Abstract. Fusion of rabies virus with membranes is triggered at low pH and is mediated by the viral glycoprotein (G). The rabies virus-induced fusion pathway was studied by investigating the effects of exogenous lipids having various dynamic molecular shapes on the fusion process. Inverted cone-shaped lysophosphatidylcholines (LPCs) blocked fusion at a stage subsequent to fusion peptide insertion into the target membrane. Consistent with the stalk-hypothesis, LPC with shorter alkyl chains inhibited fusion at lower membrane concentrations and this inhibition was compensated by the presence of oleic acid. However, under suboptimal fusion conditions, short chain LPCs, which were translocated in the inner leaflet of the membranes, considerably reduced the lag time preceding membrane merging, resulting in faster kinetics of fusion. This indicated that the rate limiting step for fusion is the formation of a fusion pore in a diaphragm of restricted hemifusion. The previously described cold-stabilized prefusion complex was also characterized. This intermediate is at a well-advanced stage of the fusion process when the hemifusion diaphragm is destabilized, but lipid mixing is still restricted, probably by a ring-like complex of glycoproteins. I provide evidence that this state has a dynamic character and that its lipid organization can reverse back to two lipid bilayers.

Key words: rhabdovirus • prefusion complex • liposome • viral fusion glycoprotein • lysophosphatidylcholine

Introduction

Entry of enveloped viruses into host cells requires fusion of the viral envelope with a cellular membrane. This step is mediated by virally encoded fusogenic glycoproteins. Activation of the fusion capacity involves structural rearrangement of these proteins upon interaction with specific triggers (e.g., low pH or specific receptors). It is generally assumed that these conformational changes result in the exposure of a fusion peptide or domain, which then interacts with and destabilizes one or both of the participating membranes.

Despite extensive work, mainly on influenza virus, the actual mechanism of the fusion process is still unknown. However, recent results have revealed striking similarities between different fusion systems. Experiments performed on viral or cellular membrane fusion have shown that inverted cone-shaped lysolipids (which promote a micellar positive spontaneous curvature) inhibit fusion when present in the contacting monolayers of membranes (Chernomordik et al., 1993, 1995; Vogel et al., 1993; Yagle et al., 1994). These results have indicated that early lipidic fusion intermediates are similar from one system to another and that membrane fusion may proceed via the formation of stalk intermediates that are local lipidic connections with negative curvatures between contacting monolayers of fusing membranes (Siegel, 1993; Chernomordik et al., 1995). This step would be followed by the formation of a transient hemifusion diaphragm. Depending on the experimental system, hemifusion might be restricted (i.e., without lipid flux between both membranes) or unrestricted (i.e., without any restriction of lipid diffusion). Unrestricted hemifusion has been shown to be the final state of fusion mediated by engineered HA ectodomain anchored into the membrane by glycosylphosphatidylinositol (GPI-HA; Kemble et al., 1994; Melikyan et al., 1995b), whereas restricted hemifusion is encountered when HA-induced fusion is reversibly blocked at 4°C (Schoch et al., 1992; Chernomordik et al., 1998). Restriction of lipid flux has been suggested to be due to a ring-like aggregate of HA surrounding the hemifusion diaphragm (Chernomordik et al., 1998). Finally, complete fusion would occur by pore formation and enlargement in the hemifusion diaphragm (Melikyan et al., 1997; Chernomordik et al., 1998).

A study characterizes the characteristics of the fusion pore and lipid dependence of fusion appear to be similar in all biological fusion events (M onck and Fernandez, 1992; Chernomordik et al.,...
1995), this model for membrane fusion (i.e., stalk formation followed by hemifusion and pore formation) has been proposed to be universal. Although it is also supported by theoretical considerations on energetics of lipidic structures (Siegel, 1993), this model is mainly based on experiments performed on influenza virus hemagglutinin (HA) \(^1\) (Chernomordik et al., 1997, 1998) and remains to be challenged in other fusion systems.

Rabies virus-induced membrane fusion is mediated by the viral transmembrane glycoprotein (G) which is organized in trimers (three monomers of 65 kDa each; Whitt et al., 1991; Gaudin et al., 1992). The fusion properties of rabies virus have been investigated (Gaudin et al., 1993). Fusion is triggered at low pH, optimal around pH 5.8–6, and is not detected above pH 6.3. Fusion of rabies virus with liposomes is preceded by a lag time, the duration of which increases with lower temperature and higher pH (up to the pH threshold for fusion). Preincubation of the virus below pH 6.75 in the absence of a target membrane leads to inhibition of viral fusion properties. However, this inhibition is reversible and readjusting the pH to above 7 leads to the complete recovery of the initial fusion activity. This is the main difference between rhabdoviruses (such as rabies virus) and other viruses fusing at low pH for which low pH-induced fusion inactivation is irreversible (Gaudin, 2000).

