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Inhibition of Reovirus Type 3 Binding to Host Cells by Sialylated Glycoproteins Is Mediated through the Viral Attachment Protein

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The interaction of mammalian reoviruses with sialylated glycoproteins was studied and found to be highly serotype specific in that attachment of type 3 Dearing reovirus to murine L cell receptors could be strongly inhibited by bovine submaxillary mucin (BSM), fetuin, and alpha$_1$ acid glycoprotein, albeit at different efficiencies, whereas attachment of type 1 Lang reovirus was inhibited only by fetuin. We subsequently demonstrated, by using reassortants between type 3 and 1 reoviruses, that inhibition of reovirus attachment to cell receptors was specified by the viral attachment protein gene *S1*. Using a solid-phase binding assay, we further demonstrated that the ability of reovirus type 3 or reassortant 1HA3 and the inability of reovirus type 1 or reassortant 3HA1 to bind avidly to BSM was a property of the viral *S1* genome segment and required the presence of sialic acid residues on BSM oligosaccharides. Taken together, these results demonstrated that there is a serotype-specific difference in the ability of the reovirus attachment protein, sigma 1, to interact with sialylated oligosaccharides of glycoproteins. Interaction of reovirus type 3 with sialylated oligosaccharides of BSM is dramatically affected by the degree of *O*-acetylation of their sialic acid residues, as indicated by the findings that chemical removal of *O*-acetyl groups stimulated reovirus type 3 attachment to BSM, whereas preferential removal of residues lacking or possessing reduced amounts of *O*-acetyl groups per sialic acid molecule with *Vibrio cholerae* sialidase abolished binding. We also demonstrated that BSM was 10 times more potent in inhibiting attachment of infectious reovirus to L cells than was *V. cholerae*-treated BSM. The results are consistent with the hypothesis that sialylated oligosaccharides on host cells or erythrocytes may act as binding sites or components of binding sites for type 3 reovirus through a specific interaction with the virus attachment protein.

Recently a β-adrenergic receptorlike membrane glycoprotein has been identified as an in vivo receptor for type 3 reovirus (5, 6). This glycoprotein is found on cells from many species and it has been proposed that its presence may account for the in vivo neural cell tropism of type 3 Dearing reovirus (5).

Several other lines of evidence suggest that oligosaccharides on host cell and erythrocyte glycoproteins and perhaps glycolipids may play a role in the interaction of mammalian reovirus type 3 with surface receptors. It has recently been shown that interaction of type 3 Dearing reovirus or cell extracts containing soluble sigma 1 polypeptide with murine L cell receptors could be dramatically decreased by mild oxidation of cells under conditions that are relatively specific for carbohydrate, suggesting a requirement for these compounds for attachment (2). In a study from this laboratory, it was found that treatment of murine L cells with bacterial sialidases (neuraminidases) dramatically reduced their ability to function as binding sites for type 3 reovirus. Both the initial attachment rate and the amount of virus bound at saturation were reduced (11, 12). In addition, it was found that purified sialic acid-containing glycoproteins, but not other glycoproteins, as well as free sialic acid and sialyllactose, were potent inhibitors of viral attachment to L cells (12). It has also been observed that the sialoglycoprotein (SGP) glycophorin is capable of inhibiting attachment of soluble type 3 reovirus sigma 1 polypeptide to L cells (R. W. Paul and P. W. K. Lee, submitted for publication).

Another line of evidence comes from studies indicating that agglutination of certain kinds of erythrocytes is sensitive to pretreatment of cells with protease or sialidase (2, 13), suggesting that erythrocyte receptors for reovirus type 3 may be sialylated glycoproteins. Furthermore, biochemical evidence has been presented that glycophorin, a major SGP of the erythrocyte membrane, is a receptor for reovirus type 3 (Paul and Lee, submitted). Among other sialic acid-binding viruses, such as certain members of the myxo- and paramyxoviruses, a strong correlation has been found between binding of the virion attachment protein to sialylated glycoconjugates of erythrocytes or purified SGPs and a requirement for utilization of sialyloligosaccharides as receptor components for infection of host cells (4, 10, 14, 16, 25, 26). Taken together, and by analogy with sialic acid-binding viruses, the available evidence is consistent with a role for oligosaccharides in reovirus attachment to host cells and erythrocytes. Whether oligosaccharides on glycoproteins or glycolipids function as receptor sites for infection of host cells by reovirus type 3 or play some other role in attachment to host cells is not clear. In this regard we have recently shown that desialylation of host cell oligosaccharides with sialidase renders these cells resistant to reovirus type 3 infection, lending support to the notion that sialoglycoconjugates play a role in the infectious process (J. R. Gentsch, manuscript in preparation).

Although available evidence suggests that host oligosaccharides play a role in reovirus type 3 binding to host cells, most of it does not address the question of the virus capsid component involved. Since the sigma 1 polypeptide is known to be the virus component involved in interaction with host cell receptors (23) and is a major determinant of viral tropism in vivo (45, 46), it was important to determine whether it was also involved in the interaction of reovirus type 3 with sialoglycoconjugates. In this study, we demon-

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strated that the reovirus attachment protein, sigma 1 polypeptide, interacts specifically and avidly with sialyloligosaccharides on sialylated glycoproteins.

MATERIALS AND METHODS

Abbreviations. AGP, alpha$_1$ acid glycoprotein; BSM, bovine submaxillary mucin; Fet, fetuin; TN buffer (25 mM Tris hydrochloride [pH 7.2], 150 mM NaCl); TN-BSA, TN containing 3% bovine serum albumin (BSA); AB (10 mM Tris hydrochloride [pH 7.2], 5 mM KCl, 150 mM NaCl, 0.5% BSA); DB, digestion buffer (10 mM Tris hydrochloride [pH 7.2], 150 mM NaCl, 1.8 mM $CaCl_2$, 0.81 mM $MgCl_2$); DB-BSA, DB containing 0.5% BSA; BB, binding buffer (140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.81 mM $MgCl_2$, 10 mM *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid buffer [pH 7.2], 1 mg of BSA per ml, 0.001% phenol red).

Chemicals and enzymes. All SGPs used in this study were purchased from Sigma Chemical Co. (St. Louis, Mo.). *Arthrobacter ureafaciens* sialidase was from Calbiochem-Behring (La Jolla, Calif.), *Clostridium perfringens* sialidase (type X) and sialic acid (98% pure) were from Sigma. Enzymobeads were from Bio-Rad Laboratories (Richmond, Calif.). L-[^{35}S]methionine was from Amersham Corp. (Arlington Heights, Ill.), and Eagle minimal essential medium (Joklik modificaton), methionine-free minimal essential medium, Dulbecco modified minimal essential medium, and medium 199 were from GIBCO Laboratories (Grand Island, N.Y.). Nitrocellulose (BA85; 0.45-μm pore size) was obtained from Schleicher & Schuell, Inc. (Keene, N.H.), and 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid was from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Scintiverse II was obtained from Fisher Scientific Co., (Pittsburgh, Pa.).

