Membrane Asymmetry Is Maintained during Influenza-induced Fusion*

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We have investigated the influence of influenza-induced membrane fusion on the transverse asymmetry of the viral and target membranes. Large unilamellar vesicles containing headgroup-labeled fluorescent phospholipid analogues in both leaflets of the membrane were treated with phospholipase D, converting all outer membrane phospholipids to phosphatidic acid and leading to the release of the fluorescent label from the outside leaflet. After fusion of virus with these liposomes, addition of the enzyme to the fusion product did not release fluorescent label again, indicating that the phospholipid analogues from the inner leaflet of the membranes had not appeared on the outer leaflet. Moreover, the integral membrane protein hemagglutinin, which is present on the outer leaflet of the viral membrane, was quantitatively digested with protease after fusion, indicating that hemagglutinin remained on the outer leaflet of the fusion product. Therefore, there is no merger of the inner with outer leaflets of the viral or the liposomal membrane during fusion, and transverse membrane asymmetry is maintained.

The constituents of biological membranes, such as the plasma membranes of cells, are distributed in an asymmetric manner between the two leaflets of the membranes (Devaux, 1991, 1992). Although these membranes continuously undergo membrane fusion and fission, their asymmetry is maintained. Thus, it is likely that membrane asymmetry is maintained even during fusion.

One of the most extensively studied membrane fusion mechanisms is that induced by the hemagglutinin (HA) glycoprotein of influenza virus. Fusion mediated by the protein is induced by low pH, leading to a conformational change in HA, which results in the insertion of the N-terminal “fusion peptide” of the HA2 subunit of the protein into the target membrane (Bentz, 1993). Although many details of the conformational change (reviewed by Hughson (1995)) and its role in fusion are now known, it is not clear how the protein achieves the merger of the viral with the target lipid bilayers. For fusion, a non-bilayer structure has to be formed, at least temporarily and locally at the site of fusion (Wilschut and Hoekstra, 1986). Little is known about these intermediate lipid structures or the role of HA in their formation. One attractive hypothesis proposes that the intermediates formed are stalks, semitoroidal (hourglass-shaped) structures composed of fused outer leaflets, but unfused inner leaflets of both membranes (Siegel, 1993a, 1993b). Stalk formation would be followed by a rupture of the inner membrane leaflets at the site of fusion, followed by their merger, which then completes bilayer fusion. There is little direct evidence for this hypothesis, but a mutant, glycosylphosphatidylinositol-anchored HA, expressed on a cell membrane, was found to fuse the outer membrane leaflets of these cells with those of red blood cells, while not inducing the merger of the inner membrane leaflets (hemifusion) (Kemble et al., 1994). These data suggested that fusion induced by the wild type HA could indeed proceed via the merger of the outer leaflets followed by that of the inner leaflets (Kemble et al., 1994; Stegmann, 1994). Therefore, in this as well as in most other proposed molecular mechanisms of membrane fusion (Ohki et al., 1987; Wilschut and Hoekstra, 1990; Bentz, 1993), there would be no merger between inner and outer leaflets, with the possible exception of a small fraction of the lipids at the site of fusion, and the transverse asymmetry of the membranes should be maintained after fusion.

Here, we have investigated whether membrane asymmetry is maintained after virus induced fusion, using asymmetric liposomes with fluorescent labels confined to the inner leaflet of the membrane. After their fusion with influenza virus, the fusion products were tested for the appearance of fluorescent probes in the outer leaflet of the fusion products. Also, the orientation of HA in the membrane of the fusion products was investigated after proteolytic treatment. It was found that neither HA nor the membrane lipids were reoriented after fusion.

