Formation of Membrane Domains Created during the Budding of Vesicular Stomatitis Virus. A Model for Selective Lipid and Protein Sorting in Biological Membranes[†]

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ABSTRACT: Vesicular stomatitis virus buds from domains of the plasma membrane that have a unique protein and lipid composition. Fluorescence digital imaging microscopy and resonance energy transfer were used to determine how the two viral envelope-associated proteins, the G and the M proteins, could alter the lateral distribution of lipids in large unilamellar vesicles and form domains. The G protein formed large domains in vesicles containing phosphatidic acid but not with phosphatidylserine, while the M protein formed domains enriched in both acidic phospholipids. Domains enriched in sphingomyelin were observed only when both the G protein and the M protein were present in vesicles containing phosphatidic acid. Phosphatidylcholine and gramicidin (chosen to represent a host membrane protein) were excluded from the domains. Cholesterol was induced to partition into the domains only in vesicles containing phosphatidic acid and sphingomyelin along with both of the proteins. Phosphatidylethanolamine was not enriched or depleted in the domains. Domains of similar composition were formed using vesicles made from dioleylphospholipids and the lipids extracted from BHK-21 plasma membranes, indicating that the fatty acid composition was not as important as the polar head groups of the phospholipids. The phospholipid and cholesterol compositions of the domains formed by the G and the M proteins in vesicles were very similar to the composition of the viral envelope, suggesting that the domains represent the areas in the plasma membrane where the virus buds. This study provides a model for selective lipid and protein sorting that occurs in biological membranes.

Vesicular stomatitis virus (VSV)[1] represents one member of the class of enveloped viruses that bud from the surface of infected cells. During the assembly and budding of the viral particle, the RNA core of the virus is surrounded by the plasma membrane, and the membrane acquires a unique composition (Pal & Wagner, 1987; Stephens & Compans, 1988). Only two virally encoded proteins are associated with the virus envelope, the G protein and the M protein. Host proteins are largely excluded from the virus. The G protein, or glycoprotein, contains a cleavable signal sequence and a single transmembrane segment. It is processed and transferred to the plasma membrane as the first component of the virus. The viral M protein, however, is synthesized on soluble ribosomes and binds to the plasma membrane at the area where the viral G protein accumulates during the budding process (Knipe et al., 1977). It has been suggested that the M protein mediates the association between the nucleocapsid and the cytoplasmic portion of the G protein in the membrane (Cartwright, 1977; Schnitzer et al., 1979; Schnitzer & Lodish, 1979). Chemical cross-linking studies suggest that the M protein is in close proximity to the viral glycoprotein (Dubovi & Wagner, 1977). One study has shown that the M protein can stabilize the interactions between G protein subunits (Lyles et al., 1992). Interactions between the G and M proteins also are suggested from experiments showing that the mobility of the G protein in the plasma membrane is modulated by the M protein (Reidler et al., 1981). Both of the proteins are indispensable for the maturation and budding of the virus.

There are major differences in the lipid composition, in addition to the protein composition, between the envelope of the virus and the host plasma membrane (Pessin & Glaser, 1980). The phospholipid composition of the virus is complex, but in general the virus is enriched in PS and Sph and depleted in PC compared to the composition of the host plasma membrane. VSV also has a high cholesterol content compared to the host plasma membrane (Patzer et al., 1978; Pessin & Glaser, 1980). Cholesterol is important for membrane fluidity, the morphology of the viral envelope, and the infectivity of the virus (Moore et al., 1978; Pal et

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[1] Abbreviations: dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; dansyl-PA, 1-acyl-2-[11-(*N*-dansylamino)undecanoyl]phosphatidic acid; dansyl-PC, 1-acyl-2-[11-(*N*-dansylamino)undecanoyl]phosphatidylcholine; dansyl-PE, 1-acyl-2-(11-(*N*-dansylamino)undecanoyl]phosphatidylethanolamine; dansyl-PS, 1-acyl-2-[11-(*N*-dansylamino)undecanoyl]-phosphatidylserine; dansyl-Sph, [*N*-[11-(*N*-dansylamino)undecanoyl]-sphingosyl]phosphocholine; DOPA, dioleoylphosphatidic acid; DOPC, dioleoylphosphatidylcholine; DOPS, dioleoylphosphatidylserine; DOPE, dioleoylphosphatidylethanolamine; G protein, the glycoprotein of vesicular stomatitis virus; M protein, the matrix protein of vesicular stomatitis virus; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; NBD-cholesterol, 22-(*N*-NBD-amino)-23,24-bisnor-5-cholen-3-ol; NBD-PA, 1-acyl-2-[6-(*N*-NBD-amino)caproyl]phosphatidic acid; NBD-PC, 1-acyl-2-[6-(*N*-NBD-amino)caproyl]phosphatidylcholine; NBD-PE, 1-acyl-2-[6-(*N*-NBD-amino)caproyl]phosphatidylethanolamine; NBD-PS, 1-acyl-2-[6-(*N*-NBD-amino)caproyl]phosphatidylserine; NBD-Sph, [*N*-[6-(*N*-NBD-amino)caproyl]sphingosyl]phosphocholine; octyl glucoside, *n*-octyl β-D-glucopyranoside; PA, phosphatidic acid; PC, phosphatidylcholine; PI, phosphatidylinositol; PM, plasma membrane; PS, phosphatidylserine; Sph, sphingomyelin; VSV, vesicular stomatitis virus.

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al., 1981; Marquardt et al., 1993). Thus, the formation of a domain on the host plasma membrane from which the virus buds represents an important part of the assembly process.

Fluorescence digital imaging microscopy provides a means to visualize and quantitate the lateral distribution of both protein and lipid components in a membrane (Rodgers & Glaser, 1993). There is considerable heterogeneity in the distribution of components in biological membranes, and membrane proteins are an important cause of lipid heterogeneity (Glaser, 1993). In a previous study, it was shown that either the G or the M protein can induce the formation of domains in phospholipid vesicles containing PA (Luan & Glaser, 1994). The two proteins together cause the domains to condense and become further enriched in PA. This paper reports further experiments to characterize the properties of the G and the M proteins in forming domains using synthetic lipids or lipids derived from the plasma membrane of BHK cells. The results show that the domains formed by the viral proteins in vesicles closely mimic the domains that occur in the plasma membrane during viral budding.

MATERIALS AND METHODS

Materials. The preparation of phospholipids and the isolation of the G and the M proteins from VSV were described previously (Luan & Glaser, 1994). NBD-Sph and NBD-cholesterol were purchased from Molecular Probes (Eugene, OR). Gramicidin (*Bacillus brevis*, Dubos) and dansyl chloride were purchased from the Sigma Chemical Co. (St. Louis, MO).

