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Membrane fusion of Semliki Forest virus requires sphingolipids in the target membrane

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Enveloped animal viruses, such as Semliki Forest virus (SFV), utilize a membrane fusion strategy to deposit their genome into the cytosol of the host cell. SFV enters cells through receptor-mediated endocytosis, fusion of the viral envelope occurring subsequently from within acidic endosomes. Fusion of SFV has been demonstrated before to be strictly dependent on the presence of cholesterol in the target membrane. Here, utilizing a variety of membrane fusion assays, including an on-line fluorescence assay involving pyrene-labeled virus, we demonstrate that low-pH-induced fusion of SFV with cholesterol-containing liposomal model membranes requires the presence of sphingomyelin or other sphingolipids in the target membrane. The minimal molecular characteristics essential for supporting SFV fusion are encompassed by a ceramide. The action of the sphingolipids is confined to the actual fusion event, cholesterol being necessary and sufficient for low-pH-dependent binding of the virus to target membranes. Complex formation of the sphingolipids with cholesterol is unlikely to be important for the induction of SFV–liposome fusion, as sphingolipids that do not interact appreciably with cholesterol, such as galactosylceramide, effectively support the process. The remarkably low levels of sphingomyelin required for half-maximal fusion (1–2 mole%) suggest that sphingolipids do not play a structural role in the SFV fusion process, but rather act as a cofactor, possibly activating the viral fusion protein in a specific manner.

Key words: cholesterol/membrane fusion/Semliki Forest virus/sphingolipids/sphingomyelin

Introduction

Membrane fusion is a key step in the infectious entry of enveloped viruses into their host cell (White, 1992; Bentz, 1993). For certain enveloped viruses, fusion occurs at the level of the host cell plasma membrane. Alternatively, fusion may occur from within the endosomal cell compartment, after uptake of intact virus particles through receptor-mediated endocytosis. In the latter process of fusion from within endosomes, the mildly acidic pH in the endosomal lumen is generally essential for activation of the fusion capacity of the virus involved.

Semliki Forest virus (SFV), a member of the alphavirus genus, represents one of the best documented examples of a virus penetrating cells through receptor-mediated endocytosis (Helenius *et al.*, 1980; Marsh *et al.*, 1983). Pioneering work of Helenius and co-workers has unravelled many characteristics of the membrane fusion and cellular entry functions of SFV, including the strict dependence of fusion activation on low pH (Helenius *et al.*, 1980; Marsh and Helenius, 1980; White *et al.*, 1980; White and Helenius, 1980; Marsh *et al.*, 1982, 1983; Kielian and Helenius, 1985). The SFV spikes, mediating the infectious host cell entry of the virus, each consist of three copies of the E2/E1 heterodimeric envelope glycoprotein. It is well established that the E1 component is solely responsible for the fusion activity of the virus (Garoff *et al.*, 1980; Omar and Koblet, 1988; Wahlberg and Garoff, 1992; Wahlberg *et al.*, 1992). Upon exposure to low pH, the E2/E1 heterodimer dissociates, and the E1 monomers rearrange to form a homotrimeric structure (Wahlberg and Garoff, 1992; Wahlberg *et al.*, 1992) that is likely to represent the fusion-active conformation of the viral spike (Wahlberg *et al.*, 1992; Bron *et al.*, 1993; Justman *et al.*, 1993).

Fusion of SFV in model systems is strictly dependent on the presence of cholesterol in the target membrane (White and Helenius, 1980; Kielian and Helenius, 1984; Bron *et al.*, 1993). The cholesterol dependence of SFV fusion has also been demonstrated in cell systems (Phalen and Kielian, 1991) while, recently, Kielian and co-workers reported that the budding of newly assembled SFV virions from the surface of infected cells requires cholesterol as well (Marquardt *et al.*, 1993).

In the present study, we demonstrate that low-pH-induced fusion of SFV in a liposomal model system is mediated specifically by remarkably low levels of sphingolipids in the target liposomes. Evidence is presented to indicate that cholesterol is primarily involved in low-pH-dependent binding of the virus to the liposomes, while the sphingolipid is required for the actual fusion event. This observation provides further insight into the lipid dependence of alphavirus membrane fusion, and is also relevant for a better understanding of the molecular mechanisms involved in the host cell entry and membrane fusion activity of other enveloped viruses.

Results

Sphingomyelin-dependent fusion of SFV with cholesterol-containing liposomes at low pH

In the course of our previous studies on SFV fusion in liposomal model systems (Wahlberg *et al.*, 1992; Bron *et al.*, 1993), we encountered a conspicuous complete lack

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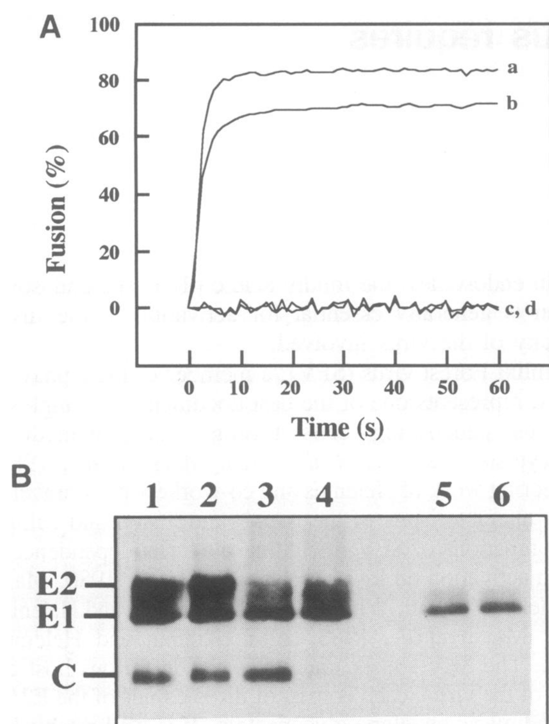

