JOURNAL OF VIROLOGY, Nov. 1971, p. 722-729
Vol. 8, No. 5

Proteins of Vesicular Stomatitis Virus and of Phenotypically Mixed Vesicular Stomatitis Virus-Simian Virus 5 Virions[1]

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Received for publication 11 August 1971

The identity of the glycoprotein of vesicular stomatitis virus (VSV) as the spike protein has been confirmed by the removal of the spikes with a protease from *Streptomyces griseus*, leaving bullet-shaped particles bounded by a smooth membrane. This treatment removes the glycoprotein but does not affect the other virion proteins, apparently because they are protected from the enzyme by the lipids in the viral membrane. The proteins of phenotypically mixed, bullet-shaped virions produced by cells mixedly infected with VSV and the parainfluenza virus simian virus 5 (SV5) have been analyzed by polyacrylamide gel electrophoresis. These virions contain all the VSV proteins plus the two SV5 spike proteins, both of which are glycoproteins. The finding of the SV5 spike glycoproteins on virions with the typical morphology of VSV indicates that there is not a stringent requirement that only the VSV glycoprotein can be used to form the bullet-shaped virion. On the other hand, the SV5 nucleocapsid protein and the major non-spike protein of the SV5 envelope were not detected in the phenotypically mixed virions, and this suggests that a specific interaction between the VSV nucleocapsid and regions of the cell membrane which contain the nonglycosylated VSV envelope protein is necessary for assembly of the bullet-shaped virion.

Vesicular stomatitis virus (VSV) is a bullet-shaped ribonucleic acid virus consisting of a helical nucleoprotein surrounded by a spike-covered lipoprotein envelope. Analysis of the proteins of VSV have revealed the presence of three to five polypeptides in the virion (2, 4, 14, 26, 28). The three major structural proteins have been identified as the nucleocapsid protein and two envelope proteins, the larger of which is a glycoprotein (2, 15, 23, 29, 30). Cartwright and co-workers (4) reported that the protein which migrated behind the nucleocapsid was associated with the spikes although they did not identify this protein as a glycoprotein. In this communication, we provide further evidence that the glycoprotein of the virion is associated with the spikes. These results are consistent with the findings that the spike proteins of influenza, parainfluenza, arboviruses, and Rous sarcoma virus are glycoproteins (5, 10, 12, 13; D. B. Rifkin and R. W. Compans, Virology, *in press*).

Recently Choppin and Compans (8) demonstrated that cells mixedly infected with the parainfluenza virus simian virus 5 (SV5) and VSV yield a population of virions which includes not only SV5 virions, which are pleomorphic but predominantly roughly spherical in shape, and bullet-shaped VSV virions, but also phenotypically mixed virions with surface properties of both parents. Of the total yield of virus from these cells, 10 to 45% was composed of hybrid particles. Most of these hybrid virions were bullet-shaped and contained VSV genomes but had the ability to adsorb to and elute from chicken red blood cells. A small number (< 2%) of these were neutralized by only SV5 antiserum, but most were neutralized by both SV5 and VSV antisera. The presence of SV5 antigens on the surface of bullet-shaped virions was demonstrated with ferritin-labeled antibody (8). These phenotypically mixed virions thus exhibited properties of both viruses in a particle with the morphology of VSV. We report here the isolation of phenotypically mixed, bullet-shaped virions and the analysis of their protein composition. Our results indicate that these hybrid particles contain the usual complement of VSV polypeptides plus the two spike proteins of SV5.

[1] Presented at the 71st Annual Meeting of the American Society for Microbiology, Minneapolis, Minn., 2-7 May 1971.

MATERIALS AND METHODS

Cells. All cell cultures were propagated as previously described (6, 7). Monolayers of the BHK-21-F line of Syrian hamster kidney cells were grown in 32-oz (ca. 960 ml) glass prescription bottles or 60-mm plastic petri dishes (Falcon Plastics, Los Angeles) in reinforced Eagle's medium (REM; reference 1) with 10% calf serum and 10% Tryptose phosphate broth. Monolayers of the MDBK line of bovine kidney cells were grown in plastic bottles in REM (1) with 10% fetal calf serum.

Viruses. The W3 strain of SV5 and the Indiana serotype of VSV were plaque-purified, propagated in MDBK cells, and assayed on BHK-21-F cells as described previously (6–8).

