JOURNAL OF VIROLOGY, Jan. 1992, p. 432–439
0022-538X/92/010432-08$02.00/0

Vol. 66, No. 1

Specific Interactions between Rotavirus Outer Capsid Proteins VP4 and VP7 Determine Expression of a Cross-Reactive, Neutralizing VP4-Specific Epitope

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Received 26 August 1991/Accepted 15 October 1991

We previously reported that the expression of rotavirus phenotypes by reassortants was affected by recipient genetic background and proposed specific interactions between the outer capsid proteins VP4 and VP7 as the basis for the phenotypic effects (D. Chen, J. W. Burns, M. K. Estes, and R. F. Ramig, Proc. Natl. Acad. Sci. USA 86:3743–3747, 1989). A neutralizing, cross-reactive VP4-specific monoclonal antibody (MAb), 2G4, was used to probe the protein-protein interactions. The VP4 specificity of 2G4 was confirmed by immunoblot analysis. MAb 2G4 reacted with both standard (SA11-Cl3) and variant rotavirus SA11 (SA11-4F) but did not react with bovine rotavirus B223 as determined by plaque reduction neutralization (PRN) and enzyme-linked immunosorbent assay (ELISA). When a panel of SA11-4F/B223 and SA11-Cl3/B223 reassortants in purified or crude lysate form that had been grown in the presence or absence of trypsin was analyzed with MAb 2G4 by PRN and ELISA, the results with some reassortants were unexpected. That is, MAb 2G4 reacted with VP4 of SA11 parental origin (4F or Cl3) when it was assembled into capsids with the homologous SA11 VP7 but failed to react with VP4 of SA11 assembled into capsids with heterologous B223 VP7. Conversely, MAb 2G4 failed to react with VP4 of B223 parental origin when it was assembled into capsids with homologous B223 VP7 but did react with B223 VP4 assembled into capsids with the heterologous SA11 VP7. Similar reactivity was observed when 2G4 was used to immunoprecipitate purified double-shelled virions. When soluble unassembled viral proteins were analyzed by ELISA, the 2G4 reactive pattern was as predicted from the parental origin of VP4. That is, 2G4 reacted with the soluble VP4 of reassortants having VP4 from SA11-Cl3 or SA11-4F and failed to react with VP4 of B223 origin, regardless of the origin of VP7. PRN and ELISA results obtained with nonglycosylated viruses revealed that the unexpected reactivity of 2G4 with virus particles was not the result of differential glycosylation of VP7 and epitope masking. These results indicate that the 2G4 epitope existed in the soluble form of VP4 encoded by SA11-Cl3 or SA11-4F but not in soluble B223 VP4. On the other hand, in assembled virions, the presentation of the 2G4 epitope on VP4 was unexpected in some reassortants and was affected by the specific interactions between VP4 and VP7 of heterologous parental origin.

Rotaviruses are members of the family *Reoviridae* and contain a genome of 11 segments of double-stranded RNA. The rotavirion is nonenveloped and contains two concentric protein coats. The outer capsid is composed of two structural proteins (VP4 and VP7) encoded by genome segments 4 and 9, respectively, in the case of the serotype 3 prototype virus SA11 (reviewed in reference 6). VP4, an 88-kDa protein, constitutes the spikes projecting from the virion surface (26) and is associated with a number of properties including hemagglutination (16), neutralization (22), protease cleavage enhancement of viral infectivity (4, 7, 9), growth restriction in culture (12, 13, 29), and viral virulence (23). VP7, an approximately 37-kDa glycoprotein, constitutes the smooth surface of the virion into which the VP4 spikes are inserted (26). VP7 induces antibodies with neutralizing and hemagglutination inhibition activities (14) and has been reported to be the viral attachment protein (19, 20, 30). VP4 and VP7 clearly interact in a highly ordered manner in the outer capsid of the rotavirion (26, 27, 36). The inner capsid of the rotavirion is composed of VP6, although it is unknown whether specific interactions occur between VP6 in the inner capsid and VP4 and VP7 in the outer capsid (26, 27, 36).

Cross-reactivity of monoclonal antibodies (MAbs) directed at both VP4 and VP7 has been reported (32–34). For example, the VP4-specific MAb 2G4 originally isolated from a mouse immunized with porcine rotavirus OSU (serotype 5) was able to neutralize rotaviruses of several serotypes (32). Cross-reactive MAbs and antigenic mutants have been intensively studied in an attempt to understand the possible role of cross-reactive epitopes in heterotypic protection following rotavirus infection. Sequence analysis of MAb escape mutants has identified the binding sites of cross-reactive MAbs (17, 33, 34), but it is not known whether factors other than amino acid sequence can affect the presentation of these sites.

We have previously reported that specific phenotypes, such as plaque size and protease dependence of growth, associated with outer capsid proteins can be affected by the recipient genetic background in rotavirus reassortants, indicating a possible role for highly specific interactions between VP4 and VP7 in phenotypic expression (4). In this study, the VP4-specific MAb 2G4 and a panel of SA11-4F/B223 and SA11-Cl3/B223 reassortants was used to study the specificity of interactions between VP4 and VP7. Here, we report that the presentation of the cross-reactive 2G4 epitope in assembled virions is indeed dependent on interactions between VP4 and VP7 in the outer capsid of the virion.

