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Sphingolipids Activate Membrane Fusion of Semliki Forest Virus in a Stereospecific Manner†

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ABSTRACT: The alphavirus Semliki Forest virus (SFV) enters cells through receptor-mediated endocytosis. Subsequently, triggered by the acid pH in endosomes, the viral envelope fuses with the endosomal membrane. Membrane fusion of SFV has been shown previously to be dependent on the presence of cholesterol in the target membrane. Recently, we have demonstrated that fusion of SFV also requires sphingolipids [Nieva, J. L., Bron, R., Corver, J., & Wilschut, J. (1994) *EMBO J. 13*, 2797−2804]. In the present paper, we show that the activation of low-pH-dependent fusion of SFV by sphingolipids is a stereospecific process. Pyrene-labeled SFV fused rapidly and extensively with liposomes consisting of a mixture of phosphatidylcholine, phosphatidylethanolamine, and cholesterol, supplemented with low concentrations of D-*erythro*-ceramide, representing the naturally occurring sphingolipid stereoisomer. Fusion was assessed by a decrease in the pyrene excimer fluorescence. L-*erythro*-, D-*threo*-, and L-*threo*-ceramide did not support fusion of the virus. Similar results were obtained with the corresponding sphingomyelin stereoisomers. The stereospecificity of SFV fusion activation was confirmed by using an assay based on degradation of the viral capsid protein by trypsin encapsulated in the target liposomes. Fusion mediated by D-*erythro*-ceramide was not affected by the additional presence in the target liposomes of ceramide stereoisomers incapable of fusion activation. Binding of the virus to the liposomes, as assessed by flotation on sucrose density gradients, was not dependent on the presence of fusion-competent or fusion-incompetent sphingolipids in the liposomes. The results of this study support the notion that a stereospecific interaction of the viral fusion protein with D-*erythro* sphingolipids in the target membrane represents an essential step in the activation of the fusion capacity of SFV.

The alphavirus Semliki Forest virus (SFV)[1] represents one of the best documented examples of a virus infecting cells through receptor-mediated endocytosis and subsequent fusion from within acidic endosomes (Marsh & Helenius, 1989;

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[1] Abbreviations: Cer, ceramide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SPM, sphingomyelin; Chol, cholesterol; SFV, Semliki Forest virus; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; NP40, Nonidet P40 [nonylphenylpoly(ethylene glycol)].

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White, 1990, 1992; Marsh & Pelchen-Matthews, 1993; Garoff *et al.*, 1994; Kielian, 1995). Many of the cellular entry and membrane fusion characteristics of SFV, including the strict dependence of fusion on low pH, have been elucidated (Helenius *et al.*, 1980; White *et al.*, 1980; White & Helenius, 1980; Marsh & Helenius, 1980; Marsh *et al.*, 1982, 1983; Kielian & Helenius, 1984, 1985). Cell entry of SFV is mediated by the E2/E1 heterodimeric envelope glycoprotein of the virus, the E1 subunit carrying the actual membrane fusion capacity (Garoff *et al.*, 1980; Omar & Koblet, 1988; Wahlberg & Garoff, 1992; Wahlberg *et al.*, 1992). At low pH the E2/E1 heterodimer dissociates, and E1 monomers rearrange to form a trypsin-resistant homotrimeric structure, which is recognized by a number of specific monoclonal antibodies (Wahlberg & Garoff, 1992; Wahlberg *et al.*,1992; Bron *et al.*, 1993; Justman *et al.*, 1993; Klimjack *et al.*, 1994). The formation of the E1 homotrimeric structure precedes the actual viral fusion reaction (Bron *et al.*, 1993), and therefore, the trimer or a larger assembly of several E1 trimers presumably represents the fusion-active conformation of the viral spike (Wahlberg *et al.*, 1992; Bron *et al.*, 1993; Justman *et al.*, 1993).

