Vesicular Stomatitis Virus Binds and Fuses with Phospholipid Domain in Target Cell Membranes

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Received September 6, 1985; Revised Manuscript Received December 5, 1985

ABSTRACT: Fusion of vesicular stomatitis virus with some cells (HELR 66, KB, and human erythrocytes, both intact and trypsinized) and liposomes made of various natural and synthetic lipids was studied with spin-labeled phospholipid. Binding of virus was assayed separately with radiolabeled and spin-labeled virus. Binding to cells and liposomes was small at neutral pH but enhanced at acidic pHs. Fusion with cells and liposomes was negligibly small at neutral pH but greatly activated at acidic pHs lower than 6.5. Activation of fusion occurred at lower pH values than enhancement of binding. Fusion occurred rapidly and efficiently, reaching a plateau at 50–80% after 3 min at 37 °C. Binding and fusion with cells were enhanced by pretreatment of cells with trypsin. Binding to liposomes was dependent on the head group 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 the efficient fusion, but there was only a small, if any, requirement for the head group. Cholesterol enhanced the fusion further. High fusion efficiency with cis-unsaturated phospholipids cannot be ascribed to the membrane fluidity but may be related to higher tail-to-head volume ratios. Possible mode of interaction of viral G glycoprotein with phospholipid is discussed. The virus cell entry mechanism is suggested as binding to the phospholipid domain in the cell surface membranes, endocytosis, and followed by fusion with the phospholipid domain in endosomes upon acidification.

The cell entry mechanism of enveloped viruses has recently been clarified (White et al., 1983; Ohnishi, 1985). Viruses possessing fusion activity only in acidic media are endocytosed, after adsorption onto susceptible cell surface membranes, and then fuse with endosomal membrane upon acidification of the intraendosomal lumen. The viral genome is released into the target cell cytoplasm on the fusion event. IFV,[1] SFV, and VSV are such examples. On the other hand, viruses capable of fusing at neutral as well as acidic pH such as HVJ can fuse directly with the target cell surface membrane to release their genome into the cytoplasm, although the intracellular uncoating route is also possible. Fusion of viral envelope with target cell membranes is thus a crucial initial step for infection of these viruses.

Viruses have a specific glycoprotein in their envelope that is responsible for binding to the cell surface receptors as well as fusion with the target membranes: HA in IFV, E in SFV, G in VSV, and gp52-gp36 in mouse mammalian tumor virus. HVJ is an exception in which HN is responsible for binding and F for fusion. These glycoproteins have been extensively studied and well characterized. On the other hand, the receptors have not been well characterized. Their binding with viral glycoprotein and its role in leading to fusion have not been clarified. Sialoglycoproteins and/or sialoglycolipids are known to be the receptors for a range of viruses such as HVJ and IFV. Human and murine histocompatibility antigens were identified as cell surface receptors for SFV (Helenius et al., 1978). However, these viruses can also bind and fuse with pure liposomes not containing the receptors (see Discussion).

The receptors for VSV have not yet been identified. The possibility for sialoglycoproteins and sialoglycolipids was ruled out by Schloemer and Wagner (1975). These authors showed that pretreatment of L cells by trypsin or neuraminidase resulted in increased binding and plaque formation. Mifune et al. (1982) observed a great enhancement of hemagglutination titer of VSV by pretreatment of human erythrocytes by protease. Moreover, Schlegel et al. (1983) have recently shown inhibition by PS liposomes of VSV binding and plaque formation in Vero cells. These results suggest that the target site for VSV is not sialoglycoproteins and sialoglycolipids nor proteins on the cell surface but phospholipids including PS. The present study is undertaken to investigate directly fusion of VSV with various liposomes by use of spin-labeled phospholipid. Fusion was assayed after removal of unadsorbed virus, and the fraction of virus fused of initially adsorbed ones was obtained from ESR peak height increase data. Binding of virus to liposomes was assayed separately. Binding and fusion with some cultured cells and erythrocytes, trypsinized or not, were also studied quantitatively. The results show that VSV can bind to PS as well as PC and efficiently fuse with phospholipids with cis-unsaturated acyl chains in acidic media.

Materials and Methods

Virus and Cells. VSV, New Jersey serotype, was grown in HELR 66 cells in MEM containing 0.2% bovine serum albumin at 36 °C for 18 h. Virus was pelleted by ultracentrifugation at 60000*g* for 50 min, resuspended in a small volume of Tris buffer (20 mM Tris-HCl, 140 mM NaCl, 5.4 mM KCl, pH 7.6), and stored at −80 °C. Virus concentration

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[1] Abbreviations: IFV, influenza virus; SFV, Semliki Forest virus; VSV, vesicular stomatitis virus; HVJ, hemagglutinating virus of Japan, synonym of Sendai virus; HA, hemagglutinin; PC, phosphatidylcholine; PC*, spin-labeled PC with 12-doxylstearate attached at the 2-position; DMPC, dimyristoyl-PC; DPPC, dipalmitoyl-PC; DOPC, dioleoyl-PC; DEPC, dielaidoyl-PC; DLPC, dilinoleoyl-PC; DAPC, diarachidoyl-PC; POPC, 1-palmitoyl-2-oleoyl-PC; PS, phosphatidylserine; DMPS, dimyristoyl-PS; DOPS, dioleoyl-PS; Pipes, 1,4-piperazinediethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; ESR, electron spin resonance.