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MATERIALS AND METHODS

Viruses and cells. Our standard laboratory wild-type SA11 clone 3 (SA11-Cl3) was isolated from an SA11 stock (8) obtained from H. Malherbe (Gull Laboratories, Salt Lake City, Utah). The SA11 variant 4F (SA11-4F) was isolated by Pereira et al. (25) from an SA11 preparation originally obtained from the Centers for Disease Control. SA11-4F has been extensively characterized (1, 4, 25). The bovine rotavirus B223 was isolated and adapted to culture by Woode et al. (35). SA11-4F/B223 and SA11-Cl3/B223 reassortants were constructed and characterized as previously described (4). All viruses were triply plaque purified and used within three passages of the final plaque purification. MA104 fetal monkey kidney cells were grown in medium 199 supplemented with 5% fetal bovine serum and were used for plaque assays and virus propagation as described previously (28).

Antibody. The cross-reactive, neutralizing VP4-specific 2G4 and neutralizing VP7-specific 159 hybridoma cell lines were originally isolated and characterized by Shaw et al. (32). The nonneutralizing VP4-specific 3D8 hybridoma cell line was isolated and characterized by Burns et al. (2). The nonneutralizing VP7-specific common 60-F2 hybridoma cell line was originally isolated and characterized by Offit et al. (24). Ascites fluids were produced by injecting 6- to 8-week-old pristane-primed BALB/c mice with 3×10^6 hybridoma cells per animal. Polyclonal antiserum was obtained from guinea pigs immunized with purified double-shelled SA11-Cl3.

Virus purification. All viruses were purified on CsCl density gradients as described previously (18). For viruses grown in the presence of 1 μg of trypsin per ml, MA104 cells were infected with a multiplicity of infection of 10 PFU per cell. For viruses grown in the absence of trypsin, MA104 cells were infected with a multiplicity of infection of 20 PFU per cell. Viruses grown in the presence of tunicamycin and the absence of trypsin were semipurified by freon extraction, pelleted through a cushion of 40% sucrose, and suspended in phosphate-buffered saline (PBS) for use directly in plaque reduction neutralization or enzyme-linked immunosorbent assay (ELISA).

Preparation of viral soluble proteins. A 150-cm^2 flask of MA104 cells was infected with a multiplicity of infection of 40 PFU per cell. After adsorption for 1 h, the inoculum was removed and the cells were fed with 25 ml of serum-free medium 199. At 5 h postinfection, the cell monolayer was washed twice with PBS. After three freeze-thaw cycles, the cell lysates were homogenized three times for 1 min each and sonicated twice for 1 min each. Finally, the cell lysates were centrifuged at 45,000 rpm for 2 h in an SW50.1 rotor. The supernatants were used as the viral soluble proteins.

Preparation of viruses grown with tunicamycin. A 150-cm^2 flask of MA104 cells was infected with a multiplicity of infection of 30 PFU per cell. After adsorption for 1 h, the inoculum was removed and the cells were fed with 25 ml of serum-free medium 199 containing 5 μg of tunicamycin per ml. The virus was partially purified as described above.

Endoglycosidase H digestion. Purified double-shelled viruses grown in the absence of trypsin were incubated with 0.1 U of endoglycosidase H (Boehringer Mannheim Biochemicals, Mannheim, Germany) per ml at 37°C for 2 h. The complete deglycosylation of VP7 was confirmed by analysis on polyacrylamide gels.

Plaque reduction neutralization assay. Plaque reduction neutralization assays were carried out with MA104 cell monolayers in 6-well plates, with serial twofold dilutions of ascites fluids or polyclonal serum. The neutralizing titers were determined by 80% reduction of 40 to 80 PFU.

ELISA. An ELISA was performed as described previously (2) with slight modification. Briefly, polyvinyl chloride 96-well plates were coated with 2G4 ascites fluid diluted 1:400 in 10 mM PBS (pH 7.2) at 4°C overnight. The plates were washed twice with PBS (200 μl per well) containing 0.05% Tween 20 and blocked with 2% bovine serum albumin in PBS for 2 h at 37°C. Purified double-shelled virus or cell lysates were added to the plates and incubated at 4°C overnight. The plates were washed five times with 0.05% Tween 20 in PBS. Hyperimmune guinea pig antiserum, prepared to purified double-shelled SA11 particles, was added to each well, and the plates were incubated at 37°C for 1 h. Following five washes, horseradish peroxidase-conjugated goat anti-guinea pig immunoglobulin G (Cappel, Malvern, Pa.) was added to each well, and the plates were incubated at 37°C for another hour. Finally, after five washes, ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); Sigma Chemical Co.] substrate was added to each well. After 30 min of incubation at room temperature, the optical density at 414 nm was determined with a Titertek Multiskan plate reader (Flow Laboratories, McLean, Va.). In all ELISAs, uninfected cell lysates were used as the negative control, and duplicate plates with the VP7 common MAb 60-F2 or VP4 common MAb 3D8 ascites as capture antibody were used as the positive control.

