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Conformational Changes and Fusion Activity of Vesicular Stomatitis Virus Glycoprotein: [^{125}I]Iodonaphthyl Azide Photolabeling Studies in Biological Membranes

Charles C. Pak, Anu Puri, and Robert Blumenthal*

Section of Membrane Structure and Function, Laboratory of Experimental and Computational Biology, Division of Basic Sciences, National Cancer Institute, National Institutes of Health, Frederick, Maryland 21702-1201

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ABSTRACT: The interaction of VSV glycoprotein (VSV G) with biological membranes was studied by photosensitized labeling. The method is based on photosensitized activation by the fluorescent lipid analog 3,3′-dioctadecyloxacarbocyanine (DiO) of a hydrophobic probe, [^{125}I]iodonaphthyl azide (^{125}INA), that rapidly partitions into the membrane bilayer of virus and cells. ^{125}INA labeling of proteins and lipids can be confined to the site of chromophore localization by photosensitized labeling. Photoactivation using visible light of target membrane labeled with DiO and ^{125}INA, to which unlabeled virions are bound, results in exclusive labeling of envelope glycoproteins inserted into the target membrane [Pak et al. (1994) *J. Biol. Chem. 269*, 14614]. In this study, we labeled lipid symmetric erythrocyte ghosts with ^{125}INA and DiO. Photosensitized activation of VSV prebound to labeled ghosts with visible light resulted in VSV G labeling under fusogenic conditions. Photoactivation of ^{125}INA by UV light, which is nonspecific, produced labeled VSV G at both acidic and neutral pH. Photosensitized labeling of VSV G by DiO-^{125}INA-ghosts was also observed at pH 5.5, 4 °C, in the absence of mixing between viral and cellular lipids, suggesting insertion of the ectodomain of VSV G. Soluble VSV G lacking the transmembrane domain inserted into DiO-^{125}INA-ghosts under the same conditions as intact VSV G. DiO inserted into intact VSV appeared to be a suitable fluorophore for continuous kinetic measurements of membrane fusion by fluorescence dequenching. Our photosensitized labeling results establish biochemical correlates for the three states of VSV G, which we had proposed based on kinetic data [Clague et al., *Biochemistry 29*, 1303]. In addition, we found that VSV G insertion into the target membrane is reversible, suggesting a "velcro"-like attachment of the fusogenic domain with the target membrane.

Entry of vesicular stomatitis virus (VSV)[1] into cells requires fusion of viral and cellular membranes (Matlin et al., 1982; White et al., 1981). The fusion event occurs after endocytosis within the low-pH environment of the endosomes, as reagents that raise the pH within endosomes inhibit membrane fusion (Schlegel et al., 1982; Matlin et al., 1982; Blumenthal et al., 1987). Experimentally, VSV can be induced to fuse with liposomes (Yamada & Ohnishi, 1986; Pal et al., 1988), or with the plasma membrane bilayer of nucleated cells (Matlin et al., 1982; Blumenthal et al., 1987) and erythrocyte ghosts (Herrmann et al., 1990; Clague et al., 1990), by exposure of prebound VSV−cell complexes to acidic conditions. Binding and fusion are known to be mediated by VSV glycoprotein (G), as it is the sole envelope glycoprotein (Thomas et al., 1985; Crimmins et al., 1983; Blumenthal et al., 1987). Expression of VSV G in cells in the absence of other VSV proteins rendered those cells capable of fusing at low pH (Riedel et al., 1984; Florkiewicz & Rose, 1984), demonstrating that VSV fusion is completely mediated by VSV G. The VSV fusion process appears to proceed through a series of steps that is initiated by exposure to low pH (Clague et al., 1990). VSV G undergoes a low-pH-induced conformational change that is observed as an increased exposure of hydrophobic domain(s) (Crimmins et al., 1983). An increase in hydrophobicity at low pH is also observed in the Rabies virus glycoprotein (G), another member of the rhabdovirus family (Gaudin et al., 1993).

The interaction of viral fusion proteins with membranes can be detected by labeling with hydrophobic photoactivatable probes. This approach was effective in the analysis of the interaction of hemagglutinin (HA) with liposomes (Durrer et al., 1996; Tsurudome et al., 1992; Stegmann et al., 1991; Harter et al., 1989; Brunner et al., 1991). Durrer et al. (1996) recently showed that the amino terminus of the HA2 transmembrane subunit inserts into liposomes during fusion. Durrer et al. (1995) have applied the hydrophobic photolabeling techique to Rabies virus and VSV using a recently developed phospholipid analogue incorporated into liposomes. Labeling of the glycoprotein strongly increased as the pH was lowered from 7.0 to 6.0, suggesting the exposure of a domain at acidic pH capable of interacting with membranes. The labeled regions were identified by generating CNBr fragments and analysis by SDS−PAGE followed by autoradiography. Labeled segments in VSV G were found to be contained within the putative fusion peptide domains predicted by hydrophobicity analysis (Ohnishi, 1988) and by site-directed mutagenesis (Fredericksen & Whitt, 1995; Zhang & Ghosh, 1994; Whitt et al., 1990).

* To whom correspondence should be addressed at NCI-FCRDC, Building 469, Room 213, P.O. Box B, Miller Dr., Frederick, MD 21702-1201. Phone: (301) 846-1446. Fax: (301) 846-6192. E-mail: blumen@helix.nih.gov.

