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Localization of Membrane-Associated Proteins in Vesicular Stomatitis Virus by Use of Hydrophobic Membrane Probes and Cross-Linking Reagents

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The location of membrane-associated proteins of vesicular stomatitis virus was investigated by using two monofunctional and three bifunctional probes that differ in the degree to which they partition into membranes and in their specific group reactivity. Two hydrophobic aryl azide probes, [^{125}I]5-iodonaphthyl-1-azide and [^{3}H]pyrenesulfonylazide, readily partitioned into virion membrane and, when activated to nitrenes by UV irradiation, formed stable covalent adducts to membrane constituents. Both of these monofunctional probes labeled the glycoprotein G and matrix M proteins, but [^{125}I]5-iodonaphthyl-1-azide also labeled the nucleocapsid N protein and an unidentified low-molecular-weight component. Protein labeling of intact virions was unaffected by the presence of cytochrome *c* or glutathione, but disruption of membrane by sodium dodecyl sulfate greatly enhanced the labeling of all viral proteins except G. Labeling of G protein was essentially restricted to the membrane-embedded, thermolysin-resistant tail fragment. Three bifunctional reagents, tartryl diazide, dimethylsuberimidate, and 4,4'-dithiobisphenylazide, were tested for their capacity to cross-link proteins to membrane phospholipids of virions grown in the presence of [^{3}H]palmitate. Only G and M proteins of intact virions were labeled with ^{3}H-phospholipid by these cross-linkers; the reactions were not affected by cytochrome *c* but were abolished by disruption of virus with sodium dodecyl sulfate. Dimethylsuberimidate, which reacts with free amino groups, cross-linked ^{3}H-phospholipid to both G and M protein. In contrast, the hydrophilic tartryl diazide cross-linked phospholipid primarily to the M protein, whereas the hydrophobic 4,4'-dithiobisphenylazide cross-linked phospholipid primarily to the intrinsic G protein. These data support the hypothesis that the G protein traverses the virion membrane and that the M protein is membrane associated but does not penetrate very deeply, if at all.

Vesicular stomatitis virus (VSV) is a negative-strand RNA-containing membrane-enveloped rhabdovirus (27) that has been extensively studied as a model membrane system (18). The virion is composed of five virus-coded proteins. N, L, and NS are associated with the nucleocapsid core; G comprises the glycoprotein spike protruding from the viral membrane; the M protein is believed to line the interior surface of the membrane and possibly serves as the "glue" that binds the nucleocapsid to the appropriate membrane site during viral budding from the host cell (8, 14). The M protein has also been implicated in control of viral transcription (5). The evidence that the glycoprotein is an intrinsic membrane protein is quite compelling (18, 23, 27). However, the assignment of M protein to a position lining the inner membrane surface is sketchier; the evidence consists mostly of resistance to proteases and surface-labeling reagents, the use of temperature-sensitive mutants, and analogy to the influenza virus M protein which appears to line the interior surface of the membrane in electron micrographs (18). Any attempt to pinpoint the exact location of VSV M protein must reconcile studies showing that M protein is not accessible to hydrophobic azido-phospholipid probes in the interior of the VSV membrane (26), although it is still close enough to the viral membrane that it can be cross-linked to phospholipids by bifunctional reagents approximately 1 nm in length (19).

Hydrophobic aryl azide probes are being increasingly used to study protein-lipid interactions in biological membranes (20). These lipophilic probes partition into membranous structures; when UV irradiated, the relatively stable azide functional group is converted into a highly reactive nitrene which will covalently bind to any proximal membrane constituents (9). Use of such radioactively labeled probes permits identification of membrane-associated proteins by determination of which proteins acquire the radioactive label.

An alternative technique for localization of membrane proteins is cross-linking of proteins to membrane phospholipids by use of defined cross-linking reagents. Identification of these cross-linked products can be established by use of radioactive phospholipids. Probes that react by different specificities and mechanisms can also yield information on the type of membrane association and the depth of membrane penetration.

We report here studies on the interaction with VSV membrane proteins of two hydrophobic aryl azide probes: [^{125}I]5-iodonaphthyl-1-azide ([^{125}I]INA) (2) and [^{3}H]pyrenesulfonylazide ([^{3}H]PySA) (22), as well as three bifunctional reagents which can cross-link membrane lipids with VSV proteins: dimethylsuberimidate (DMS), tartryl diazide (TDA), and dithiobisphenylazide (DTBPA).

MATERIALS AND METHODS

Chemicals and radiochemicals. Glutathione (GSH), horse heart cytochrome *c*, and thermolysin (protease type X; EC 3.4.24.4) were purchased from Sigma Chemical Co., St. Louis, Mo. Acrylamide was from Bio-Rad Laboratories, Richmond, Calif., and Corning UV filters were from Swift Glass Co., Elmira, N.Y. Na^{125}I (specific activity, 2,000 Ci/mmol) was from Amersham Corp., Arlington Heights, Ill., and [^{3}H]palmitate (specific activity, 18 Ci/mmol) was purchased from New England Nuclear Corp., Boston, Mass. PySA was purchased from Molecular Probes, Inc., Plano, Tex.; DMS and DTBPA were from Pierce Chemical Co., Rockford, Ill. Tartryl dihydrazide was previously prepared in this laboratory by E. Dubovi (7) according to the method of Lutter et al. (13); 5-aminonaphthyl-1-azide was a gift from Tuvia Bercovici of the Weizmann Institute, Rehovot, Israel.

