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Sphingolipid-Dependent Fusion of Semliki Forest Virus with Cholesterol-Containing Liposomes Requires both the 3-Hydroxyl Group and the Double Bond of the Sphingolipid Backbone

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Low-pH-induced membrane fusion of Semliki Forest virus (SFV) in a model system is mediated by sphingolipids in the target membrane; ceramide is the sphingolipid minimally required (J. L. Nieva, R. Bron, J. Corver, and J. Wilschut, EMBO J. 13:2797–2804, 1994). Here, using various ceramide analogs, we demonstrate that sphingolipid-dependent fusion of SFV with cholesterol-containing liposomes exhibits remarkable molecular specificity, the 3-hydroxyl group and the 4,5-*trans* carbon-carbon double bond of the sphingosine backbone being critical for the sphingolipid to mediate the process. This observation supports the notion that sphingolipids act as a cofactor in SFV fusion, interacting directly with the viral fusion protein to induce its ultimate fusion-active conformation.

The alphavirus Semliki Forest virus (SFV) enters its host cell through receptor-mediated endocytosis, routing the virus particles to the endosomal cell compartment. Subsequently, induced by the mildly acidic pH in the lumen of the endosomes, the viral envelope fuses with the endosomal membrane, through which the viral genome gains access to the cell cytosol (6, 9, 16, 18–20). This fusion process is mediated by the E1 subunit of the E2/E1 heterodimeric envelope glycoprotein of the virus. At low pH, this protein undergoes a sequence of conformational changes (2, 11, 14, 15, 29, 30), including dissociation of the heterodimer and eventual formation of a new oligomer consisting of three copies of the E1 subunit. It has been proposed that this E1 homotrimer represents the fusion-active configuration of the viral spike (2, 11, 29).

It is well established that cholesterol is required for fusion of SFV with model (2, 14, 30, 31) as well as cellular (25) membranes. Recently, we have shown that this cholesterol-dependent fusion process is mediated specifically by low concentrations of sphingolipids in the target membrane (24). While cholesterol sufficed for low-pH-dependent binding of SFV to target liposomes, sphingolipids were required for induction of lipid mixing and release of the viral nucleocapsid into the lumen of the liposomes. Ceramide was found to represent the sphingolipid minimally required for supporting the SFV fusion process (24).

In this study, we examined a number of ceramide analogs for their abilities to mediate fusion of SFV with cholesterol-containing liposomes. Fusion of SFV was assessed on the basis of dilution of pyrene-labeled phospholipids from the viral membrane into the liposomes, which results in a decrease of the pyrene excimer fluorescence at 480 nm (2, 24, 28, 29). This decrease was recorded continuously in an Aminco-Bowman Series 2 fluorometer (SLM/Aminco, Urbana, Ill.) at an excitation wavelength of 343 nm. Pyrene-labeled SFV was produced from baby hamster kidney cells (BHK-21), cultured beforehand in the presence of 1-pyrenehexadecanoic acid (Molecular Probes, Eugene, Oreg.), as described previously (2, 24, 29). Liposomes (large unilamellar vesicles) were prepared in 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid–150 mM NaCl–0.1 mM EDTA, pH 7.4 (HNE buffer), by subjection of lipid dispersions to 5 cycles of freezing and thawing (10) and subsequent extrusion (21) through Unipore polycarbonate filters (0.2-μm pore size; Nuclepore Inc., Pleasanton, Calif.) with a high-pressure extruder (Lipex Biomembranes, Vancouver, Canada). Liposomes consisted of a mixture of phosphatidylcholine (PC) from egg yolk, phosphatidylethanolamine (PE) prepared by transphosphatidylation of egg PC, and cholesterol (Chol) in a molar ratio of 1:1:1.5, by itself or supplemented with 10 mol% ceramide or ceramide analog. PC and PE were from Avanti Polar Lipids (Alabaster, Ala.), and Chol was from Sigma Chemical Co. (St. Louis, Mo.). D-*erythro*-Ceramide, with either a saturated C_8- or a saturated C_{18}-*N*-acyl chain (C_8-Cer and C_{18}-Cer, respectively), was synthesized essentially as described elsewhere (27). C_{18}-(3-deoxy)-ceramide (C_{18}-3-deoxy-Cer) was synthesized in a scheme that started with the reaction of (*R*)-benzylglycidyl ether with lithium pentadecyne (1). C_8-(3-methoxy)-ceramide (C_8-3-OMe-Cer) was synthesized by a modification of a method described by Kan et al. (12). C_8-(4,5-dihydro)-DL-*erythro*-ceramide (C_8-dihydro-Cer) was synthesized as described elsewhere (13). The structural formulas of the ceramides and ceramide analogs used are shown in Fig. 1.

