Formation of Membrane Domains by the Envelope Proteins of Vesicular Stomatitis Virus†

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Received September 20, 1993; Revised Manuscript Received January 7, 1994®

ABSTRACT: The properties of the two envelope-associated proteins of vesicular stomatitis virus, the glycoprotein (G) and the matrix protein (M), were investigated in order to understand the mechnanism of virus budding and domain formation in membranes. Fluorescence resonance energy transfer was used to study the interaction between the G protein and specific phospholipids. The protein had the highest affinity for phosphatidic acid among the phospholipids tested. Fluorescence digital imaging microscopy also was used to determine how the protein could alter the lateral distribution of phospholipids in membranes. Large domains enriched in phosphatidic acid were observed when the protein was incorporated into phospholipid vesicles. The G protein colocalized with the phosphatidic acid-enriched domains. Similar experiments carried out with the M protein showed that the M protein induced the formation of domains enriched not only in phosphatidic acid but also in phosphatidylserine. The phosphatidic acid-enriched domains induced by either the G or M proteins were similar in terms of the degree of enrichment of phosphatidic acid and the size of the domains. When the two proteins were reconstituted in vesicles at the same time, the domains were condensed. There was a greater degree of phosphatidic acid enrichment, and the size of the domains was reduced. The formation of domains enriched in the viral proteins and specific phospholipids may mimic the first steps that occur during budding of the virus from the plasma membrane of infected cells.

In many cases, the distributions of lipid and proteins in a biological membrane are very heterogeneous (Glaser, 1993). The budding of enveloped viruses, such as vesicular stomatitis virus (VSV),[1] from the plasma membrane of infected cells represents a clear case of the formation of a distinct lipid and protein domain (Pessin & Glaser, 1980). These viruses mature at the cell surface by a budding process that involves pinching off a piece of cell membrane containing cellular lipids and virally coded proteins (Pal & Wagner, 1987; Stephans & Compans, 1988). Studies on several enveloped RNA viruses suggest the following general model. First, the viral glycoproteins (the G protein for VSV) are inserted into the plasma membrane. The glycoproteins assemble into a patch or domain, and host proteins are excluded. At this time the nucleocapsid aligns under the glycoprotein domain, and some unspecified interactions perhaps involving the nucleocapsid, the overlying modified membrane, and (in some cases) a virally coded M protein between the nucleocapsid and the membrane drive the budding process to completion. In a number of respects the process resembles the sorting of membrane proteins during vesicle formation and endocytosis or exocytosis.

For VSV, the M and G proteins are the only two virally coded proteins associated with the viral envelope, and they are essential for budding to occur. Only trace amounts of host proteins are found in the mature virus. Virus mutants with a defective M protein do not form virus particles at the nonpermissive temperature (Knipe et al., 1977), and virus mutants with a temperature-sensitive G protein form particles at a reduced yield but contain the G protein during the budding process. The G protein is then cleaved to give spikeless virus particles (Metsikko & Simons, 1986). The role of membrane lipids in the process of viral morphogenesis is as uncertain as the role of the viral proteins. If the rate of incorporation of phospholipid polar head-group analogues into VSV, Rous sarcoma virus, and the plasma membrane of the infected cells is followed, it is clear that the viruses do not simply reflect the lipid composition of the plasma membrane. Instead, the viruses bud from regions of the plasma membrane with a distinctive lipid composition (Pessin & Glaser, 1980). VSV has a higher PS and Sph content and a lower PC content in its envelope compared to the phospholipid composition of the plasma membrane of infected cells. These observations are consistent with the existence of membrane domains at least the size of the viruses, approximately 0.1 μm, during the budding process.

The G protein forms the spikelike projections of the virus, and it has a transmembrane domain as well as covalently bound fatty acids. No particular lipid specificity has been reported for the G protein, but it can alter bilayer properties such as the gel to fluid phase transition and the enthalpy of the transition (Pal & Wagner, 1987). The M protein is a highly basic, nonglycosylated protein that interacts with acidic phospholipids. The M protein has been shown to induce a

† This work was supported in part by a grant from the American Cancer Society (BE-167).

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® Abstract published in *Advance ACS Abstracts,* March 15, 1994.

[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, dioleylphosphatidic acid; DOPC, dioleylphosphatidylcholine; DOPS, dioleylphosphatidylserine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); M protein, the matrix protein of VSV; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; 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; PS, phosphatidylserine; Sph, sphingomyelin; Tris, tris(hydroxymethyl)-aminomethane; VSV, vesicular stomatitis virus.

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lateral reorganization of membrane lipids, but primarily during the phase transition (Wiener et al., 1985).

The purpose of the present study was to investigate the possible role of the G and M proteins in the process of domain formation in the plasma membrane prior to budding. The hypothesis is that the G and M proteins by themselves are capable of forming domains and sequestering specific lipids.

In this study two methods were utilized to examine the distribution of components in the membrane. Fluorescence resonance energy transfer was used to provide information about the relative distribution of different phospholipids around the G protein (Wang et al., 1987), and fluorescence digital imaging microscopy was used to directly visualize the membrane domains caused by both the M and G proteins. The latter method has been used for studying, for example, phospholipid domain formation caused by proteins such as cytochrome *c* (Haverstick & Glaser, 1989) and for determining the heterogeneous distributions of phospholipids that exist in the erythrocyte membrane (Rodgers & Glaser, 1993a,b). The results presented in this paper show that both the G and M proteins are capable of forming domains enriched in specific phospholipids.