Virus. VSV Indiana serotype was grown on BHK-21 cells at 31°C and purified from the cell culture media according to the procedure of McSharry and Wagner (15). [^{3}H]palmitate-labeled VSV was purified similarly from cultures grown in media containing 2.5 μCi of [^{3}H]palmitate per ml from time of cell passage until virus harvest. Protein was estimated by the method of Lowry et al. (12).

Preparation of probes. All operations with PySA, INA, and DTBPA were performed in the dark with red-lamp illumination. Nonradioactive PySA was tritiated by ICN, Inc., Irvine, Calif., according to the procedure of Wilzbach (28) and purified by thin-layer chromatography (TLC) on 2-mm-thick activated Silica Gel G, using 100% chloroform as the solvent (22). The band migrating at R_f = 0.75 was visualized by exposure of a small part of the TLC plate to a Westinghouse Sterilamp UV source. The unirradiated portion of the TLC plate containing the [^{3}H]PySA was scraped off, and the [^{3}H]PySA was eluted with chloroform through a sintered-glass funnel. The sample was dried under nitrogen and dissolved in acetone. The [^{3}H]PySA concentration was estimated with a Cary 219 spectrophotometer, using extinction coefficients calculated from the cold, authentic PySA; purity was determined by TLC in chloroform on Quanta-Gram Q1 Silica Gel G. The specific activity was 14.3 Ci/mol.

The [^{125}I]INA was synthesized from 5-aminonaphthyl-1-azide by method 3 of Bercovici and Gitler (2). Purity of the final [^{125}I]INA product was determined by TLC on activated Whatman K1F Silica Gel G with fluorescent indicator, using *n*-hexane as the solvent, and by UV spectroscopy. Results agreed with those of Bercovici and Gitler (2).

Tartryl diazide was synthesized immediately before use by the method of Dubovi and Wagner (7). The final TDA concentration was 200 mM, assuming that the reaction went to completion.

Reaction conditions. (i) PySA and INA. Suspensions of 1.0 ml of a 1-mg/ml concentration of VSV in phosphate-buffered saline (PBS) were incubated in the dark with either 5 μl of [^{125}I]INA (~4 × 10^{6} cpm) in ethanol or 10 μl of [^{3}H]PySA (~4 × 10^{5} cpm) in acetone. Samples containing [^{125}I]INA were then UV irradiated for 60 s by an Osram HBO lamp at 10-cm distance through Corning 0-54 and 7-54 filters; samples containing [^{3}H]PySA were UV irradiated for 5 min at 3-cm distance from the long-wavelength lamp of a Mineralight UVSL-25 with the blue filter removed. Immediately after UV irradiation, most samples were added to 10 ml of cold acetone and the protein was precipitated. The pellets after low-speed centrifugation were acetone precipitated a second time, dried, and suspended in the gel electrophoresis sample buffer. Samples to be thermolysin treated by the method of Schloemer and Wagner (23) were not acetone precipitated at this point but incubated with 25 U of thermolysin for 30 min at 37°C, layered on top of a 0 to 40% sucrose gradient in 10 mM Tris, pH 7.5, and centrifuged at 250,000 × *g* for 2 h. The band of virus was collected and then acetone precipitated as described above.

(ii) DMS. Cross-linking reactions of VSV by DMS were performed similarly to the procedure of Pepinsky and Vogt (19). A suspension of 0.81 ml of 0.25-mg/ml [^{3}H]palmitate-labeled VSV in 50 mM triethanolamine, 50 mM NaCl, pH 8.0, was incubated with 80 μl of 40 mM DMS in the same buffer for 2 h at room temperature. The solution was then Folch extracted with 4 volumes of chloroform-methanol (2:1, vol/vol) to remove most of the excess phospholipid; the protein interface was collected, dried under nitrogen, and dissolved in 0.4 ml of gel electrophoresis sample buffer.

(iii) TDA. The TDA cross-linking reaction was similar to that described by Dubovi and Wagner (7). A 0.5-ml solution of [^{3}H]palmitate-labeled VSV at 1 mg/ml in 50 mM triethanolamine–50 mM NaCl (pH 8.5) was mixed with 10 μl of 200 mM TDA which had been synthesized immediately before use. The reaction proceeded for 30 min at room temperature and was stopped by addition of 50 μl of 1 M methylamine, pH 7.7. A 0.44-ml portion of gel electrophoresis sample buffer was added before electrophoresis.

(iv) DTBPA. The procedure for reaction of DTBPA with VSV was modified from the procedure of Mikkelsen and Wallach (16). A solution of 0.5 ml of 1-mg/ml [^{3}H]palmitate-labeled VSV in PBS was preincubated with 5 μl of 30-mg/ml DTPBA in 95% ethanol for 5 min in the dark. This sample was then

UV irradiated for 5 min at a distance of 3 to 4 cm from the long-wavelength lamp of a Minerlight UVSL-25 UV lamp with the blue filter removed. Native, free sulfhydryl groups were then immediately blocked by addition of 125 μl of 12% sodium dodecyl sulfate (SDS) and 40 mM *N*-ethylmaleimide for 15 min. The sample was Folch extracted with 4 volumes of chloroform-methanol (2:1, vol/vol), and the dried protein pellet was redissolved in 0.5 ml of gel electrophoresis sample buffer without dithiothreitol present, which would have cleaved the DTBPA disulfide bond.