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[1] Abbreviations: VSV, vesicular stomatitis virus; VSV G, VSV glycoprotein; ^{125}INA, [^{125}I]iodonaphthyl azide; DiO, 3,3′-dioctadecyloxacarbocyanine; DiO-^{125}INA-ghosts, lipid symmetric ghosts labeled with DiO and ^{125}INA.

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Since liposomes are inherently more susceptible to destabilization and protein insertion than biological membranes, we examined membrane insertion using biological membranes as targets. We have developed a method to study such insertion in the case of influenza hemagglutinin (HA) (Pak et al., 1994). The method is based on photosensitized activation by fluorescent lipid analogs of a hydrophobic probe, [^{125}I]iodonaphthyl azide (^{125}INA), that rapidly partitions into the membrane bilayer of cells (Bayley & Knowles, 1978). Irradiation of cells containing ^{125}INA with ultraviolet (UV) light results in the formation of a reactive nitrene group capable of covalently labeling adjacent proteins and lipids (Bayley & Knowles, 1980). ^{125}INA labeling of proteins and lipids can be confined to the site of chromophore localization by photosensitized labeling. This method relies upon the ability of certain chromophores, when excited by visible light, to activate ^{125}INA by an energy transfer mechanism (Raviv et al., 1987, 1989). Photosensitized labeling requires collisional contact between the chromophore and ^{125}INA, thereby limiting labeling to the site of chromophore localization (Rosenwald et al., 1991; Raviv et al., 1990). Using this approach, we effectively labeled influenza HA in biological membranes under fusogenic conditions (Pak et al., 1994).

In this study, we used photosensitized labeling to monitor the conformational changes leading to insertion of VSV G into biological membranes. We find optimal labeling at pH 5.5 and 37 °C (fusogenic condition), significant labeling at pH 5.5 and 4 °C, and no labeling at pH > 6.4 and 37 °C. Soluble VSV G lacking the transmembrane domain was labeled at pH 5.5, indicating that some portion of the extracytoplasmic domain was labeled. Our results on photolabeling of VSV G are in agreement with those of Durrer et al. (1995), who used a completely different system consisting of a phospholipid analogue with a carbene reactive group and liposomes as targets. We obtained additional information concerning the reversibility of insertion. Our photolabeling results confirm the three-state model for VSV G, which we had proposed based on kinetic data (Puri et al., 1992; Clague et al., 1990).

EXPERIMENTAL PROCEDURES

Materials. All proteolytic enzymes were purchased from Sigma (St. Louis, MO) with the exception of bromelain, which was bought from Calbiochem (La Jolla, CA). [^{125}I]-iodonaphthyl azide (^{125}INA) (~1 mCi/μmol) was obtained from Lofstrand Laboratories (Gaithersburg, MD) and 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO) from Molecular Probes (Eugene, OR). Purified VSV (Indiana serotype) was prepared by J. Brown and B. Newcomb at the University of Virginia as described (Thomas et al., 1985). Fresh blood was obtained from the NIH blood bank. Anti-VSV G antibodies were generated as described previously (Blumenthal et al., 1987). Antibodies against the carboxyl tail of VSV G were kindly donated to us by Heinz Arnheiter. This antibody was generated against a peptide encoding for amino acids 501−511 (Arnheiter et al., 1984).

DiO Fluorescence Dequenching Assay. DiO-labeled VSV was prepared by incubating 1 mL of VSV in PBS (0.38 mg/mL) with DiO (5 μL of 3 mg/mL in DMSO) for 10 min at room temperature. Unincorporated DiO was removed by applying the DiO−VSV mixture to a Sephadex G25 PD10 column (Pharmacia, Piscataway, NJ). Eluate was pelleted by ultracentrifugation at 55000*g* for 50 min, 4 °C. Any aggregated virus was removed by a low-speed centrifugation at 3000 rpm for 3−5 min in an Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, NY). This procedure resulted in VSV loaded with DiO at quenched concentrations. Lipid symmetric erythrocyte ghosts were prepared as previously described (Clague et al., 1990). Preparation of erythrocyte ghosts in the presence of Ca^{2+} results in the symmetric redistribution of membrane phospholipids (Williamson et al., 1985), and an enhanced binding and fusion with VSV (Herrmann et al., 1990). Erythrocyte ghosts were incubated with DiO−VSV for 40 min on ice to promote virus binding, washed twice with PBS, and resuspended in PBS. DiO fluorescence dequenching was monitored with an SLM 8000 spectrofluorometer (SLM Instruments Inc., Urbana, IL) with excitation and emission wavelengths set at 475 and 510 nm, respectively. Fifty microliters of VSV−ghost complexes was added to a cuvette containing 2 mL of PBS preadjusted to the desired pH and temperature. Fusion extents were normalized to the maximal fluorescence dequenching, determined by the addition of Triton X-100 (0.05% final concentration).

Direct Photolabeling of VSV by UV Activation of ^{125}INA. Ten micrograms of VSV per sample was loaded with ^{125}INA (0.1 mCi/0.1 mL/10 mg of VSV) under subdued light. Virus was exposed to UV light irradiation for 2 min on ice. Samples were then incubated with or without cathepsin D for 4 h at 37 °C as described (Crimmins et al., 1983). We modified the protocol by using 2 M Tris−maleate and 15.6 mg/mL cathepsin D in the following ratio: 2.5:0.16:0.09 VSV:Tris−maleate:cathepsin D. All samples were boiled in reducing sample buffer and separated on 10−20% Tricine SDS−PAGE (Novex, San Diego, CA). Gels were either stained with Coomassie blue, dried, and analyzed by phosphorimager or transferred to nitrocellulose for immunoblotting with anti-VSV G carboxyl-terminal antibodies (1:200) and visualized with alkaline phosphatase conjugated secondary antibody + substrate (Bio-Rad, Melville, NY).

