantly increased over wt SIN in their ability to infect in the absence of cellular cholesterol, with increases of ~500-fold and 60-fold, respectively. The SKM mutant showed no increase from wt SIN in infectivity on depleted cells. Similarly, the presence of the wt SFV sequence PGM in the SIN background caused a highly cholesterol-dependent phenotype, with a difference of ~5.5 logs in infectivity of the PGM mutant between control and depleted cells. Thus, while a strong increase in virus infection of cholesterol-depleted cells resulted from the combination of serine^{226} with either glycine^{227}-methione^{228} or glycine^{227}-asparagine^{228}, the serine was not strictly required for increased cholesterol independence as the glycine^{227}-methione^{228} substitution also increased virus ability to infect sterol-depleted cells. A requirement for the serine hydroxyl group in conferring cholesterol independence was thus not observed. Neither serine^{226} nor glycine^{227} alone was sufficient to reduce cholesterol dependence, since both the SKN and PGM mutants were highly cholesterol dependent. These results indicate that, similar to SFV, sequences in the SIN 226 region affect virus cholesterol dependence, and they suggest that the overall conformation of this region, rather than specific amino acid side chains, is involved in the virus sterol requirement.

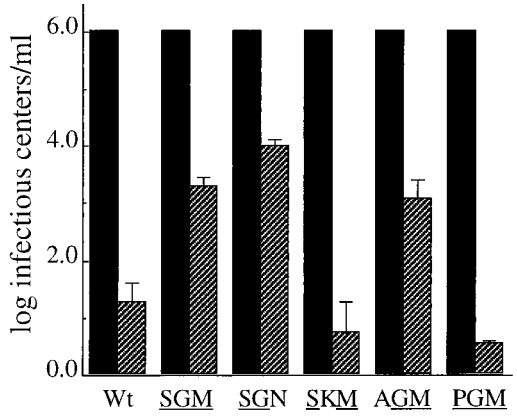

FIG. 7. Infection by wt and mutant SIN virus in control and cholesterol-depleted C6/36 cells. Infection of control (solid bars) and cholesterol-depleted (hatched bars) C6/36 cells with wt and mutant SIN virus stocks was quantitated as described in the legend for Fig. 3A. Infection was normalized to 10^6 infectious centers/ml on control cells. Data are the averages of two independent experiments, and error bars show the ranges.

DISCUSSION

Virus membrane fusion and virus budding are two critical steps in the life cycles of enveloped animal viruses and represent possible sites for intervention by antiviral therapy. Membrane fusion is catalyzed by the virus spike proteins, which change their conformation to a fusogenic form in which the viral hydrophobic fusion peptide is exposed and interacts with the target membrane (11). While this general scheme appears common to all virus fusion reactions, different viral fusion proteins use different mechanisms to trigger fusion. For example, the fusogenic conformational changes in the influenza virus spike protein require low pH (13), those of avian leukosis and sarcoma virus require spike-receptor interaction (12), and in the case of HIV-1, virus fusion is triggered by a mechanism that requires interaction with both the CD4 receptor and a 7-transmembrane, G protein-coupled coreceptor (1, 7). Similarly, virus budding is the general means by which viruses acquire a membrane envelope, but this reaction varies markedly in the intracellular site of budding, the viral components required to support budding, and the mechanisms involved (8). Alphaviruses are similar to many other viruses in having a fusion reaction that is triggered by low pH and a budding reaction that occurs at the plasma membrane. Interestingly, both of these steps in the alphavirus life cycle are greatly enhanced by the presence of cholesterol in the cell membrane. Thus, studies of alphaviruses have identified lipids as possible cofactors for critical steps in infection and progeny virus production.

We have here characterized the cholesterol dependence of SIN, the prototype member of the alphaviruses. Similar to SFV, SIN strongly required cellular cholesterol for fusion, infection, and virus exit. The SIN strain we used in this study is derived from the Toto 1101 infectious clone, a hybrid of HRsp strain-derived sequences (nsP1, partial nsP2, C-terminal region of PE2, 6K, and E1) and HR strain-derived sequences (partial nsP2, nsP3, nsP4, capsid, and most of PE2) (25). Previous studies showed that infection of cells by Toto 1101 occurs via acid-pH-induced fusion, with an apparent fusion threshold that is somewhat more acidic than that of wt SFV and therefore with a lower K_i for two endosomal acidification inhibitors, ammonium chloride and bafilomycin A-1 (10). Although the precise pH value required for fusion and infection thus differs between wt SFV and the SIN strain used here, the cholesterol dependence of virus fusion and exit was significant and comparable between the two viruses. Moreover, as predicted from the location of the SFV *srf-3* mutation, mutations in the E1 226 region of SIN decreased the cholesterol dependence of virus fusion, suggesting that this region is also involved in the SIN cholesterol requirement. Interestingly, the SGM mutant, which is less cholesterol dependent for growth, infection, and fusion than wt SIN, is also less cholesterol dependent for virus exit. This is in keeping with our previous results, which suggested a correlation between the relative cholesterol dependence of virus fusion and virus exit (22, 28). In SFV, the cholesterol requirement for exit can be localized to a late stage of virus exit, subsequent to spike protein arrival at the plasma membrane (20), and similarly, we presume that SIN also needs cholesterol for a late step in virus exit from the plasma membrane. Although the exact relationship between the sterol requirement for fusion and exit is as yet unclear, the results to date suggest that sterol-E1 interactions are involved in both virus fusion and exit and that mutations that decrease the stringency of the sterol requirement for fusion similarly affect the requirement for virus exit.