Fusion of SFV has been shown previously, in both model system and cells, to be dependent on cholesterol (White & Helenius, 1980; Kielian & Helenius, 1984; Phalen & Kielian, 1991; Wahlberg *et al.*, 1992; Bron *et al.*, 1993; Nieva *et al.*, 1994). Recently, we have observed that fusion requires the additional presence of sphingolipids in the target membrane (Nieva *et al.*, 1994; Wilschut *et al.*, 1995). Cholesterol appears to be necessary and sufficient for low-pH-dependent binding of the virus to liposomes, while the sphingolipid is essential for induction of the actual fusion event (Nieva *et al.*, 1994). Fusion of SFV requires only low concentrations of sphingolipid in the target liposomes (Nieva *et al.*, 1994). Furthermore, the action of the sphingolipid in the fusion process exhibits remarkable molecular specificity, both the 4,5-*trans* double bond and the 3-hydroxyl group of the sphingosine backbone being crucial for the sphingolipid to support fusion of SFV (Corver *et al.*, 1995; Wilschut *et al.*, 1995).

The critical role of the sphingolipid 3-hydroxyl group raises the question about whether the stereochemistry at C-3, and also at C-2, of the sphingosine backbone is an important parameter determining the capacity of sphingolipids to mediate fusion of SFV. In this study, using various ceramide and sphingomyelin stereoisomers, we demonstrate that low-pH-activated fusion of SFV with liposomal membranes is mediated exclusively by D-*erythro* sphingolipids.

MATERIALS AND METHODS

Lipids. Phosphatidylcholine (PC) from egg yolk, phosphatidylethanolamine (PE), prepared by transphosphatidylation of egg PC, and sphingomyelin (SPM) from bovine brain or egg yolk were obtained from Avanti Polar Lipids (Alabaster, AL). Cholesterol (Chol) was from Sigma Chemical Co. (St. Louis, MO). D-*erythro*-, L-*erythro*-, L-*threo*-, and D-*threo*-ceramide, all with a C_8 acyl chain, were synthesized as described previously (Karasavvas *et al.*, 1995). C_{18}-D-*erythro*-, C_8-D-*threo*-, and C_8-L-*erythro*-sphingomyelin were synthesized as described (Byun *et al.*, 1994). For structural formulas of the Cer and SPM stereoisomers used, see Figure 1.

Cells and Virus. Baby hamster kidney (BHK-21) cells were grown in Glasgow's modification of Eagle's minimal essential medium (Flow Laboratories, Irvine, Ayrshire, Scotland), supplemented with 10% tryptose phosphate broth, 5% fetal calf serum, and 2 mM glutamine.

SFV was purified from the medium of infected cells, as described before (Wahlberg *et al.*, 1992; Bron *et al.*, 1993; Stegmann *et al.*, 1993). Pyrene-labeled SFV was obtained from cells cultured beforehand in the presence of the fluorescent fatty acid 16-(1-pyrenyl)hexadecanoic acid (Molecular Probes, Eugene, OR). The fatty acid is biosynthetically incorporated in the cellular membrane lipids. Thus, virus particles budding from these cells have pyrene-labeled phospholipids in their envelopes (Wahlberg *et al.*, 1992; Bron *et al.*, 1993; Stegmann *et al.*, 1993; Nieva *et al*, 1994; Corver *et al.*, 1995). [^{35}S]Methionine-labeled SFV was grown and purified, as described before (Wahlberg & Garoff, 1992). Viral phospholipid was determined, after extraction of membrane lipids (Bligh & Dyer, 1959), by phosphate analysis (Böttcher, 1961).

Liposomes. Liposomes (large unilamellar vesicles) were prepared by a freeze−thaw/extrusion procedure, as described before (Bron *et al.*, 1993; Nieva *et al.*, 1994; Corver *et al.*, 1995). Briefly, lipid mixtures, dried from chloroform/methanol, were hydrated in HNE buffer (150 mM NaCl, 5 mM HEPES, 0.1 mM EDTA, pH 7.4) and subjected to five cycles of freeze−thawing (Mayer *et al.*, 1985). The vesicles were sized by extrusion (Hope *et al.*, 1985) through two stacked Unipore polycarbonate filters with a pore size of 0.2 μm (Nucleopore, Inc., Pleasanton, CA) in a high-pressure extruder (Lipex Biomembranes, Vancouver, BC, Canada). The liposomes consisted of a mixture of PC, PE, and Chol (molar ratio 1:1:1.5), supplemented with Cer or SPM as indicated. Liposomes containing trypsin were prepared by freeze−thaw/extrusion in HNE buffer, pH 7.4, in the presence of 10 mg/mL trypsin (Merck, Darmstadt, FRG). The trypsin-containing liposomes were separated from unencapsulated trypsin by gel filtration on Sephadex G-100 (Pharmacia, Uppsala, Sweden) in HNE buffer. Lipid concentrations of liposome suspensions were determined by phosphate analysis (Böttcher *et al.*, 1961).