Fig. 1. Sphingomyelin-dependent fusion of SFV with cholesterol-containing liposomes at low pH. (**A**) Fluorescence recordings of the dilution of viral envelope pyrene-labeled lipids into target liposomes. On-line fusion measurements were performed at 37°C as described in Materials and methods. Fusion was recorded as a decrease in the pyrene excimer fluorescence. Curve a, PC/PE/SPM/cholesterol (molar ratio 1:1:0.35:1.5) liposomes, pH 5.55; curve b, PC/SPM/cholesterol (1:0.35:1.5) liposomes, pH 5.55; curve c, PC/PE/cholesterol (1:1:1.5) liposomes, pH 5.55; curve d, PC/PE/SPM/cholesterol (1:1:0.35:1.5) liposomes, pH 7.4. (**B**) Degradation of the capsid protein by liposome-encapsulated trypsin after release of the viral nucleocapsid into the liposomal lumen. The trypsin assay was carried out as described in Materials and methods. Lanes 1 and 2, incubation at pH 7.4 with PC/PE/cholesterol (1:1:1.5) and PC/PE/SPM/cholesterol (1:1:0.35:1.5) liposomes, respectively; lanes 3 and 4, incubation at pH 5.55 with PC/PE/cholesterol (1:1:1.5) and PC/PE/SPM/cholesterol (1:1:0.35:1.5) liposomes, respectively. In all cases, after the incubation, the samples were further incubated for 1 h at pH 8.0 and 37°C. Lanes 5 and 6 represent controls in which the virus was incubated at pH 5.55, in the absence of trypsin inhibitor, with PC/PE/cholesterol (1:1:1.5) or PC/PE/SPM/cholesterol (1:1:0.35:1.5) liposomes, followed by incubation for 1 h at pH 8.0 in the presence of Triton X-100 (0.5%, v/v).

of fusion when SFV was exposed to low pH in the presence of liposomes consisting of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and cholesterol. In an earlier study on fusion of SFV in a liposomal model system by White and Helenius (1980), such a lack of fusion with cholesterol-containing zwitterionic liposomes had not been observed. In the search for a possible explanation for this apparent discrepancy, we discovered that inclusion of bovine-brain sphingomyelin (SPM) in the liposomal membrane resulted in the restoration of fast and virtually complete fusion of the virus with the liposomes.

This observation is illustrated in Figure 1. Fusion of SFV, biosynthetically labeled in its envelope phospholipids with the fluorophore pyrene, was measured in the presence of target liposomes on the basis of dilution of labeled phospholipids from the viral into the liposomal membrane, resulting in a decrease in the pyrene excimer fluorescence. Panel A shows fusion curves obtained with pyrene-labeled SFV and PC/PE/cholesterol (molar ratio 1:1:1.5), PC/PE/SPM/cholesterol (1:1:0.35:1.5) or PC/SPM/cholesterol (1:0.35:1.5) liposomes as target membrane vesicles, at pH 5.55. No decrease in SFV pyrene excimer fluorescence was observed in the absence of SPM in the liposomal membrane (curve c), whereas in the presence of SPM fusion was fast and extensive (curves a and b). PE in the liposomes was not required for fusion to occur, although fusion with liposomes containing PE was slightly faster and more extensive than fusion with PC/SPM/cholesterol liposomes (curve a versus curve b). PC was not required either, as fusion with PE/SPM/cholesterol liposomes was fast and extensive (results not shown). Fusion did exhibit the well-documented requirement for cholesterol: no decrease in pyrene excimer fluorescence was observed with PC/PE/SPM (1:1:0.35) liposomes (results not shown). Finally, as expected, fusion with the SPM-containing liposomes required low pH: at neutral pH there was no decrease in the pyrene excimer fluorescence (Figure 1A, curve d).

In order to ensure that the change in pyrene excimer fluorescence reflected fusion, as opposed to other modes of lipid mixing, we determined whether during the process the viral core was released into the aqueous interior of the liposomes, utilizing an assay described before (White and Helenius, 1980; White *et al.*, 1982). Trypsin was encapsulated in the liposomes, while a trace amount of [^{35}S]methionine-labeled SFV was mixed with the fluorescently labeled SFV. Fusion was assessed on the basis of degradation of the internal viral capsid protein in the presence of trypsin inhibitor in the external medium. In agreement with the fluorescence fusion data, degradation of the capsid protein was observed only with liposomes containing both cholesterol and SPM at low pH (Figure 1B, lane 4). In the controls (lanes 5 and 6), involving solubilization in Triton X-100 of virus–liposome mixtures after low-pH treatment in the absence of trypsin inhibitor, the residual band represents the E1 protein which has become trypsin-resistant due to the exposure to acid pH (Kielian and Helenius, 1985; Wahlberg and Garoff, 1992; Wahlberg *et al.*, 1992; Bron *et al.*, 1993).

Taken together, the results in Figure 1 demonstrate that the presence of cholesterol in target liposomes, although required, is not sufficient for the induction of fusion of SFV at low pH, and that SPM has the capacity to mediate rapid and extensive fusion of the virus with cholesterol-containing liposomes.

Fusion of SFV with liposomes: SPM concentration dependence

In order to assess the concentration dependence of the induction of SFV–liposome fusion by SPM, we measured the rate and extent of fusion of the virus with PC/PE/cholesterol (1:1:1.5) liposomes containing different amounts of bovine-brain SPM. Figure 2 shows that a fusion response of SFV was elicited at very low concentrations of SPM in the liposomal membrane. Panel A presents fusion curves obtained with liposomes containing increasing concentrations of SPM. Panel B presents initial rates of fusion and fusion extents after 5 min as a function of the SPM concentration in the liposomes. Even at the lowest concentration tested (0.3 mole%), SPM clearly mediated