Low pH-induced conformational changes of the glycoprotein and their relationships with fusion activity have been studied. It has been demonstrated that G can assume at least three different states (Gaudin et al., 1993): the native (N) state detected at the viral surface (pH 7); the activated (A) hydrophobic state; and the fusion inactive conformation (I). There is a pH-dependent equilibrium between these states that is shifted toward the I state at low pH. The A state is detected immediately after acidification, induces the formation of viral aggregates stabilized at low pH and low temperature (Gaudin et al., 1993), and interacts with the target membrane as a first step of the fusion process (Durrer et al., 1995). The I state is detected after prolonged incubation at low pH. In the I conformation, G is longer than in the N conformation, but also antigenically distinct and more sensitive to proteases. The exact role of I during the fusion process is unclear, but it has been proposed that the role of the I state is to avoid fusion during transport of G in the acidic Golgi vesicles (Gaudin et al., 1995). For vesicular stomatitis virus (VSV), another rhabdovirus, the same three states have been postulated on the basis of kinetics studies (Clague et al., 1990; Puri et al., 1992).

The domain of rabdoviral G interacting with the target membrane (the so-called fusion peptide) has been located using hydrophobic photolabeling (Durrer et al., 1995) and by directed mutagenesis (Li et al., 1993; Zhang and Hosh, 1994; Fredericksen and Whitt, 1995, 1996). These results have suggested that an uncharged domain of VSV G from amino acid 118 to 136 and the homologous domain of rabies virus constitute the fusion peptide of the glycoprotein.

Recent structural studies of the fusion glycoprotein of influenza virus, retroviruses, Ebola virus, and Simian Virus 5 (a paramyxovirus) have indicated remarkable similarities (Bolluough et al., 1994; Fass et al., 1996; Chan et al., 1997; Weissenhorn et al., 1997, 1998; Baker et al., 1999; Kóbe et al., 1999). All these fusion protein ectodomains form rod-shaped α-helical bundles. In the structural studies, both the COOH-terminal membrane anchor and the NH₂-terminal fusion peptide were removed (to have a soluble molecule), but the results strongly suggest that these peptides are colocalized at one end of the rod (Skehel and Wiley, 1998). As it has been demonstrated for the HA, this conformation is not the metastable native conformation of the fusion protein, but most probably represents the structure of this protein at the end of the fusion process. A common feature of all these fusion glycoproteins is that they are synthesized as a precursor that must be cleaved to generate the NH₂-terminal fusion peptide. This is not the case for rhabdoviruses for which there is no activating cleavage of the fusion protein. Furthermore, no coiled-coil formation is predicted for the rhabdovirus glycoprotein and a careful look over the amino acid sequence of G does not reveal any obvious heptad repeats. These observations, together with the reversibility of the low pH-induced conformational changes, suggest that rhabdoviral G may define another category of fusogenic proteins and thus, is a good candidate to challenge the proposed model for membrane fusion.

In the present study, using a liposome assay, I have investigated the sensitivity of rabies virus-induced membrane fusion to lysophosphatidylcholine (LPC) having aliphatic chains of varying length, and to oleic acid (OA). The results are consistent with the fusion model proposed for influenza virus (Chernomordik et al., 1998). The data also indicate that under suboptimal fusion conditions the rate limiting step for membrane fusion is the formation of a fusion pore in a diaphragm of restricted hemifusion. Furthermore, the lipidic structure in the previously described cold-stabilized prefusion complex (Gaudin et al., 1993; Durrer et al., 1995) was characterized. It appeared that this intermediate is at an advanced stage of the fusion process when a putative hemifusion diaphragm is already destabilized, but lipid mixing is still restricted, most probably by a ring-like complex of glycoproteins. This state has a dynamic character and its lipid organization can reverse back to two lipid bilayers. Finally, although the data support the idea that the lipidic fusion intermediates are the same for influenza and rabies virus-induced membrane fusion, they also reveal some subtle differences that will be discussed and have to be taken into consideration for a global understanding of virus-induced membrane fusion.

### Materials and Methods

#### Chemicals

N-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine (RHO-PE), N-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine (NBD-
NaCl, 1 mM EDTA at 25,000 rpm in a SW 28 (Beckman Coulter) at 4° for 50 min through 30% glycerol in 10 mM Tris-HCl, pH 7.5, 50 mM Tris-HCl, pH 7.5). Viral purification was achieved by another centrifugation step using a BB12 rotor (Pharmacia Biotech).

If not indicated otherwise, all solutions were prepared in organic solvents. The initial fluorescence intensity of TMRD-3000 or calcein-loaded liposomes was measured with a spectrofluorimeter. Then, 10 μl of a 0.02 pH units were used. Experiments presented here were repeated at least three times and all qualitative results reported in this paper were observed in each experiment.

Preparation of Liposomes and Assay for Fusion
A total of 700 μg of PC, 300 μg of PE, and 100 μg of gangliosides dissolved in organic solvents were mixed with 10 μl of RHO-PE and 10 μg of NBD-PE and left in vacuo. The lipidic film was suspended in 1 ml of buffer A (150 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 25 mM Tris-HCl, pH 7.5). Viral purification was achieved by another centrifugation step of 50 min through 30% glycerol in 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA at 25,000 rpm in a SW 28 (Beckman Coulter) at 4°C.