Fusion Assays. Fusion of pyrene-labeled SFV with liposomes was detected as a *decrease* of the pyrene excimer fluorescence due to dilution of the pyrene-labeled lipids from the viral into the liposomal membrane (Wahlberg *et al.*, 1992; Bron *et al.*, 1993; Nieva *et al.*, 1994; Corver *et al.*, 1995; Wilschut *et al.*, 1995). On-line measurements were carried out in an Aminco Bowman Series 2 fluorometer (SLM/Aminco, Urbana, IL) at excitation and emission wavelengths of 343 and 480 nm, respectively. Virus (final concentration 0.5 μM phospholipid) and liposomes (final concentration 0.2 mM phospholipid) were mixed in the cuvette of the fluorometer in a final volume of 0.7 mL of HNE buffer, pH 7.4. The content of the cuvette was stirred and kept at a temperature of 37 °C. Fusion was initiated by injection of a small pretitrated volume of 0.1 M MES and 0.1 M acetic acid, pH 4.9, to achieve a final pH of 5.5. The fusion scale was calibrated such that 0% fusion corresponded to the initial excimer fluorescence level and 100% fusion to the fluorescence after addition of the detergent octa(ethylene glycol) *n*-dodecyl monoether (Fluka, Buchs, Switzerland) to a final concentration of 10 mM, resulting in infinite dilution of the fluorophore.

Transfer of the viral nucleocapsid into the liposomal lumen was assessed by the degradation of the viral capsid protein by liposome-encapsulated trypsin (White & Helenius, 1980; White *et al.*, 1982; Nieva *et al.*, 1994) in the presence of soybean trypsin inhibitor in the external medium. A mixture of a trace amount of [^{35}S]methionine-labeled virus, unlabeled virus (0.5 μM phospholipid), and trypsin-containing liposomes (0.2 mM phospholipid) in a total volume of 0.2 mL of HNE buffer containing 125 μg/mL soybean trypsin inhibitor (Sigma) was adjusted to pH 5.5 by addition of a small pretitrated volume of 0.1 M MES and 0.1 M acetic acid (pH 4.9) and incubated for 15 s at 37 °C. Then, the pH was adjusted to 8.0 by addition of a pretitrated volume of 0.1 M Tris (pH 10.7), and the incubation was continued for 15 min at 37 °C. NP40 (Fluka) was added to the samples to a concentration of 1% (w/v), and subsequently, proteins were precipitated with trichloroacetic acid (5% w/v). The protein samples were analyzed by SDS−PAGE and subsequent autoradiography. Prior to electrophoresis, the samples were incubated for 3 min at either 37 or 70 °C, as indicated. The intensities of the protein bands were estimated by densitometric analysis of the autoradiograms, using an UltroScan XL laser densitometer (Pharmacia).

Virus−Liposome Binding. Binding of virus to liposomes was assessed by flotation analysis on sucrose density gradients (Kielian & Helenius, 1984; Wahlberg *et al.*, 1992; Bron *et al.*, 1993; Nieva *et al.*, 1994; Wilschut *et al.*, 1995). A mixture of a trace amount of [^{35}S]methionine-labeled SFV, unlabeled SFV (0.5 μM phospholipid), and liposomes (1 mM lipid) in a total volume of 0.2 mL was adjusted to pH 5.5 with a pretitrated volume of 0.1 M MES and 0.1 M acetic acid (pH 4.9), incubated at pH 5.5 for 1 min at 37 °C, and subsequently adjusted to pH 8.0 with a pretitrated volume of 0.1 M Tris (pH 10.7). Alternatively, the mixtures were incubated at neutral pH, upon which a pretitrated volume of 0.1 M Tris (pH 10.7) was added to adjust the pH to 8.0. Samples of 50 μL were subjected to sucrose density gradient centrifugation, as described before (Wahlberg *et al.*, 1992; Bron *et al.*, 1993; Nieva *et al.*, 1994), the liposome-bound virus floating to the top of the gradient. The gradients were fractionated from the top and the radioactivity distributions determined by liquid scintillation counting.

