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Effect of Erythrocyte Transbilayer Phospholipid Distribution on Fusion with Vesicular Stomatitis Virus

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ABSTRACT: To identify the specific component(s) in the target membrane involved in fusion of vesicular stomatitis virus (VSV), we examined the interaction of the virus with human erythrocyte membranes with asymmetric and symmetric bilayer distributions of phospholipids. Fusion was monitored spectrofluorometrically by the octadecylrhodamine dequenching assay. Fusion of VSV with lipid-symmetric erythrocyte ghosts was rapid at 37 °C and low pH, whereas little or no fusion was observed with lipid-asymmetric ghosts. Conversion of phosphatidylserine in the lipid-symmetric ghost membrane to phosphatidylethanolamine by means of the enzyme phosphatidylserine decarboxylase did not alter the target membrane's susceptibility to VSV fusion. Spin-labeled phospholipid analogues with phosphatidylserine, phosphatidylethanolamine, and phosphatidylcholine headgroups incorporated into the outer leaflet of lipid-asymmetric erythrocytes did not render those membranes fusogenic. Electron spin resonance spectra showed an increased mobility of a phosphatidylcholine spin-label incorporated into the outer leaflet of lipid-symmetric erythrocyte ghosts as compared to that of lipid-asymmetric ghosts. These results indicate that the susceptibility to VSV fusion is not dependent on any particular phospholipid but rather is related to packing characteristics of the target membrane.

The envelope of vesicular stomatitis virus (VSV)[1] consists of a bilayer membrane with a single type of spike glycoprotein, the G protein, which mediates attachment to the cell surface and induces fusion between viral and target membranes (Pal et al., 1987). pH-dependent fusion of VSV with cells has been studied by a variety of methods (White et al., 1981; Matlin et al., 1983; Yamada & Ohnishi, 1986; Blumenthal et al., 1987). However, it is not clear what components are necessary in target membranes to render them susceptible to VSV fusion. On the basis of inhibition studies, Schlegel et al. (1983) and Mastromarino et al. (1988) support the notion of phospholipid or sialoglycolipid specificity for VSV penetration. On the other hand in studies of fusion of VSV with liposomes Yamada and Ohnishi (1986) showed no phospholipid specificity.

We examine the issue of target membrane specificity using the octadecylrhodamine (R18) assay (Hoekstra et al., 1984), which directly monitors fusion between the virus and a biological membrane. As the biological target we chose the erythrocyte membrane, whose phospholipid arrangement can readily be modified. The phospholipids of normal erythrocytes are arranged asymmetrically across the plasma membrane; phosphatidylcholine (PC) and sphingomyelin are predomiantly on the outer surface, whereas others such as phosphatidylserine (PS) and phosphatidylethanolamine (PE) are predominantly restricted to the inner leaflet (Zwaal et al., 1975). However, erythrocytes can be lysed and resealed under conditions where the asymmetric distribution of phospholipids is lost or retained (Williamson et al., 1985). We have recently shown that

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[1] Abbreviations: VSV, vesicular stomatitis virus; R18, octadecylrhodamine B chloride; PBS, phosphate-buffered saline; ESR, electron spin resonance; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; (0,2)PC, 1-palmitoyl-2-(4-doxylpentanoyl)-PC; (0,2)PS, 1-palmitoyl-2-(4-doxylpentanoyl)-PS; (0,2)PE, 1-palmitoyl-2-(4-doxylpentanoyl)-PE; PSD, PS decarboxylase; FDQ, fluorescence dequenching; RBC, red blood cell(s).

erythrocyte ghosts with a lipid-symmetric phospholipid bilayer distribution are susceptible to fusion with VSV (Grimaldi et al., 1988). In this study we examine what factor(s) will render the erythrocyte membrane fusogenic after symmetrization.