Our mutagenesis results indicate that several sequences in the 226 region can increase SIN cholesterol independence, including ^{226}SGM, ^{226}SGN, and ^{226}AGM. Interestingly, a subset of alphaviruses, including strains of eastern equine encephalitis virus and Venezuelan equine encephalitis virus, contain ^{226}AG in combination with alternative amino acids at position 228 and elsewhere in the 224 to 235 region (28). The alphavirus Barmah Forest virus contains the sequence ^{226}SGN and has the same sequence as the SIN SGN mutant throughout the 224 to 235 region save for the single exception of alanine at position 225. Given the significant overall sequence divergence among alphaviruses and the limited available functional information, we cannot draw conclusions about the cholesterol dependence of other alphaviruses. It is clear, however, that SIN and SFV, which are distantly related members of the alphaviruses (27), are both highly cholesterol dependent, suggesting that all members of the alphaviruses may have a cholesterol requirement for virus entry and exit. The reason for the cholesterol requirement in SFV and SIN infection is not known, but it is interesting that although selection for growth on cholesterol-depleted cells yields SFV mutants with strikingly lower cholesterol requirements, such mutants still have a strong preference for cholesterol-containing membranes as fusion targets (3, 28). In contrast, unrelated viruses, such as vesicular stomatitis virus, can support low-pH-dependent fusion, infection of both mammalian and insect cells, and plasma membrane-localized budding in the absence of a sterol requirement (4, 22, 24).

Previous studies of SFV fusion with liposomes demonstrated that sterol analogues could sustain efficient fusion even though the available biophysical evidence indicated that these analogues were inactive in affecting membrane fluidity or in associating with phospholipids (17). The key sterol structural feature for supporting fusion was the sterol 3β-hydroxyl group (17, 24). Experiments with the E1 ectodomain of SFV revealed a critical role for 3β-hydroxysterol in potentiating low-pH-dependent conformational changes in E1, E1 trimer formation, and E1 insertion into lipid bilayers (19). Recent biochemical studies of *srf-3* have demonstrated that the P226S mutation acts to increase the relative cholesterol independence of the fusogenic conformational changes in E1, including homotrimer formation (2). Taken together, these results suggest that the alphavirus cholesterol requirement probably reflects a role for cholesterol in inducing or maintaining the optimal E1 conformation(s) necessary for virus fusion and exit.

ACKNOWLEDGMENTS

We thank Anna Ahn for technical assistance, the members of our lab for helpful discussions and suggestions, and Duncan Wilson and the members of our lab for critical readings of the manuscript. We thank Ellen Strauss, Milton Schlessinger, and Richard Kuhn for their generosity in providing antibodies to the SIN spike protein and Richard Kuhn for providing Toto 1101 and helpful suggestions.

This work was supported by grants to M.K. from the American Cancer Society (RPG-93-013-07-MBC), the National Institutes of Health (R01 GM57454), and the Hirschl Charitable Trust; by the Jack K. and Helen B. Lazar fellowship in Cell Biology; and by Cancer Center Core Support grant NIH/NCI P30-CA13330. T.C. was supported by funds from the Einstein summer research program for undergraduate students.