RESULTS AND DISCUSSION

Only the D-*erythro Stereoisomers of Cer or SPM Mediate Fusion of SFV with Liposomes.* In a previous study, we have shown that sphingolipid-mediated fusion of SFV with liposomes critically depends on the 4,5-*trans* carbon−carbon double bond and the 3-hydroxyl group of the sphingosine backbone (Corver *et al.*, 1995; Wilschut *et al.*, 1995). Now, we examined the importance of the stereochemistry at the two asymmetric carbon atoms of the sphingosine backbone, using C_8-Cer stereoisomers (for structural formulas, see Figure 1). As demonstrated previously (Corver *et al.*, 1995), the length of the acyl chain, at least in the range from C_{18} to C_8, has no detectable effect on the capacity of Cer to mediate fusion of SFV.

Figure 2A shows that, at pH 5.5, pyrene-labeled SFV fused rapidly and extensively with PC/PE/Chol liposomes containing 10 mol % of D-*erythro*-Cer (curve a). Fusion was monitored on-line as a decrease of the pyrene excimer fluorescence due to dilution of pyrene-labeled phospholipid

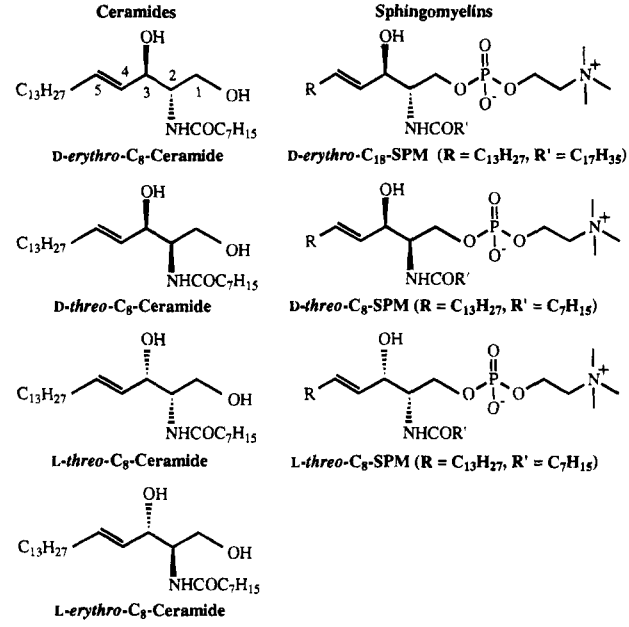

FIGURE 1: Structural formulas of the stereoisomers of Cer and SPM used.

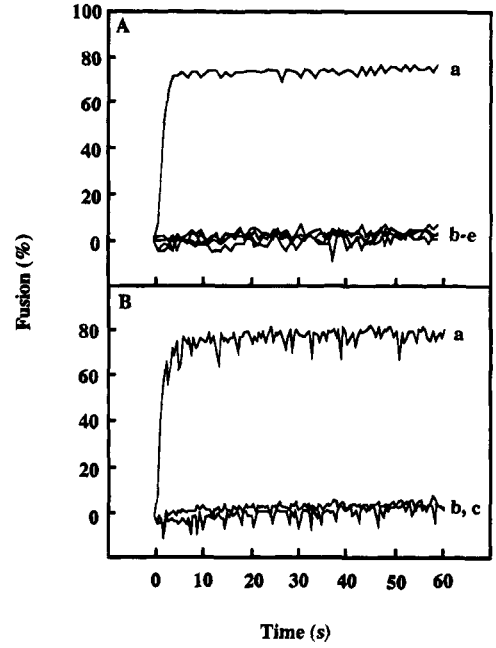

FIGURE 2: Effect of Cer and SPM chirality on fusion of pyrene-labeled SFV with PC/PE/Chol/Cer liposomes. On-line fusion measurements were performed at 37 °C and pH 5.5, as described in Materials and Methods. Fusion was recorded as a *decrease* of the pyrene excimer fluorescence. Liposomes consisted of PC/PE/Chol (molar ratio 1:1:1.5), supplemented with 10 mol % of Cer or SPM. Panel A: curve a, D-*erythro*-C_8-Cer; curve b, L-*erythro*-C_8-Cer; curve c, D-*threo*-C_8-Cer; curve d, L-*threo*-C_8-Cer; curve e, no Cer. Panel B: curve a, D-*erythro*-C_{18}-SPM; curve b, D-*threo*-C_8-SPM; curve c, L-*erythro*-C_8-SPM.

from the viral into the liposomal membrane. No fusion occurred at neutral pH (results not shown). At pH 5.5, maximal fusion (>70%) was reached within 10 s after acidification. Fusion required the Cer component in the target liposomes: no fusion was observed with liposomes consisting of PC/PE/Chol alone (Figure 2, curve e), in agreement with earlier observations (Nieva *et al.*, 1994; Wilschut *et al.*, 1995).