Inhibition of Fusion By LPC and OA
Stock solutions of lauroyl (5 mM), myristoyl (1 mM), and palmitoyl (1 mM) LPC were freshly prepared as aqueous dispersions. Stock solution of stearoyl LPC (1 mM) and OA (1 mM) were freshly prepared as ethanolic solutions. In fusion experiments, the lipids were added into the cuvette containing the liposomes diluted in phosphate-citrate buffer at the required pH 2 min before addition of the virus. This time was largely sufficient to reach the equilibrium of their partition between the aqueous medium and the liposome membrane as judged by the increase of NBD fluorescence (due to exogenous lipid incorporation), which had reached a plateau after a few seconds (see Fig. 2 A).

The increase of NBD fluorescence due to lipid incorporation into the liposomes was used to estimate the amount of lipid incorporated. To evaluate how various dilutions of NBD-PE and RHO-PE affect the extent of NBD-PE fluorescence, a 1:1 (wt/wt) mixture of NBD-PE and RHO-PE was diluted into different amounts of a 7:3:1 (wt/wt) mixture of PC, PE, and gangliosides in organic solvents. Liposomes were then made as described in the previous paragraph. Fluorescence of NBD was measured before (F) and after (F₀) 0.8% Triton X-100 addition. A plot of F₀/F versus the fluorescent probe dilution gave a calibration curve that was used to estimate the incorporation of lipids in the liposomes. This approach assumes that the fluorescent probes and the added lipids are homogeneously and independently distributed in the liposome membrane.

Liposome Binding to Virus
For these experiments, liposomes were made as described above, but contained 40 μCi of [³H]PC per mg of lipid. For binding experiments, 15 μl of liposomes were mixed with 75 μl of phosphate-citrate buffer at pH 6.45 or 150 mM NaCl, 30 mM Tris-HCl, pH 8, and 10 μl of virus (50 μg of viral proteins). The mixture was incubated for 3 min on ice, layered onto a cold 25% glycerol solution in 150 mM NaCl, 50 mM Tris-HCl, pH 8, or in phosphate-citrate buffer pH 6.45, and then centrifuged for 30 min at 40,000 rpm in an SW 55 rotor (Beckman). The amount of liposomes associated with virus in the pellet was determined by liquid scintillation counting. To study their effect on liposome binding to virus, exogenous lipids were added to the mixture before addition of the virus.

Results
Effect of LPC on Rabies Virus-induced Membrane Fusion
A lipidic membrane lipid composition by addition of lipids that affect the curvature of lipid monolayers has been used to study the different stages of membrane fusion (Chernomordik et al., 1993, 1997; Vogel et al., 1993; Y eagle et al., 1994). Of particular interest are the inverted cone-shaped LPC that promote a micellar positive spontaneous curvature, and the cone-shaped cis-unsaturated fatty acids that promote inverted hexagonal H₃ phase. Thus, stalk formation should be promoted by cis-unsaturated fatty acids and inhibited by LPC when these lipids are present in the outer leaflet of the membranes. On the other hand, pore formation and expansion should be promoted by LPC and inhibited by cis-unsaturated fatty acids when these lipids are present in the inner leaflet of the membranes (Chernomordik et al., 1998).

The dependence of fusion on the presence of various concentrations of LPC with saturated aliphatic chains of varying lengths (from 12 to 18 hydrocarbon groups) was studied. Under optimal fusion conditions (pH 5.8), the extent of fusion decreased in a dose dependent manner (Figs. 1 and 2 D). The LPC concentrations used in these experiments did not solubilize the liposomes: as shown in Fig. 2 A, upon LPC addition, an increase of NBD fluorescence was observed that was much smaller than the increase of NBD fluorescence upon addition of solubilizing lipids.
concentrations of Triton X-100 (0.8%). This slight increase of NBD fluorescence was due to incorporation of LPC into the membrane of the liposomes resulting in dilution of RHO-PE and NBD-PE. The increase of NBD fluorescence was measured as described in Materials and Methods. Fluorescence; A.U., arbitrary units.

Potential leakage induced by LPC was also investigated. For palmitoyl (C16) and stearoyl (C18) LPC, no leakage was observed when fusion-inhibiting concentrations were used. For these lysolipids, three to four times higher concentrations than those used in the inhibition experiments were necessary for the encapsulated dyes to leak out (Fig. 2, B and C). A slight leakage of TMRD-3000, occurring much slower than in the presence of solubilizing concentrations of LPC, was detected when fusion-inhibiting concentrations of lauroyl (C12) and myristoyl (C14) LPC were used (Fig. 2 C). This leakage was not detected when calcein-loaded liposomes were used (although calcein is smaller than TMRD-3000), but slight increase of calcein fluorescence could have been hidden by the noisy signal (Fig. 2 B). Taken together, these control experiments indicated that the inhibitory effect of LPC was neither due to solubilization nor to lysis of liposomes.