Cells and viruses. Growth of cells and viruses and preparation of ^{35}S-labeled reoviruses have been described in detail previously (30). The genotypes of parental and reassortant viruses were verified by analysis of their RNA segments by polyacrylamide gel electrophoresis as described previously (30) except that segments were visualized by silver staining (27).

Attachment inhibition experiments. Experiments were done essentially as described previously (12). Briefly, L cells growing in suspension were pelleted and washed once in BB complete, suspended in BB, distributed to 1.5-ml microcentrifuge tubes (4×10^5 cells per tube), and pelleted for 3 s; the supernatant was removed, and the cell pellets were equilibrated at 4°C for 15 min. To each tube was added 50 μl of BB containing 0.5 or 1.0 μg of ^{35}S-labeled reovirus type 1, 3, 1HA3, or 3HA1 and various concentrations of SGP inhibitors. Virus-inhibitor mixtures were made ahead of time and also preincubated for 15 min at 4°C before addition to cells. Tubes were then vortexed and incubated at 4°C for 1 h with frequent mixing. Samples were diluted to 0.3 ml with BB which had been pre-equilibrated at room temperature (RT), pelleted, and washed once with 0.5 ml of BB, and cell-associated, trichloroacetic acid (TCA)-precipitable radioactivity was measured as described previously (12). Although not measured for every experiment, the counts per minute recovered in the supernatant plus those in the pellet were greater than or equal to 93% of the total added counts per minute. The specific activity of virus preparations generally varied from 20,000 to 60,000 cpm/μg of viral protein and was 90% TCA precipitable. Control binding generally varied from 50 to 80% of the total TCA-precipitable counts per minute added, except for clone 3HA1, with which 20 to 25% became cell associated.

Alkaline hydrolysis of BSM. Sialic acid residues of BSM are *O*-acetylated at hydroxyl groups on carbon atoms C7, C8, and C9 (36, 43). To remove these acetyl groups we dissolved BSM in H_2O and added 0.4 N NaOH to final concentrations of 0.1 N NaOH and 5 mg BSM per ml and then incubated the mixture at 4°C for 45 min and neutralized it with 0.4 N HCl (34, 36). This solution was dialyzed for 18 h against 4 liters of deionized H_2O with one change of the water and then lyophilized and stored desiccated at −20°C until used. Mock-treated BSM was incubated in 0.1 M NaCl and then dialyzed and lyophilized as described above.

Effect of SGPs on reovirus type 3 infectious-center formation. For infectious-center formation, de-*O*-acetylated or *V. cholerae*-treated BSM was suspended in BB and adjusted to pH 7.2, and a series of 10-fold dilutions were made in the same buffer. Sufficient type 3 reovirus was then added to produce 10^6 PFU/0.1 ml of virus-inhibitor mixture. After 15 min of preincubation at 4°C, 0. 1 ml of the mixture was added to cell pellets containing 5×10^5 murine L cells, and incubation was continued for 60 min at 4°C with frequent mixing. The samples were then centrifuged for 3 s in an Eppendorf microcentrifuge, the supernatant was removed, and the pellet was washed once with 0.5 ml of cold BB and once with 0.5 ml of RT binding buffer. We then suspended the pellet in 1 ml of gel-saline, carried out a series of 10-fold dilutions in the same buffer, and plated the samples on murine L cell monolayers (9) to determine the number of cells acting as infectious centers (1).

Sialic acid content of inhibitors. We dissolved inhibitors in deionized water, diluted 50- to 500-μg samples to 0.25 ml with deionized H_2O, mixed them with an equal volume of 0.2 N sulfuric acid, and then hydrolyzed them for 60 min at 80°C to release bound sialic acid. Sialic acid standards were treated identically. Free sialic acid was then measured by a modification of the thiobarbituric acid method (31, 44) as described previously (12). The following values, expressed in nanomoles of sialic acid per mg of inhibitor, represent the mean of two determinations: BSM, 174; AGP, 265; Fet, 137; transferrin, 35; and BSA, 3; ovalbumin was not detectable in this assay. Glycophorin has a reported content of 809 nmol/mg (22). A second lot of BSM, reported by the manufacturer to have an increased sialic acid content (388 nmol/mg) had a content of 374 nmol of sialic acid per mg by the Jourdian assay (21) or 332 nmol/mg by the Warren assay, in basic agreement with the estimate of the manufacturer. We treated sialic acid residues of bovine mucin with 0.1 N NaOH for 45 min at 4°C and then neutralized them with HCl before acid hydrolysis (34, 36) to remove *O*-acetyl groups which interfere with the assay of sialic acid.

Sialidase treatment of BSM. BSM (32 mg; 374 nmol of sialic acid per mg) was suspended in 2.4 ml of DB, 500 international mU (1 mU = 1 nmol of product released per min at 37°C and optimal pH) of *V. cholerae* sialidase was added, and the pH was adjusted to 7.2. Treatment was then carried out for 8 h at 37°C, the enzyme was inactivated by heating in a boiling water bath for 3 min, and aliquots were frozen, lyophilized, and stored desiccated at −20°C until use. The glycosidically bound sialic acid content of this preparation was 226 nmol/mg. For estimation of sialic acid removed in situ by *A. ureafaciens* sialidase, BSM was spotted on nitrocellulose (10 μg/4 μl spotted), air dried, and digested with 7 ml of DB containing 10 mU of the enzyme per ml for 90 min at 37°C. A trace of [^{14}C]sialic acid was then added to the supernatant fraction, which was then lyophilized, suspended in 1 ml of distilled water, and desalted on Biogel P2 (10) as described previously (12). Recovery was

determined from the amount of [^{14}C]sialic acid recovered and generally varied from 75 to 95%. We estimated with the thiobarbituric acid assay that about 70% of the glycosidically bound sialic acid of BSM was released by this in situ digestion procedure.