Figure 2A also shows that pyrene-labeled SFV did not fuse with PC/PE/Chol liposomes containing 10 mol % of L-*erythro*-, D-*threo*-, or L-*threo*-Cer (curves b−d, respectively). This indicates that only the naturally occurring D-*erythro* stereoisomer of Cer has the capacity to activate membrane fusion of SFV. The absolute stereospecificity of SFV fusion activation by Cer was confirmed in three independent other experiments, involving not only PC/PE/Chol liposomes containing 10 mol % of Cer but also liposomes containing 20 mol % of Cer (results not shown).

We investigated not only Cer but also SPM stereoisomers for their capacity to mediate fusion of SFV. Previously, we have demonstrated that natural SPMs (from either brain or egg) are potent activators of SFV fusion (Nieva *et al.*, 1994). Figure 2B (curve a) shows that pyrene-labeled SFV fused efficiently with PC/PE/Chol liposomes containing 10 mol % of C_{18}-D-*erythro*-SPM. On the other hand, no fusion was observed with liposomes containing either C_8-L-*erythro*-SPM (curve b) or C_8-D-*threo*-SPM (curve c). This lack of fusion is not due to the shorter length of the acyl chain of these SPM stereoisomers, since, as noted above, in the C_8−C_{18} range, the acyl chain length does not influence the capacity of sphingolipids to support fusion of SFV. Therefore, SFV fusion mediated by SPM exhibits the same stereospecificity as fusion mediated by Cer.

To confirm that the decrease in pyrene excimer fluorescence reflected fusion, as opposed to transfer of the pyrene label between the viral and liposomal membranes, we determined the release of the viral nucleocapsid into the lumen of Cer-containing liposomes, using an assay involving [^{35}S]methionine-labeled SFV and trypsin encapsulated in the liposomes (White & Helenius, 1980; White *et al.*, 1982; Nieva *et al.*, 1994). Fusion was assessed on the basis of degradation of the viral capsid protein after exposure of virus−liposome mixtures to pH 5.5 in the presence of soybean trypsin inhibitor in the medium. The results, shown in Figure 3, are in agreement with the results of the fluorescence fusion measurements in Figure 2A. Only after low-pH incubation of SFV with liposomes containing D-*erythro*-Cer was degradation of the viral capsid protein observed (Figure 3, lanes 4 and 8), whereas with liposomes containing either L-*threo*-, L-*erythro*-, or D-*threo*-Cer, the capsid protein remained intact (Figure 3, lanes 1−3 and 5−7, respectively). As a control, liposomes with L-*threo*-, L-*erythro*-, D-*threo*-, or D-*erythro*-Cer were also incubated with SFV at pH 7.4. As expected, with none of the liposomes was the capsid protein degraded (Figure 3, lanes 9−12, respectively), indicating that no fusion had occurred.

The results in Figure 3 (lanes 1−4) also demonstrate that, upon incubation of the SFV−liposome mixtures at low pH, the E1 envelope glycoprotein is partly converted to an NP40-resistant trimer with an apparent MW of about 150 000. The E1 trimer band appears in SDS−PAGE analysis provided that the samples are not heated prior to electrophoresis (Wahlberg *et al.*, 1992). Accordingly, when the samples were incubated for 3 min at 70 °C before separation, the trimer band was not detected (Figure 3, lanes 5−8). It is interesting to note that the observed extent of E1 homotrimer formation (E1 trimer relative to total E1) was independent of whether the liposomes contained the fusion-supporting or one of the fusion-inactive Cer stereoisomers. This indicates that the formation of the E1 homotrimer, the supposed fusion-active conformation of the viral spike

