Sialoglycoprotein of Vesicular Stomatitis Virus: Role of the Neuraminic Acid in Infection

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Neuraminidase free of proteolytic activity substantially reduced the infectivity of vesicular stomatitis (VS) virus but less effectively than trypsin. The only sugar residue hydrolyzed by neuraminidase was *N*-acetyl neuraminic acid, ~89% of which was liberated from virion glycoprotein and the rest from virion glycolipid. Desialylation of virion glycoprotein but not of glycolipid resulted in progressive loss of infectivity. Sialyl transferase prepared and partially purified from BHK-21 cells catalyzed resialylation by CMP-[¹⁴C]sialic acid of the glycoprotein of neuraminidase-treated VS virions and supersialylation of unhydrolyzed VS viral glycoprotein. Resialylation of desialylated VS virions resulted in substantial (26-fold) restoration of their infectivity. We conclude that terminal neuraminic acids of VS viral sialoglycoprotein play an important role in initiation of infection with this virus.

Vesicular stomatitis (VS) virus is a single-stranded RNA virus composed of a helical nucleocapsid surrounded by a lipoprotein envelope which is covered with spikelike projections (16). The VS virion contains five structural polypeptides: a nucleoprotein (N), a large protein (L) which is required for polymerase activity (5), and a phosphoprotein (NS), all of which are associated with the nucleocapsid (9, 22, 23). A membrane protein (M) constitutes the structural matrix of the envelope, and a glycoprotein (G) comprises the spikes (14, 24). Kelley et al. (10) demonstrated that the isolated G protein is the major antigen that stimulates the production of and reacts with neutralizing antibody. The ability to hemagglutinate goose erythrocytes and to inhibit cellular RNA and protein synthesis has recently been shown to be the property of isolated glycoproteins (J. J. McSharry and P. W. Choppin, personal communication). In addition, it seems likely that the VS viral glycoprotein provides the attachment site of the virus to cell surfaces. VS virus loses infectivity when treated with trypsin which specifically removes the surface spike projections of the viral envelope (3).

Carbohydrates which are constituents of viral glycoproteins and glycolipids (11) account for as much as 13% of the total mass of the VS virion (15) and are presumably derived from host cells (2, 7). Chemical analysis of the sugar composition of glycopeptides isolated from VS virions grown in mouse L cells revealed the presence of

at least five monosaccharides: *N*-acetyl neuraminic acid, mannose, glucose, galactose, and *N*-acetyl glucosamine. *N*-acetyl neuraminic acid is the major sugar component representing about 37% of the total carbohydrate content of the glycoprotein (15) and is also present in the major glycolipid found in VS virus propagated in BHK cells (11). However, the functions of these carbohydrates in either maintaining the structural integrity of the virion or participating in any biological activity of the virus have not been demonstrated.

The purpose of the present investigation was to determine whether the carbohydrate moiety of the VS viral glycoprotein contributes to infectivity. To this end, the terminal *N*-acetyl neuraminic acid residues were first removed from the virion by enzymatic hydrolysis and then reattached by means of a sialyl transferase. The results support the contention that the terminal sialic acids of the viral glycoprotein are in part responsible for viral infection.

MATERIALS AND METHODS

Chemicals and radiochemicals. Trypsin (3× crystallized) was purchased from Worthington Biochemical Corp., Freehold, N.J., and *Vibrio cholerae* neuraminidase was from Behringwerke, Marburg, West Germany. Fetuin, purified by the Spiro procedure, was obtained from Gibco, Grand Island, N.Y., and bovine submaxillary mucin was supplied by Sigma Chemical Co., St. Louis, Mo. [³H]leucine (60 Ci/mmol) and [³H]tyrosine (44 Ci/mmol) were procured from Schwarz/Mann, Orangeburg, N.Y. D-[6-

^3H]glucosamine-hydrochloride (7.3 Ci/mmol), CMP-[4,5,6,7,8,9- ^{14}C]neuraminic acid (221 to 230 mCi/mmol), and [^{14}C]amino acid hydrolysate (51 mCi/matom) were purchased from New England Nuclear Corp., Boston, Mass.: *N*-acetyl-[4,5,6,7,8,9- ^{14}C]neuraminic acid (270 mCi/mmol) was from Amersham/Searle, Arlington Heights, Ill.

Virus and cell culture. The Indiana serotype of VS virus was used in all experiments. Recently cloned stocks of virus were propagated in confluent monolayers of BHK-21 cells cultivated in 75-cm² Falcon plastic flasks (9). Cell cultures were infected at a multiplicity of ~0.1 PFU/cell, which resulted in yields of only B virions. For the production of labeled virus, the media contained [^3H]glucosamine (2 $\mu\text{Ci/ml}$), [^{14}C]amino acid hydrolysate (1 $\mu\text{Ci/ml}$), or 5 $\mu\text{Ci/ml}$ each of [^3H]leucine and [^3H]tyrosine.

Virus that had been released into the medium 18 to 20 h post-infection was purified according to the procedure of Emerson and Wagner (4). Virions were harvested from the media which had been clarified by centrifugation at $800 \times g$ for 10 min. The media were then centrifuged for 90 min at $80,000 \times g$ in an SW27 rotor through a 2-ml pad of 50% glycerol in Earle balanced salt solution (BSS). The pellet was suspended in BSS, sonicated, layered on top of preformed 0 to 40% sucrose gradients, and centrifuged in an SW25.1 rotor for 90 min at $35,000 \times g$. The visible band of B particles was removed by side puncture of the tube and diluted in BSS, and the virions were pelleted in an SW50L rotor at $80,000 \times g$ for 60 min. The purified virions were resuspended in 0.01 M Tris-hydrochloride buffer (pH 7.0), disaggregated by ultrasonic vibration, and stored at either 4 C or -80 C. Infectivity of B particles was determined by plaque assay on monolayers of L cells and expressed as plaque-forming units (PFU) (21).