Determination of *O*-acetyl content of BSM preparations. The *O*-acetyl content of sialic acid residues on BSM were estimated by the Ludowieg and Dorfman (24) modification of the Hestrin (20) procedure as described by Schauer (35). Briefly, samples containing 0.2 to 2.0 μmol of ester (5 mM ethyl acetate in MeOH:H_2O [1:1] was used as the standard) were suspended in 0.4 ml of distilled H_2O, combined with 0.4 ml of alkaline hydroxylamine (0.175 M hydroxylamine hydrochloride, 0.75 M NaOH prepared fresh each time by mixing equal volumes of 0.35 M hydroxylamine hydrochloride and 1.5 M NaOH), vortexted, and incubated for 10 min at RT. To this mixture was added 0.4 ml of 0.75 M perchloric acid, the solutions were vortexted, and 0.2 ml of 0.07 M ferric perchlorate in 0.4 M perchloric acid was added. After vortexing, optical densities were read at 520 nm on a Gilford model 250 spectrophotometer. For reactions in which glycoprotein was present, we centrifuged the reactions mixtures at 1,000 × *g* for 20 min at 4°C to remove denatured protein. In reactions in which there was significant turbidity due to denatured glycoprotein in the reaction, we made a background subtraction by preparing an identical set of glycoprotein samples and leaving out the hydroxylamine reagent (but not NaOH). The optical density readings of these samples were subtracted from that of the corresponding reaction containing hydroxylamine.

Iodination of purified reoviruses. Purified viruses were dialyzed for 15 to 18 h against 0.2 M sodium phosphate, pH 7.2, and 1 mg of each was iodinated with 1 mCi of Na^{125}I (New England Nuclear Corp., Boston, Mass.) by the lactoperoxidase method with Bio-Rad enzymobeads. The procedure was as the manufacturer recommended except that iodination was for 15 min at RT. Virus was separated from unreacted iodine on PD-10 columns (Pharmacia Fine Chemicals, Piscataway, N.J.), which had been pre-equilibrated in 0.2 M sodium phosphate buffer containing 0.5% BSA and then re-equilibrated in the same buffer without BSA. Recovery was about 70%. Viruses were stored at 4°C and used within 2 weeks. Specific activities were approximately 8 × 10^5 and 4 × 10^5 TCA-precipitable counts per minute per μg of viral protein for type 1 and 3 reoviruses, respectively.

Binding of reoviruses to immobilized SGPs. We used a modification of the antigen spot test to bind reoviruses to immobilized SGPs (7, 18). SGPs were dissolved in TN buffer at 5 to 50 mg/ml, dilutions were made in the same buffer, the samples were spotted onto dry nitrocellulose paper (3 or 4 μl per spot) and air dried, and excess protein-binding sites were blocked with TN-BSA for 1 to 2 h at RT or 15 to 17 h at 4°C. The strips or sheets of nitrocellulose were then rinsed once with AB, 5 × 10^5 to 5 × 10^6 cpm of ^{35}S- or ^{125}I-labeled virus (diluted in AB) was added, and incubation was carried out for 1 to 5 h at RT. The samples were then washed three times for 10 min each with BSA-free AB, air dried, and subjected to autoradiography at −80°C with Kodak XAR-5 medical X-ray film. For sialidase treatment of BSM before binding assays, 9- or 10-μg samples of BSM were spotted on nitrocellulose, air dried, blocked for 2 h with TN-BSA, and then treated with AB containing 0 to 50 mU of *A. ureafaciens* sialidase per ml for 90 min at 37°C. The individual strips were incubated with 3 × 10^5 cpm of type 3 reovirus in 3 ml of AB for 2 h at RT, washed as described above, dried, and autoradiographed. It was estimated that concentrations of sialidase which resulted in complete abolition of subsequent type 3 reovirus attachment (10 mU/ml) removed about 1 nmol of sialic acid per 10 μg of BSM spotted (approximately 68% of the total glycosidically bound sialic acid). When 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid was used as a competitive inhibitor of sialidase, the inhibitor stock solution was made in DB-BSA and adjusted to pH 7.2, dilutions were made in the same buffer, and sialidase was added at 0.5 mU/ml. After 15 min at 37°C, the sialidase–2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid mixture was added to tubes containing nitrocellulose strips with BSM spotted on them, and digestion was carried out as described above. Strips were then washed five times and probed with type 3 reovirus as described above. For binding to modified BSM (see Fig. 5), we treated native BSM with 0.1 N NaOH to deacetylate sialic acid residues (36) or treated it with *V. cholerae* sialidase either alone or followed by treatment with 0.1 N NaOH, all as described above, before spotting it onto dry nitrocellulose. We quantitated nonspecific and specific binding by cutting the nitrocellulose into squares (approximately 10 to 12 mm^2), dissolving it in Scintiverse II scintillation fluid, and counting it in a Beckman LS 7500 scintillation counter.

RESULTS

Genetic mapping of the interaction of reoviruses with SGPs. Recently, we showed that the outer capsid of reovirus type 3 interacts with sialyloligosaccharides on host cells and purified glycoproteins (12), but we were not able to determine the virus capsid protein(s) involved. The glycoproteins we used were chosen based on the similarity of their sialyloligosaccharide components to those found on mammalian cell membrane glycoproteins. To determine the capsid protein specificity of these interactions, we searched for SGPs that would not inhibit type 1 reovirus attachment to L cells among those previously demonstrated to be effective inhibitors of type 3 reovirus attachment (12). One such glycoprotein is BSM, a highly sialylated glycoprotein whose major carbohydrate component is an *O*-linked disaccharide, sialyl alpha-2,6-galactosamine (15). Reovirus type 1 attachment was inhibited very little (maximum inhibition was 25% at 20 mg/ml, the highest concentration tested in any experiment) at concentrations up to 10 mg/ml, whereas type 3 reovirus attachment was effectively inhibited at concentrations below 2 μg/ml (Fig. 1). Note that BSM concentrations were expressed in milligrams per milliliter since an accurate molecular weight for this compound was not available. We calculated that the amount of BSM required to reduce type 3 reovirus attachment to 50% of maximal was at least 500- to 1,000-fold less in most experiments than that required for type 1 reovirus (the endpoint for type 1 reovirus was not reached in this or any other experiment). As observed previously, maximal inhibition was 80 to 90% for BSM versus type 3 reovirus.