Photosensitized Labeling of VSV G. Labeling of VSV G upon interaction with target membranes was performed as described for influenza virus HA (Pak et al., 1994). DiO-labeled erythrocyte ghosts were prepared by incubating packed erythrocytes with DiO (3 μg/mL final concentration) in the presence of Diluent C from the PKH26 fluorescent dye kit (Sigma Chemical Co., St. Louis, MO) as directed in the manufacturer's instructions. After labeling, erythrocyte ghosts were prepared as described above and resuspended in PBS at a 50% hematocrit concentration. ^{125}INA was incorporated into the cell membrane by rapid mixing of 1 μCi of ^{125}INA with 2.5×10^8 RBC ghosts per sample under subdued light. One hundred microliters of 0.38 mg/mL VSV was bound to DiO-^{125}INA-ghosts for 40 min on ice, after which the pH was lowered in samples by the addition of 0.05 M citric acid. At the indicated times, VSV−cell complexes were immediately irradiated with visible light and solubilized with Triton X-100 lysis buffer (1% Triton X-100 in 300 mM NaCl, 50 mM Tris buffer, pH 7.6, containing protease inhibitors). Cell lysate was collected and incubated with anti-VSV G antibodies (1:40) for 1 h. Protein G−agarose was added to mixtures for 30 min, after which samples were washed, boiled, and loaded onto 10% SDS−PAGE and visualized by exposure to Phosphor-Imager screens. Quantitation of autoradiography was done using Molecular Dynamics (Sunnyvale, CA) software. To quantitate binding, VSV was labeled with DiO as described above and bound

to unlabeled lipid symmetric erythrocyte ghosts. Samples were subsequently placed in PBS buffer preadjusted to pH 7.4 or 5.5 at 37 or 4 °C. Some samples were reneutralized following incubation at pH 5.5, 4 °C. All samples were washed 3 times with the appropriate pH buffer, lysed by the addition of Triton X-100, and the associated fluorescence was monitored.

Preparation of Soluble VSV G. Soluble VSV G was prepared by incubating 1.2 mg of VSV with 2 M Tris−maleate and cathepsin D (1.56 mg/mL) at the following ratio: 1.5:0.53:0.45, respectively, for 4 h at 37 °C. This differs from the previously described protocol (Crimmins et al., 1983) which set the incubation pH at 4. These conditions were chosen to prevent acid denaturation of the protein without reducing the enzymatic activity of cathepsin D (unpublished observation). Subsequently the VSV/cathepsin D mixture was subjected to ultracentrifugation at 100000*g* (1 h, 4 °C). The pelleted virus was incubated with 5 mM phosphate buffer, pH 8, to induce release of the cathepsin D treated VSV G. Following a second ultracentrifugation step, the supernatant was harvested. Analysis by SDS−PAGE confirmed that the supernatant consisted almost exclusively of soluble VSV G; ~20 μg of soluble VSV G was incubated with 10^9 DiO-^{125}INA-ghosts and monitored for photosensitized labeling as described above.

RESULTS

DiO−VSV Fusion with Erythrocyte Ghosts. The fusion of VSV with liposomes (Yamada & Ohnishi, 1986; Pal et al., 1988), nucleated cells (Matlin et al., 1982; Blumenthal et al., 1987), and erythrocyte ghosts (Herrmann et al., 1990; Clague et al., 1990) has been described by observing the dequenching of spin-labeled or fluorescent probes. We have modified the fluorescence dequenching assay by using DiO as the fluorophore. VSV was loaded with DiO in quenched concentrations, and then bound to erythrocyte ghosts. Fluorescence dequenching was monitored after addition of bound virus−cell complexes to buffer preadjusted to the desired pH. Figure la shows that VSV fuses with erythrocyte ghosts in a pH-dependent manner, with maximal fusion occurring at pH 6.0 or less. The threshold of membrane fusion appears to be at or near pH 6.2, with no detectable fusion at pH 6.4 or above. The absence of fluorescence dequenching at the higher pH values confirms the specificity of the fusion assay.

It has been reported that influenza virus undergoes low-pH-induced fusion with liposomes and erythrocyte ghosts at 0 °C, albeit after a 4−8 min lag period (Schoch et al., 1992; Stegmann et al., 1991). In order to determine if VSV was also capable of undergoing fusion with erythrocyte ghosts at the lower temperature, virus−cell complexes were incubated at 37 or 4 °C under pH 7.4 or 5.5 conditions. In contrast to influenza virus, VSV displays no ability to fuse at 4 °C, even in the presence of pH 5.5 buffer and after incubating for more than 1 h at the reduced temperature (Figure 1b). Apparently at 4 °C at least one of the steps necessary for VSV fusion does not occur.