**MATERIALS AND METHODS**

Phospholipase D from Streptomyces spec. AA586, with a specific activity of 180 units/mg from Genzyme, Cambridge, MA, was kindly donated to us by Dr. Peter Walde, Eidgenössische Technische Hochschule, Zürich, Switzerland. Protease K from Tritrichromum album was obtained from Boehringer (Mannheim, Germany), and lipids were from Avanti Polar Lipids (Alabaster, AL). Large unilamellar vesicles, containing phosphatidyicholine (PC) and phosphatidylethanolamine (PE) (mixed acyl chains, form egg yolk) in a 2:1 ratio, and 0.6 mol% each of N- (lissamine rhodamine B sulfonyl)phosphatidylethanolamine (N-Rh-PE) and N-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (N-NBD-PE) were made by extrusion through 0.1-μm defined pore poly carbonate filters (Nuclepore, Pleasanton, CA) (Mayer et al., 1986) as described before (Nefel et al., 1995) and stored under argon at 4°C. Phospholipid phosphate was determined according to Bottcher (1961). Influenza virus, strain X-31, was grown for us by the Schweizerische Serum- und Impfstoffinstitut (Bern, Switzerland) and purified and stored as described before (Nefel et al., 1995).

Fusion of virus with liposomes was measured by the resonance energy transfer assay (Struck et al., 1981). Fluorescence was recorded at excitation and emission wavelengths of 465 and 530 nm, respectively, using a 515-nm long pass filter between cuvette and emission monochromator. For calibration of the fluorescence scale, the initial residual level of fluorescence was set to 0% and the fluorescence at infinite probe dilution to 100%. The latter value was obtained after lysis of the liposomes with Triton X-100 (0.25% v/v) and correction for the effect of...
Fusion of influenza virus with symmetric or asymmetric liposomes. Virus was injected into a cuvette containing symmetric liposomes (a) or asymmetric liposomes (b and c) at pH 5.1 (a and b) or pH 7.4 (c), 37 °C. At the arrows, 2 units of phospholipase D were added. The fluorescence of N-NBD was measured, and the fluorescence scale calibrated, as described under “Materials and Methods.” Final concentration of liposomes and virus was 5 μM phospholipid each.

Triton on the quantum yield of N-NBD-PE (Struck et al., 1981). Liposomes (350 μM), labeled with N-NBD-PE and N-Rh-PE in both leaflets of the membrane, were made asymmetric by digesting the fluorescent probes and other phospholipids in the outer leaflets with phospholipase D (200 μg/ml) for 60 min at pH 7.5 and 37 °C. Subsequently the liposomes were separated from the enzyme by gel filtration on a Sepharose 4B column. The phospholipid concentration of fractions was assayed as described above, and the presence of phospholipase checked by SDS-PAGE. For thin layer chromatography, lipids were extracted according to Bligh and Dyer (1959), run on silica-coated glass plates, and eluted with chloroform/methanol/water (65:35:5, v/v/v). Lipids and digestion products were visualized using specific stains as described elsewhere (New, 1990) and identified by comparison to known standards purchased from Avanti Polar Lipids (Alabaster, AL).

RESULTS

Lipid Asymmetry Is Maintained after Fusion—Large unimellar vesicles (0.1 μm diameter), containing PC and PE (2:1 ratio) and the fluorescent phospholipid analogues N-NBD-PE and N-Rh-PE in both leaflets of the membrane were made by extrusion (Mayer et al., 1986). The incorporation of these probes allows the measurement of membrane fusion by the resonance energy transfer method (Struck et al., 1981). Upon mixing these liposomes with virus at low, but not at neutral pH, an increase in fluorescence was seen (Fig. 1), which is caused by membrane fusion (Stegmann et al., 1986).