Figure 2 shows that pyrene-labeled SFV fused efficiently with PC-PE-Chol liposomes containing either C_{18}-Cer (Fig. 2A, curve a) or C_8-Cer (Fig. 2B and C, curves a) at pH 5.5 and 37°C. The fusion reaction reached a final extent of approximately 80%. In agreement with our previous observations (24), fusion required the ceramide component in the liposomes, as

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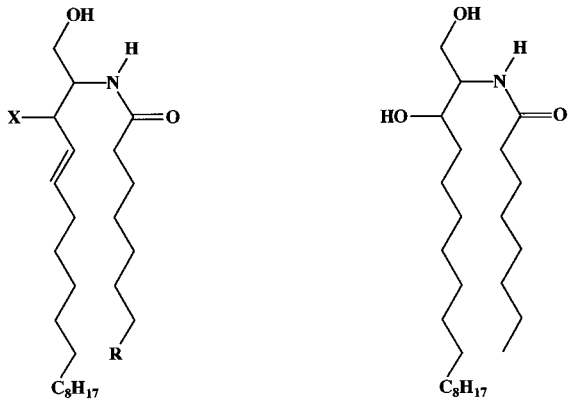

C_{18}-Cer: R = $C_{10}H_{21}$, X = OH

C_8-Cer: R = CH_3, X = OH

C_{18}-3-deoxy-Cer: R = $C_{10}H_{21}$, X = H

C_8-3-OMe-Cer: R = CH_3, X = OCH_3

C_8-dihydro-Cer

FIG. 1. Structural formulas of the ceramides and ceramide analogs used.

the virus did not fuse with liposomes consisting of the PC-PE-Chol mixture alone (results not shown). Since C_{18}-Cer and C_8-Cer were equally efficient, it can be concluded that the acyl chain length is not an important parameter determining the capacity of ceramides to mediate fusion of SFV.

In contrast to liposomes with C_{18}-Cer or C_8-Cer, liposomes containing the ceramide analogs C_{18}-3-deoxy-Cer (which lacks the 3-hydroxyl group at the sphingosine backbone) or C_8-3-OMe-Cer (in which the 3-hydroxyl group is methylated) did not support fusion of SFV (Fig. 2A and B, curves b). These results indicate that the 3-hydroxyl group at the sphingosine backbone plays a crucial role in sphingolipid-mediated fusion of SFV.

We also examined the importance of the 4,5-*trans* carbon-carbon double bond in the sphingosine backbone. Figure 2C (curve b) shows that the virus did not fuse with PC-PE-Chol liposomes containing C_8-dihydro-Cer (in which the double bond is replaced by a single bond). This indicates that, in addition to the 3-hydroxyl group, the double bond in the sphingosine backbone is also essential for ceramide to support fusion of SFV.

To ensure that not only the fusion-active but also the fusion-inactive ceramide analogs were properly incorporated in the liposomal membrane, we extracted the lipids from the liposome preparations according to the method of Bligh and Dyer (1a) and analyzed the extracts by thin-layer chromatography using the following solvent systems: chloroform-methanol (9:1) (detection of C_{18}-Cer, C_8-Cer, and C_8-dihydro-Cer), chloroform-ethanol (98:2) (detection of C_8-3-OMe-Cer), and hexane-ethyl acetate (4:5) (detection of C_{18}-3-deoxy-Cer). In all cases, the ceramide contents of the liposomal lipid extracts were the same as the ceramide contents of the corresponding original lipid mixtures (results not shown). This eliminates the possibility that the lack of SFV fusion with the ceramide analog-containing liposomes was due to a lack of incorporation of the analogs in the liposomal membrane.