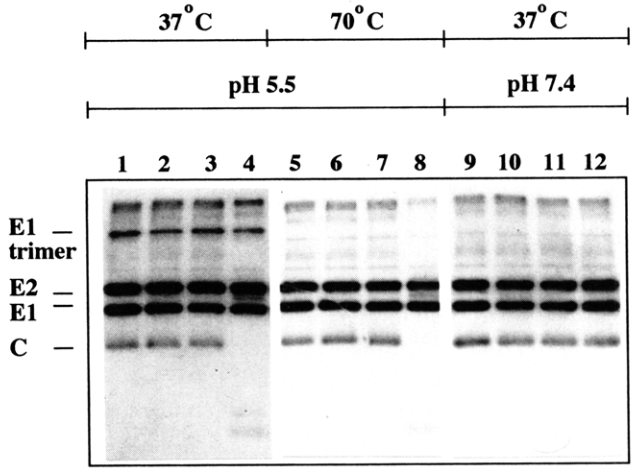

FIGURE 3: Effect of Cer chirality on fusion of SFV with trypsin-containing PC/PE/Chol/Cer liposomes, as assessed by degradation of the viral capsid protein the viral capsid protein C. Fusion of [^{35}S]methionine-labeled SFV and trypsin-containing liposomes at 37 °C, in the presence of trypsin inhibitor in the external medium, was determined as described in Materials and Methods. Lanes: 1−8, incubation at pH 5.5; 9−12, incubation at pH 7.4. Liposomes consisted of PC/PE/Chol (molar ratio 1:1:1.5), supplemented with 10 mol % of L-*threo*-C_8-Cer (lanes 1, 5, 9), L-*erythro*-C_8-Cer (lanes 2, 6, 10), D-*threo*-C_8-Cer (lanes 3, 7, 11), or D-*erythro*-C_8-Cer (lanes 4, 8, 12). Prior to electrophoresis, the protein samples were incubated for 3 min at either 37 °C (lanes 1−4 and 9−12) or 70 °C (lanes 5−8). The estimated extents of E1 homotrimer formation (intensity of the trimer band relative to the sum of the intensties of the E1 trimer and monomer bands) were 28% (lane 1), 25% (lane 2), 29% (lane 3), and 26% (lane 4).

(Wahlberg *et al.*, 1992; Bron *et al.*, 1993; Justman *et al.*, 1993), by itself does not require the presence of sphingolipids. Although the estimated extents of trimer formation in the experiment of Figure 3 were comparatively low, ranging from 25% to 29% [see also Bron *et al.* (1993)], the value in the case of lane 4 (26%) indicates that the fusion-supporting D-*erythro*-Cer does not significantly stimulate E1 trimer formation. The lack of effect of fusion-active sphingolipids in target liposomes on the extent of E1 trimer formation has been observed in several other independent experiments (results not shown). While the extent of trimer formation varied depending on the pH, temperature, and time of incubation [see also Bron *et al.* (1993)], at any one specific condition we have been unable to detect stimulation (or inhibition) of trimer formation by sphingolipid in the liposomes.

In summary, the above results demonstrate that the activation of SFV membrane fusion by sphingolipids is a strictly stereospecific phenomenon. Only D-*erythro* sphingolipids mediate rapid and extensive fusion of the virus.

Binding of SFV to Liposomes Is Not Inhibited by Cer Stereoisomers Which Do Not Support Fusion. The fusion of SFV and liposomes consists of at least two steps: (i) binding of the virus to the liposomes and (ii) merging of the viral envelope with the liposomal membrane. To examine the possibility that the lack of fusion of SFV with liposomes containing L-*threo*-, L-*erythro*-, or D-*threo*-Cer was due to inhibition of virus−liposome binding, as opposed to an effect on the fusion process *per se*, we determined the extent of virus−liposome binding in a direct manner. [^{35}S]Methionine-labeled SFV was incubated at pH 5.5 and 37 °C with PC/PE/Chol liposomes containing 10 mol % of one of the four Cer stereoisomers. Liposome-associated virus was