Growth and purification of virus. The procedures for growth and purification of VSV and SV5 have been previously described in detail (3, 16, 22). MDBK cells were infected with VSV or SV5 at a multiplicity of ~ 10 plaque-forming units (PFU) per cell. After a 2-hr adsorption period at 37 C, 10 ml of REM with 2% calf serum was added. VSV cells were harvested ~ 18 hr after infection and SV5 at ~ 40 hr. Released virus was harvested from the infected medium, clarified by centrifugation at 2,000 × *g* for 30 min, and then precipitated with 7% polyethylene glycol 6000 (PEG) and 2.3% NaCl at 4 C for 10 to 16 hr (21). The precipitated virus was pelleted at 1,000 × *g* for 20 min, resuspended in Eagle's medium containing 1% bovine serum albumin (BSA), and further purified by isopycnic centrifugation in a linear 15 to 40% (w/w) potassium tartrate gradient at 90,000 × *g* for 2.5 hr in a Beckman SW27 rotor. Usually a single band formed with each virus; occasionally two bands formed in the VSV preparation, in which case only the upper band was collected (17). The virus band was collected and dialyzed overnight at 4 C against water, REM, or 0.1 M tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 7.2) depending on the analysis to be performed.

Propagation of phenotypically mixed SV5-VSV particles. Phenotypically mixed SV5-VSV virions were propagated as described previously (8). MDBK cell monolayers were infected with SV5 at 10 PFU per cell, and 18 to 24 hr later they were superinfected with VSV at the same multiplicity. After an additional 18 to 20 hr at 37 C, released virus was harvested and purified as described above.

Chemicals and isotopes. Chemicals and isotopes were obtained as follows: components for polyacrylamide gels, Canal Industrial Corp., Rockville, Md.; ^{3}H- and ^{14}C-reconstituted protein hydrolysates (Schwarz mixture), Schwarz BioResearch, Orangeburg, N.Y.; D-glucosamine-*6*-^{3}H-hydrochloride (specific activity 3.6 Ci/mole), New England Nuclear Corp., Boston, Mass.; *Streptomyces griseus* protease type VI, bromelain, chymotrypsin, trizma-HCl, and trizma-base, Sigma Chemical Co., St. Louis, Mo.; twice crystallized trypsin, Worthington Biochemical Corp., Freehold, N.J.; crystalline BSA Armour Pharmaceutical Co., Chicago, Ill.; PEG 6000, Amend Drug and Chemical Co., New York, N.Y., and Triton X-100, Beckman Instruments, Inc., Fullerton, Calif.

Radioactive labeling of VSV and SV5. Confluent monolayers were inoculated as described above, and after the adsorption period 10 ml of the appropriate medium containing the isotope was added. For amino acid labeling, the medium consisted of three parts REM without amino acids and one part complete REM, 2% calf serum, and either 5 μCi of ^{3}H-amino acid per ml or 1 μCi of ^{14}C-amino acid-labeled protein hydrolysate per ml. For labeling of carbohydrate, the medium consisted of complete REM, 2% calf serum, and 5 μCi of ^{3}H-glucosamine per ml. Virus was propagated at 37 C, harvested, and purified as described above.

Polyacrylamide gel electrophoresis. Viral proteins were dissociated in the presence of 1% sodium dodecyl sulfate (SDS) and 1% 2-mercaptoethanol (2-ME) by heating at 100 C for 1 min (24). Acrylamide gels (7.5%), approximately 10 cm long with a 0.5-cm stacking gel of 2.5% acrylamide, were used. In coelectrophoresis experiments, samples were mixed before treatment with SDS and 2-ME. Procedures employed in the preparation of polyacrylamide gels, electrophoresis, and staining with Coomassie brilliant blue (3), and the processing of gels for determination of radioactivity (25) were described previously.

Enzyme treatment. After purification, virus was dialyzed against 0.1 M Tris buffer (*p*H 7.2), and to nine parts of virus was added one part of a solution of *S. griseus* protease type VI (1 mg/ml) in 0.1 M Tris buffer. The virus-enzyme mixture was incubated at 37 C for 1 to 2 hr and monitored by electron microscopy to determine when the spikes had been digested. Control virus preparations were incubated at 37 C without the enzyme solution. When the digestion of spikes was judged to be essentially complete, the reaction was stopped by repurification of the enzyme-treated and control virus preparations in potassium-tartrate gradients followed by collection and dialysis of the virus as described above.