Immunoprecipitation of double-shelled virus. Purified double-shelled virus was incubated with MAb 2G4 at 4°C overnight. Twenty microliters of protein A conjugated to agarose beads (Calbiochem Corp., La Jolla, Calif.) was added, and the mixture was incubated at room temperature for 15 min. The beads were pelleted and washed one time each with A buffer (0.5 M NaCl, 0.05% Nonidet P-40 [NP-40], 10 mM Tris [pH 7.2]), B buffer (0.1 M NaCl, 0.05% NP-40, 10 mM Tris [pH 7.2]), and C buffer (0.05% NP-40, 10 mM Tris [pH 7.2]). The bound virus was removed from the resin by being boiled for 2 min in 50 μl of sample buffer (60 mM Tris [pH 6.8], 5% 2-mercaptoethanol, 1% sodium dodecyl sulfate, 10% glycerol). The viral proteins were resolved by polyacrylamide gel electrophoresis (PAGE) and detected by immunoblot.

Immunoblot. Immunoblotting was performed as previously described (1) except that 5% 2-mercaptoethanol was used in the sample buffer and the horseradish peroxidase-DAB (3,3',4,4'-tetraaminobiphenyl) system was used for staining.

RESULTS

When constructing SA11-4F/B223 reassortants for use in a prior study (4), we found that progeny viruses containing VP4 from SA11-4F were apparently not neutralized as expected by the VP4-specific MAb 2G4, whereas progeny containing VP4 from B223 were unexpectedly neutralized by 2G4 when the antibody was used in agar overlay for selection of the progeny. The unexpected results when antibody selection was used led to the study reported here.

Construction and characterization of reassortants. The majority of the reassortants used were constructed and characterized previously (4). Four previously undescribed reassortants were constructed specifically for this study. Reassortant R-A32 was isolated from the cross of reassortant R-197 and SA11-Cl3 parental viruses. Reassortants

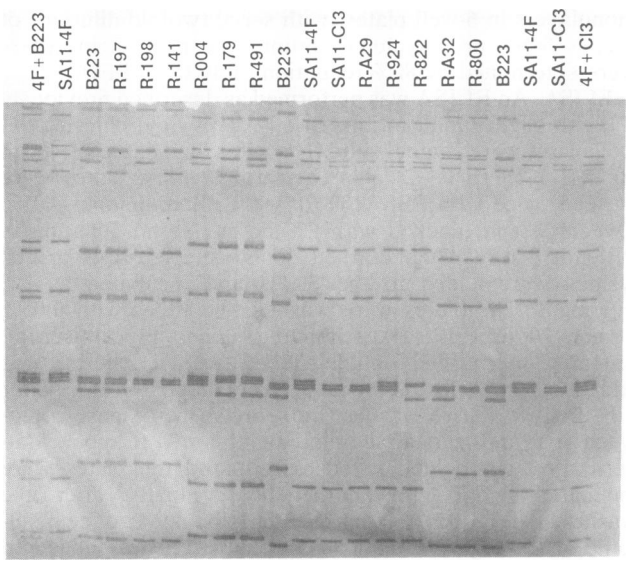

FIG. 1. Electrophoresis of genome RNAs of SA11-4F, SA11-Cl3, and B223 and reassortants derived from these viruses. Single-shelled particles from CsCl gradients were treated with proteinase K (1 mg/ml) at 37°C overnight. The samples then were subjected to electrophoresis in a 12% polyacrylamide gel at 20 mA for 21 h. The genome segments were visualized by being stained with silver. In the original gel, the distance between B223 segment 1 and segment 11 was 11.9 cm. The deduced parental origins of the genome segments of each recombinant are listed in Table 1.

R-924, R-800, and R-822 were isolated from a cross of reassortant R-A32 and SA11-4F as the parents. Nonselective isolation and characterization were done as previously described (4, 11). The parental origin of each genome segment of all reassortants was determined by electrophoresis (Fig. 1), and the genotypes are listed in the tables for ease of interpretation.

Verification of MAb 2G4 specificity. The unexpected neutralization obtained with MAb 2G4 as a selecting antibody in previous studies (4) suggested that either the specificity of the MAb had changed or the wrong MAb had been used. New ascites fluids were prepared, and the specificity of 2G4 was tested by immunoblot (Fig. 2). Probing immunoblots with hyperimmune polyclonal anti-SA11 serum revealed that all viruses tested were transferred to the membrane. The 2G4 antibody bound specifically to VP4 and VP5* (a protease cleavage product of VP4) from SA11-4F, SA11-Cl3, and reassortant R-197, all of which were viruses that contained SA11 VP4 although R-197 was not neutralized by 2G4. In contrast, B223 and reassortant R-004 that contained B223 VP4 did not bind 2G4 in the blot, although R-004 was neutralized by 2G4. The presence of VP4 in all lanes was confirmed by use of the 3D8 nonneutralizing MAb in the immunoblots, with 3D8 reacting with VP4 from all viruses although the reactions with B223 and R-004 were quite weak. The VP7-specific MAb 60-F2 reacted with VP7 from all viruses. These results indicate that the 2G4 MAb is indeed specific for VP4 from SA11-Cl3 and SA11-4F. Under the conditions of these immunoblots, in which protein-protein interactions were disrupted, 2G4 reacted with reassortant VP4 as predicted from the parental origin of the protein. This contrasted with neutralization, in which assembled structures were intact and 2G4 neutralized in a nonpredictable fashion (see below).

