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Influenza Viruses Select Ordered Lipid Domains during Budding from the Plasma Membrane*

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During the budding of enveloped viruses from the plasma membrane, the lipids are not randomly incorporated into the envelope, but virions seem to have a lipid composition different from the host membrane. Here, we have analyzed lipid assemblies in three different viruses: fowl plague virus (FPV) from the influenza virus family, vesicular stomatitis virus (VSV), and Semliki Forest virus (SFV). Analysis of detergent extractability of proteins, cholesterol, phosphoglycerolipids, and sphingomyelin in virions showed that FPV contains high amounts of detergent-insoluble complexes, whereas such complexes are largely absent from VSV or SFV. Cholesterol depletion from the viral envelope by methyl-β-cyclodextrin results in increased solubility of sphingomyelin and of the glycoproteins in the FPV envelope. This biochemical behavior suggests that so-called raft-lipid domains are selectively incorporated into the influenza virus envelope. The "fluidity" of the FPV envelope, as measured by the fluorescence polarization of diphenylhexatriene, was significantly lower than compared with VSV or SFV. Furthermore, influenza virus hemagglutinin incorporated into the envelope of recombinant VSV was largely detergent-soluble, indicating the depletion of raft-lipid assemblies from this membrane. The results provide a model for lipid selectivity during virus budding and support the view of lipid rafts as cholesterol-dependent, ordered domains in biological membranes.

The hypothesis that lipids from the plasma membrane are not randomly included into the viral envelope, but that budding could occur from specialized domains of the membrane, was put forward several years ago (1, 2). Comparing the lipid composition of various viruses with the composition of the host cell membrane, clear cut differences have been reported, although caution has to be exercised because viruses can be isolated practically pure, whereas plasma membrane fractions cannot be (2–4). Thus, the role of lipid domains has remained controversial and has not so far been given functional significance.

New insights into the mechanisms of domain formation in biological membranes have come from the analysis of detergent-resistant membrane fractions (5). Recent evidence suggests that laterally associating sphingolipids and cholesterol form small ordered domains, called rafts, which are resistant to extraction with Triton X-100 at 4 °C. These domains can incorporate specific proteins and function as platforms for intracellular sorting and signal transduction events (6, 7). Recently, the concentration of GPI[1]-anchored proteins in microdomains has been confirmed in living cells by biophysical and biochemical methods without the use of detergents (8, 9). However, when GPI-anchored proteins on the apical surface of epithelial cells were analyzed, no such concentration could be observed (10). The reason for this discrepancy is not known, yet one explanation could be that this membrane represents a continuous raft domain in contrast to the plasma membrane of nonpolarized cells, where raft domains would be noncontinuous (6, 10, 11). Recent studies have shown that cholesterol promotes the detergent insolubility of GPI-anchored proteins or transmembrane proteins both in cellular membranes and in artificial lipid vesicles (12–15). Lipids recovered in detergent-insoluble glycolipid-enriched complexes (DIGs) were shown to have higher melting temperatures than the average lipids from the plasma membrane, suggesting that raft-domains might be formed from lipids with preferentially saturated acyl chains (16). Interestingly, for artificial lipid vesicles detergent insolubility could be correlated with the formation of a liquid-ordered phase in the membrane (15, 17), suggesting that phase separation might be the basis for the formation of insoluble lipid rafts in biological membranes (7, 18).

Also, specific viral glycoproteins have been described to be associated with raft domains during transport to the cell surface. Both the influenza virus neuraminidase and hemagglutinin are recovered in DIGs after entering the Golgi complex (19, 20). Efficient surface transport of influenza virus HA requires cholesterol, and the protein stays raft-associated at the plasma membrane (14, 21).

Here we used membranes of baby hamster kidney (BHK) cells and the viral envelopes of influenza fowl plague virus (FPV), vesicular stomatitis virus (VSV), and Semliki Forest virus (SFV) as model systems to analyze the formation of detergent-resistant lipid domains in a biological membrane. Our results demonstrate that the viruses contain lipid raft assemblies to a very different extent. Furthermore, we show that in all of the membranes analyzed cholesterol is required for the formation of detergent-insoluble lipid complexes and that such complexes are highly ordered. Our results therefore

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[1] The abbreviations used are: GPI, glycosylphosphatidylinositol; DIG, detergent-insoluble glycolipid-enriched complex; HA, hemagglutinin; FPV, fowl plague virus; VSV, vesicular stomatitis virus; SFV, Semliki Forest virus; BHK, bovine hamster kidney; SM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PAGE, polyacrylamide gel electrophoresis; CD, methyl-β-cyclodextrin; DPH, diphenylhexatriene; DPPC, dipalmitoylphosphatidylcholine.

support a model in which cholesterol promotes the formation of lipid domains that become selectively incorporated into the influenza envelope.