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was determined as protein weight by Lowry et al. (1951), with bovine serum albumin as a standard. For ^{32}P labeling of virus, virus-bound cells were incubated in MEM not containing phosphate for 1 h and then in MEM containing [^{32}P]phosphate at 20 μCi/mL for 10 h. After ultracentrifugation, the pellet was resuspended in Tris buffer and centrifuged in a stepwise sucrose density gradient. The fraction containing virus was collected and dialyzed against Tris buffer. The specific activity was 2200 cpm/μg of virus protein. Spin-labeling of virus was carried out as described previously (Maeda et al., 1975; Kuroda et al., 1986) with a slight modification. Virus was incubated with PC* vesicles in the presence of human erythrocyte lysate (4%) at 37 °C for 1 h. The lysate contained PC transfer protein (Kuroda & Ohnishi, 1983), which catalyzed incorporation of PC* into the viral envelope. After incubation, virus was washed with Tris buffer containing bovine serum albumin and then with Tris buffer.

Trypsinization of cells (HELR 66 or KB cells) was done by treatment with 0.1% trypsin (Difco) for 20 min at 37 °C, followed by several washings with phosphate-buffered saline. Trypsinization of human erythrocytes was carried out for a longer period (1 h). Ghosts were prepared by incubating intact or trypsinized erythrocytes in a hypotonic buffer (5 mM Tris-HCl, 2 mM EDTA, pH 7.6) at 4 °C.

Phospholipid. DMPC, DPPC, DOPC, DEPC, DLPC, DAPC, and POPC were purchased from Sigma and used without further purification. DMPS and DOPS were prepared by enzymatic conversion of DMPC and DOPC, respectively, according to Comfurius and Zwaal (1977) and purified. Egg yolk PC (Singleton et al., 1965) and bovine brain PS (Sanders, 1967) were prepared as described. Total lipid was extracted from human erythrocyte ghosts according to Folch et al. (1951). PC* was synthesized according to Hubbell and McConnell (1971). Phospholipids in benzene or chloroform were stored at −20 °C. Phospholipid concentration was determined by the method of Bartlett (1959).

Liposomes were prepared by agitation of phospholipid in a Pipes buffer (5 mM Pipes, 140 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, pH 7.6) with a thermomixer at a temperature higher than the phase transition [at room temperature (23 °C) or 45 °C].

Binding to Cells. ^{32}P-Labeled virus (8 μg) or PC*-labeled virus (40 μg) was added to 2 × 10^6 HELR cells or 25 μL of 10% (v/v) human erythrocytes and incubated at 4 °C for 1 h in a MES buffer (140 mM NaCl, 40 mM MES) at various pH values. The cells were washed with the same buffer and centrifuged. The amount of virus in the pellet was determined at a room temperature by radioactivity measurements with a liquid scintilation counter or by ESR peak height measurements after pH readjustment to 7.5.

Hemagglutination and Hemolysis. Virus and trypsinized erythrocytes were mixed and diluted with MES buffer at various pH values. Hemagglutination unit was determined according to Salk (1944). For hemolysis, virus was mixed with 25 μL of 10% (v/v) trypsinized erythrocytes and incubated for 15 min at 4 °C. Then, 1 mL of MES buffer at various pH values was added and incubated for 30 min at 37 °C. After centrifugation, the optical density of the supernatant at 520 nm was measured.

Binding to Liposomes. Virus (40 μg) was mixed with various concentrations of liposomes in 0.16 mL of citrate or phosphate buffer at various pH values (140 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, and 80 mM citrate for pH 5.5–6.0 or 80 mM phosphate for pH 6.2–7.5) and incubated at 4 °C for 30 min. Virus bound to liposomes was separated from unbound virus by a stepwise sucrose density gradient centrifugation as follows. An equal volume of 60% (w/w) sucrose was added to the incubation mixture and layered on 50 μL of 60% sucrose in citrate or phosphate buffer. Then, 1 mL of 25% sucrose was put on the sample layer, and finally, 0.2 mL of the buffer was put on the 25% layer. After centrifugation at 4 °C for 1 h at 9000g, virus-bound liposomes and free liposomes floated to the 0–25% interface and unbound virus sedimented to the 30–60% interface. The upper and lower fractions were collected and mixed with 10% n-octyl β-D-glucopyranoside to solubilize liposomes and then with 0.2 M Hepes at pH 7.5. The fluorescence intensity of the fractions at 330 nm with excitation at 280 nm was measured to quantitate protein. Fraction of bound virus was obtained by $I_u/(I_u + I_l)$ where I_u and I_l are the fluorescence intensity of upper and lower fractions, respectively. In the absence of liposomes, only a negligible fraction of virus (<3%) floated to the upper fraction.