To determine whether this effect was due to a differential interaction between BSM and the virion sigma 1 polypeptide, we measured the ability of BSM to inhibit the attachment of reassortant reoviruses 3HA1 and 1HA3. The origin of these viruses has been described previously (46). The attachment of reassortment 1HA3, which possesses nine genes derived from a type 1 reovirus parent and the attachment (HA) protein gene from a type 3 reovirus parent, was strongly inhibited by BSM, whereas attachment of the reassortant containing nine type 3 reovirus genes and the attachment protein from type 1 reovirus (3HA1) was not

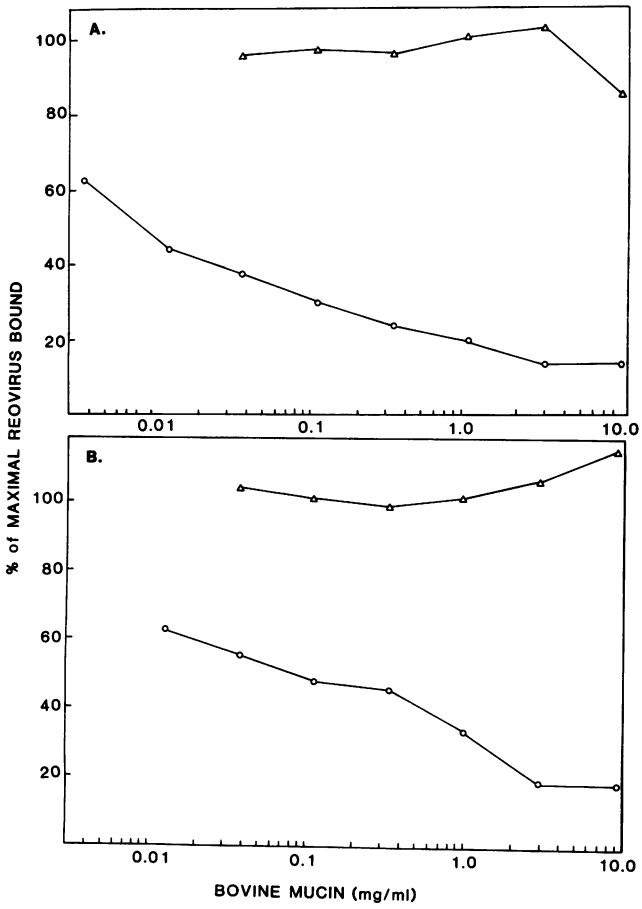

FIG. 1. Inhibition of reovirus attachment to murine L cells by BSM. Samples of [^{35}S]methionine-labeled reoviruses (0.5 μg/4 × 10^5 cells) were mixed with different concentrations of BSM (0 to 10 mg/ml) and preincubated for 15 min at 4°C and then 50 μl of each mixture was added to two tubes, each containing 4 × 10^5 cells. Samples were then mixed and incubated for 2 h at 4°C with frequent mixing, diluted to 300 μl with binding buffer, pelleted, and washed once with the same buffer, and the TCA-precipitable and cell-associated radioactivity was measured as described previously (12). Each experiment was performed two to four times. Control binding varied from 4,000 to 30,000 cpm depending on the specific activity of the virus preparation. Although not measured in all experiments, the recoverable counts per minute in the supernatant plus the cell pellet were usually greater than 93% of the total added counts per minute. Panel A: ^{35}S-labeled reovirus type 1 (△); ^{35}S-labeled reovirus type 3 (○). Panel B: ^{35}S-labeled reovirus 1HA3 (○); ^{35}S-labeled reovirus 3HA1 (△).

inhibited at all (panel B). This result demonstrated that it is primarily the virion sigma 1 polypeptide that specifies the ability to interact with SGPs. Previous results demonstrated that it is primarily the sialyloligosaccharide residues that virus particles interact with and not the protein backbone (12).

It is worth noting that in this series of experiments the inhibition endpoints (50 % of maximal) were not identical for type 3 and 1HA3 reovirus. Although a very small difference was observed (Table 1) in subsequent experiments, we cannot rule out that the *M2* and *S4* genes could have a modulating effect on the interaction of sigma 1 polypeptide with BSM. Nevertheless, these experiments taken together

TABLE 1. Effect of Fet, AGP, and BSM on reovirus type 1, 2, 3, 1HA3, and 3HA1 attachment to murine L cells

Reovirus	nmol of inhibitor sialic acid for 50% inhibition of maximal attachment[a]		
	Fet	BSM	AGP
Type 1	170	≥159[b]	≥547[b]
3HA1	153	≥159[c]	≥547[b]
Type 3	75	0.1 (1.8)[d]	130
1HA3	91	0.8 (2.5)	99

[a] Represents the amount of glycosidically bound glycoprotein inhibitor sialic acid required to inhibit the attachment of 1 μg of virus to 4 × 10^5 L cells by 50%. Counts per minute bound to control cells are given in the legends to Fig. 1 and 2.

[b] Maximum inhibition was 10 to 25%.

[c] There was no inhibition at the highest concentration used.

[d] Values in parentheses were determined in an independent experiment by using different virus and BSM preparations.

showed that only type 3 and 1HA3 reovirus attachment to murine L cell receptors are significantly inhibited by BSM.

In further experiments, we analyzed the ability of other soluble SGPs to inhibit attachment of type 1 and 3 reoviruses to L cells (Fig. 2). The results shown in panel A demonstrated that AGP acts similarly to BSM in that it inhibits type 3 and 1HA3 reovirus but not type 1 and 3HA1 reovirus

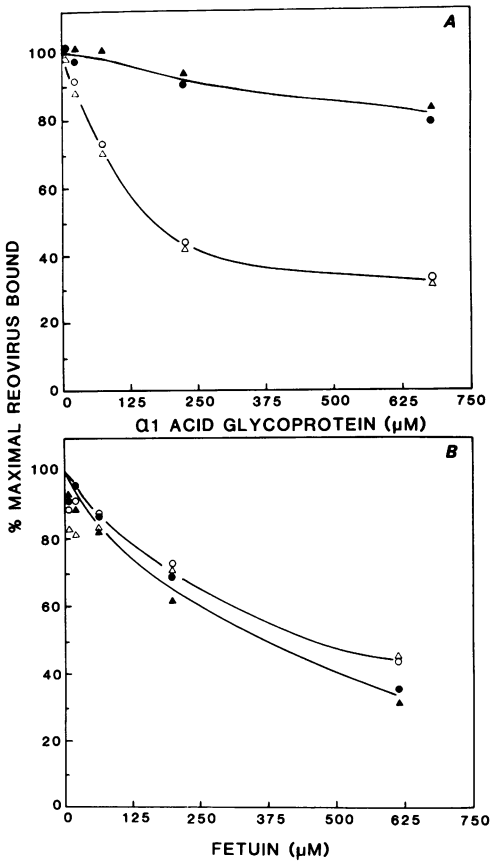

FIG. 2. Inhibition of reovirus attachment by Fet and AGP. The experiments were carried out essentially as described in the legend to Fig. 1. Control binding varied from 10,000 to 13,000 cpm for the different virus preparations. Panel A: type 1 reovirus plus AGP, (●); type 3 reovirus plus AGP (○); 1HA3 reovirus plus AGP (△); 3HA1 reovirus plus AGP (▲). The symbols in panel B designate the same viruses, but Fet was the SGP inhibitor.

attachment. In contrast to these results, Fet acts as an inhibitor of type 1, type 3, and both reassortant reoviruses. This result was important because it demonstrated that the type 1 reovirus sigma 1 polypeptide is capable of direct interaction with SGPs. Type 2 reovirus interaction with SGPs was also examined and found to produce an inhibition pattern quite similar to that of type 1 and 3HA1 reoviruses (data not shown).