As in other systems (Chernomordik et al., 1997), the inhibiting concentrations were dependent on the length of the hydrocarbon chains: LPC with longer chains have an inhibiting effect at lower concentrations (Figs. 1 and 2 D, Table I). However, as it was well documented that partitioning of LPCs into membranes increases with their hydrophobicity (i.e., with their hydrocarbon chain length), the concentration of LPC in the membranes was evaluated using NBD dequenching (due to LPC incorporation into the membrane), and the calibration curve was obtained as described in Materials and Methods. Estimates of the membrane concentrations required to cause 50% inhibition of fusion are indicated in Table I. It appeared that LPC with shorter hydrocarbon chains (and thus a more pronounced inverted cone shape) required lower membrane concentrations to inhibit fusion.

Under suboptimal fusion conditions (pH 6.2), palmitoyl (C16) and stearoyl LPC (C18) were still inhibiting fusion in a dose dependent manner at concentrations similar to those inhibiting fusion at pH 5.85 (Fig. 3). However, al-
Table 1. Total and Membrane LPC Concentrations Giving 50% Fusion Inhibition

| LPC   | Total LPC concentration giving 50% fusion inhibition | Membrane concentration of LPC giving 50% fusion inhibition |
|-------|--------------------------------------------------------|-----------------------------------------------------------|
| Lauroyl | 240 ± 50                                                | 4.1 ± 0.5                                                 |
| Myristoyl | 23 ± 2                                                 | 5.8 ± 0.5                                                 |
| Palmitoyl | 6.5 ± 1                                                | 8.7 ± 2                                                   |
| Stearyl  | 4.1 ± 2                                                 | 9.9 ± 2                                                   |

The total LPC concentration in the medium giving 50% of fusion inhibition was calculated from the graph in Fig. 2. The amount of LPC incorporated in the membrane, and thus the membrane concentration of LPC giving 50% of inhibition, were calculated as indicated in Materials and Methods.

though still inhibiting fusion at high concentrations, lauroyl (C12) and myristoyl (C14) LPC increased the number of fusion events at low concentrations (Fig. 3). Furthermore, in the presence of lauroyl and myristoyl LPC, the lag time preceding fusion at pH 6.2 disappeared and the initial kinetics of fusion were faster.

The simplest explanation of these first results is that incorporation of LPC in the outer leaflet of the membranes resulted in inhibition of stalk formation as demonstrated by the results obtained at pH 5.85. Furthermore, Fujii et al. (1985) have shown that the rate of transbilayer movement of LPC increases with decreasing acyl chain length (consistent with the slight leakage of liposomes observed in the presence of lauroyl and myristoyl LPC; Fig. 2 C), the results obtained at pH 6.2 strongly suggest that lauroyl and myristoyl LPC, but not palmitoyl or stearoyl LPC, were translocated into the inner leaflet and thus were present in both monolayers. Indeed, the presence of lauroyl and myristoyl LPC in the inner leaflet would promote pore formation and expansion, and would explain the increase in fusion extent, together with the faster kinetics of fusion. Finally, at pH 6.2, the lag time preceding fusion was no longer detected in the presence of lauroyl and myristoyl LPC. This indicates that this lag time is not due to slow binding of the virions to the liposomes, but rather that the rate limiting step of fusion under suboptimal conditions is the formation and the expansion of a fusion pore in a hemifusion diaphragm.

**LPC Does Not Inhibit Fusion Peptide Insertion in the Target Membrane**

Although the inhibiting effect of LPC on rabies virus-induced membrane fusion could be explained by their effect on the outer monolayer curvature, it was also possible that part of their inhibiting action was due to binding to the hydrophobic fusion peptide which could then no longer insert into the target membrane. Such a mechanism for LPC-induced fusion inhibition has been proposed for the case of HIV gp120/gp41-mediated fusion (Günther, A usborn and Stegmann, 1997).

Thus, a potential inhibition of rabies virus binding to target liposomes was investigated. For this, rabies virus was incubated for three minutes at pH 6.45 and 0°C with liposomes containing tritiated PC in the presence of LPCs. Under these conditions, although no fusion is detected, the virions interact in a hydrophobic manner with the target membrane via their fusion peptides. These conditions previously have been defined as prefusion conditions (Gaudin et al., 1993; Durrer et al., 1995). A Pfizer incubation, the mixture was centrifuged through a 30% glycerol cushion, above which unbound liposomes were floating, and the radioactivity associated with the virus in the pellet was assayed by liquid scintillation counting. The results are presented in Fig. 4. They show that the presence of 300 μM lauroyl, 30 μM myristoyl, 10 μM palmitoyl, or 10 μM stearoyl LPC was without effect on virus binding to the liposomes, indicating that it was indeed their ability to induce a micellar positive curvature of the outer leaflet, which was responsible for their ability to inhibit fusion.