Envelope Fusion Assay. Fusion of virus with cells or liposomes was assayed by transfer of PC* from the virus envelope to the target membranes as described previously (Kuroda et al., 1986). Spin-labeled virus (80–100 μg) was mixed with 2 × 10^6 KB or HELR cells in MES buffer at pH 6.5 and kept for 20 min at 4 °C for adsorption. The mixture was then added with MES buffer at various pH values and centrifuged at 500g for 30 s. Spin-labeled virus (60 μg) was mixed with 0.1 mL of trypsinized ghosts (50% v/v) in hemolysis buffer, kept for 10 min at 4 °C, and centrifuged at 12000g for 30 s. The resulting pellet was resuspended in 20 μL of MES buffer at various pH values and sucked into a capillary tube at 4 °C, and the ESR spectrum was measured repeatedly at 37 °C. Reason for the use of ghosts instead of intact erythrocytes was that the transfer of PC* in this case was solely due to envelope fusion since ghosts do not contain PC transfer protein (Kuroda et al., 1986). Fusion with liposomes was assayed by mixing spin-labeled virus (40 μg) with liposomes (0.4 μmol) at neutral pH for 10 min at 4 °C, followed by centrifugation at 12000g for 90 s to remove unadsorbed virus. The pellet was resuspended in 20 μL of citrate or phosphate buffer at various pH values, and the ESR spectrum was measured at 37 °C.

The fraction of fused virus was obtained by $(H - H_V)/(H_M - H_V)$ where H, H_V, and H_M were the normalized ESR central peak height of the sample at appropriate time, that of spin-labeled virus, and that of cells or liposomes containing a small concentration of PC*. In the present assay, the concentration of PC* in virus was ca. 10% of viral lipid and that of PC* in ghost and in liposome membranes after complete fusion was ca 0.2%, which is indeed very dilute with negligible spin–exchange interactions.

Results

Binding to Cells. Binding of virus to cells was not inhibited by trypsin treatment of cells but rather enhanced. The enhancement was quite pronounced for human erythrocytes; the percentage of bound virus increased from 4 to 36% at pH 6.5. Accordingly, virus agglutinated trypsinized erythrocytes but not intact erythrocytes. On the other hand, the enhancement was slight for HELR cells; the binding to untreated and trypsinized cells was respectively 17 and 21% at pH 5.5, 16 and 25% at pH 6.5, and 4 and 4% at pH 7.5.

The virus binding was small at neutral pH but enhanced at pH values lower than 7.0 (Figures 1a and 2Aa). At still lower pH values (<6.0), the binding became small again, thus yielding an optimum at around pH 6.5 for both trypsinized HELR cells and erythrocytes. Trypsinization did not modify the pH dependence for HELR cells. The virus-induced hem-

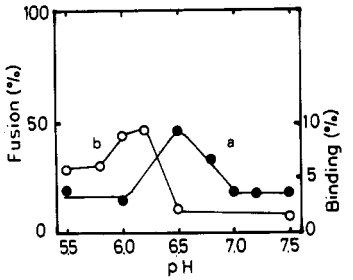

FIGURE 1: pH dependence of binding (a) and fusion (b) of VSV with HELR 66 cells. Binding was assayed after incubation of ^{32}P-labeled virus (8 μg) with trypsinized cells (2×10^6) at 4 °C for 1 h at various pH values. Envelope fusion was assayed with spin-labeled virus (100 μg) adsorbed onto trypsinized cells (2×10^6). Unadsorbed virus was removed by centrifugation. Percentage of fused virus of initially adsorbed virus was obtained after 10 min at 37 °C by the ESR peak height as described under Materials and Methods.

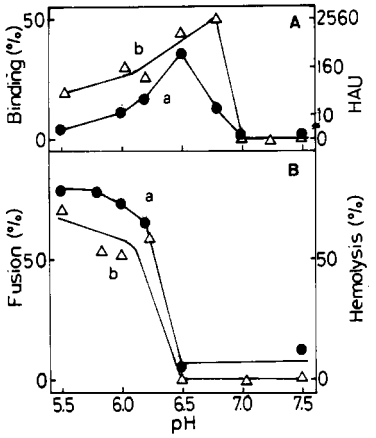

FIGURE 2: pH dependence of binding (A) and fusion (B) of VSV with trypsinized erythrocytes. (A) Data for both binding (a) and hemagglutination (b) are given. Binding was assayed after incubation of ^{32}P-labeled virus (8 μg) with 25 μL of trypsinized erythrocytes (10% v/v) at 4 °C for 1 h. The hemagglutination titer was assayed as described under Materials and Methods. (B) Data for both envelope fusion (a) and hemolysis (b) are given. Envelope fusion was assayed with spin-labeled virus (60 μg) adsorbed onto 0.1 mL of trypsinized erythrocyte ghosts (50% v/v). Percentage of fused virus after 10 min at 37 °C or that of hemolyzed erythrocytes after incubation at 37 °C for 30 min is presented.

agglutination had a pH dependence similar to that of binding as expected (Figure 2Ab).