To compare the relative inhibitory potency of these SGPs directly, we expressed their potency as the amount of inhibitor, glycosidically bound sialic acid, required to inhibit reovirus attachment by 50%. When this was done, it was found that BSM was at least 73- and 40-fold more potent than AGP as an inhibitor of type 3 and 1HA3 reovirus attachment, respectively (Table 1). A similar result was found when the potency of BSM for inhibition of type 3 or 1HA3 reovirus attachment was compared with that of Fet for inhibition of the same two viruses. The potency of Fet for inhibition of type 1 or 3 HA1 reovirus attachment was essentially the same (within two-fold) as the potency of Fet and AGP as inhibitors of type 3 or 1HA3 reovirus attachment. The estimates involving the inhibitor potency of BSM represent a minimum difference in potency since the highest BSM endpoints for the type 3 and 1HA3 reoviruses (1.8 and 2.5 nmol) were used to calculate the differences.

Binding of reovirus types 1 and 3 to SGPs. To determine whether the inhibitory effects of these SGPs were due to stable binding between inhibitor and virus, we developed a solid-phase binding assay which was a modification of the antigen spot test (18). In this assay, inhibitors were spotted on nitrocellulose paper and then probed with different ^{35}S- or ^{125}I-labeled reoviruses. For BSM, we observed that there was a direct correlation between potency as an attachment inhibitor and ability of parental and reassortant reoviruses to bind stably to immobilized BSM (Fig. 3A). Whereas significant type 3 and 1HA3 reovirus binding above background could be detected at BSM concentrations less than or equal to 0.1 μg (0.015 nmol glycosidically bound; inhibitor, sialic acid), little type 1 and 3HA1 reovirus binding could be detected at concentrations up to 240 times greater. Maximal specific type 1 or 3HA1 reovirus binding was only 7% of maximal type 3 or 1HA3 reovirus binding. Thus, the type 3 virion sigma 1 polypeptide interacts with BSM much more avidly than does the type 1 sigma 1 polypeptide.

To determine whether removal of terminal sialic acid residues affects this interaction, we measured type 3 reovirus binding to BSM which had been treated with *A. ureafaciens* sialidase (panel B). We found that treatment with as little as 0.5 mU of sialidase per ml reduced subsequent attachment 80%, whereas higher concentrations reduced specific attachment 95%. This result is consistent with earlier findings that sialic acid residues are essential for type 3 reovirus interaction with glycoproteins (12).

We also measured the relative efficiency of attachment of ^{125}I-labeled type 3 or 1 reovirus to other SGP inhibitors by the solid-phase binding assay (Fig. 4). Consistent with its reduced potency as a cell attachment inhibitor compared with BSM, stable binding of type 3 reovirus to Fet was much less efficient. Type 3 reovirus also bounds effectively to human glycophorin, a highly sialylated (22), integral membrane glycoprotein of erythrocytes. A single dose of glycophorin inhibited type 3 attachment to murine L cells by 70%, but we did not construct an inhibition curve for this protein (Gentsch, unpublished data). The limits of detection of type 3 reovirus binding to Fet and glycophorin in this experiment were approximately 9 and 1 nmol of glycosidically bound inhibitor sialic acid, respectively (66.7 and 1.4 μg spotted). The limit of detection for type 3 reovirus binding to BSM was 0.01 nmol (data not shown), suggesting that the virus has a much higher affinity for BSM oligosaccharides. Also see Fig. 3A, where the endpoint for type 3 reovirus binding to BSM is 0.1 μg (≤0.015 nmol). Type 3 reovirus bound very inefficiently to AGP and not at all to transferrin, both of which are sialylated, and not at all to a poorly sialylated glycoprotein, ovalbumin, or to BSA, a nonglycosylated protein. Results similar to those shown in Fig. 4 were obtained with [^{35}S]methionine-labeled virus preparations (data not shown). On some occasions, stable binding to AGP was observed, but it was always very weak compared with type 3 reovirus binding to BSM. The reason that AGP works as efficiently as Fet in an attachment inhibition assay (Table 1) but binds to reovirus type 3 less well in a solid-phase binding assay is not known.

Type 1 reovirus bound efficiently only to Fet, in keeping with its being the only SGP that significantly inhibited type 1