Neutralization of parental and reassortant viruses. To confirm the unexpected neutralization of certain reassortants with 2G4, the panel of parental and reassortant viruses was subjected to neutralization tests with antibodies of different specificities. The SA11 parental viruses (SA11-4F and SA11-Cl3) were neutralized by 2G4 to high titers, although SA11-Cl3 was neutralized to a significantly higher titer (Table 1). This difference in neutralization behavior most likely was the

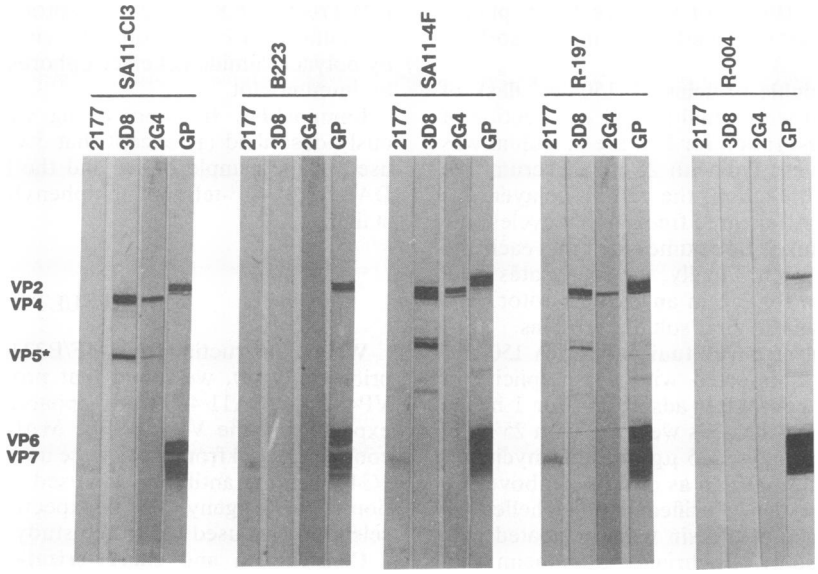

FIG. 2. Immunoblot analysis of SA11-Cl3, B223, SA11-4F, and reassortants R-197 and R-004. Purified, double-shelled virus (35 μg) grown in the absence of trypsin was electrophoresed in a 12% polyacrylamide gel. The proteins were immunoblotted to a nitrocellulose membrane, and proteins in each identical lane were probed in an immunoperoxidase reaction with the VP7-specific MAb 60-F2 (2177 in the figure), the VP4-common MAb 3D8, the VP4-specific neutralizing MAb 2G4, or guinea pig polyclonal anti-SA11 serum. The positions of the viral proteins are indicated on the left.

TABLE 1. Neutralization of parental and reassortant viruses with VP4-specific MAb 2G4 and control antibodies

Virus	Parental origin of genome segment[a]											Neutralization titer of[b]:				
												MAb 2G4 with:				
												Infected cell lysates		Semipurified virus without trypsin, with TM	MAb 159 without trypsin	Polyclonal anti-SA11 with trypsin
	1	2	3	4	5	6	7	8	9	10	11	With trypsin	Without trypsin			
Parental viruses																
SA11-4F	F	F	F	F	F	F	F	F	F	F	F	3,200	6,400	6,400	>512,000	>3,200
B223	B	B	B	B	B	B	B	B	B	B	B	<50	<50	<200	<4,000	<500
SA11-Cl3	S	S	S	S	S	S	S	S	S	S	S	12,800	12,800	51,200	>512,000	>3,200
SA11-4F/B223 reassortants																
R-141	B	B	B	F[c]	B	B	B	B	F[c]	B	B	3,200	12,800	3,200	>512,000	>3,200
R-197	B	B	B	F[c]	B	B	B	B	B	B	B	<50[d]	<50[d]	<200[d]	<4,000	<500
R-198	B	B	B	B	B	B	B	B	F[c]	B	B	3,200[d]	6,400[d]	6,400[d]	>512,000	>3,200
R-491	F	F	F	B[c]	F	F	F	F	B[c]	F	F	<50	<50	ND[e]	<4,000	<500
R-004	F	F	F	B[c]	F	F	F	F	F	F	F	3,200[d]	3,200[d]	3,200[d]	>512,000	>3,200
R-179	F	F	F	F	F	F	F	F	B[c]	F	F	<50[d]	<50[d]	<200[d]	<4,000	<500
Other reassortants																
R-A29	S	S	S	F[c]	S	S	S	S	S	S	S	6,400	3,200	ND	>512,000	>3,200
R-924	F	F	F	S[c]	F	F	F	F	F	F	F	12,800	12,800	ND	ND	ND
R-822	F	F	F	S[c]	F	F	F	F	B[c]	F	F	50[d]	<50[d]	ND	ND	ND
R-A32	B	S	B	S[c]	B	B	B	B	B	B	B	50[d]	50[d]	ND	<4,000	500
R-800	B	S	B	S[c]	B	B	B	B	F[c]	B	B	6,400	12,800	ND	512,000	>3,200

[a] F, SA11-4F; B, B223; S, SA11-Cl3.