Liposomes of this size, made by extrusion, have their phospholipids symmetrically distributed over both leaflets of the membrane (Balch et al., 1994). To produce vesicles that would have the fluorescent probes confined to the inner leaflet only, the liposomes were treated with phospholipase D. This enzyme hydrolyzes the bond between the phosphates of phospholipids and their headgroups, converting phospholipids to phosphatic acid. If the liposomes were treated with phospholipase at pH 7.5 and 37 °C, while the fluorescence of N-NBD-PE was being monitored, a continuous decrease in fluorescence was observed, until a level corresponding to about half the original fluorescence was reached (Fig. 2). Further addition of phospholipase did not cause a further decrease in fluorescence. Although the NBD label is present on the headgroup of this fluorescent phospholipid, it is well known to loop back into the hydrophobic inner region of the membrane due to its hydrophobicity (Chattopadhyay, 1990). Therefore, the fluorescence of the NBD headgroup, after its removal from the phospholipid, most likely decreased because of the considerably reduced quantum yield of NBD in the aqueous phase (Chattopadhyay, 1990; Ferry-Forgues et al., 1993). Moreover, it is possible that the fluorophore aggregates in an aqueous environment, and in that situation self-quenching could further add to the decrease.

To check the action of the phospholipase, a sample was taken after the fluorescence decrease had leveled off. The lipids were extracted and analyzed by thin layer chromatography. It was found that 49 ± 12% of the total liposomal phospholipids were converted to phosphatic acid. Further addition of phospholipase after the fluorescence decrease had leveled off (Fig. 2) did not lead to the production of more phosphatic acid. Together, the above data indicate that phospholipase D digests the outer membrane phospholipids quantitatively, producing liposomes that have PC, PE and fluorescent probes confined to the inner leaflet and only phosphatic acid in the outer leaflet. Thus, the enzyme does not cross the membrane, and fluorescence can be conveniently used to monitor its activity.

To determine if these liposomes were capable of fusing with influenza virus, phospholipase D was first removed from the liposomes by chromatography on a Sephadex 4B column. The efficiency of resonance energy transfer between the N-NBD-PE and the N-Rh-PE of the asymmetric liposomes was found to be somewhat lower (5-10%) than between those of symmetric liposomes. This is most likely because, although asymmetric vesicles have the same concentration of fluorescent probes in the inner leaflet, they lack the resonance energy transfer that takes place between probes in the inner and outer leaflet of symmetric vesicles.

If virus was added to the asymmetric liposomes, fusion occurred, as reported by decreased resonance energy transfer between the inner membrane leaflet probes (Fig. 1). Fusion with these liposomes was faster than with symmetric vesicles, most likely because their outer leaflet consisted of the negatively charged phosphatic acid, and fusion with liposomes made of negatively charged phospholipids is faster than with zwitterionic liposomes (Stegmann et al., 1989). Since some negatively charged liposomes fuse with virus at neutral pH in a non-physiological fashion (Stegmann et al., 1996), fusion was also tested at neutral pH. No increase in fluorescence was seen at that pH (Fig. 1), indicating that fusion of the virus with these asymmetric liposomes is low-pH-dependent. Note that these liposomes, which are negatively charged on the outside and zwitterionic on the inside, are essentially “inside out” with respect to the physiological target membranes for influenza fusion.

To analyze if the membrane asymmetry was changed after fusion, the appearance of fluorescent probes from the inner leaflet on the outer leaflet was tested by adding phospholipase to fusion products after their fusion with virus. No decrease in fluorescence was seen (Fig. 1). However, a decrease in fluorescence was detected if we used asymmetric liposomes that had been stored at 4°C for 4 days (not shown). During this time, transmembrane flip-flop of the lipids should have returned fluorophores to the outside of the vesicles (Devaux, 1991, 1992).
In order to further test if we would have been able to determine fluorescent phospholipid analogues, should they have appeared on the outside of the liposomes, asymmetric liposomes were subjected to several freeze-thaw cycles to induce lipid “scrambling.” A decrease in fluorescence was seen upon addition of phospholipase to these liposomes (Fig. 2). Therefore, the above data indicate that target membrane phospholipid asymmetry is maintained after the fusion of influenza virus with liposomes.