Fusion with Cells. Fusion of VSV with cells was negligibly small at neutral pH but markedly enhanced at acidic pH. Figure 3 shows time course of fusion with trypsinized erythrocyte ghosts (a), intact KB cells (b), and intact HELR cells (c). Fusion with trypsinized erythrocytes proceeded very rapidly and efficiently, reaching a plateau at 80% fusion after 3 min at pH 5.5. Fusions with KB and HELR cells were also rapid but less efficient, 30 and 20% after 5 min at pH 5.5, respectively.

Fusion with intact erythrocytes was negligibly small. Trypsinization therefore greatly enhanced the fusion to an efficiency of 80%. Trypsinization of KB cells also enhanced the fusion. However, the enhancement was not so large; the fusion efficiency after 5 min at 37 °C at pH 5.5 was 30% for intact cells, 34% for cells trypsinized for a shorter period (10 min), and 45% for cells treated for a longer period (20 min). Trypsinization of HELR cells also caused a slight enhancement. It also modified the time course of fusion. Fusion continued to occur for longer period, instead of reaching a clear plateau.

The pH dependence of fusion is shown in Figures 1b and 2Ba. The threshold pH was 6.5 for both HELR cells and

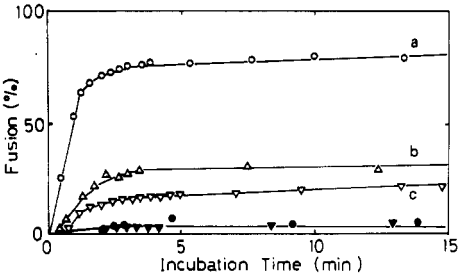

FIGURE 3: Time course of fusion of VSV with cells: trypsinized erythrocyte ghosts (a), intact KB cells (b), and intact HELR cells (c). Spin-labeled virus was mixed with respective cells at 4 °C for the appropriate time and centrifuged to remove unadsorbed virus. The ESR spectrum of the virus-bound cells resuspended in buffer at pH 5.5 (open symbols) or pH 7.5 (closed symbols) was measured at 37 °C repeatedly. Percentage of fused virus was calculated from the ESR peak height as described under Materials and Methods.

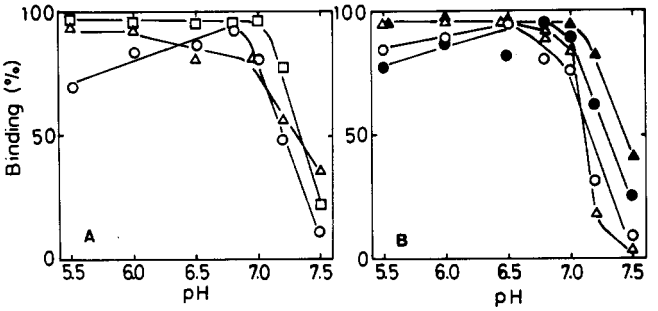

FIGURE 4: pH dependence of binding of VSV to liposomes: (A) erythrocyte total lipid (□), ox brain PS (△), and egg yolk PC (○); (B) DOPS (▲), DOPC (●), DMPS–cholesterol (1:1) (△), and DMPC–cholesterol (1:1) (○). Percentage of bound virus was assayed after incubation of virus (40 μg) with lipid (0.2 μmol of phospholipid) for 30 min at 4 °C.

trypsinized erythrocytes. Fusion with HELR cells became less efficient at still lower pH values (<5.8), thus yielding a broad optimum around pH 6.0–6.2 (Figure 1b). On the other hand, fusion with erythrocytes did not decline at lower pH values (Figure 2Ba). The virus-induced hemolysis showed the same pH dependence as that of fusion Figure 2Bb).

At low temperatures, 4–7 °C, fusions with HELR cells and with ghosts and also the virus-induced hemolysis did not occur at pH 5.5. This justifies assignment of cell-associated viruses after incubation at 4 °C to those bound but not fused.

The pH dependence of fusion was different from that of binding. Both binding and fusion for HELR cells had an optimum, but the optimum as well as the threshold for fusion shifted to lower pH values (Figure 1). Binding to erythrocytes had an optimum, but fusion did not have such an optimum (Figure 2Aa,Ba). The threshold pH for fusion (6.5) was again lower than that for binding (7.0).

Pretreatment of virus at acidic pH caused irreversible loss of hemolytic activity as in the case for IFV (Sato et al., 1983). The decrease in activity occurred by pretreatment at pH values lower than 7.0 in MES buffer for 30 min at 4 °C, and the complete loss occurred at pH values lower than 6.0 (data not shown).

Binding to Liposomes. Binding of VSV to liposomes was quite large at acidic pHs and decreased at pH values higher than 7.0 (Figure 4). The pH dependence was similar both for natural and synthetic phospholipids, irrespective of difference in the head group and the acyl chain composition and also of the presence and absence of cholesterol.