To exclude the possibility that the lack of SFV fusion with the ceramide analog-containing liposomes was due to inhibition of virus binding to the target membrane, as opposed to an effect on the fusion process per se, we examined the binding of

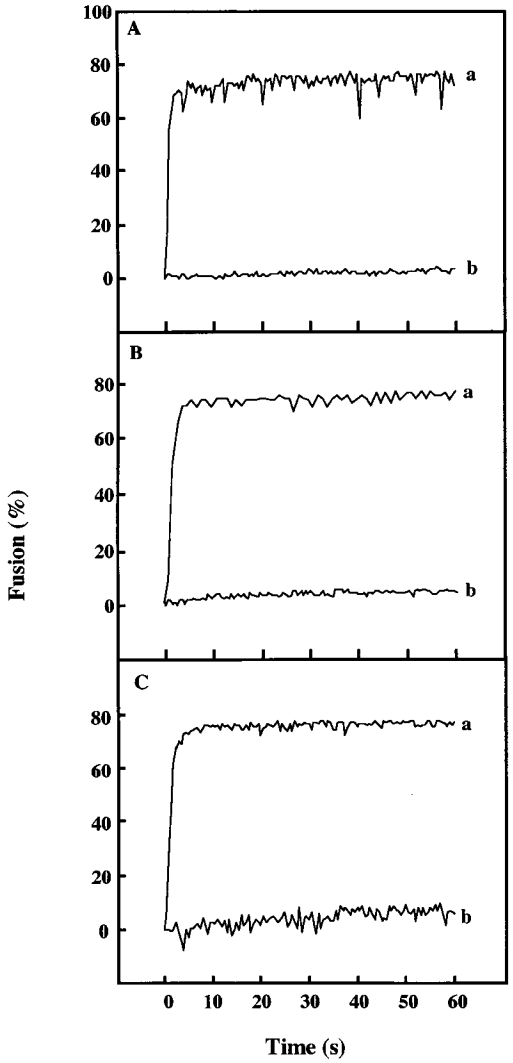

FIG. 2. Effects of the 3-hydroxyl group and the 4,5-*trans* carbon-carbon double bond of the sphingosine backbone on sphingolipid-mediated fusion of SFV with cholesterol-containing liposomes. Fusion of pyrene-labeled SFV with liposomes was monitored as a decrease of the pyrene excimer fluorescence at 480 nm. Virus and liposomes were mixed in a thermostated (37°C) cuvette in a continuously stirred volume of 0.7 ml (final volume) of HNE buffer (pH 7.4) at concentrations of 0.5 and 200 μM of viral and liposomal lipid, respectively. At time = 0 s, the mixture was acidified to pH 5.5 by injection of a small pretitrated volume of 0.1 M morpholineethanesulfonic acid–0.1 M acetic acid, pH 4.9. The fusion scale was set such that the initial excimer fluorescence at 480 nm represented 0% fusion and the residual fluorescence after addition of the detergent octaethylene glycol-monododecyl ether to a concentration of 10 mM (representing infinite dilution of the fluorophore) corresponded to 100% fusion. Liposomes consisted of a PC-PE-Chol mixture (molar ratio, 1:1:1.5) and contained 10 mol% of the ceramide or ceramide analogs, specified below. (A) Curve a, C_{18}-Cer; curve b, C_{18}-deoxy-Cer. (B) Curve a, C_8-Cer; curve b, C_8-3-OMe-Cer. (C) Curve a, C_8-Cer; curve b, C_8-dihydro-Cer.

