The Rhesus Rotavirus Outer Capsid Protein VP4 Functions as a Hemagglutinin and Is Antigenically Conserved When Expressed by a Baculovirus Recombinant

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Rhesus rotavirus (RRV) gene 4 was cloned into lambda bacteriophage, inserted into a polyhedrin promoter shuttle plasmid, and expressed in Sf9 cells by a recombinant baculovirus. The baculovirus-expressed VP4 protein made up approximately 5% of the *Spodoptera frugiperda*-infected cell protein. Monoclonal antibodies that neutralize the virus bound to the expressed VP4 polypeptide, indicating that the expressed VP4 protein was antigenically indistinguishable from viral VP4. In addition, we have determined that the baculovirus-expressed VP4 protein bound to erythrocytes and functions as the RRV hemagglutinin. The endogenous hemagglutinating activity of the VP4 protein, like the virus, was inhibited by guinea pig antirotavirus hyperimmune serum and by VP4-specific neutralizing monoclonal antibodies. The human erythrocyte protein, glycophorin, also inhibited hemagglutination by RRV or the expressed VP4 protein and appears to be the rotavirus erythrocyte receptor. The baculovirus-expressed VP4 protein was conserved functionally and antigenically in the absence of other outer or inner capsid rotavirus components and represents a logical candidate for future immunological studies.

Rotaviruses contain 11 double-stranded RNA segments inside a double protein capsid icosahedral shell (13, 22). The outer capsid is composed of proteins VP7 and VP4 in a molar ratio of 13 to 1 (40). VP7 is the viral glycoprotein (37 kilodaltons [kDa]) which specifies the serotype of each rotavirus (5, 9, 18, 20, 26, 33). Rhesus rotavirus (RRV) VP4 protein is 86.5 kDa and is encoded by genomic RNA segment 4 (3, 25, 32). Gene reassortment studies have demonstrated that gene 4 segregates with viral phenotypes for hemagglutination (HA) and trypsin-enhanced plaque formation (25). In the presence of trypsin, VP4 is cleaved to VP5 (60 kDa) and VP8 (28 kDa), resulting in the conversion of noninfectious rotavirus to an infectious form (12). Trypsin-activated virus enters cells rapidly by direct cell membrane penetration (16, 28, 47, 48), and portions of the VP4 protein have been implicated in the entry process (32).

In addition to trypsin-enhanced infectivity, the gene 4 product has been associated with viral HA and neutralization (4, 20, 23, 25, 27, 36). The ability of most rotaviruses to agglutinate erythrocytes and the ability of antirotavirus serum to inhibit HA have been well documented (4, 19, 27, 51). Antibodies directed toward the VP7 or VP4 proteins neutralize the virus, inhibit HA, and passively protect mice against rotavirus challenge in vivo (5, 18, 20, 23, 26, 33, 36–38, 45; S. M. Matsui, P. A. Offit, P. T. Vo, E. R. Mackow, D. A. Benfield, R. D. Shaw, L. Padilla-Noriega, and H. B. Greenberg, J. Clin. Microbiol., in press). By using a number of neutralizing monoclonal antibodies (MAbs) and a corresponding set of escape mutants, the sites involved in rotavirus neutralization have recently been located on the VP7 and VP4 proteins (10, 31, 32, 45, 49, 50). In VP7, one primary conformationally determined neutralization domain comprised of two discontinuous epitopes has been identified (10, 31). A second, apparently minor, neutralization domain has also been recognized by one MAb (10). The primary VP7 neutralization domain contains both homotypic and heterotypic discontinuous epitopes (31). In contrast, the VP4 heterotypic and homotypic neutralization regions were located in five sites on VP8 and in three sites on VP5 which appear to be continuous (32).