EXPERIMENTAL PROCEDURES

Cell Culture, Virus Stocks—BHK cells (strain CCL10, American Culture Collection) were maintained in G-MEM (5% FCS, 10% tryptose-phosphate 10 mM Hepes, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin) in 5% CO_2 at 37 °C in a humidified incubator. Virus stocks of influenza virus (strain A/FPV), VSV, SFV, and the recombinant VSV-HA (22) were produced as described before (23, 24).

Preparation of Membrane Fractions—From BHK cells, a light membrane fraction was prepared essentially as described (25). Briefly, cells were homogenized with a syringe under a nitrogen atmosphere. A postnuclear supernatant was adjusted to 1.5 M sucrose and overlayered with 1.2 and 0.8 M sucrose. After centrifugation for 4 h (45,000 rpm, 4 °C in a SW40 rotor, Beckman), membranes were recovered from the 1.2/0.8 M interface. For radioactive labeling, cells on one 10-cm culture dish were incubated for 4 h with 25 μCi of [1α,2α-^{3}H]cholesterol (48 Ci/mmol) in infection medium (see below) or for 36 h with 80 μCi of [^{32}P]orthophosphate in G-MEM without tryptose phosphate. To analyze membranes after viral infection, the cells were harvested 6 h postinfection and processed as above.

Purification of Viruses—For the analysis of proteins, lipids, and DPH polarization, the viruses were grown in BHK cells. For the production of nonlabeled viruses, cells from three overconfluent 80-cm^2 flasks were trypsinized and seeded in 10 275-cm^2 flasks. After 36 h, viruses were adsorbed to the 90% confluent cells (2×10^8 plaque-forming units/flask) in infection medium (MEM; 0.2% BSA, 10 mM Hepes, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin) for 1 h. The inoculum was removed and replaced by infection medium. After 24 h, the medium was collected and centrifuged two times to remove cell debris (1500 rpm, 4 °C, Heraeus centrifuge). The supernatant was then layered on top of a step gradient in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl (TN) consisting of 3 ml of 55% (w/v) sucrose and 10 ml of 10% (w/v) sucrose in a SW28 centrifuge tube. After a 2-h centrifugation at 25,000 rpm and 4 °C, the 55/10% interface was collected, diluted 3-fold in TN, and then applied on an 8-ml 50% (w/v) glycerol cushion in TN. After a 2-h spin (25,000 rpm, 4 °C) in a SW28 rotor, the supernatant was discarded, and the virus pellet was resuspended in TN buffer overnight at 4 °C. A preparation from 10 275-cm^2 flasks routinely yielded 1–2 mg of viral protein for FPV and VSV and about 0.2 mg for SFV.

Radiolabeled viruses were produced following essentially the same protocol in smaller scale using two 10-cm tissue culture dishes. Two hours postinfection, the cells were labeled with 0.5 mCi of [^{35}S]methionine in medium containing 5 μM unlabeled methionine. For labeling with [1α,2α-^{3}H]cholesterol (48 Ci/mmol), cells were preincubated for 4 h with 50 μCi of [^{3}H]cholesterol/dish in Dulbecco's modified Eagle's medium (0.1% ethanol, 10 mM Hepes, 2 mM glutamine, 100 units/ml, and 100 μg/ml streptomycin). Subsequently, cells were washed twice with infection medium, and viruses were adsorbed and collected as described above.

For labeling with ^{32}P, cells on two 10-cm tissue culture dishes were incubated with 750 μCi of [^{32}P]orthophosphate/dish for 30 h in G-MEM without tryptose phosphate. Then cells were washed, and viruses were adsorbed and collected.

Detergent and Cyclodextrin Extractions—Aliquots of virus or membrane preparations with a total lipid content smaller than 1 μg were extracted with 50 μl of 1% (w/v) Triton X-100, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA (TXNE) on ice for 20 min. In pelleting experiments, the extract was spun in a TLA-100 centrifuge (Beckman) in a TLA100 rotor for 20 min at 80,000 rpm and 4 °C. Proteins in supernatant and pellet fractions were analyzed by SDS-PAGE. Alternatively, lipids from pellet and supernatant fractions were extracted according to Bligh and Dyer (26) and subsequently analyzed by thin layer chromatography on silica plates (Kieselgel 60; Merck) with chloroform/methanol/water (60:35:8, v/v/v) as a solvent. Radiolabeled ^{32}P-containing lipids were visualized by autoradiography and quantified by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA). Relative amounts of [^{3}H]cholesterol were quantified by scintillation counting.

For flotation gradients, virus aliquots containing 10 μg of protein were extracted with 250 μl of TXNE on ice for 20 min. Extracts were then brought to 40% Optiprep by the addition of 500 μl of Optiprep stock solution (Nycomed Pharma, Oslo, Norway), overlayered with 1.2 ml of 30% Optiprep in TXNE and 250 μl of TXNE. Samples were spun for 2 h in a TLS 55 rotor (50,000 rpm, 4 °C), and six fractions were collected from the top of the gradient. Proteins were precipitated from the fractions by the addition of one volume of 20% trichloroacetic acid and analyzed by SDS-PAGE and Western blotting.