[b] Neutralization titer expressed as reciprocal of dilution yielding 80% reduction of 40 to 80 PFU. Specificities of MAbs 2G4 and 159 were VP4 and VP7, respectively. Hyperimmune polyclonal anti-SA11 serum was raised against purified double-shelled SA11-Cl3. TM, tunicamycin.

[c] Donor segment.

[d] Neutralization behavior was not predicted on the basis of the parent of origin for VP4 and the VP4 specificity of MAb 2G4.

[e] ND, not done.

result of the SA11-4F variant possessing a bovinelike genome segment 4 and VP4 (1, 21). In contrast, the B223 parental virus was not neutralized by 2G4. When reassortants were tested, neutralization appeared to segregate with segment 9 and its product VP7 (Table 1). This result was unexpected since MAb 2G4 was shown to be VP4 specific. Examination of genotypes revealed that when the outer capsid protein species were of homologous origin (both SA11 or both B223), the reassortant (e.g., R-141, R-491, R-A29, R-924, or R-800) was neutralized as predicted from the parental origin of VP4. On the other hand, if the outer capsid proteins were of heterologous parental origin, the parental origin of VP4 did not predict neutralization. Specifically, reassortants (e.g., R-197, R-179, R-822, and R-A32) that contained VP4 from SA11-4F or SA11-Cl3 (the neutralized parent) in combination with VP7 from B223 (the nonneutralized parent) were not neutralized. In addition, reassortants (e.g., R-198 and R-004) that contained VP4 from B223 (the nonneutralized parent) and VP7 from SA11 (the neutralized parent) were neutralized. This result indicated that viruses with outer capsids composed of heterologous combinations of VP4 and VP7 were, for some reason, behaving in an unexpected fashion in neutralization tests. Growth of the viruses in the absence of trypsin, so that VP4 was not cleaved, did not qualitatively affect the neutralization results (Table 1).

VP7 of rotavirus is glycosylated, and VP7 in strains of different viruses has been demonstrated to contain different numbers of carbohydrate side chains. SA11 VP7 contains a single carbohydrate attachment site (15), whereas bovine viruses are characterized by multiple glycosylation sites (10). Furthermore, the carbohydrate side chains on VP7 have been shown to alter the antigenicity of the virus (3). To test the possible role of differential glycosylation of VP7 in the unexpected neutralization results, the viruses were grown in the presence of tunicamycin and partially purified. Electrophoresis of the partially purified virions and Western blot (immunoblot) analysis with polyclonal anti-SA11 guinea pig serum revealed that virions contained no detectable glycosylated VP7 (data not shown). Lack of glycosylation of VP7 had no effect on the relative neutralization of the viruses (Table 1).

Neutralization tests with the VP7-specific MAb 159 revealed that it neutralized the SA11 parent but did not neutralize the B223 parent and that it neutralized the reassortants as predicted on the basis of the origin of VP7 (Table 1). In addition, hyperimmune polyclonal anti-SA11 serum neutralized all the viruses on the basis of the parental origin of VP7 as expected for hyperimmune serum with predominately VP7-neutralizing activity (Table 1). Taken together, these results indicate that the 2G4 epitope on VP4 was differentially expressed, depending on the parental origin of the VP7 with which it interacted in the outer capsid of the virion. However, the converse was not true, and the expression of the 159 epitope on VP7 was not affected by the parental origin of VP4.

ELISA of parental and reassortant viruses. To determine whether MAb 2G4 was able to bind, in a nonneutralizing fashion, to viruses that were not neutralized in plaque reduction neutralization, an ELISA was performed with 2G4 as the capture antibody and purified viruses or soluble viral proteins as antigen. As shown in Table 2, purified viruses

TABLE 2. ELISA reactivity of assembled and unassembled VP4 from parental and reassortant viruses with VP4-specific MAbs