MATERIALS AND METHODS

Phospholipids. All the phospholipids used in this study were obtained and stored as described by Haverstick and Glaser (1987, 1988). NBD-PS and NBD-PA were synthesized from NBD-PC using the procedure developed recently by Comfurius et al. (1990). In brief, 1 mL of 50% L-serine, 0.1 M $CaCl_2$, 0.1 M sodium acetate pH 5.6, and 2% octyl glucoside were added to 1 μmol of NBD-PC in the form of a lipid film. The mixture was incubated at 45 °C for 10 min with vigorous shaking. Cabbage phospholipase D (6 units) were added and the mixture incubated at 45 °C for 3 h with gentle shaking. An additional aliquot of phospholipase D (6 units) was added halfway through the incubation. The reaction was stopped by the addition of 0.3 mL of 0.5 M EDTA, pH 8.0, and the NBD-PS and NBD-PA were purified the same way as described by Haverstick and Glaser (1987).

Isolation of VSV G Protein. Vesicular stomatitis virus (Indiana serotype) was grown by infecting baby hamster kidney 21 cells with 0.1 PFU/cell followed by incubation at 37 °C for 18–24 h. The virus was purified from the culture medium by different centrifugation followed by equilibrium sedimentation in a sucrose gradient (Lyles, 1979). The viral glycoprotein was released from the purified virus by 30 mM octyl glucoside. The glycoprotein was further purified by sedimentation into a 15–30% sucrose gradient containing 60 mM octyl glucoside, 0.5 M NaCl, and 50 mM Tris (pH 7.6) as described by Petri and Wagner (1979). The fractions from the gradient containing the glycoprotein were pooled and dialyzed against 50 mM Tris, pH 7.6, and 0.1 M NaCl to remove the sucrose and detergent.

Fluorescent Labeling of the G Protein. The viral glycoprotein was fluorescently labeled with dansyl chloride by using a method similar to that used for cytochrome b_5 labeling as described by Gilmore and Glaser (1982). A mixed micelle of octyl glycoside and dansyl chloride was prepared by dissolving 6 μmol of dansyl chloride in 1 mL of 60 mM octyl glucoside, 0.1 M NaCl, and 50 mM Tris, pH 7.6, with the help of vortexing and bath sonication. One milliliter of purified virus (2 mg of protein/mL) was added to the detergent–dansyl chloride dispersion, and the pH value of the mixture was adjusted to 8.0. The solution was stirred at room temperature for 1 h, and the dansyl-labeled glycoprotein was purified from the mixture by the same method used for the purification of the unlabeled protein.

Isolation of the VSV M Protein. The M protein was purified from VSV based on the method employed by Carroll and Wagner (1979). Briefly, purified virus was solubilized in 10 mM Tris (pH 8.0) containing 1% Triton X-100, 0.25 M NaCl, and 1.2 mM dithiothreitol to a final protein concentration of 1 mg/mL. The solution was stirred for 30 min at room temperature and then centrifuged at 200000*g* for 2 h. The supernatant containing mostly the G and M proteins was removed and diluted with an equal volume of 10% glycerol in 10 mM Tris, pH 7.5. The solution was dialyzed against 0.1 M NaCl, 10% glycerol, 0.2% Triton X-100, and 10 mM Tris, pH 7.5, overnight. The M protein was purified by chromatography on a 1 × 6 cm Whatman P11 phosphocellulose column which had been equilibrated with 0.1 M NaCl, 10% glycerol, 10 mM Tris, pH 7.5, and 0.2% Triton X-100. The column was loaded and thoroughly washed by the equilibration buffer, followed by washing with the equilibration buffer minus Triton X-100 until the absorbance at 280 nm fell to a low level. The M protein was eluted with 0.65 M NaCl, 10% glycerol, and 10 mM Tris, pH 7.5. The pooled M protein fractions were dialyzed against 0.1 M NaCl, 10% glycerol, and 10 mM Tris, pH 7.5, and then loaded onto a new Whatman P11 phosphocellulose column for further purification. The column was eluted by 10% glycerol and 10 mM Tris, pH 7.5, with a linear gradient from 0.1 to 1 M NaCl.

Vesicle Preparation for the Microscopy Experiments. Giant unilamellar vesicles for viewing with the fluorescence microscope were made as described by Haverstick and Glaser (1987). The G protein was incorporated into the vesicles using a protein solution in 10 mM Tris, pH 7.6, to hydrate the dried phospholipid film at a molar ratio of G protein to phospholipids at 1:500. The incorporation of the M protein was done in a similar manner to give a molar ratio of M protein to phospholipids of 1:100. When the two proteins were reconstituted together, the same ratio was used to give a G protein:M protein:phospholipid mole ratio of 1:5:500.

Instrumentation and Image Processing. The fluorescence microscopy and image processing operations were carried out as previously described (Rodgers & Glaser, 1993). The excitation wavelengths were between 340 and 380 nm for NBD imaging and 420 and 460 nm for dansyl imaging. The emission wavelengths were >430 and >515 nm for dansyl and NBD imaging, respectively. The background illumination was subtracted from all images, and the resulting images were normalized to a mean radiance value of 100 ± 4. A pseudo color scheme was applied to the radiance values, which ranged from blue (0) to yellow to red (255) (Figure 2G).