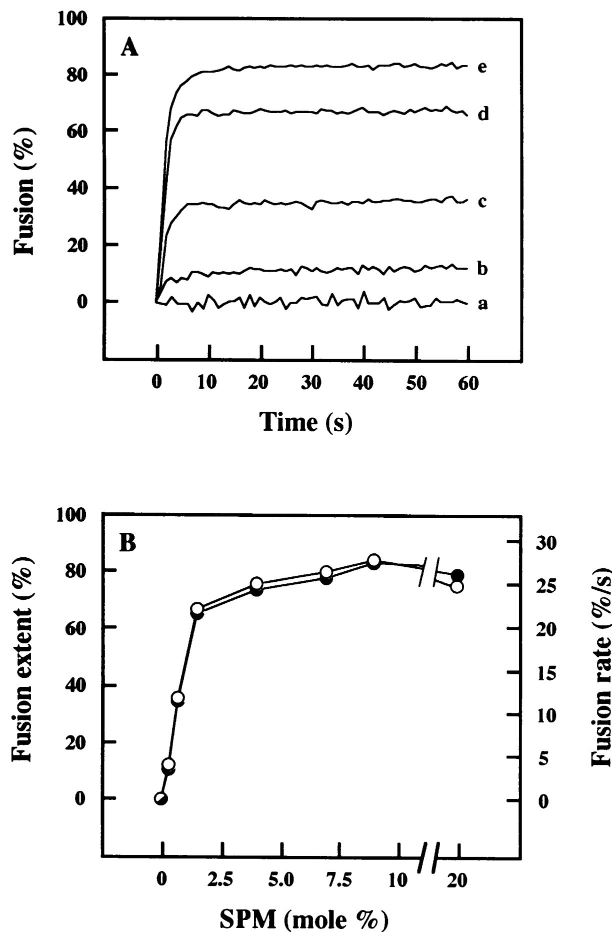

Fig. 2. Concentration dependence of the effect of SPM on fusion of SFV with cholesterol-containing liposomes. Liposomes were prepared of a PC/PE/cholesterol (1:1:1.5) mixture, supplemented with increasing concentrations of bovine-brain SPM. Fusion of the liposomes with pyrene-labeled SFV was measured at 37°C, as described in the legend to Figure 1A. In (A), curves a, b, c, d and e represent 0, 0.3, 0.7, 1.5 and 9 mole% SPM, respectively. In (B), the open symbols represent the extents of fusion after 5 min, and the closed symbols the initial rates of fusion determined from the tangents to the steepest initial part of the fusion curves.

Table I. SFV–liposome fusion mediated by different sphingolipids

Lipid	Initial rate of fusion (%/s)	Extent of fusion after 5 min (%)
Brain SPM	28	84
Egg-yolk SPM	26	77
Brain cerebrosides	25	64
Brain ceramide	23	70
Sphingosine	0	0
Control (no sphingolipid)	0	0

Liposomes were made of a PC/PE/cholesterol (1:1:1.5) mixture, supplemented with the indicated sphingolipids at 10 mole% concentrations. Fusion with pyrene-labeled virus was monitored, as described in the legends to Figures 1A and 2. Initial rates were determined from the tangents to the steepest initial part of the fusion curves.

significant fusion of the virus with the liposomes, while half-maximal fusion was reached with liposomes containing < 1 mole% of SPM.

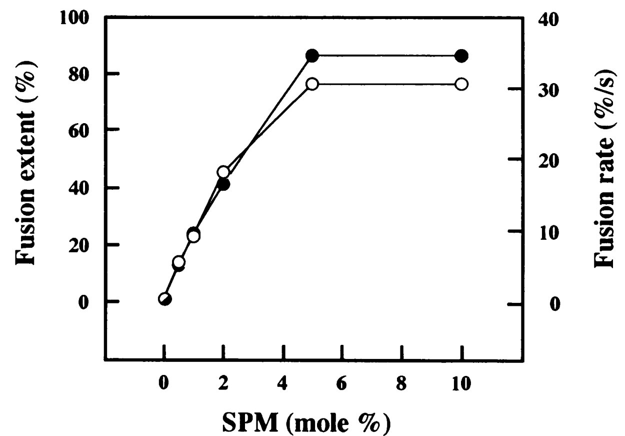

Fig. 3. Concentration dependence of the effect of C_{18}-SPM on fusion of SFV with cholesterol-containing liposomes. Liposomes were prepared of a PC/PE/cholesterol (1:1:1.5) mixture, supplemented with C_{18}-SPM at the indicated concentrations. Fusion of the liposomes with pyrene-labeled SFV was measured as described in the legends to Figures 1A and 2.

Effects of the fatty-acyl composition and the polar head group of SPM: ceramide as the sphingolipid minimally required for SFV fusion

The results in Table I delineate the minimum molecular requirements for the induction of fusion of SFV with cholesterol-containing liposomes at low pH. Several SPMs or SPM analogs were investigated for their capacity to mediate the process. SPM from either egg or bovine brain supported SFV–liposome fusion to similar extents. Egg and brain SPM differ in their fatty-acyl composition, the former containing mostly palmitic acid (16:0), and the latter stearic acid (18:0) as well as small amounts of the longer chain behenic (22:0), lignoceric (24:0) and nervonic (24:1) acids. Also, the semisynthetic *N*-stearoyl-SPM (C_{18}-SPM) fully supported fusion of SFV. Figure 3 shows the initial rates and extents of SFV fusion with PC/PE/cholesterol liposomes containing increasing concentrations of C_{18}-SPM. Like bovine-brain SPM, C_{18}-SPM mediated SFV fusion at remarkably low concentrations. Half-maximal fusion, in terms of both initial rate and fusion extent, was reached at ~2 mole% C_{18}-SPM. The initial rates of fusion observed at higher levels of C_{18}-SPM were slightly higher than the corresponding rates seen with liposomes containing brain SPM. These results indicate that the nature of the fatty-acyl chain in SPM is not crucial for the capacity of SPM to support fusion of SFV. We do note, however, that with brain SPM half-maximal fusion was achieved at a ~2-fold lower concentration in the liposomal membrane (Figure 2B) than with C_{18}-SPM (Figure 3).