Enzyme treatment. Purified B particles of VS virus that had been labeled with [^3H]glucosamine and [^{14}C]amino acids were incubated in a reaction mixture which contained, in a final volume of 0.5 ml, 30 μmol of Tris-hydrochloride buffer (pH 7.0) and either 5 μmol of CaCl_2 and trypsin or 0.5 μmol of CaCl_2 and *V. cholerae* neuraminidase. After incubation at 37 C for the specified times, the reaction was terminated by centrifugation in an SW65 rotor at $100,000 \times g$ for 1 h at 4 C to yield a pellet and a supernatant fraction. The pellet was suspended in 0.1 M Tris-hydrochloride buffer (pH 7.0), sonicated for 20 s in a Raytheon sonicator at 50 V, and assayed for infectivity. Levels of infectivity were compared with those of virions treated identically, except that the incubation mixtures lacked trypsin and neuraminidase. The amount of radioactivity solubilized by enzyme treatment was measured by pipetting 50 to 100 μl of the supernatant fraction into scintillation vials containing 0.5 ml of Nuclear-Chicago solubilizer diluted 1:1 with water. Ten milliliters of toluene-based scintillation fluors was added, and the samples were counted in a scintillation spectrometer. Background ^3H and ^{14}C radioactivity of 50 to 100 counts/min present in the supernatant fluid of the control virus mixtures was subtracted from each experimentally determined value.

Preparation of glycoprotein acceptors. Sialic acid was removed from fetuin by mild acid hydrolysis. Fetuin (100 mg) was dissolved in 5 ml of water, and the pH was adjusted to 1.0 with 2 N H_2SO_4 . The mixture was then heated to 85 C for 2 h, cooled, and neutralized by the addition of 0.1 N NaOH. Desialylated fetuin was dialyzed extensively against water until all soluble sialic acid was removed. Cold 95% ethanol was added to dialyzed fetuin to a final concentration of 50% ethanol, followed by sufficient 0.5 M sodium phosphate buffer (pH 6.0) to achieve a molarity of 0.1. After storage at -20 C for 16 h, the precipitate which had formed was dissolved in 0.05 M sodium phosphate buffer (pH 6.0), dialyzed against water for 24 h, and lyophilized. The dry powder was stored at -20 C. Acid hydrolysis removed 0.17 μmol of sialic acid per 0.5 mg of fetuin. Bovine submaxillary mucin (BSM) was purified by the method of Tsuiiki et al. (20). Cethyltrimethylammonium bromide was added to 500 mg of BSM dissolved in 50 ml of water until precipitation was complete. The precipitate was collected by centrifugation, dissolved in water, and fractionated with cold 95% ethanol. The precipitate formed at 60 to 70% ethanol was collected by centrifugation, dialyzed against water, and lyophilized. To remove sialic acid, 100 mg of purified BSM was subjected to acid hydrolysis and neutralized with NaOH in a manner identical to that for fetuin. Acid hydrolysis liberated 0.21 μmol of sialic acid per 0.5 mg of BSM.

When purified virions were used as acceptors, neuraminic acid was removed just prior to use in the sialyl transferase reaction. Approximately 2×10^{11} PFU of virus suspended in 100 mM Tris-hydrochloride buffer (pH 7.0) was incubated in a mixture consisting of 50 U of neuraminidase and 10 mM CaCl_2 at 37 C for 2 h. The virus-enzyme mixture was centrifuged at $100,000 \times g$ for 1 h. The resulting pellet was washed with 100 mM sodium phosphate buffer (pH 7.0) and resuspended in 0.1 M sodium phosphate buffer (pH 7.0). A portion of the buffer (pH 7.0). A portion of the virion preparation was assayed for contaminating neuraminidase activity by incubation with fetuin and CaCl_2 at 37 C for 4 h. In all cases no neuraminidase activity was detected.

Preparation and partial purification of sialyl transferase. Sialyl transferase was prepared from confluent monolayers of uninfected BHK-21 cells cultivated in 75-cm² Falcon plastic flasks. Cell monolayers from 60 flasks (approximately 4×10^8 cells) were each washed twice with 10 ml of BSS and drained. Ten milliliters of BSS was added to each flask, and cells were removed with the aid of a magnetic stirring bar. Cells were pooled, centrifuged at $800 \times g$ for 10 min, and washed with BSS. The washed cell pellet was suspended in a volume of 0.1 M sodium phosphate buffer (pH 6.9), 0.01 M NaCl, and 0.0015 M MgCl_2 to give 0.5 ml of packed cells per 5 ml of buffer. After swelling for 20 min at 4 C with occasional mixing to maintain a homogeneous suspension, cells were disrupted in a Dounce tissue homogenizer employing a tight-fitting pestle. For optimal cell breakage, 25 to 30 strokes were employed. The cell homogenate was centrifuged at $800 \times g$ for 10 min to

remove nuclei and cellular debris. The supernatant fraction was removed, and the pellet was washed with 10 ml of homogenization buffer. After centrifugation at $800 \times g$ for 10 min, the supernatant and the wash fractions were combined and centrifuged in an SW27 rotor at $96,000 \times g$ for 1 h. The resulting pellet was suspended by homogenization in 1.5 ml of 100 mM sodium phosphate buffer (pH 6.9); 1 ml of resuspended pellet was centrifuged in an SW65 rotor at $100,000 \times g$ for 2 h through 9 ml of 20% sucrose in 100 mM sodium phosphate buffer (pH 6.9). The resulting pellet was dissolved in 1 to 2 ml of 100 mM sodium phosphate buffer (pH 6.9). The sialyl transferase at this stage of purification is stable on storage at 4 C for 2 weeks without loss of enzyme activity. Prior to use in the sialyl transferase reaction, the enzyme preparation was sonicated in a Sonifier Cell Disrupter (Model W140D, Ultrasonic, Inc.) at maximal voltage utilizing a microtip probe. Seven sonic bursts of 30-s duration were applied, and after each of these the temperature was allowed to return to 4 C and was not permitted to exceed 9 C during this procedure. The sonicated enzyme was centrifuged in a Sorval SSA34 rotor at $27,000 \times g$ for 10 min; the resulting supernatant fraction served as the source of enzyme.

