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COMMUNICATIONS

Published on Web 10/20/2006

Spontaneous Membrane Fusion Induced by Chemical Formation of Ceramides in a Lipid Bilayer

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Although membrane fusion is an essential and fundamental event in living organisms, the molecular mechanism of the process is not yet fully understood. As structures of biomembranes are asymmetric and heterogeneous, lipid composition appears to play a significant role in morphological changes of the membranes.[1−3] However, the direct relationship between dynamic alterations of the lipid composition of a membrane and dynamic morphological transformation of membrane structures has not been elucidated previously. We report here, for the first time, that mere chemical generation of ceramide and related double-chain lipids in the membrane of small unilamellar vesicles (SUVs) induces fusion of the vesicles.

Morphological changes in cellular membranes actually occur through the cooperation of multiple factors,[2,3] most likely with fusogenic proteins controlling these events.[4] Because the process involves such a complex biological system, it has been difficult to investigate the function of individual factors, particularly the role of lipids, on fusion or fission events. To elucidate lipid function, it is necessary to employ a simple model system that excludes any extra biological factors, even identified factors such as enzymes, in which composition of membrane lipids can be altered by chemical reactions with minimal perturbation. Thus, we have successfully developed an artificial system in which the synthesis of double-chain lipids of the ceramide family occurs by dehydrocondensation of fatty acids and a sphingosine or its analogues at the surface of a liposome membrane.

As shown in Figure 1, we designed the reaction at the membrane surface based on 1,3,5-triazine dehydrocondensing agents, which enable us to conduct coupling of carboxylic acids and amines in micelles as well as in an aqueous solvent by catalysis of a tertiary amine.[5] When SUVs containing fatty acid salts **1**, sphingosine **2**, and an amphiphilic tertiary amine catalyst **3** are treated with 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT), ceramide **6** can be efficiently produced via a three-step reaction, because all of the reactants and intermediates (**1**−**4**) are amphiphilic, with the reacting site at the polar headgroup. Only the polar headgroups of lipids change significantly; the composition of the hydrophobic moieties is maintained during the reaction. Thus, inverted cone- or cylindrical-shaped lipids (**1** and **2**) are converted into cone-shaped lipids (**6**). We hypothesized that the reduced surface charge of the liposomes may cause their aggregation by desolvation or elimination of counterions at the surface. Furthermore, the presence of cone-shaped lipids in the outer monolayer of liposomes is believed to promote membrane fusion, whereas inverted cone-shaped lipids inhibit it.[3] We were therefore curious whether the resulting liposome, with a ceramide-enriched outer monolayer, would undergo spontaneous fusion.

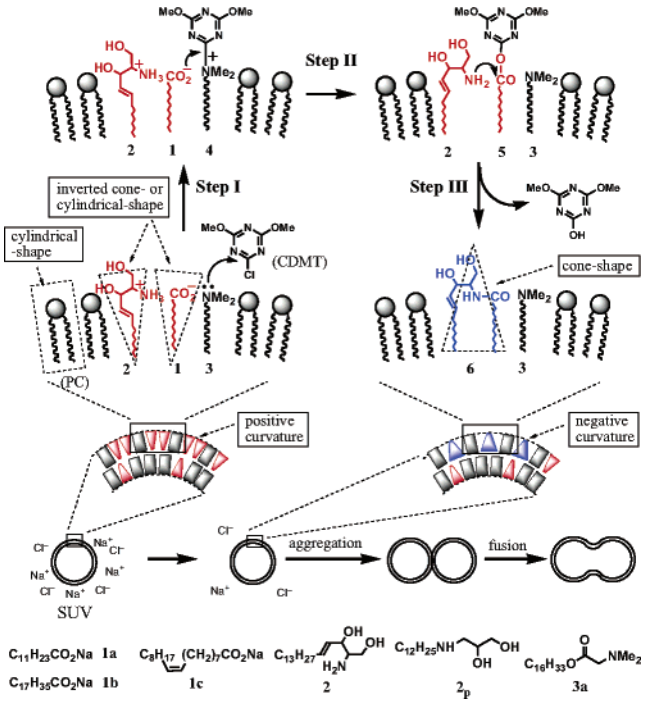

Figure 1. Concept of the membrane fusion by ceramide synthesis.

To that end, we employed 1,2-dihydroxy-4-azahexadecane ($\mathbf{2_p}$, pseudo-sphingosine with a dodecyl group at the nitrogen atom) instead of sphingosine **2**. With its two hydroxyl groups and one amino group, the chemical properties of $\mathbf{2_p}$ would be similar to those of **2**. Dimethylglycine hexadecyl ester **3a** was used as a tertiary amine catalyst. Liposome fusion was monitored by a probe mixing assay or a probe dilution assay based on fluorescence resonance energy transfer between NBD-PE and Rh-PE.[6]

The results of the probe mixing assay are shown in Table 1. Fusion was estimated from the efficiency of energy transfer value, *E* (%),[7] which should increase upon fusion. As we expected, a large increase in *E* value (65%) was observed, strongly suggesting the induction of membrane fusion when liposomes that include phosphatidylcholine (PC), laurate **1a**, $\mathbf{2_p}$, and **3a** are treated with a large excess of CDMT for 3 h (run 1).[8] In a control experiment without **3a**, no increase in *E* value (<1%) was observed. Similar results were obtained with stearate **1b** and oleate **1c** with longer alkyl chains (runs 2 and 3). The larger *E* value with **1c** (78%) over that with **1b** (41%) could be due to a higher fluidity of the membrane composed of **1c**, with a *cis*-olefin, than **1b**, with a saturated alkyl group. No significant further increase in *E* values with **1a** and **1c** was observed by extending the reaction time to 24 h, meaning that the membrane fusion event with these fatty acids could almost finish within 3 h.