The individual contributions of antibodies to VP4 and VP7 to protective immunity have not been fully determined. The importance of the immune response to VP4 was emphasized by Ward et al. (52) who have determined that 80% of the serum neutralizing antibodies detected following rotavirus infection of adult volunteers are directed toward the VP4 protein. The important role of VP4 in inducing a neutralizing response after oral infection, as opposed to systemic immunization, has also been recently documented by Shaw et al. (43). The significance of the VP4 protein in the immune response to rotavirus infection and the relative stability of the neutralizing epitopes involved suggest that expression of VP4 may be useful for the analysis of functional, antigenic, and immunogenic determinants of the VP4 polypeptide.

One study (done by Arias et al. [2]) has presented the partial expression of 45% of VP4 in an *Escherichia coli* β-galactosidase fusion protein. They reported that in rotavirus seropositive mice, the partial VP4 polypeptide induced antibodies that neutralize the virus and inhibit HA, but they did not report functional aspects of the protein.

In this study, we have cloned gene 4 of RRV and expressed the complete VP4 protein. RRV gene 4 was placed under control of the baculovirus polyhedrin promoter and inserted into baculovirus by homologous recombination of cotransfected DNAs. The VP4-baculovirus recombinant expressed high levels of VP4 protein in the cytoplasm of Sf9 cells. Antigenically, the expressed VP4 protein was indistinguishable from the VP4 protein on the rotavirus icosahedral outer capsid. Expressed VP4 protein functioned in HA and hemagglutination inhibition (HI) assays. Finally, the human erythrocyte protein, glycophorin, inhibited HA by VP4

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protein or RRV and appears to be the erythrocyte receptor for rotavirus.

MATERIALS AND METHODS

Cells and viruses. MA104 cells are a continuous line of rhesus monkey kidney cells and were grown in medium 199 as previously described (44). RRV (ATCC VR 954) was grown on MA104 cells and purified on CsCl gradients (44). *Spodoptera frugiperda* cells (Sf9 and ATCC CRL 1711) were grown in serum-free medium at 28°C in Grace medium (JR Scientific, Woodland, Calif.) supplemented with 5% fetal calf serum. *Autographa californica* nuclear polyhedrosis virus (AcNPV) (*Baculoviridae*) strain E_2 was a gift from Max Summers and was propagated as previously described (46). *E. coli* XL1-Blue {*endA1 hsdR17 supE44 thi-1* λ⁻ *recA1 gyrA96 relA1 lac* [F' *proAB lacI*�absqZΔM15, Tn*10* (Tetʳ)]}, Lambda Zap *Eco*RI arms, and filamentous helper bacteriophage R408 were obtained from Stratagene Inc. LB medium and agar plates were used for bacterial growth and supplemented as needed with 0.2% maltose, 10 mM $MgSO_4$, 100 μg of ampicillin per ml, or 12.5 μg of tetracycline per ml.

cDNA synthesis and cloning. Plus-stranded RNAs were synthesized from single-shelled RRV cores essentially as described by Flores et al. (15). Oligonucleotides (18 bases long) representing the 5' end of each strand of gene 4 double-stranded RNA were synthesized on an Applied Biosystems oligonucleotide synthesizer. The negative-strand oligonucleotide was used to prime first-strand cDNA synthesis on 10 μg of plus-stranded RRV RNA in a 100-μl reaction. Briefly, the RNA and primer were boiled for 1 min, annealed at 50°C for 15 min, cooled to room temperature for 5 min, and placed in a solution containing 50 mM Tris hydrochloride (pH 8.3), 75 mM KCl, 5 mM $MgCl_2$, 10 mM dithiothreitol, 100 U of RNAsin (Promega Biotec, Madison, Wis.), and 500 mM of each deoxynucleoside triphosphate with 50 U of avian myeloblastosis virus reverse transcriptase (Seikegaku America). The reaction was incubated at 42°C for 1 h and then at 50°C for 15 min. At the end of the incubation, reactions were phenol extracted and ethanol precipitated. The recovered first-strand reaction was annealed to the plus-stranded primer as described above, and second-strand cDNA synthesis was performed as described by the D'Alessio et al. (8) modification of the Gubler and Hoffman procedure (21). Following second-strand synthesis, custom adapters described by Bevins et al. (6) were ligated to the cDNA. cDNA was separated from the adapters on low-melting-point 1% agarose gels and an ethidium bromide-stained band of 2.4 kilobases was excised and extracted by standard techniques. cDNAs were phosphorylated with polynucleotide kinase (New England BioLabs, Inc., Beverly, Mass.), ligated to *Eco*RI-digested dephosphorylated Lambda Zap arms, and packaged in vitro. Lambda plaques were screened for the insertion of gene 4 by hybridization. Lambda Zap contains an f1 origin of replication and termination sequences which flank the polylinker insertion site (Stratagene). Inserts were excised from Lambda Zap into filamentous phagemids by coinfection with a helper filamentous phage (R408) and subsequently recovered as pSK BlueScript plasmids by selection on LB ampicillin plates. Full-length clones were determined by (i) sequencing the termini of inserts (42) and (ii) transcribing RNA from the insert DNA template in vitro with T3 or T7 RNA polymerase, followed by translating the mRNA in a rabbit reticulocyte system (Stratagene) and analysis by polyacrylamide gel electrophoresis.