Sialyl transferase assay. Sialylation of glycoprotein acceptors was accomplished by a modification of the method of Grimes (6). The standard incubation mixture contained, in a volume of 0.18 ml, 1.8 μ mol of EDTA solution which was adjusted to pH 6.9 with 1.0 N NaOH, 0.5 nmol of CMP- 14 C]sialic acid (specific activity, 180 to 200 counts per min per pmol), 5 to 10 μ mol of sodium phosphate buffer (pH 6.9), 0.05 ml of glycoprotein acceptor, and 0.1 to 0.15 mg of enzyme preparation. When either fetuin or BSM was employed as the neuraminic acid acceptor, 0.5 mg in 0.05 ml of 100 mM sodium phosphate buffer (pH 6.9) was used per assay. The reaction was initiated by the addition of enzyme, allowed to proceed at 37 C for the specified time, and terminated by pipetting 50 μ liters of the assay mixture onto a disk of Whatman no. 3 filter paper. The disks were immediately transferred to a beaker of stirring 10% trichloroacetic acid and then were processed as previously described (1). Radioactivity was counted in 10 ml of toluene-based scintillation fluid.

Measurements of protein and sialic acid concentrations. Protein concentrations were determined according to the Lowry procedure (13) using crystalline bovine plasma albumin as a standard. Sialic acid was measured by using the thiobarbituric acid assay of Warren (25). Absorbancies were routinely monitored at 549 and 532 nm, and contributions of lipids absorbing at 532 nm were eliminated as suggested by Warren (25).

Polyacrylamide gel electrophoresis. Samples of the sialyl transferase reactions were prepared for gel electrophoresis by precipitating proteins after the addition of 50 μ g of bovine plasma albumin with 1 vol of cold 20% trichloroacetic acid. The contents of the tubes were mixed thoroughly and cooled on ice for 15 min. After centrifugation, the pellet was washed three times with 5 ml of cold 10% trichloroacetic acid, followed by 10 vol of cold acetone. The acetone-

washed pellet was dissolved in 10 mM sodium phosphate buffer (pH 7.4) containing 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol. Samples were placed in a boiling water bath for 2 min prior to transfer to gels. The procedure for polyacrylamide gel electrophoresis has been previously described (24).

RESULTS

Loss of infectivity following treatment of VS virions with trypsin or neuraminidase. We chose to compare the effects of trypsin and neuraminidase on infectivity of VS-B virions for several reasons. First, since trypsin can selectively remove viral glycoprotein, resulting in marked reduction in infectivity (3), it was of interest to determine whether the amount of glycoprotein liberated corresponded with loss of infectivity. Secondly, we wished to determine the function of the terminal sialic acids of the viral glycoprotein in the infective process by employing a preparation of protease-free neuraminidase.

To examine the role of the glycoprotein and neuraminic acid residues of the VS virion in the infective process, B virions labeled with 3 H]glucosamine and 14 C]amino acids were divided into two equivalent sets of samples, one of which was treated with either trypsin or neuraminidase and the other of which served as the control. Individual tubes of each series contained 8×10^9 PFU of VS virus in 0.5 ml of incubation mixture. After incubation at 37 C for the desired time intervals, the reaction mixtures were centrifuged at $100,000 \times g$ for 1 h. Under these conditions, the virions in the pellet were separated from the enzymes. Infectivity of control virus preparations decreased slightly with time of incubation at 37 C; the infectivity of enzyme-treated virions is expressed as percent of that of the control virus processed at each time point.

Figure 1 shows the amount of radioactivity solubilized by treatment of VS virions with each enzyme and the corresponding loss in infectivity of the virus. An increase in the amount of 14 C]protein and 3 H]glucosamine solubilized by trypsin was accompanied by a rapid loss in infectivity followed by a slower decline to 0.01% (Fig. 1A). The ratio of 3 H: 14 C counts in the supernatant was relatively constant at approximately 3:1, a value which agreed well with that ratio found in the undigested viral glycoprotein as determined by polyacrylamide gel electrophoresis (data not shown). Furthermore, analysis of trypsinized virions by gel electrophoresis revealed progressive reduction only in the amount of virion glycoprotein concomitant with the progressive loss of infectivity.

FIG. 1. Comparative effects of trypsin and neuraminidase on infectivity and release of carbohydrate and protein from purified B particles of VS virus. Virions (8×10^8 PFU) labeled with [^3H]glucosamine and [^{14}C]amino acids (165,500 and 143,000 counts/min, respectively) were incubated in 0.5 ml of a reaction mixture consisting of either (A) 6×10^{-2} M Tris-hydrochloride buffer (pH 7.0), 10^{-2} M CaCl_2 , and 5 μg of trypsin or (B) 6×10^{-2} M Tris-hydrochloride buffer (pH 7.0), 10^{-3} M CaCl_2 , and 0.075 U of neuraminidase. The virus-enzyme mixtures were incubated at 37 C for the specified times, and the reactions were terminated by centrifugation in an SW65 rotor at $100,000 \times g$ for 1 h. The supernatant and pellet fractions were separated, and the pellet was suspended in 0.2 ml of 100 mM Tris-hydrochloride buffer (pH 7.0), sonicated, and titered by plating on L-cell monolayers. Infectivity of enzyme-treated virus is expressed as percentages of that of control virus treated identically, except that neuraminidase and trypsin were replaced in the reaction mixture with 100 mM Tris-hydrochloride buffer (pH 7.0). Solubilized ^3H and ^{14}C radioactivity was counted by scintillation spectrometry using 100 μliters of the supernatant fractions. Background radioactivity in supernatants of untreated virus was subtracted at each time point.

VS virions incubated with neuraminidase exhibited a similar but somewhat slower loss in infectivity coincident with solubilization of [^3H]carbohydrate counts (Fig. 1B). However, no

[^{14}C]protein counts were released by neuraminidase treatment, indicating that the loss of infectivity was not due to the removal of a protein but only to carbohydrate. It is also noteworthy that the maximal decline in infectivity of VS virus treated with trypsin was 2 to 3 logs greater than that of virions exposed to neuraminidase. Moreover, neuraminidase liberated only 20% of the [^3H]glucosamine label as compared to that solubilized by trypsin.