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10.1021/ja0652969 CCC: $33.50

Table 1. *E* Values after Definite Times in Probe Mixing Assay[a]

run	fatty acid salt	product	*E* (%) 1 h	2 h	3 h	24 h
1	**1a**	$\mathbf{6a_p}$	1	39	65	68
2	**1b**	$\mathbf{6b_p}$	0	13	41	66
3	**1c**	$\mathbf{6c_p}$	7	63	78	73

[a] The reaction was performed with **1**, $\mathbf{2_p}$, **3a**, and CDMT. **1a**, sodium laurate; **1b**, sodium stearate; **1c**, sodium oleate.

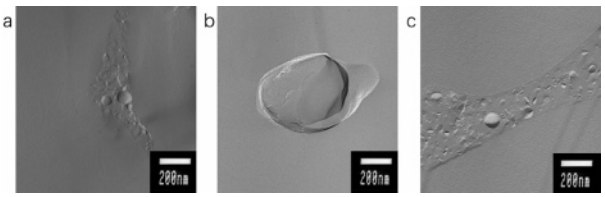

Figure 2. Freeze−fracture transmission electron microscopy (TEM) of liposome. Scale bar = 200 nm. 1.3 mM each of PC, **1a**, and $\mathbf{2_p}$, and 0.26 mM of **3a**. (a) Liposomes are present with vesicle size ranging from 50 to 100 nm before addition of CDMT. (b) Large to giant vesicles with diameters up to about 1 μm are formed at 3 h after addition of CDMT (6.5 mM). (c) A control experiment during the same period without CDMT.

When a water-soluble, dehydrocondensing agent 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM),[5a] available in water, was employed instead of a combination of catalyst **3a** and CDMT in the reaction using **1a**, the *E* value was very low, even after 72 h (22%).

Since the probe mixing assay is known to be sensitive to aggregation of vesicles, a probe dilution assay that is generally insensitive to the mere aggregation of vesicles was also employed.[6] This assay confirmed that, for experiments using **1a**, membrane fusion involving lipid mixing did indeed occur (see Supporting Information).

The formation of pseudo-ceramide $\mathbf{6_p}$ appears to be essential for inducing membrane fusion in this system, in that it apparently occurs prior to the increase in the *E* value. Thus, the yields of $\mathbf{6a_p}$, which were determined by ESI-MS, were 48% and 77% at 1 and 2 h, respectively, in the reaction under the same conditions as in Table 1, run 1. On the other hand, no significant formation of $\mathbf{6a_p}$ (2%) was observed in the above system using DMT-MM after 24 h, and under these conditions the *E* value was only 12%.

The change in size of the liposomes during the reaction was measured by dynamic light scattering. The mean volume diameter of the liposome, including **1a** with CDMT, increased from 46 to 166 nm over the course of 3 h, whereas no change in the diameter was observed without addition of CDMT. We also observed liposomes prepared from lipids of a higher concentration (20 times) by freeze−fracture transmission electron microscopy (TEM). The liposomes containing **1a** were in the range of 50−100 nm before reaction (Figure 2a). When CDMT was added to start dehydrocondensation, large to giant vesicles with diameters up to about 1 μm were formed after 3 h (Figure 2b). No change in the size of liposomes was observed without CDMT during the same period (Figure 2c). The increase in the diameter (up to 20 times the original in the largest examples) indicates that hundreds of SUVs fused one after another.

Finally, we have found that the membrane fusion proceeded with an SUV comprised of naturally occurring lipids, in which sphingosine **2** was used instead of $\mathbf{2_p}$, leading to the formation of ceramide **6a**. Interestingly, under the same conditions as those shown in Table 1, run 1, the *E* value increased moderately (36% after 24 h), despite the formation of a large amount of **6a** (87%), indicating, as one would expect, that membrane stability is very sensitive to the chemical structure of the composite lipids. Several reports concerning dynamic morphological changes of a molecular assembly induced by chemical reaction of lipids have appeared in the literature;[9] however, to the best of our knowledge, there has been no report of successful spontaneous membrane fusion of liposomes by synthetic chemical transformation (dehydrocondensation) of naturally occurring lipids.

It is well known that membrane fusion of liposomes is readily induced by the addition of various fusogenic molecules, like polyethyleneglycol and alkali earth ions.[1c] These methods can be classified as external chemical stimulation, in which the fusogenic molecules force the membrane to fuse by interaction with a lipid headgroup or the surface of vesicles. In marked contrast, in our system the SUV spontaneously undergoes membrane fusion upon internal chemical stimulation by the product of our artificial enzyme system.

Ceramide has been reported to serve a variety of functions in inducing morphological changes of membranes; for example, it acts as a signal molecule and a constituent of a microdomain (a lipid raft).[10] The present work introduces the possibility that endogenous mere formation of ceramides from single-chain lipids (sphingosine and fatty acids) may induce membrane fusion, without requiring fusogenic proteins or other biological factors. Our system should be very useful for elucidating the dynamic functions of a variety of lipids in membranes.

Acknowledgment. This work was supported partially by a Grant-in-Aid for Science Research (No. 18659006) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Supporting Information Available: Synthetic methods, experimental details, and further discussion. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA0652969