The phosphorylcholine moiety, the polar head group that SPM shares with PC, appeared not to be essential for SPM to mediate fusion of SFV, as bovine-brain cerebrosides and ceramide could substitute for SPM in the process (Table I). On the other hand, the single-chain sphingosine was ineffective. It thus appears that a double-chain sphingosine-based lipid molecule, represented by a ceramide, constitutes the minimal element required for mediating the low-pH-induced fusion of SFV with cholesterol-containing liposomes.

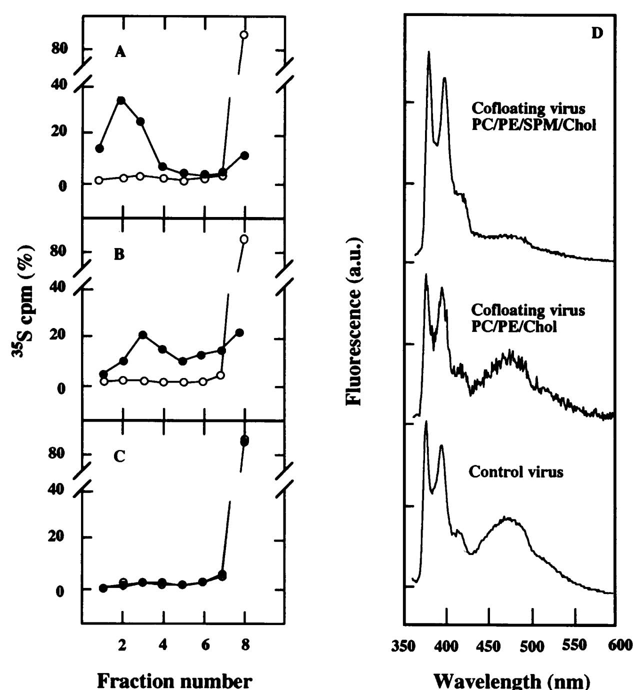

Fig. 4. Differential effects of cholesterol and SPM on low-pH-dependent binding and fusion of SFV to liposomes. SFV (a mixture of ^{35}S- and pyrene-labeled virus, 2.5 μM lipid) and liposomes (1 mM lipid) were incubated at pH 5.85 and 20°C, essentially as described before (Bron *et al.*, 1993). Virus–liposome interaction was initiated by acidification of the mixture to pH 5.85 through the addition of a pre-titrated volume of 0.3 M MES (pH 5.2). After 1 min, the pH was taken back to 8.4 by the addition of a pre-titrated volume of 1 M NaOH. Attachment of the virus to the liposomes was assessed by co-flotation analysis on sucrose gradients, as described in Materials and methods. (**A**) PC/PE/SPM/cholesterol (1:1:0.35:1.5) liposomes; (**B**) PC/PE/cholesterol (1:1:1.5) liposomes; (C) PC/PE/SPM (1:1:0.35) liposomes. In all cases, the closed circles represent samples treated at pH 5.85 for 1 min and the open circles samples incubated at pH 7.4. Fraction 1 is the top of the gradient. (**D**) presents pyrene fluorescence emission spectra of SFV, cofloating with the liposomes. Fractions 1–4 from the gradients were pooled and spectra were taken from 350 to 600 nm, with excitation at 343 nm. Top spectrum, virus cofloating with PC/PE/SPM/cholesterol (1:1:0.35:1.5) liposomes (A); middle spectrum, virus cofloating with PC/PE/cholesterol (1:1:1.5) liposomes (B); bottom spectrum, untreated control virus.

Cholesterol is required for low-pH-dependent binding of SFV to liposomes, sphingolipids mediate the actual fusion event

Fusion of SFV with liposomes at low pH consists of at least two distinct steps: the initial binding of the virus to the liposomes and the subsequent fusion of the viral envelope with the liposomal membrane. Figure 4 demonstrates that sphingolipids exert their effect specifically at the level of the fusion step, cholesterol being critically involved in the binding preceding the actual fusion event.

A mixture of fluorescently and radioactively labeled virus was incubated with liposomes of various compositions at pH 5.85 and 20°C. This condition had been found to be optimal for the determination of pH-dependent virus–liposome binding (Bron *et al.*, 1993). Subsequently, the liposome-associated virus was separated from non-bound virus by flotation on a sucrose density gradient (Kielian and Helenius, 1984; Wahlberg *et al.*, 1992; Bron *et al.*, 1993). The virus bound not only to PC/PE/SPM/cholesterol liposomes (Figure 4A), but also to PC/PE/cholesterol liposomes (Figure 4B), albeit to a somewhat lesser extent (83% versus 53%). Virtually no binding was observed with PC/PE/SPM liposomes (Figure 4C). Binding was strictly dependent on low pH: at neutral pH binding to the liposomes, irrespective of their lipid composition, was negligible (open circles in 4A–C).

Upon analysis of the fluorescence profile of the co-floating virus, it appeared that only the virus bound to the PC/PE/SPM/cholesterol liposomes had actually fused, since in this fraction the pyrene excimer fluorescence at 477 nm had largely disappeared (Figure 4D, top spectrum). The virus associated with the PC/PE/cholesterol liposomes exhibited a relative excimer fluorescence intensity (Figure 4D, middle spectrum), identical to that of the original virus preparation (Figure 4D, bottom spectrum), corresponding to a complete lack of mixing of the viral and liposomal lipids. These results indicate that cholesterol is essential for low-pH-dependent binding of the virus to target liposomes, the sphingolipid being required for the fusion process *per se*.

Is SPM involved in the initial SFV–liposome binding?

The above results are highly suggestive of divergent roles of cholesterol and sphingolipids in the overall process of SFV–liposome fusion, cholesterol being required for low-pH-dependent binding of SFV to the liposomes and sphingolipids for the actual fusion process. Yet, it cannot be excluded that the sphingolipids play a role in the binding process as well, while, likewise, cholesterol may be involved in the actual fusion reaction.