Experimental Procedures

Materials. Octadecylrhodamine B chloride (R18) was obtained from Molecular Probes (Junction City, OR), 2,4,6-trinitrobenzenesulfonic acid was from Sigma (St. Louis, MO), and thin layer plates (Silica 60) were from E. Merck (Cincinnati, OH). Fresh blood from healthy donors was collected from the NIH blood bank.

Labeling of Virus. Purified VSV (Indiana) was obtained from J. Brown and B. Newcomb at the University of Virginia (Charlottesville, VA). The virus was grown on monolayer cultures of baby hamster kidney (BHK-21) cells and purified by sucrose velocity and density gradients, yielding approximately 1 mg/mL VSV protein (Thomas et al., 1985). The fluorescent probe was inserted into the viral bilayer by injecting 10 μL of a 2 mM R18 solution in ethanol under vortexing into 2 mL of NaCl (145 mM) and Hepes (10 mM), pH 7.4, containing 1 mg of virus protein. After a 10-min incubation at room temperature, unbound probe was removed by harvesting the virus from the interface after centrifugation for 40 min at 40 000 rpm at 4 °C on a 70% sucrose cushion using an LM8 ultracentrifuge (Beckman Instruments, Palo Alto, CA) with a 70 Ti rotor, or by elution from a Sephadex G-25 PD-10 column (Pharmacia, Piscataway, NJ).

Measurement of Membrane Fusion. R18-labeled VSV was incubated for 40 min at 4 °C with 5 mL of RBC or ghost suspension, containing 3×10^9 cells as determined by a Coulter Multisizer. The suspension was then washed twice in PBS and stored as a resuspended pellet of 5-mL volume. R18 VSV–RBC suspensions (20 μL) were transferred into a cuvette containing 2 mL of NaCl–Hepes buffer, pH 7.4, prewarmed at 37 °C. The pH was changed to the desired value by injecting into the cuvette the appropriate amount of a solution of 1 M 2-(*N*-morpholino)ethanesulfonic acid. The pH was always remeasured at the end of the experiment. The R18 fluorescence dequenching (% FDQ) rate was measured in a Model 8000 spectrofluorometer (SLM Instruments Inc., Urbana, IL) with 1-s time resolution at 560- and 585-nm excitation and emission wavelengths, respectively. The percentage fluorescence dequenching was calculated according to (Blumenthal et al., 1987)

$$\% \text{ FDQ} = (100)(F - F_0)/(F_t - F_0) \quad (1)$$

where F, F_0, and F_t are fluorescence values at a given time, at zero time, and after disruption of the VSV–erythrocyte complexes with Triton X-100, respectively.

Preparation of Erythrocyte Ghosts with Lipid-Asymmetric or -Symmetric Membranes. Erythrocytes from fresh human blood were lysed against 15 volumes of 10 mM Tris and 0.1 mM EGTA (pH 7.2) containing 1 mM $CaCl_2$ (lipid-symmetric ghosts) or 1 mM $MgCl_2$ (lipid-asymmetric ghosts). Resealing both kinds of ghosts was performed by adding concentrated buffer solution consisting of 150 mM Na_2HPO_4, 50 mM KH_2PO_4, 1.22 M NaCl, 30 mM KCl, 1 mM $CaCl_2$, and/or 1 mM $MgCl_2$ at 37 °C for 40 min and subsequently washing three times in PBS. The procedure is based on that of Williamson et al. (1985), who reported that the Ca^{2+} ghosts lose the asymmetric distribution of membrane lipids, characteristic to the human erythrocyte, whereas the Mg^{2+} ghosts retain membrane asymmetry. We deviate from their procedure, primarily in using a higher lysis volume (Clague et al., 1990). To restore and maintain the phospholipid asymmetry of Ca^{2+} ghosts, ATP (1.5 mM final concentration) as well as an ATP-generating system, consisting of 100 IU/mL porcine heart creatine phosphokinase and 10 mM creatine phosphate (Calbiochem, San Diego, CA), was included in the resealing buffer.