Preparation of Fluorescently Labeled G and M Proteins. The G protein of VSV was labeled with dansyl chloride as described previously (Luan & Glaser, 1994). To label the M protein, 0.1 mg of lissamine rhodamine B sulfonyl chloride (from a stock solution of 10 mg/mL in water) was added per milligram of protein of the whole virus in a final volume of 0.5 mL in 10 mM Tris, pH 7.5. An equal volume of 0.5 M NaCl and 2% Triton X-100 in 10 mM Tris, pH 8.0, was added. The pH value of the solution was adjusted to 9.0 with 1.0 M Na_2CO_3, and the solution was stirred in the dark at room temperature for 1 h. The solution was centrifuged at 200000*g* for 2 h, and the supernatant containing the lissamine rhodamine-labeled M protein was loaded on a Whatman P11 phosphocellulose column. This step and the further purification of the labeled M protein were carried out using the same procedure that was used for the unlabeled M protein (Luan & Glaser, 1994).

Purification of the Plasma Membrane from BHK-21 Cells, Lipid Extraction, and Lipid Analysis. The plasma membrane of BHK-21 cells was isolated using the method described by Esko et al. (1977). The purity of the preparation was determined by assaying the activity of the oubain-sensitive (Na^+,K^+)ATPase as described by Schimmel et al. (1973) with the modifications made by Esko et al. (1977). The purity was comparable to that obtained by Esko et al. (1977). Total lipids from the plasma membrane were extracted by the method of Rose and Oklander (1965). The phospholipids were separated by two-dimensional thin-layer chromatography on RediCoat 2D plates (250 μm, Supelco, Inc.) using $CHCl_3/CH_3OH/NH_4OH$ (65/25/5) in the first dimension and $CHCl_3/CH_3COCH_3/CH_3OH/CH_3COOH/H_2O$ (6/8/2/2/1) in the second dimension as described by Pessin and Glaser (1980). The spots containing the phospholipids were scraped from the plate, and the quantity of phospholipid in a spot was determined by the procedure of Ames and Dubin (1960). The cholesterol concentration was measured with a reagent kit obtained from the Sigma Chemical Co. (St. Louis, MO).

Preparation of Large Unilamellar Vesicles for Microscopy. Large unilamellar vesicles suitable for viewing with the fluorescence digital imaging microscope were prepared basically using the procedure described by Darszon et al. (1980). Specifically, a total of 250 nmol of phospholipids in chloroform was placed in a clean 15 × 45 mm screw cap vial with a flat bottom. The chloroform was evaporated using a gentle stream of nitrogen to form a very thin and uniform layer of lipid along the bottom of the vial. Then 0.5 mL of 10 mM Tris buffer, pH 7.6, was gently placed on top of the lipid film. The vial was kept in a dark, vibration-free place for 48 h. The clear layer of solution containing the large vesicles was carefully removed, and the vesicles were used for microscopy (Haverstick & Glaser, 1987).

Reconstitution of the Viral Proteins into Vesicles for Microscopy. The reconstitution of the G protein and the M protein into large unilamellar vesicles was achieved by hydrating the dried lipid film with the purified, detergent-free proteins in 10 mM Tris, pH 7.6, for 48 h as previously described (Luan & Glaser, 1994). The molar ratio of G protein:M protein:phospholipid was 1:5:500, which represents the approximate ratio in the virus. Purification of the G protein using nonionic detergents, as done in this study, retains the functional state of the protein since the protein partitions into bilayers in its native configuration (Petri & Wagner, 1980), it causes vesicles to fuse (Eidelman et al., 1984), and it can restore infectivity to spikeless virions (Bishop et al., 1975).

Dansylation of Gramicidin and Its Incorporation in Vesicles for Microscopy. Gramicidin was fluorescently labeled with dansyl chloride using the procedure of Veatch and Blout (1976) as modified by Haverstick and Glaser (1989). Specifically, 50 mg of dansyl chloride was added to 4 mL of acetone containing 25 mg of gramicidin. Then 2 mL of 0.1 M sodium bicarbonate was added with vigorous stirring, and the mixture was incubated for 3 h at room temperature. The solvent was evaporated under a stream of nitrogen, and the residue was dissolved in methanol. The sample was loaded onto a Sephadex LH 20 column (1 × 25 cm) and eluted with methanol. Dansylgramicidin was excluded from the column. The product was characterized by tryptophan and dansyl absorbance and their fluorescence spectra. The incorporation of gramicidin into large unilamellar vesicles was done by forming the lipid film containing the peptide and then hydrating the film as described above. The procedure to form the lipid film was repeated several times by dissolving the film and drying it again until a uniform, thin film had been formed on the bottom of the vial.

Microscopy. In addition to the dansyl and NBD imaging carried out previously (Luan & Glaser, 1994), the lissamine rhodamine imaging was done using excitation wavelengths between 530 and 560 nm and emission wavelengths greater than 570 nm. The background illumination was subtracted from all the images, and the resulting images were normalized to a mean radiance value of 100 ± 4. A pseudo color scheme, which ranged from blue (0) to yellow to red (255), was applied to the radiance values in order to display the images as shown in Figure 1.