Direct UV Labeling of VSV with ^{125}INA. Prior to monitoring VSV G interaction with erythrocyte ghosts, we wished to determine if ^{125}INA could label VSV G, and if so, what portion of the protein was labeled by the probe. The labeling of VSV proteins by direct UV activation of ^{125}INA has been previously described (Zakowski & Wagner, 1996; Mancarella & Lenard, 1981). Those studies demonstrated that direct activation of ^{125}INA by UV irradiation predominantly labeled VSV G, although other viral proteins were also labeled. The protease cathepsin D has been previously used to generate a soluble form of VSV G that leaves the extracellular portion intact (Crimmins et al., 1983). To show that ^{125}INA labeling of VSV G under these conditions was associated with a small hydrophobic fragment that contains the transmembrane domain of the protein, we cleaved VSV G by cathepsin D and identified the carboxyl tail of VSV G by immunoblotting with specific antibodies. VSV containing ^{125}INA was exposed to UV light irradiation to induce direct labeling of proteins, after which virus was treated with cathepsin D in order to generate the soluble and transmembrane fragments. Coomassie blue staining shows cathepsin D treatment of ^{125}INA-labeled VSV is effective in generating a cleaved form of VSV G ("soluble VSV G"), as evidenced by a slightly faster migrating form of the protein (Figure 2a). Immunoblotting with antisera against the ectodomain of VSV G confirms the identity of the faster migrating protein following

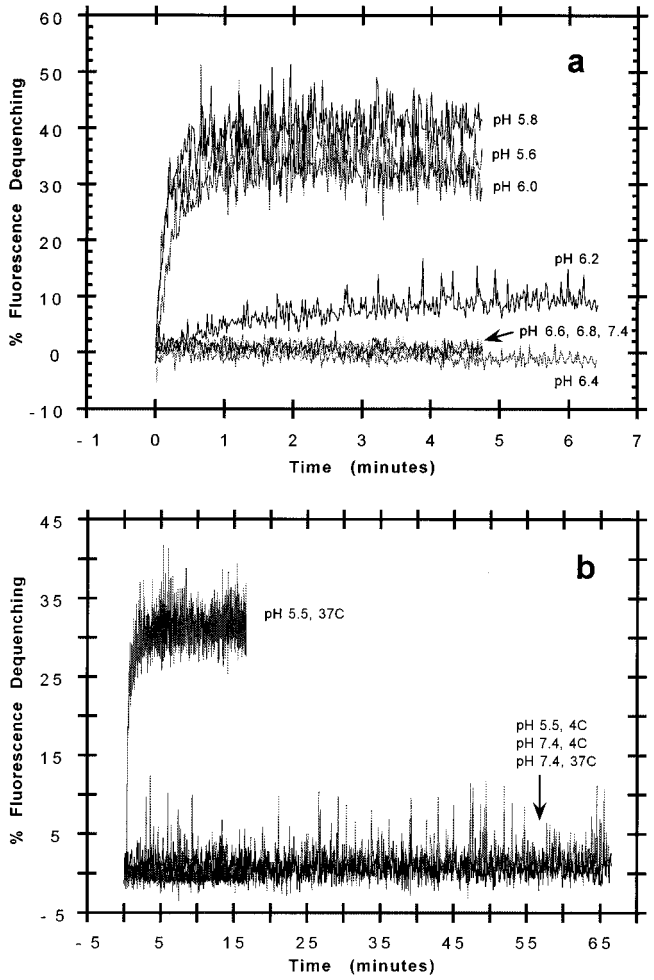

FIGURE 1: Fusion of VSV with erythrocyte ghosts. DiO-labeled viruses were prebound to erythrocyte ghosts on ice for 40 min, washed, and then added to a cuvette containing PBS at the desired pH and temperature. Fluorescence was monitored immediately after addition of the virus−cell mixture. Percent fluorescence dequenching was determined as $100 \times [(F - F_o)/(F_t - F_o)]$, where F_o and F are fluorescence intensities at time zero and at a given time point, respectively, and F_t is the fluorescence after disruption of cells by Triton X- 100. (a) pH dependence of fluorescence dequenching was determined by adding DiO-VSV + erythrocyte ghost complexes to PBS preadjusted to a given pH. (b) Effect of temperature on fusion was performed by adding DiO-VSV + erythrocyte ghosts to pH 7.4 or 5.5 PBS preadjusted to 37 or 4 °C.

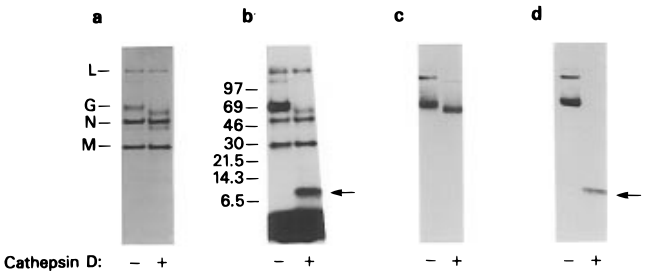

FIGURE 2: Direct UV labeling of VSV with ^{125}INA and cathepsin D digestion. VSV containing ^{125}INA was exposed to UV light to induce photoactivation of ^{125}INA. VSV was subsequently treated with or without cathepsin D for 4 h. Samples were loaded onto 10−20% Tricine SDS−PAGE. Gels were stained with (a) Coomassie blue and analyzed by (b) autoradiography. Identical samples were transferred onto nitrocellulose and probed with (c) antisera against VSV G or (d) antibodies recognizing the carboxyl terminus of VSV G. Arrows signify the position of the transmembrane ~7 kDa fragment. Molecular mass markers (kDa) are shown to the left of panel b. L, polymerase; G, glycoprotein; N, nucleocapsid; M, matrix protein.