SDS-polyacrylamide gel electrophoresis (PAGE). Samples containing 50 μg of viral protein (200 μg for [^{3}H]PySA-labeled VSV) in SDS-PAGE sample buffer (2% SDS, 25 mM Tris, 100 mM dithiothreitol, and 20% glycerol, pH 6.8, with bromophenol blue added as tracking dye) were incubated at 100°C for 5 min and electrophoresed at 100 V per slab gel until stacked and then at 200 V for 2.5 h. The running gels contained 12.5% acrylamide, 0.1% bisacrylamide, 0.1% SDS, and 0.375 M Tris, pH 8.7, overlaid with stacking gels containing 4% acrylamide, 0.2% bisacrylamide, 0.1% SDS, and 0.125 M Tris, pH 6.8, according to the method of Laemmli (10) as modified by Carroll and Wagner (4). Thermolysin-treated samples were electrophoresed as above but in running gels containing 17.5% acrylamide, 0.07% bisacrylamide, 0.1% SDS, 7 M urea, and 0.375 M Tris, pH 8.7, by the procedure described by Petri and Wagner (21). Gels were stained with Coomassie brilliant blue, destained, and sliced into 1.3-mm segments. Radioactivity of samples labeled with ^{125}I was determined by counting in a Beckman Biogamma 4000; ^{3}H radioactivity was determined by scintillation spectrometry as previously described (23).

RESULTS

Structure and characterization of probes. The five probes used in this study are diagramed in Fig. 1 and represent several different types of covalent binding reagents. The INA and PySA are a monofunctional aryl azide and a sulfonyl azide, respectively. They are radiolabeled and sufficiently hydrophobic that they will partition almost exclusively into membrane structures (2, 22). The PySA sulfonyl azide functional group is more polar than the INA aryl azide, and the product nitrene after UV irradiation is more reactive. Of the three cross-linking reagents used, DTPBA is the most hydrophobic; it is an aryl azide like the INA but is bifunctional and cleavable by sulfhydryl compounds. TDA is a nonhydrophobic bifunctional acyl azide with a cleavable glycol structure; it has a reaction mechanism and selectivity similar to that of the INA and DTBPA aryl azides. DMS is a noncleavable, bifunctional bisimidate with selectivity for binding to primary amino groups.

[^{125}I]INA and [^{3}H]PySA were prepared as described in Materials and Methods. TLC of [^{125}I]INA was performed on activated Silica Gel G with fluorescent indicator, using *n*-hexane as the solvent. Subsequent detection under UV light showed one spot migrating at $R_f \sim 0.75$ which was permanently darkened after UV exposure. Samples of [^{125}I]INA which were UV irradiated before TLC remained at the origin. These results as well as the UV absorbancy spectrum (data not shown) of the [^{125}I]INA sample agree with those of Bercovici and Gitler (2). TLC of [^{3}H]PySA, authentic cold PySA, and [^{3}H]PySA preirradiated with UV light was performed on activated Silica Gel G without fluorescent indicator, using chloroform as the solvent. Spots were visualized under UV light which permanently darkened them, and both [^{3}H]PySA and cold authentic PySA ran identically at $R_f \sim 0.93$. Preirradiated [^{3}H]PySA remained at the origin; UV absorbancy spectra were run and agreed with the results of Sator et al. (22), as did the TLC results.

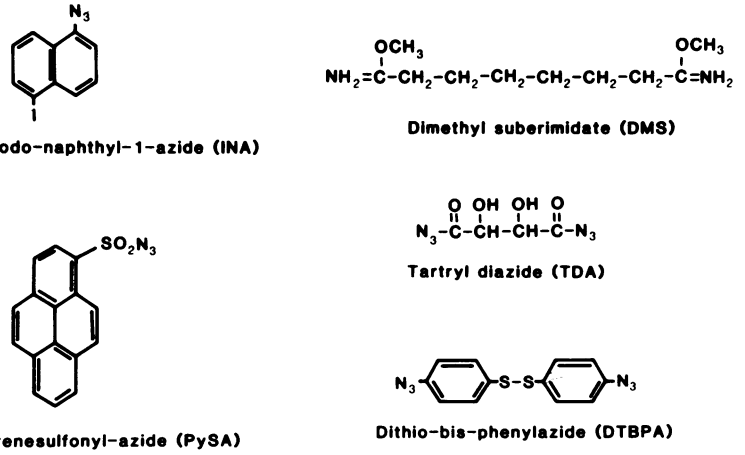

FIG. 1. *Chemical structures and names of probes used in subsequent experiments.*

The ability of INA and PySA to label VSV was tested in the following manner. Equal portions of either 5 μl of [^{125}I]INA in ethanol or 10 μl of [^{3}H]PySA in acetone were incubated with 1 ml of a 1-mg/ml suspension of VSV in PBS (<1% final concentration of organic solvent) for 5 min in the dark, after which samples were UV irradiated from an appropriate source. Samples were removed from irradiation at specified times and acetone precipitated. ^{125}I and ^{3}H radioactivities were determined for both the protein pellets after resuspension into 1.0 ml of PBS and the acetone supernatant after being dried down. [^{125}I]INA had maximal incorporation into the VSV pellet fraction at 1.5 min, whereas the [^{3}H]PySA was slower and did not attain maximal incorporation until 5 min. The level of incorporation into the pellet for [^{3}H]PySA was only 5%, whereas that for [^{125}I]INA was about 30% (data not shown). The low incorporation of [^{3}H]PySA into VSV was sufficient for further experimentation but explains the low level of radioactivity to be seen in individual proteins after SDS-PAGE. Subsequent electrophoresis showed that much of the incorporated probe in both cases was attached to viral lipid rather than to protein (data not shown).