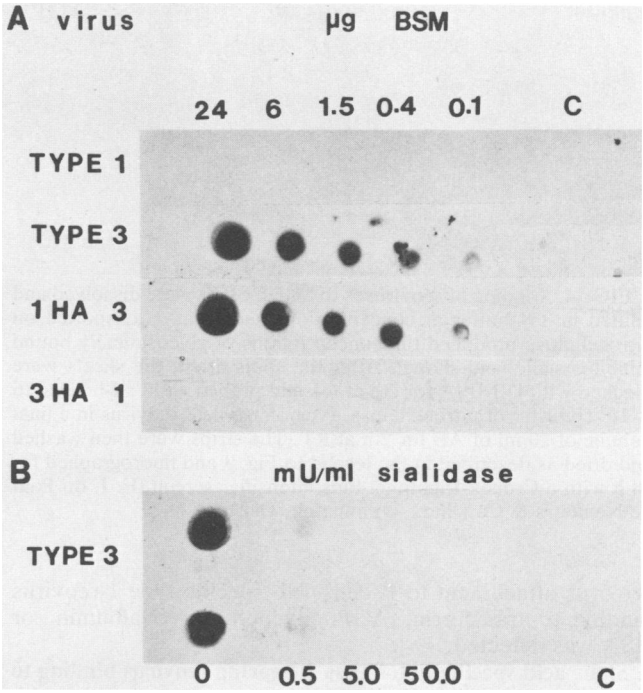

FIG. 3. Binding of reoviruses to BSM. (A) BSM was dissolved in TN buffer at 8 mg/ml, and a series of fourfold dilutions were prepared in the same buffer. Each dilution (3 μl) was spotted on nitrocellulose and air dried, and excess protein-binding sites were blocked with DB-3% BSA overnight at 4°C. The nitrocellulose strips were then washed once with AB and incubated for 4 h at RT with 5 ml of AB containing 10 μg (specific activities of these preparations varied from 1.8×10^4 to 4.1×10^4 cpm/μg) of individual virus preparations per ml. Nitrocellulose strips were then washed in AB three times for 10 min each on a rocker platform, air dried, and autoradiographed for 60 h. Maximal specific binding, 4,915 cpm/11 mm^2; mean background binding, 203 cpm/11 mm^2. (B) Samples (9 μg) of BSM were spotted on nitrocellulose, air dried, and blocked as described above. Identical strips containing two BSM spots each were then cut, and the strips were incubated with DB-BSA containing various amounts of *A. ureafaciens* sialidase for 90 min at 37°C. The strips were then washed five times in AB, probed with type 3 reovirus, washed, dried, and autoradiographed for 120 h, all as described above. C, Control (area of sheet where no BSM was spotted). Maximal specific binding, 1,633 cpm; background binding, 123 cpm per strip of nitrocellulose.

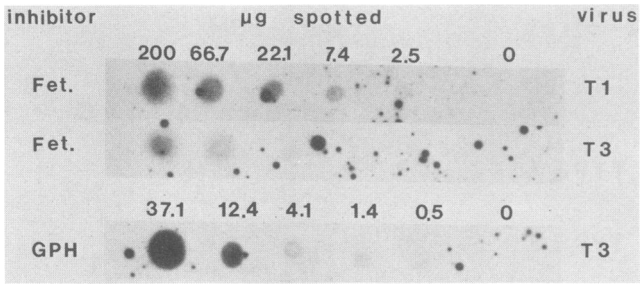

FIG. 4. Binding of reoviruses to SGPs. SGPs were dissolved and diluted in TN buffer at concentrations such that 3 μl spotted on nitrocellulose produced the concentrations of glycosidically bound inhibitor sialic acid shown. After the spots dried, the sheets were blocked with DB-BSA for 2 h at RT and probed with ^{125}I-labeled (6×10^5 cpm/cm^2 of nitrocellulose) type 1 or type 3 reovirus in a final volume of 20 ml of AB for 2 h at RT. The strips were then washed and dried as described in the legend to Fig. 3 and fluorographed for 48 h with a Cronex Lightning-Plus intensifier screen (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.).

reovirus attachment to L cells. No specific type 1 reovirus binding to transferrin, AGP, glycophorin, ovalbumin, or BSA was detected.

Sialic acid specificity for type 3 Dearing reovirus binding to BSM. We observed (Fig. 3B) that treatment of BSM with very low concentrations of sialidase completely abolished its affinity for type 3 reovirus, suggesting that the presence of sialic acid on inhibitor oligosaccharides is essential for this interaction. However, when we determined the amount of sialic acid removed at *A. ureafaciens* sialidase concentrations that would cause this effect (10 mU/ml in this case) we estimated that about 70% of the total was removed (this would leave about 1,600 pmol of sialic acid per 9 μg of BSM spotted). Although this suggests that sialic acid is important in this interaction, we were nonetheless surprised that this resulted in complete abolition of type 3 reovirus binding, since the experiment shown in panel A demonstrated that binding could be detected at concentrations as low as 0.1 μg of BSM (15 pmol of sialic acid). Since BSM contains a high percentage of *O*-acetylated sialic acid residues which are partially resistant to hydrolysis by bacterial sialidases (36, 43), it is plausible that the conditions of our treatment were not extensive enough to remove *O*-acetylated sialic acid residues completely and that type 3 reovirus may not bind or only bind weakly to oligosaccharides in which the terminal sialic acid residues contain *O*-acetyl groups.

To test this hypothesis, we analyzed the effect of removal of *O*-acetyl groups from BSM sialic acid residues on subsequent reovirus attachment (Fig. 5, rows 1 and 2). Mild base treatment of BSM resulted in a dramatic increase in its affinity for type 3 reovirus. For native BSM, the least amount of inhibitor which could be spotted and still detect type 3 reovirus binding above background corresponded to approximately 10 pmol of glycosidically bound sialic acid, whereas for deacetylated BSM it was ≤1.0 pmol. Furthermore, at low concentrations of BSM (10^2 or 10^1 pmol spotted), the amount of type 3 reovirus binding to native BSM was only 13 and 2% of binding to corresponding amounts of deacetylated BSM as determined by scintillation counting of 10-mm^2 pieces of nitrocellulose (data not shown). At higher concentrations (10^3 or 10^4 pmol spotted), binding to native BSM was approximately 50% of binding to de-*O*-acetylated BSM.

To gain additional evidence, we treated a sample of BSM with *V. cholerae* sialidase, which has been reported to be able to remove a maximum of 55% of BSM sialic acid because of its slow rate of hydrolysis of *O*-acetylated residues (43). In those same experiments, after treatment with *V. cholerae* sialidase this enzyme preferentially removed sialic acid residues lacking *O*-acetyl groups or mono-*O*-acetylated sialic acid residues while demonstrating very little activity against de-*O*-acetylated residues (43). In the sample used here, we estimated that about 40% of the total sialic acid was removed (data not shown). When we probed this BSM sample with type 3 reovirus (row 3), we observed no specific binding, even though the highest concentration spotted was equivalent in glycosidically bound sialic acid to native or deacetylated BSM. To determine whether it was the decrease in sialic acid content which resulted in the failure of type 3 reovirus to bind, we treated a portion of the *V. cholerae*-treated sample with 0.1 N NaOH to de-*O*-acetylate the remaining sialic acid residues, spotted in on nitrocellulose, and then probed it with type 3 reovirus. Type 3 reovirus bound almost as avidly to base-treated, *V. cholerae* sialidase-treated BSM (row 4) as it did to native, base-treated BSM. In quantitative terms, type 3 reovirus binding to sialidase-treated, deacetylated BSM varied from