Virus	Parental origin of segment[a]			ELISA reactivity of[b]:					
				MAb 2G4 with:					MAb 3D8 with soluble lysate proteins
				Purified virus		Semipurified virus without trypsin with TM	Purified virus with Endo H	Soluble lysate proteins	
	4	9	Other	With trypsin	Without trypsin				
Parental viruses									
SA11-4F	F	F	F	>2,000	1,852	1,446	1,490	183.5 (33.8)	255.8 (11.6)
B223	B	B	B	47	62	40	42	31.8 (10.6)	177.8 (11.0)
SA11-Cl3	S	S	S	>2,000	1,909	581	ND[e]	182.1 (58.9)	318.8 (35.4)
SA11-4F/B223 reassortants									
R-141	F[c]	F[c]	B	>2,000	1,981	1,576	ND	116.4 (13.3)	313.8 (20.9)
R-197	F[c]	B	B	54[d]	52[d]	96[d]	30[d]	156.5 (32.3)	309.3 (20.8)
R-198	B	F[c]	B	>2,000[d]	1,951[d]	1,394[d]	ND	33.5 (12.3)	156.2 (12.2)
R-491	B[c]	B[c]	F	39	55	131	62	7.3 (17.9)	151.3 (15.3)
R-004	B[c]	F	F	>2,000[d]	>2,000[d]	1,767[d]	ND	10.8 (16.5)	143.5 (22.0)
R-179	F	B[c]	F	74[d]	48[d]	160[d]	29[d]	107.4 (29.4)	414.8 (8.1)
Other reassortants									
R-A29	F[c]	S	S	>2,000	1,640	ND	ND	148.6 (18.5)	344.5 (18.5)
R-924	S[c]	F	F	>2,000	1,852	ND	ND	134.8 (10.9)	480.5 (48.7)
R-822	S[c]	B[c]	F	58[d]	40[d]	ND	ND	150.0 (38.0)	333.3 (31.9)
R-A32	S[c]	B	B	55[d]	50[d]	ND	ND	133.1 (10.2)	325.0 (19.5)
R-800	S[c]	F[c]	B	>2,000	1,987	ND	ND	160.3 (17.5)	427.5 (27.1)
Mock-infected lysate control				ND	ND	ND	ND	11.0 (12.3)	43.8 (2.9)

[a] F, SA11-4F; B, B223; S, SA11-Cl3.
[b] ELISA was performed with MAb 2G4 or 3D8 as the capture antibody and polyclonal SA11-Cl3 serum as the detector serum. All tests with purified double-shelled virus utilized 1 μg of antigen per well. Results are expressed as $A_{414} \times 1{,}000$. Data shown are representative of two to three determinations. TM, tunicamycin; Endo H, endoglycosidase H. For soluble lysate proteins, data are presented as the means (standard deviations) for eight determinations on a single plate.
[c] Donor segment.
[d] ELISA reactivity was not predicted on the basis of the parental origin of VP4, the target antigen for MAb 2G4.
[e] ND, not done.

grown in the presence or absence of trypsin reacted with 2G4 in an ELISA in a manner completely analogous to that seen in neutralization tests (Table 1). That is, when it was present in heterologous combination with VP7 in the outer capsid, VP4 reacted in a nonpredicted fashion. Thus, when the 2G4 was bound, as detected by the ELISA, the reassortant was also neutralized. Analysis of nonglycosylated viruses either grown in the presence of tunicamycin (Table 2) or deglycosylated by endoglycosidase H digestion after purification (Table 2) showed that the glycosylation state of VP7 had no effect on the ELISA reactivity. For both methods of producing nonglycosylated virus, PAGE analysis and immunoblotting showed VP7 to be completely deglycosylated (data not shown). This indicated that carbohydrate residues on VP7 were not differentially masking the 2G4 epitope. When soluble viral proteins were prepared by high-speed centrifugation of lysates to remove particulate structures, a different pattern of ELISA reactivity was observed (Table 2). In this case, proteins reacted in an ELISA as predicted by the parental origin of VP4 in the virus, regardless of the parental origin of VP7. This latter result indicates that the 2G4 epitope is present on unassembled VP4 from SA11-4F or SA11-Cl3 but is absent from unassembled VP4 from B223. This is in contrast to the results obtained for an ELISA with purified particles or neutralization, in which heterologous combinations of VP4 and VP7 in assembled particles gave nonpredicted results. In all ELISAs, the use of the VP7-specific MAb 60-F2 (data not shown) or the VP4 common MAb 3D8 (Table 2) as capture antibody served as a positive control for the presence of sufficient viral antigen.

Immunoprecipitation of purified viruses with MAb 2G4. To examine the effect of assembly of VP4 and VP7 in the outer capsid on the reactivity of VP4 with MAb 2G4, either purified virus or soluble viral proteins were immunoprecipitated with 2G4. The immunoprecipitates were resolved on PAGE, and the viral proteins were blotted to nitrocellulose and detected by reaction with polyclonal anti-SA11 serum in an immunoperoxidase reaction. When purified viruses were immunoprecipitated with 2G4, SA11-4F and SA11-Cl3 were precipitated and complete viral protein profiles were observed (Fig. 3). B223 was not precipitated by 2G4. When the panel of reassortants was examined, the results of immunoprecipitation were similar to the results obtained by neutralization or ELISA of virions. That is, nonpredicted results were obtained when VP4 and VP7 were of heterologous origin. In contrast, when viral soluble proteins were tested, we found that VP4 of none of the viruses could be precipitated, regardless of whether the lysate was prepared from parental or reassortant virus. Thus, immunoprecipitation of virions confirmed the results from the ELISA, which indicated that nonpredicted 2G4 reactivity of VP4 accompanied assembly of heterologous VP4 and VP7 into viral particles. However, because of the failure of immunoprecipitation, we were unable to confirm the ELISA results indicating that unassembled, soluble VP4 reacted as predicted by the parent of origin.