Reaction of [^{125}I]INA with VSV. A 5-μl sample of [^{125}I]INA in ethanol was preincubated with 1 ml of a 1-mg/ml suspension of VSV in PBS in the dark for 5 min. The concentration of ethanol was always maintained at <1%. UV irradiation was then performed for 5 min, at which time the sample was acetone precipitated twice, blown dry under a stream of nitrogen to remove the last traces of acetone, and then dissolved in 1.0 ml of SDS-PAGE buffer. After electrophoresis of [^{125}I]INA-labeled VSV on 12.5% acrylamide, gels were stained for protein with Coomassie brilliant blue and then sliced into 1.3-mm pieces and counted for ^{125}I.

Figure 2 shows the distribution of [^{125}I]INA incorporated into the proteins of intact virions and VSV disrupted with 1% SDS. Most of the [^{125}I]INA was incorporated into the G protein of the intact virions, with smaller amounts incorporated into the N and M proteins (Fig. 2A). Identification of peaks was done by matching the slice number to the location of the Coomassie brilliant blue protein stain. The area at the dye front exhibited the expected large number of ^{125}I counts incorporated into VSV membrane phospholipid which had been linked to [^{125}I]INA and not removed by the acetone washing. An unidentified peak between the M protein and the dye front was consistently seen to migrate with an R_m corresponding to a molecular weight of ~10,000 but was deemed to be an artifact of the INA labeling since no stainable protein could

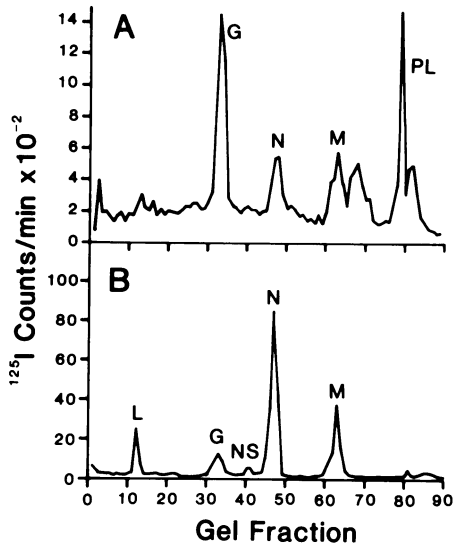

FIG. 2. *SDS-PAGE of VSV proteins after reaction with [^{125}I]INA of (A) intact virus or (B) virus previously disrupted with 1% SDS. VSV (1 mg in 1 ml of PBS) was incubated with 5 μl of [^{125}I]INA in the dark, UV irradiated for 5 min, and acetone precipitated twice. The protein pellet was redissolved in SDS-PAGE sample buffer, and a 50-μg portion was electrophoresed on a slab gel (running gel: 12.5% acrylamide, 0.1% N,N-methylenebisacrylamide, 0.1% SDS, and 0.375 M Tris, pH 8.7; stacking gel: 4% acrylamide, 0.2% N,N-methylenebisacrylamide, 0.1% SDS, 10% glycerol, and 0.125 M Tris, pH 6.8) run at 100 V until the sample stacked and then at 200 V for 2.5 h. Gels were stained for protein with Coomassie brilliant blue and then sliced and counted for radioactivity. Viral proteins L, G, NS, N, and M were identified by matching position of protein stain to peak of radioactivity. PL designates the phospholipid at the dye front.*

be seen in this region of the gel (data not shown). The labeling of the N protein was also unexpected. Mixtures of [^{125}I]INA and VSV which were not UV irradiated but otherwise treated in the same manner showed, after electrophoresis, that no ^{125}I was incorporated into any of the viral proteins. This control indicates that the binding of ^{125}I to viral protein is a specific result of UV irradiation which converts the azide group into a highly reactive nitrene (9).

That the [^{125}I]INA probe did not label hydrophilic protein regions after UV irradiation was shown by the addition of 1 mg of cytochrome *c* per ml to the incubation mixture; no ^{125}I could be detected by SDS-PAGE at a position corresponding to cytochrome *c* (data not shown). Similarly, if [^{125}I]INA were free in solution or if the reactive azide group were protruding from the membrane, it would be reduced by sulfhydryl compounds to the corresponding amine

which has no labeling capability (25). Addition of 10 mM GSH in the incubation mixture had no effect on the pattern of incorporated ^{125}I counts (data not shown).

Studies were undertaken to determine whether the [^{125}I]INA probe predominantly labels that portion of the G protein that is embedded in the virion membrane. It has been shown that proteases, such as thermolysin, remove about 90% of G protein from intact VSV and leave a small (molecular weight ≈ 5,800) hydrophobic tail piece which remains in the membrane (17, 23). VSV that had first been reacted with [^{125}I]INA and then treated with thermolysin retained in a rapidly migrating fraction more than two-thirds of the ^{125}I incorporated into the whole G protein (Fig. 3). The thermolysin-resistant glycoprotein tail piece represented less than 10% of the molecular weight of the native G protein. No residual intact glycoprotein could be detected in thermolysin-treated virions subjected to electrophoresis on SDS-PAGE gels stained with Coomassie brilliant blue, indicating that proteolysis was complete (data not shown).

When VSV was disrupted in 1% SDS, the membrane structure was destroyed and the proteins were completely denatured into a linear configuration with SDS bound as a shell surrounding each protein molecule. In this situation, all of the viral proteins presumably have a hydrophobic shell which should bind [^{125}I]INA and allow labeling of all proteins after UV irradiation. Reaction of [^{125}I]INA with VSV in the presence of 1% SDS labeled all five of the viral proteins (Fig. 2B). The ^{125}I artifact peak shown in Fig. 2A is not present in Fig. 2B. The G protein showed an anomalously low level of labeling in the presence of SDS as compared with the N and M proteins; the amount of ^{125}I incorporated into G remained unchanged in the presence or absence of 1% SDS. The N and M proteins, on the other hand, showed 10- to 15-fold increases in the extent of labeling in the presence of SDS. The L and NS proteins, which were not labeled at all in the intact virion, became labeled to the same relative extent as the N and M proteins on a per-milligram-of-protein basis when the labeling was performed in 1% SDS. The low level of labeling of the G protein could be due to the fact that G is an amphipathic glycoprotein and the carbohydrate chains possibly are either not allowing proper binding of the SDS to the entire length of the polypeptide or are somehow interfering with proper binding of the [^{125}I]INA before UV irradiation.