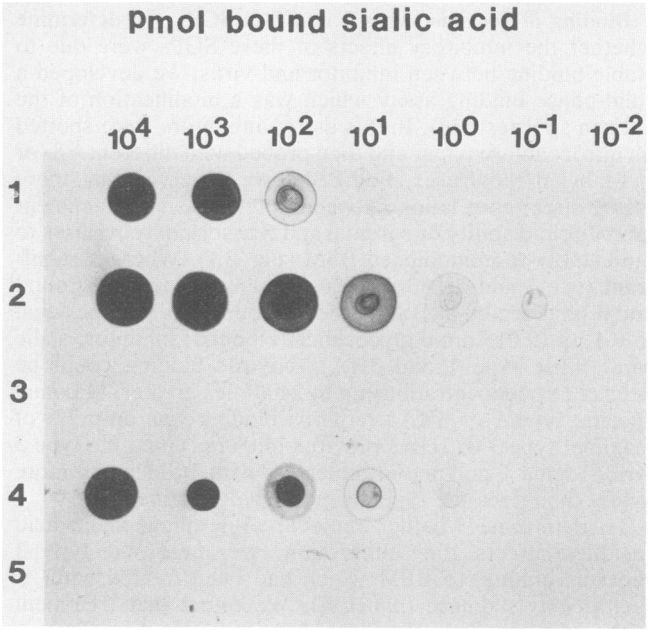

FIG. 5. Binding of type 3 reovirus to modified BSM. Native or modified BSM preparations were dissolved in TN at a concentration sufficient to produce 10^4 pmol of glycosidically bound sialic acid per 4 μl. A series of 10-fold dilutions of each were made in TN, and 4 μl of each dilution was spotted onto dry nitrocellulose. After air drying, the sheet of nitrocellulose was blocked for 1 h at RT with TN-BSA, rinsed once with AB buffer, and probed with 20 ml of AB containing 5×10^6 cpm of ^{35}S-labeled reovirus type 3 (31,000 cpm/μg of protein). After 90 min at RT, the sheet was washed, dried, and autoradiographed for 92 h as described in the legend to Fig. 3. Row no: 1, Native BSM; 2, BSM treated with 0.1 N NaOH for 45 min at 4°C; 3, BSM treated with *V. cholerae* sialidase; 4, the same *V. cholerae* sialidase-treated BSM preparation as in row 3 subsequently treated with 0.1 N NaOH for 45 min at 4°C (details of the treatments are described in Materials and Methods); 5, no sample spotted (used as a control for nonspecific binding of type 3 reovirus to nitrocellulose). Mean nonspecific binding (row 5), 299 cpm/11 mm^2. maximal specific binding: 6,427 cpm (row 1); 12,979 cpm (row 2); 0 cpm (row 3); and 10,568 cpm (row 4).

TABLE 2. Effect of modified BSM on infectious reovirus type 3 binding to murine L cells

Inhibitor (concn [mg/ml])	No. of infectious centers per 5×10^5 cells in expt no.[a]:				Mean (±SEM) $\log_{10}$ reduction in infectious centers
	1	2	3	4	
None	2.6×10^5	1.8×10^5	2.4×10^5	1.0×10^5	
De-BSM[b] (5)	1.9×10^3	2.9×10^2	1.5×10^3	2.1×10^3	2.2 ± 0.5
VC-BSM[c] (5)	2.8×10^4	4.5×10^4	2.3×10^4	1.4×10^4	0.9 ± 0.2

[a] Inhibitors were mixed with BB and 10^6 PFU of type 3 reovirus per 100 μl of mixture and then 100 μl was added to 5×10^5 L cells. At the end of 60 min, inhibitors and unbound virus were washed away, and the cells were diluted and plated on L cell monolayers as described in Materials and Methods.
[b] De-*O*-acetylated (base-treated) BSM.
[c] *V. cholerae*-treated BSM.

13 to 81% of binding to native, deacetylated BSM. Finally, at comparable concentrations, type 3 reovirus bound less well to native BSM than to sialidase-treated, deacetylated BSM (compare rows 1 and 4; 10^2 and 10^1 pmol spotted). Taken together, these results suggested that reovirus type 3 binds preferentially to sialic acid residues on BSM which lack or possess reduced amounts of *O*-acetyl groups. Consistent with this interpretation, the deacetylated BSM preparations used by us possessed about 10% of the *O*-acetyl groups per mol of sialic acid (0.1 versus 1.0) present in native BSM (data not shown). However, these results need confirmation by a detailed analysis of *O*-acetyl content of free and bound sialic acid residues before and after sialidase treatment.

To determine whether binding to deacetylated and *V. cholerae* sialidase-treated, deacetylated BSM required sialic acid on BSM oligosaccharides, we spotted samples of each preparation on nitrocellulose, treated them with *A. ureafaciens* sialidase, and probed them with type 3 reovirus. Binding was completely abolished, suggesting that sialic acid residues are important for interaction with deacetylated BSM preparations (data not shown).

Effect of deacetylated or *V. cholerae*-treated BSM on binding of infectious reovirus to murine L cells. To determine whether there was a correlation between inhibition of radiolabeled type 3 reovirus binding to L cells, stable binding to inhibitors, and the ability of infectious virions to interact with these glycoproteins, we measured the ability of deacetylated or *V. cholerae*-treated BSM to inhibit reovirus type 3 binding to murine L cells by an infectious-center assay. In four independent experiments done on four different days with two different preparations of deacetylated and *V. cholerae*-treated BSM, we observed that in most experiments, deacetylated BSM reduced virus binding to L cells by 99% on average and was about 10 times more effective than *V. cholerae*-treated BSM (Table 2). Furthermore, in two experiments in which an endpoint was calculated, it was found that at least about 100 times more *V. cholerae*-treated BSM was required to inhibit infectious virus attachment by 50% (data not shown). Thus, these results are consistent with attachment inhibition and direct-binding assays and demonstrated that de-*O*-acetylated BSM can compete effectively for attachment of infectious virions to host cell receptors.

DISCUSSION

In this study, we analyzed the specificity of the interaction of reovirus type 3 Dearing and, to a lesser extent, that of type 1 Lang with several purified SGPs which were chosen based on the similarity of their sialyloligosaccharide side chains to those found on mammalian cell plasma membrane glycoproteins (12). One of the most important findings of this study was that the ability of type 3 and 1HA3 reoviruses to interact avidly with several different sialylated glycoproteins and the inability of type 1 and 3HA1 reoviruses to interact significantly with any glycoprotein except Fet is a specific property of the virus attachment protein, sigma 1 polypeptide. This was an important result because it ruled out the possibility that nonspecific interactions mediated by μ1C and σ3 polypeptides played a major role in the interaction of type 3 reovirus with SGPs. Together with other recent reports (2, 12), these results raise the possibility that the virion attachment protein may use such structures on host cells as receptor components for reovirus attachment. The finding (Table 2) tha BSM is a potent and specific attachment inhibitor for infectious type 3 reovirus lends support to this hypothesis. Nevertheless, it is important to note that modulatory effects of μ1C and σ3 polypeptides on these interactions cannot be ruled out until a large panel of reassortants are examined.

