Cell entry mechanisms of alphaviruses
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Chapter 2
Sphingolipid and Cholesterol Dependence of Alphavirus Membrane Fusion
Lack of Correlation with Lipid Raft Formation in Target Liposomes

Barry-Lee Waarts, Robert Bittman, and Jan Wilschut

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2.1 Abstract

Semliki Forest virus (SFV) and Sindbis virus (SIN) are enveloped viruses that infect their host cells by receptor-mediated endocytosis and subsequent fusion from within acidic endosomes. Fusion of the viral envelope requires the presence of both cholesterol and sphingolipids in the target membrane. This is suggestive of a possible involvement of sphingolipid-cholesterol microdomains, or “lipid rafts,” in the membrane fusion and cell entry process of the virus. In this study, large unilamellar vesicles (LUVs) were prepared from synthetic sphingolipids and sterols that vary with respect to their capacity to promote microdomain formation, as assessed by gradient flotation analysis in the presence of Triton X-100 (TX-100). SFV and SIN fused with LUVs irrespective of the presence or absence of TX-100 insoluble microdomains. These results suggest that SFV and SIN do not require the presence of lipid rafts for fusion with target membranes. Furthermore, it is not necessary for sphingolipids to reside in a detergent-insoluble complex with cholesterol in order to promote SFV or SIN fusion.
2.2 Introduction

Semliki Forest virus (SFV) and Sindbis virus (SIN) are enveloped positive-strand RNA viruses belonging to the genus Alphaviruses of the family Togaviridae (reviewed in 1). Alphaviruses enter their host cell by receptor-mediated endocytosis, a process which delivers the virus particles to the endosomal cell compartment. The viral RNA subsequently gains access to the cytosol through fusion of the viral envelope with the endosomal membrane from within the lumen of the endosome (2). The fusion process is mediated by the viral spike protein E1 (3), which prior to fusion rearranges from its heterodimeric complex with E2 (4, 5) into a homotrimeric configuration (6). The trigger for the rearrangement of the viral spike protein is the mildly acidic pH in the lumen of the endosome (7-9).

It has been established that the fusion of SFV in model systems is strictly dependent on low pH and requires the presence of cholesterol (Chol) and sphingolipids in the target membrane (10-17). The Chol-dependence of SFV fusion has also been demonstrated in cells (18), while in addition budding of SFV from the infected-cell surface appears to require Chol as well (15, 19). Recently, it has been shown that low-pH-dependent fusion of the alphavirus SIN, similar to that of SFV, also requires the presence of Chol and sphingolipids in the target membrane (20). This suggests that a requirement for Chol and sphingolipids is a general characteristic of the membrane fusion activity of alphaviruses. For SFV, it has been shown that Chol mainly mediates the binding of the viral envelope to the target membrane, whereas sphingolipids are involved in the actual fusion event (13, 14).

The dependence of SFV and SIN fusion on the presence of both Chol and sphingolipids in the target membrane is suggestive of a possible involvement of sphingolipid-Chol microdomains or “lipid rafts,” which are present in mammalian cellular membranes as small entities (21, 22). These lipid rafts are thought to exist in bilayers in the liquid-ordered L_0 phase as complexes consisting of Chol and (glyco)sphingolipids with predominantly saturated acyl chains (23). The extended conformation of the saturated acyl chains of (glyco)sphingolipids allows these lipids to pack optimally with Chol (21, 24, 25). Lipid rafts appear to be insoluble in non-ionic detergents such as Triton X-100 (TX-100) at 4°C. Thus, lipid rafts can be isolated from cells as detergent-insoluble complexes (DICs), together with the proteins recruited into the rafts (26). There is extensive evidence for the function of lipid rafts in a variety of lipid and protein sorting events and signal transduction cascades (23, 26, 27).

Importantly, lipid rafts have been shown to play an important role in the cell entry of viruses. Simian virus 40 (SV40) enters cells via caveolae, which are special invagination sites rich in lipid rafts (28, 29). Human immunodeficiency virus type 1 (HIV-1) also appears to use rafts as a platform for host cell entry (reviewed in 30). Glycosphingolipids, which are abundantly present in lipid rafts, interact with the HIV-1 envelope glycoprotein gp120 and are required for expression of viral membrane fusion activity (31, 32). It has been suggested that gp120 induces lateral reorganization of rafts, bringing complexes of gp120 with CD4, a receptor also present in rafts, together with rafts containing the chemokine co-receptor (33). HIV-1 virions pass a barrier of
mucosal epithelial cells by a process called transcytosis, which requires the presence of galactosylceramide in lipid rafts to mediate virion binding (34).