Recombinant baculovirus construction. Gene 4 was excised by digestion with *Not*I and filled in with T4 DNA polymerase (New England BioLabs) in the presence of 200 mM of each deoxynucleoside triphosphate. Baculovirus shuttle plasmid pACYM1 (34) was kindly provided by David Bishop. The plasmid was linearized by *Bam*HI digestion, filled in as described above, and blunt end ligated to gene 4. Transformants were screened for orientation, and the plasmid pAC435 was cotransfected into Sf9 cells with isolated Ac NPV genomic DNA (46) at a ratio of 10 to 1 by a lipofection method (14). Briefly, plasmid and AcNPV DNA were mixed together with 1,2 dioleyl-oxy-propyl trimethylammonium chloride and phosphatidylethanolamine, and the lipid-DNA complexes were added to Sf9 cells which were previously seeded for 1 h in 25-cm² flasks in the absence of serum (3 × 10^6 cells). After 90 min, the transfection medium was replaced with 5% fetal calf serum containing Grace medium and the culture fluid was harvested 3 days later. Recombinants were selected by visual inspection of polyhedron-negative plaques and plaque purified three times to obtain recombinant virus that was devoid of wild-type virus.

VP4 protein expression. Sf9 cells were infected at a multiplicity of infection of 1 with baculovirus recombinants or AcNPV controls. Recombinant VP4-Sf9 extracts were sonicated for 30 s at 30 W, assayed for protein concentration by the method of Lowry et al. (30) and used subsequently in HA and HI assays and for sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis and enzyme-linked immunosorbent assay (ELISA) (27, 29).

HA, HI, and ELISA. VP4-Sf9 sonicates, RRV, or control AcNPV-Sf9 sonicates were used in HA assays (27). RRV VP4 protein was titrated in HA tests, and 8 HA units were used for HI assays. HI assays and ELISA were performed with VP4-specific neutralizing MAbs M11, A1, A15, 1A9, 5D9, M14, 5C4, 7A12, M2, 2G4, and M7 that have been previously described (32, 45). Guinea pig hyperimmune anti-RRV serum and VP7-specific MAbs 159 and 60 have been used as positive and negative HI controls, respectively. Chicken ovalbumin grade VI, turkey ovalbumin grade VI, human glycophorin type MN, bovine submaxillary gland mucin, fetal calf fetuin, and bovine serum albumin along with *N*-acetyl neuraminic acid were purchased from Sigma Chemical Co., St. Louis, Mo.) and used in HI assays. In ELISA, recombinant VP4-Sf9 or AcNPV-Sf9 sonicates diluted in phosphate-buffered saline (PBS) were coated on Immulon-2 plates, then coated with 1% bovine serum albumin, and identified using the above sera and goat anti-mouse or anti-guinea pig alkaline phosphatase conjugates.