Another interesting finding of this study was the dramatic difference in the ability of type 3 Dearing reovirus to bind to different SGPs. The reason that type 3 reovirus binds avidly to BSM and glycophorin and poorly to other SGPs such as transferrin is not known. As noted previously (12), it cannot be explained by the sialic acid content of these glycoproteins since the BSM, AGP, and Fet preparations used here differed by twofold or less in their sialic acid contents. One possible explanation is that type 3 reovirus exhibits linkage specificity in its interaction with oligosaccharides. For example, certain strains of influenza A virus bind preferentially to sialyl α2,3-galactosyl compared with sialyl α2,6-galactosyl residues on glycoproteins or vice-versa (33). Whereas the major *O*-linked disaccharide of BSM is present on glycophorin as part of an *O*-linked tetrasaccharide (42, 47), it is also present on Fet (28, 37), which interacts weakly with type 3 reovirus compared with BSM and glycophorin. Thus, we have no direct evidence that type 3 reovirus exhibits linkage specificity. It must also be considered that properties of the polypeptide backbone could greatly influence interaction of the sigma 1 protein with sialyoligosaccharides on glycoproteins. For example, the molecular size, arrangement, or density of oligosaccharide side chains or a tendency of the inhibitor to aggregate in solution could be involved (14).

The finding that type 3 reovirus interacts preferentially with BSM whose sialic acid residues lack or contain reduced amounts of *O*-acetyl residues is an intriguing result. In agreement with previous studies (12), this finding offers further evidence that the interaction between the viral attachment protein and sialyloligosaccharides on glycoproteins is not a simple charge effect due to carboxyl groups on sialic acid but may be highly specific for the structure of the sialic acid molecule and the other sugar(s) of the oligosaccharide chain. Since sialic acid residues of BSM are variously acetylated at carbon atoms C7, C8, and C9 (36, 43), it

is interesting to determine which, if any, of these acetyl groups are most important in regulating the interaction of type 3 reovirus with BSM. In this regard, treatment of BSM with influenza C receptor-destroying enzyme, which specifically hydrolyzes only 9-*O*-acetyl groups on BSM sialic acid residues without releasing sialic acid from the rest of the oligosaccharide chain (19), increases its activity as a hemagglutination inhibitor of reovirus type 3 as efficiently as does base treatment. This result suggests that *O*-acetylation at C9 is important in this interaction (manuscript in preparation).

The role of acetylation of sialic acid residues in the interaction of type 3 reovirus with host cell oligosaccharides is completely unknown. It is known that mammalian species differ widely in the degrees to which and the positions at which they *O*-acetylate sialic acid on cell surface glycoconjugates (36). It will be interesting to determine whether host cells which apparently lack receptors for type 3 reoviruses (8) have a high degree of *O*-acetylation of their membrane sialic acid residues. In this regard, studies in progress indicate that reovirus interacts poorly with cells which possess 9-*O*-acetylated sialic acid on their glycoconjugates, suggesting that this hypothesis has merit (manuscript in preparation). It is worth noting that precedent for a modulating effect of *O*-acetylation of sialic (*N*-acetylneuraminic) acid residues in recognition of receptor determinants occurs among influenza A viruses. For example, among those strains which recognize sialyl alpha-2,6-galactosyl residues as receptor determinants on cell membrane glycoconjugates, derivatization of cellular oligosacchardies with 9-*O*-acetyl-*N*-acetylneuraminic acid in place of *N*-acetylneuraminic acid abolished virus-mediated hemagglutination (17). In contrast, influenza C virus binds to cellular sialoglycoconjugates containing 9-*O*-acetyl-*N*-acetylneuraminic acid but not to those containing *N*-acetylneuraminic acid (32).

It is of interest to compare the results reported here with the recent results of others. Except for binding to BSM, type 3 reovirus bound most avidly to glycophorin A (Fig. 4), a highly sialylated integral membrane protein of erythrocytes. Very recently, it has been reported that glycophorin A is the erythrocyte receptor which mediates hemagglutination by reovirus type 3. This conclusion was based in part on the finding that purified glycophorin inhibits type 3 reoviral or soluble sigma 1-mediated hemagglutination of erythrocytes, whereas another sialylated glycoprotein, Fet, worked only at very high concentrations (Paul and Lee, submitted). Furthermore, glycophorin could inhibit attachment of soluble sigma 1 polypeptide to murine L cells, and sigma 1 could bind directly to glycophorin immobilized or microtiter plates. These activities could be abolished by pretreating glycophorin with sialidase to remove sialic acid from its oligosaccharide components (Paul and Lee, submitted). Thus, our results are in good agreement with those of these investigators and are consistent with the hypothesis that sialylated glycoproteins have a biological role in reovirus interaction with host cells.

Although there is evidence that the reovirus attachment protein interacts specifically with sialyloligosaccharide components of host cells and erythrocytes (2, 12, 13), biological evidence for a direct role of sialyloligosaccharides as receptor components for reovirus type 3 infection of host cells has been lacking. In this regard, we have recently demonstrated that desialylation of the oligosaccharide components of certain host cells resulted in dramatically increased resistance to reovirus type 3 infection, lending support to the hypothesis that host cell sialyloligosaccharide components may play a role in reovirus infection of host cells (Gentsch, in preparation). However, proof of this hypothesis will require specific restoration of susceptibility to infection using, for example, ganglioside incorporation (26, 40, 41) or specific resialylation experiments (4, 10, 25) as described for other viruses which use sialyloligosaccharide structures as receptor components. Alternatively, selection of reovirus type 3 variants resistant to SGP-mediated inhibition of infection of host cells could help to delineate a biological role for cellular oligosaccharides in reovirus infections.

The relationship between the evidence for oligosaccharides as receptor components and the demonstration that a 67-kilodalton glycoprotein is an in vivo receptor for reovirus type 3 is not known (5, 6). Since there are host cells which may lack this glycoprotein in their plasma membrane and bind type 3 reovirus efficiently (29), it is plausible that there are alternate binding and entry pathways for reoviruses. Alternatively, both oligosaccharides and the 67-kilodalton glycoproteins may function as receptor determinants in productive binding and entry into host cells by reovirus type 3. In this same vein, both biological (3, 38) and biochemical (39) data indicate that the viral attachment protein possesses distinct host cell-binding (neutralization) and erythrocyte-binding (hemagglutination) domains. The finding that the erythrocyte-binding domain of the attachment protein may use a heavily sialylated integral membrane protein, glycophorin, as an erthyroctye attachment site (Paul and Lee, submitted), taken together with other evidence, is not inconsistent with a hypothesis that alternate host cell surface structures may be used in attachment by type 3 reovirus.

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