Reconstitution of the G Protein into Phospholipid Vesicles for Energy Transfer Measurements. Unilamellar phospholipid vesicles were prepared by sonication (Wang et al., 1988). A total of 2.5 μmol of egg yolk phosphatidylcholine and 37.5 nmol of a phospholipid, which was labeled with a dansyl group at the end of a C11 fatty acid on the 2 position of the phospholipid, in organic solvents was mixed and dried under a stream of nitrogen at 37 °C. The dried lipid film was hydrated at 37 °C for 30 min by addition of 5 mL of 10 mM HEPES, pH 7.4, and 100 mM KCl. The mixture was dispersed by vigorous vortexing for 1 min and sonicating for 1 min with a bath sonicator. The dispersion was further sonicated under nitrogen by using the semi-microprobe of a Heat System-Ultrasonics W-375 sonicator for 15 min in an ice bath. Multilamellar liposomes were removed by centrifugation at 100000*g* for 1 h. The reconstitution of the glycoprotein was

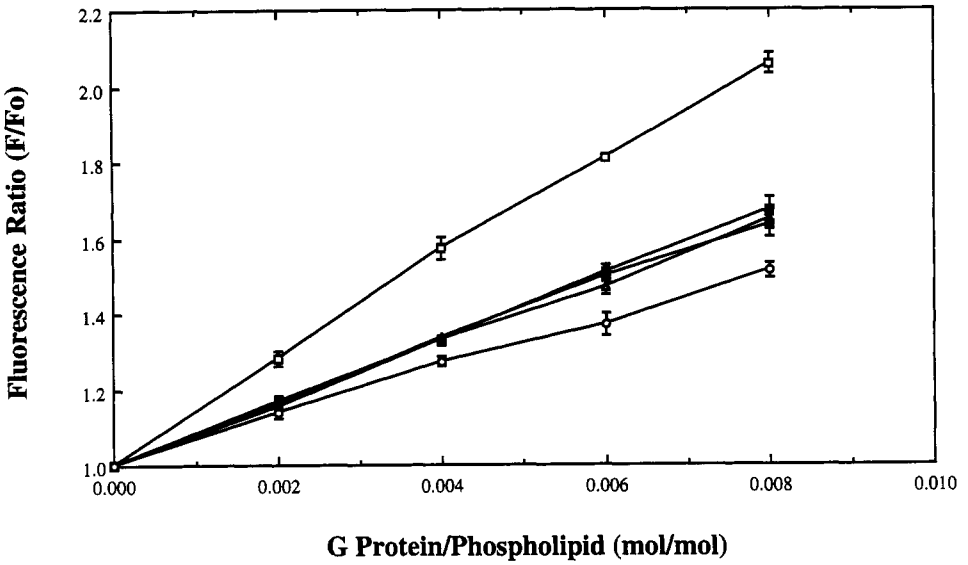

FIGURE 1: Energy transfer from the G protein to dansyl-PA (□), dansyl-PC (▲), dansyl-PE (△), dansyl-PS (■), and dansyl-Sph (○). The samples were excited at 280 nm, and dansyl fluorescence enhancement was measured. F_0 and F are the dansyl fluorescence before and after the addition of the G protein, respectively. The phospholipid concentration was 500 nmol/mL, and the dansylphospholipid to phospholipid molar ratio was 1.5:100.

carried out by addition of an appropriate amount of detergent-free protein to the preformed vesicles, followed by a 30-min incubation at 37 °C as described by Petri and Wagner (1980).

Resonance Energy Transfer Measurements. Fluorescence measurements were performed by the method described by Wang et al. (1988). The amount of fluorescence energy transfer from the tryptophan residues of the glycoprotein to the dansyl groups on specific phospholipids was determined from the enhancement of the dansyl fluorescence due to the incorporation of the protein into the vesicles. The tryptophan residues were excited at 280 nm, which is a wavelength where the absorbance of the dansyl groups was negligible under the experimental conditions.

The difference in energy transfer efficiencies between the tryptophan residues of the protein to the dansyl group on different phospholipids could be estimated by comparing the difference in the slopes from the graph of F/F_0 versus the concentration of the donor in the vesicles, where F and F_0 are the dansyl emission intensities in the presence and absence of the energy transfer donor, respectively.

The G protein contains 12 tryptophan residues, and quenching of tryptophan fluoresence by the dansyl groups under the conditions of these experiments was very low (<1%) and did not allow accurate measurements.

The samples used in the energy transfer experiments also were excited at 350 nm to directly excite the dansyl groups. There was no significant change (<2%) of the fluorescence emission upon the incorporation of protein, indicating that that there was no change in quantum yield under the different conditions.

RESULTS

Ability of the G Protein To Form Domains Enriched in Specific Phospholipids. To determine if the G protein showed a specific affinity for certain phospholipids, the extent of fluorescence energy transfer was measured between the protein's tryptophan residues and different phospholipids labeled with a dansyl moiety on the acyl chains. This method has been used previously to show that different phospholipids are not randomly distributed around D-β-hydroxybutyrate dehydrogenase (Wang et al., 1988). Figure 1 illustrates the increase in dansyl fluorescence corresponding to increasing amounts of the glycoprotein in the vesicles. In this experiment the overall phospholipid composition of the vesicles was the same, and only the type of dansyl-labeled phospholipid was changed. If the glycoprotein had no preferential affinity for any individual phospholipid in the membrane, every phospholipid would be randomly distributed around the glycoprotein molecules, and there would be no differences in the energy transfer between the protein and the different phospholipids. However, the energy transfer data showed that the glycoprotein had the largest energy transfer efficiency for dansyl-PA. The energy transfer to dansyl-PS, dansyl-PE, and dansyl-PC was similar, while the energy transfer to sphingomyelin was slightly lower.