**Effect of OA on Rabies Virus-induced Membrane Fusion**

The effect of cone-shaped lipids on fusion was then studied, and liposomes were incubated with various OA concentrations before addition of the virus. The effect of OA was dependent on pH. At pH 6.2, OA inhibited the fusion reaction. A concentration of 5 μM OA decreased the ex-
tent of fusion by a factor of 2.5 and a concentration of 10 μM virtually completely inhibited fusion (Fig. 5, pH 6.2). At lower pH, OA was much less inhibiting, and at pH 5.85 (Fig. 5), a concentration of 20 μM of OA was without effect on the fusion reaction. Potential inhibition of virus-liposome binding by OA was also assayed. A gain (Fig. 4) it was shown that under prefusion conditions, OA was without effect on virus binding to the liposomes. Thus, as OA is known to be easily translocated in the inner leaflet (Borring et al., 1989), inhibition of the fusion reaction under suboptimal fusion conditions was probably due to inhibition of pore formation and expansion in the hemifusion diaphragm.

Compensation of the Inhibiting Effect of LPC by Addition of OA

The inhibiting effect of LPC at pH 5.85 could be compensated at least partially by addition of OA into the membranes (Fig. 6). The compensatory effect was stronger with palmitoyl and stearoyl LPC (Fig. 6, B and C), but was still detected with myristoyl LPC (Fig. 6 A). No compensatory effect of OA on fusion inhibition by lauroyl LPC was observed. However, presence of OA affected the incorporation of lauroyl LPC into the membranes as judged by the larger increase of NBD fluorescence (data not shown).

These results are in agreement with the additivity of lipid effects on the spontaneous curvature of lipid monolayer (Madden and Cullis, 1982), and reinforce the idea that the inhibiting effect of LPC is due to its ability to induce a positive curvature of the outer membrane leaflet.

Structure of the Prefusion Complex

As mentioned above, we have previously demonstrated the existence of a stable prefusion complex (Gaudin et al., 1993; Durrer et al., 1995) that is formed when the virus is preincubated between pH 6.3 and 6.7 with liposomes on ice. The kinetics of formation of the rabies virus prefusion complex (RVPC) at pH 6.45 and 0°C were investigated (Fig. 7). Virions were incubated for increasing periods in the presence of liposomes at pH 6.45 and 0°C. The mixture was then diluted in the spectrofluorimeter cuvette containing a pH 6.2 phosphate-citrate buffer at 22°C. In the absence of this preincubation step, the fusion curve at pH 6.2 and 22°C had a typical sigmoidal shape and the increase of NBD fluorescence was preceded by a lag time of about 30 s (Fig. 7, but also see Fig. 2). Preincubation of the virus with the liposomes at pH 6.45 and 0°C resulted in faster fusion kinetics, disappearance of the lag time, and increase of fusion extent. A significant modification of the aspect of the fusion curve was already detected after a 20 s preincubation (Fig. 7), and the effect was maximal after about 2 min as longer preincubations did not result in faster fusion kinetics or larger increase of fusion extent (data not shown).

As LPCs were able to arrest fusion after hydrophobic interaction of the glycoprotein, but before lipid mixing, it was interesting to test the effect of LPC on fusion once RVPC is made. For this, virions were incubated with lipids.
liposomes at 0°C, either at pH 6.45 or 7.4 for three minutes. The prefusion complex was made at pH 6.45, but not at pH 7.4. The mixture was then diluted in the spectrofluorimeter cuvette containing phosphate-citrate buffer at pH 5.85 in the presence or absence of 10 μM LPC (palmitoyl or stearoyl LPC). After a preincubation of the virions and the liposomes at pH 7.4, lipid mixing was still sensitive to LPC (Fig. 8, pH 7.4), whereas when the prefusion complex was previously formed at pH 6.45 and 0°C, lipid mixing was then largely insensitive to LPC (Fig. 8, pH 6.45). The results of these experiments indicated that at pH 6.45 and 0°C, fusion is blocked at a stage downstream of the palmitoyl and stearoyl LPC-sensitive stage.

Finally, the disappearance of the lag time at pH 6.20 indicates that once RVPC is made, most of the events preceding lipid mixing have already occurred. As experiments performed with lauroyl and myristoyl LPC have indicated that the rate limiting step of the fusion process at pH 6.2 could be the formation of a fusion pore and its expansion in a hemifusion diaphragm, this suggests that at pH 6.45 and 0°C, fusion is blocked at a well-advanced stage when a putative hemifusion diaphragm is already destabilized, but lipid mixing is still restricted.

**Merger of Membranes Inside RVPC Is Reversible**

As already demonstrated (Gaudin et al., 1993; Durrer et al., 1995), a second protonation step lowering the pH below 6.3 is necessary to induce the transition from the RVPC state to complete fusion. Indeed, incubation of RVPC at pH 6.45 and temperature above 20°C did not induce any increase of NBD fluorescence (data not shown). Furthermore, after incubation of RVPC at pH 6.45 and 30°C (Fig. 9 A), the rate and the extent of fusion induced at pH 6.2 were lower than the rate and extent observed when RVPC was directly diluted at pH 6.2 and 0°C, indicating a progressive disruption of RVPC under such conditions.