This very high labeling of certain viral proteins with [^{125}I]INA in the presence of SDS could possibly be due not only to linkage of the ^{125}I to the protein but also to [^{125}I]INA linking

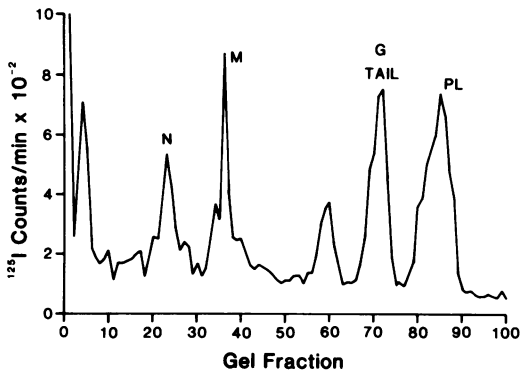

FIG. 3. *SDS-PAGE of proteins of VS virions exposed to thermolysin after having been treated with [^{125}I]INA. VSV (1 mg in 1 ml of PBS) was incubated with 5 μl [^{125}I]INA in the dark, UV irradiated for 5 min, and then incubated with 25 U of thermolysin at 37°C for 30 min. Virus was purified by centrifugation on a gradient of 0 to 40% sucrose and 10 mM Tris, pH 7.5, at 250,000 × g for 2 h; the band of virus was collected, acetone precipitated twice, and redissolved in SDS-PAGE sample buffer, and a 50-μg sample was run on SDS-PAGE as described in Fig. 1 except that the running gel contained 17.5% acrylamide, 0.07% N,N-methylenebisacrylamide, 0.1% SDS, 7 M urea, and 0.375 M Tris, pH 8.7. Gels were stained for protein with Coomassie brilliant blue and then sliced and counted for radioactivity. Identification of radiolabeled viral proteins N, M, and G TAIL (as the thermolysin-resistant, hydrophobic G tail piece) was by matching position of protein stain to peak of radioactivity. PL designates the phospholipid at the dye front.*

to the SDS molecule and then not being washed off the protein or not being exchanged off during electrophoresis. To test this possibility, a portion of this sample was incubated at 100°C for 10 min in a fivefold excess of added SDS and electrophoresed. There was essentially no change in the level of incorporated counts (data not shown), and the high level of labeling in the presence of SDS is considered to result from [^{125}I]INA binding to protein and not to SDS.

Reaction of [^{3}H]PySA with VSV. A second hydrophobic probe, [^{3}H]PySA, was tested similarly to [^{125}I]INA for its capacity to covalently bind to VSV proteins. A 10-μl sample of [^{3}H]PySA in acetone was added to a 1-mg suspension of intact or SDS-disrupted VSV in 1 ml of PBS (final acetone concentration was 1%) and incubated in the dark for 5 min under a nitrogen atmosphere; this was followed by UV irradiation for 5 min also under nitrogen, after which the samples were acetone precipitated twice, dried, and dissolved in 0.5 ml of SDS-PAGE buffer. Figure 4 shows the comparative results of SDS-PAGE of the proteins of intact or SDS-disrupted VSV labeled with [^{3}H]PySA. The levels of

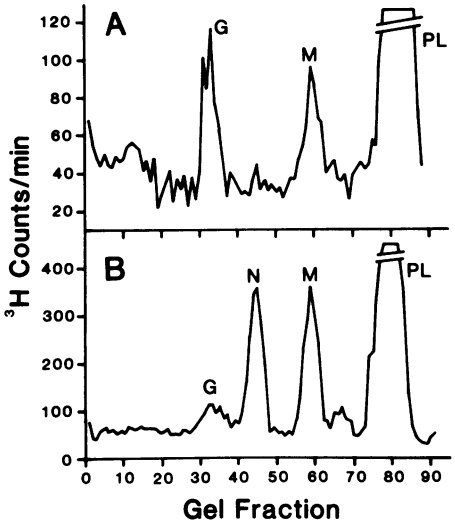

FIG. 4. *SDS-PAGE of VSV proteins after reaction with [^{3}H]PySA of (A) intact virus or (B) virus previously disrupted with 1% SDS. The protocol was essentially the same as that in Fig. 2 except that [^{3}H]PySA was substituted for [^{125}I]INA, a different UV source was used for irradiation, and 200-μg samples were applied to gels.*

[^{3}H]PySA incorporation were low, as expected, but G and M were the only proteins of intact VSV labeled to any significant extent (Fig. 4A). There was no detectable labeling of proteins when the reaction mixture was not UV irradiated or when a solution of [^{3}H]PySA was UV irradiated and then immediately incubated in the dark with VSV for 5 min without further irradiation (data not shown).

The artifact seen to migrate faster than the M protein after reaction with [^{125}I]INA was not detected when [^{3}H]PySa was used; moreover, [^{3}H]PySA did not detectably label the N protein. As was the case with [^{125}I]INA, the presence in the reaction mixture of either 10 mM GSH or a 1-mg/ml concentration of cytochrome *c* did not affect labeling of VSV proteins with [^{3}H]PySA (data not shown).