Enzymatic Treatment of the Target Membrane. The enzyme phosphatidylserine decarboxylase (PSD) from *Escherichia coli* catalyzes the formation of phosphatidylethanolamine (PE) by decarboxylating PS. The purified enzyme (Li & Dowhan, 1988) was kindly provided by Dr. William Dowhan. To convert PS in the lipid-symmetric ghost preparation, 30 μL of PSD (51 000 units/mL, 0.8 mg/mL in a buffer containing 10 mM potassium phosphate, 0.1% Triton X-100, 10% glycerol, 10 mM β-mercaptoethanol, and 0.6 M NaCl) was added to 9×10^8 ghosts in 400 μL of NaCl–Hepes buffer and incubated at 37 °C for 30 min. Control ghosts were incubated with the same PSD buffer without the enzyme. At the end of the incubation the ghosts were washed two times and stored on ice. To quantify the amount of conversion, the lipids were extracted with methanol/chloroform (Bligh & Dyer, 1959). The extract was applied to a silica gel thin-layer plate, developed in $CHCl_3/CH_3OH/H_2/CH_3OOH$ (65:43:3:1), and stained with ninhydrin.

Insertion of Spin-Labeled Phospholipid Analogues into Erythrocyte Membranes and Electron Spin Resonance (ESR) Measurements. We have used the spin-labeled phospholipid analogues 1-palmitoyl-2-(4-doxylpentanoyl)-PC [(0,2)PC], 1-palmitoyl-2-(4-doxylpentanoyl)-PS [(0,2)PS], and 1-palmitoyl-2-(4-doxylpentanoyl)-PE [(0,2)PE], synthesized according to Seigneuret and Devaux (1984). The concentration of spin-labeled phospholipid corresponds to about 1% erythrocyte membrane lipid for mobility measurements using ESR and 5% for the studies designed to examine the effect of phospholipid headgroup on VSV fusion. Prior to labeling, cells were incubated for 5 min with 5 mM diisopropyl fluorophosphate to minimize hydrolysis of the spin-labeled lipid. Erythrocytes were labeled by drying the desired amount of spin-labeled lipid from chloroform/methanol (1:1 v/v), resuspending in 1 mL of PBS by vigorously vortexing, adding 9 mL of packed erythrocytes or ghosts, and gently mixing. The spin-labeled lipids immediately incorporate into the membrane. It has been shown (Seigneuret et al., 1984) that the spin-labels are originally incorporated in the outer leaflet and redistribute differently after incubation. In order to prevent translocation of the PS and PE by the aminophospholipid translocase (Seigneuret & Devaux, 1984), the erythrocyte ghosts were incubated in the presence of 0.2 mM sodium orthovanadate and 1 mM EDTA (Bitbol et al., 1987).

ESR spectra of (0,2)PC incorporated into the outer leaflet of erythrocytes or ghosts were recorded by using a Varian spectrometer E-109 X-band with 100-kHz magnetic field modulation equipped with the Laboratory Data Acquisition System built at DCRT, NIH. The capillary tube containing sample was surrounded by a single-jacketed quartz Dewar flask through which dry nitrogen gas at constant temperature was passed. Spectra were measured at a microwave power of 20 mW and a modulation amplitude of 2.5 or 1.0 G at 7 and 37 °C, respectively. Although inward motion of (0,2)PC is slow (Seigneuret & Devaux, 1984; Calvez et al., 1988), ESR spectra were recorded immediately after labeling to make sure that the probe is on the outer leaflet. At least five spectra per sample were averaged to improve the signal to noise ratio.