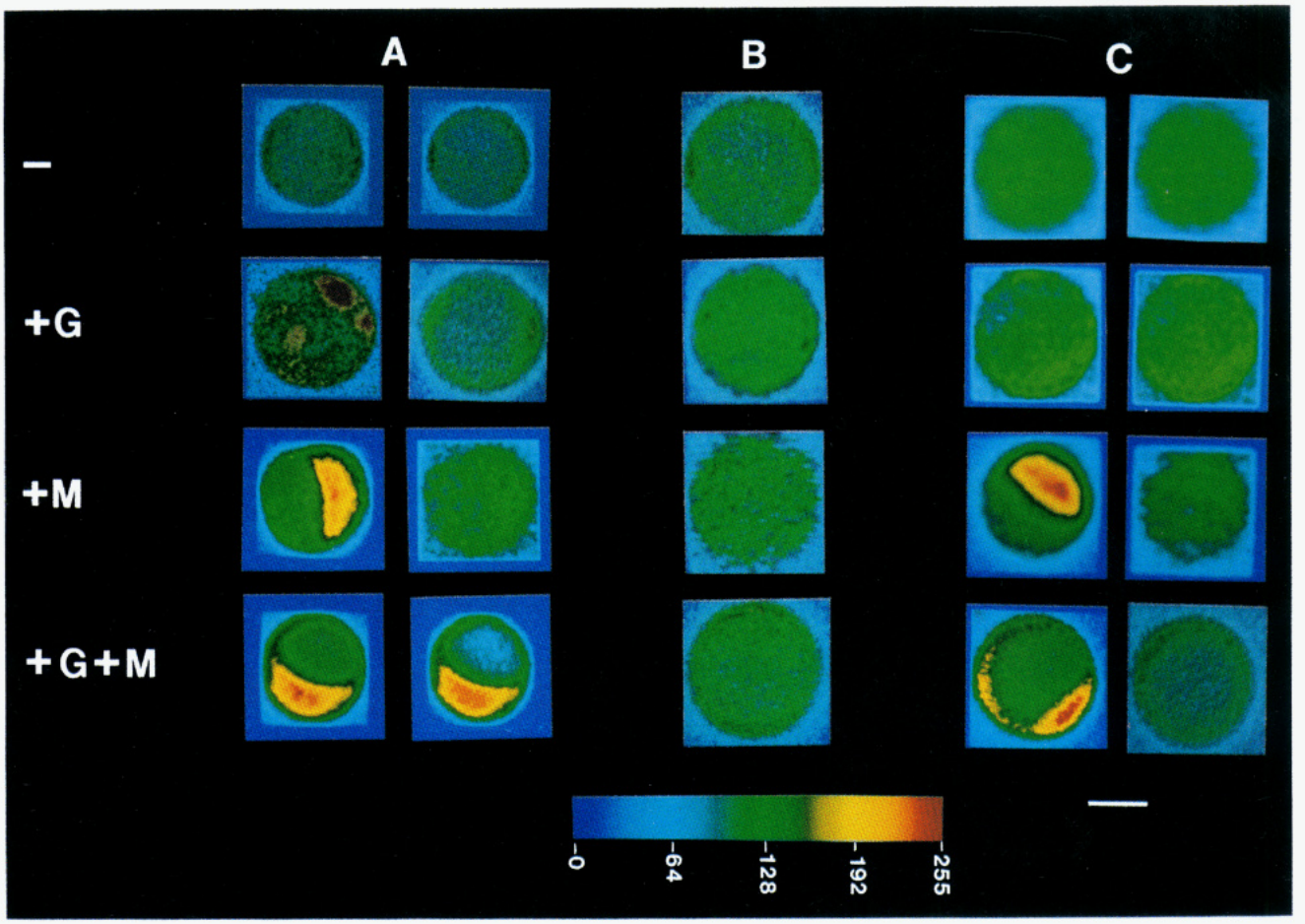

FIGURE 1: Formation of membrane domains induced by the G and the M proteins of VSV in large unilamellar vesicles. Shown in panel A are images of double-labeled DOPC vesicles containing 10 mol % DOPA, 2 mol % dansyl-PA, 10 mol % Sph, and 0.5 mol % NBD-Sph. Panel B shows NBD-Sph images of DOPC vesicles containing 10 mol % Sph and 0.5 mol % NBD-Sph. Panel C shows images of double-labeled vesicles similar to those in panel A except DOPA and dansyl-PA were replaced by DOPS and dansyl-PS, respectively. In both panel A and panel C, the vesicles were visualized for dansyl fluorescence (images on the left) and NBD fluorescence (images on the right). Either no protein, the G protein, the M protein, or both proteins together were added to the vesicles shown in the first, second, third, or fourth rows of the three panels, respectively. The images were normalized to a mean radiance value of 100 ± 4. The pseudo color scheme applied to the images is shown with the equivalent radiance values at the bottom of the figure. The white bar equals 4 μm.

Measurement of Fluorescence Resonance Energy Transfer. The energy transfer between the tryptophan residues of the G protein and different dansyl-labeled phospholipids was carried out in small unilamellar vesicles prepared by sonication. The reconstitution of the G and the M protein with the vesicles and the measurements were carried out by the same methods as used previously (Luan & Glaser, 1994). The tryptophan residues were excited at 280 nm, and the increase in dansyl fluorescence due to energy transfer was determined. The efficiency of energy transfer can be estimated by comparing the slope of the graph of F/F_0 versus the concentration of the G protein in vesicles, where F and F_0 are the dansyl emission intensities in the presence and absence of the G protein, respectively. The efficiency of energy transfer from the M protein to the dansyl groups of the phospholipids was not significant in these experiments.

RESULTS

Formation of Domains Enriched in Acidic Phospholipids by the G and the M Proteins of VSV. Previously it was shown that both the G and the M proteins can form domains in large unilamellar vesicles that are enriched in acidic phospholipids. However, both proteins do not act equivalently. The G protein will form domains enriched in PA but not PS, while the M protein will form domains enriched in either PA or PS. These results are illustrated in Figure 1 using DOPC vesicles with either 10 mol % DOPA plus 2 mol % dansyl-PA (panel A, images on the left) or 10 mol % DOPS plus 2 mol % dansyl-PS (panel C, images on the left). (The vesicles in Figure 1 also contained Sph, but Sph did not influence these results.) When both proteins were reconstituted into the vesicles at the same time, the domains were condensed on the basis of the quantitation of domains in 70 vesicles (Luan & Glaser, 1994).

Formation of Sph-Enriched Domains Induced by the Combination of the G Protein and the M Protein. One objective of this research was to determine how closely the composition of the domains formed by the viral proteins resembled the composition of the viral envelope. Toward this end, the next phospholipid examined was Sph since this phospholipid is also enriched in the virus versus the plasma membrane of the infected cell. In DOPC vesicles containing only 10 mol % Sph and 0.5 mol % NBD-Sph, neither the two proteins individually nor the combination of the G and the M proteins induced the formation of domains (Figure 1, panel B). When PA was included in the vesicles, the result was quite different. As before, when DOPC vesicles containing 10 mol % DOPA, 2 mol % dansyl-PA, 10 mol % Sph, and 0.5 mol % NBD-Sph were reconstituted with only one of the two proteins, Sph was not significantly enriched in the domains (Figure 1, panel A images on the right). Only when both the G and the M proteins were

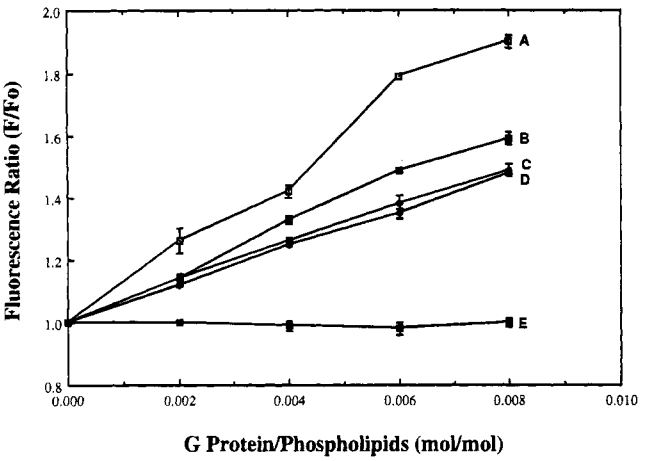

FIGURE 2: Energy transfer from the tryptophan residues of the G protein to dansyl-Sph in vesicles in the presence of the M protein (A). The phospholipid concentration was 500 nmol/mL, and the composition of the vesicles was egg PC with 10 mol % DOPA and 1.5 mol % dansyl-Sph. The molar ratio of G/M was maintained at 1/5 when the M protein was added. The samples were excited at 280 nm, and dansyl fluorescence enhancement was measured. F_0 and F are the dansyl fluorescence before and after the addition of the proteins, respectively. Curves C and D show the energy transfer from the G protein to dansyl-Sph in the absence of the M protein but with 10 mol % DOPA (C) and without DOPA (D). Energy transfer between the G protein and dansyl-PC (1.5 mol %) in vesicles with no other components is shown by curve B. The lack of energy transfer from the M protein to dansyl-Sph (1.5 mol %) with 10 mol % DOPA, but without the G protein, is shown in curve E.

incorporated into the PA-containing vesicles at the same time was the NBD-labeled Sph sequestered in the domains. The NBD-Sph domains colocalized with the dansyl-PA domains.