Whereas only two-thirds of the associated ^{125}I in the G protein could be found in the membrane-associated tail piece after thermolysin treatment of VSV which had been reacted with [^{125}I]INA (Fig. 3), more than 90% of the [^{3}H]PySA label in the G protein could still be seen in the tail piece after thermolysin treatment of [^{3}H]PySA-treated VSV (Fig. 5). The specificity of [^{3}H]PySA for membrane regions would thus appear to be somewhat higher than that of [^{125}I]INA. This greater membrane specificity may also explain why the [^{3}H]PySA did not label the N protein, whereas the [^{125}I]INA did.

Reaction of [^{3}H]PySA with VSV which had been disrupted with 1% SDS produced the SDS-PAGE pattern shown in Fig. 4B. The N and M proteins were heavily labeled in the presence of SDS, whereas the G protein again showed no change in the extent of labeling in the presence or absence of SDS. Neither the L nor NS protein appeared to be labeled by [^{3}H]PySA in the presence of SDS.

Reaction of cross-linking reagents with VSV. Bifunctional reagents that can covalently link proteins to adjacent membrane components were tested to determine whether, and the degree to which, VSV proteins are associated with the virion membrane phospholipids. In these experiments, we used the three cross-linking reagents DMS, TDA, and DTBPA, the chemical structures of which are shown in Fig. 1. Membrane phospholipids were labeled by growing VSV in the presence of [^{3}H]palmitate, which produces the SDS-PAGE pattern seen in Fig. 6A. As expected, the only detectable ^{3}H radioactivity was found in the G protein and in the phospholipids that migrated to the dye front. The labeling of G by [^{3}H]palmitate was the result of a covalent attachment of fatty acid to the protein (24).

DMS at a concentration of 2 mM was allowed to react for 2 h with VSV (0.25 mg/ml) that had been labeled with [^{3}H]palmitate; the sample was then Folch extracted to remove most of the non-cross-linked, labeled phospholipid. The protein pellet was dissolved in SDS-PAGE buffer and electrophoresed in 12.5% polyacrylamide gels.

Figure 6B reveals significant incorporation of [^{3}H]palmitate-labeled phospholipid only into the G and M proteins of virions exposed to the DMS cross-linker. Additionally, protein-protein

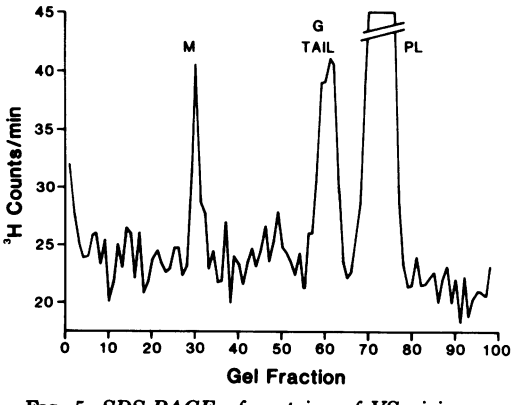

FIG. 5. *SDS-PAGE of proteins of VS virions exposed to thermolysin after having been treated with [^{3}H]PySA. The protocol was essentially the same as that in Fig. 3 except that [^{3}H]PySA was substituted for [^{125}I]INA, a different UV source was used, and 200-μg samples were applied to gels.*

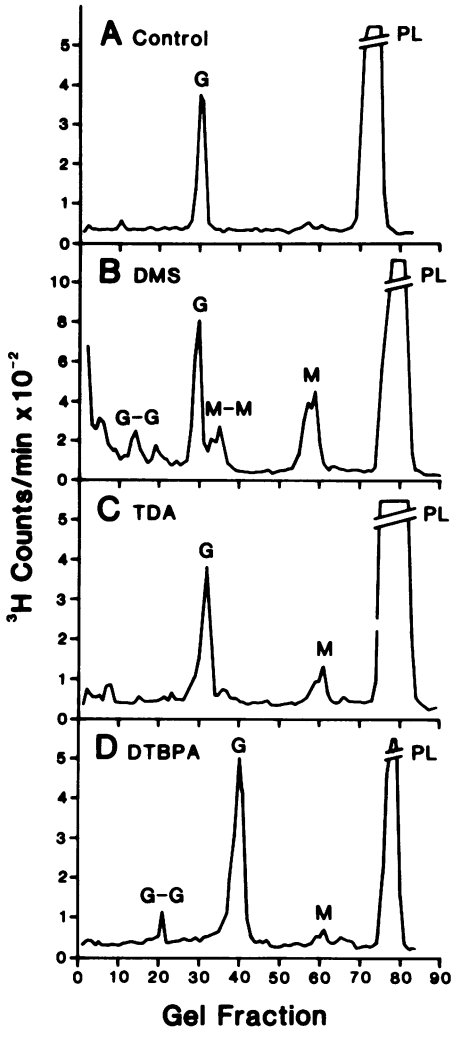

FIG. 6. *SDS-PAGE of proteins of intact VSV labeled with [^{3}H]palmitate and the effects of three cross-linking reagents. VSV was grown in the presence of 2.5 μCi of [^{3}H]palmitate per ml, and 50-μg samples of control virus (A) and virus cross-linked with DMS (B), TDA (C), or DTBPA (D) were electrophoresed identically as in Fig. 2 and 4 except that the SDS-PAGE sample buffer for the VSV cross-linked with DTBPA did not contain dithiothreitol. Reaction conditions for cross-linking of [^{3}H]palmitate-labeled VSV with DMS, TDA, and DTBPA were as described in Materials and Methods. Identification of the radiolabeled viral proteins G and M was by matching position of Coomassie brilliant blue protein stain to peak of radioactivity. The cross-linked homodimers G-G and M-M were identified by estimating molecular weight. PL designates the phospholipid at the dye front.*

cross-linking had occurred and produced G-G and M-M dimers which also had acquired the [^{3}H]palmitate label. The unlabeled peak between the G and G-G peaks could well be the G-M heterodimer (7). The protein pattern revealed by Coomassie brilliant blue staining showed a large amount of high-molecular-weight cross-linked products, most of which did not correlate with any of the peaks of ^{3}H radioactivity in Fig. 6B (data not shown).