Results

Interaction of VSV with Human Erythrocyte Ghosts. Maintainance of the phospholipid asymmetry in erythrocyte

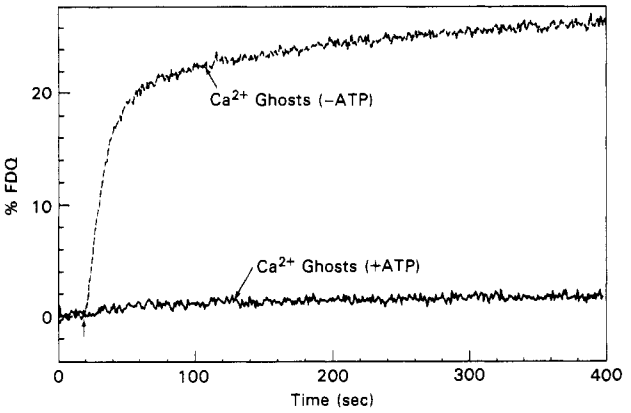

FIGURE 1: Role of phospholipid distribution in fusion of VSV with erythrocyte ghosts. Erythrocyte ghosts were prepared in the presence of Ca^{2+} + ATP or Ca^{2+} − ATP (marked on the curves) as described under Experimental Procedures. R18 VSV was bound to the erythrocyte ghosts, washed, and incubated in 2 mL of PBS, pH 7.4, at 37 °C. About 20 s later the pH in the medium was changed to 5.5 as indicated by the arrow. % FDQ was calculated according to eq 1.

Table I: Effect of Incorporation of Spin-Labeled Phospholipids into Erythrocyte Ghosts on Fusion with VSV

target	% FDQ
intact erythrocytes	1.4
+(0,2)PC	2.1
+(0,2)PS	1.5
+(0,2)PS + ascorbate	1.0
+(0,2)PE	1.9
lipid-symmetric ghosts[a]	30.1
+(0,2)PS	31.7

[a] Prepared in the presence of Ca^{2+}.

membranes is due to rapid translocation of PS and PE across the erythrocyte membrane by an aminophospholipid translocase that leads to their disposition in favor of the inner leaflet (Seigneuret & Devaux, 1984; Calvez et al., 1988). This effect is reversibly inhibited after ATP depletion of the erythrocytes and could be restored in resealed erythrocyte ghosts if hydrolyzable ATP was included in the internal medium. Figure 1 shows rapid fusion of VSV with lipid-symmetric ghosts prepared in the presence of Ca^{2+}, whereas little fusion activity was seen with ghosts prepared in the presence of Ca^{2+} and an ATP-generating system. Since the aminophospholipid translocase is functioning in the presence of intracellular Ca^{2+} and ATP (Bitbol et al., 1987), we surmise that ghosts prepared under those conditions are lipid-asymmetric. The presence of ATP in the resealing buffer either restored the asymmetry or prevented establishment of a symmetric bilayer distribution. In either case, the experiment shows that the fusion activity of VSV with erythrocyte ghosts was not due to effects of intracellular Ca^{2+}.

Headgroup Specificity. Since PS and PE are expressed on the outer leaflet of the erythrocyte membrane after symmetrization, it might be that either headgroup serves as a necessary component for VSV fusion. To test that notion, we incorporated the phospholipid analogues (0,2)PC, (0,2)PS, and (0,2)PE into the outer leaflet of membranes of intact erythrocytes and tested their effect on VSV fusion. Incorporation was about 5% of total membrane phospholipid, which corresponds to the % PS in lipid-symmetric membranes. Table I shows that incorporation of the spin-labels with PS, PE, and PC headgroups did not enhance fusion of VSV with lipid-asymmetric erythrocyte membranes. Erythrocyte membranes loaded with (0,2)PS showed the same low level of fluorescence