PA appeared to have a special role in forming the domains since its function could not be replaced by another acidic phospholipid, PS. Not only was PA necessary to form domains when the G protein alone was added to vesicles, but PA also was critical to form domains enriched in Sph. When the G protein was added to double-labeled vesicles containing PS and Sph, no significant domains were formed (Figure 1, panel C). When the M protein was added to the vesicles in place of the G protein, domains were formed that were enriched in PS, but Sph remained uniformly distributed in the vesicles. When both the G and M proteins were added together, the results were similar in that the domains were enriched in PS while the distribution of Sph was uniform.

Measurements using fluorescence resonance energy transfer were carried out in order to confirm the fluorescence microscopy results using a second method. The energy transfer between the tryptophan residues of the G protein and dansyl-labeled Sph in egg PC vesicles with or without PA is shown in curves C and D of Figure 2, respectively. The results are presented as the dansyl fluorescence in the presence of the G protein over the fluorescence in the absence of the G protein as a function of the G protein concentration. The slopes of the two curves were very close to that obtained for the G protein-reconstituted control vesicles containing only PC and dansyl-labeled PC (Figure 2, curve B), which demonstrated a random distribution of labeled phospholipid around the G protein (Luan & Glaser, 1994). Thus, the results indicate a random distribution of PC and Sph in the vesicles whether or not PA was present. Although the enhancement of the dansylphospholipid fluorescence due to energy transfer was appreciable, the quenching of the tryptophan fluorescence was small. Since resonance energy transfer depends on the sixth power of the distance between the energy donor and acceptor, the results indicate that only a limited number of the 12 tryptophan residues in the G protein were involved in the energy transfer.

The M protein binds to the surface of membranes, and no energy transfer is observed with dansyl-labeled PA, for example, even though the protein forms domains with PA (Luan & Glaser, 1994). There also was no energy transfer between the M protein and dansyl-Sph in vesicles with only the M protein incorporated (Figure 2, curve E). The lack of energy transfer may be due to the distance between the tryptophan residues of the M protein and the dansyl groups, and the low concentration of dansylphospholipid in the vesicles.

When the M protein and the G protein were added together to egg PC vesicles containing PA and Sph, there was an increase in fluorescence energy transfer from the G protein to dansyl-Sph (Figure 2, curve A). These results suggest that the addition of the M protein to the G protein-containing vesicles caused enrichment of Sph around the G protein. When PA was eliminated from the vesicles, no such an effect was observed. These results correspond to the results obtained using fluorescence microscopy as shown in Figure 1.

Depletion of PC and Gramicidin from Domains Formed by the G and the M Proteins. VSV is depleted in PC versus the plasma membrane of the host cell, and in addition, host proteins normally present in the plasma membrane are largely excluded from the virus. The distribution of PC was studied in vesicles labeled with dansyl-PC and reconstituted with both the G protein and the M protein. The vesicles also contained PA, PS, and Sph for optimal domain formation. Panel A of Figure 3 shows images of three double-labeled vesicles containing 67.5 mol % DOPC, 10 mol % DOPA, 10 mol % DOPS, 10 mol % Sph, 2 mol % dansyl-PC, and either 0.5 mol % NBD-PA (row 1), 0.5 mol % NBD-PS (row 2), or 0.5 mol % NBD-Sph (row 3). The vesicles were viewed for NBD fluorescence (images on the left) and dansyl fluorescence (images on the right). As shown in the figure, the NBD image and dansyl image of the vesicles were always complementary to each other, suggesting the exclusion of PC from the PA-, PS-, and Sph-enriched domains induced by the combination of the G protein and the M protein.

Dansyl-labeled gramicidin was chosen to represent a typical host membrane protein with no lipid specificity. The same experiments were carried out using dansylgramicidin as were done to demonstrate the exclusion of PC from the domains. Shown in panel B of Figure 3 are images of three double-labeled vesicles containing 67.5 mol % DOPC, 10 mol % DOPA, 10 mol % DOPS, 10 mol % Sph, 2 mol % dansylgramicidin, and either 0.5 mol % NBD-PA (row 1), 0.5 mol % NBD-PS (row 2), or 0.5 mol % NBD-Sph (row 3). The vesicles were reconstituted with the G protein and the M protein and viewed for NBD fluorescence (images on the left) and dansyl fluorescence (images on the right). The same results were obtained as for the vesicles labeled with dansyl-PC; i.e., dansyl-labeled gramicidin was excluded from the PA-, PS-, and Sph-enriched domains induced by the G and M proteins.

In an effort to investigate the distribution of the G and the M proteins when domains enriched in PA, PS, and Sph were formed, advantage was taken of triple-labeled vesicles reconstituted with a dansyl-labeled G protein, a lissamine