To address the question as to whether lipid rafts are involved in the sphingolipid- and Chol-dependence of alphavirus fusion, we used synthetic sphingolipids and sterols that vary in their ability to support formation of DICs. We found that fusion of SFV and SIN is rapid and extensive in the absence or presence of DICs, indicating that fusion of these viruses does not require the sphingolipids to reside in raft-like microdomains with Chol.

2.3 Experimental Procedures

**Lipids** - The fluorescent probe 16-(1-pyrenyl)hexadecanoic acid (pyrene fatty acid) was obtained from Molecular Probes Europe BV (Leiden, The Netherlands). Phosphatidylcholine (PC) from egg yolk, phosphatidylethanolamine (PE) prepared by transphosphatidylation of egg PC, sphingomyelin (SPM) from brain tissue, N-oleoyl ceramide, and Chol were obtained from Avanti Polar Lipids (Alabaster, AL). 5-Androsten-3β-ol, ergosterol, and stigmasterol were purchased from Steraloids (Newport, RI). N-Oleoyl-, N-stearoyl-, and N-elaïdoyl-SPM were synthesized by N-acylation of D-erythro-sphingosylphosphocholine with the corresponding p-nitrophenyl esters, as described previously (35). D-erythro-Sphingosylphosphocholine was prepared by acid-catalyzed methanolation of egg SPM as described previously (35). 20(R)-Isooctyl-5-pregnen-3β-ol (abbreviated as C4 sterol) was synthesized as described before (36).

**Cells and Virus** - SFV and SIN TR339 were propagated on baby hamster kidney cells (BHK-21) cultured in Glasgow’s modification of Eagle’s minimal essential medium (Gibco/BRL, Breda, The Netherlands), supplemented with 5% fetal calf serum, 10% tryptose phosphate broth, 200 mM glutamine, 25 mM HEPES, and 7.5% sodium bicarbonate. Pyrene-labeled virus was isolated from the medium of infected BHK-21 cells, cultured beforehand in the presence of pyrene fatty acid, essentially as described previously (6, 12, 13, 20). Briefly, BHK-21 cells were grown in medium containing 15 µg of pyrene fatty acid per ml for 48 h. The cells were infected at a multiplicity of infection of 4. At 24 h postinfection, pyrene-labeled virus was harvested from the medium by ultracentrifugation in a Beckman type 19 rotor for 2.5 h at 100,000 x g at 4°C. The particles were further purified by ultracentrifugation on a 20 to 50% (w/v) sucrose density gradient in a Beckman SW41 rotor for 16 h at 100,000 x g at 4°C.

**Liposomes** - Large unilamellar vesicles (LUVs) were prepared by a freeze-thaw/extrusion procedure (37, 38). Briefly, lipid mixtures were dried from a chloroform-methanol solution under a stream of nitrogen and further dried under vacuum for at least 1 h. The lipid films were hydrated in 5 mM HEPES, 150 mM NaCl, 0.1 mM EDTA (pH 7.4) (HNE) and subjected to five cycles of freezing and thawing. Subsequently, the vesicles were sized by extrusion 21 times through a polycarbonate filter with a pore size of 0.2 µm (Nuclepore, Whatman, Clifton, NJ) in a mini-extruder (LiposoFast, Avestin, Ottawa, Canada). Radiolabeled LUVs were prepared as described above except a trace amount (1-1.5 µCi) of [7(n)-3H]Chol ([3H]Chol) (3.5 Ci/mmol,
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Amersham Pharmacia Biotech, Roosendaal, The Netherlands) was added to the lipid mixtures before drying.