Electron microscopy. A drop of virus suspension was placed on a grid with a carbon-coated Formvar film, stained with 2% phosphotungstate (*p*H 6.2), and examined in a Philips EM300 microscope.

Protein determinations. Protein was estimated by the method of Lowry et al. (19) with BSA as a standard.

RESULTS

Spike protein of VSV. To determine which of the two VSV envelope proteins is associated with the spikes on the virion, various proteolytic enzymes were tested for their ability to remove selectively one of the two surface proteins without affecting the other structural proteins of the virion. *S. griseus* protease type VI (Pronase) was found to be more satisfactory than trypsin, chymotrypsin, or bromelain. VSV, doubly labeled with ^{3}H-glucosamine and ^{14}C-amino acids, was prepared; one portion was treated with enzyme as described above, and the remainder served as control. After incubation at 37 C, both the control and treated samples were rebanded in potassium tartrate and dialyzed against distilled water, and the virion polypeptides were separated by SDS-

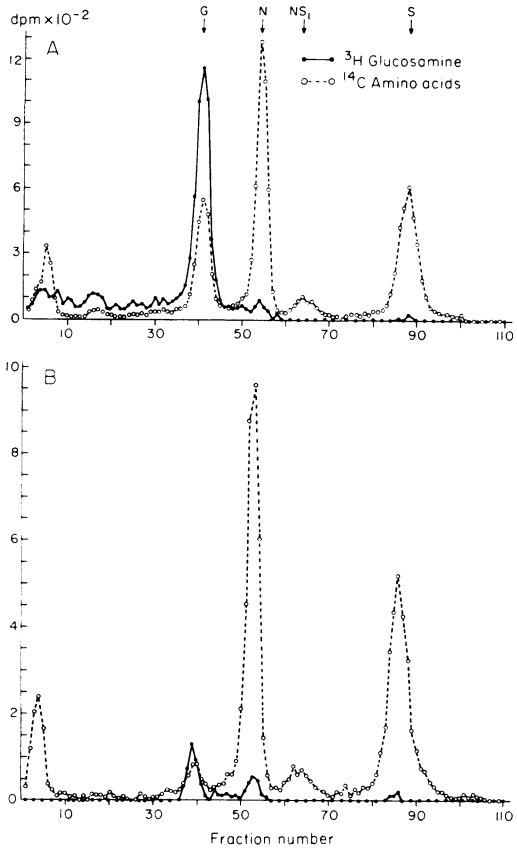

FIG. 1. *Removal of the VSV glycoprotein by protease treatment. Virions were labeled with ³H-glucosamine and ¹⁴C-amino acid mixture and treated with protease as described in the text. The electropherogram of a control virus preparation is shown in A, and protease-treated virus, in B. G, glycoprotein; N, nucleocapsid protein; NS1, protein of unknown function; and S, nonglycosylated envelope protein. In this and subsequent figures electrophoresis was on 7.5% gels at 3 v/cm for 14 to 16 hr. The origin is on the left.*

polyacrylamide gel electrophoresis. Figure 1A shows the electropherogram of the control virus preparation. Five ¹⁴C-amino acid-labeled peaks are present. One of these, the glycoprotein, is also labeled with ³H-glucosamine. Using the nomenclature of Wagner et al. (30), four of these VSV proteins are identified as follows: G, the glycoprotein; N, the nucleocapsid protein; NS1, a protein which we consistently find and which has been reported to be a nonstructural protein (30); and S, the nonglycosylated surface protein. The exact nature of the peak near the origin of the gel is uncertain, but it may be an aggregate or a large viral protein (29). The electropherogram of the enzyme-treated VSV is shown in Fig. 1B. The G protein peak has been almost completely removed, but no changes in the other proteins, including NS1, are seen.