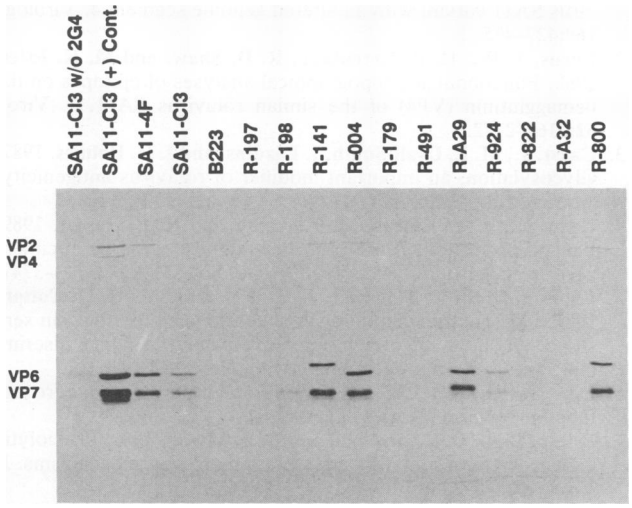

FIG. 3. Immunoprecipitation of purified parental and reassortant virions with the VP4-specific MAb 2G4. Purified, double-shelled virus (3.6 μg) grown in the absence of trypsin was mixed with a 1:100 dilution of 2G4 ascites fluid and incubated overnight at 4°C. The immune complexes were collected by incubation with protein A-agarose at room temperature for 15 min. The viral proteins bound to the protein A-agarose were released and resolved on polyacrylamide gels, blotted to nitrocellulose membrane, and probed with polyclonal anti-SA11 serum in an immunoperoxidase reaction. The positions of the viral proteins are indicated on the left.

DISCUSSION

Highly specific interactions between VP4 and VP7 in the rotavirion have long been assumed to be based on the observations that (i) these two proteins comprise the highly ordered outer capsid of the virus (19), (ii) anti-VP7 MAb can inhibit the hemagglutination activity of VP4 (14), and (iii) trypsin cleavage of VP4 abolishes reactivity of the virus with certain nonneutralizing VP7-specific MAbs (5). This assumption gained further support from the determination of the three-dimensional structures of rotavirus (26, 27, 36). These three-dimensional reconstructions revealed an outer capsid with a smooth surface and holes, from which complex spikes longer than 10 nm projected. Reconstructions with Fab fragments of the VP4-specific MAb 2G4 bound to the particles revealed the spikes to be composed of VP4 with the antibody bound at the distal ends of the spikes (26). This indicated that the carboxy-terminal VP5 trypsin cleavage fragment of VP4, containing the 2G4 epitope (17), was situated at the distal end of the spike. It is unclear whether the amino-terminal VP8 fragment of VP4 anchors the spike into the smooth outer capsid composed of VP7 and mediates protein-protein interactions between VP4 and VP7, or whether the arrangement of VP4 in the spike is more complex. However, the experiments described here and our prior work (4) lend additional support to the notion that highly specific physical interactions occur between VP4 and VP7 in the outer capsid of the rotavirion. Furthermore, our results indicate that the specific interactions between VP4 and VP7 can be influenced in subtle ways by interactions of VP4 and VP7 of heterologous origin and that these subtle changes in interaction can influence important phenotypes associated with VP4.

The results presented here demonstrate that heterologous combinations of VP4 and VP7, derived from SA11-4F or SA11-Cl3 and B223, expressed the 2G4 epitope on VP4 in a fashion not predicted by the parental origin of VP4 in reassortants. However, the nonpredicted reactivity of 2G4 with reassortant VP4 was observed only when the VP4 was assembled into virus particles (Tables 1 and 2; Fig. 3) and not when the antibody was reacted with soluble, unassembled viral protein (Table 2). This requirement for the assembly of VP4 with heterologous VP7 for nonpredicted reactivity of 2G4 provides strong evidence for the interaction between VP4 and VP7 in the outer capsid as the mediator of the nonpredicted expression of the 2G4 epitope. Furthermore, the differential expression of the 2G4 epitope in assembled versus nonassembled VP4 of reassortants with heterologous VP4 and VP7 indicates that the VP4 of SA11-4F, SA11-Cl3, and B223 all potentially contain the 2G4 epitope. Thus, the 2G4 epitope in VP4 of SA11-4F and SA11-Cl3 is normally expressed in the parental viruses but can be rendered cryptic by altered conformation induced through interaction with the heterologous VP7 from B223 in reassortants. In a similar fashion, VP4 of B223 does not express a 2G4 epitope in the parental virus, but the interaction of B223 VP4 with heterologous VP7 in reassortants can result in expression of a previously cryptic 2G4 epitope.