In the next experiment, 0.5 ml of VSV (1 mg/ml) in triethanolamine buffer was allowed to react with 10 μl of freshly prepared TDA (200 mM) for 30 min, after which time the reaction was stopped by addition of 0.1 M methylamine, pH 7.7, and electrophoresed after addition of SDS-PAGE buffer. Figure 6C shows that M was the only protein apparently cross-linked by TDA to phospholipid, but Table 1 indicates a small degree of phospholipid cross-linking to G protein after subtracting the background covalent [^{3}H]palmitate in control G protein. The extent of cross-linking appears to be less since the incorporation of ^{3}H label was lower, there were no discernible G-G or M-M dimers, and the Coomassie brilliant blue staining indicated lesser formation of high-molecular-weight products (data not shown).

DTBPA in 95% ethanol was preincubated in the dark at a final concentration of 1 mM with 0.5 ml of VSV (1 mg/ml) in PBS for 5 min and then UV irradiated for 5 min similarly to the [^{3}H]PySA reaction. Since DTBPA is cleavable by free sulfhydryls, protein sulfhydryl groups were blocked after irradiation by derivatization with 8 mM *N*-ethylmaleimide in the presence of 2% SDS. Samples were then Folch extracted and the protein pellet was dissolved in SDS-PAGE buffer in the absence of dithiothreitol. Addition

TABLE 1. *Comparative quantitation of the degree to which the G and M proteins in the membrane of intact VSV are covalently linked to [^{3}H]palmitate-labeled phospholipids of control VSV or VSV exposed to cross-linker DMS, TDA, or DTBPA*

VSV treatment	cpm of [^{3}H]palmitate associated with[a]:			
	Total G[b]	Total M[b]	G minus control[c]	M minus control[c]
Control	800	35		
DMS	2,020	2,261	1,220	2,226
TDA	934	357	134	322
DTBPA	1,632	80	832	45

[a] Counts per minute above background determined from data shown in Fig. 6 by pooling those gel slices which contained the entire protein peak identified by radioactivity and Coomassie brilliant blue staining.

[b] Sum of monomer and dimer peaks.

[c] Total counts per minute in cross-linked G and M protein after subtraction of the counts per minute in the control (not cross-linked) VSV M and G protein which contained covalently linked [^{3}H]palmitate (24).

of any such reducing compounds would have cleaved the DTBPA disulfide cross-link. Figure 6D shows the SDS-PAGE pattern of [^{3}H]palmitate-labeled VSV reacted with DTBPA. As noted, the G protein and a G-G dimer both ran slightly faster on this gel because of the absence of any reducing agent. Only the G monomer and dimer were significantly cross-linked by DTBSA to ^{3}H-phospholipid.

Table 1 summarizes the data on protein-phospholipid cross-linking by the three bifunctional reagents reacted with VSV labeled with [^{3}H]palmitate; also shown is background radioactivity of control VSV, the G protein of which normally contains significant covalently linked [^{3}H]palmitate, as previously reported by Schmidt and Schlesinger (24). Minimal cross-linking of ^{3}H-phospholipid to G protein by TDA and to M protein by DTBPA is probably not significant; hydrophilic TDA did not appear to be a very effective cross-linker. DMS clearly linked ^{3}H-phospholipid to both G and M proteins, whereas the highly lipophilic DTBPA preferentially linked ^{3}H-phospholipid to the G protein.

Coomassie brilliant blue staining of the gels shown in Fig. 6 revealed that all three bifunctional reagents produced some protein-protein cross-links as well as protein-phospholipid cross-links. When any of these reagents was reacted with virus in the presence of 1 mg of cytochrome *c* per ml, there was no labeling of the cytochrome *c* (data not shown), indicating that this system of cross-linking to labeled membrane phospholipid is measuring only membrane-associated proteins.

In contrast to the results with [^{125}I]INA and [^{3}H]PySA, addition of GSH abolished all evidence of cross-linking by TDA and DTBPA (data not shown). This was expected for the DTBPA since this reagent is a disulfide and is cleaved by sulfhydryl groups. The result with TDA indicates that this reagent's functional groups are accessible to reduction by the freely soluble GSH and therefore that the TDA is a relatively hydrophilic compound that is not labeling from the interior of the membrane. Addition of 1% SDS during any of the cross-linking reactions also abolished evidence of protein-phospholipid cross-linking (data not shown).

DISCUSSION

There is general agreement on membrane composition of VSV. The G protein is an integral membrane protein extending through the membrane, whereas the M protein lines the interior face of the membrane with the nucleocapsid contained inside (27). The evidence for the membrane location of the G protein is extensive (18), but there is little biochemical verification for association of the M protein with the VSV membrane. Stoffel et al. (26), using 16-azido-[^{3}H]palmitic acid and UV irradiation to covalently label membrane constituents in close association with this membrane probe, were able to show extensive labeling of the G protein but no labeling of the M protein. These results implied either that the M protein was not membrane associated or, more likely, that M protein was not inserted to a very great depth into the membrane since the reactive 16-azido group resided in the most central portion of the membrane. Pepinsky and Vogt (19) were able to show that the M protein, as well as the G protein, reside within or not more than 1 nm from the VSV membrane based on the ability to cross-link these proteins with DMS to radioactively labeled phosphatidylethanolamine.