cathepsin D digestion as truncated VSV G (Figure 2c). Autoradiography shows labeling of VSV proteins as previously reported (Zakowski & Wagner, 1996; Mancarella & Lenard, 1981), with the ^{125}INA label primarily associated with VSV G. Cathepsin D treated VSV demonstrates a ~7 kDa labeled fragment (Figure 2b, arrow) that coincides with the disappearance of labeled VSV G. Although the soluble VSV G is labeled to some extent, quantitative analysis showed that the majority of the label is associated with the ~7 kDa fragment. Antibodies specific for the carboxyl tail of G protein (Arnheiter et al., 1984) identify this fragment as the transmembrane domain (Figure 2d, arrow). It appears that ^{125}INA is capable of labeling VSV G via the transmembrane domain with only minimal labeling of the ectodomain region.

Photosensitized Labeling of VSV G by DiO-^{125}INA-Ghosts. We have previously studied the interaction of influenza virus HA with erythrocyte ghosts by ^{125}INA photosensitized labeling (Pak et al., 1994). The same methodology was applied to study the interaction of VSV G with erythrocyte ghosts. Photosensitized activation was compared to direct UV activation for the ability to selectively label VSV G. ^{125}INA photosensitized activation by visible light irradiation resulted in VSV G labeling only after incubation with erythrocyte ghosts under fusogenic conditions (pH <6.4 , 37 °C) (Figure 3). VSV G labeling displays a sharp threshold of pH dependence between pH 6.2 and 6.4. At pH 6.2 or less, nearly maximal labeling was observed, whereas VSV incubated at pH 6.4 or higher did not yield significant VSV G labeling. This indicates that VSV G is not able to insert into the target membrane at pHs >6.4. The lack of VSV G labeling when virus−cell mixtures were incubated at pH > 6.4 demonstrates the specificity of photosensitized labeling. In contrast, direct UV irradiation induced ^{125}INA to label VSV G at both pH 5.5 and pH 7.4 (data not shown). This nonspecific labeling of VSV G, due to the transfer of ^{125}INA to unfused virions, demonstrates the necessity of photosensitized activation for selective labeling of VSV G.

Since there was no membrane fusion at low pH, 4 °C (Figure 1b), we were interested to determine if the block in VSV G labeling was at the level of VSV G interaction with the erythrocyte membrane bilayer. Incubation of VSV with DiO-^{125}INA-ghosts at 4 °C, pH 5.6, results in significant labeling of VSV G (Figure 4, lane 2), although not to the

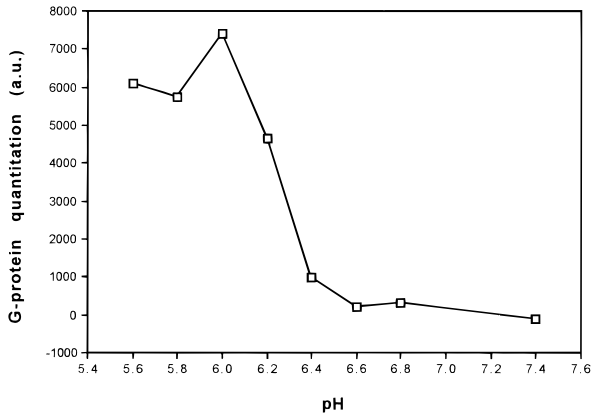

FIGURE 3: pH dependence of photosensitized labeling VSV G by DiO-^{125}INA-ghosts. VSV prebound to DiO-^{125}INA-ghosts were incubated at the given pH, 37 °C, for 15 min prior to irradiation with visible light. The intensity of the VSV G band was quantified by volume integration using Molecular Dynamics software.

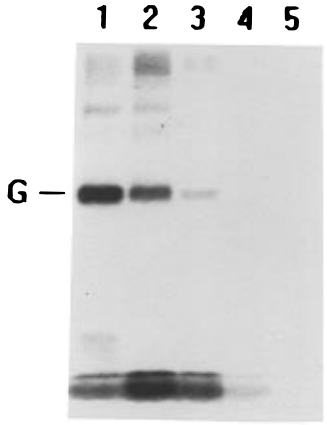

FIGURE 4: Photosensitized labeling of VSV G at 4 °C and reversibility of ^{125}INA labeling. Prebound VSV+DiO-^{125}INA-ghost complexes were incubated at 37 or 4 °C, pH 5.5, for 15 min and irradiated with visible light. For reversibility experiments, samples were incubated at pH 5.5, 4 °C, for 15 min, reneutralized, and immediately irradiated. Lanes: 1, pH 5.5, 37 °C; 2, pH 5.5, 4 °C; 3, pH 5.5, 4 °C (+reneutralization); 4, pH 7.4, 37 °C; 5, without DiO. G, VSV G.

levels observed when the incubation was at 37 °C (Figure 4, lane 1).

VSV differs from influenza virus in that the activation of fusion is reversible (Puri et al., 1988; Blumenthal et al., 1987). However, it is not known if insertion of VSV G is also a reversible event. Therefore, VSV−DiO-^{125}INA-ghost complexes were incubated at pH 5.5, 4 °C, after which samples were reneutralized and incubated at pH 7.4, 37 °C. The interaction of VSV G with erythrocyte membranes is a reversible event at 4 °C, since reneutralization of the low-pH-treated sample reduced the level of VSV G labeling to background levels (Figure 4, lane 3). The reversibility occurred within 8 min, the minimum time necessary to photoactivate the ^{125}INA for labeling.