To confirm that only the G protein of VSV is affected by the enzyme treatment, ³H-amino acid-labeled virus was treated with the protease and then mixed with ¹⁴C-amino acid-labeled control virus and subjected to coelectrophoresis on polyacrylamide gels. Figure 2 shows that only the G protein was affected by the enzyme treatment. The relative amounts and the electrophoretic mobilities of proteins N, NS1, and S are similar in the treated and untreated virus. Although the results in Fig. 2 are compatible with a small amount of NS1 being removed by the enzyme, the significance of this is doubtful due to the low level of radioactivity. Further, there was no loss of NS1 in other experiments (Fig. 1). The small amount of glycoprotein remaining on the enzyme-treated virus comigrates with the glycoprotein of the marker virus; thus, this represents a small portion of the G protein molecules which have been unaltered by the enzyme and is not a fragment of each of these molecules.

These results indicate that the protease has selectively removed the G protein from the virions without affecting the other viral proteins, presumably because the other proteins were protected from the action of the enzyme by the lipid in the viral envelope. That this was the case was indicated by the finding that, if the virions labeled with radioactive amino acids were treated first with 1% Triton X-100 and then with protease under the same conditions used above, 100% of the radioactivity was rendered acid-soluble. In addition, the protease-treated material was subjected to polyacrylamide gel electrophoresis, and

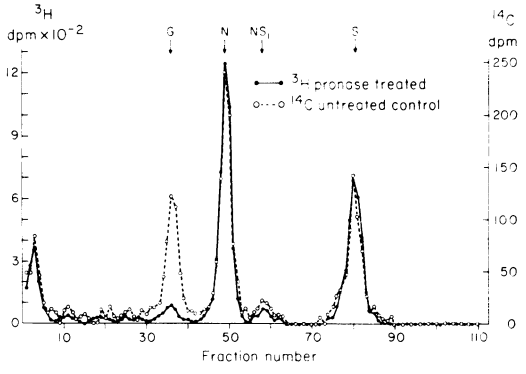

FIG. 2. *Lack of effect of protease on the nonglycosylated proteins of VSV. ³H-amino acid-labeled VSV was treated with protease, mixed with ¹⁴C-amino acid-labeled, untreated VSV, and analyzed by coelectrophoresis on a polyacrylamide gel.*

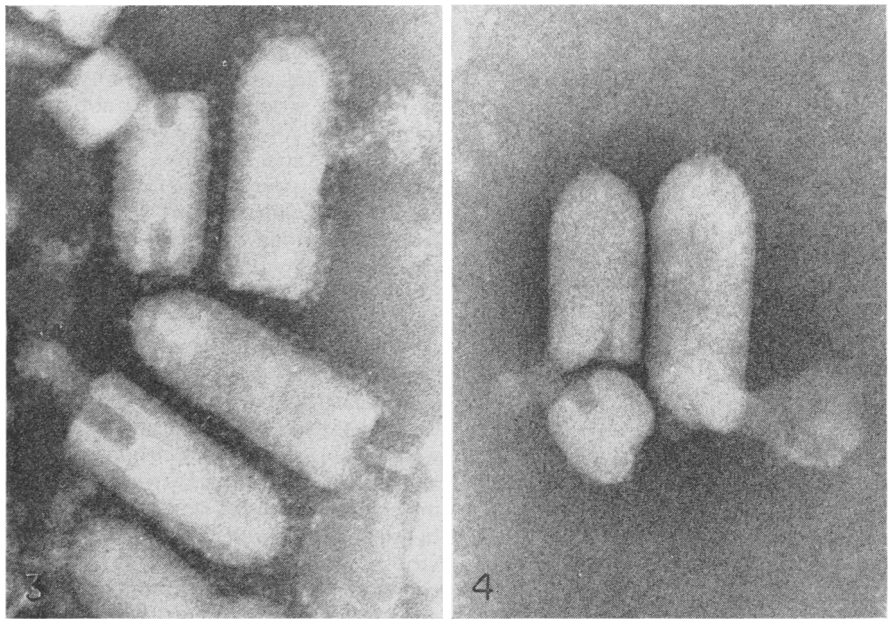

FIG. 3. *Untreated VSV negatively stained with 2% sodium phosphotungstate, pH 6.8. The spikes are clearly visible on the surface of the virions.* ×*190,000.*

FIG. 4. *Protease-treated VSV negatively stained with 2% sodium phosphotungstate, pH 6.8. The spikes have been removed, leaving smooth-surfaced, bullet-shaped particles.* ×*190,000.*

there was no evidence of any remaining protein in the position of any of the virion proteins. Thus, all of the proteins were susceptible to digestion after the virion was disrupted by the detergent.