Similar results were obtained when RVPC was treated with proteinase K at pH 6.45 and 0°C (Fig. 9 B). Thus, proteolysis of G also resulted in breaking RVPC without inducing complete fusion (as a control, dilution of RVPC in the fluorimeter cuvette thermostated at 0°C containing phosphate-citrate buffer at pH 6.45 and proteinase K did not induce any fluorescence increase).

These results indicate that the transition from RVPC to complete fusion is still a glycoprotein-dependent process and that local membrane merger (and maybe even initial pore formation in a restricted hemifusion diaphragm) is not sufficient to allow spontaneous complete fusion of membrane lipid bilayers. The corollary is that fusion pore expansion is still a high-cost energy step.

As a conclusion, in RVPC, although fusion is blocked at a stage subsequent to the LPC-sensitive stage. Virions and liposomes were incubated for 3 min on ice at pH 6.45 or 7.4 before dilution in the spectrofluorimeter cuvette containing phosphate-citrate buffer at pH 5.85 in the absence or presence of 10 μM palmitoyl or stearoyl LPC. The prefusion complex was made at pH 6.45, but not at pH 7.4. Fluor., Fluorescence; A.U., arbitrary units.

**Figure 8.** In RVPC, fusion is blocked at a stage subsequent to the LPC-sensitive stage. Virions and liposomes were incubated for 3 min on ice at pH 6.45 or 7.4 before dilution in the spectrofluorimeter cuvette containing phosphate-citrate buffer at pH 5.85 in the absence or presence of 10 μM palmitoyl or stearoyl LPC. The prefusion complex was made at pH 6.45, but not at pH 7.4. Fluor., Fluorescence; A.U., arbitrary units.

As a conclusion, in RVPC, although fusion is blocked at a stage downstream of the LPC-sensitive stage (Fig. 8), the
lipid organization is still sensitive to prolonged incubations in the presence of LPC. This reveals the dynamic character of this fusion intermediate and confirms that this stage can still reverse back to two membranes.

**Discussion**

Lipids of different shape, such as lysolipids or cis-unsaturated fatty acids, have proved to be useful tools to study the structural intermediates occurring during the fusion process (Chernomordik et al., 1993, 1995, 1997, 1998; Vogel et al., 1993; Yagle et al., 1994). In the present study, I have used LPC and OA to determine the rabies virus-induced fusion pathway. The effects of these lipids on rabies virus-induced fusion are similar to those observed in other fusion systems and reinforce the idea that the fusion pathway is the same in all biological fusion events.

**LPC Incorporated in the Outer Leaflet of Membranes Inhibits Rabies Virus-induced Membrane Fusion Consistently with the Stalk Hypothesis**

Under optimal fusion conditions (pH 5.85), LPCs with aliphatic chains having 12–18 hydrocarbon groups inhibit fusion in a range of inhibitory concentrations in solution that is similar to that observed for the influenza virus (Chernomordik et al., 1997). This result is in agreement with the predictions of the stalk model for fusion as presence of LPC in the outer leaflet would inhibit stalk formation (Chernomordik et al., 1999). However, the mechanism of LPC inhibition of fusion is often debated. Particularly, one alternative interpretation is that LPC directly binds to the fusion peptide of fusogenic glycoproteins and inhibits its interaction with the target membrane (Günther-A usborn and Stegmann, 1997). The results presented here rule out this hypothesis for rabies virus as the presence of LPC does not inhibit virus binding to liposomes under prefusion conditions. Furthermore, addition of OA was found to compensate the inhibitory effect of LPC in agreement with its complementary cone shape. This result indicates that it is membrane-incorporated LPC rather than LPC in solution that is responsible for fusion inhibition. Finally, as for the influenza virus, it appears that although the aqueous concentration of lauroyl LPC giving a twofold inhibition is ~60 times higher than that of stearoyl LPC, the corresponding membrane concentration of lauroyl LPC is two times lower than that of stearoyl LPC. As lauroyl LPC has a more pronounced inverted cone shape, here again, this result is consistent with the stalk hypothesis.

In the case of influenza virus-induced fusion, presence of OA in the outer leaflet of the fusing membranes promoted fusion, presumably because it promoted stalk formation (Chernomordik et al., 1997). Interestingly, rabies virus-induced fusion was not promoted in the presence of OA (except when LPC was added previously in the membranes). This suggests that stalk formation is an efficient process in this fusion system, which cannot be further enhanced by presence of OA in the outer leaflet. This difference with the influenza virus may be due to intrinsic properties of the fusion machinery of both viruses. However, it may also be due to the different experimental procedures, as Chernomordik et al. (1997) were working with cells expressing HA fusing with red blood cells, whereas I used viruses fusing with liposomes. Both the density of fusogenic glycoproteins (much higher at the viral surface) and different constraints on cellular and liposomal membranes may explain this difference of efficiency in stalk formation.