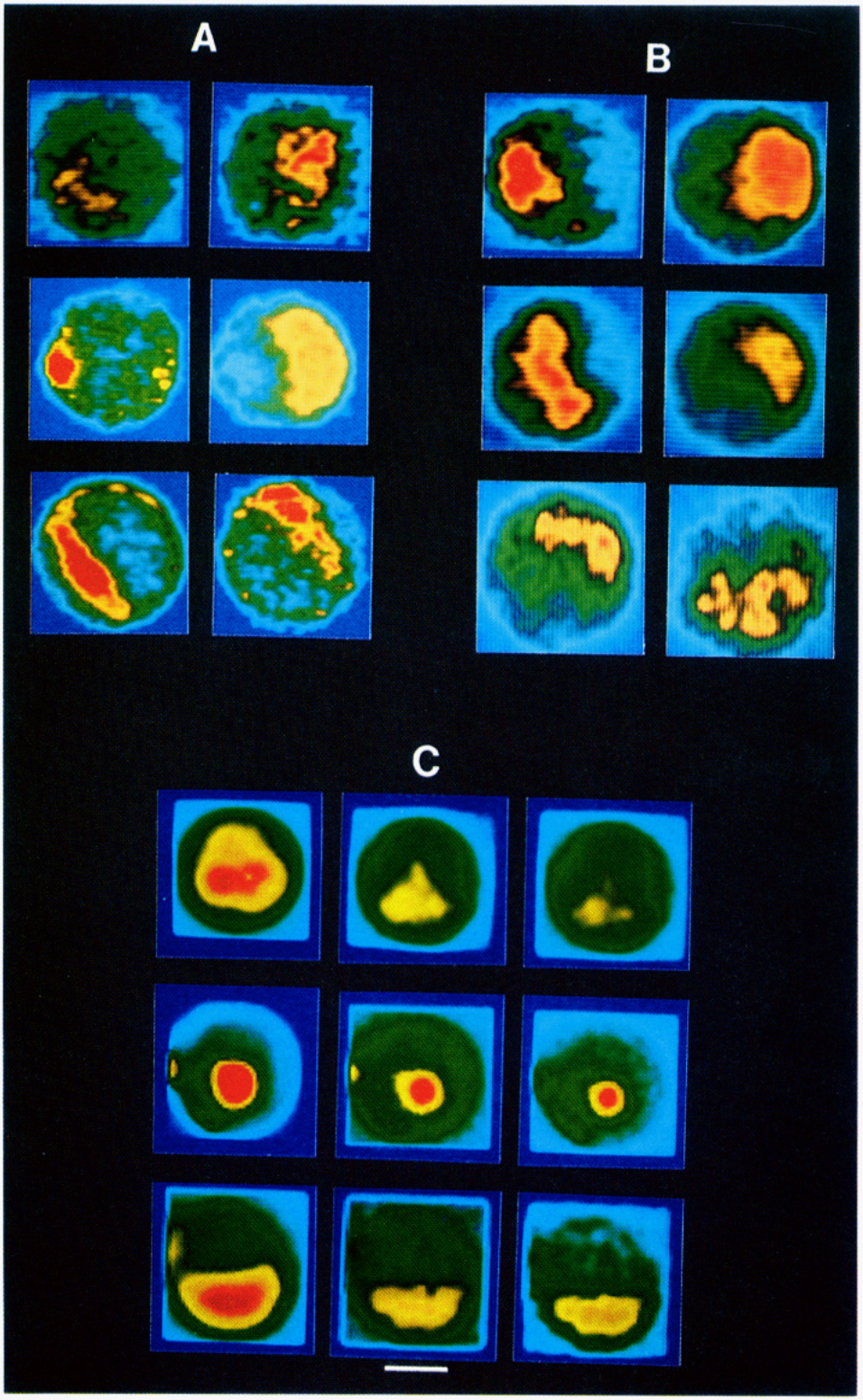

FIGURE 3: Exclusion of PC and gramicidin and the colocalization of the G and the M protein in the PA-, PS-, and Sph-enriched domains induced by the two proteins in the vesicles. Shown in panel A are images of three double-labeled DOPC vesicles containing 10 mol % DOPA, 10 mol % DOPS, 10 mol % Sph, 2 mol % dansyl-PC, and either 0.5 mol % NBD-PA (row 1), 0.5 mol % NBD-PS (row 2), or 0.5 mol % NBD-Sph (row 3). The vesicles were reconstituted with the G protein and the M protein and viewed for NBD fluorescence (images on the left) and dansyl fluorescence (images on the right). Shown in panel B are vesicles similar to those in panel A, except that 2 mol % dansyl-PC was replaced by 2 mol % dansylgramicidin in all three vesicles. Images of NBD fluorescence are shown on the left, and images of dansyl fluoresence are shown on the right. Shown in panel C are images of three triple-labeled DOPC vesicles containing 10 mol % DOPA, 10 mol % DOPS, 10% Sph, and either 0.5% NBD-PA (row 1), 0.5 mol % NBD-PS (row 2), or 0.5 mol % NBD-Sph (row 3) with the incorporation of the fluorescently labeled G and M proteins. The vesicles were viewed for NBD fluorescence (left image), dansyl G protein fluorescence (middle image), and lissamine rhodamine M protein fluorescence (right image). The white bar equals 4.5 μm.

rhodamine-labeled M protein, and NBD-labeled phospholipids. In panel C of Figure 3, row 1 shows a vesicle composed of 69.5 mol % DOPC, 10 mol % DOPA, 10 mol % DOPS, 10 mol % Sph, and 0.5% NBD-PA, with the incorporation of the two fluorescently labeled viral proteins. The vesicle was viewed separately for NBD fluorescence (left image), dansyl fluorescence (middle image), and lissamine rhodamine fluorescence (right image). When NBD-

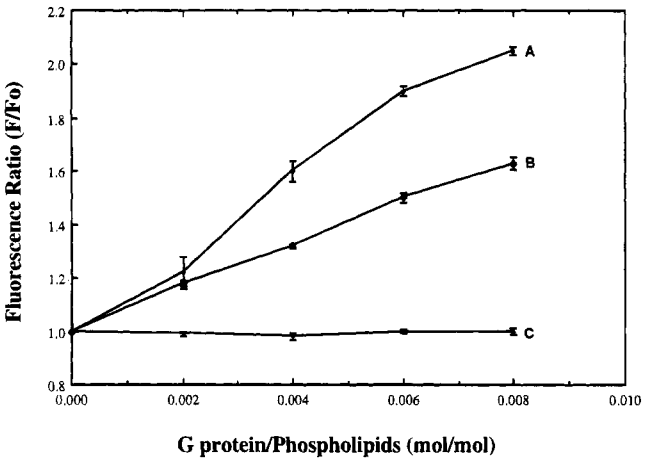

FIGURE 4: Energy transfer from the tryptophan residues of the G protein to dansyl-PS in vesicles in the presence of the M protein (A) and the absence of the M protein (B). The egg PC vesicles were made at a concentration of 500 nmol/mL with 1.5 mol % dansyl-labeled phospholipid. The molar ratio of G/M was maintained at 1/5 when the M protein was incorporated into the vesicles. The data collecting and processing were the same as described in Figure 2. The lack of energy transfer from the M protein to dansyl-PS under the same conditions but with no G protein is shown by curve C.

PA was induced to form a domain in the vesicle, both the dansyl-G protein and the rhodamine-M protein were found in the same area where PA was enriched. Although both the G protein and the M protein individually can induce the formation of PA-enriched domains, the two proteins together colocalized in the domains rather than forming separate domains.

Row 2 and row 3 of panel C show triple images of two vesicles with the same compositions as the vesicle in row 1 except that 0.5 mol % NBD-PA was replaced by either 0.5 mol % NBD-PS (row 2) or 0.5 mol % NBD-Sph (row 3), respectively. In all cases the G and M proteins colocalized in the domains.

The association of the G protein with PS-enriched domains induced by the M protein was supported by fluorescence energy transfer measurements. The energy transfer from the tryptophan residues of the G protein to dansyl-PS (Figure 4, curve B) was similar to the energy transfer to dansyl-PC when only the G protein was reconstituted into vesicles (Figure 2, curve B), indicating no enrichment of PS around the G protein. When the M protein was added to the vesicles containing the G protein and dansyl-PS, energy transfer between the G protein and dansyl-PS increased (Figure 4, curve A). Since the tryptophan residues of the M protein were too far from the dansyl groups in the bilayer to contribute to the energy transfer (Figure 4, curve C), the enhancement of energy transfer between the G protein and dansyl-PS indicated that dansyl-PS was enriched around the G protein in the presence of the M protein.