Extractions with methyl-β-cyclodextrin (CD; Sigma) were performed for 15 min at 37 °C in TN containing 5 mM EDTA and 10 μg/ml defatted BSA with gentle agitation. Subsequently, samples were cooled on ice and spun for 30 min in a TLA100 rotor (80,000 rpm, 4 °C).

Electron Microscopy—Virions were sedimented by centrifugation in a TLA45 rotor (20 min, 30,000 rpm, 4 °C), and negative staining was performed using glow-discharged carbon-coated Formvar grids and a 1% (w/v) solution of uranyl acetate (Sigma).

DPH Polarization Measurements—Polarization measurements were performed essentially as described (16). Virus preparations or lipid vesicles were resuspended in 100 μl of TN buffer, and 1 μl of DPH (Sigma) dissolved in tetrahydrofuran was added. Samples were incubated for 1 h in the dark, and DPH fluorescence intensity was measured at 25 °C in an AB2 luminescence spectrometer (SLM Aminco) equipped with Glan-Thompson polarizers (excitation wavelength, 359 nm; emission at 427 nm; band pass 4 nm). Lipid vesicles were prepared by sonication of 2 ml of 0.5 mg/ml PC from egg yolk (Sigma) or 0.5 mg/ml DPPC (Sigma) in TN buffer. Similar results were obtained using viruses or lipid vesicles at concentrations of 0.05–0.5 μg/ml and DPH at final concentrations of 50 nM to 1 μM. Fluorescence detected without DPH or without the addition of viruses was negligible.

RESULTS

Glycoproteins in the Influenza Virus Envelope, but Not in VSV or SFV, Are Recovered in DIGs—To study raft domains in different envelopes, the viruses were produced in BHK fibroblasts and isolated by gradient centrifugation. The obtained preparations were essentially pure as judged by SDS-PAGE and silver staining (Fig. 1*A*). The detergent solubility of the viral glycoproteins was analyzed by extraction with Triton X-100 on ice and centrifugation in Triton-containing density gradients. Influenza virus HA was floating to low density as observed before (14), whereas the VSV G or the SFV glycoproteins stayed in the bottom of the gradient, indicating that they were solubilized by the detergent (Fig. 1*B*). Therefore, association of a glycoprotein with rafts in the viral envelope is specific for influenza viruses and not a general phenomenon.

Extractability of Cholesterol in Viral Envelopes—Cholesterol is strongly enriched in detergent-insoluble fractions from cellular membranes (5). We therefore compared the Triton solubility of cholesterol in the different viral envelopes and the host cell membrane. BHK cells were labeled with [^{3}H]cholesterol, and viruses were produced and purified. The radiolabeled membranes were extracted with Triton X-100 on ice and centrifuged, and radioactivity in supernatant and pellet fractions was quantified by scintillation counting (material pelleted by this centrifugation at 178.000 $\times$ *g* will be considered "insoluble"). For comparison, a [^{3}H]cholesterol-labeled light membrane fraction from BHK cells was prepared and detergent-extracted. Strikingly, the cholesterol solubility showed large differences between the viruses analyzed (Fig. 2*A*). In the influenza viruses (FPVs) 41 $\pm$ 8% of the cholesterol was pelleted, in VSV 13 $\pm$ 6%, and in SFV only 5 $\pm$ 5%. Compared with the BHK light membrane fraction (enriched for the plasma membrane, endosomes, and Golgi membranes (27)), cholesterol solubility in the influenza virus envelope is decreased and increased in VSV and SFV. It is important to note that the cholesterol solubility in membranes from virally infected BHK cells was not significantly changed in comparison with uninfected cells (data not shown). We next used CD to further analyze the extractability of cholesterol in the different viral envelopes. In contrast to detergents, incubation with low concentrations of CD leaves membranes intact and mediates selective efflux of sterols from the bilayer (28, 29). When [^{3}H]cholesterol-labeled influenza viruses and VSV were treated with increasing concentrations of CD, cholesterol was more easily removed from the VSV envelope (Fig. 2*B*). For example, 1 mM