A third experimental approach to the study of membrane protein-lipid associations, which has been receiving increased attention of late, is the use of exogenous hydrophobic membrane probes, such as [^{125}I]INA and [^{3}H]PySA (2, 22). Both of these probes partition into membranous structures to an extent greater than 97% and, when UV-irradiated, covalently attach to any adjacent membrane constituents. Electrophoresis patterns presented here showed that G and M proteins in the membrane of intact VSV were labeled by both of these probes.

Further evidence for the membrane association of the M as well as the G protein comes from the cross-linking of these proteins to radiolabeled membrane phospholipids by use of DMS, TDA, and DTBPA. These three reagents differ in hydrophobicity and reaction mechanism both among themselves and in comparison with INA and PySA. DMS readily cross-linked ^{3}H-phospholipid to both G and M proteins, but only the M protein was cross-linked to ^{3}H-phospholipid by TDA and only the G protein by DTBPA (see Table 1).

The very low level of cross-linking of M protein by DTBPA may reflect a situation similar to that encountered by Stoffel et al. (26) with the 16-azido-[^{3}H]palmitic acid. The DTBPA may be so hydrophobic that it seeks the most interior portion of the membrane and is therefore not accessible to the M protein on or near the membrane surface. The hydrophobic [^{125}I]INA probe, however, labeled the M as well as the G protein despite its presumed location in the membrane interior. Figure 2A shows that the M protein is labeled by [^{125}I]INA to only about one-half the extent of the G protein, and this low labeling of M may be due to its inaccessibility to the [^{125}I]INA in the membrane interior. The greater labeling of M relative to G

protein by the [^{3}H]PySA probe could be explained by the fact that the sulfonyl azide moiety is more polar than the aryl azide and would orient so that the reactive azide group is closer to the membrane surface. It is not sufficiently polar, however, so as to significantly protrude from the membrane surface or it would have been affected by either the hydrophilic cytochrome *c* or GSH incorporated in the reaction mixture.

The sensitivity of labeling by TDA to inhibition by GSH indicates that the TDA is accessible in aqueous solution and does not partition into membranes. This would explain the lack of labeling of the G protein, the membrane-associated region of which is embedded within the membrane in a nonaqueous environment. The M protein becomes cross-linked with TDA to form M homodimers (7), presumably because its membrane association is at the surface of the membrane and accessible to the nonhydrophobic TDA.

Reaction of VSV with the [^{125}I]INA, in addition to labeling of the G and M proteins, also produced labeling of the N protein and of some artifact that migrated with a mobility corresponding to a molecular weight of ~10,000 (Fig. 2A). None of the other probes labeled either of these species, which stresses the importance of not relying upon the results from only one type of probe. This labeling of the N protein is probably due to the presence of hydrophobic pockets in the protein which bind the [^{125}I]INA and then become labeled upon UV irradiation. The N protein is experimentally difficult to work with in solution because of problems of aggregation and precipitation which may reflect this very hydrophobic character (S. U. Emerson, personal communication). The presence of the labeled peak running at ~10,000 daltons was at first thought to be an artifact of the [^{125}I]INA probe similar to that found by Cerletti and Schatz (6). Another possibility, as recently reported by Lodish and Porter (11), could be a low-molecular-weight host cell membrane protein which is specifically incorporated into the VSV membrane during viral maturation. Such a protein, however, should have been detected by the other probes as well; therefore, the identity of this peak labeled by [^{125}I]INA remains obscure.

Arguments have been raised against the efficacy and specificity of aryl azide probes such as the [^{125}I]INA and [^{3}H]PySA used here (1), but our control studies seem to rebut the arguments. Analysis of the thermolysin-treated samples (Fig. 3 and 5) showed that the labeling of the G protein was confined almost exclusively to the membrane-embedded hydrophobic tail piece portion of that protein with only minimal labeling on the protruding protease-sensitive spike portion. That these probes were not accessible to labeling of hydrophilic non-membrane-associated regions was also shown by the inability of either GSH or cytochrome *c* to compete for labeling by the aryl azide probes. Indiscriminate labeling of all viral proteins by [^{125}I]INA and [^{3}H]PySA occurred only when the virus sample had been disrupted in 1% SDS, thus destroying virus membrane integrity and enveloping all of the proteins in a hydrophobic film of SDS. Conversely, addition of 1% SDS during the reaction of VSV with cross-linking reagents abolished all labeling by removal of the labeled phospholipid from the vicinity of the proteins. The argument that such aryl azide probes are perturbing the membrane and creating a nonphysiological state can best be countered by comparing the results of studies on the cytochrome *c* oxidase system. Cerletti and Schatz (6), using the aryl azide probes [^{125}I]INA and *S*-(4-azido-2-nitrophenyl)-[^{35}S]thiophenol, and Bisson et al. (3), who used azide-derivatized phosphatidylcholine probes, obtained essentially identical subunit labeling patterns. The aryl azide probes would thus seem to be at least as nondisruptive of the membrane as is added phosphatidylcholine and are legitimate probes of membrane structure.

The results from these studies with five different probes of viral membrane protein-phospholipid interaction show that, in addition to the intrinsic G protein, the M protein is in close association with the VSV membrane. This association, however, appears to be on the surface of the membrane; the M protein apparently does not penetrate the viral membrane to any great depth.

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