RESULTS

Construction of VP4-baculovirus recombinants. The prime objectives of this study were to determine whether the RRV outer capsid protein VP4 could be expressed at high levels using insect cells infected with a baculovirus vector and whether the expressed VP4 protein retains the antigenic and functional characteristics of the viral VP4. The study was initiated by cloning RNA transcripts of double-stranded RNA segment 4 of RRV into lambda phage. The longest gene 4 insert obtained lacks the first 4 bases at the 5' terminus but contains an intact translational start site and the complete 3' terminus. The missing terminal bases probably represent the priming of second-strand cDNA synthesis by a short RNA oligonucleotide generated by RNAse H, rather than priming by the added 5'-end-specific DNA primer.

Gene 4 was excised and inserted into the blunt-ended *Bam*HI site of the AcNPV shuttle plasmid, pACYM1 (Fig.
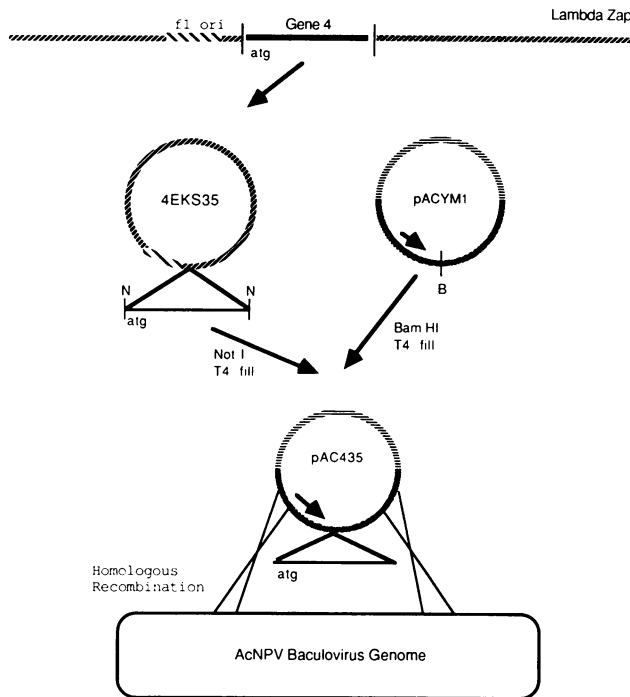
FIG. 1. Construction of recombinant VP4-baculovirus. A Lambda Zap gene 4 recombinant was coinfected into *E. coli* XL1-Blue along with a filamentous helper phage, R408. A filamentous phage containing the insert DNA was recovered and grown as an ampicillin-resistant colony containing the 4EKS35 plasmid. Gene 4 was released from the plasmid by digestion with *Not*I and blunt end ligated into the *Bam*HI-linearized pACYM1 baculovirus shuttle plasmid. The resultant pACYM1435 plasmid was cotransfected with AcNPV DNA into Sf9 cells, and homologous recombinants were selected by visual inspection of polyhedra-minus plaques.

1). The plasmid and wild-type AcNPV genomic DNA were cotransfected into Sf9 cells. Homologous recombination of the plasmid and genomic DNA resulted in the concomitant insertion of gene 4 under the control of the polyhedrin promoter and the deletion of polyhedrin coding sequences. Recombinant viral plaques were screened initially for the absence of occlusion bodies formed by the polyhedrin protein.

Expression and antigenicity of VP4 protein. Recombinant occlusion-minus viruses were plaque purified three times and screened for VP4 expression by ELISA (Table 1), Coomassie blue staining of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2), Western blot (immunoblot) analysis (data not shown), and immunoprecipitation of VP4 protein (Fig. 3). The highest level of VP4 expression was obtained from one clone which was designated M1RRV4C3 and was used throughout these experiments. The gene 4-baculovirus recombinant expressed an 86.5-kDa protein in Sf9 cells and lacked the polyhedrin protein (Fig. 2). The VP4 protein was detected in the cytoplasm of VP4-AcNPV recombinant-infected Sf9 cells by indirect immunofluorescence (data not shown). A densitometric scan of a Coomassie blue-stained gel indicated that 5% of the total cellular protein was expressed in an 86.5-kDa VP4 protein band compared with a 15% expression level for the polyhedrin protein in wild-type AcNPV-infected cells (Fig. 2).