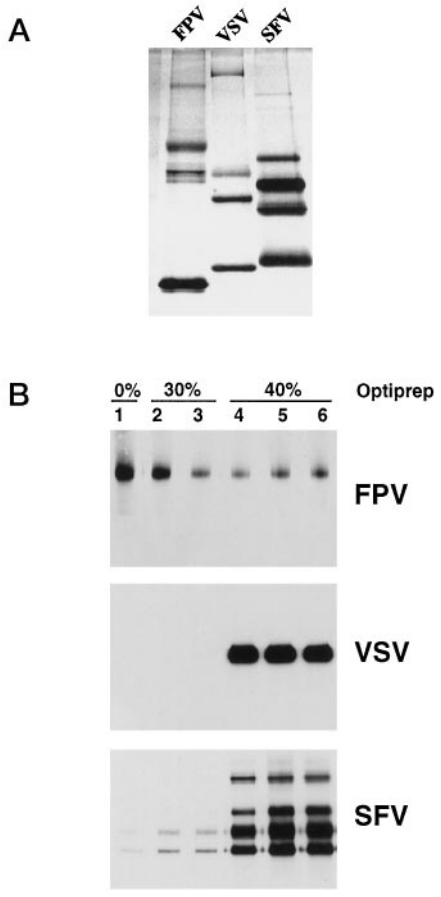

FIG. 1. *A*, purification of viruses from BHK cell cultures. BHK cells were infected with FPV, VSV, or SFV, and viruses were collected for 24 h. 3 μg of protein of each preparation obtained by gradient centrifugation were analyzed by SDS-PAGE and silver staining. *B*, glycoproteins in FPV, but not VSV and SFV, are associated with DIGs. Viral preparations were extracted with 1% Triton X-100 on ice, and extracts were adjusted to 40% Optiprep. The extract (750 μl) was overlayered with 1.2 ml of 30% Optiprep and 250 μl of buffer, both containing 1% Triton X-100, and centrifuged for 2 h at 55,000 rpm (TLS 55 rotor, 4 °C). Fractions were collected from the top of the gradient (*lanes 1–6*) and analyzed by Western blotting with antibodies against the viral glycoproteins.

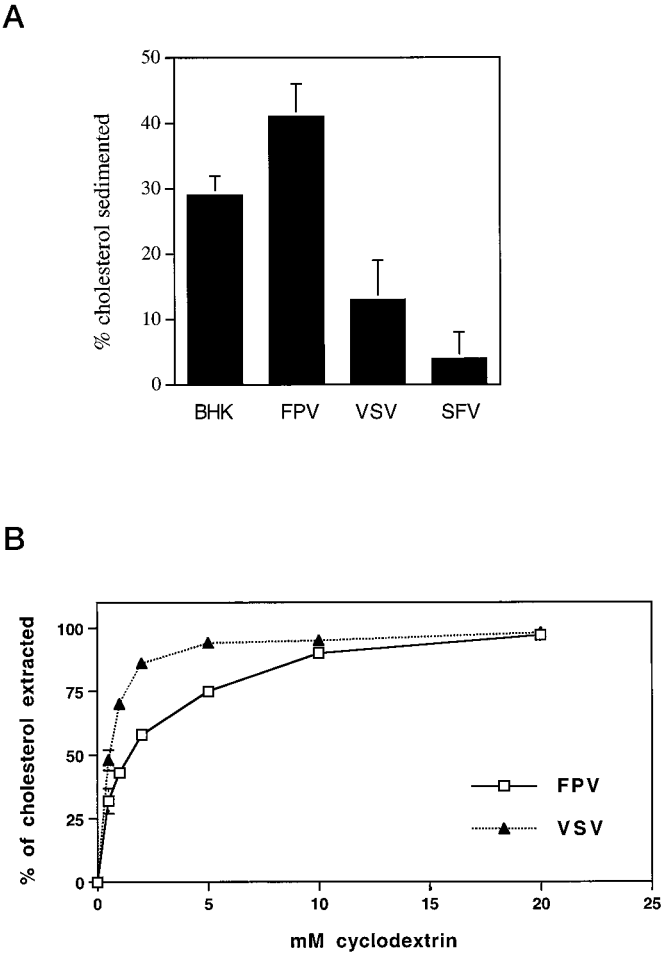

FIG. 2. **Cholesterol extractability in viral and cellular membranes.** *A*, [^{3}H]cholesterol-labeled virions or BHK membranes were extracted with 1% Triton X-100 on ice and centrifuged (30 min, 80,000 rpm, 4 °C, TLA100). Relative amounts of [^{3}H]cholesterol in supernatant and pellet were quantified by scintillation counting. Free counts not sedimented without detergent extraction (3–10% of total) were subtracted from the supernatant samples. *B*, [^{3}H]cholesterol-labeled virions were extracted with increasing amounts of methyl-β-cyclodextrin for 15 min at 37 °C. Viruses were sedimented by centrifugation (30 min, 80,000 rpm, 4 °C, TLA100), and relative amounts of [^{3}H]cholesterol in supernatant and pellet were quantified by scintillation counting. Free counts not sedimented without cyclodextrin treatment (3–10% of total) were subtracted from the supernatant samples.

CD extracted 70 ± 3% of cholesterol from VSV but only 43 ± 2% from the influenza virus particles. It should be noted that practically no further cholesterol efflux was observed when the extraction times were extended beyond 15 min (data not shown). During the extraction, the viral particles remained intact and did not show any dramatic structural changes as judged by negative staining and electron microscopy (Fig. 3). The extractability of cholesterol by CD from the SFV envelope was found to be intermediate compared with the VSV and FPV envelope; however, the results were somewhat variable (data not shown). One possible explanation came from the morphological analysis of the extracted particles. In contrast to FPV and VSV, the structure of the SFV particles was affected upon cholesterol removal, and the particles were penetrated by the stain (Fig. 3*F*). This might indicate a perturbation of the membrane and result in fragmentation of the particles, especially at higher CD concentrations. Instability of SFV particles has been observed before, when virions were produced in cholesterol-free insect cells (30). However, clearly cholesterol in influenza viruses is in a detergent-resistant state, whereas this is not the case for VSV and SFV.