Identification of the [^3H]glucosamine-labeled product released from virions by neuraminidase. Since the substance released by neuraminidase treatment of VS virus appears to influence the degree to which virions can infect cells, it was essential to identify the product of the neuraminidase reaction. The lack of proteolytic activity in the *V. cholerae* neuraminidase preparation was determined by the sensitive dimethyl casein assay (12), as well as by the absence of solubilized [^{14}C]amino acid following incubation of virus with neuraminidase (see Fig. 1B). The neuraminidase-released product was assumed to be a carbohydrate, presumably *N*-acetyl neuraminic acid, but it was essential to establish the identity of the sugar with certainty. Therefore, virions that had been labeled with [^3H]glucosamine were incubated with neuraminidase and pelleted by centrifugation; the resulting supernatant fraction was subjected to descending paper chromatography in *n*-butanol-pyridine-water (6:4:3) solvent system for 48 h.

Figure 2 demonstrates that all of the [^3H]glucosamine label released by neuraminidase chromatographed as *N*-acetyl neuraminic acid; no ^3H -radioactivity was observed in the regions to which other sugars found in the VS viral glycoprotein would be expected to migrate. Similar results were obtained when the solvent system was ethyl acetate-pyridine-water (72:20:23).

However, since the only sugars which contain ^3H -label derived from glucosamine are *N*-acetyl neuraminic acid and *N*-acetyl glucosamine (19), the neuraminidase preparation might also contain contaminating glycosidases which could liberate unlabeled monosaccharides not detected under these conditions. Therefore, B virions were labeled with either [^3H]glucose, [^3H]galactose, [^3H]fucose, or [^3H]mannose, purified by differential and rate-zonal centrifugations and subjected to neuraminidase treatment. The supernatants obtained after centrifugation of the virus-enzyme mixtures were chromatographed as described in the legend to Fig. 2. No radioactivity was detected in the paper chromatograms, which indicates the lack of

FIG. 2. Paper chromatogram of the supernatant fraction following centrifugation of purified B particles of VS virus treated with neuraminidase. Virions labeled with [^3H]glucosamine were incubated for 2 h at 37 C in a reaction mixture containing 50 U of neuraminidase as described in Materials and Methods. After centrifugation, the supernatant fraction was partitioned from the pellet and evaporated at 56 C under a stream of N_2 gas. The residue was dissolved in 50% ethanol, mixed with various ^{14}C -marker sugars and transferred to Whatman no. 1 chromatography paper. Descending chromatography was carried out in a 1-butanol-pyridine-water (6:4:3) solvent system for 48 h. After drying, the chromatogram was cut into 1-cm sections and transferred to scintillation vials containing 10 ml of toluene-based scintillation fluid. The arrows indicate the positions of ^{14}C -marker sugars. Abbreviations: NANA, N-acetyl neuraminic acid; Gal, galactose; Glu, glucose; Man, mannose; N-Ac-gal, N-acetyl galactosamine; Fuc, fucose; and N-Ac-glu, N-acetyl glucosamine.

conversion of these sugars to N-acetyl neuraminic acid and the absence of contaminating glycosidases in the *V. cholerae* neuraminidase preparation.

Polyacrylamide gel electrophoresis of VS virions treated with trypsin and neuraminidase. To confirm that the glycoprotein is the site of attack by trypsin and neuraminidase, equivalent amounts of virions labeled with [^3H]glucosamine and [^{14}C]amino acids were incubated for 2 h with either trypsin or neuraminidase. After partitioning the pellet and supernatant by centrifugation, the viral proteins in the pellet were extracted with sodium dodecyl sulfate and analyzed by electrophoresis on 7.5% polyacrylamide gels. Figure 3A depicts the electrophoretic profile of proteins extracted from control virus preparations. Of the five [^{14}C]amino acid-labeled proteins only one, protein G, contained ^3H -label. In addition, a second region of [^3H]glucosamine label is present in a small peak which migrates slightly ahead of the tracking dye, to a position which is consist-

ent with the migration of glycolipids (17). The electropherogram of trypsin-treated virus (Fig. 3B) shows complete and selective degradation of only the G protein; the ratio of radioactivity of the four remaining proteins (L, N, NS, and

FIG. 3. Electropherograms of proteins of untreated VS virus (A) and VS virus treated with trypsin (B) or with neuraminidase (C). Equal amounts of purified B virions that had been labeled with [^3H]glucosamine and [^{14}C]amino acids were incubated at 37 C for 2 h in the presence of 5 μmol of CaCl_2 , 30 μmol of Tris-hydrochloride buffer (pH 7.0), and either 0.5 mg of trypsin or 50 U of neuraminidase. After incubation, the reaction mixtures were centrifuged at $100,000 \times g$ for 1 h. Viral proteins were extracted in 10 mM sodium phosphate buffer (pH 7.4) containing 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol. Samples were boiled for 2 min and subjected to electrophoresis for 9 h at 5 mA per gel on 7.5% polyacrylamide gels. Gel slices of 1.25 mm were dissolved in Nuclear-Chicago solubilizer and toluene-based fluors and counted in a scintillation spectrometer. Arrows mark the position of VS viral proteins L, G, N, NS, and M.

M) was identical to that found in the control virus (Fig. 3A). Trypsin appeared to have no effect on the distribution of ^3H -label in the glycolipids (Fig. 3B). Neuraminidase removed 15% of the ^3H -label from the glycoprotein and also caused a reduction in the peak of ^3H -label in the glycolipids (Fig. 3C). Since the major glycolipid in virions grown in BHK cells is the hematoside, neuraminosyl-galatosyl-glucosyl-ceramide (11), this datum indicates that some of the sialic acid of the glycolipids is susceptible to neuraminidase attack.

Lack of influence of glycolipid neuraminic acid on infectivity. Inasmuch as neuraminidase can remove the neuraminic acid residues of both glycolipids and glycoproteins (see Fig. 3C), it was of interest to determine whether glycolipid sialic acids are involved in viral infectivity. The approach used was to remove the neuraminic acids of the glycoprotein by incubation with trypsin, which attacks only the glycoprotein but does not affect the glycolipids. The role in infectivity of the sialic acid residue in glycolipids can then be ascertained following neuraminidase treatment of trypsinized VS virions.