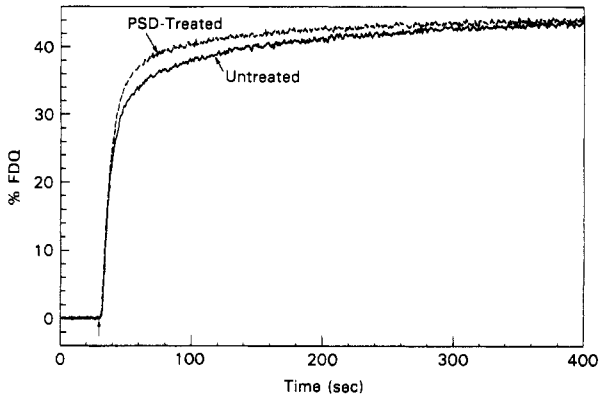

FIGURE 2: PS decarboxylase treatment of lipid-symmetric ghosts. Preparation of the ghosts and treatment with PSD were as described under Experimental Procedures. Control ghosts were incubated with the same PSD buffer without the enzyme (marked "untreated"). Fluorescence dequenching of R18 VSV was measured at pH 5.5 and 37 °C for untreated and PSD-treated erythrocyte ghosts (marked on the curves) as described in the legend to Figure 1.

dequenching in the presence and absence of ascorbate, indicating that the low fluorescence level was not due to quenching of R18 by the nitroxide radical.

To exclude the possibility that the low level of dequenching was due to an inhibitory effect of the spin-labels transferred from erythrocytes to VSV during the preincubation stage, we incorporated the spin-label into the lipid-symmetric ghosts and examined their effect on dequenching. As shown in Table I, the fluorescence increase was about the same with lipid-symmetric ghosts in the presence or absence of (0,2)PS, indicating that this compound did not have an inhibitory effect.

The failure of incorporated (0,2)PS to render lipid-asymmetric erythrocyte membranes fusogenic might be due to removal of the PS analogue by the bound virus during preincubation. However, this seems unlikely since we add about 40 virions bound per cell, which yields a total viral to erythrocyte surface area ratio of about 0.03:1 (Clague et al., 1990). Moreover, the R18 dequenching indicates a significant dilution of the dye from the virions to the erythrocyte rather than vice versa.

In Situ Treatment of Phospholipids. Treatment of lipid-symmetric ghosts with the aminophospholipid reagent trinitrobenzenesulfonic acid had little effect on fusion (data not shown). However, the experiment was compromised by quenching of R18 by the trinitrophenyl moiety, especially under conditions where low amounts of R18-labeled virus was present. A more specific reagent for phospholipids is PS decarboxylase, which converts PS to PE. Figure 2 shows that treatment of lipid-symmetric ghosts with PS decarboxylase (PSD) did not affect VSV fusion. This indicates that PS is not a necessary determinant in the fusion of VSV with target membranes. Thin-layer chromatograms of extracted erythrocyte lipids after PSD treatment indicate that all the erythrocyte PS is converted to PE (data not shown). Since the PSD treatment was carried out in the presence of 0.01% Triton X-100, the erythrocyte membranes were not intact and all of the PS was accessible to the enzyme. The detergent was removed after the treatment. Control experiments with erythrocyte membranes treated with 0.01% Triton X-100 alone followed by removal of the detergent indicate that the Triton X-100 treatment alone did not affect the fusogenic properties of the target membrane (see curve marked "untreated" in Figure 2).

If the PE headgroup were a critical factor in VSV fusion with membranes, then we would expect an enhancement of