Therefore, using flotation analyses as in Figure 4, we further investigated whether SPM has the capacity to mediate low-pH-dependent binding of SFV to liposomes lacking cholesterol. However, in agreement with and extending the results in Figure 4C, binding of the virus to liposomes in the absence of cholesterol was marginal even at SPM concentrations in PC/PE (1:1) liposomes of up to 50 mole% (results not shown). This strongly suggests that under the conditions employed, SPM, in the absence of cholesterol, lacks the capacity to mediate significant binding of SFV to liposomes at low pH.

Then, in order to establish whether SPM might facilitate cholesterol-dependent binding of SFV to liposomes, we determined the binding of the virus to liposomes, with and without SPM, as a function of the cholesterol concentration in the liposomal membrane. The results are shown in Figure 5. Again, irrespective of whether SPM was present in the liposomes, very little binding occurred in the absence of cholesterol. With increasing concentrations of cholesterol, binding increased in a sigmoidal fashion. For both PC/PE/SPM and PC/PE liposomes, binding was almost complete at 50 mole% cholesterol, again underlining the notion that SPM is not essential for binding. However, at intermediate cholesterol concentrations, binding to SPM-containing liposomes was higher than binding to PC/PE liposomes, indicative of a stimulatory role of SPM on the cholesterol-dependent binding of the virus to the liposomes.

It is difficult to distinguish between an effect of SPM on the cholesterol-dependent binding *per se* and a possible indirect effect due to the additional occurrence of fusion in the case of the SPM-containing liposomes. As shown in Figure 5, with the PC/PE/SPM/cholesterol target liposomes, binding of the virus in each case was paralleled by an almost

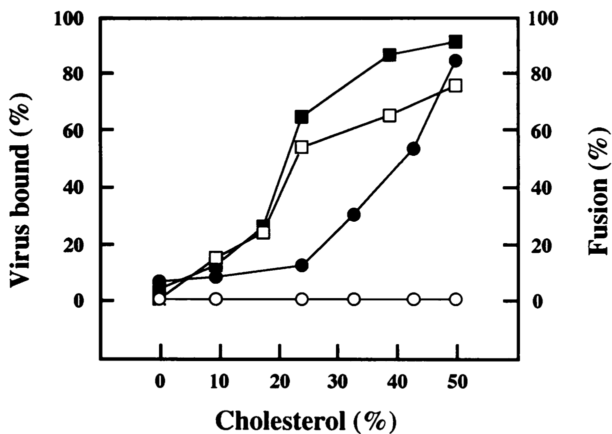

Fig. 5. Binding and fusion of SFV to PC/PE/cholesterol and PC/PE/SPM/cholesterol liposomes as a function of the cholesterol mole fraction. Liposomes were made of PC/PE (1:1) or PC/PE/SPM (1:1:0.35) mixtures, each supplemented with the indicated amounts (mole%) of cholesterol. Binding of radioactively labeled SFV to these liposomes was assessed as described in the legend to Figure 4, fusion of pyrene-labeled SFV was measured as in Figures 1A and 2. Closed symbols, binding; open symbols, extent of fusion after 5 min; squares, SPM-containing liposomes; circles, liposomes without SPM.

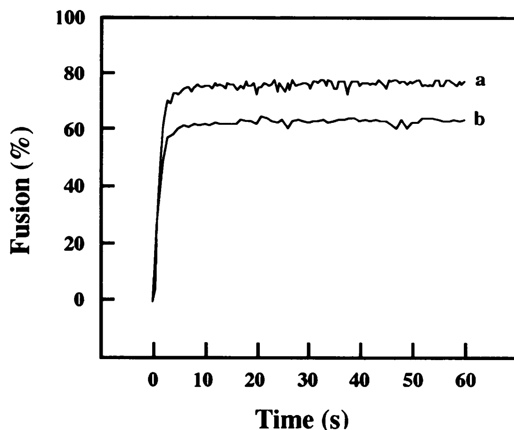

Fig. 6. Fusion of SFV with liposomes containing galactosyl-ceramide. Liposomes were prepared of a PC/PE/cholesterol (1:1:1.5) mixture, supplemented with 10 mole% bovine-brain SPM (curve a) or Gal-Cer (curve b). Fusion was measured as in Figures 1A and 2.

equal extent of fusion, implying that the virus that was found associated with these liposomes had in fact almost completely fused. On the other hand, with the PC/PE/cholesterol liposomes, no fusion occurred (Figure 5), implying that in this case the binding values represent binding *per se*. It is not unlikely that, due to the very occurrence of fusion in the presence of SPM, SFV−liposome binding, which may initially be reversible to some extent, becomes irreversible and, thus, appears more extensive than in the absence of fusion.

Is cholesterol required for the actual SFV fusion process?

Finally, we investigated whether cholesterol, besides being involved in the low-pH-dependent binding of SFV to liposomes, plays an essential role in the actual fusion process as well. Clearly, cholesterol lacks the capacity to mediate the fusion process *per se* in the absence of sphingolipids (Figures 1−5). However, this does not exclude the possibility that sphingolipids can only mediate the actual fusion process in obligatory conjunction with cholesterol. It is well established that sphingolipids readily engage in complex formation with cholesterol (Fugler *et al.*, 1985; Lund-Katz *et al.*, 1988; Grönberg and Slotte, 1990; Needham and Nunn, 1990; McIntosh *et al.*, 1992; Slotte, 1992). Such complexes might constitute specific targets for the actual SFV fusion reaction.

Therefore, we compared two sphingolipids, with diverging capacities to form a specific complex with cholesterol, for their ability to support SFV−liposome fusion. Studies on lipid monolayers at the air−water interface have provided evidence for the formation of a high-affinity complex between SPM and cholesterol (Lund-Katz *et al.*, 1988; Grönberg and Slotte, 1990) with a 1:2 stoichiometry (Slotte, 1992), whereas galactosyl-ceramide has been shown not to interact appreciably with cholesterol in mixed monolayers (Slotte *et al.*, 1993). The results in Figure 6 show that the fusion-supporting capacities of these sphingolipids are virtually identical. This is strongly suggestive of the notion that the initiation of low-pH-dependent SFV−liposome fusion by sphingolipids, after binding of the virus to cholesterol-containing vesicles, is independent of the presence of cholesterol.