Analysis of DIC Formation - Detergent-insoluble complexes (DICs) were isolated from LUVs (300 µM phospholipid) by treatment with 1% TX-100 (v/v, Sigma) in HNE buffer at 4°C for 30 min. Subsequently, the detergent-LUV mixture was mixed with 60% (w/v) Optiprep (Axis-Shield, Oslo, Norway) to a final concentration of 45% Optiprep in a final volume of 1 ml and placed on the bottom of an ultracentrifuge tube. For the isolation of DICs from LUVs containing [3H]Chol the LUV extract was overlaid with a 2.9-ml layer of 10% Optiprep (w/v), 1% TX-100 (v/v) in HNE, and a 1-ml layer of 5% Optiprep (w/v) and 1% Triton X-100 (v/v) in HNE. The extracted LUVs were overlaid with a 1-ml layer of 15% Optiprep (w/v), 1% TX-100 (v/v) in HNE, a second layer of 1.9-ml with 10% Optiprep (w/v) in HNE without TX-100, and a third layer of HNE alone. These gradients were used to minimize the interference of TX-100 in the phosphate assay and thin-layer chromatography (TLC) analysis, which were used to estimate the formation and content of DICs. The gradients were subjected to ultracentrifugation in a Beckman SW55 Ti rotor for 18 h at 100,000 x g at 4°C. After centrifugation, fractions of 275 µl were collected, starting from the top of the gradient. Aliquots were removed to determine the amount of [3H]Chol in each fraction by liquid-scintillation counting. When non-radiolabeled LUVs were used, the amount of phosphate present in the fractions was determined by a standard phosphate assay (39). In order to compare DIC formation with the different sterols used, the amount of phosphate present in the pooled fractions 4-8, the DIC-containing fractions, was determined by phosphate analysis after extraction of the lipids (40). For the TLC analysis of the DICs, fractions 4-8 were pooled, and the lipids were extracted and spotted on silica gel 60 HPTLC plates (Merck) developed with CHCl3/MeOH/NH4OH (25% in water) 65:25:5 (v/v). The lipid spots were stained with iodine.

Fusion Assay - Pyrene-labeled SFV or SIN (1 µM viral phospholipid) and LUVs (100 µM phospholipid) were mixed in 0.665 ml of HNE in a magnetically stirred and thermostatted (37°C) quartz cuvette in an AB2 fluorometer (SLM/Aminco, Urbana, IL). Fusion was triggered by the addition of 35 µl of 0.1 M MES, 0.2 M acetic acid, pre-titrated with NaOH to achieve a final pH of 5.5 for SFV or 5.0 for SIN, and the decrease of pyrene excimer fluorescence intensity (excitation and emission at 340 and 480 nm, respectively) was monitored continuously. The fusion scale was calibrated such that 0% fusion corresponded to the initial excimer fluorescence intensity and 100% fusion to complete dilution of the probe (6, 12, 13, 20, 41).

2.4 Results

Evaluation of the Presence of DICs in LUVs

To evaluate the presence of DICs in liposomes, LUVs consisting of PC/PE/SPM/Chol (molar ratio, 1:1:1:1.5) were treated with TX-100 at 4°C and subjected to a density gradient flotation analysis, as described in “Experimental Procedures.” DICs floating to the top of a 45-10-5% density gradient were detected by the [3H]Chol initially incorporated in the LUVs. With PC/PE/SPM/Chol LUVs, as much as 30% of the total amount of Chol present in the gradient floated to low density
Lack of correlation between raft formation and membrane fusion

(Fig. 1A). When SPM was excluded from the LUVs, no radiolabeled Chol was detected at the top of the gradient (Fig. 1B).

To investigate the effect of Chol on DIC formation we used the same flotation procedure, but with a slightly different gradient in order to avoid interference from TX-100 in the phospholipid determination assay. Again, efficient DIC formation was observed when both SPM and Chol were present in the LUVs (Fig. 1C). Approximately 30% of the total amount of phospholipid was found in the floating DIC fraction. In the absence of Chol, no phospholipid was detected at the top of the gradient (Fig 1D). About 90% of the total amount of phospholipid in the DIC-containing fractions isolated from LUVs prepared from PC/PE/SPM/Chol consisted of SPM, as determined by phospholipid analysis of the lipid spots separated by TLC (Fig. 2). PC and PE were largely excluded from these complexes, in agreement with the composition of cellular rafts reported previously (21, 42).