Interaction of Soluble VSV G with Erythrocyte Ghosts. The labeling of VSV G under nonfusogenic conditions such as pH 5.5, 4 °C suggests we are observing the insertion of the fusion peptide sequence into cellular membranes prior to membrane fusion. However, it is possible that this labeling may be due to nonspecific labeling of VSV G via the transmembrane domain. To determine if this was the case, soluble VSV G was generated as described by Crimmins et al. (1983). Generation of soluble VSV G was

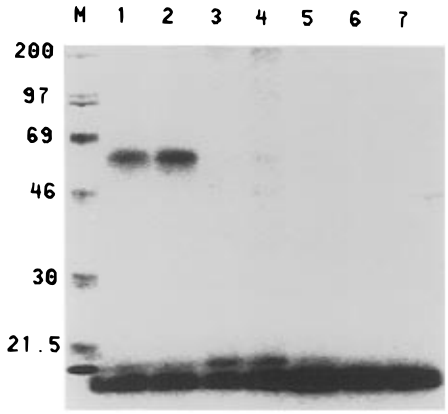

FIGURE 5: Photosensitized labeling of soluble VSV G by DiO-^{125}INA-ghosts. Soluble VSV G was prepared by cathepsin D digestion of VSV as described under Experimental Procedures. ~20 μg of soluble VSV G was incubated with 10^9 DiO-^{125}INA-ghosts per sample for 40 min, on ice, and then treated as follows for 10 min. Lanes: 1, pH 5.5, 37 °C; 2, pH 5.5, 4 °C; 3, pH 7.4, 37 °C; 4, pH 5.5, 4 °C (+reneutralization at 37 °C); 5, pH 5.5, 4 °C (+reneutralization at 4 °C); 6, without DiO; 7, without visible light irradiation. Molecular weight standards are shown in the lane marked M.

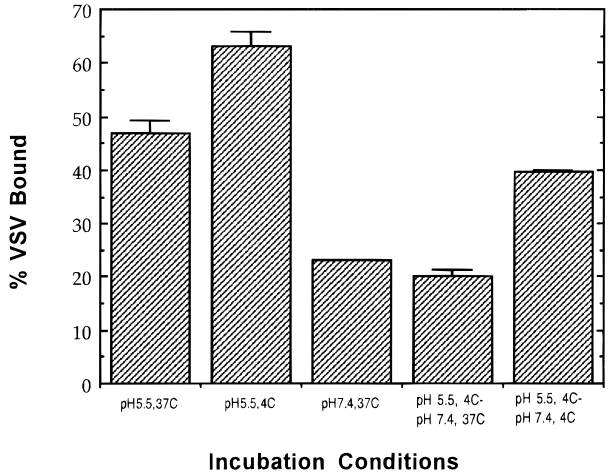

FIGURE 6: Binding of DiO-containing VSV to erythrocyte ghosts. VSV was labeled with DiO, washed, and incubated with erythrocyte ghosts as described under Experimental Procedures. Samples were washed at pH 5.5 or 7.4, 4 °C, following the incubation and lysed, and the associated fluorescence was monitored.

confirmed by SDS−PAGE (Figure 2a). Soluble VSV G bound to DiO-^{125}INA-ghosts was labeled following exposure to low pH, regardless of the temperature (Figure 5, lanes 1 and 2). However, samples that were either maintained at neutral pH (Figure 5, lane 3) or reneutralized prior to irradiation (Figure 5, lanes 4 and 5) displayed no labeling of the protein, demonstrating the labeling of soluble VSV G required a low-pH-induced interaction. Since soluble VSV G lacks the transmembrane domain, the observed labeling is attributed to insertion of the fusion peptide region (Durrer et al., 1995) into the erythrocyte ghosts and not as a result of nonspecific labeling of the transmembrane domain.

The correlation of VSV G labeling with pH 5.5 incubation raises the possibility that the labeling is due merely to the enhanced binding observed for VSV at acidic pH (Yamada & Ohnishi, 1986). Therefore, we compared VSV binding with VSV G photolabeling under identical conditions to determine if a correlation could be established. Figure 6 shows that incubation of VSV−erythrocyte ghost complexes at acidic pH does result in enhanced virus binding, correlating with the appearance of VSV G labeling (Figure 4). However, reneutralization of samples at 4 °C resulted in loss of VSV G labeling (Figure 4) despite significant VSV binding to cells (Figure 6). Furthermore, the labeling of intact VSV G was greater after 37 °C treatment at pH 5.5 than 4 °C (Figure 4, lanes 1, 2) even though more VSV was bound to cells under the latter condition. These results indicate that VSV G labeling in the fusogenic state is not due to enhanced binding.