Discussion

Fusion of SFV requires sphingolipids in the target membrane

The most important outcome of this study is that low-pH-induced fusion of SFV in a model system requires the presence of sphingolipids in the target membrane (Figures 1−5). While our present results fully confirm earlier results on the critical role of cholesterol in the expression of the membrane fusion activity of SFV (White and Helenius, 1980; Kielian and Helenius, 1984; Phalen and Kielian, 1991; Wahlberg *et al.*, 1992; Bron *et al.*, 1993), it appears that cholesterol is involved primarily in the steps leading up to the actual fusion event. Cholesterol clearly promotes low-pH-dependent attachment of SFV to target liposomes (Figures 4 and 5). However, it does not suffice for completion of the fusion event, since with PC/PE/cholesterol liposomes the process is aborted at a stage just prior to membrane merging (Figures 4 and 5). The fusion event *per se* is critically dependent on the presence of sphingolipid in the target liposomes, the minimal requirement being encompassed by a ceramide (Table I).

It is remarkable that the sphingolipid dependence of SFV fusion had not been noted in the early pioneering study by White and Helenius (1980) on SFV fusion in a liposomal model system. A possible explanation may be found in the remarkable concentration dependence of the sphingolipid effect on SFV fusion. Half-maximal fusion was observed at SPM concentrations of 1−2 mole% in the target liposome bilayer (Figures 2 and 3). At the time the early studies were carried out, highly purified natural phospholipids were not readily available. Indeed, we have detected small amounts of SPM in old PC preparations similar to the PC used by White and Helenius (1980) (results not shown), which could explain why the critical role of SPM in mediating the fusion of SFV with liposomes has gone unnoticed at the time.

Different roles for cholesterol and sphingolipids in the overall SFV–liposome fusion process

As noted above, cholesterol and sphingolipids appear to play divergent roles in the process of low-pH-induced SFV–liposome interaction and fusion, the presence of cholesterol in the liposomal bilayer being necessary and sufficient for binding of the virus to the liposomes, and the sphingolipids being essential for induction of membrane merging (Figures 4 and 5). Yet, it is interesting to consider the possibility that cholesterol and sphingolipids act in concert, at the level of either the initial binding or the actual fusion event, or both.

It is well established that SPM interacts tightly with cholesterol. The evidence includes (i) a comparatively strong condensation of SPM monolayers by cholesterol (Lund-Katz *et al.*, 1988; Grönberg and Slotte, 1990; Slotte, 1992), resulting in protection of the 3β-OH group of the sterol from the action of cholesterol oxidase (Grönberg and Slotte, 1990; Slotte, 1992); (ii) a slower rate of cholesterol desorption from SPM bilayers than from other phospholipid bilayers (Fugler *et al.*, 1985; Lund-Katz *et al.*, 1988); and (iii) a higher compressibility of SPM/cholesterol bilayers as compared to PC/cholesterol bilayers (Needham and Nunn, 1990; McIntosh *et al.*, 1992). The cohesion between SPM and cholesterol is governed primarily by Van der Waals interactions between the largely saturated acyl chains of the SPM and the steroid nucleus (McIntosh *et al.*, 1992), while specific hydrogen bond formation does not appear to be involved (Grönberg *et al.*, 1991; McIntosh *et al.*, 1992). The tight interaction between SPM and cholesterol has been suggested to result in the formation of microdomains enriched in SPM and cholesterol (Van Blitterswijk *et al.*, 1987; Slotte, 1992), although direct evidence for the existence of such domains in model systems or cell membranes is lacking. Sphingolipid/cholesterol microdomain formation has been proposed as a basis for a variety of cellular sorting events (Simons and Van Meer, 1988; Brown, 1992; Brown and Rose, 1992).

The sigmoidal dependence of SFV–liposome binding on the cholesterol concentration in the liposomal bilayer (Figure 5) suggests a certain degree of co-operativity between the sterol molecules in the binding process. This may be a reflection of an involvement of cholesterol-enriched microdomains within the liposomal membrane. Accordingly, the shift in the binding curve in the case of the SPM-containing liposomes toward lower cholesterol concentrations (Figure 5) may be due to a more pronounced tendency of lipid bilayers containing SPM, as opposed to membranes containing other phospholipids, to form cholesterol-enriched microdomains. On the other hand, it is equally possible that the relatively high extent of SFV binding to SPM-containing liposomes (Figure 5) is simply due to the very fact that, in this case, not only binding but also fusion occurs, whereas with liposomes lacking SPM the interaction is limited to binding *per se*. Fusion effectively renders the interaction irreversible, which may account for the apparent stimulation of the binding by SPM. In conclusion, therefore, it appears that while sphingolipids and, thus, sphingolipid/cholesterol microdomain formation are not required for SFV–liposome binding, the results in Figure 5 leave the question unanswered as to whether such microdomains in the target membrane would facilitate the binding process.

A similar conclusion can be drawn with respect to the possible role of sphingolipid/cholesterol microdomains in the actual fusion event. Clearly, the observation that both Gal-Cer and SPM effectively support SFV–liposome fusion (Figure 6), while Gal-Cer does not interact with cholesterol in mixed monolayers (Slotte *et al.*, 1993) and SPM interacts very strongly (Grönberg and Slotte, 1990; Slotte, 1992), essentially rules out the possibility that sphingolipid/cholesterol microdomain formation represents a structural requirement for SFV–liposome fusion. On the other hand, it is likely that if microdomains enriched in both cholesterol and sphingolipid exist in the target membrane for SFV, such domains would facilitate the fusion process. Indeed, when the virus binds to a cholesterol-rich membrane domain, the simultaneous enrichment of this domain with sphingolipid would provide for a favorable condition for activation of the viral membrane fusion activity by the sphingolipid. It is possible that the small difference between bovine-brain SPM (Figure 2) and C_{18}-SPM (Figure 3) with respect to the concentration dependence of their fusion-supporting capacities is a reflection of their abilities to engage in complex formation with cholesterol, the nature of the fatty-acyl chain of SPM being an important determinant for the affinity of SPM for cholesterol (Grönberg *et al.*, 1991; McIntosh *et al.*, 1992).