Since the energy transfer data indicated that glycoprotein could cause the preferential enrichment of PA around the protein molecules, it was of interest to find out if the protein could induce the formation of domains containing PA and if the domains would be of sufficient size to be visualized by fluorescence microscopy. Large unilamellar DOPC vesicles were made, containing different NBD-labeled phospholipids with the NBD moiety attached to the end of the acyl chain in the 2 position of the phospholipids. The NBD fluorescence images of vesicles containing 0.5 mol % NBD-PC and 0.2 mol % viral glycoprotein showed that PC was uniformly distributed across the vesicles (Figure 2A) whether or not the glycoprotein was present. The G protein also had no effect on vesicles containing earlier 10 mol % PS (0.5 mol % NBD-PS) (Figure 2B), 10 mol % Sph (0.5 mol % NBD-Sph) (Figure 2C), or 10 mol % PE (0.5 mol % NBD-PE) (Figure 2D). The left image in Figure 2E shows the uniform distribution of NBD-PA in vesicles containing 10 mol % PA (0.5 mol % NBD-PA) without the addition of the G protein. When the G protein was incorporated into the NBD-PA-labeled vesicles, the NBD-PA was no longer uniformly distributed in the vesicles, but rather it was sequestered into patches or domains (Figure 2E, right image). Figure 2F shows two images of a vesicle containng NBD-PA and the G protein labeled with dansyl chloride viewed for NBD (image on the left) and the dansylglycoprotein (image on the right). The domain of the dansyl-G protein and NBD-PA colocalized in the same areas

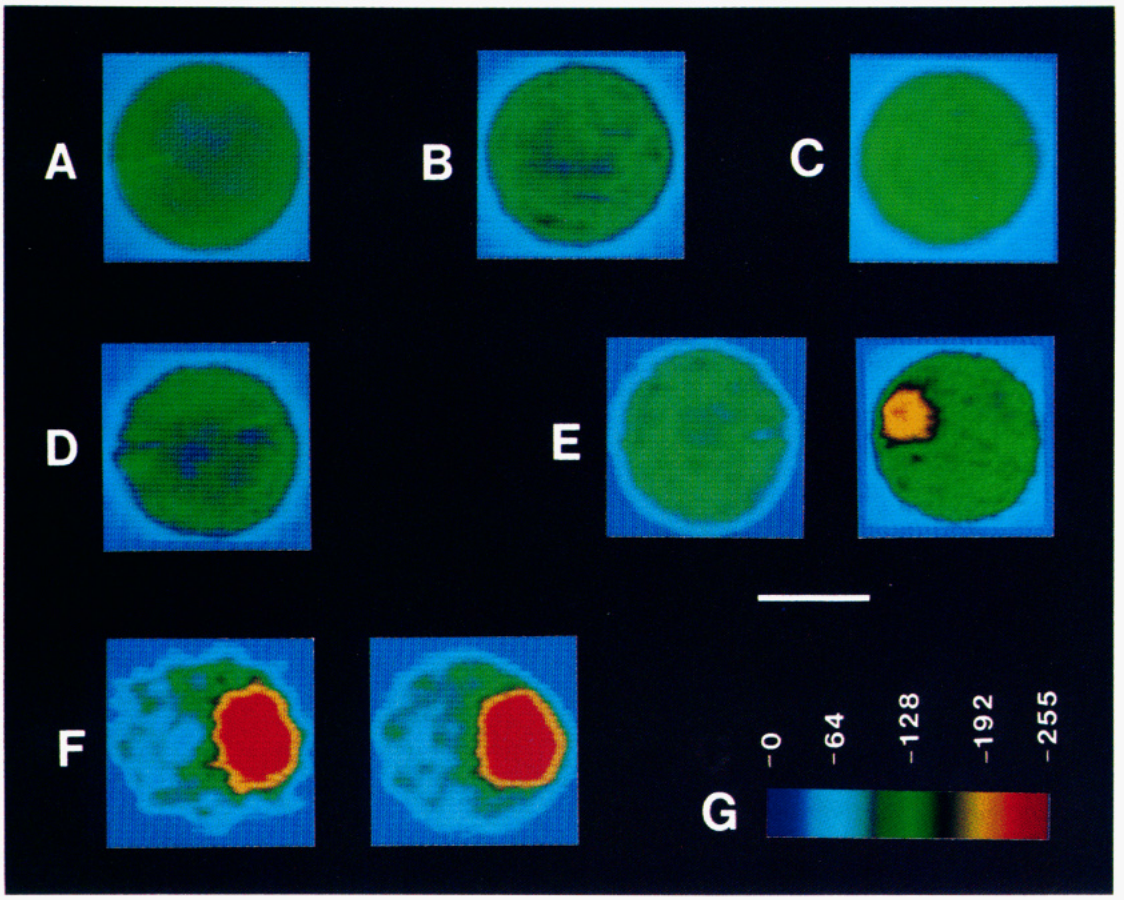

FIGURE 2: Visualization of the formation of PA-enriched domains induced by the G protein in large unilamellar vesicles. Shown in panels A–D are NBD images of DOPC vesicles containing either 0.5 mol % NBD-PC (A), 0.5 mol % NBD-PS and 10 mol % DOPS (B), 0.5 mol % NBD-Sph and 10 mol % Sph (C), or 0.5 mol % NBD-PE and 10 mol % DOPE (D), all reconstituted with 0.2 mol % viral G protein. Shown in panel E are NBD images of two DOPC vesicles containing 0.5 mol % NBD-PA and 10 mol % DOPA before (left image) and after (right image) the incorporation of 0.2 mol % viral glycoprotein. Shown in panel F are two images of the same DOPC vesicle containing 0.5 mol % NBD-PA, 10 mol % DOPA, and 0.2 mol % G protein labeled with dansyl chloride and viewed for either NBD (left image) or the dansylglycoprotein (right image). The images were normalized to a mean gray value of 100 ± 4. The pseudo color scheme applied to the images is shown in panel G with the equivalent radiance values. The white bar equals 4 μm.

in the vesicles, indicating that the viral glycoprotein was responsible for the redistribution of PA into the domains. The domains in each vesicle differed in terms of size and degree of enrichment. Both PA and the G protein were enriched in the domains.