The Influenza Envelope Is Enriched for Cholesterol-dependent Detergent-insoluble Lipid Assemblies—The observed differences in cholesterol extractability could result from the inclusion of raft domains into the viral envelopes to differing extents. To test this hypothesis, viruses were produced in ^{32}P-labeled BHK fibroblasts and detergent-extracted at 4 °C. Lipids from the soluble and insoluble fractions were separated by thin layer chromatography and quantified by PhosphorImager analysis. The major ^{32}P-labeled lipids detected in the BHK light membrane fraction were phosphatidylethanolamine (PE), phosphatidylcholine (PC), and sphingomyelin (SM) (Fig. 4*A*). While PE and PC were largely solublized by Triton X-100, a fraction of the SM (41 ± 7%) was insoluble (as for the cholesterol extractability, no significant changes in the Triton X-100 solubility of ^{32}P-labeled lipids were observed 6 h after viral infection; data not shown). However, the SM solubility in the viral envelopes showed dramatic differences. While in influenza viruses 70 ± 5% of the SM could be sedimented after detergent extraction, only 27 ± 7 or 15 ± 8% could be pelleted from VSV or SFV, respectively (Fig. 4*A*). To analyze the cholesterol dependence of the lipid solubility, membranes or virus particles were preextracted with 5 mM CD. We chose this con-

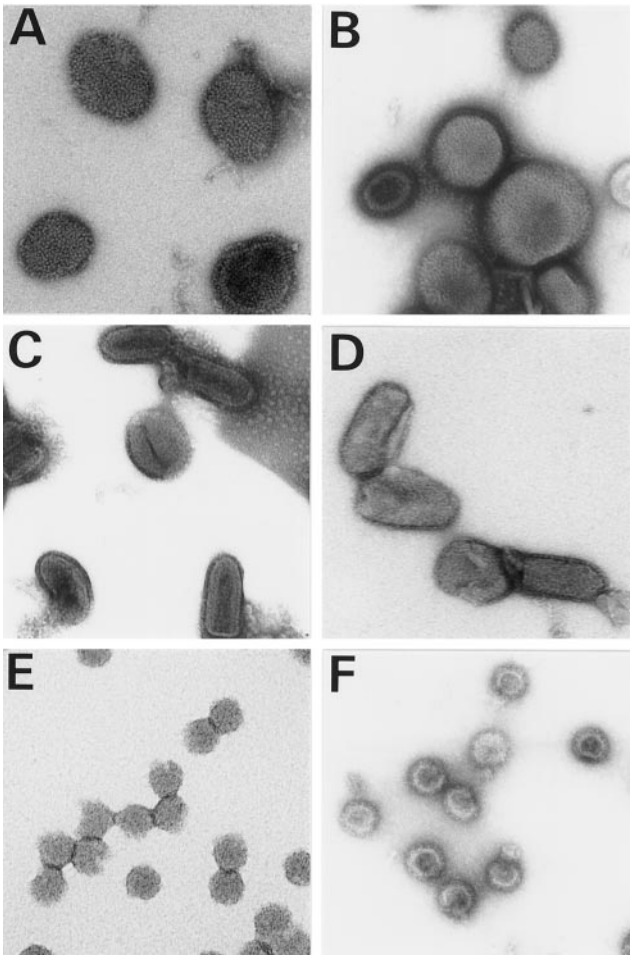

FIG. 3. **Morphological analysis of viral particles after cholesterol depletion.** FPV (*A* and *B*), VSV (*C* and *D*), and SFV (*D* and *E*) were incubated without (*A*, *C*, and *E*) or with 5 mM CD (*B*, *D*, and *F*) for 20 min at 37 °C and subsequently pelleted by centrifugation for 30 min (80,000 rpm, 4 °C, TLA100). The preparations were then observed in an electron microscope after negative staining.