Formation of Cholesterol-Enriched Domains by the G Protein and the M Protein. In order to study the possibility that cholesterol-enriched domains would be induced by the viral proteins, the G and M proteins were reconstituted either individually or together in DOPC vesicles containing 10 mol % cholesterol and 0.5 mol % NBD-cholesterol. The NBD images of vesicles showed that cholesterol remained uniformly distributed in the vesicle after the addition of the G and the M proteins (Table 1). In addition, the two proteins had no effect on the cholesterol distribution in vesicles

Table 1: Roles of the G and M Proteins in the Formation of Cholesterol-Enriched Domains in Vesicles Made with Different Phospholipids[a]

vesicle composition						
phospholipid				protein		cholesterol domain formation[c]
PE	PS	Sph	PA	G	M	
−	−	−	−	−	−	−
−	−	−	−	+	−	−
−	−	−	−	−	+	−
−	−	−	−	+	+	−
+	−	−	−	+	+	−
−	+	−	−	+	+	−
−	−	+	−	+	+	−
−	−	−	+	+	+	−
−	−	+	+	+	−	−
−	−	+	+	−	+	−
−	−	+	+	+	+	+
−	+	+	−	+	+	−
plasma membrane lipids[b]				−	−	−
plasma membrane lipids[b]				+	−	−
plasma membrane lipids[b]				−	+	−
plasma membrane lipids[b]				+	+	+

[a] DOPC vesicles containing 10 mol % cholesterol, 0.5 mol % NBD-cholesterol, and 10 mol % of the specified phospholipid were reconstituted with the G and M proteins as indicated. [b] Vesicles were made from lipids extracted from the plasma membrane of BHK cells plus 0.5 mol % NBD-cholesterol and reconstituted with the G and M proteins as indicated. [c] Vesicles were viewed for NBD-cholesterol, and the absence (−) or the presence (+) of cholesterol-enriched domains was determined.

containing either 10 mol % DOPA, 10 mol % DOPS, 10 mol % DOPE, or 10 mol % Sph.

The inability of the viral proteins to induce domains enriched in cholesterol in these vesicles contrasted to the cholesterol domains observed when vesicles were made from lipids extracted from the plasma membrane of BHK-21 cells (Table 1). Both the G and the M proteins were necessary to induce cholesterol to partition into the domains. The fact that cholesterol-enriched domains formed with the extracted lipids suggested that participation of more than one phospholipid might be necessary in order to induce cholesterol to partition into the domains. Since the combination of the G protein and the M protein induced Sph-enriched domains in vesicles only when the vesicles contained PA, the combination of PA and Sph was used to examine the formation of cholesterol-enriched domains. When DOPC vesicles containing 10 mol % DOPA, 10 mol % Sph, 10 mol % cholesterol, and 0.5 mol % NBD-cholesterol were used, a uniform distribution of NBD-cholesterol was observed in the vesicles without the addition of the proteins or with the addition of only the G protein or the M protein. However, when both the G protein and the M protein were incorporated into the vesicles together, NBD-cholesterol was no longer uniformly distributed in the vesicles, but rather it was sequestered into domains. PS would not substitute for PA in inducing cholesterol to partition into the domains.

The colocalization of cholesterol-enriched domains with the PA-, PS-, and Sph-enriched domains induced by the combination of the G protein and the M protein was demonstrated using double-labeled and triple-labeled vesicles, and the results are presented in Figure 5. Panels A−C show images of double-labeled vesicles made from lipids extracted from the plasma membrane of BHK-21 cells plus 0.5 mol % NBD-cholesterol and either 2 mol % dansyl-PA (A), 2 mol % dansyl-PS (B), or 2 mol % dansyl-Sph (C) and reconstituted with both the G and M proteins. The vesicles

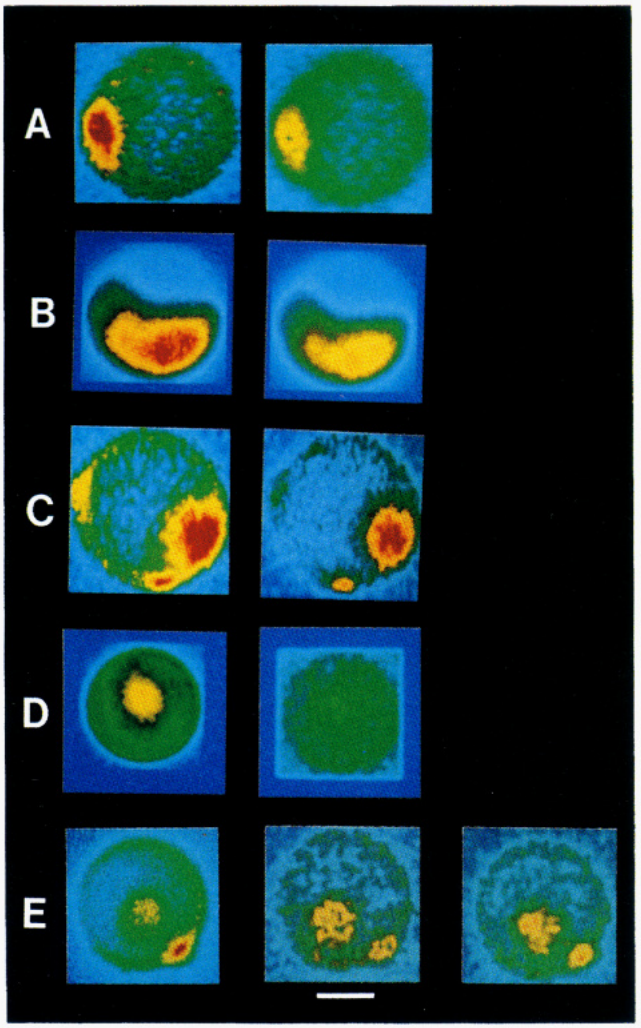

FIGURE 5: Colocalization of cholesterol with PA, PS, Sph, the G protein, and the M protein in the domains induced by the combination of the two proteins. Shown in panels A−D are images of double-labeled vesicles containing lipids extracted from the plasma membrane of BHK-21 cells, plus 0.5 mol % NBD-cholesterol and either 2 mol % dansyl-PA (A), 2 mol % dansyl-PS (B), 2 mol % dansyl-Sph (C), or 2 mol % dansyl-PE (D), and reconstituted with the combination of the G and M proteins. The vesicles were viewed separately for NBD fluorescence (images on the left) and dansyl fluorescence (images on the right). Shown in panel E are images of the similar vesicle containing extracted lipids and triple-labeled with 0.5 mol % NBD-cholesterol with the incorporation of dansyl-labeled G protein and lissamine rhodamine-labeled M protein. The vesicle was viewed separately for NBD fluorescence (left image), dansyl fluorescence (middle image), and lissamine rhodamine fluorescence (right image). The white bar equals 4 μm.

were viewed separately for NBD fluorescence (images on the left) and dansyl fluorescence (images on the right). Once again, large NBD-cholesterol-enriched domains colocalized with the dansyl-PA-, dansyl-PS-, and dansyl-Sph-enriched domains in the vesicles.