Ability of the M Protein To Form Domains Enriched in Specific Phospholipids. The M protein has specific interactions with negatively charged phospholipids, and it was expected that domains would be formed similar to those with the G protein and PA. When the M protein was added to DOPC vesicles containing dansyl-PA, no fluorescence energy transfer was observed between the tryptophan residues and the dansyl groups. Since binding of the M protein to the vesicles should have occurred under these conditons, the result indicates that the tryptophan residues are too far away from the dansyl groups for significant energy transfer to occur, which is not an unreasonable result for a peripheral membrane protein. The efficiency of energy transfer decreases as a function of the distance to the sixth power, and the theoretical distance for 50% energy transfer between a tryptophan residue and a dansyl group is approximately 22 Å (Wang et al., 1988).

The M protein is capable of forming domains in vesicles containing acidic phosholipids, however. In vesicles without any acidic phospholipids, the M protein had no effect on the uniform distribution of the phospholipids in the vesicles (Figure 3A–C). Figure 3D shows vesicles containing 89.5% DOPC, 10% DOPA, and 0.5% NBD-PA which were viewed for NBD fluorescence without the addition of the M protein (left image) and with the incorporation of M protein (right image). After the M protein was added to the vesicles, the fluorescent PA migrated into large domains in the vesicles. Thus, the M protein had the same ability as the G protein to induce PA-enriched domains in vesicles. PS was also used in the experiments since PS is another negatively charged phospholipid, and a previous study (Pessin & Glaser, 1980) had shown that the PS concentration was higher in the envelope of vesicular stomatitis virus compared to the plasma membrane of the host cells. Figure 3E shows vesicles containing 89.5% DOPC, 10% DOPS, and 0.5% NBD-PS, viewed for NBD fluorescence for the vesicle without (left image) and with (right image) the addition of the M protein.

Combination of the G and M Proteins Together To Form Domains. In order to determine the effect of both the G and M proteins together in forming domains, the proteins were reconstituted with PA-containing vesicles. The composition of the vesicles was 89.5% DOPC, 10% DOPA, and 0.5% NBD-PA, with the ratio G protein:M protein:total lipids = 1:5:500. As with the two proteins individually, large domains enriched in PA were observed (Figure 3F). The domains in each vesicle had a distinctive pattern with different sizes and degrees of enrichment. A large number of vesicles that had been reconstituted with the proteins individually and with both of the proteins together were studied in order to compare the differences between the domains. A total of 70 images were