Figure 1. Flotation of DICs after treatment of LUVs with TX-100 at 4°C. LUVs consisted of PC, PE, SPM, and Chol (A, C); PC, PE, and Chol (B); or PC, PE, and SPM (D). Flotation of Chol was determined on the basis of the [3H]Chol content of each fraction of the density gradient after ultracentrifugation (A and B). Flotation of phospholipid was determined by inorganic phosphate analysis of the gradient fractions (C and D).
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Effect of Acyl Chain Structure of SPM on DIC Formation

We next used the flotation assay with [3H]Chol described above to assess the ability of synthetic SPMs with different N-acyl chains to form DICs with Chol. These sphingolipid analogs were incorporated into LUVs with PC, PE, and Chol (molar ratio PC/PE/sphingolipid/Chol, 1:1:1:1.5). A high extent of DIC formation was observed with stearoyl- and bovine-brain SPMs (Table I); in the former the N-acyl chain is saturated, and in the latter the saturated : unsaturated acyl chain ratio is 93:7 (mol:mol) (43). On the other hand, when N-oleoyl- or N-linoleoyl-SPM were incorporated in PC, PE, Chol bilayers, no flotation of [3H]Chol occurred. The latter SPMs have one and two cis unsaturated bonds, respectively, which interfere with an efficient alignment with Chol (21, 24, reviewed in 25). These results are consistent with the recent findings of Samsonov et al. (44), who observed that rafts could not be formed with N-oleoyl-SPM in planar membranes, in contrast to egg SPM and N-stearoyl SPM. Table I also shows that when SPM has a trans C9-C10 double bond in the N-acyl chain (N-elaidoyl SPM), 16% of the total [3H]Chol floated to a low density. Thus 18:1-Δ9-trans-SPM does assemble into microdomains with Chol to a limited extent, in contrast to the corresponding Δ9-cis-SPM.

Effects of Modification of Sterol Structure on DIC Formation

Next, we evaluated the ability of four different sterols (stigmasterol, ergosterol, androstenol, and C4-sterol) to support DIC formation in our model system. In other systems, these sterols had been shown to differ from one another with respect to their ability to participate in lipid raft formation (45, 46). The sterols were incorporated into LUVs (PC/PE/sterol molar ratio of 1:1:1:1.5). Flotation analysis after extraction with TX-100 indicated that DICs were formed to a greater extent with stigmasterol and ergosterol than with Chol (Table II). A lower extent of DIC formation was observed with C4-sterol, whereas androstenol failed to support significant DIC formation. This observation is consistent with the report of Patra et al. (47), who found that 16-androsten-3β-ol, which resembles the structure of androstenol (5-androsten-3β-ol), does not promote the formation of DICs in liposomes. The sterol-dependence of DIC formation noted in Table II is also in accord with the results of Xu et al. (45) (see Discussion).

Effects of Different Sphingolipids and Sterols on SFV Fusion

To assess the potential correlation between the fusion of SFV and DIC formation in target liposomes, we studied the interaction of pyrene-labeled virus with LUVs
Lack of correlation between raft formation and membrane fusion

### TABLE I

**Flotation of [3H]Chol after TX-100 extraction of LUVs containing different sphingolipids.** LUVs contained PC, PE, sphingolipid, and Chol (molar ratio 1:1:1:1.5) and a trace amount of [3H]Chol. After treatment of the LUVs with 1% TX-100 (v/v) at 4°C, the detergent-LUV mixture was mixed with Optiprep to a final concentration of 45% Optiprep (w/v) and overlaid with layers of 10% and 5% Optiprep in an ultracentrifuge tube. After centrifugation (100,000 x g, 4°C), the gradient was fractionated and the amount of [3H]Chol in each fraction was determined by liquid-scintillation counting. The percentage of [3H]Chol that floated to low-density relative to the total amount of [3H]Chol present in the gradient is given for LUVs containing the sphingolipids indicated (mean ± S.D.). Results represent an average of triplicate experiments.

<table>
<thead>
<tr>
<th>Sphingolipid</th>
<th>[3H]Chol in DICs</th>
<th>% of total</th>
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<tbody>
<tr>
<td>Brain SPM</td>
<td>31.6 ± 2.2</td>
<td></td>
</tr>
<tr>
<td>N-stearoyl-SPM</td>
<td>42.4 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>N-elaidoyl-SPM</td>
<td>16.0 ± 3.1</td>
<td></td>
</tr>
<tr>
<td>N-oleoyl-SPM</td>
<td>0.1 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>N-linoleoyl-SPM</td>
<td>0.1 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>No sphingolipid</td>
<td>0.1 ± 0.0</td>
<td></td>
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</table>

### TABLE II

**Flotation of phospholipid after TX-100 extraction of LUVs containing different sterols.** LUVs contained PC, PE, brain SPM, and sterol (molar ratio 1:1:1:1.5). After treatment of the LUVs with 1% TX-100 (v/v) at 4°C the detergent-LUV mixture was mixed with Optiprep to a final concentration of 45% Optiprep (w/v) and overlaid with layers of 15%, 10%, and 5% Optiprep in an ultracentrifuge tube. After centrifugation (100,000 x g, 4°C), the gradient was fractionated and the amount of phospholipid in fractions 4-8 was determined by phosphate analysis. The percentage of phospholipid that floated to low density relative to the total amount of phospholipid present in the gradient is given for LUVs containing the sterols indicated (mean ± S.D.). Results represent an average of triplicate experiments.