The new protein band was identified to be VP4 by Western blots with guinea pig hyperimmune anti-RRV serum (data not shown) and by immunoprecipitation with hyperimmune anti-RRV serum and a library of monoclonal antibodies (MAbs) to VP4 (Fig. 3). The expressed protein comigrated with authentic VP4 from RRV-infected MA104 cell lysates and was not present in wild-type AcNPV-Sf9 lysates or immunoprecipitated by VP7-specific MAbs (Fig. 3). Additional protein bands below the complete VP4 protein probably represent incompletely synthesized VP4 or degradation products of VP4 and are present in both RRV cell lysates and VP4-Sf9–expressing cells. Finding that a variety of neutralizing MAbs and anti-RRV hyperimmune serum identify this protein indicates that the baculovirus VP4 protein is antigenically similar to VP4 on the virion.

In an ELISA, the VP4 product was bound to microtiter plates and then identified with guinea pig hyperimmune anti-rotavirus serum or 11 neutralizing monoclonal antibodies. Each VP4-specific antibody preparation recognized the

TABLE 1. Antibody recognition of RRV virus- and baculovirus-expressed VP4 protein

Antibody or serum tested	Amino acid no. at VP4 neutralization site	Optical density at 405 nm showing binding[a] to:		HI titer versus:[b]	
		RRV virion	Expressed VP4	RRV	Expressed VP4
M11	87	0.22	0.26	1,600	1,600
A1	88	0.26	0.31	3,200	3,200
A15	89	0.33	0.35	12,800	25,600
1A9	100	0.11	0.15	800	400
5D9	114	0.19	0.29	800	400
M14	148	0.18	0.13	6,400	6,400
5C4	150	0.26	0.11	1,600	800
7A12	188	0.19	0.48	25,600	12,800
M2	388	0.16	0.27	200	25,600
2G4	393	0.13	0.24	25,600	12,800
M7	393	0.14	0.21	1,600	25,600
Anti-RRV hyperimmune serum		1.21	2.31	102,400	102,400
60[c]	No site	0.23	0.018	<200	<200
159[c]	No site	0.68	0.015	51,200	<200

[a] Values from alkaline phosphatase ELISA reactions are shown. ELISA was performed as previously described (44). Expressed VP4 protein was generated from VP4-Sf9 cell lysates harvested 32 h postinfection.

[b] HI assays were performed as previously described (27) using 8 HA units of RRV virus or VP4-Sf9 lysates. Titers indicate the dilution of antisera that resulted in HI.