Purified B virions that had been labeled with [^3H]glucosamine and [^{14}C]amino acids were divided into three fractions, each containing 8×10^9 PFU. One fraction was incubated with trypsin, the second with neuraminidase, and the last fraction, which served as a control, was incubated with buffer. After incubation for 2 h, the supernatant fractions of the reaction mixtures were isolated by centrifugation and as-

sayed for radioactivity. The pellets were resuspended in buffer and one half was used to measure infectivity. The remainder was employed in subsequent incubation with the alternative enzyme, i.e., trypsinized virions were incubated with neuraminidase and desialylated virus was treated with trypsin.

The results of a typical experiment are shown in Table 1. Virus which was treated with trypsin, thereby removing the viral G protein and its neuraminic acid residues, exhibited 0.007% of the infectivity possessed by the control virions. However, further incubation of these trypsinized virions with neuraminidase did not result in a further loss of infectivity, although neuraminic acid residues of virion glycolipids were hydrolyzed. These sialic acid residues could have originated only from glycolipids, since no residual glycoproteins were present in the virions as judged by polyacrylamide gel electrophoresis. Therefore, it is reasonable to conclude that only the neuraminic acid residues of the viral glycoprotein influence infectivity. In addition, these data indicate that the glycolipids contain about 11% of the total neuraminic acid content of the virion.

Basic enzymatic requirements and substrate specificity of BHK-cell sialyl transferase. If any significance is to be attributed to the apparent infectivity function of neuraminic acid residues of the VS viral glycoprotein, then resialylation of neuraminidase-treated virions should result in restoration of their infectivity. To this end, sialyl transferase was isolated from

TABLE 1. Comparative effect on infectivity of the removal of neuraminic acid from glycoprotein and glycolipid of VS virus^a

Pretreatment	Treatment	Radioactivity (counts/min) in supernatant fraction		Infectivity ^b	
		[^3H]glucosamine	[^{14}C]amino acids	PFU/ml	% of control
None	None	50	35	4.2×10^{10}	100
None	Trypsin	26,103	9,955	2.9×10^6	0.007
None	Neuraminidase	8,588	30	4.0×10^9	1
None	None	65	45	2.2×10^{10}	100
Trypsin	Neuraminidase	963	47	1.5×10^6	0.007
Neuraminidase	Trypsin	19,776	9,153	1.8×10^9	0.008

^a Purified B virions (8×10^9 PFU/0.2 ml) that had been labeled with [^3H]glucosamine and [^{14}C]amino acids were pretreated for 2 h at 37°C in 0.5 ml of an incubation mixture consisting of Tris-hydrochloride buffer (pH 7.0) and CaCl_2 in the presence or absence of 0.5 mg of trypsin or 50 U of neuraminidase. After centrifugation at $100,000 \times g$ for 1 h, radioactivity was measured using 50 μl of the supernatant fractions. The pellets were washed with 100 mM Tris-hydrochloride buffer (pH 7.0), centrifuged at $100,000 \times g$ for 1 h, sonicated, and suspended in 0.2 ml of 100 mM Tris-hydrochloride buffer (pH 7.0). One half of the pellet was used to determine infectivity; the remainder was incubated with either trypsin or neuraminidase exactly as described above. After termination of the reaction by centrifugation, the pellets were resuspended in 0.1 ml of the above buffer and plated on L cells. Radioactivity was monitored using 100 μl of the supernatant.

^b Plaque assays were performed on monolayers of L cells.

BHK-21 cells. A problem arose, however, when it was found that crude homogenates of BHK cells contained sialyl transferases of low activity and high levels of endogenous acceptors (80% of the total exogenous acceptor activity). In addition, the crude BHK-cell sialyl transferase was found to be dependent upon Triton X-100 for activity; this requirement for Triton X-100 was particularly unacceptable since the viral glycoprotein is dissociated from the viral envelope by Triton X-100 (10), thus making impossible experiments designed to reconstitute infectivity. These obstacles were overcome by subsequent enzyme purification by means of differential centrifugation and sonication as the main steps (see Materials and Methods). These procedures resulted in a 25-fold purification; the resulting enzyme preparation was soluble, contained only low levels of endogenous sialic acid acceptors, and did not depend upon detergent for activation. Before this sialyl transferase could be used in an attempt to restore viral infectivity, it was necessary to characterize the activity of the enzyme and its substrate requirements.

Table 2 depicts some requirements of the partially purified BHK-cell sialyl transferase whose activity was monitored by the incorporation of [^{14}C]sialic acid into acid-precipitable protein substrates. There was insignificant incorporation of [^{14}C]sialic acid when the enzyme had been heated at 100 C or when the exogenous acceptor was omitted from the reaction mixture. Addition of 10^{-2} M EDTA stimulated incorporation of radioactivity into glycoproteins, whereas 0.1% Triton X-100 inhibited enzyme activity by 40%. No specific metal requirement for activity was observed. Native, unhydrolyzed BSM and fetuin were poor acceptors. Somewhat surprisingly, VS virus which had not been desialylated with neuraminidase also incorporated moderate amounts of sialic acid. The presence of desialylated viral or nonviral glycoprotein acceptors resulted in a 10- to 20-fold increase in acid-precipitable radioactivity over that observed when no exogenous acceptor was added and about a fivefold increase over the glycoprotein substrates not desialylated with neuraminidase.

Transfer of sialic acid to VS viral glycoprotein. Although the G protein was the likely candidate to serve as the virion acceptor in the sialyl transferase reaction, it seemed essential to determine the substrate specificities of both membrane proteins and other virion components. Other possibilities were that the viral glycolipids could incorporate sialic acid or that the presence of virions in the sialyl transferase

TABLE 2. *Functional requirements and glycoprotein acceptor specificity of BHK-cell sialyl transferase and CMP-[^{14}C]sialic acid*

Reaction conditions ^a	[^{14}C]sialic acid incorporation (counts per min per 50 μ liters) ^b	
	Expt I	Expt II
Requirements for sialylation:		
Complete reaction mixture ^c	3,954	4,096
– desialylated fetuin	304	315
– enzyme	84	75
– enzyme, plus boiled enzyme ^d	106	115
– 10^{-2} M EDTA	1,914	1,925
+ 10^{-3} M MgCl_2	3,873	3,998
+ 10^{-3} M MnCl_2	3,987	3,903
+ 0.1% Triton X-100	2,478	2,519
Exogenous glycoprotein acceptors ^e :		
None	240	258
Bovine submaxillary mucin	672	643
Fetuin	716	747
VS virus	865	893
Desialylated BSM	3,363	3,259
Desialylated fetuin	4,015	3,987
Desialylated VS virus	4,104	4,083

^a All reactions were incubated for 2 h at 37 C.