There appeared to be no distinct preferability for the head group as well as the acyl chain composition in the binding (see Table I). The percentage of bound virus at acidic pH was

Table I: Binding and Fusion of VSV with Liposomes[a]

lipid	binding (%)		fusion (%)	
	pH 5.5	pH 7.5	pH 5.5	pH 7.5
erythrocyte total lipid	97	22	80	4
PC				
egg yolk	67	15	42	7
dimyristoyl	95	16	8	4
+cholesterol	73	9		
dipalmitoyl			12	10
dielaidoyl			25	4
dioleoyl	82	30	52	6
+cholesterol	87	26	89	13
dilinoleoyl			74	5
+cholesterol			79	19
1-palmitoyl-2-oleoyl			42	3
+cholesterol			65	6
diarachidoyl + dimyristoyl (2:3)			39	29
+cholesterol			29	6
PS				
ox brain	78	27	52	6
dimyristoyl			47	24
+cholesterol	90	10	27	5
dioleoyl	97	55	63	6
+cholesterol	84	31	85	10

[a] Binding was assayed after incubation of VSV (40 μg) with liposomes (0.2 μmol of phospholipid) at 4 °C for 30 min. Percentage of fused virus of initially adsorbed virus was obtained from the ESR peak height increase after 10 min at 37 °C. Cholesterol was contained at a 1:1 molar ratio when indicated.

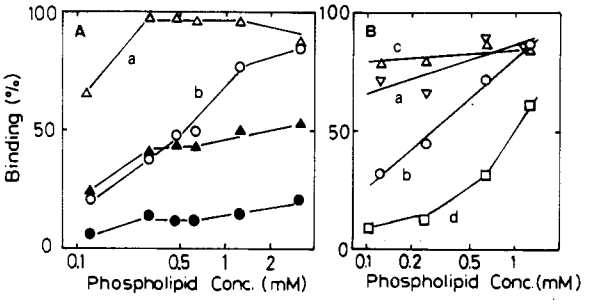

FIGURE 5: Binding of VSV to liposomes as a function of the latter concentration: (A) DOPS (a) and DOPC (b); (B) 1:1 mixtures of cholesterol and DOPS (a), DOPC (b), DMPS (c), and DMPC (d). Binding was assayed after incubation of virus (40 μg) in 0.16 mL of the indicated concentration of phospholipid at pH 5.5 for 30 min at 4 °C. (A) Data for binding at pH 7.5 (closed symbols) are also presented.

97% for erythrocyte total lipid, 67% for egg yolk PC, and 78% for bovine brain PS. Similarly, the binding to synthetic phospholipids with homogeneous acyl chains was 96% for DMPC, 83% for DOPC, and 97% for DOPS. However, when the amount of liposomes was made smaller, a distinct preferability for the head group, phosphorylserine to phosphorylcholine, was disclosed (Figure 5A). The binding to DOPS was nearly the same as that to DOPC in the presence of a large amount of liposomes (0.2 μmol/40 μg of virus) but much larger than that to DOPC (66% vs. 21%) at a smaller amount of phospholipid (0.02 μmol/40 μg of virus). The same tendency was observed when liposomes contained cholesterol (Figure 5B). Binding to DOPS–cholesterol and to DMPS–cholesterol did not decrease when the amount of liposomes was reduced, while binding to DOPC–cholesterol and to DMPC–cholesterol greatly decreased. Such difference in the concentration dependence may represent a larger binding constant of virus to PS than that to PC. In the presence of larger amounts of liposomes, the binding appeared to saturate for both classes of phospholipids.

Binding to liposomes at neutral pH was small. However, a preferability for DOPS to DOPC in the binding at pH 7.5

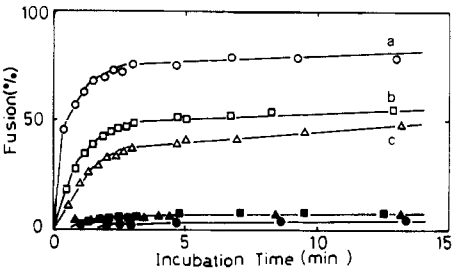

FIGURE 6: Time course of fusion of VSV with liposomes: erythrocyte total lipid (a), ox brain PS (b), and egg yolk PC (c). Spin-labeled virus (40 μg) was mixed with 0.4 μmol of phospholipid at 4 °C for 10 min and centrifuged. The ESR spectrum of the pellet resuspended in buffer at pH 5.5 (open symbols) or pH 7.5 (closed symbols) was measured at 37 °C.

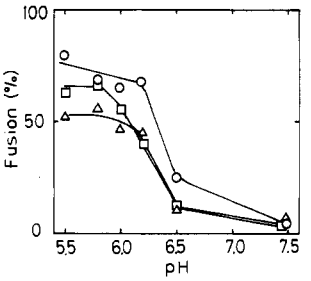

FIGURE 7: pH dependence of fusion of VSV with liposomes: erythrocyte total lipid (○), DOPS (□), and DOPC (△). Percentage of fused virus was obtained after 10 min at 37 °C. The assay condition was the same as that in Figure 6.

was observed as shown in Figure 5A.

The pH dependence of binding to liposomes was different from that of binding to cells. While the latter had an optimum pH at around 6.5, binding to liposomes did not decrease at lower pH values.