Figure 3 shows an electron micrograph of untreated virions which have the characteristic bullet shape and are covered with spikes about 10 nm in length. Figure 4 shows that the enzyme-treated virions are still bullet-shaped, but are smooth-surfaced, having lost their spikes. The protease-treated, spikeless particles are thus sufficiently stable to withstand rebanding in potassium tartrate gradients without disruption. However, concomitant with the loss of spikes, there was a four- to five-log drop in infectivity of the enzyme-treated virus, e.g., from 3.5×10^9 to 7.5×10^4 PFU/ml.

The above results clearly demonstrate that the VSV glycoprotein is the spike protein of the virion, and that the envelope protein S is not affected by the enzyme. Protein S thus appears to be present in the viral envelope in such a location that it is protected from proteolytic action by the lipid in the membrane.

Isolation and analysis of bullet-shaped, phenotypically mixed VSV-SV5 particles. To determine which proteins were present in the phenotypically mixed virions which are produced by cells mixedly infected with SV5 and VSV, and which have surface properties of both parents (8), it was necessary to separate SV5 and VSV virions. VSV has a density of 1.16 to 1.18 g/ml (20), and the density of SV5 is 1.23 g/ml (16); it was found that the two types of virions could be separated by isopycnic centrifugation in potassium tartrate. ^{3}H-amino acid-labeled VSV and ^{14}C-amino acid-labeled SV5 virions were mixed, co-precipitated with polyethylene glycol, and then separated by centrifugation at 90,000 × *g* for 2.5 hr in a linear 20 to 30% (w/w) potassium tartrate gradient. Equal fractions were collected from the bottom and assayed for radioactivity, hemagglutinating activity, and infectivity (Fig. 5). VSV virions banded at a density which was gravimetrically determined to be 1.16, and electron microscopy of this band revealed only typical, bullet-shaped virions. There was a very small amount of ^{3}H radioactivity at a density of 1.23. All of the ^{14}C-labeled SV5 virions were found in a single band at a density of 1.23; there was no evidence of SV5 contaminating the VSV band. Furthermore, the hemagglutinating activity coincided with the SV5 radioactivity, and plaque assays of the infectivity in the two bands in the presence and absence of VSV antiserum as described by Choppin and Compans (8) also showed that there was less than 0.1% contamination of the VSV band by SV5. These results indicate that the

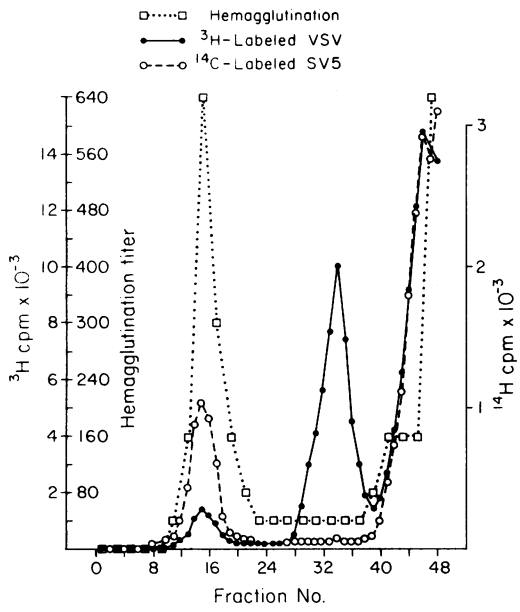

FIG. 5. *Separation of SV5 and VSV by isopycnic centrifugation in a potassium tartrate gradient. ^{3}H-amino acid-labeled VSV and ^{14}C-amino acid-labeled SV5 were mixed, precipitated with polyethylene glycol, and centrifuged to equilibrium in a 20 to 30% potassium tartrate gradient. Fractions were analyzed for radioactivity and hemagglutinating activity.*