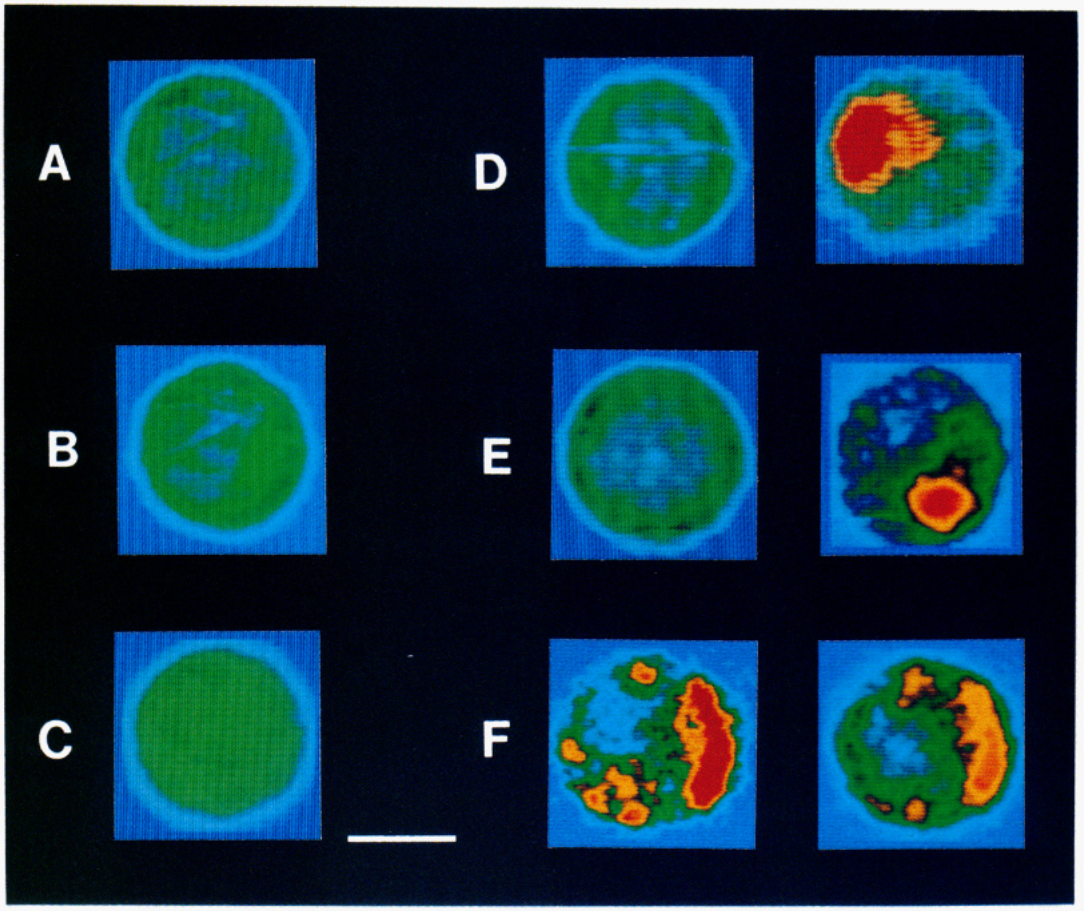

FIGURE 3: Visualization of domains induced by the M protein in large unilamellar vesicles. Shown in panels A–C are NBD images of DOPC vesicles containing either 0.5 mol % NBD-PC (A), 0.5 mol % NBD-PE and 10 mol % DOPE (B), 0.5 mol % NBD-Sph and 10 mol % Sph (C), all in the presence of the viral M protein. Similar vesicles containing 0.5 mol % NBD-PA and 10 mol % DOPA (B) or 0.5 mol % NBD-PS and 10 mol % DOPS (E) were viewed for NBD before (left images) or after (right images) the addition of the M protein. Panel F shows two images of the same vesicle containing 0.5 mol % NBD-PA, 10 mol % DOPA, and 0.2 mol % G protein labeled with dansyl chloride and viewed for either NBD (left image) or the dansylglycoprotein (right image) in the presence of the M protein. The white bar equals 4 μm.

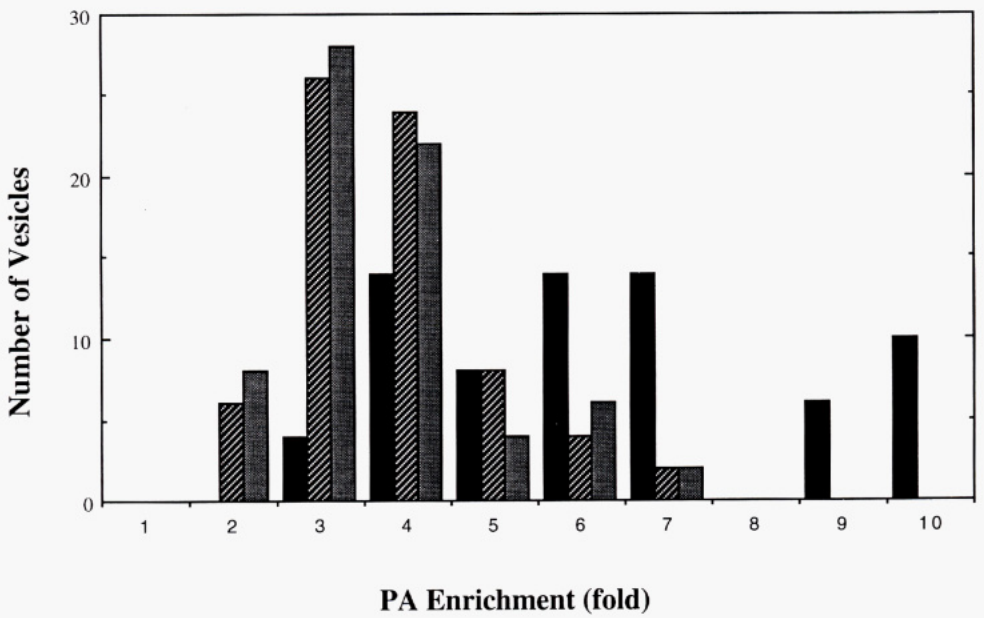

FIGURE 4: Effect of the G protein and the M protein on the enrichment of PA in the domains. Shown in the graph are three populations of PA-containing vesicles reconstituted with either the G protein (hatched bars), the M protein (shaded bars), or both the G and M proteins together (solid bars). All the images used for processing were normalized to a mean radiance value of 50 ± 2. The enrichment of PA was defined as the ratio of the highest radiance value within the domain to the lowest radiance value within the vesicle. The sample size was 70 for each population of vesicles.

analyzed in each case, and the enrichment of PA in the domains is given in Figure 4. The degrees of PA enrichment for the vesicles reconstituted with either the G or M protein alone were very similar to each other. Increasing the amount of the M protein in the vesicles 2-fold or the amount of the G protein in the vesicles 2-fold did not affect the distribution of PA