The lipid dependence of the SFV fusion process

From the point of view of structural preferences of lipids, the results in Figure 1 on the lipid dependence of SFV fusion are quite remarkable. The virus fuses avidly with membranes consisting of SPM, PC and cholesterol. Both SPM and PC are known for their tendency to adopt a lamellar organization in an aqueous environment (Cullis *et al.*, 1991). SPM, particularly in combination with cholesterol, forms very stable bilayer structures (Needham and Nunn, 1990; McIntosh *et al.*, 1992). Also, sphingolipids and cholesterol are major constituents of rigid lamellar structures with a pronounced barrier function, such as the myelin sheaths surrounding nerve axons (Hakomori, 1981) and the stratum corneum of the skin (Parrott and Turner, 1993). This particular property of sphingolipids is difficult to reconcile with a role in membrane fusion, as fusion involves bilayer destabilization and a local disruption of the lamellar organization of the lipids in the apposed membranes (Wilschut, 1991; Wilschut and Bron, 1993). Recently, Siegel (1993a,b) has proposed that membrane fusion processes, including viral fusion, may well proceed via a modified 'stalk' mechanism (Chernomordik *et al.*, 1987). The extent to which such a mechanism is energetically favorable strongly depends on the lipid composition of the apposed membranes, or, more specifically, on the intrinsic radius of curvature of the local lipid–water interfaces and on the tendency of the lipids contained in the fusion intermediate to undergo a lamellar-inverted phase transition. In this respect, it is remarkable that the bilayer-stabilizing SPM supports SFV fusion while, on the other hand, PE, which has the ability to adopt an inverted geometry, notably the hexagonal (H_{II}) configuration (Cullis *et al.*, 1991), is relatively inert (Figure 1A).

In view of these considerations, it would appear that the specific function of sphingolipids in the SFV fusion reaction is not at a structural level, stabilizing the fusion intermediate. It is more likely that sphingolipids play the role of a cofactor required for activation of the fusion function of the viral

envelope, possibly through induction of a specific conformational change in the viral fusion protein. The notion that sphingolipids play a cofactor role in SFV fusion is supported by the remarkably low concentrations of SPM that suffice to mediate the process (Figures 2 and 3). Currently, we are investigating the effects of cholesterol and sphingolipids on the low-pH-induced conformational changes in the SFV spike protein.

The fact that the fusion of SFV requires the presence of sphingolipids in the target membrane indicates that the virus has optimally adapted to the lipid composition of the leaflet of the cell target membrane it is facing. The external half of the plasma membrane, and likewise the lumenal half of the endosomal membrane, are rich in bilayer-stabilizing lipids such as PC and SPM (Cullis *et al.*, 1991; Devaux, 1991). Also, the SFV envelope itself is rich in SPM (Renkonen *et al.*, 1971; Allan and Quinn, 1989). Furthermore, although it had been reported that the majority of the SPM would be located on the inner leaflet of the SFV bilayer (Van Meer *et al.*, 1981), in a more recent study it was concluded that the SPM and PC in the SFV envelope are exposed at the outer surface (Allan and Quinn, 1989). Clearly, enveloped viruses have developed strategies to circumvent the relative refractoriness to fusion of the exoplasmic leaflet of cell membranes and their own envelopes. It is interesting that SFV even capitalizes on the very presence of SPM or other sphingolipids in the exoplasmic leaflet for activation of its fusion capacity.

Role of sphingolipids in other viral fusion events?

Finally, the involvement of sphingolipids and cholesterol may not be restricted to fusion of alphaviruses. A role for cholesterol in cellular entry has been suggested for a number of enveloped viruses other than SFV (Malvoisin and Wild, 1990; Cervin and Anderson, 1991), including HIV (Sarin *et al.*, 1985; Hansen *et al.*, 1990; Aloia *et al.*, 1988, 1993). Like that of SFV, the envelope of HIV is rich in SPM and cholesterol (Aloia *et al.*, 1988, 1993). Moreover, there is evidence to indicate that the membrane of HIV is enriched in both SPM and cholesterol relative to the plasma membrane of the cells the virus is budding from (Aloia *et al.*, 1993), although in the specific study involved surprisingly low SPM contents were found in the plasma membrane of the H9 cells used.

An involvement of sphingolipids in HIV fusion is also supported by the observations that galactosyl-ceramide (Bhat *et al.*, 1991; Harouse *et al.*, 1991; Fantini *et al.*, 1993) and a glycosylphosphatidylinositol-linked form of CD4 (Jasin *et al.*, 1991), which may be associated with SPM/cholesterol microdomains (Brown, 1992; Brown and Rose, 1992), mediate host cell entry of HIV.

Materials and methods

Lipids

PC from egg yolk, PE prepared by transphosphatidylation of egg PC, SPM from bovine brain or egg yolk, and cerebrosides (a mixture of galactosyl-ceramide and glucosyl-ceramide) were obtained from Avanti Polar Lipids Inc. (Birmingham, AL). Ceramide, galactosyl-ceramide (Gal-Cer), sphingosine and cholesterol were from Sigma Chemical Co. (St Louis, MO). The fluorescent fatty acid 16-(1-pyrenyl)hexadecanoic acid was purchased from Molecular Probes (Eugene, OR).