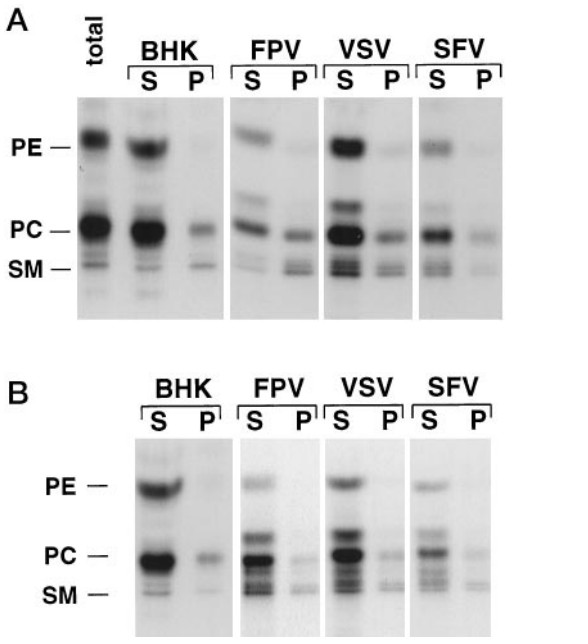

FIG. 4. **Triton X-100 solubility of lipids in FPV, VSV, SFV, and BHK membranes.** *A*, BHK cells were labeled with [^{32}P]orthophosphate, and a light membrane fraction or radiolabeled viruses were isolated. Samples were treated with 1% Triton X-100 in TNE buffer on ice and centrifuged (30 min, 80,000 rpm, 4 °C, TLA100). Lipids from the supernatant (*S*) and the pellet (*P*) fractions were extracted and analyzed by thin layer chromatography. Labeled lipids were detected by autoradiography and quantified by PhosphorImager analysis. The positions of PE, PC, and SM are indicated. *B*, for cholesterol depletion, the membranes were treated with 5 mM cyclodextrin, pelleted, subsequently extracted with Triton X-100, and analyzed as in *A*.

TABLE I

Triton X-100 solubility of cholesterol (Chol), SM, PC, and PE in BHK membranes and viral envelopes, without or after (+CD) cholesterol depletion by 5 mM cyclodextrin

The sedimentability of each lipid at 279,000 × g after Triton X-100 extraction is listed. Results from at least three independent experiments with three independent determinations are given with S.D. values.

Membrane	Sedimentability of lipid			
	Chol	SM	PC	PE
		%		
BHK	29 ± 3	41 ± 7	11 ± 2	4 ± 3
+CD		21 ± 2	10 ± 2	5 ± 2
FPV	41 ± 8	70 ± 5	37 ± 4	12 ± 3
+CD		20 ± 8	10 ± 5	2 ± 3
VSV	13 ± 6	27 ± 7	14 ± 2	5 ± 2
+CD		19 ± 5	10 ± 3	5 ± 4
SFV	4 ± 5	15 ± 8	14 ± 9	5 ± 5
+CD		24 ± 6	13 ± 5	3 ± 5

centration because it allows the removal of 70–90% of the cholesterol but leaves the particles intact and results only in a minimal loss of ^{32}P-labeled lipids (<5%) from the samples (data not shown). Insolubility of SM was cholesterol-dependent, since CD preextraction of the membranes resulted in increased solubilization, both in the FPV and VSV envelopes and in the BHK membrane fraction (Fig. 4*B*, Table I). Also, the solubility of PC in the FPV envelope was strongly increased upon cholesterol removal (Table I). Therefore, cholesterol is not only required for the association of proteins with DIGs, but generally for the formation of detergent-insoluble lipid assemblies in a biological membrane. This also confirms that DIGs are not complexes coalescing from intrinsically insoluble components but that an interplay between different lipid species is required to form a detergent-resistant membrane.

DPH Fluorescence Polarization Reveals Ordered Domains in the FPV Envelope—Studies with artificial lipid vesicles demonstrated that membranes in the liquid-ordered phase and GPI-anchored proteins incorporated into these membranes are insoluble in Triton X-100 at 4 °C (16, 17). It was therefore proposed that DIGs isolated from biological membranes might also be derived from domains in the liquid-ordered phase (7). Fluorescence polarization of the dye DPH incorporated into membranes has been widely used as a measure of acyl chain order. Lipid vesicles containing detergent-insoluble lipids showed significantly higher fluorescence polarization than lipid vesicles from detergent-soluble lipid mixtures, supporting the idea of ordered domains as a basis for insolubility (16). While cellular membranes are heterogenous, viral envelopes provide a unique possibility to analyze DPH fluorescence polarization in a biological lipid bilayer. When DPH was incorporated into the envelopes of FPV, VSV, and SFV, the influenza virus showed the highest fluorescence polarization, whereas the lowest values were obtained for VSV (Table II). For comparison, DPPC and egg yolk PC vesicles prepared by sonication were analyzed. Clearly, all viral membranes show a higher fluorescence polarization than the egg yolk PC lipid vesicles, which are in the liquid crystalline phase. At 25 °C, the DPPC vesicles are in the gel phase (T_m = 41 °C) and accordingly show a very high fluorescence polarization (0.382). The values obtained for the FPV envelope are only slightly lower, suggesting

TABLE II
Polarization of DPH fluorescence in viruses or lipid vesicles at 25 °C

Fluorescence polarization of DPH (50 nM) in viruses or lipid vesicles (each 0.05 μg/ml) was measured. Values are averaged from at least three independent experiments, and S.E. values were maximally 0.020.

Sample	Polarization
FPV	0.365
VSV	0.247
SFV	0.303
ePC[a]	0.144
DPPC	0.382

[a] Egg yolk PC.

a high degree of order in this envelope and strongly supporting the hypothesis that DIGs are derived from ordered domains in biological membranes. Rather surprisingly, the SFV envelope showed intermediate values between the VSV and FPV indicating a limited "fluidity," although according to the detergent solubility of the lipids highly ordered lipid complexes were not detected (Table I). Most likely, this can be explained by the fact that SFV is an alphavirus, which is structurally different from the two negative strand RNA viruses FPV and VSV. Alphaviruses have a very high protein content, and the icosahedral lattices of the spike and capsid proteins are connected by strong protein-protein interactions (31, 32). These tightly packed protein assemblies might cause the ordered structure of the membrane, sensed by the lipid probe.