Fusion with Liposomes. VSV fused with liposomes as rapidly and efficiently at acidic pH but negligibly at neutral pH as with cell membranes. Figure 6 shows the time course of fusion with liposomes made of natural lipids, erythrocyte total lipid, bovine brain PS, and egg yolk PC. The fusion reactions came to near saturation after 3 min at 37 °C at pH 5.5. The fusion efficiency at saturation was very high for erythrocyte lipid (80%) and moderate for bovine PS (53%) and egg PC (41%). The threshold pH for the fusions was 6.5 (Figure 7), nearly the same as that for fusions with cells. The pH dependence was similar to that for trypsinized erythrocytes but different from that for HELR cells that had an optimum.

Fusion with liposomes made of various synthetic phospholipids was studied to determine whether there are any specific requirements for the head group and/or the acyl chains as the target. VSV fused rapidly and efficiently with phospholipids with cis-unsaturated acyl chains, independent of the head group, phosphoserine or phosphocholine (see Table I). The rate, efficiency, and pH dependence of fusion were quite similar to those for natural lipids (Figure 7).

Fusions with DOPC, DLPC, and DOPS at pH 5.5 were efficient (52, 74, and 63%, respectively), but those with DMPC and DPPC were negligibly small (8 and 12%, respectively). Binding to these saturated and unsaturated phospholipids was not different from each other as described above. Fusion with PC with trans-unsaturated acyl chains (DEPC) occurred but to a small extent (25%). Fusion with PC with saturated acyl chain at the 1-position and cis-unsaturated acyl chain at the 2-position (POPC) was also efficient (42%). Inclusion of cholesterol to these cis-unsaturated phospholipids enhanced the fusion to various extents (see Table I). DMPS appears

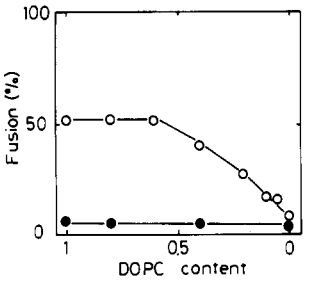

FIGURE 8: Fusion of VSV with liposomes made of mixed DOPC and DMPC as a function of the mixing ratio. Percentage of fused virus was obtained after 10 min at 37 °C at pH 5.5 (○) or at pH 7.5 (●).

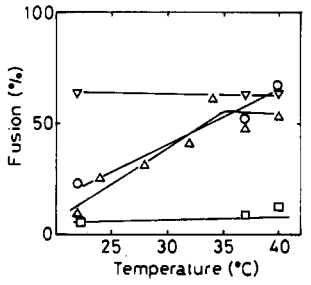

FIGURE 9: Temperature dependence of fusion of VSV with liposomes: DOPS (▽), DOPC (○), DMPS (△), and DMPC (□). Percentage of fused virus was obtained after 10 min at pH 5.5 at the indicated temperature. Other experimental conditions are the same as in Figure 6.

as an exception; in contrast to saturated PCs, it can be a target for VSV to fuse with, though to a small extent (47%). Fusion at neutral pH also occurred though to a still smaller extent (24%).

Fusion with liposomes made of a mixture of active DOPC and inactive DMPC was studied as a function of the mixing ratio (Figure 8). Fusion at pH 5.5 occurred as efficiently as that of pure DOPC liposomes until the DOPC content was 60%, below which the efficiency decreased. DAPC was chosen as a target with four cis unsaturations. Since it will not form stable liposomes by itself, a mixture with DMPC at a molar ratio of 2:3 was used. Fusion occurred at an efficiency of 39% at pH 5.5 (Table I). Fusion also occurred at neutral pH, though to a smaller extent (29%). Occurrence of fusion at neutral pH is a different characteristic from that of fusions with natural membranes and lipids.

The temperature dependence of fusion with some synthetic phospholipids was studied in a range from 20 to 40 °C (Figure 9). Fusion with DOPS did not appreciably change in that temperature range, whereas fusion with DOPC decreased with decrease in temperature. Fusion with DMPS also decreased with temperature. At low temperatures (4 °C) where the binding assay was made, there were no fusions even with DOPS at pH 5.5, justifying the assay.

Discussion

VSV bound to cells and liposomes. The binding was enhanced at acidic pHs lower than 7.0, but there was also a small amount of binding at neutral pH. Binding to the cell surface increased on pretreatment of cells with trypsin. Binding to liposomes was dependent on the head group, stronger to PS than to PC, but not much dependent on the acyl chain composition. VSV can therefore bind to phospholipid domains in the cell surface membrane, more strongly to PS-rich domain but also to PC-rich domain. In erythrocytes, the cell surface proteins apparently prevent access of virus to phospholipid domains. This is also true for other cells, but the inhibition was much weaker. Our results on PS binding are consistent with inhibition of binding and infection of VSV in Vero cells by PS liposomes (Schlegel et al., 1983). These authors observed failure of complete inhibition by various phospholipids other than PS and suggested plasma membrane PS to be a binding site for VSV. However, their data also showed that higher amounts of PC liposomes inhibited VSV binding, though much weakly. We emphasize that PC-rich domains are also the binding site for VSV. This may be biologically significant since PS is located in the inner layer of cell surface membranes as observed for several instances (Op den Kamp, 1979). Schloemar and Wagner (1975) showed that neuraminidase and/or trypsin treatments of VSV resulted in loss of its binding to L cells. The sialoglycopeptide segment in G may therefore be involved in the virus binding to phospholipid domains.