Virus

The membrane phospholipids of SFV were labeled biosynthetically with the fluorophore pyrene, as described before (Wahlberg *et al.*, 1992; Bron *et al.*, 1993). This labeling procedure relies on the production of virus from cells prior cultured in the presence of pyrene-labeled hexadecanoic acid. Since the pyrene-labeled fatty acid is readily incorporated in the cellular membrane lipids, a virus preparation is obtained that contains a significant amount of pyrene-labeled phospholipids in its envelope. Briefly, BHK-21 cells, prior cultured for 48 h on medium containing 10 μg/ml pyrene-labeled hexadecanoic acid, were infected with SFV at a multiplicity of infection of 10. After a further incubation for 24 h, labeled progeny virus was harvested and purified following standard methodology (Wahlberg *et al.*, 1992; Bron *et al.*, 1993). Labeling of SFV with the pyrene fluorophore according to this procedure does not affect the infectivity of the virus (Bron *et al.*, 1993).

[^{35}S]Methionine-labeled SFV was grown and purified as described previously (Wahlberg and Garoff, 1992).

Viral phospholipid was determined, after extraction of membrane lipids (Bligh and Dyer, 1959), by phosphate analysis (Böttcher *et al.*, 1961).

Liposomes

Large unilamellar vesicles (LUV) were prepared by a freeze–thaw extrusion procedure. Briefly, lipid mixtures were dried from a solution in chloroform/methanol (1:1) under a stream of nitrogen, and subsequently for 1 h at high vacuum. Lipids were hydrated in 150 mM NaCl, 0.1 mM EDTA, 5 mM HEPES, pH 7.4 (HNE) at 50°C, and subjected to 10 cycles of freeze–thawing (Mayer *et al.*, 1985). The vesicles were sized by extrusion (Hope *et al.*, 1985), at 50°C, through two stacked Unipore polycarbonate filters with a pore size of 0.2 μm (Nuclepore, Inc., Pleasanton, CA) in an Extruder (Lipex Biomembranes, Inc., Vancouver, BC, Canada).

The mean diameter of the vesicles was determined by quasi-elastic light-scattering analysis in a model 370 Particle Sizer (Nicomp Particle Sizing Systems, Santa Barbara, CA) and found to range between 165 and 170 nm for vesicles of different lipid compositions. The extrusion procedure gave high lipid recoveries (>80%) and did not detectably affect the lipid composition of the liposomes, as assessed by TLC analysis of lipid extracts from liposome samples taken before and after extrusion.

Trypsin-containing liposomes were also prepared by freeze–thaw extrusion in HNE in the presence of 10 mg/ml trypsin (Merck, Darmstadt, Germany). The vesicles were separated from unencapsulated trypsin by gel filtration (White and Helenius, 1980).

Lipid concentrations of liposome suspensions were determined by phosphate analysis (Böttcher *et al.*, 1961).

Fusion assays

Fusion of SFV was examined in a liposomal model system, involving pyrene-labeled virus (Wahlberg *et al.*, 1992; Bron *et al.*, 1993). Upon excitation at 343 nm, the pyrene probe forms excimers (excited dimers), which fluoresce at 480 nm, some 100 nm higher than the emission wavelength of excited monomers (Galla and Hartmann, 1980). Excimer formation in the labeled virus is proportional to the surface density of labeled phospholipids. Upon fusion of a pyrene-labeled virus particle with an unlabeled target membrane, the pyrene phospholipids dilute into the target membrane, resulting in a decrease in the pyrene excimer fluorescence intensity. This decrease was monitored on-line at 480 nm in an Aminco Bowman Series 2 fluorometer (SLM/Aminco, Urbana, IL). Unless indicated otherwise, virus (final concentration, 0.5 μM phospholipid) and liposomes (final concentration, 200 μM phospholipid) were mixed in the cuvette of the fluorometer in a final volume of 2.0 ml HNE at pH 7.4. The content of the cuvette was stirred magnetically and maintained at a temperature of 37°C. Fusion was initiated by injection of a pre-titrated volume of 0.3 M MES (pH 5.2) to achieve a final pH of 5.55. The fluorescence data were processed using the software supplied with the Aminco Bowman Series 2 fluorometer. The fusion scale was calibrated such that 0% fusion corresponded to the initial excimer fluorescence level and 100% fusion to the fluorescence level obtained after the addition of Triton X-100 to a final concentration of 0.5% (v/v).

Transfer of the viral nucleocapsid to the liposomal lumen upon fusion of the virus with the liposomes was assessed as degradation of the capsid protein by liposome-encapsulated trypsin (White and Helenius, 1980; White *et al.*, 1982; Kielian and Helenius, 1984) in the presence of soy-bean trypsin inhibitor in the external medium. The trypsin assay was carried out under conditions identical to those of the fluorescence experiments. Briefly, pyrene-labeled SFV was mixed with a trace amount of [^{35}S]methionine-labeled virus. Samples were incubated for 5 min at 37°C and pH 5.55, in the presence of 125 μg/ml soy-bean trypsin inhibitor (Sigma) in the medium, returned to pH 8.0 by the addition of a pre-titrated volume of 0.1 M NaOH,

and further incubated for 1 h at 37°C. The samples were precipitated with trichloroacetic acid (TCA) (5% w/v) and the proteins analyzed by SDS–PAGE and subsequent fluorography (Chamberlain, 1979).

Virus–liposome binding

Binding of virus to liposomes was assessed by flotation analysis on sucrose density gradients (Kielian and Helenius, 1984; Wahlberg *et al.*, 1992; Bron *et al.*, 1993). Mixtures of [^{35}S]methionine- and pyrene-labeled SFV with liposomes, pre-incubated at low pH and subsequently neutralized as indicated, were mixed with 150 μl 46% (w/v) sucrose to yield a final sucrose concentration of 44% (w/v). On top of this, sucrose solutions of 25% (w/v) (350 μl) and 5% (w/v) (200 μl) were layered. After centrifugation in a Beckman TLS55 rotor at 150 000 *g* for 2 h at 4°C, the gradients were fractionated from the top. The distribution of the [^{35}S]methionine label in the gradient was quantified by liquid scintillation counting. Where desired, fractions of co-floating virus were pooled as indicated, and pyrene fluorescence emission spectra were taken in the Aminco Bowman Series 2 fluorometer at an excitation wavelength of 343 nm.

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