^b [^{14}C]sialic acid incorporation into glycoproteins was determined by pipetting 50 μ liters onto filter paper disks which were immediately placed in a bath of 10% trichloroacetic acid. The processed disks were then counted in a scintillation spectrometer.

^c Complete reaction mixture of 10 mM EDTA (pH 6.9), 0.5 nmol of CMP-[^{14}C]sialic acid (200 counts per min per pmol), 0.5 mg of acid-hydrolyzed fetuin in 5 μ mol of sodium phosphate buffer (pH 6.9), 0.13 mg of enzyme, and sufficient 100 mM sodium phosphate buffer (pH 6.9) to give a final volume of 0.18 ml. When additions or omissions occurred, the volume was maintained at 0.18 ml by adjusting the volume of the sodium phosphate buffer.

^d 100 C for 60 s.

^e Five-hundred micrograms of BSM, fetuin, desialylated BSM, or desialylated fetuin were used per assay, whereas 100 μ g of either VS virus or desialylated VS virus were employed. Sialic acid was removed from BSM and fetuin by acid hydrolysis and from purified B virions by neuraminidase treatment.

reaction could cause stimulation of sialic acid incorporated into nonviral endogenous acceptors. Therefore, we examined by polyacrylamide gel electrophoresis the distribution of the ^{14}C label among all the potential VS viral substrates of the transferase reaction.

Identical amounts of [^3H]amino acid-labeled B virions or desialylated B virions were incubated with CMP-[^{14}C]sialic acid in the standard sialyl transferase mixtures for 2 h at 37 C and then were precipitated with trichloroacetic

acid. Viral proteins were extracted with sodium dodecyl sulfate and subjected to electrophoresis. The results are shown in Fig. 4. The [^{14}C]sialic acid was found, presumably covalently linked to substrates, in three regions of the gels when virus, either untreated with neuraminidase (Fig. 4A) or desialylated (Fig. 4B), were used as acceptors. A slow-moving product of high molecular weight which migrates just ahead of the L protein was identified as the endogenous acceptor in the enzyme preparation (Fig. 4C). A second peak comigrated with the glycoprotein (G), and a labeled product is present in the region where glycolipids are found. No ^{14}C label was detected in the membrane protein M or in any of the other viral proteins. Virions previously desialylated with neuraminidase incorporated approximately five times as much [^{14}C]sialic acid into the glycoprotein as did virions untreated with neuraminidase. Moreover, twice the amount of sialic acid was found attached to the glycolipids of the untreated control virus (Fig. 4A and 4B). However, it is uncertain that glycolipids of untreated virions incorporated additional neuraminic acid, since the amounts of ^{14}C radioactivity found in the glycolipid-like component of the endogenous acceptor (Fig. 4C) and of the untreated virions (Fig. 4A) were approximately the same.

The preceding experiment demonstrated that virions which had not been subjected to neuraminidase treatment incorporate sialic acid which became covalently linked to the viral glycoprotein. This observation suggests that the viral glycoprotein is not fully sialylated during maturation unless the *in vitro* incorporation of sialic acid involved an isotope exchange reaction. If so, virus labeled with [^3H]glucosamine should exchange its [^3H]sialic acids for exogenously added CMP-[^{14}C]sialic acid, resulting in the formation of either [^3H]sialic acid or CMP-[^3H]sialic acid. To test this possibility, [^3H]glucosamine-labeled VS-B virions were incubated in the standard sialyl transferase reaction mixture under conditions which resulted in the incorporation of approximately 20% of the exogenously added [^{14}C]sialic acid. The reaction was terminated by the addition of cold acetone, and the supernatant fraction was partitioned by centrifugation. The supernatant fraction was evaporated, reconstituted in 50% ethanol, and subjected to descending paper chromatography in 95% ethanol-1 M ammonium acetate (pH 7.5) (75:30, vol/vol) for 42 h. Neither [^3H]sialic acid nor CMP-[^3H]sialic acid was present in the chromatogram, indicating that the attachment of sialic acid residues to the virion glycoprotein was probably not merely

FIG. 4. Polyacrylamide gel electrophoresis of [^{14}C]sialic acid-labeled products of undesialylated VS virions (A), of VS virions desialylated with neuraminidase (B), and of endogenous receptor in the absence of virus (C). Equivalent amounts of B virions or desialylated B virions which had been labeled with [^3H]leucine and [^3H]tyrosine were incubated in the sialyl transferase reaction mixture as described in Table 2. An identical reaction mixture was prepared except virus was not added (C). After incubation for 2 h at 37°C, proteins were precipitated in the presence of 50 μg of bovine plasma albumin with cold 20% trichloroacetic acid. The precipitate was washed with cold 10% trichloroacetic acid and cold acetone and dissolved in 10 mM sodium phosphate buffer (pH 7.4), 1% sodium dodecyl sulfate, and 1% 2-mercaptoethanol. The samples were electrophoresed on 7.5% polyacrylamide gels for 8 h. Sample C was coelectrophoresed with added, untreated ^3H -viral protein marker. The arrows mark the positions of the viral proteins L, G, N, and M. Symbols: O, [^{14}C]sialic acid; ●, [^3H]protein.

an isotope exchange reaction.

Restoration of infectivity of neuraminidase-treated VS virus by sialylation. We sought to determine if the introduction of neuraminic acid into desialylated virions would result in restoration of the infectivity which was lost upon neuraminidase treatment. We also explored the possibility that normal infectivity of VS virions might be enhanced by addition of further sialic acid above the normal level present in the glycoprotein of native, untreated VS virions.