SFV to the liposomes by flotation analysis on sucrose density gradients, as described previously (2, 14, 24, 29). Incubation of the virus for 1 min at pH 5.5 and 37°C with liposomes composed of the PC-PE-Chol mixture alone or supplemented with either C_{18}-Cer, C_{18}-3-deoxy-Cer, C_8-3-OMe-Cer, or C_8-dihydro-Cer resulted in almost complete (80 to 90%) binding, whereas at neutral pH binding was negligible (results not shown). This indicates that SFV does retain the capacity to bind to cholesterol-containing liposomes in a low-pH-depen-

dent manner, irrespective of the presence of fusion-supporting or fusion-inactive ceramides. Therefore, it can be concluded that the fusion-supporting ceramides exert their effect specifically at the level of the fusion process itself.

The results presented in this report demonstrate that low-pH-induced fusion of SFV with cholesterol-containing liposomes exhibits remarkable molecular specificity. Not only does the process require sphingolipids in the target membrane (24); in addition, it appears that specific functional features of the sphingolipid are essential. These include the 3-hydroxyl group and the 4,5-*trans* carbon-carbon double bond of the sphingosine backbone. This extraordinary specificity is difficult to explain in terms of a structural function of the sphingolipids in the SFV fusion process, since, relative to the corresponding native sphingolipids that do support SFV fusion, the fusion-inactive 3-deoxy, 3-methoxy, and dihydro derivatives are likely to have very similar structural characteristics. For example, sphingomyelin and its 3-deoxy derivative exhibit almost identical structural behavior both in vesicles (12), and in monolayers (7). Yet, sphingomyelin efficiently supports SFV fusion (24) while its 3-deoxy analog (like C_{18}-3-deoxy-Cer in Fig. 2A) is completely inactive (results not shown). Therefore, the present results strongly support the notion that sphingolipids act as a molecular cofactor, activating the SFV fusion function in a highly specific manner. A cofactor role is also consistent with the very low concentrations of sphingolipid in target liposomes that suffice to support the fusion reaction (24). Furthermore, we emphasize that sphingolipids are considered to enhance rather than destabilize the lamellar organization of lipid bilayers (4, 22, 23), a property which is difficult to reconcile with a structural role of these lipids in a membrane fusion reaction (3, 26, 32).

It is tempting to speculate that the cofactor role of sphingolipids in membrane fusion of SFV involves the induction of a conformational change in the viral envelope glycoprotein as a result of a direct interaction between the sphingolipid and the protein. It is well established that at low pH, the E2/E1 heterodimeric spike protein of SFV undergoes a number of conformational changes (2, 11, 14, 15, 29, 30). These include dissociation of the heterodimer, exposure of new epitopes on E1 recognized by conformation-specific monoclonal antibodies, emergence of a trypsin-resistant phenotype of E1, and formation of an E1 homotrimeric structure. The E1 homotrimer is likely to represent the spike structure involved in the fusion reaction (2, 11, 29). At least part of these conformational changes can occur in the absence of target membrane lipids, i.e., when virus particles alone are exposed to low pH (29). On the other hand, recently, Kielian and coworkers have shown that acid-induced conversion of a soluble form of E1 (E1*) is strongly promoted by liposomes containing sphingomyelin (17). We propose that, after initiation of spike conversion on isolated virions and subsequent binding of the virus to cholesterol-containing target membranes, additional conformational changes are involved in the formation of the ultimate fusion-active conformation of the viral spike. These conformational changes would then require a specific interaction of the acid-converted E1 protein with sphingolipids.

There are few published examples of specific sphingolipid-protein interactions. However, we do note that, as in our present study, an absolute specificity for ceramide as against dihydro-ceramide has also been observed in the case of protein phosphatase activation in ceramide-mediated signal transduction (33). In addition, it is interesting that cellular entry and fusion of human immunodeficiency virus type 1 can be mediated by galactosyl ceramide (5, 8, 34). Galactosyl ceramide also supports fusion of SFV (24). In future structure-function studies, it will be of interest to investigate further details of the molecular features required for interaction of sphingolipids with membrane fusion proteins.

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