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MEMBRANE FUSION OF INFLUENZA VIRUS WITH PHOSPHATIDYLCHOLINE LIPOSOMES CONTAINING VIRAL RECEPTORS

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SUMMARY: pH-dependent membrane fusion of influenza virus with liposomes made of phosphatidylcholine was studied by the spin-labelling method. Efficiency of viral fusion with liposomes composed of dimyristoyl phosphatidylcholine or dipalmitoyl phosphatidylcholine was considerably lower compared to dioleoyl phosphatidyl choline or egg yolk phosphatidylcholine, suggesting importance of unsaturation of acyl chains of lipid bilayers. Reconstitution of specific viral receptors such as Glycophorin or sialylparagloboside strongly enhanced fusion with liposomes composed of dimyristoyl phosphatidylcholine. A direct comparison between the activities of the receptors showed that Glycophorin was about 50 times more effective than sialylparagloboside at the same receptor / phosphatidylcholine molar ratio.

HA protein of influenza virus is the best-characterized among fusogenic membrane proteins (for reviews, see from 1 to 6). Fusion activity of the protein is expressed only at acidic conditions. Influenza virus utilizes the acidic environment of endosomes of host cells to liberate viral genomes by membrane fusion. In addition to the importance for the mechanism of viral infection, HA is regarded as a useful model system for studies of protein-induced membrane fusion.

Abbreviations: HA ; hemagglutinin, RBC ; red blood cell, PC ; phosphatidylcholine, DMPC ; dimyristoyl phosphatidylcholine, DPPC ; dipalmitoyl phosphatidylcholine, DOPC ; dioleoyl phosphatidylcholine, egg PC ; egg yolk phosphatidylcholine, SiaPG ; sialylparagloboside.

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Liposomes are convinient tools for studies of the molecular mechanism of viral fusion, because their composition is simple and can be easily modified (7, 8, and 9). In our study, liposomes composed of PC with various acyl chains were used as target membranes of influenza virus and viral fusion with them was examined by the spin-labelling method (10). The virus fused much more efficiently with DOPC or eggPC liposomes than with DMPC or DPPC liposomes. To clarify the role of specific viral receptors in membrane fusion, Glycophorin and SiaPG of human RBC were reconstituted in DMPC liposomes which are originally "less fusible".

MATERIALS AND METHODS

Influenza virus A/PR/8(H1N1) grown in embryonated chicken eggs was purified as described (10). Glycophorin was purified from human RBCs as described (11). Gangliosides (SiaPG, GM1a, and GM3) were kind gift from Dr. Y. Suzuki (University of Sizuoka, School of Pharmaceutical Sciences). DMPC, DPPC, and DOPC were purchased from Sigma Chemical Co. Egg PC (12) and spin-labeled PC (13) were prepared as described. Buffers used were PIPES buffer (5mM PIPES, 145mM NaCl, pH7.5) and acidic buffers (20mM sodium citrate, 130mM NaCl, pH5.0 - 6.5).

Spin-labeled PC was included in the viral membrane as described (10) and the virus was suspended in 145mM NaCl. Liposomes containing (or not containing) Glycophorin or gangliosides were prepared according to MacDonald and MacDonald (14) and suspended in 145mM NaCl. The liposomes were multi- or quasi-lamellar large vesicles as observed by quick-frozen-replica techniques (15). Viral fusion with liposomes was assayed by PC transfer from viral membrane to liposomes as described before (7) with slight modifications. Spin-labeled virus (37μg of total protein) and liposomes (150μg of lipid) were mixed in 250μl of 145mM NaCl and stood for 10min at 4°C. The mixture was centrifuged at 4°C for 90sec at 15,000Xg, and the pellet was suspended in 20μl of acidic buffer at 4°C. The sample was packed in a quartz capillary tube at 4°C, and ESR spectrum was recorded at 37°C repeatedly. The ESR signals for spin-labeled virus are broadened by the spin-spin interactions because of a high concentration of spin-labeled PC in the viral membrane. Viral fusion with liposomes causes dilution of spin-labeled PC in the fused membrane and leads to an increase in the ESR peak height. Fusion efficiency (%) was estimated from normalized ESR peak height per unit number of spin labels, essentially as described before (16). An equation used for calculation of the efficiency is 100 (P-V) / (L-V), where V,L, and P are normalized ESR peak heights for virus, for liposomes containing 0.5% spin-labeled PC, and for the reacting system at any time,respectively.