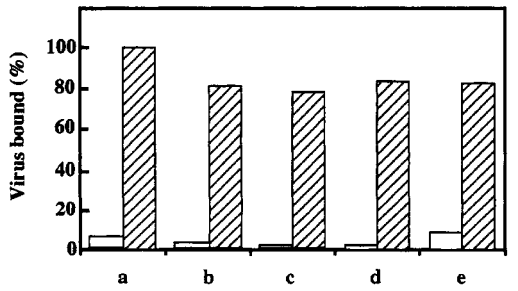

FIGURE 4: Binding of SFV to PC/PE/Chol/Cer liposomes. Binding of [^{35}S]methionine-labeled SFV to liposomes at pH 5.5 or pH 7.4 was determined by coflotation analysis on sucrose density gradients, as described in Materials and Methods. Liposomes consisted of PC/PE/Chol (molar ratio 1:1:1.5), supplemented with 10 mol % of Cer. Bars: a, D-*erythro*-C_8-Cer; b, L-*erythro*-C_8-Cer; c, D-*threo*-C_8-Cer; d, L-*threo*-C_8-Cer; e, no Cer. Open bars, pH 7.4; hatched bars, pH 5.5.

separated from nonbound virus by flotation on sucrose density gradients (Wahlberg *et al.*, 1992; Bron *et al.*, 1993; Nieva *et al.*, 1994).

Figure 4 (bars a–d) shows that, at low pH, the virus bound to the liposomes to similar high extents, irrespective of whether the liposomes contained D-*erythro*-, D-*threo*, L-*erythro*-, or L-*threo*-Cer. Consistent with earlier observations (Nieva *et al.*, 1994; Corver *et al.*, 1995; Wilschut *et al.*, 1995), the virus also bound extensively to PC/PE/Chol liposomes without sphingolipid (bar e). There was no binding at neutral pH (open bars). These results indicate that the lack of fusion of SFV with liposomes containing D-*threo*-, L-*erythro*-, or L-*threo*-Cer is not due to inhibition of virus–liposome binding. Rather, the fusion-inactive Cer stereoisomers appear to lack the capacity to activate fusion of the virus at a stage of the process after the initial binding.

L-erythro-, L-threo-, and D-threo-Cer Do Not Inhibit SFV Fusion Activation by D-erythro-Cer. In order to determine whether the fusion-inactive L-*erythro*, L-*threo*, or D-*threo* stereoisomers affect fusion induced by the fusion-active D-*erythro*-Cer in the target membrane, we determined the rates and extents of SFV fusion with liposomes containing increasing concentrations of D-*erythro*-Cer, both in the absence and presence of 10 mol % of L-*erythro*-Cer.

Figure 5 (open symbols) shows that low levels of D-*erythro*-Cer in the target liposomes sufficed for activation of SFV fusion, significant fusion already occurring at the lowest concentration tested (0.6 mol %) and half-maximal fusion requiring a concentration of approximately 3 mol % in the liposome membrane. Importantly, throughout the concentration range tested, the fusion-supporting capacity of D-*erythro*-Cer was not influenced by the presence of 10 mol % of L-*erythro*-Cer in the target membrane (Figure 5, closed symbols). The initial rate (panel A) and the final extent of fusion (panel B) were neither inhibited nor stimulated by L-*erythro*-Cer. In similar independent experiments, we observed that the two other fusion-inactive Cer stereoisomers, L-*threo*- and D-*threo*-Cer, did not affect the fusion-supporting capacity of D-*erythro*-Cer either (results not shown).

In conclusion, D-*threo*-, L-*threo*-, and L-*erythro*-Cer are inert in the fusion process and do not affect the ability of D-*erythro*-Cer to support fusion of SFV. This lack of competitive inhibition argues strongly against a structural role of fusion-supporting sphingolipids in SFV fusion. Rather, it would appear that the fusion-inactive Cer stereoisomers simply lack the specificity required for interaction