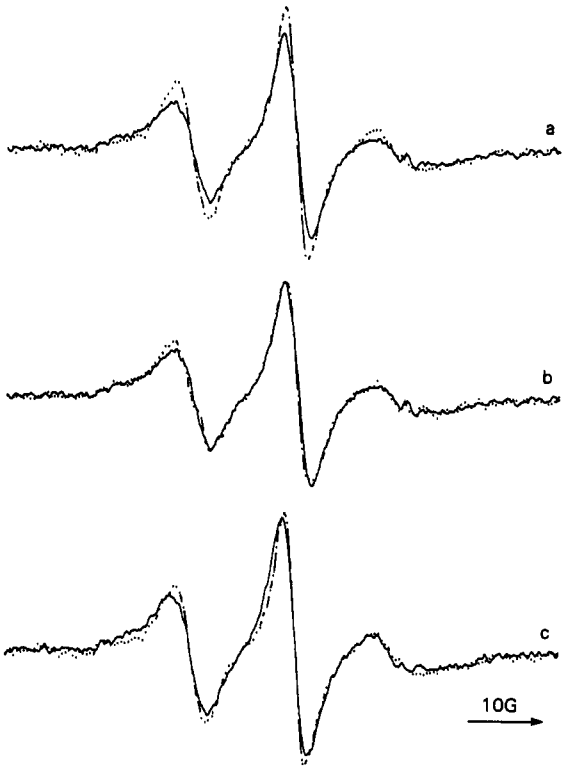

FIGURE 3: Electron spin resonance spectra of a spin-labeled phospholipid incorporated into erythrocyte ghosts. Preparation of erythrocyte ghosts with symmetric and asymmetric bilayer distributions, incorporation of the spin-labeled phospholipid (0,2)PC, and ESR measurements are described under Experimental Procedures. All spectra were in PBS, pH 7.4 at 7 °C. (a) Spectra of (0,2)PC in intact erythrocytes (—) and ghosts (···) prepared in the presence of Ca^{2+}. (b) Spectra of (0,2)PC in intact erythrocytes (—) and ghosts (···) prepared in the presence of Mg^{2+}. (c) Spectra of (0,2)PC in erythrocyte ghosts prepared in the presence of Ca^{2+} and in the presence (—) or absence of ATP (···).

fluorescence dequenching with more PE in the target membrane. Figure 2 shows that after PSD treatment there was no enhancement of fusion, indicating that the increased PE content did not have much of an effect.

Role of Lipid Packing. Since it appears that phospholipid headgroups do not play a significant role in mediating VSV fusion, we examined the role of acyl chain mobility. It has been shown that the compositional asymmetry of RBC is associated with differences in acyl chain mobility of inner and outer leaflets (Tanaka & Ohnishi, 1976; Seigneuret et al., 1984). We examined acyl chain mobility of lipid-symmetric ghosts using the spin-labeled analogue (0,2)PC. The ESR spectra at 7 °C of (0,2)PC incorporated in the outer leaflet of intact erythrocyte and ghosts membranes are shown in Figure 3. The spectrum of (0,2)PC incorporated in intact RBC is characteristic of a more restricted motion than that of the spin-label incorporated into lipid-symmetric ghosts (Figure 3a). This indicates an increase in acyl chain mobility of the outer leaflet of the lipid-symmetric ghosts, since the probe is only incorporated into the outer leaflet of the erythrocyte membranes. On the other hand, only slight differences with intact erythrocytes were seen in the spectra of (0,2)PC incorporated into lipid-asymmetric ghosts (Figure 3b) or into Ca^{2+} ghosts formed in the presence of ATP (Figure 3c). This indicates that the latter two preparations have similar outer leaflet fluidity as intact RBC. Spectra taken at 37 °C showed a similar pattern as at 7 °C. Although the line shape of the spectra is a complicated function of order parameters and rates of probe motion, one can express physical differences between membrane preparations in a semiquantitative form. By use of a formula of Keith et al. (1970), apparent correlation times for isotropic motion at 37 °C of 1.20, 0.78, and 1.02 ns for intact RBC, lipid-symmetric ghosts, and ATP-treated ghosts, respectively, are calculated.

Discussion

Although the VSV G protein has been very well studied and characterized, little is known about the exact nature of the cellular receptor for VSV on the target membrane. The extremely wide host range of VSV suggests that its cellular receptor is a component of virtually all cell plasma membranes. A number of studies have been carried out to identify receptor activity for VSV by identifying components on the target membrane that inhibit binding and infectivity. Such studies have implicated phosphatidylserine (Schlegel et al., 1983) and phosphatidylinositol or the ganglioside GM3 (Mastromarino et al., 1988) as important components involved in VSV attachment and fusion. However, an "antagonist", which inhibits the activity of a given protein, is not necessarily part of the functional activity. To study the involvement of a given membrane component in the fusion reaction, that component needs to be incorporated into the target membrane, and the fusion reaction with the modified target should then be examined directly.