HA Incorporated into the VSV Envelope Is Detergent-soluble—In vitro detergent-resistant membranes can be formed solely by lipids (16). However, it is not understood how proteins influence these lipid assemblies. Proteins might be required to induce or stabilize such domains in biological membranes. We used a recombinant vesicular stomatitis virus, which incorporates influenza virus HA into the envelope (VSV-HA (22)) to analyze the detergent solubility of HA in the lipid environment of VSV. BHK cells were infected with VSV-HA, and [^{35}S]methionine-labeled viruses were collected. The ratio of HA to VSV G in the envelope was approximately 1:5, as previously reported (Ref. 22, Fig. 5*A*). Virions were extracted with Triton X-100 on ice and centrifuged. Proteins in the supernatant and pellet were analyzed by autoradiography or Western blotting with HA or VSV G antibodies (Fig. 5*A*). Strikingly, both glycoproteins were largely solubilized by the detergent. Consistently, no significant amounts of HA were floating in density gradients after Triton X-100 extraction (Fig. 5*B*, compare with Fig. 1*B*). Furthermore, cholesterol in the VSV-HA envelope was efficiently solubilized in Triton X-100 (8 ± 6% insoluble), indicating that the presence of HA in a viral envelope is not sufficient to create insoluble domains and that the lipid environments incorporated into VSV and FPV during budding are different.

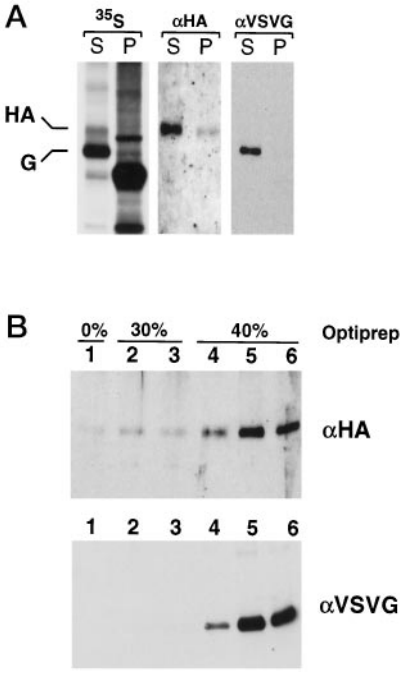

FIG. 5. **Triton X-100 extractability of HA in the envelope of VSV.** *A*, VSV-HA virions labeled with [^{35}S]methionine were produced in BHK cells, purified, and subsequently extracted with Triton X-100 on ice. Proteins of the supernatant (*S*) and pellet (*P*) fractions were analyzed by autoradiography (^{35}S) or Western blotting with antibodies against HA (*αHA*) or VSV G (*αVSVG*). *B*, VSV-HA virions were extracted with 1% Triton X-100 on ice, and extracts were adjusted to 40% Optiprep. The extract (750 μl) was overlayered with 1.2 ml of 30% Optiprep and 250 μl of buffer, both containing 1% Triton X-100, and centrifuged for 2 h at 55,000 rpm (TLS 55 rotor, 4 °C). Fractions were collected from the top of the gradient (*lanes 1–6*) and analyzed by Western blotting with antibodies against HA or VSV G, respectively.

DISCUSSION

Viruses as Model System to Study Membrane Domains—We have analyzed detergent-resistant lipid complexes in cellular membranes and different viral envelopes. The use of the viral envelope as a model membrane has several advantages over artificial lipid vesicles. Since the envelope is derived from the cellular plasma membrane, it has a natural lipid composition. Furthermore, bilayer asymmetry is preserved. Finally, the membrane contains proteins that will have a profound influence on the membrane structure. Our results demonstrate that the three viral envelopes contain lipid raft domains to a very different extent as judged by the detergent solubility of their constituent proteins and lipids. For artificial lipid vesicles, it was demonstrated that such detergent-insoluble complexes are derived from lipids in the liquid-ordered or gel phase (15, 17). Several of our observations suggest that influenza viruses contain lipids in a state similar to the liquid-ordered phase. First, cholesterol, sphingomyelin, and phosphatidylcholine in the influenza envelope are detergent-insoluble to a large extent. Second, cholesterol is required for the insolubility of the lipids. We have previously demonstrated that cholesterol is required for detergent insolubility of HA in cells as well in the isolated virions (14). The cholesterol dependence of SM and PC insolubility shows that cholesterol is generally required for the formation of insoluble complexes in biological membranes rather than only for a specific interaction between HA and the sterol. This is consistent with the requirement for cholesterol for the formation of ordered domains in the membrane (33). Finally, the DPH fluorescence polarization measurements demonstrate that acyl chains in the influenza virus envelope are highly ordered, whereas acyl chains in the VSV envelope are more mobile. In lipid vesicles, detergent-resistant membranes in the gel phase can be formed independently of cholesterol, simply by the addition of high amounts of sphingolipid (15). Instead, the lipid composition of the membranes in mammalian cells appears to be such that cholesterol is essential to promote the formation of the liquid-ordered phase, whereas a gel phase does not exist in cell membranes (34). However, it has been observed that mammalian cells can remodel their lipid acyl chains when grown under sterol-limiting conditions. Insect cells, on the other hand, remain viable when cellular cholesterol levels are reduced to 1–3% (35). Therefore, cells appear capable of changing their lipid composition to allow the formation of laterally organized domains even in the absence of cholesterol as has been observed for glycosphingolipids. However, the properties of such cholesterol-poor domains have not been characterized