Two identical samples, each containing 10^{11} PFU of purified B virions labeled with [^3H]amino acids, were incubated in a mixture containing Tris-hydrochloride buffer (pH 7.0) and 1 mM CaCl_2 . Virions in one sample were desialylated by neuraminidase (50 U). After incubation for 2 h, the VS-B virions in both samples were partitioned by centrifugation, washed, suspended in equal volumes of 100 mM sodium phosphate buffer (pH 6.9), and sonicated. Control virus and desialylated virions (each possessing equivalent [^3H]protein counts) were incubated in a series of tubes containing CMP-[^{14}C]sialic acid in standard sialyl transferase mixtures. [^{14}C]sialic acid incorporation into desialylated fetuin served to monitor enzyme activity while the endogenous acceptor activity was also measured.

Figure 5 and Table 3 show the detailed results of total [^{14}C]sialic acid incorporation, infectivity, and distribution of label in the viral glycoprotein, glycolipids, and endogenous acceptor at each time point. In Fig. 5A, the total sialic acid incorporation into acid-hydrolyzed fetuin and desialylated virions is approximately equal, indicating that the enzyme is the rate-limiting step in these experiments. The decline in enzyme activity after 2 h demonstrates the instability of the sonicated enzyme at 37 C. Infectivity of virions subjected to sialylation, depicted in Fig. 5B, is expressed as percent of that of control virions incubated in the transferase reaction mixture in the absence of CMP-[^{14}C]sialic acid, i.e., virions which did not incorporate exogenous sialic acid. As shown previously, neuraminidase treatment of B particles of VS virus caused a 98% decrease in infectivity. Upon resialylation of neuraminidase-treated virions, infectivity increased from 2 to 45% of the original virus preparation, which represents a 26-fold increase in infectivity (Table 3). Concomitant with this rise in infectivity was an increase in total incorporation of sialic acid into the virions (Fig. 5A and 5B). More importantly, this restoration of infectivity parallels the increase in the number of neuraminic acid residues attached to the glycoproteins of previously

FIG. 5. Effect of *in vitro* sialylation (A) on infectivity (B) of previously desialylated and control unsialylated VS virions. Purified VS-B virions (10^{11} PFU) that had been labeled with [^3H]leucine and [^3H]tyrosine were desialylated by neuraminidase (50 U). After incubation for 2 h at 37 C, the reaction was terminated by centrifugation at $100,000 \times g$ for 1 h. The pellet and supernatant fractions were partitioned, and the pellet was washed with 100 mM sodium phosphate buffer (pH 6.9), recentrifuged, suspended in the sodium phosphate buffer, and sonicated for 20 s. An equal amount of B virions was processed in an identical manner except that these virions were incubated in the absence of neuraminidase. Either 4×10^8 PFU of control virus or 8×10^7 PFU of desialylated virions in 50 μl of 100 mM sodium phosphate buffer (pH 6.9) were incubated in the standard sialyl transferase reaction mixture, which contained 0.15 mg of enzyme and 0.5 nmol of CMP-[^{14}C]sialic acid (217 counts per min per pmol). When exogenous acceptor was omitted, the volume was maintained at 0.18 ml by addition of sodium phosphate buffer (pH 6.9). Five-hundred micrograms of fetuin was used per assay. Incubations were performed at 37 C for the specified times. The incorporation of [^{14}C]sialic acid (A) was measured as described in Materials and Methods. Duplicate assays of incorporation were reproducible with an error of less than 10%. When virus was the acceptor, 100 μl from each reaction tube was used to determine infectivity (B). Total incorporation of [^{14}C]radioactivity (A) was calculated by the formula: counts per min per 50 μl $\times 3.6$. Symbols: $\bullet$, desialylated virus; $\circ$, untreated virus; $\blacksquare$, desialylated fetuin; Δ , no exogenous acceptor. Infectivity (B) is expressed as percent of the infectivity of control virus processed in an identical manner but incubated in a sialyl transferase reaction which lacked CMP-[^{14}C]sialic acid.

TABLE 3. Increased infectivity as a function of [^{14}C]sialic acid incorporated into glycoprotein of VS virus incubated with BHK-cell sialyl transferase and CMP-[^{14}C]sialic acid^a

Substrate	Time (h)	Total incorporation of [^{14}C]sialic acid (pmol) ^b	[^{14}C]sialic acid incorporation into various acceptors ^c (pmol)			Infectivity			
			Endogenous (nonviral)	Glycolipid	Viral glycoprotein	% of control virus ^d		Fold increase	
Desialylated VS virus	0					1.9	2.3	0	
	0.5	14.5	4.1	2.1	8.2	2.1	2.3	0	1.2
	1.0	29.0	4.4	3.5	21.2	9.8	9.4	4.4	5.9
	1.5	43.2	3.8	6.5	32.8	20.9	29.7	12.9	18.3
	2.0	56.5	4.0	5.7	47.0	37.1	38.3	13.7	20.0
	3.0	66.0	4.0	8.6	53.5	43.0	45.0	20.3	25.7
Untreated virus	0					100			
	0.5	10.8	4.2	2.2	4.4	103	98		
	1.0	19.9	4.1	2.5	13.3	93	106		
	1.5	25.1	4.5	3.1	17.6	104	109		
	2.0	24.7	4.0	3.7	17.0	97	100		
	3.0	25.7	3.8	3.6	18.2	107	92		

^a Preparation of desialylated VS virus and the sialyl transferase reaction mixture was performed exactly as described in the legend to Fig. 5. Equivalent amounts of virus and desialylated virus labeled with [^3H]tyrosine and [^3H]leucine were incubated in triplicate in the standard sialyl transferase reaction mixture at 37°C. At the specified time intervals, 50 μl from each tube of the triplicate series was withdrawn and used to determine the incorporation of [^{14}C]sialic acid. Infectivity was measured in duplicate employing 100 μl . The contents of the third tube of the set were used for gel electrophoresis.

^b Picomoles of [^{14}C]sialic acid incorporated were calculated from the formula: ^{14}C counts per min per 50 μl $\times 3.6 \div 217$ ^{14}C counts per min per pmol. Results were normalized to [^3H]protein counts. Triplicate assay values varied by less than 5%.

^c Distribution of [^{14}C]sialic acid-labeled products was determined by polyacrylamide gel electrophoresis as described in the legend to Fig. 4. The percent of ^{14}C counts in various regions of the gel were used to estimate the picomoles of [^{14}C]sialic acid incorporated into each acceptor by multiplying the percent of ^{14}C counts in each region of the gel by the total incorporation of sialic acid.