DISCUSSION

Insertion of VSV G into Biological Target Membranes. Fusion of VSV with that of the host cell is mediated by VSV G and triggered by the low-pH environment within the endosome. Kinetic fusion studies of fluorescently labeled virus have been performed to dissect the events which occur following the low-pH triggering of HA (Puri et al., 1992; Clague et al., 1990). These studies have revealed a number of intermediates in the fusion cascade before the final coalescence of membranes and aqueous spaces. A crucial step following the conformational change is insertion of some portion of the envelope glycoprotein into the target membrane, which has been detected by labeling with hydrophobic photoactivatable probes. This approach was effective in the analysis of the interaction of hemagglutinin (HA) with liposomes (Durrer et al., 1996; Tsurudome et al., 1992; Stegmann et al., 1991; Harter et al., 1989; Brunner et al., 1991).

We have developed a novel approach to characterize viral envelope protein insertion as a result of fusion (Pak et al., 1994). It is based on a technique called "photosensitized labeling" which has been previously developed to study recognition phenomena in membrane and cell biology (Raviv et al., 1989). By this methodology, lipophilic aryl azides are photoactivated in situ by energy transfer from a variety of chromophores which are excited by visible light. Using this approach, proteins and lipids involved in multidrug resistance of tumor cells were identified (Raviv et al., 1990). This technique appears to be well-suited for the detection of VSV G insertion into biological membranes because of the confinement of the activating photosensitizer, DiO, to the target membrane. Our results are in complete agreement with those of Durrer et al. (1995), who used 1-*O*-hexadecanoyl-2-*O*-[9-[[[2-[^{125}I]iodo-4-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyl]oxy]carbonyl]nonanoyl-*sn*-glycero-3-phosphocholine incorporated into liposomes, which served as target membranes for Rabies virus and VSV fusion. We detected photosensitized labeling of VSV G only upon lowering the pH below 6.4 (Figure 3), whereas UV activation produced labeled VSV G at both acidic and neutral pH. The VSV G labeling by DiO-^{125}INA-ghosts observed at pH 5.5, 4 °C (Figure 4) in the absence of mixing between viral and cellular lipids (Figure lb) strongly suggests insertion of the ectodomain of VSV G. We confirmed this observation by showing that soluble VSV G inserts into DiO-^{125}INA-ghosts under the same conditions as intact VSV G (Figure 5). We had performed similar studies with the soluble form of influenza HA (Pak et al., 1994). The absence of the transmembrane domain ensures that any labeling of this protein must be due to interaction of the VSV G ectodomain with the erythrocyte membrane. Removal of the transmembrane domain is confirmed by immunoblotting with an antibody specific for a peptide within the carboxyl tail region (Figure 2d). The equivalent amount of soluble VSV G labeling after pH 5.5, 37 °C and 4 °C incubation with DiO-^{125}INA-ghosts (Figure 5) is expected since the only interaction that is possible for

this protein with erythrocyte membranes is mediated via the ectodomain. In contrast, labeling of VSV G in the intact virus results in different levels of incorporation of ^{125}INA whether the virus was incubated with DiO-^{125}INA-ghosts at 37 °C or at 4 °C, pH 5.5 (Figure 4), because of the availability of the transmembrane region of VSV G following membrane fusion at the higher temperature whereas at 4 °C only the ectodomain of inserted viral fusion proteins is available for labeling.

A New Lipid Probe To Monitor Membrane Fusion. The use of DiO for photosensitized labeling fortuitously led us to a new fluorophore suitable for continuous kinetic measurements of membrane fusion. Octadecylrhodamine has been used previously to monitor fusion of VSV with Vero cells (Puri et al., 1988; Blumenthal et al., 1987). Unfortunately, this dye induces aggregation of VSV (Puri et al., 1988), perhaps due to its positive charge. In addition, a recent study raised several reservations about the use of octadecylrhodamine to rigorously monitor membrane fusion (Stegmann et al., 1993). We found that VSV could be labeled with DiO in quenched concentrations without any effect on aggregation (results not shown) or on its fusogenic potential (Figure 1). There was no spontaneous transfer of DiO from virus to cell under pH 7.4, 37 °C or pH 5.5, 4 °C conditions (Figure 1), indicating the fluorophore remains associated with the virus under nonfusogenic conditions. Furthermore, the fluorescence dequenching observed at pH 5.5, 37 °C could be abrogated by the addition of anti-VSV G antibodies or when heat-treated virus was used (results not shown), indicating the fluorescence increase is VSV G dependent and is not due to nonspecific transfer of DiO from VSV to cells. The kinetics of DiO−VSV fusion with erythrocyte ghosts (Figure l) were comparable to those previously observed using octadecylrhodamine (Herrmann et al., 1990; Clague et al., 1990). Therefore, it appears DiO can be successfully used to measure membrane fusion.

The Three-State Model for VSV G. Based on our kinetic data, we had formulated model which relates presumed conformational transitions of the VSV G protein to mechanisms of viral fusion (Puri et al., 1992; Clague et al., 1990). According to the model (Scheme 1), VSV G undergoes a proton-driven shift (Blumenthal, 1988) from a "T" (tense) state at neutral pH either to the "R" (relaxed) state, which is fusion-active, or to the "D" (desensitized) state, which is fusion-inactive (Clague et al., 1990). Our analysis of the kinetic data indicates that R and D represent a series of states (Clague et al., l990), but for simplicity we will designate them as one state each. We had hypothesized that the conformational changes result in movement of the fusion active region of the protein either into the target membrane (R state) or without inserting into the membrane (D state) (Puri et al., 1992). The three-state model differs from that of influenza HA where the fusogenic state occurs *en route* to inactivation (Blumenthal et al., 1994; Ludwig et al., 1995; Ramalho-Santos et al., 1993), and all processes are irreversible.