<table>
<thead>
<tr>
<th>Sterol</th>
<th>Phospholipid in DICs</th>
<th>% of total</th>
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<tbody>
<tr>
<td>Cholesterol</td>
<td>31.0 ± 3.3</td>
<td></td>
</tr>
<tr>
<td>Stigmasterol</td>
<td>40.9 ± 8.6</td>
<td></td>
</tr>
<tr>
<td>Ergosterol</td>
<td>41.2 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>Androstenol</td>
<td>2.4 ± 3.4</td>
<td></td>
</tr>
<tr>
<td>C4-sterol</td>
<td>25.9 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>No sterol</td>
<td>0.4 ± 0.6</td>
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containing different SPMs or sterols. The pyrene fusion assay monitors fusion on-line as a decrease of pyrene excimer fluorescence intensity upon dilution of pyrene-labeled phospholipids from the viral into the liposomal target membrane (6, 12).

SFV underwent efficient fusion with LUVs containing Chol and SPM, irrespective of the degree of unsaturation of the SPM N-acyl chain. For example, very similar kinetics and extents of fusion were observed with LUVs containing either N-oleoyl SPM or brain SPM (Fig. 3A, curves a and b, respectively), whereas there was no fusion with PC/PE/Chol LUVs lacking SPM (Fig. 3A, curve c). Fig. 4A presents a survey of the extents of SFV fusion with LUVs containing various sphingolipids, together with PC, PE, and Chol. These sphingolipids included SPMs with different N-acyl chains as well as N-oleylceramide. For reasons of comparison, Fig. 4A also presents the extents of DIC formation in these LUVs (black bars). Clearly, fusion of SFV was rapid and extensive with LUVs containing sphingolipids that lack the ability to assemble into DICs with Chol. This finding also holds for LUVs containing N-oleylceramide, which fused extensively with SFV but did not form DICs. It is noteworthy that fusion supported by N-stearoyl-SPM, a DIC-promoting lipid, was slower (results not shown) and 20% less extensive than that supported by brain SPM (Fig. 4A).

Similar results were obtained with target LUVs containing various sterols, together with PC, PE, and brain SPM. Fig. 3B shows, as an example, a comparison between the fusion of SFV with LUVs containing stigmasterol (curve a) or androstenol (curve b). The extents as well as the kinetics of fusion are virtually indistinguishable.

Figure 3. Low-pH-induced fusion of pyrene-labeled SFV with LUVs. Fusion was measured on-line at 37°C as a decrease of viral pyrene excimer fluorescence, as described in Experimental Procedures. Panel A: curve a, PC/PE/Chol/N-oleoyl-SPM (molar ratio, 1:1:1:5:1); curve b, PC/PE/Chol/brain SPM (molar ratio, 1:1:1:5:1); curve c, PC/PE/Chol (molar ratio, 1:1:1:5). Panel B: curve a, PC/PE/stigmasterol/brain SPM (molar ratio, 1:1:1:5:1); curve b, PC/PE/androstenol/brain SPM (molar ratio, 1:1:1:5:1).

Fig. 4B summarizes the results obtained with all five of the sterols we used with respect to their efficacy in supporting SFV fusion. On the other hand, the sterols varied considerably in their ability to assemble into DICs with brain SPM. Particularly,
androstenol did not promote DIC formation, yet it strongly supported fusion SFV with LUVs (Fig. 4B). It has been demonstrated previously, in a different fusion assay, that 5α-androstan-3β-ol, which is structurally similar to androstenol (5-androsten-3β-ol), promotes SFV-liposome fusion to the same extent as does Chol (11).