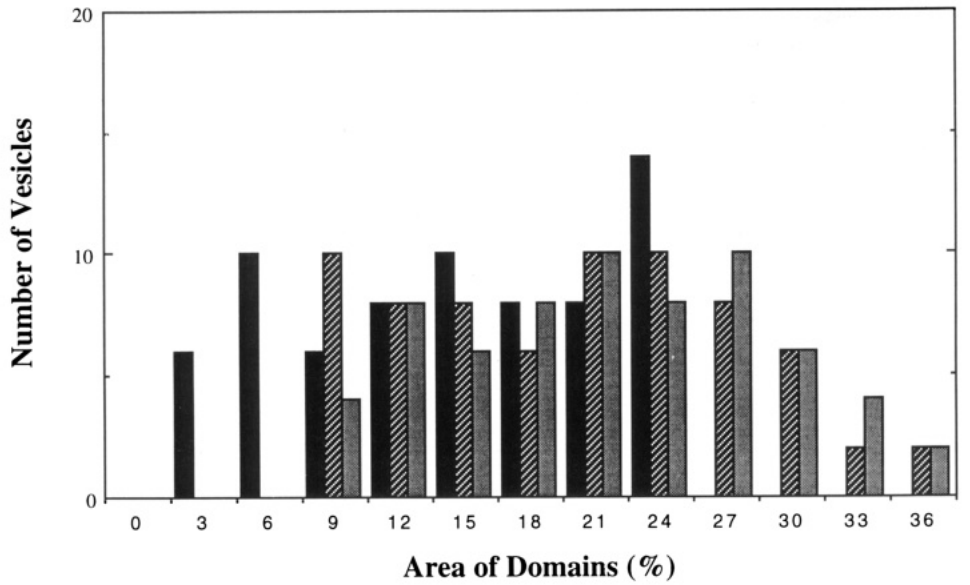

FIGURE 5: Effect of the G protein and the M protein on the size of PA-enriched domains. Shown in the graph are three populations of PA-containing vesicles reconstituted with either the G protein (hatched bars), the M protein (shaded bars), of both the G and M proteins together (solid bars). All the images used for processing were normalized to a mean radiance value of 50 ± 2. The domains were defined as all the pixels with radiance values larger than 75. The sizes of the domains are given as the percentage of domain size versus the total area of the image. The sample size was 70 for each population of vesicles.

enrichment (data not shown). However, the vesicles with both the G and M proteins together had domains with a greater enrichment of PA. The center of the distribution shifted from a 3–4-fold enrichment to a 6–7-fold enrichment.

Another parameter used to characterize the domains is their sizes, which were defined as the total areas of the image with radiance values greater than 150% of the average radiance value for the whole image. The effect of the combination of the two proteins on the sizes of the PA-enriched domains in comparison with the two proteins individually is shown in Figure 5. Once again, the distributions for the vesicles reconstituted with either the G or M proteins individually were basically the same. Significant changes were observed in the sizes of the domains induced by the combination of the two proteins, however. The size distribution for the domains shifted to a lower range. The center of the distribution shifted from approximately 22% for the vesicles containing only one of the proteins to approximately 12% for the vesicles containing both proteins. Thus, the sizes of the domains were reduced by approximately a factor of 2 when both the G and M proteins were incorporated into the vesicles. Once again, this effect could not be achieved by just increasing the amount of a single protein in the vesicles.

The increase in PA enrichment observed when both of the proteins were incorporated into the domains together was supported by fluorescence energy transfer measurements. When the M protein was added to vesicles containing the G protein and dansyl-PA, there was an increase in energy transfer (Figure 6). Since the tryptophan residues of the M proteins were too far from the dansyl groups to contribute to the energy transfer, the results suggest that the domains containing the G protein and dansyl-PA were further enriched by the presence of the M protein. Consequently, there was a higher extent of energy transfer between the tryptophan residues of the G protein and dansyl-PA.

It is also possible that the addition of the M protein caused a conformational change in the G protein, and the enhanced energy transfer was partly due to a change in the orientation factor. The results of the microscopy experiments showed that the PA-enriched domains were condensed, however. In any case, the results suggest an interaction between the two proteins.

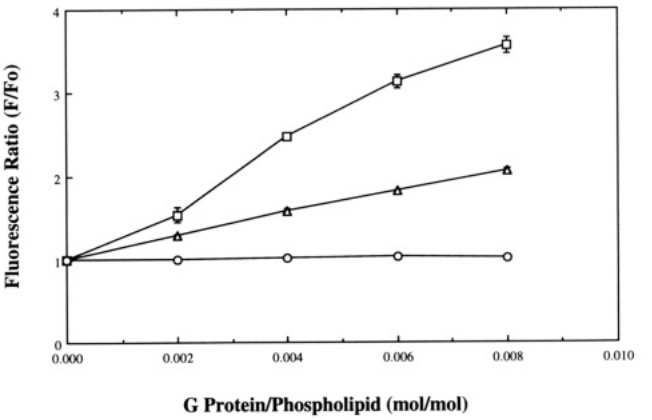

FIGURE 6: Energy transfer from the G protein to dansyl-PA in the presence (□) and the absence (△) of the M protein. All conditions are the same as in Figure 1, and the molar ratio of the G protein to the M protein was 1:5. The lack of energy transfer from the M protein to dansyl-PA under the same conditions is also shown in the graph (○).

DISCUSSION

The fluorescence resonance energy transfer experiments with dansyl-labeled phospholipid vesicles reconstituted with the glycoprotein of VSV showed a different distribution of PA around the G protein, compared with the distribution of the other phospholipids. The higher extent of energy transfer between the G protein and PA indicated a preferential affinity for PA. This result was consistent with the ability of the G protein to form domains enriched in PA and the glycoprotein as observed by fluorescence microscopy.

The glycoprotein is one of the two envelope-associated proteins in VSV. One of the critical steps in the budding process of the enveloped RNA viruses is the clustering of the viral glycoproteins after they are inserted into the plasma membrane. Most studies on the quaternary structure of the G protein indicate it is a timer, but its oligomerization state is in a dynamic equilibrium that changes under different

conditions (Lyles et al., 1990). The formation of the G protein domains along with a particular phospholipid (PA) visualized in this study suggests that the G protein clustering on the plasma membrane may be favored by some particular lipid environment. Only small amounts of PA are found in the mature virus, but it seems possible that locally high concentrations of PA might trigger the clustering of the G protein and be involved in the initial steps of virus budding.