Figure 5E shows images of a triple-labeled vesicle made from extracted BHK-21 plasma membrane lipids plus 0.5 mol % NBD-cholesterol and the incorporation of the two fluorescently labeled viral proteins. The vesicle was viewed separately for NBD-cholesterol fluorescence (left image), dansyl-G protein fluorescence (middle image), and lissamine rhodamine-M protein fluorescence (right image). When NBD-cholesterol was induced to form a domain in the vesicle, both the dansyl-G protein and the lissamine rhodamine-M protein were found in the exact same areas where cholesterol was enriched.

Table 2: Phospholipid Composition of the Plasma Membrane BHK-21 Cells, the Envelope of VSV, and the Domains Induced by the G and M Proteins in Vesicles[a]

membrane preparation	phospholipid composition (mol %)						
	PC	PE	Sph	PS	PI	PA	others
plasma membrane	43.0	27.3	13.9	6.3	6.7	1.9	1.0
VSV envelope	28.5	27.0	21.6	13.3	4.1	4.3	1.4
domain (average)	29.9	27.3	19.0	9.6	−[b]	3.6	−
domain (maximum)	13.2	27.3	28.7	14.7	−	5.1	−

[a] The standard deviation associated with the determination of the phospholipid composition was less than ± 2%, and the standard deviation associated with determining the composition of the domains was less than ±5.0%. Domain (average) refers to the composition of the domains determined from the average radiance value of the domains, while domain (maximum) refers to the composition of the domains determined from the maximum radiance value in the domains. For PC, which is excluded from the protein-induced domains, the minimum radiance value was used. PE was not significantly enriched or depleted in the domains. [b] −, not determined.

Table 3: Ratio of Cholesterol/Phospholipid in the Plasma Membrane of BHK-21 Cells, the Envelope of VSV, and the Domains Induced by the G and M Proteins in Vesicles[a]

membrane preparations	cholesterol/phospholipid (mol/mol)
plasma membrane	0.49 ± 0.02
VSV envelope	0.83 ± 0.02
domain (average)	1.10
domain (maximum)	2.40

[a] The standard deviation associated with determining the composition of the domains was less than ±5.0%. Domain (average) refers to the cholesterol to phospholipid ratio determined from the average radiance values of the domains, while domain (maximum) refers to the cholesterol to phosholipid ratio determined from the maximum radiance values in the domains.

Uniform Distribution of Phosphatidylethanolamine in Vesicles Containing Domains Induced by the G and the M Proteins. The only other major phospholipid found in VSV is PE, and the concentration of PE in the virus is similar to the concentration of PE in the host cell plasma membrane. Figure 5D shows a double-labeled vesicle made from lipids extracted from the plasma membrane of BHK-21 cells. The vesicle also contained 0.5 mol % NBD-cholesterol and 2 mol % dansyl-PE, and it was reconstituted with both the G and the M proteins. A domain enriched in cholesterol was observed, but the distribution of PE remained uniform.

Phospholipid and Cholesterol Compositions of the Plasma Membrane of BHK-21 Cells and of the Envelope of VSV. Total phospholipids and cholesterol were extracted from the plasma membrane of BHK-21 cells and VSV, and their compositions were analyzed. Compared to the plasma membrane of the cells, the envelope of VSV had a higher concentration of PS and Sph and a lower concentration of PC (Table 2). The PE concentration was similar for both samples. Interestingly, the VSV envelope appears to contain more PA than the plasma membrane, but because of the low amount of PA in the samples, a large error was associated with these measurements.

Cholesterol analysis of the samples showed that the envelope of VSV also had a much higher cholesterol concentration than the plasma membrane of the host cells (Table 3).

Phospholipid and Cholesterol Compositions of the Domains Induced by the G Protein and the M Protein. Fluorescence digital imaging microscopy permits quantitative

analysis of the concentration of a fluorescent phospholipid in a vesicle. Because of the linear output of the system, it is possible to construct a standard curve of the radiance value of an image versus the concentration of a fluorescent phospholipid in the vesicle. The concentration of a fluorescent phospholipid in a domain can be determined from the radiance value of the domain using the standard curve. The radiance values have to be proportional to the concentration of the fluorophore, and photobleaching has to be avoided. If the initial concentration of the fluorescent phospholipid is known, the fold enrichment in the domain and the concentration in the domain can be calculated. Alternatively, the fold enrichment in the domain versus the average radiance value of the vesicle can be determined directly. Because of the heterogeneity of the vesicle preparations, it is necessary to collect images of a population of vesicles. A complete compositional analysis was carried out for vesicles made from the extracted lipid from the plasma membrane of BHK-21 cells and containing the G and M proteins. The vesicles were labeled with either 0.2 mol % NBD-PC, 0.2 mol % NBD-PA, 0.2 mol % NBD-PS, or 0.2 mol % NBD-Sph and viewed for NBD fluorescence. A total of 50 images were collected and analyzed for each population of vesicles. A domain was defined as the areas of a vesicle with radiance values larger than 150% of the average radiance value of the image of the whole vesicle. The average radiance value of the domain was determined, and the value was used to calculate the concentration of the fluorescent phospholipid in the domain. Both methods of calculating the composition gave comparable results.

For vesicles labeled with NBD-PC, a domain was defined as the areas of a vesicle with radiance values lower than 50% of the average radiance value of the image of the whole vesicle.

In vesicles containing the extracted lipids from the plasma membrane of BHK-21 cells, labeled with NBD-PE and reconstituted with both the G and M proteins together, no domains with enrichment or depletion of NBD-PE were observed. Therefore, the fold enrichment of PE in the domains induced by the G and M proteins was 1.0.

As discussed above, the average radiance value of a domain was used to calculate the concentration of a particular phospholipid in the domain, which is based on an arbitrary definition of a domain. Since the domains are not uniform, it is also instructive to consider the maximum concentration of a phospholipid in a domain by using the highest radiance value within a domain to determine the enrichment.

The phospholipid composition of the domains induced by the G and the M proteins in vesicles made from the lipids extracted from the plasma membrane of BHK-21 cells is presented in Table 2. Included in the table are molar percentages of PA, PS, PC, PE, and Sph determined for the average composition and the maximum concentration of a phospholipid (or the minimum concentration for PC) in the domains determined by fluorescence digital imaging microscopy. The agreement between the composition of the domains and the actual composition of VSV was surprisingly good. The values for the average composition of the domains were closer to the actual composition for PC, Sph, and PA while the maximum values of the domains showed slightly better agreement for PS. Since PE was not enriched or depleted in the domains, the result was the same as the actual composition.