**Figure 4. Lack of correlation between SFV fusion with LUVs and the degree of DIC formation in the LUVs.** Fusion was measured on-line at 37°C as a decrease of viral pyrene excimer fluorescence, as described in Experimental Procedures. For reasons of comparison the extents of fusion (gray bars) are given together with the extents of DIC formation (black bars). Panel A: LUVs were prepared from PC/PE/Chol/sphingolipid (molar ratio, 1:1:1.5:1); A, brain SPM; B, N-stearoyl-SPM; C, N-ε-laidoyl-SPM; D, N-oleoyl-SPM; E, N-linoeoyl-SPM; F, N-oleoylceramide; G, control without sphingolipid. Panel B: LUVs were prepared from PC/PE/sterol/brain SPM (molar ratio, 1:1:1.5:1); A, Chol; B, ergosterol; C, stigmasterol; D, androstenol; E, C4-sterol; F, control without sterol. Error bars represent the standard deviation between three experiments.

**Concentration Dependence of SFV Fusion on DIC-promoting and Non-promoting Lipids**

The above results indicate that the presence of DICs is not essential for SFV fusion. Previous work has shown that there are optimal concentrations of Chol and SPM for binding and fusion of SFV with LUVs (13). We then explored the possibility that if suboptimal SPM or Chol concentrations are used in LUVs the presence of DIC-promoting lipids may exert an influence on the kinetics of SFV fusion. Thus we determined the dependence of SFV fusion on the concentration of brain SPM versus N-oleoyl-SPM. Similarly, the dependence of SFV fusion on the concentration of Chol in target liposomes was investigated using LUVs with a fixed ratio of PC/PE/SPM or PC/PE/N-oleoyl-SPM. Fig. 5A shows that the extent of fusion is the same in LUVs prepared with brain SPM and N-oleoyl-SPM at suboptimal concentrations (0.2, 0.5, 1.0, 1.5, 2.0, and 5.0 mol%). Additionally, the dependence of SFV fusion on Chol content was not influenced by the presence of N-oleoyl-SPM (Fig. 5B). In fact, the extents of fusion at 10 and 20 mol% Chol were even higher with PC/PE/N-oleoyl-
SPM/Chol liposomes. This suggests that even at suboptimal lipid concentrations the presence of DIC-promoting lipids is not more favorable for fusion than the presence of lipids which do not promote formation of DICs.

**Fig. 5. Sphingolipid and Chol concentration dependence of pyrene-labeled SFV fusion with LUVs.** Fusion was measured on-line at 37°C as a decrease of viral pyrene excimer fluorescence, as described in Experimental Procedures. Panel A: LUVs were prepared with PC/PE/Chol (molar ratio, 1:1:1.5) and an increasing concentration of N-oleoyl-SPM (squares) or brain SPM (circles). Panel B: LUVs were prepared with PC/PE/N-oleoyl-SPM (molar ratio, 1:1:0.35) (squares) or PC/PE/brain SPM (1:1:0.35) (circles) and an increasing concentration of Chol.

**Effect of the Presence of DICs in the Target Membrane on SIN Fusion**

Finally, we addressed the question whether another alphavirus, SIN, is able to fuse with LUVs prepared from lipids that do not induce formation of DICs. SIN fusion has recently shown to be, like SFV fusion, dependent on the presence of Chol and sphingolipids in the target membrane (20). As shown in Fig. 6, SIN underwent efficient fusion with LUVs prepared from PC/PE/N-oleoyl-SPM/Chol or PC/PE/SPM/androstenol. Moreover, the kinetics were similar to that found for SIN fusion with PC/PE/SPM/Chol LUVs, indicating that the lipid dependence of alphavirus fusion does not involve the participation of DICs.
2.5 Discussion

This paper addresses questions as to whether sphingolipid-Chol microdomains or lipid rafts are involved in the membrane fusion process of alphaviruses. Sphingolipid and Chol analogs were evaluated for their ability to promote DIC formation in LUVs, on the one hand, and for their fusion-supporting capacity in a liposomal model system, on the other. Remarkably, irrespective of their ability to promote DIC formation, the lipids all efficiently supported low-pH-induced fusion of SFV and SIN in this liposomal model system, indicating that sphingolipid-Chol microdomains or lipid rafts are not required for fusion of these two alphaviruses.

Analysis of DIC Formation

To evaluate the presence of Chol-sphingolipid complexes in liposomes, LUVs with different lipid compositions were treated with TX-100 at 4°C followed by gradient flotation analysis. Similar detergent extraction methods have been used before to assess lipid domain formation in model membranes and cells (26, 48-50). It is important to note that the detection of sphingolipid-Chol complexes is not restricted to low-temperature conditions, although the extent of microdomain formation may be temperature-dependent. Recent studies, using fluorescent methods and atomic force microscopy, have shown that the presence of DICs in both model and cell membranes, as assessed by detergent extraction in the cold, closely corresponds to the formation of lipid microdomains at physiological temperatures (22, 44-46, 51, 52).