Scheme 1

$$D \rightleftharpoons T \rightleftharpoons R \rightharpoonup F$$

The photosensitized labeling allows us to examine the biochemical correlates of the states we had hypothesized based on kinetic data: the F state represents fusion and is accessible by a variety of biophysical techniques based on fluorescence dequenching (Puri et al., 1988, 1992, 1993; Herrmann et al., 1990; Clague et al., 1990; Blumenthal et al., 1987), or electron spin resonance using spin-labeled phospholipid probes (Yamada & Ohnishi, 1986). We find that the F state is reached at pH < 6.2 (Figure l) and temperatures >10 °C (Puri et al., 1988). At pH 5.5 and 4 °C, there is no fusion (Figure lb), but the VSV G is not desensitized under these conditions (Puri et al., 1988). In fact, preexposure of the virus at pH 5.5 and 4 °C leads to activation of fusion activity. We therefore conclude that these conditions drive VSV G into the R state. In this paper, we show that the extracytoplasmic domain of VSV G is labeled in the R state (Figure 5), in agreement with Durrer et al. (1995). We not only found labeling under these conditions but also made the new observation that the insertion is reversible (Figures 4 and 5). This is quite different from results with influenza HA where low pH and 4 °C lead to a state committed to fusion at neutral pH which cannot be reversed even by treatment with trypsin or DTT, which removes the major portion of the envelope glycoprotein (Schoch et al., 1992). The reversibility in the case of VSV G suggests a "velcro"-like attachment with the target membrane. Studies with peptides representing the NH_2 terminus of influenza HA2 (Rafalski et al., 1996; Wharton et al., 1988) and of Sendai F_1 (Rapaport & Shai, 1996) indicate a strong interaction with the lipid bilayer, leading to insertion, membrane destabilization, and vesicle fusion. Based on our reversibility data, we do not expect peptide analogues from the fusogenic region of VSV G or Rabies G to interact so forcefully with lipid bilayers.

In our analysis of the rapid kinetics of VSV membrane fusion (Clague et al., 1990), we had proposed a D state to account for the fact that the extents of fusion are pH-dependent. We confirmed the existence of the D state by showing that fusion at optimal pH was decreased by preexposure of VSV−cell complexes to pH 6.6 and 37 °C. The D state could be reversed by returning to pH 7.4 (Clague et al., 1990). When we treated VSV with neuraminidase, we found an increase in the extent which was due to an inability to go into the D state rather than speeding up the transition to the R state (Puri et al., 1992). The photolabeling data in this study (Figure 3) and those by Durrer et al. (1995) confirm our hypothesis that VSV G in the D state (pH 6.6, 37 °C) fails to insert into target membranes (Puri et al., 1992). This is the case in spite of the fact that in the D state VSV G becomes more hydrophobic as determined by liposome binding (Yamada & Ohnishi, 1986). There was no photosensitized labeling of neuraminidase-treated VSV after incubation at pH 6.6 and 37 °C (data not shown), consistent with the notion that the D state becomes unavailable for VSV G after such treatment (Puri et al., 1992). Moreover, there was no increased photosensitized labeling in the R state (pH 5.5 and 37 °C) of neuraminidase-treated VSV G (data not shown), consistent with the notion that the enhanced fusion activity of VSV is due to lack of desensitization of neuraminidase-treated virus and not to enhancement of the fusion reaction (Puri et al., 1992).

The three states have also been characterized in the Rabies virus G protein by a variety of biochemical, biophysical, immunological, and morphological criteria and denoted as neutral, activated, and inactive (Gaudin et al., 1993, 1996). Since the shift to the fusion-inactive state is reversible, we prefer to call it desensitized in accordance with receptorology (Blumenthal, 1988). As with VSV G, Gaudin et al. (1993)

found reduced fusion activity by shifting to the D state, and enhanced fusion activity by shifting to the R state. In the D state, the spikes of Rabies G became 32 Å longer than in the T state and were no longer recognized by Mabs directed against a particular antigenic site (Gaudin et al., l 993). Moreover, as in VSV G, there was increased hydrophobicity as determined by ANS fluorescence. As with VSV G, there was photolabeling of the extracytoplasmic domain in the R state but not in the D state (Durrer et al., 1995). Kinetic analysis of the rate of desensitization for different strains of rabies virus suggests that the structural transition toward the D state is irrelevant to the fusion process but plays a role in avoiding unspecific fusion during transport of G in the acidic Golgi vesicles (Gaudin et al., 1995). The use of Mabs specific for the D state led to the isolation of mutants which became resistant to acid-induced desensitization (Gaudin et al., 1996), in the same way that neuraminidase-treated VSV G became resistant to desensitization (Puri et al., 1992). Using these mutants, Gaudin et al. (1996) demonstrated a hexagonal lattice of glycoproteins at the viral surface and the importance of lateral interactions between spikes of the rhabdovirus.

Using photosensitized labeling, we confirmed our working hypothesis based on kinetic studies of VSV G-mediated fusion which was in remarkable agreement with Rabies G data gathered by Gaudin and co-workers (Gaudin et al., 1993, 1995, 1996). The photosensitized labeling technique is very accessible and could easily be applied to VSV G mutants. Moreover, it can be used for viral envelope glycoproteins which have an absolute requirement for cell surface receptors to induce fusion.

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