**Rabies Virus Induced-membrane Fusion Proceeds Via the Formation of a Lipidic Pore in a Hemifusion Diaphragm**

Another critical issue for fusion mediated by a protein machinery is the nature of the fusion pore: is it a proteinaceous pore whose expansion leads to lipid merger (Lindau and Almers, 1995) or a lipidic pore in a hemifusion diaphragm (Zimmerberg et al., 1993; K Emble et al., 1994; Melikyan et al., 1995b, 1997; Chernomordik et al., 1998)? In general, inhibition of fusion by membrane-incorporated LPC is considered as a strong argument in favor of the latter hypothesis (Chernomordik et al., 1998, 1999), but two other observations presented here are also consistent with a lipidic nature of the fusion pore.

First, under suboptimal fusion conditions, OA, which is known to be translocated in the inner leaflet (Broring et al., 1989), inhibits fusion as expected from a cone-shaped lipid promoting negative curvature of the inner leaflet. Second, one striking and unexpected result was the disappearance of the lag time preceding fusion and the faster kinetics of fusion in the presence of lauroyl and myristoyl LPC under suboptimal fusion conditions (pH 6.20). This demonstrates...
that the events preceding membrane continuity were sensitive to the shape of the lipids present in the membranes. Thus, the lag time detected before the onset of fusion was not due to slow binding of the virions to the liposomes, but rather to slow reorganization of the lipids in the fusion complex. The faster fusion kinetics were not observed with LPC having longer hydrocarbon chains. As the rate of transbilayer movement of LPC increases with decreasing acyl chain length (Fujii et al., 1985) consistently with the results presented here on LPC-induced leakage, this suggests that lauroyl and myristoyl LPC were translocated in the inner leaflet and, in agreement with the lipodic pore model, would promote pore formation in a hemifusion diaphragm. Thus, the fusion enhancement observed in experiments of lauroyl and myristoyl LPC under suboptimal conditions is probably due to an increased proportion of hemifusion diaphragms that transformed into complete fusion although less stalks were made. Finally, the disappearance of the lag time in the presence of lauroyl and myristoyl LPC indicates that the rate limiting step for fusion under suboptimal conditions would be the formation of a fusion pore and its enlargement into a hemifusion diaphragm.

In the Prefusion Complex, the Hemifusion Diaphragm Is Already Destabilized, but Lipid Mixing Is Still Restricted

Previously, we had demonstrated that when viruses and liposomes are preincubated at slightly acidic pH (between pH 6.4 and 6.7) and 0°C, although no membrane continuity is detected, rabies virions bind to the liposomes in a hydrophobic manner and their fusion peptides are inserted in the target membrane (Gaudin et al., 1993; Durrer et al., 1995). Here, the structure of this RVPC was investigated. The results indicated that once RVPC is made, most of the events preceding membrane continuity are already achieved. In fact, arguments presented in the results section suggest that in RVPC, fusion is blocked at an advanced stage when a fusion pore is already present in the hemifusion diaphragm, but lipid mixing is still restricted.

Restriction of lipid diffusion was first demonstrated in HA-induced membrane fusion (Chernomordik et al., 1998). This restriction is supposed to be due to the formation of a ring-like complex made of several low pH-activated HA (Blumenthal et al., 1996; Damieli et al., 1996). In the case of the rhabdoviruses, it has been shown that more than one trimer of G is required to build a competent fusion site (Bundo-Morita et al., 1988; Gaudin et al., 1993). Furthermore, we have recently described two rabies mutants with mutations in their glycoprotein for which the conformational change toward the inactivated state is considerably slowed down. Interestingly, these mutants presented an unexpected phenotype: they showed a hexagonal lattice of G at their surface under prefusion conditions (pH 6.6 and 0°C). Each angle of a hexagon seemed to be made up by a trimer of G and the lattice was not observed when spikes in the inactivated conformation were detected (Gaudin et al., 1996). We have proposed that one hexagon, once associated with a target membrane, constitutes a minimal prefusion complex for rabies virus (Gaudin et al., 1999). If this is true, diffusion of the lipids inside this hexagon might be restricted either by the transmembrane domains or by membrane-inserted fusion peptides of the activated glycoproteins making up a hexagon. The fact that, in photolabeling experiments performed under prefusion conditions (6.4 and 0°C), labeling was found only in the fusion peptide and not in the transmembrane domain (Durrer et al., 1995) suggests that lipid diffusion is restricted by fusion peptides inserted in the target membrane. This could also be the case for influenza virus because GP1-HA lacking HA transmembrane domain has been shown to induce restricted hemifusion under suboptimal fusion conditions (Chernomordik et al., 1998).

The properties of RVPC resemble those of the so-called frozen intermediate of fusion (FIF; also called committed state) observed for influenza HA-induced fusion (Schoch et al., 1992; Chernomordik et al., 1998). However, it has been shown that FIF is a restricted hemifusion that precedes fusion pore formation (Chernomordik et al., 1998), whereas the kinetics presented here suggest that pore formation has already occurred in RVPC. In fact, the lipidic organization in RVPC is probably similar to the structure of the flickering pore observed in influenza virus-induced fusion (Mélikyan et al., 1993, 1995a; Tse et al., 1993; Zim-merberg et al., 1994), which is also a dynamic intermediate.