Our inability to immunoprecipitate VP4 with 2G4 contrasts with the reported ability of this MAb to precipitate an 84-kDa protein from virus-infected cell lysates (32). We were unable to effect precipitation in a systematic survey of assay conditions that included (i) precipitation from lysates made with a number of different viruses reactive with 2G4, (ii) solid-phase antigen capture versus liquid precipitation techniques, (iii) different immunoglobulin G collecting beads, and (iv) different salt and detergent washing methods for removal of nonspecifically bound viral proteins. Since 2G4 is reported to be a highly avid MAb (32), we expected precipitation from lysates of soluble proteins, and the inability to immunoprecipitate VP4 is not understood. However, the ability to precipitate assembled VP4 in the form of virions suggests that there may be conformational differences affecting reactivity of the 2G4 epitope between assembled and unassembled VP4, in addition to the differences in 2G4 reactivity that are mediated by interactions between VP4 and VP7 of heterologous origin reported here. In contrast to our inability to immunoprecipitate soluble VP4, 2G4 is reported to be nonreactive in immunoblots (32), and we were able to demonstrate immunoblot reactivity when large amounts of antigen (35 μg of virus) were used (Fig. 2).

The observation of transition between expression and cryptic presentation of an epitope may have major implications for the use of MAbs in the characterization and typing of viruses and for the use of reassortants for vaccination, since protein-protein interactions have now been demonstrated to affect epitope presentation. However, we are currently unable to determine the generality of the effects of protein-protein interactions between VP4 and VP7 on the expression of epitopes. The phenomenon may be rare, as we note that the presentation of the VP7-specific epitopes defined by MAbs 159 and 60-F2 was not affected by the interaction of heterologous VP4 and VP7 in the reassortants. On the other hand, the phenomenon may be highly significant. MAb 2G4, for which the phenomenon was demonstrated, was generated against the porcine rotavirus OSU and was of interest primarily because it neutralized multiple rotavirus serotypes (32). The importance of this epitope was emphasized by the demonstration that 56% of children vaccinated with a candidate serotype 3 vaccine responded to the cross-reactive 2G4 epitope as determined by epitope-blocking assays (31).

Several other aspects of our data merit comment. (i) The antigenicity of rotavirus has been demonstrated to be affected by glycosylation of VP7 (3), and bovine rotaviruses have been shown to contain multiple potential glycosylation sites on VP7, whereas SA11 has a single glycosylation site (reviewed in reference 6). The analysis of viruses prepared so that VP7 was not glycosylated revealed that the glycosylation state of VP7 did not play a role in the unexpected presentation of the 2G4 epitope (Tables 1 and 2). Thus, we were able to conclude that carbohydrate residues on VP7 did not simply mask the 2G4 epitope on VP4, nor did they introduce the change in conformation that resulted in the unexpected expression of the 2G4 epitope. (ii) The work of others (34) has shown that some cross-reactive, neutralizing VP4-specific MAbs can bind to certain viruses without neutralizing them. The concordance of our results by neutralization (Table 1), ELISA (Table 2), and immunoprecipitation (Fig. 3) of assembled virus particles indicated that among our parental and reassortant viruses, when a virus was not neutralized by 2G4, it did not detectably bind the antibody. Thus, the altered protein-protein interactions in reassortants containing heterologous VP4 and VP7 did not result in conversion of a binding that led to neutralization to a nonneutralizing interaction with the MAb or vice versa. (iii) The effects of heterologous VP4 and VP7 interaction on the presentation of epitopes defined by anti-VP4 or anti-VP7 MAbs were seen with only one (2G4) of the several MAbs used here. We have not yet screened a panel of MAbs representing epitopes at other sites on VP4 and VP7 to determine whether the phenomenon is limited to the 2G4 epitope or whether it will be seen with other epitopes. However, we do note that the effects of heterologous VP4 and VP7 interaction are not limited exclusively to the phenotype of 2G4 epitope expression. We have previously reported that the phenotypes of (a) formation of small clear plaques in the absence of exogenous protease, (b) formation of large plaques in the presence of exogenous protease, and (c) ability to grow to significantly higher titer in the presence of protease, characteristic of the SA11-4F variant, were affected by the interaction of heterologous VP4 and VP7 in SA11-4F/B223 reassortants (4). Thus, the effects mediated by interaction of heterologous VP4 and VP7 elucidated here for expression of MAb epitopes clearly extend to other phenotypes associated with VP4. (iv) Finally, we note that the 2G4 MAb binds to VP4 at the distal ends of the spikes, whereas VP4 and VP7 presumably interact at the base of the spike some 8 to 10 nm distant from the affected epitope (26). If this understanding of the structure of the rotavirus outer capsid is correct, the altered interaction of heterologous VP4 and VP7 in reassortants must somehow be transmitted through the VP4 spike and manifested at the distal antibody-binding site. We are currently probing the interaction of VP4 and VP7 in our panel of reassortants with additional antibodies and other reagents in an attempt to elucidate the molecular nature of the altered interaction of heterologous VP4 and VP7 in reassortants.

ACKNOWLEDGMENTS

We thank Harry Greenberg and his collaborators for providing hybridoma cell lines 2G4, 159, and 60-F2.

This work was supported by Public Health Service grants AI16687 (R.F.R.) and DK30144 (M.K.E.).

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