Effect of the SPM N-acyl Chain Structure and Sterol Structure on the Formation of DICs

DICs were present in the membranes of LUVs consisting of PC/PE/Chol/SPM and absent if either Chol or SPM was not included in the bilayer (Fig. 1A-D). PC and PE were largely excluded from DICs as shown by TLC analysis of the DICs (Fig. 2). When SPMs with at least one cis unsaturation in the N-linked acyl chain were used, DIC formation with Chol was disrupted (Table I). With N-elaidoyl-SPM, in which the double bond is trans, DIC formation did occur, albeit to a lesser extent than with brain.
and N-stearoyl-SPM. This supports the idea that an elongated conformation of the acyl chain, at least between the second and the tenth carbon atom, is needed for optimal SPM-Chol microdomain formation (24, 53, 54). Although a double bond interferes with efficient SPM-Chol interaction, a trans unsaturation has an elongated conformation and therefore does result in a limited degree of microdomain formation. These findings are completely consistent with the current understanding that formation of microdomains mainly depends on the structural properties of the lipids involved. A long saturated N-acyl chain in SPM supports a tight interaction with Chol, resulting in the formation of microdomains that are detergent-insoluble in the cold. These liquid-ordered SPM-Chol microdomains are likely to exist next to liquid-crystalline areas rich in cis-unsaturated lipids (50, 55, 56). Unsaturated PC and PE, but also cis-unsaturated SPMs, are excluded from liquid-ordered complexes because the kinked chain structure limits the ability of these phospholipids to align with Chol (25, 42, 57, 58).

To evaluate the effect of sterol structure on DIC formation in LUVs, four sterols were used in this study with differing ability to engage in ordered lipid microdomain formation (45-47). In agreement with observations of Xu and London (46), who used a fluorescence quenching assay involving multilamellar lipid vesicles containing an unsaturated quencher phospholipid and a fluorescent probe, we found that androstenol has a very limited ability to support microdomain formation. On the other hand, we observed that stigmasterol efficiently promotes domain formation, whereas Xu et al. (45) found stigmasterol to be less efficient than Chol at least at 23°C. The comparatively high extents of domain formation we found with stigmasterol probably arise from the lower temperature (4°C) at which we treated the LUVs with TX-100. At lower temperatures, the differences between Chol and stigmasterol in their ability to promote domain formation become smaller (45). Likewise, in the fluorescence quenching assay at 23°C, C4-sterol has a limited ability to engage in formation of domains with dipalmitoyl-PC (45), whereas in the context of PC/PE/sterol/SPM (molar ratio 1:1:1.5:1) LUVs at 4°C, C4-sterol does promote the formation of DICs to significant extents (Table II).

**Fusion of SFV and SIN with LUVs in the Absence of DICs**

Fusion of SFV and SIN has been shown to be strictly dependent on the simultaneous presence of Chol and sphingolipids in the target membrane (13-17). Remarkably, all of the sphingolipids and sterols used in this study, irrespective of their ability to support DIC formation in LUVs, were able to promote SFV fusion to approximately the same extent (Fig. 4). Specifically, fusion was fast and extensive in the presence of N-oleoyl-SPM or androstenol, but these lipids were completely unable to support DIC formation in our LUV system. These results clearly indicate that fusion of SFV, which is critically dependent on the simultaneous presence of cholesterol and sphingolipid in the target membrane, does not require that these lipids are organized in rafts. Similar results were obtained for SIN, which also fused efficiently with liposomes containing N-oleoyl-SPM or androstenol (Fig. 6). This suggests that fusion of alphaviruses in general does not require the presence of lipid rafts in the target membrane.