^d Infectivity is expressed as the percent PFU of virus incubated in the sialyl transferase reaction mixture with (experimental) or without (control) CMP-[^{14}C]sialic acid. Duplicate results are recorded.

desialylated virions (Table 3). Surprisingly, no increase in infectivity was observed after 30 min of incubation, although 8 pmol of sialic acid had been incorporated into the G proteins of neuraminidase-treated virions.

The fact that 54 pmol of sialic acid can be incorporated into the glycoproteins of 3.6×10^{10} desialylated virions (an amount of physical particles equivalent to 4×10^9 PFU of untreated virus) indicates that one particle of VS virus contains approximately 900 molecules of sialic acid located on the glycoproteins or ~ 3 molecules per glycoprotein, assuming 300 glycoprotein molecules per virion (S. U. Emerson, unpublished data). In other experiments, as much as five molecules of sialic acid could be attached to one glycoprotein. Although VS virions which were not pretreated with neuraminidase can incorporate up to 18 pmol of sialic acid (1 molecule per glycoprotein), no significant increase in infectivity ensued (Table 3).

These data support the hypothesis that sialic acid residues of the viral glycoprotein are in-

involved in the infective process. Although the VS viral G protein can be supersialylated, there appears to exist a limit to the number of sialic acid residues that can contribute to infectivity.

DISCUSSION

The data presented here indicate that the terminal sialic acids of the VS viral glycoprotein are responsible in part for initiation of infection. In support of this contention are the following observations: (i) the progressive release of sialic acid from virions by incubation with protease-free neuraminidase is accompanied by a concomitant decrease in infectivity; and (ii) the transfer of *N*-acetyl neuraminic acid onto desialylated virions resulted in reconstitution of most of the infectivity. Those sialic acid residues which are instrumental in the infective process are presumably located on the viral glycoprotein rather than on the envelope glycolipids. This conclusion is suggested by the finding that neuraminidase treatment of VS

virions, whose glycoproteins had been completely removed by trypsinization, liberated neuraminic acid from glycolipid but did not induce a further decrease in infectivity. The data also indicated that ~11% of the neuraminic acid is present in the glycolipids, whereas ~89% is protein-bound; these values are in agreement with those reported by Klenk and Coppin (11). In contrast, another envelope virus, influenza, contains neuraminidase activity, lacks sialic acid, and yet is infectious. Moreover, sialic acid could be attached to the influenza viral glycoproteins with a sialyl transferase without affecting infectivity (18).

It is reasonable to assume that the restoration of infectivity of neuraminidase-treated virions by resialylation is directly attributable to increase in amounts of sialic acid attached to the viral glycoprotein; the gradual increase in infectivity roughly paralleled the progressively greater incorporation of sialic acid into the viral glycoprotein. These neuraminic acid residues are presumably covalently linked to the glycoprotein, since sodium dodecyl sulfate and mercaptoethanol did not dissociate the sialic acid from the glycoprotein. The sialic acid transferred from CMP-[¹⁴C]sialic acid to the viral glycoprotein could be selectively removed only with neuraminidase.

The exact function of neuraminic acid residues in the infective process is still not completely clear. Since the initial event in viral infection is adsorption of virus to cell surfaces mediated by the viral glycoprotein (3), the assumption is not unreasonable that sialic acid participates in this process. The loss of infectivity upon removal of neuraminic acid residues could result either from loss of the viral carbohydrate attachment site or from change in conformation of the polypeptide portion which could itself provide the viral attachment site. The latter interpretation is not inconsistent with our results. Removal of many neuraminic acid residues lowered infectivity by only 99%, whereas trypsin treatment produced a 99.99% reduction in infectivity. Moreover, the subsequent treatment of desialylated VS virions with trypsin resulted in a further 100-fold decline in infectivity (Table 1). These data are difficult to reconcile with the supposition that neuraminic acid residues function as the sole cell attachment sites on the virion. Conceivably, the sialic acid content of the viral glycoprotein could influence the penetration of virions into cells, but several observations make this hypothesis unlikely. Heine and Schnaitman (8) have demonstrated that after VS virus cell incubation for 15 min at 37 C, the parental virion glycoprotein was found attached to cellular membranes and

did not penetrate into the cytoplasm. In addition, neuraminidase was able to lower the adsorbing capacity of VS virus to influenza virus, a reaction which would be reversed by treatment of VS virus with neuraminidase (P. Ackell and R. R. Wagner, unpublished data). The process by which glycoprotein neuraminic acid influences VS virus infectivity will be studied further by adsorption and hemagglutination.

The current hypothesis of the synthesis of viral glycoproteins is that the peptide moiety is specified by the viral genome and attachment of the carbohydrate portion is mediated by host cell glycosyl transferases. Glycoproteins of VS virus not pretreated with neuraminidase also exhibited the ability to incorporate sialic acid, which suggests that during maturation in the highly permissive BHK cell the virus is not fully sialylated. The reason for this observation is not clear, especially in view of the fact that the BHK cells in which the virus was grown contained the necessary enzyme. Variation of sialic acid content of glycopeptides has been found in VS virions propagated in different cell lines (2; S. A. Moyer and D. F. Summers, personal communication). In addition, the VS viral glycoprotein is devoid of fucose when virions are grown in L cells, whereas virus propagated in BHK cells contain fucose as part of the glycoprotein (R. H. Schloemer, unpublished data). This variability in the carbohydrate content of VS viral glycoprotein is presumably the result of virus modification by host cells.

It is interesting to speculate on the biological significance of host cell modification of the viral glycoprotein. The carbohydrate content of the G protein may influence the efficiency of viral attachment and infectivity. For example, since infectivity is related to the number of sialic acid residues on the viral glycoprotein, it is possible that the infectivity of virions produced in different cells may be proportional to the sialic acid content of the virion glycoprotein. Furthermore, the extent of hemagglutinating activity of VS virions propagated in different cell lines varies at least tenfold (J. J. McSharry, personal communication), the cause of which could reside in the carbohydrate moiety of the glycoprotein since only this portion is host-cell specified. Experiments are currently being conducted to determine if a correlation exists between the degree of biological activity associated with the viral glycoprotein and the sugar composition of the glycoprotein from virions grown in various cells.

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