VSV adsorbed onto phospholipid domains in the target membrane can then fuse rapidly and efficiently with them upon acidification of the environments. Fusion with cells and liposomes was rapid and efficient at acidic pH values lower than 6.5. Studies using synthetic phospholipids disclosed a distinct requirement for cis-unsaturated acyl chains for the efficient fusion but only a small, if any, requirement for the head group, in contrast to the requirement for binding. Not all acyl chains are needed to be cis unsaturated, but about 50% unsaturation appears to be sufficient [see results on POPC (Table I) and on mixed DMPC–DOPC (Figure 8)]. Cholesterol enhanced the fusion efficiency further. Rapid and efficient fusion with natural lipids and cell membranes can therefore be well understood since they consisted of various classes of phospholipids with more than 50% of cis-unsaturated acyl chains, and plasma membranes contained cholesterol at high contents [see White (1973)].

Eidelman et al. (1984) have studied fusion of reconstituted G-containing vesicles with various liposomes. The method does not dissociate binding and fusion processes. They observed faster fusion with PS or PA but only slow fusion with PC. However, the pH dependence largely shifted to lower pH, and the threshold was at about pH 5.

Cis-unsaturated phospholipids have a markedly lower phase transition temperature than saturated or trans-unsaturated phospholipids. However, this does not mean a large difference in the liposome membrane fluidity compared to that at the same temperature in the liquid-crystalline phase. ESR spectra of PC* incorporated at a small concentration in various liposomes such as DMPC, DEPC, DOPC, POPC, DLPC, DMPS, and DOPS were quite similar to each other at 37 °C. Moreover, the lateral diffusion constants in various synthetic and natural phospholipid membranes were not very different from each other when compared at the same temperature. Cholesterol markedly decreased the membrane fluidity; the overall splitting value of the ESR spectra for the above liposomes was greatly increased from ca. 33 G to 42–45 G at 37 °C on inclusion of cholesterol at a 1:1 molar ratio in them. On the other hand, it enhanced fusion for cis-unsaturated phospholipid liposomes. Therefore, the membrane fluidity alone cannot be a major factor to govern the fusibility.

High fusion efficiency with cis-unsaturated phospholipids may be related to a larger volume occupied by the acyl chains than trans-unsaturated and saturated phospholipids. Higher tail-to-head volume ratios favor the hexagonal phase or inverted micelle formation (Israelachvili et al., 1980), and involvement of inverted micelles in fusion is proposed with strong evidence (Cullis & Hope, 1978). Hexagonal phase and inverted micelles have been observed for phosphatidylethanolamine, phosphatidic acid, and cardiolipin in the presence of

Ca^{2+} and glycerol monooleate (Cullis & de Kruijff, 1979). However, no hexagonal phase has been observed for cis-unsaturated PCs nor PSs. It is speculated, therefore, that interaction of G with target membranes may locally induce formation of inverted micelles consisting mainly of cis-unsaturated phospholipids. The effect of cholesterol may be consistent with this idea since cholesterol, another molecule with a high tail-to-head volume ratio, was shown to favor hexagonal phase formation in certain situations.

Can any specific segment(s) in G glycoprotein be responsible for the interaction with target membrane lipid domains to cause fusion? All fusogenic viral glycoproteins so far examined have a hydrophobic sequence consisting of about 20 amino acid residues: the amino-terminal segment in HA_2 in IFV, in F_1 in HVJ, and in gp36 in mouse mammalian tumor virus and residues from 80 to 101 in E_1 in SFV. By analogy, a hydrophobic sequence from 107 to 140 within G may be responsible for the interaction with target membranes (Asano & Asano, 1984). Schlegel and Wade (1985) have recently shown that a peptide corresponding to the N-terminal 25 amino acids of G had a pH-dependent hemolytic activity. Therefore, this terminal peptide can also be a candidate for the interacting segment. However, the peptide contained five basic residues, which may inhibit interaction with lipid bilayer because of the charge. They also showed that a much smaller peptide consisting only of six residues had hemolytic activity.

Binding and fusion with cells and liposomes were enhanced on lowering the pH. However, the response was different between binding and fusion; enhancement of binding occurred at higher pH values than activation of fusion. This is true for both cells and liposomes. This suggests a domain structure in G protein; segment(s) with or surrounded by amino acid residues of higher p*K* values are responsible for the binding and others with or surrounded by lower p*K* values for the fusion. The binding domain may contain sialoglycopeptide, and the fusogenic domain may include the hydrophobic sequence 107–140 as discussed above. Such domain structure has been demonstrated for HA in IFV; the receptor-binding domain is located near the distal ridge, and the putative fusogenic segment, N-terminal segment of HA_2, is buried deep inside the molecule (Wilson et al., 1981).