The results of the SPM and Chol concentration dependence of SFV fusion (Fig. 5) provide further support for our conclusion that rafts are not required for the fusion process. Brain SPM, which efficiently promotes microdomain formation, and N-oleoyl-
Lack of correlation between raft formation and membrane fusion

SPM, which does not promote microdomain formation, were equally effective in supporting SFV fusion under suboptimal conditions in terms of SPM (panel A) or Chol (panel B) concentrations in the target liposomes. Furthermore, in the presence of an excess of Chol, fusion was quite efficient at very low concentrations of SPM, with both brain and \( N \)-oleoyl-SPM (see also Ref. 13). Although the presence of very small complexes of brain SPM and Chol cannot be excluded under these conditions, it is unlikely that raft-like domains are formed, simply because there is too little SPM present in a single liposome to produce even a single raft, assuming that the smallest raft has a radius of about 13 nm (22). The identical dependence of SFV fusion on the concentration of brain SPM or \( N \)-oleoyl-SPM in target liposomes strongly suggests that in the concentration range involved both lipids are homogeneously distributed in the liposomal membrane and have an equal capacity to support fusion of the virus.

While our present data clearly demonstrate that the presence of lipid rafts in the target membrane is not required for fusion of SFV or SIN, the results obtained with PC/PE/Chol/\( N \)-stearoyl-SPM LUVs (Fig. 4A) suggest that the presence of rafts may even be inhibitory. Fusion with these LUVs was slower and had an extent of about 42% (Fig. 4A), about 20% lower than the extent of fusion found for the other SPM species. Since the degree of DIC formation was highest for \( N \)-stearoyl-SPM, there may in fact be an inverse correlation between fusion and DIC formation. Indeed, when we incorporated a lower concentration of \( N \)-stearoyl SPM (5 mol%), so as to reduce the extent of DIC formation in the LUVs, the kinetics and extent of fusion became higher (results not shown). Likewise, DIC formation was very high with ergosterol, whereas the extent of SFV fusion with LUVs containing ergosterol was relatively low (Fig. 4B), again providing circumstantial support for an inverse correlation between fusion and microdomain formation.

**Biological Implications for the DIC-Independent Alphavirus Membrane Fusion Activity**

Whereas our present data indicate that the presence of lipid rafts in bilayer membranes is not essential for alphavirus fusion and may even be inhibitory, we cannot exclude the possibility that rafts are involved in the cell entry process of alphaviruses. Clearly, fusion of SFV was not completely blocked when SPM-Chol microdomains were present in the target liposomes. There is convincing evidence to indicate that lipid rafts are abundantly present in endosomal membranes (59-61). These rafts represent potential targets for alphavirus fusion, after receptor-mediated uptake of the virus particle via the clathrin-dependent endocytosis pathway (2, 7-9, 62, 63). On the other hand, it is possible that the virus may avoid entering into the raft domains within the endosomal membrane. It is likely that there will be “free” sphingolipid molecules, which are not associated with rafts, just as there are also non-raft Chol molecules (21, 42). The latter might serve as anchoring molecules for the virus. In this regard, it is important to note that SFV fusion requires only very low concentrations of sphingolipid in the target membrane (Fig. 5 and Ref. 13).

While this work was in progress, Ahn and coworkers reported (64) that E1 ectodomains (E1*) are associated with sterol-rich membrane domains in liposomes. These observations support the idea that SFV does utilize raft domains in the endosomal membrane as a target for fusion. On the other hand, Ahn et al. (64) found that androstanol did not mediate association of E1* with DICs, presumably because
androstanol is not able to promote DIC formation altogether (see Fig. 4B), while in earlier studies androstanol had been found to efficiently support SFV fusion (11). Taken together, these observations suggest that SFV E1 has the capacity to interact with rafts and that the virus may also be able to fuse with rafts. However, in agreement with our present observations, the data of Ahn et al. (64) support the notion that the fusion activity of SFV is not dependent on the presence of rafts in the target membrane.

Rafts may be used as a platform for cell entry of other viruses. For example, Simian virus 40 (SV40) binds to MHC class I molecules, and the virus-receptor complexes translocate to caveoalae (invagination sites on the cell surface enriched in lipid rafts). Agents that disrupt raft formation specifically block the entry of SV40 into cells (28, 29). Very recently, it was shown that the cell entry of Ebola virus is dependent on the integrity of lipid rafts, since raft-destabilizing agents significantly inhibit infection (65). Furthermore, HIV-1 is thought to enter its host cell via lipid rafts (30-34). Disruption of the rafts by methyl-β-cyclodextrin completely blocked transepithelial transcytosis of HIV-1 (34), and reduced HIV-1 envelope fusion (33), strongly suggesting that HIV-1 uses a cell entry pathway involving rafts. In future work, we plan to disrupt sphingolipid-Chol domains in target cells in order to establish whether or not rafts are involved in the infectious cell entry of SFV or SIN.

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2.7 References

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