In the experiments, the binding of the G protein was independent of the vesicle composition. The dansyl-labeled G protein was added to vesicles containing different phospholipids, and images were collected of a population of the vesicles. The average intensities of the images (total radiance in the image per pixel) from different populations were similar, indicating that the vesicles contained comparable amounts of the G protein.

Labeling of the G protein also did not affect the ability of the protein to form domains. The unlabeled and dansyl-labeled proteins were reconstituted with PA-containing vesicles, and the enrichments of PA and the sizes of the domains were measured. The results from the unlabeled and labeled proteins were similar; the enrichments of PA and the sizes of the domains were not affected by labeling the G protein.

Substantial work also has been done with the second envelope-associated protein, the M protein, of VSV in terms of the interactions of the protein with phospholipids (Wiener et al., 1983, 1985; Zakowski et al., 1981). All these studies concluded that the M protein had specific interactions with acidic phospholipids. In the present report, the visualization of domain formation using fluorescence digital imaging microscopy demonstrated directly that not only did the M protein preferentially interact with negatively charged phospholipids but such interactions also lead to the formation of large domains enriched in PA and PS in the vesicles (Figure 3).

The M protein is synthesized on soluble ribosomes and binds to the plasma membrane during the budding process. Chemical cross-linking studies suggest that the M protein is the close proximity to the viral glycoprotein (Dubovi & Wagner, 1977), and the M protein can stabilize the interactions of the G protein subunits (Lyles et al., 1992). Since both of the proteins individually could induce the formation of domains enriched in PA, further experiments were carried out to determine the effect of the two proteins combined in the same vesicles. The protein and lipid composition of the vesicles was chosen to represent the composition of the envelope of VSV. Accordingly, the molar ratio of G protein:M protein:total phospholipids was chosen to be 1:5:500 for the vesicle preparation (Altstiel & Landsberger, 1981; Wagner et al., 1975). The combination of the G and M proteins caused the domains to condense considerably. There was a further enrichment of PA in the domains and also a reduction in the size of the domains. Both effects were about 2–3-fold (Figures 4 and 5).

The findings of this study indicate that the G and M proteins can cause the formation of domains and the selective enrichment of certain phospholipids in the domains. This may represent the first step toward understanding the budding process of the virus from the plasma membrane of the infected cells.

REFERENCES

Altstiel, L. D., & Landsberger, F. R. (1981) *Virology 115*, 1–9.

Carroll, A. R., & Wagner, R. R. (1979) *J. Virol. 29*, 134–142.

Comfurius, P., Bevers, E. M., & Zwaal, R. F. A. (1990) *J. Lipid Res. 31*, 1719–1721.

Dubovi, E. J., & Wagner, R. R. (1977) *J. Virol. 22*, 500–509.

Gilmore, R., & Glaser, M. (1982) *Biochemistry 21*, 1673–1680.

Glaser, M. (1993) *Curr. Opin. Struct. Biol. 3*, 475–481.

Haverstick, D., & Glaser, M. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4475–4479.

Haverstick, D., & Glaser, M. (1988) *J. Cell Biol. 106*, 1885–1892.

Haverstick, D., & Glaser, M. (1989) *Biophys. J. 55*, 677–682.

Knipe, D. M., Baltimore, D., & Lodish, H. F. (1977), *J. Virol. 21*, 1149–1158.

Lyles, D. S. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5621–5625.

Lyles, D. S., Varela, V. A., & Parce, J. W. (1990) *Biochemistry 29*, 2442–2449.

Lyles, D. S., McKenzie, M., & Parce, J. W. (1992) *J. Virol. 66*, 349–358.

Metsikko, K., & Simons, K. (1986) *EMBO J. 5*, 1913–1920.

Pal, R., & Wagner, R. R. (1987) in *The Rhabdoviruses* (Wagner, R. R., Ed.) pp 75–128, Plenum Press, New York.

Pessin, J. E., & Glaser, M. (1980) *J. Biol. Chem. 255*, 9044–9050.

Petri, W. A., Jr., & Wagner, R. R. (1979) *J. Biol. Chem. 254*, 4313–4316.

Petri, W. A., Jr., & Wagner, R. R. (1980) *Virology 107*, 543–547.

Rodgers, W., & Glaser, M. (1993a) in *Optical Microscopy: Emerging Methods & Applications* (Herman, B., & Lemasters, J. J., Eds.) pp 263–283, Academic Press, New York.

Rodgers, W., & Glaser, M. (1993b) *Biochemistry 32*, 12591–12598.

Stephans, E. B., & Compans, R. W. (1988) *Annu. Rev. Microbiol. 42*, 489–516.

Wagner, R. R., Emerson, S. U., Imblum, R. L., & Kelley, J. M. (1975) *Negative Strand Virus* (Mahy, B. W. J., Barry, R. D., Eds.) Vol. I, pp 1–19, Academic Press, New York.

Wang, S., Martin, E., Cimino, J., Omann, G., & Glaser, M. (1988) *Biochemistry 27*, 2033–2039.

Wiener, J. R., Wagner, R. R., & Freire, E. (1983) *Biochemistry 22*, 6117–6123.

Wiener, J. R., Pal, R., Barenholz, Y., & Wagner, R. R. (1985) *Biochemistry 24*, 7651–7658.

Zakowski, J. J., Petri, W. A., & Wagner, R. R. (1981) *Biochemistry 20*, 3902–3907.