bullet-shaped VSV virions can be efficiently purified without a significant amount of contamination by SV5 virions. It thus became feasible to separate the bullet-shaped, phenotypically mixed VSV-SV5 particles from SV5-like virions. Since particles with the sedimentation properties of SV5 are sometimes contaminated by a small amount of VSV (see Fig. 5), the possible presence of phenotypically mixed particles with the sedimentation properties of SV5 virions could not be accurately determined. Therefore, the present report is confined to studies of those phenotypically mixed virions which are bullet-shaped, sediment at the density of VSV, and contain the VSV genome. Isolation and purification of these particles was accomplished by subjecting the yield from MDBK cells mixedly infected with SV5 and VSV to isopycnic centrifugation in potassium tartrate. The band at a density of 1.16 was collected and found by electron microscopy to consist entirely of bullet-shaped virions. Plaque assays indicated $< 0.1\%$ contamination with SV5 virions, a result similar to that described above. After dialysis against phosphate-buffered saline, the hybrid virus particles possessing SV5 hemagglutinin were then separated from normal VSV particles by adsorption to chicken erythrocytes at 0 C for 1 hr and elution at 37 C for 1 hr as described previously (8). The eluted virions consisted of bullet-shaped, phenotypically mixed virions. The virions which did not adsorb to the erythrocytes consisted of normal VSV virions, with the possible addition of some hybrids that might not possess enough SV5 hemagglutinin to adsorb. The eluted hybrid virions and the nonadsorbing virions were then further purified and concentrated by rebanding in potassium tartrate, followed by dialysis against water prior to analysis of proteins.

Polyacrylamide gel electrophoresis of the polypeptides of VSV-SV5 hybrid particles. VSV-SV5 hybrid particles labeled with ^{3}H-amino acids were isolated as described above and their polypeptides were analyzed by coelectrophoresis with ^{14}C-amino acid-labeled marker viruses on polyacrylamide gels. Figure 6A shows coelectrophoresis of hybrid virions with VSV marker. The hybrid virions contain six polypeptides, four of which comigrate with VSV proteins G, N, NS1, and S, and two additional polypeptides, designated 2 and 4, which do not coincide with the VSV marker proteins. One of these precedes the VSV glycoprotein, and the other precedes the VSV nucleoprotein. Figure 6B shows that these two additional polypeptides in the hybrid virions comigrate precisely with two polypeptides in the SV5 marker, i.e., SV5 proteins 2 and 4. The SV5 nucleocapsid protein, 3, and protein 6, which is thought to be the major nonspike protein of the SV5 envelope, are not present in the hybrid particles. Although SV5 protein 6 and VSV protein NS1 migrate at similar rates, their migration is not identical, and this finding has been confirmed by repeated experiments. Further, the amount of protein in the NS1 peak relative to proteins N and S is the same in the hybrid as in standard VSV virions. Therefore, a significant amount of SV5 protein 6 is not present in the hybrid virions. The presence or absence of SV5 protein 5 in the hybrids cannot be adequately evaluated since protein 5 is a minor component even in standard SV5 virions, and it migrates in a position under the large VSV nucleoprotein peak. That VSV protein N is present in the hybrid virions is indicated by the precise comigration of peak N in the hybrid with that in standard VSV, by the similarity in the amounts of protein N in these peaks in the hybrid and standard VSV virions, and by the presence of nucleocapsid in the hybrid virions as shown by electron microscopy.

These results with amino acid labels indicated that the bullet-shaped, phenotypically mixed VSV-SV5 particles contain the four VSV proteins, plus SV5 proteins 2 and 4. SV5 proteins 2 and 4