RESULTS

Effect of unsaturation of acyl chains Viral fusion with liposomes made of PCs with different acyl chain composition were assayed at pH5.2 and 37°C. The ESR peak height for spin-labeled virus increased very rapidly and almost saturated within 2 min for fusion with any liposomes examined. Fusion efficiency for various PC liposomes measured at a 5 min reaction point is shown in Table 1. The virus fused with DOPC and egg PC liposomes very efficiently at acidic pH (61% and 50%, respectively). Only small change in ESR signals was observed at neutral pH, demonstrating that the change at pH5.2 was attributable to biological activity of HA. On the other hand, fusion with DPPC or DMPC liposomes was restricted to lower level (28% and 18%). Since the head groups of phospholipids used are common, the difference in fusion efficiency is due to the acyl chain composition.

Reconstitution of viral receptors in DMPC liposomes As described above, DMPC liposome was essentially unsuitable for a target membrane of influenza virus. We included Glycophorin and SiaPG in DMPC liposomes and examined their effect on the fusion efficiency. It is well established that HA binds to sialyl-oligosaccharides and the recognizable structure of carbohydrate chains is specific for each viral strain (17). Glycophorin and SiaPG of human RBCs are known to be specific receptors for influenza A viruses (18). Both of the receptors effectively enhanced the viral fusion with DMPC liposomes (Fig. 1). The fusion efficiency for

Table 1 Effect of unsaturation of acyl chains of PCs on viral fusion with PC liposomes. Efficiency of viral fusion 5 min after the start of reaction with PC liposomes with different acyl chain compositions were determined by the spin-labelling method at 37°C and pH5.2 or pH7.4.

	pH7.4	pH5.2
DPPC	18%	28%
DMPC	14%	18%
DOPC	10%	61%
eggPC	15%	50%

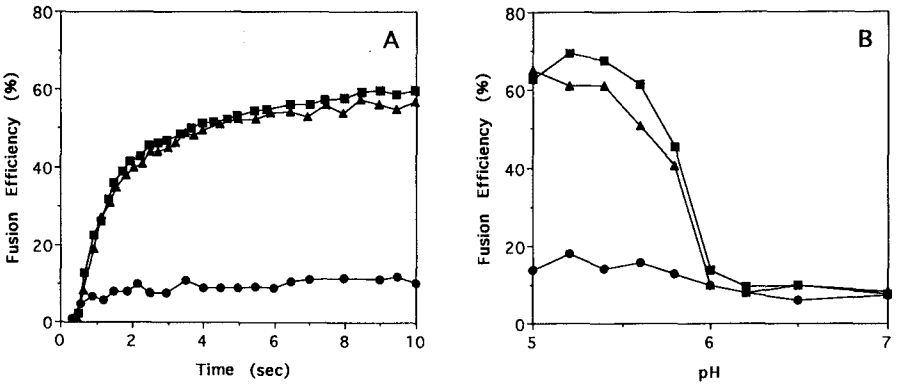

Figure 1. Time course and pH dependence of viral fusion with DMPC liposomes. (A) Time course of viral fusion with DMPC liposomes at pH5.2 and 37°C and (B) pH dependence of fusion efficiency 5 min after acidification of the samples to pH5.2 at 37°C. Pure DMPC liposome (circles) and DMPC liposomes containing Glycophorin (triangles) or SiaPG (squares) were used as target membranes.

DMPC liposomes was raised from 10 - 20% to 50 - 70% when the receptors were included. Time course and pH dependence of the viral fusion was almost identical between the receptors (Fig. 1A, B).

To compare potencies between Glycophorin and SiaPG, liposomes containing the receptors at various concentrations were prepared and viral fusion with them was examined. Fig. 2 shows dependence of fusion efficiency on the concentrations of Glycophorin and SiaPG. The fusion efficiency of 17% for DMPC liposomes was increased by addition of Glycophorin to attain 60% at the receptor / DMPC molar ratio of about $1X10^{-4}$ (Fig. 2A). To reach a half enhancement point where the fusion efficiency is about 40%, the receptor / DMPC ratio of about $1X10^{-5}$ was necessary. For SiaPG, the receptor / DMPC ratio of about $5X10^{-3}$ was needed for the fusion efficiency of 60% and about $5X10^{-4}$ for 40% (Fig. 2B). Thus Glycophorin was approximately 50 times more effective than SiaPG to enhance viral fusion in terms of the receptor / DMPC molar ratio.