Reversibility of Membrane Merger at the RVPC-arrested Stage

In the case of influenza HA-induced membrane fusion, once FIF is made, fusion can be achieved at pH 7.4 and 37°C. This is not the case with RVPC for which a second protonation step (occurring below pH 6.3) is absolutely required for fusion to be triggered. In fact, increasing the temperature without lowering the pH simply results in slow disruption of RVPC and progressive recovery of the sigmoidal shape of the fusion curves when fusion is further induced at pH 6.2 and 23°C. It could be tempting to relate this result to the reversibility of the rhabdovirus glycoprotein conformational changes (Crimmins et al., 1983; Doms et al., 1987; Puri et al., 1988, 1992; Clague et al., 1990; Gaudin et al., 1991, 1993; Pak et al., 1997). However, as treatment of RVPC by proteinase K also results in RVPC disruption without inducing any fusion, this indicates that the transition from RVPC to complete fusion is a high-cost energy step that depends on the integrity and the correct refolding of the glycoproteins directly involved in the fusion process. This also rules out the hypothesis that local membrane connections are sufficient to allow spontaneous fusion of lipid bilayers in agreement with recent results obtained by Leikina and Chernomordik (2000) using influenza HA to induce membrane fusion. Finally, together with the fact that prolonged incubation of RVPC with LPC at pH 6.45 and 0°C also resulted in its disruption, proteolysis experiments also demonstrate that the lipid organization in RVPC can reverse back to its initial state (i.e., two bilayers) and that the energy cost of local membrane merger in RVPC is compensated by the energy provided by G conformational changes.

Toward a Universal Model for Fusion

Despite the important differences between their fusion machineries (Gaudin, 2000), rabies virus and influenza vi-
rus-induced membrane fusion follow a similar pathway (Fig. 11). In both cases, the first step in the fusion process is the insertion of the fusion peptide into the target membrane (Stegmann et al., 1991; Tsurudome et al., 1992; Dur rer et al., 1995) and maybe also into the viral membrane (Weber et al., 1994; Wharton et al., 1995). This insertion would induce the formation of a stalk structure that connects target and viral membranes. The initial stalk structure would then expand and the inner leaflets of the membranes would form a hemifusion diaphragm. This step would be followed by pore formation and expansion leading to complete fusion. This fusion model is also supported by data obtained from other fusion systems: stalk formation is consistent with the lysolipid-induced inhibition of calcium-triggered exocytosis, GTP dependent fusion of rat liver microsomes, pH-induced fusion of insect cells infected by baculovirus, and Sendai virus fusion with liposomes (Chernomordik et al., 1993, 1995; Yeagle et al., 1994). Hemifusion has been detected not only in the case of fusion induced by GPI-HA (Kemble et al., 1994; Melikyan et al., 1995b), but also in the case of fusion induced by HA having a mutation in position 1 of HA2 (Qiao et al., 1999), and in the case of paramyxovirus SV5 fusion protein lacking its intraviral COOH-terminal domain (Bagai and Lamb, 1996). Finally, for both the influenza and rabies virus, complete fusion is preceded by a stage at which lipid diffusion is strongly restricted. This step seems to involve a ring-like structure made up of activated fusogenic glycoproteins. Such a structure is also encountered in the case of baculovirus GP64-induced membrane fusion (Plonsky and Zimmerberg, 1996; Markovic et al., 1998) suggesting that the supramolecular organization of the fusion machinery is similar from one system to another.

Although the mechanism of membrane rearrangements leading to fusion is probably universal, depending on the experimental conditions, fusion may be blocked at different stages that are not the same from one system to another. For example, the membrane organization is probably not the same in RVPC and FIF, as RVPC seems to be at a more advanced stage on the fusion pathway. Thus, dissecting the fusion pathway of various fusion systems and identifying stable intermediates will certainly be useful if one wants to get a detailed cinematic view of the fusion mechanism and to know how proteins induce this complex process.

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Figure 11. Rabies virus-induced fusion pathway. A, The initial low pH-induced conformational change results in exposition of the glycoprotein fusion peptide that interacts with the target membrane. B, The result of this insertion is the formation of a stalk that connects the outer leaflets of the fusogenic membranes inside a ring of glycoproteins. Stalk formation is inhibited by LPC present in the outer leaflet. C, This is followed by the formation of a hemifusion diaphragm in which lipid diffusion is restricted. D, The next step is the formation of a fusion pore in the hemifusion diaphragm. Lipid diffusion is still restricted. This is the stage arrested at pH 6.45 and 0°C (RVPC). A t this stage, the lipid organization can still reverse back to two membranes. E, A mother stage of protonation would break the ring of glycoproteins and would induce membrane fusion.
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