(36).

Influence of Proteins on Membrane Domain Organization—Viral envelopes have a very high protein:lipid ratio. It is therefore conceivable that, in addition to the lipid composition, proteins exert an influence on the membrane structure. Influenza virus HA contains in its membrane-spanning domain a determinant that is required for the association with lipid raft domains (14, 37). Also, the palmitoylation of HA might contribute to the detergent insolubility, because a mutant lacking the cytoplasmic tail with the palmitoylation sites (38) is fully soluble.[2] Both determinants interact with the lipid bilayer and might affect the order of the lipids. However, the fact that HA incorporated into the VSV envelope was mostly soluble suggests that this membrane does contain fewer lipids in an ordered phase and that the inclusion of HA is not sufficient to organize such a detergent-resistant environment. Most likely, the VSV and influenza viruses acquire membranes with different lipid composition from the plasma membrane. The cholesterol to phospholipid ratios in the envelopes of influenza viruses, VSV, and SFV appear to be similar (1, 3, 39). Since sphingolipids and saturated phospholipids are known to promote the formation of raft domains (15, 16), these lipids might be preferentially incorporated into influenza viruses. In the future, a detailed analysis of the lipid head groups and acyl chains in the different viral envelopes should clarify this issue. Such an analysis might also allow the identification of raft-promoting lipids without the use of detergents.

It has recently been shown that raft domains in the plasma membrane are small and dispersed but that they can coalesce upon cross-linking of their lipid or protein constituents (8, 9, 40). Similarly, large oligomeric proteins like caveolin could stabilize rafts into macroscopic domains in the liquid-ordered phase. Inversely, the size and stability of the caveolin complexes are modulated by raft domains, illustrating an interdependence of raft size and protein oligomerization (41). Similarly, rafts could be stabilized by proteins binding to the cytoplasmic leaflet of the domains (42). We speculate that lipids in cellular membranes are kept in an equilibrium state in which specific protein interactions can modulate the dynamics of rafts, leading to coalescence or dispersion of domains in the liquid-ordered phase, depending on the oligomeric state of the protein. Coalescence of domains in the liquid-ordered phase could induce bending of the membrane (43). In agreement with this view, caveolae form invaginations on the cell surface but are flattened out when cholesterol is removed (44). Similarly, coalescence of a liquid-ordered phase might facilitate budding of influenza viruses from the plasma membrane. In this latter case, the membrane is curved outwards, whereas in the former it is curved inwards. The topology of the invagination might depend on the properties of the specific proteins involved.

Lipid Domains in Virus Assembly—We have shown that lipids in the influenza virus envelope are in an ordered state and less ordered in the VSV envelope. This suggests that influenza virus is assembled from raft domains in the plasma membrane. For VSV, fluorescence digital microscopy has revealed the formation of domains during the budding step. Both the glycoprotein and the matrix protein were shown to induce lateral organization of lipid within the membrane (39, 45). Consistently, the lipid composition of the VSV envelope was shown to differ from the host cell membrane, indicating selective inclusion of specific lipid components (2). For influenza viruses, a detailed comparison of the lipid head groups and the acyl chains in the envelope and the host cell membrane has not been performed. The incorporation of raft domains into the viral envelope may explain some puzzling features of influenza virus assembly. It has been observed that lateral cross-linking of HA by antibodies creates large patches with raft characteristics (40). Concomitantly, cytoplasmic proteins with raft affinity can be recruited to the raft patches without requiring direct interactions with the influenza spike protein (40). It has been a surprising finding that influenza viruses in which the cytoplasmic tails of both glycoproteins have been deleted still form infectious progeny (46). This could be explained by connecting the influenza spike proteins over the rafts with the cytoplasmic matrix protein. Assuming that the spike proteins interact laterally with each other, this would lead to exclusion of cellular glycoproteins. A role for raft domains during virus assembly is also highlighted by the fact that the membrane-spanning domain of influenza HA is required for both its incorporation into the envelope and raft association (14, 37, 47). Therefore, coalescing lipid domains might be employed at sites of particle assembly to facilitate the exclusion of cellular proteins and to favor specific incorporation of the viral components.

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[2] P. Scheiffele and K. Simons, unpublished data.

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