REFERENCES

1. **Binley, J., and J. P. Moore.** 1997. HIV-cell fusion: the viral mousetrap. Nature **387:**346–348.
2. **Chatterjee, P., and M. Kielian.** 1998. Unpublished results.
3. **Chatterjee, P., and M. Kielian.** 1998. Unpublished results.
4. **Cleverley, D. Z., H. M. Geller, and J. Lenard.** 1997. Characterization of cholesterol-free insect cells infectible by baculoviruses: effects of cholesterol on VSV fusion and infectivity and on cytotoxicity induced by influenza M2 protein. Exp. Cell Res. **233:**288–296.
5. **DeTulleo, L., and T. Kirchhausen.** 1998. The clathrin endocytic pathway in viral infection. EMBO J. **17:**4585–4593.
6. **Duffus, W. A., P. Levy-Mintz, M. R. Klimjack, and M. Kielian.** 1995. Mutations in the putative fusion peptide of Semliki Forest virus affect spike protein oligomerization and virus assembly. J. Virol. **69:**2471–2479.
7. **Feng, Y., C. C. Broder, P. E. Kennedy, and E. A. Berger.** 1996. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science **272:**872–877.
8. **Garoff, H., R. Hewson, and D.-J. E. Opstelten.** 1998. Virus maturation by budding. Microbiol. Mol. Biol. Rev. **62:**1171–1190.
9. **Garoff, H., J. Wilschut, P. Liljestrom, J. M. Wahlberg, R. Bron, M. Suomalainen, J. Smyth, A. Salminen, B. U. Barth, and H. Zhao.** 1994. Assembly and entry mechanisms of Semliki Forest virus. Arch. Virol. **9:**329–338.
10. **Glomb-Reinmund, S., and M. Kielian.** 1998. The role of low pH and disulfide shuffling in the entry and fusion of Semliki Forest virus and Sindbis virus. Virology **248:**372–381.
11. **Hernandez, L. D., L. R. Hoffman, T. G. Wolfsberg, and J. M. White.** 1996. Virus-cell and cell-cell fusion. Annu. Rev. Cell Dev. Biol. **12:**627–661.
12. **Hernandez, L. D., R. J. Peters, S. E. Delos, J. A. T. Young, D. A. Agard, and J. M. White.** 1997. Activation of a retroviral membrane fusion protein: soluble receptor-induced liposome binding of the ALSV envelope glycoprotein. J. Cell Biol. **139:**1455–1464.
13. **Hughson, F. M.** 1995. Structural characterization of viral fusion proteins. Curr. Biol. **5:**265–274.
14. **Kielian, M.** 1995. Membrane fusion and the alphavirus life cycle. Adv. Virus Res. **45:**113–151.
15. **Kielian, M., and A. Helenius.** 1985. pH-induced alterations in the fusogenic spike protein of Semliki Forest virus. J. Cell Biol. **101:**2284–2291.
16. **Kielian, M., M. R. Klimjack, S. Ghosh, and W. A. Duffus.** 1996. Mechanisms of mutations inhibiting fusion and infection by Semliki Forest virus. J. Cell Biol. **134:**863–872.
17. **Kielian, M. C., and A. Helenius.** 1984. The role of cholesterol in the fusion of Semliki Forest virus with membranes. J. Virol. **52:**281–283.
18. **Kielian, M. C., S. Keranen, L. Kaariainen, and A. Helenius.** 1984. Membrane fusion mutants of Semliki Forest virus. J. Cell Biol. **98:**139–145.
19. **Klimjack, M. R., S. Jeffrey, and M. Kielian.** 1994. Membrane and protein interactions of a soluble form of the Semliki Forest virus fusion protein. J. Virol. **68:**6940–6946.
20. **Lu, Y. E., and M. Kielian.** 1999. Unpublished results.
21. **Marquardt, M. T., and M. Kielian.** 1996. Cholesterol-depleted cells that are relatively permissive for Semliki Forest virus infection. Virology **224:**198–205.
22. **Marquardt, M. T., T. Phalen, and M. Kielian.** 1993. Cholesterol is required in the exit pathway of Semliki Forest virus. J. Cell Biol. **123:**57–65.
23. **Nieva, J. L., R. Bron, J. Corver, and J. Wilschut.** 1994. Membrane fusion of Semliki Forest virus requires sphingolipids in the target membrane. EMBO J. **13:**2797–2804.
24. **Phalen, T., and M. Kielian.** 1991. Cholesterol is required for infection by Semliki Forest virus. J. Cell Biol. **112:**615–623.
25. **Rice, C. M., R. Levis, J. H. Strauss, and H. V. Huang.** 1987. Production of infectious RNA transcripts from Sindbis virus cDNA clones: mapping of lethal mutations, rescue of a temperature-sensitive marker, and in vitro mutagenesis to generate defined mutants. Virology **61:**3809–3819.
26. **Smith, T. J., R. H. Cheng, N. H. Olson, P. Peterson, E. Chase, R. J. Kuhn, and T. S. Baker.** 1995. Putative receptor binding sites on alphaviruses as visualized by cryoelectron microscopy. Proc. Natl. Acad. Sci. USA **92:**10648–10652.
27. **Strauss, J. H., and E. G. Strauss.** 1994. The alphaviruses: gene expression, replication, and evolution. Microbiol. Rev. **58:**491–562.
28. **Vashishtha, M., T. Phalen, M. T. Marquardt, J. S. Ryu, A. C. Ng, and M. Kielian.** 1998. A single point mutation controls the cholesterol dependence of Semliki Forest virus entry and exit. J. Cell Biol. **140:**91–99.
29. **White, J., and A. Helenius.** 1980. pH-dependent fusion between the Semliki Forest virus membrane and liposomes. Proc. Natl. Acad. Sci. USA **77:**3273–3277.
30. **Wilschut, J., J. Corver, J. L. Nieva, R. Bron, L. Moesby, K. C. Reddy, and R. Bittman.** 1995. Fusion of Semliki Forest virus with cholesterol-containing liposomes at low pH: a specific requirement for sphingolipids. Mol. Membr. Biol. **12:**143–149.