VSV bound and fused with liposomes made of pure lipid. This is not exceptional since HVJ (Haywood & Boyer, 1984), IFV (Maeda et al., 1981; White et al., 1982), and SFV (White & Helenius, 1980), which normally bind to the cell surface receptors, can also bind and fuse with liposomes not containing receptors. Fusion of these viruses bound to the cell surface receptors would also take place with phospholipid domains in the membranes. Role of the receptors may therefore be to increase efficiency of fusion by catching and keeping virus close to the cell surface. In the presence of high concentrations of target lipids, as in most model systems, virus has ample opportunity to encounter and bind to them. Or, in the present system, the bound virus was pelleted down together with target lipids.

This study suggests that VSV binds to phospholipid domains in target cell surface membrane, is endocytosed, and fused with the phospholipid domains in endosomes when the intraendosomal lumen was acidified to pH values lower than 6.5. Endocytosis of virus particles adsorbed onto phospholipid domains would then be an interesting problem.

ACKNOWLEDGMENTS

We express our sincere thanks to Dr. M. Ohuchi for HELR 66 cells.

Registry No. DMPC, 18194-24-6; DPPC, 63-89-8; DEPC, 56782-46-8; DOPC, 4235-95-4; DLPC, 998-06-1; POPC, 26853-31-6; DAPC, 61596-53-0; DMPS, 64023-32-1; DOPS, 70614-14-1; cholesterol, 57-88-5.

REFERENCES

Asano, A., & Asano, K. (1984) *Tumor Res. 19*, 1–20.
Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466–468.
Comfurius, P., & Zwaal, R. F. A. (1977) *Biochim. Biophys. Acta 488*, 36–42.
Cullis, P. R., & Hope, M. J. (1978) *Nature (London) 271*, 672–674.
Cullis, P. R., & de Kruijff, B. (1979) *Biochim. Biophys. Acta 559*, 399–420.
Eidelman, O., Schlegel, R., Tralka, T. S., & Blumenthal, R. (1984) *J. Biol. Chem. 259*, 4622–4628.
Folch, J., Ascoli, I., Lees, M., Meath, J. A., & LeBaron, F. N. (1951) *J. Biol. Chem. 191*, 833–841.
Haywood, A. M., & Boyer, B. P. (1984) *Biochemistry 23*, 4161–4166.
Helenius, A., Morin, B., Fries, E., Simmons, K., Robinson, P., Schirrmacher, V., Terhorst, C., & Strominger, J. L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3846–3850.
Hubbel, W. L., & McConnell, H. M. (1971) *J. Am. Chem. Soc. 93*, 314–326.
Isaraelachvili, J. N., Marcelja, S., & Horn, R. G. (1980) *Q. Rev. Biophys. 13*, 121–200.
Kuroda, K., & Ohnishi, S. (1983) *J. Biochem. (Tokyo) 94*, 1809–1913.
Kuroda, K., Kawasaki, K., & Ohnishi, S. (1986) *Biochemistry* (in press).
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 192*, 265–275.
Maeda, T., Asano, A., Ohki, K., Okada, Y., & Ohnishi, S. (1975) *Biochemistry 14*, 3736–3741.
Maeda, T., Kawasaki, K., & Ohnishi, S. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4133–4137.
Mifune, K., Ohuchi, M., & Mannen, K. (1982) *FEBS Lett. 137*, 293–297.
Ohnishi, S. (1985) in *Biomolecules: Electronic Aspects* (Nagata, C., Hatano, M., Tanaka, J., & Suzuki, H., Eds.) pp 227–252, Japan Scientific Societies Press, Tokyo, and Elsevier, Amsterdam.
Op den Kamp, J. A. F. (1979) *Annu. Rev. Biochem. 48*, 47–71.
Salk, J. E. (1944) *J. Immunol. 79*, 87–98.
Sanders, H. (1967) *Biochim. Biophys. Acta. 144*, 485–487.
Sato, S. B., Kawasaki, K., & Ohnishi, S. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3153–3157.
Schlegel, R., & Wade, M. (1983) *J. Virol. 53*, 319–323.
Schlegel, R., Tralka, T. S., Willingham, M. C., & Pastan, I. (1983) *Cell (Cambridge, Mass.) 32*, 639–646.
Schloemer, R., & Wagner, R. R. (1975) *J. Virol. 15*, 882–893.
Singleton, W. S., Gray, M. S., Brown, M. L., & White, J. L. (1965) *J. Am. Oil Chem. Soc. 42*, 53–56.
White, D. A. (1973) in *Form and Function of Phospholipid* (Ansell, G. B., Hawthorne, J. N., & Dawson, R. M. C., Eds.) Chapter 16, Elsevier Scientific, Amsterdam, New York, and London.
White, J., & Helenius, A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3273–3277.
White, J., Kartenbeck, J., & Helenius, A. (1982) *EMBO J. 1*, 217–222.
White, J., Kielian, M., & Helenius, A. (1983) *Q. Rev. Biophys. 16*, 151–195.
Wilson, I. A., Skehel, J. J., & Wiley, D. C. (1981) *Nature (London) 289*, 366–373.