Two kinds of experiments were undertaken to acertain the effect being specific for the receptors. At first, other monosialyl gangliosides, GM1a and GM3, were

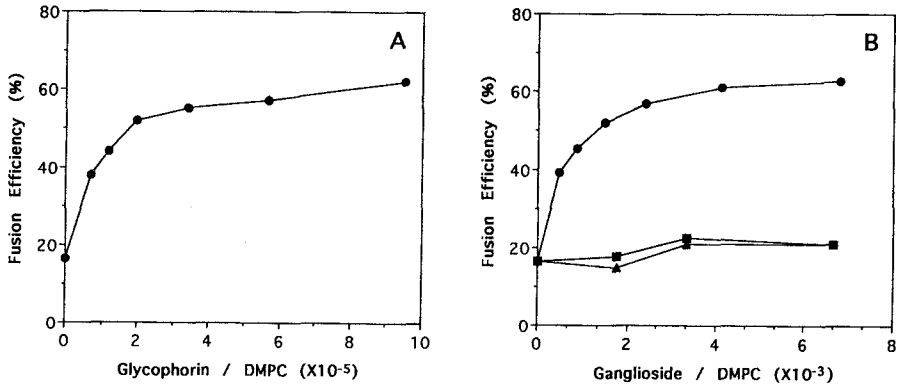

Figure 2. Enhancement of viral fusion with DMPC liposomes by viral receptors. Effect of Glycophorin (A) and gangliosides (B) on viral fusion with DMPC liposomes at pH5.2 and 37°C were determined. Fusion efficiency after 5min of acidification was ploted against receptor concentrations. Gangliosides used were SiaPG (circles), GM1a (triangles), and GM3 (squares).

included in DMPC liposomes instead of SiaPG. As shown in Fig. 2B, both of them were ineffective to enhance fusion in the range of the receptor / DMPC ratio examined ($<7X10^{-3}$). These results are consistent with the previous report that SiaPG is more effective than GM1a and GM3 as a receptor for A/PR/8(H1N1) (18). Secondly, DMPC liposomes containing Glycophorin were treated with neuraminidase to distroy the receptor structure and used for the fusion assay. This pretreatment reduced the fusion efficiency to the same level with pure DMPC liposomes (data not shown).

DISCUSSION

Influenza virus could fuse in a pH-dependent manner with any PC liposomes used (Table 1). But the fusion efficiency was strongly dependent on unsaturation of acyl chains of PC. Requirement of unsaturated acyl chains was also reported in vesicular stomatitis virus fusion with liposomes studied by Yamada and Ohnishi (19).

The requirement cannot be ascribed to membrane fluidity. DMPC liposomes (T_c = 23°C) are in the liquid-crystalline state at 37°C, whereas DPPC liposomes (T_c = 41°C) in the gel state. Although being in such different physical states, both of them

showed low fusion efficiency. In the previous study using DMPS liposomes, the fusion efficiency was not affected by T_c of DMPS, also denying involvememt of membrane fluidity (7). Thus the reason for the requirement for unsaturated acyl chains remains to be elucidated. However, It is likely that lipid bilayers of liposomes containing PCs with unsaturated acyl chains have higher deformability than those with only saturated acyl chains, and are more easily transformed to the highly curved intermediate structure of membrane fusion which was observed by quick-frozen-replica techniques (15, 20).

Introduction of specific viral receptors efficiently enhanced viral fusion with DMPC liposomes (Figs. 1and 2). The enhancement by the receptors was not an artifact but based on the specific binding of the receptors to HA by biological activities. Reconstitution of the receptors into liposomes enabled us a direct comparison of fusion enhancement activity between membrane glycoprotein and glycolipid receptors. Glycophorin enhanced fusion much more efficiently than SiaPG. It has been reported that SiaPG has the highest binding activity to HA of A/PR/8(H1N1) among gangliosides of human RBC membranes (17). These facts incicate that Glycophorin is more effective to enhance fusion of the viral strain than any glycolipid of the membrane, and therefore that the protein is the most effective receptor in the membrane.

Insertion of the "fusion peptide" of HA into lipid bilayers of target membranes is regarded as a key step of membrane fusion (7). HA actually can insert its "fusion peptide" into bare liposome membranes without the receptors (21, 22), obviously owing to simple collisions of viral particles and liposomes. Pre-binding of HA to the receptors at neutral pH can achieve stable contact of the protein with surfaces of target membranes. When acidified, the "fusion peptide" of receptor-bound HA might be inserted into the apposed lipid bilayers more efficiently than that of unbound HA because of the contact through the receptors.

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