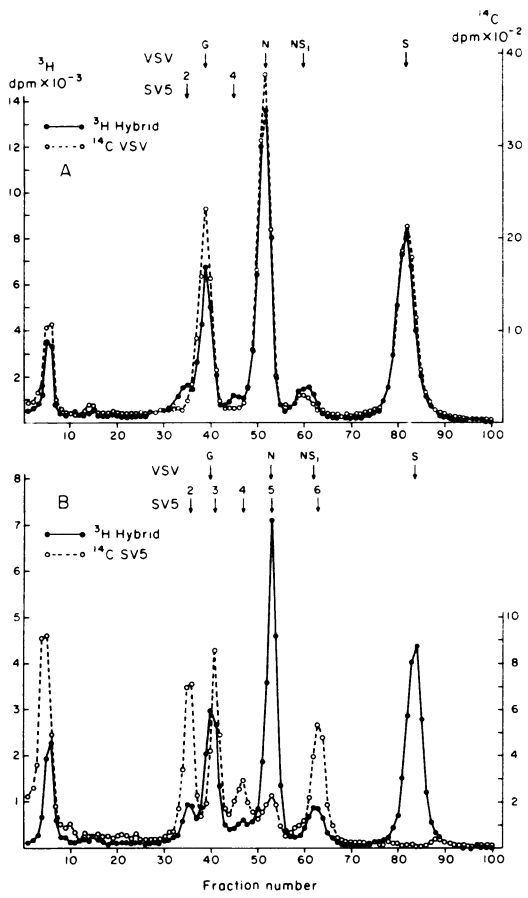

FIG. 6. *Polypeptides of phenotypically mixed VSV-SV5 virions. (A) Coelectrophoresis on a polyacrylamide gel of ^{3}H-amino acid-labeled VSV-SV5 hybrid virions with ^{14}C-amino acid-labeled VSV as a marker. (B) Coelectrophoresis of ^{3}H-amino acid-labeled VSV-SV5 hybrid virions with ^{14}C-amino acid-labeled SV5 as a marker.*

are glycoproteins (18) which form the spikes on the surface of the virion and possess neuraminidase and hemagglutinating activities (5). To provide further evidence that these two SV5 glycoproteins were present in the VSV-SV5 hybrid virions, ^{3}H-glucosamine-labeled hybrid virions were analyzed by coelectrophoresis with ^{14}C-amino acid-labeled marker virus. Figure 7A shows coelectrophoresis of the hybrids with VSV marker virus. Three glucosamine-containing peaks are seen in the hybrid pattern, one coinciding with the VSV glycoprotein and two others in the positions of the two additional protein peaks described above. Figure 7B shows that these two glucosamine-labeled proteins comigrate precisely with the two SV5 glycoproteins 2 and 4.

Polyacrylamide gel analysis of the bullet-shaped virions which banded at a density of 1.16 but did not adsorb to erythrocytes revealed a protein pattern indistinguishable from that of normal VSV virions. Thus, this population either consisted entirely of normal VSV virions, or contained an insufficient quantity of hybrids for the SV5 proteins to be detected by gel electrophoresis.

The above results indicate that bullet-shaped, phenotypically mixed VSV-SV5 virions contain the four VSV structural proteins, plus the two spike glycoproteins of SV5. These two SV5 spike proteins enable the phenotypically mixed particles to adsorb to and elute from chicken erythrocytes. Assay of the neuraminidase activity of hybrid virions by the thiobarbituric acid method of Warren (31) revealed that they possessed this enzyme, as would be expected from the presence of SV5 proteins 2 and 4 which have been shown to possess hemagglutinin and neuraminidase ac-

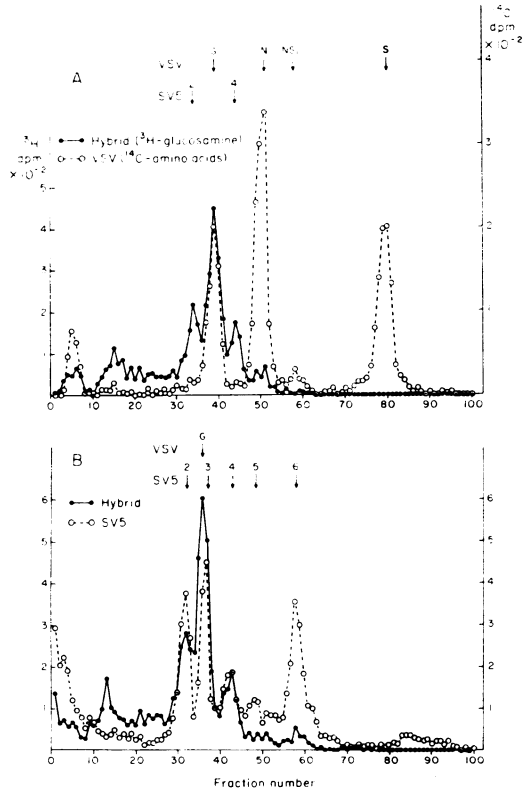

FIG. 7. *Glycoproteins of phenotypically mixed VSV-SV5 virions. (A) Coelectrophoresis on a polyacrylamide gel of ^{3}H-glucosamine-labeled VSV-SV5 hybrid virions with ^{14}C-amino acid-labeled VSV as a marker. (B) Coelectrophoresis of ^{3}H-glucosamine-labeled VSV-SV5 hybrid virions with ^{14}C-amino acid-labeled SV5 as a marker.*

tivities (5). The present results also indicate that neither the nucleocapsid protein 3 nor the major nonspike envelope protein 6 of SV5 is incorporated into the bullet-shaped, phenotypically mixed particles which contain the VSV genome.