HA Remains on the Outer Leaflet—The integral membrane protein HA is present on the outer leaflet of the viral membrane. To test whether the asymmetric distribution of this protein was maintained after fusion, we exploited the fact that HA, which is resistant to a variety of proteases, becomes sensitive to protease K after the low-pH-induced conformational change in the protein. Virus was incubated at pH 4.8 or 7.4 for 15 min at 37 °C in the absence or presence of an excess of liposomes, followed by neutralization and digestion with protease K. The internal matrix and nucleocapsid proteins of the intact virus were digested. These data indicate that after fusion, HA does not cross the membrane. In samples of intact virus that were not treated at low pH, none of the proteins were digested. These data indicate that after fusion HA remains accessible to protease and therefore must be on the outside of the fusion products.

**DISCUSSION**

The results presented in this paper indicate that membrane asymmetry is maintained after the low pH-induced fusion of influenza virus with large unilamellar vesicles. Experiments with asymmetric liposomes containing fluorescent label on the inside leaflet indicate that there is no merger of the liposomal inner leaflet with either of the outer membrane leaflets. The fusion experiments were done at a 1:1 ratio of virus to liposomes, and virus particles and liposomes have roughly the same size and therefore the same amount of membrane lipids. Earlier quantitative analyses of fusion in this system have shown that one virion fuses with slightly more than one liposome on average, and that there is no further fusion of virus with fusion products or fusion of virus with virus (Nir et al., 1986; Stegmann et al., 1989). Thus, in the fusion products approximately equal amounts of membrane material are contributed by the virus and the liposomes. Considering this degree of lipid mixing, and knowing that the liposomal lipids did not move from their inner leaflet to the outer leaflet of the membrane of the fusion product, it is most likely that the viral lipids maintained their transverse asymmetry as well.

Moreover, the viral integral membrane protein HA remained on the outer leaflet after fusion. The final level of fluorescence increase that was reached in the fusion experiments (Fig. 1) was about 10%, meaning that approximately 20% of the virus underwent one round of fusion with liposomes (Nir et al., 1986; see above). By loading different quantities of HA on gels, we found that after silver-stain, using a laser-driven gel scanner, we could still detect around 1% of the HA loaded on the gel shown in Fig. 3. Since no HA could be detected after fusion and protease digestion at all, 95–100% of the HA was still present on the outer leaflet of the fusion products.

Membrane-impermeant small molecules such as trinitro-benzene sulfonic acid or dithionite are more commonly used to produce and probe membrane asymmetry than phospholipase D (McIntyre and Sleight, 1991; Gruber and Schindler, 1994). However, we found that the viral membrane is quite permeable to these substances. Convenielly, the fluorescence of N-NBD-PE present in the outer leaflet was lost after its digestion with the phospholipase, but the enzyme did not penetrate the viral membrane. Thus, this probe-enzyme combination provided a fast and efficient method for the determination of the asymmetry of these membranes, and it is potentially useful for other membranes that are permeable to small molecules also.

Biological membranes continuously undergo membrane fusion and fission. For example, fibroblasts can recycle 50% of their plasma membranes (Steinman et al., 1983). Using a short chain fluorescent sphingomyelin analogue inserted into the outer leaflet of the plasma membrane, Koval and Pagano (1989) found that the probe was internalized by endocytosis, and then recycled to the plasma membrane with a half-time of 15–20 min. After 60 min, all non-metabolized probe could be extracted from the outer leaflet of the membrane again. Thus, although cells possess amino phospholipid translocases and other “flipases” that could restore membrane asymmetry (Devaux, 1991, 1992), it seems more logical to assume that asymmetry is maintained during fusion. In line with this, in most models for the molecular mechanism of biological membrane fusion (Ohki et al., 1987; Wilschut and Hoekstra, 1990; Bentz, 1993) it is proposed that fusion involves merger of the outer leaflets of the participant membranes followed by that of the inner leaflets, maintaining the asymmetry of the bulk of the lipids. This paper presents the first confirmation of that assumption.

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