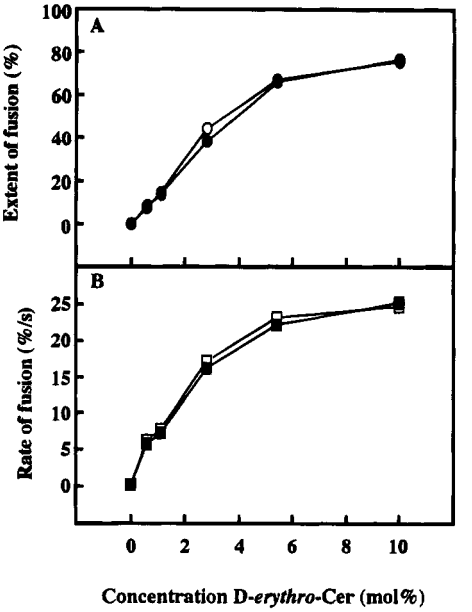

FIGURE 5: Lack of influence of L-*erythro*-Cer on fusion of SFV mediated by D-*erythro*-Cer. On-line fusion measurements were performed at 37 °C and pH 5.5, as in the experiment of Figure 1. Liposomes consisted of PC/PE/Chol (molar ratio 1:1:1.5) or PC/PE/L-*erythro*-C_8-Cer/Chol (molar ratio 1:0.65:0.35:1.5), supplemented with increasing concentrations of D-*erythro*-C_8-Cer as indicated. The extent of fusion (panel A) was determined 60 s after acidification (pH 5.5). The initial rate of fusion (panel B) was determined from the tangent to the first part of the curve. Closed symbols, liposomes with L-*erythro*-C_8-Cer; open symbols, liposomes without L-*erythro*-C_8-Cer.

with a component of the virus involved in fusion. This, in turn, provides support for the notion that D-*erythro* sphingolipids activate SFV fusion through direct binding to the E1 fusion protein.

CONCLUDING REMARKS

The results of this study clearly demonstrate that the activation of SFV fusion by sphingolipids is a stereospecific process. Only D-*erythro* sphingolipids, representing the naturally occurring sphingolipid stereoisomer, support fusion of SFV in a liposomal model system, while the other three stereoisomers are completely inert.

In a previous study (Corver *et al.*, 1995), we have shown that specific molecular features are required for sphingolipids to mediate fusion of SFV. These include the 3-hydroxyl group and the 4,5-*trans* carbon–carbon double bond of the sphingosine backbone. This molecular specificity, and the low concentrations of sphingolipids that suffice for fusion activation (Nieva *et al.*, 1994), lends support to the hypothesis that sphingolipids act as a molecular cofactor in the fusion process. Our present results provide further strong support for this notion. In terms of structural properties, the chirality of phospholipid molecules is not an important factor in determining lipid order of membranes or interactions with cholesterol (Ghosh *et al.*, 1971). Therefore, the absolute stereospecificity of SFV fusion activation by sphingolipids essentially eliminates the possibility of a nonspecific structural role of sphingolipids in the fusion process.

The D-*threo*, L-*threo*, and L-*erythro* sphingolipid stereoisomers not only lack the ability to support fusion of SFV but they also lack the ability to interfere with the SFV fusion activation by the D-*erythro* isomer. This complete absence

of competitive inhibition by the three fusion-incompetent stereoisomers provides strong independent support for the idea that D-*erythro* sphingolipids mediate the process through a specific interaction with a viral component involved in the fusion process, presumably the E1 fusion protein. If sphingolipids independent of their chirality had the capacity to interact with E1 (the D-*erythro* isomer being the only one capable of inducing, for example, a subsequent conformational change in the protein), then one would expect the fusion-incompetent stereoisomers to inhibit fusion activation by the D-*erythro* isomer. Since such an inhibition does not occur, the specificity of the sphingolipid action appears to be located at the level of the binding of the lipid to the viral fusion protein. Currently, attempts to obtain direct proof for stereospecific binding of sphingolipids to E1 are underway.

It remains to be established whether a low-pH interaction of sphingolipids with SFV or E1 induces a conformational change in the protein essential for expression of its membrane fusion activity. Previous studies have indicated that a low-pH-induced homotrimeric form of E1 may well represent the fusion-active structure of the viral spike (Wahlberg & Garoff, 1992; Wahlberg *et al.*, 1992; Bron *et al.*, 1993; Justman *et al.*, 1993; Klimjack *et al.*, 1994). Our results shown in Figure 3 do not provide any support for a possible critical role of D-*erythro* sphingolipids in the formation of the E1 homotrimer *per se*. By contrast, the extent of E1 trimerization was the same in the presence of D-*erythro*-Cer or any one of the other Cer stereoisomers or even in the absence of Cer altogether (results not shown). One possibility is that D-*erythro* sphingolipids interact with trimeric E1, inducing further conformational changes in the trimer structure that are critical for fusion. On the other hand, Klimjack *et al.* (1994), using a soluble truncated ectodomain of E1 (E1*), observed that oligomerization of the protein required membranes containing both cholesterol and sphingolipids. In future studies, it will be of interest to further investigate the abilities of cholesterol and sphingolipids to bind to and induce conformational changes in E1, both in intact virions and in model systems involving the isolated E1 ectodomain.

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