DISCUSSION

The selective removal of the VSV glycoprotein by the *S. griseus* protease concomitant with the morphological disappearance of the spikes from the virus surface has confirmed and extended the observation of Cartwright and co-workers (4) that this protein forms the spikes. The finding that the VSV spikes are composed of glycoproteins is in agreement with the results with four other groups of enveloped RNA viruses in which the spike proteins, but no other proteins, were found to contain carbohydrate. These included influenza (12, 27), parainfluenza (5), arbo- (10, 13), and Rous sarcoma viruses (Rifkin and Compans, Virology, *in press*).

The finding that none of the nonglycosylated VSV proteins was affected by the protease treatment of the intact virus, although the enzyme digested these proteins after the virus was treated with Triton X-100 to remove the lipid, suggests that the nonspike envelope protein S does not occupy an external position on the virion surface, but is associated with the lipid in such a way that it is protected from enzyme action.

The function of protein NS1 is still not clear. Wagner and co-workers (30) have found this protein in infected cells, but not regularly in purified virions, and thus have interpreted it to be a nonstructural protein which contaminates virus preparations which are not sufficiently purified. Mudd and Summers (26) found this protein in purified virus, and we have also consistently found this protein, but no evidence of other contaminants, in our preparations of purified VSV. It appears significant that this protein was not removed by protease treatment, as would be expected if it were a nonstructural protein contaminating the external surface of the virus. This suggests that NS1, although present in small amount, may be a component of the virion which is protected from the action of the enzyme by lipid. Further studies are required to establish the exact function of this protein.

The present and previous (8) studies have shown that virions with the typical bullet shape of VSV may contain SV5 proteins. The demonstration, that such phenotypically mixed virions contain, in addition to the VSV proteins, the two SV5 glycoproteins but neither the nucleocapsid protein nor the major nonspike envelope protein of SV5, has several interesting implications. The presence of the two SV5 glycoproteins indicates that between the paramyxoviruses and the rhabdoviruses there are no stringent restrictions on the type of glycoproteins or spikes that can be incorporated into the virion. The fact that high yields of phenotypically mixed virions can be produced, i.e., $> 10^9$ PFU/ml, indicates that the addition of SV5 glycoproteins to particles containing the VSV membrane protein S is an efficient process. Furthermore, the finding that both SV5 glycoproteins can be present on such virions without significantly altering the VSV morphology suggests that spike glycoproteins have little influence on the shape of the virion. This supports the conclusion that the bullet shape is determined by the nucleocapsid or protein S, or both. Wagner and co-workers (29) previously concluded that the bullet shape is due to the nucleocapsid since the nucleocapsid coils retained their shape after stripping off the viral envelope with digitonin. Finally, the absence of SV5 protein 6 in the phenotypically mixed virions suggests that the VSV nucleocapsid does not associate with regions of cell membrane which contain this SV5 membrane protein but that such association requires the VSV membrane protein S. The available data are compatible with the hypothesis that in the assembly of VSV a specific interaction must occur between the VSV nucleocapsid and regions of cell membrane which contain the envelope protein S. Previous work from our laboratory suggested that a specific interaction occurs between influenza and parainfluenza virus nucleocapsids and regions of cell membrane which contain virus-specific proteins (9, 11). The present results suggest that a similar interaction occurs with rhabdoviruses and that this interaction is between the nucleocapsid and the nonglycosylated membrane protein.

ACKNOWLEDGMENTS

We thank Cathleen O'Connell and Ann Erickson for excellent technical assistance.

This investigation was supported by Public Health Service research grant AI-05600 from the National Institute of Allergy and Infectious Diseases and by contract AT(30-1)-3983 from the U.S. Atomic Energy Commission.

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