Yamada and Ohnishi (1986) have studied fusion of VSV with liposomes made of various natural and synthetic lipids using spin-labeled phospholipid. They found that binding to liposomes was somewhat dependent on the headgroup of the phospholipid, stronger to phosphatidylserine than to phosphatidylcholine, but not much dependent on the acyl chain composition. On the other hand, cis-unsaturated acyl chains were required for efficient fusion, but there was only a small, if any, requirement for the headgroup.

Since some doubts might exist as to the relevance of virus–liposome fusion to penetration of virus by biological membranes (Sarkar & Blumenthal, 1988), we decided to examine this issue with biological membranes. We chose erythrocyte membranes the disposition of whose phospholipids is readily modified. We found that lipid-symmetric erythrocyte ghosts are suitable targets for VSV fusion whereas lipid-asymmetric ghosts are not. There was no phospholipid specificity for VSV fusion as indicated by a variety of experiments which involve either incorporation of spin-labeled phospholipid analogues into lipid-asymmetric ghosts or treatment of phospholipid headgroups in lipid-symmetric ghosts with chemical reagents or the enzyme PS decarboxylase. The presence of naturally occurring sialoglycolipids on the outer surface of lipid-asymmetric ghosts appears not to be sufficient for VSV fusion.

Our results with natural membranes are basically in agreement with those of Yamada and Ohnishi (1986) using liposomes, namely, that no specific phospholipid is required for VSV fusion. The congruence between liposomal and biological targets indicates that, under the proper experimental conditions, the liposome is an appropriate target for VSV fusion. The two leaflets of the normal erythrocyte bilayer differ in lipid packing, with the outer leaflet being more tightly packed than the inner leaflet (Tanaka & Ohnishi, 1976; Seigneuret & Devaux, 1984), presumably as a result of higher levels of unsaturated fatty acids in the aminophospholipids which are concentrated in the inner leaflet (Williams et al., 1966). In the lipid-symmetric ghosts, those unsaturated acyl chains have presumably moved to the outer monolayer as indicated by our ESR measurements (Figure 3). The requirement for a high proportion of cis-unsaturated phospholipids in the liposomal targets (Yamada & Ohnishi, 1986) is

consistent with our finding that VSV will only fuse with lipid-symmetric erythrocyte ghosts. Moreover, incorporation of cholesterol into the liposome appeared to enhance fusion. This is consistent with our observation that removal of cholesterol from lipid-symmetric erythrocytes by incubation with PC liposomes decreased VSV fusion (unpublished observations).

Tullius et al. (1989) have recently shown that poly(ethylene glycol) induced fusion occurred more readily with erythrocyte ghosts, where both fusing partners had a symmetric distribution of phospholipids. The enhanced PEG-induced fusion of lipid-symmetric ghosts is attributed to a decrease in packing of the exterior lipids as judged by a large increase in staining with the fluorescent dye merocyanine 540 (Tullius et al., 1989). Moreover, cell partitioning in a two-phase aqueous polymer system suggests that the surface of lipid-symmetric erythrocytes is more hydrophobic than the surface of lipid-asymmetric cells (McEvoy et al., 1986). In cellular membranes, many barriers or repulsive forces, e.g., steric, electrostatic, deformation, and hydration, exist to prevent membranes from fusing [for a review see Blumenthal (1987)]. Catalysis of membrane fusion involves interactions designed to overcome those barriers. Ohki (1988) proposed that increased "hydrophobicity" of the membrane surface, which is related to membrane interfacial tension and phospholipid packing, is a critical factor in lowering those barriers. Catalytic action of viral spike glycoproteins might then involve the generation of hydrophobic interactions between membranes.

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