The cholesterol composition of the domains was determined using similar vesicles labeled with 0.2 mol % NBD-cholesterol, and the results are presented in Table 3. The molar ratio of cholesterol to total phospholipids was 1.1 for the average composition of the domains induced by the G protein and the M protein. This value is closer to the actual ratio for the envelope of VSV (0.83) than the maximum concentration of cholesterol found in the domains (2.4).

An attempt was made to examine the enrichment of the G and the M proteins in the domains. A total of 20 double-labeled images were collected of vesicles made from the lipids extracted from the BHK-21 plasma membrane and reconstituted with the dansyl-labeled G protein and the lissamine rhodamine-labeled M protein. Unfortunately, due to the low fluorescence level generated by the two proteins in the vesicles, a low signal to noise ratio was associated with all the images. This made the analysis of the data unreliable.

DISCUSSION

The G and the M proteins are the only viral proteins associated with the viral envelope, and host proteins are largely excluded. The G protein contains a classic signal sequence, and after its synthesis in the endoplasmic reticulum the protein is processed and transported to the plasma membrane. In the plasma membrane, the G proteins subsequently cluster into a domain at the site of viral budding. At this time the nucleocapsid and the M protein, which is made as a soluble protein and eventually resides between the nucleocapsid and the membrane, drive the budding process to completion. Since the factors that control this process or the mechanism of this process are not known, a starting hypothesis for the present research was that the two viral proteins were all that were necessary for the formation of the domain on the plasma membrane where budding occurs.

Previous experiments suggested that the two proteins cooperate in forming condensed domains enriched in PA (Luan & Glaser, 1994). That is, the two proteins together cause the formation of domains that are different than the domains caused by the proteins individually. This was amply illustrated by the results reported here. For example, both proteins were necessary in order to induce Sph and cholesterol to be enriched in the domains. The G protein or the M protein alone was not effective.

Direct evidence for interaction between the two proteins also was observed in 100 mol % DOPC vesicles containing no negatively charged phospholipid. The M protein by itself did not bind to these vesicles. However, the M protein did bind to the vesicles when the G protein was incorporated (data not shown).

Even though the concentration of PA was low in the plasma membrane and in the virus, the results suggest that PA played an important role in the formation of the viral domains. The G protein did not form domains by itself unless PA was present. Also, when PA was eliminated from the vesicles, even the combination of the two proteins was not able to induce the formation of Sph-enriched and cholesterol-enriched domains. In these experiments PS did not substitute for PA. PA is an important signaling molecule in the cell, and it is possible the formation of a PA-enriched domain may be important for the initiation of virus budding.

The combination of the G and M proteins caused the formation of domains not only enriched in PA but also

enriched in PS, Sph, and cholesterol. The results from vesicles triple-labeled with NBD-lipids, the dansyl-G protein, and the lissamine rhodamine-M protein demonstrated that the proteins colocalized with PA, PS, and Sph in the same domains. When domains enriched in PA, PS, and Sph were induced in the vesicles by the combination of the G and M proteins, dansyl-labeled gramicidin and dansyl-PC were excluded from the domains.

The formation of cholesterol-enriched domains not only required the combination of the G protein and the M protein but also required the presence of PA and Sph. The interaction between cholesterol and phospholipids has been the subject of a number of studies leading to sometimes controversial results (Yeagle, 1985; Tampe et al., 1991).

Some studies have suggested a preferential affinity of cholesterol for Sph over other phospholipids (Demel et al., 1977; van Dijik, 1979; Sankaram & Thompson, 1990; Demel & de Kruyff, 1976; Blume, 1980; Housley & Stanley, 1982; Bittman et al., 1994), while another study has not (Calhoun & Shipley, 1979). No other study has shown the degree of lipid selectivity and partitioning of lipids into domains as reported here. The development and use of fluorescence digital imaging microscopy to directly measure the lateral distribution of membrane lipids have made these experiments feasible.

Early experiments on the composition of VSV and Rous sarcoma virus grown on cells where the lipid composition had been modified showed that the virus selected its phospholipid components on the basis of the polar head group of the phospholipid and not on the fatty acid composition (Pessin & Glaser, 1980; Pessin, 1980). Consequently, the strategy followed in the present experiments was to put the fluorophore on the acyl chain, maintain the same fatty acid composition, and vary the head groups. Previous studies using this strategy have shown that using different fluorophores produces no significant effect on the induction of domains using calcium or different membrane proteins (Rodgers & Glaser, 1993). In these experiments, the temperature was always above the transition temperature of the phospholipids so that the lipids were in the liquid crystalline phase. In the present experiments the fatty acid composition also did not produce any observable effect on domain formation with the G and the M proteins. Vesicles made from dioleylphospholipids gave similar results to vesicles made from lipids extracted from the plasma membrane of BHK-21 cells. When the head group was varied, it was found that the viral proteins induced domains enriched in PA, PS, and Sph, while PE was distributed uniformly and PC was excluded from the domains.

In addition, dansylgramicidin was excluded from the domains induced by the viral proteins, and NBD-cholesterol was enriched in the domains. Dansylgramicidin was used to represent a "typical host membrane protein" with no lipid specificity. Another assumption in this study is that NBD-cholesterol behaves like cholesterol, which it appears to do (Craig et al., 1981). The NBD moiety is attached to the side chain of cholesterol leaving the 3-OH group free. Chattopadhyay and London (1988) showed that a similar NBD analog of cholesterol inserts into the bilayer with the same orientation as free cholesterol.

Analysis of the lipid composition showed that the envelope of VSV contained more PS, Sph, and cholesterol, equal amounts of PE, and less PC compared with the BHK-21 plasma membrane (Tables 2 and 3). This is similar to the results that were obtained using chicken embryo fibroblasts as the host cell (Pessin & Glaser, 1980). In this earlier study, a careful analysis was carried out to demonstrate that the virus had a different composition than the plasma membrane, and this was not due to contamination of the membrane preparations nor other reasons. Table 2 also shows that the viral envelope had a higher PA concentration compared to the plasma membrane. However, because of the low content of PA, the data were not accurate enough to draw a definite conclusion.

Analysis of the images showed that the domains induced by the G protein and the M protein had a composition very similar to that of the viral envelope (Tables 2 and 3). This result gives credence to the methods used in this study and strongly suggests that the domains formed in vesicles by the viral envelope-associated proteins really represent the areas in the host plasma membrane where the virus buds.

Cellular vesicles with unique lipid and protein compositions appear to be formed as a normal part of membrane traffic within a cell and in the endocytotic and exocytotic pathways. The selective sorting of lipids and proteins into membrane domains where these vesicles are formed may occur by a mechanism similar to that observed here for the formation of domains during viral budding, at least to some extent. Since most membrane proteins and lipids are made in the endoplasmic reticulum and selectively distributed to other cellular membranes, such a mechanism of domain formation may have general significance for vesicle formation as well as for the heterogeneity normally found within membranes (Glaser, 1993).

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