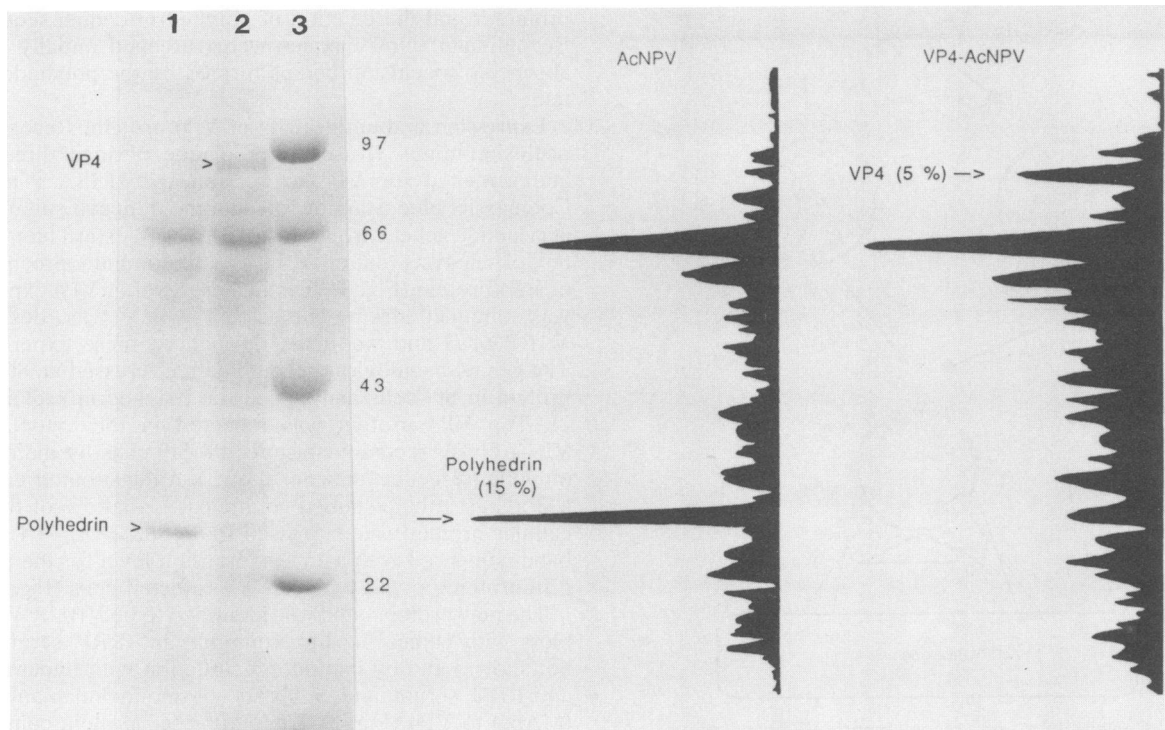[c] MAbs 60 and 159 bind to the RRV VP7 protein.

FIG. 2. Baculovirus expression of VP4. Sf9 cells were infected with wild-type AcNPV or VP4-baculovirus recombinants at a multiplicity of infection of 1. Infected-cell monolayers were harvested 32 h later and sonicated in PBS. Approximately 20 μg of each lysate was boiled in sodium dodecyl sulfate sample buffer and run on a sodium dodecyl sulfate-10% polyacrylamide gel. The gel was fixed with 50% methanol–10% acetic acid and stained in Coomassie blue R-250. Lane 1, AcNPV-Sf9–infected cell lysate; lane 2, VP4-baculovirus–infected Sf9 cell lysate; lane 3, molecular weight markers. Arrowheads indicate the position of the 29-kDa polyhedrin protein (lane 1) and the 86.5-kDa VP4 protein (lane 2). Gel lanes were scanned with a densitometer (right) to quantitate the polyhedrin and VP4 protein expression levels. Peaks were integrated by using a Gaussian analysis program (Hoeffer Scientific), and the relative contribution of protein peaks are expressed as a percentage of total Sf9 infected-cell protein.

expressed VP4 protein, while VP7-specific MAbs 60 and 159 did not react in the ELISA (Table 1). The 11 VP4-specific N-MAbs used in this study are directed at six previously described neutralization sites (32).

HA and HI of expressed VP4. RRV has the ability to agglutinate erythrocytes, and in a genetic analysis of reassortant viruses, this function cosegregates with gene segment 4 (25). To determine whether HA is purely a function of the gene 4 product, VP4, we have used baculovirus-expressed VP4 protein in HA tests with human erythrocytes. In contrast to AcNPV-Sf9 lysates, VP4-Sf9 cell lysates agglutinated erythrocytes (Fig. 4). HA by approximately 1 μg of expressed VP4 protein was found to represent the HA activity of 2.5×10^7 RRV PFU. Apparently, the baculovirus-expressed VP4 protein requires no other rotavirus protein to bind to and agglutinate erythrocytes.

Since the VP4 protein was able to HA, we determined whether N-MAbs which inhibit RRV HA could also inhibit HA by the VP4 protein. Four to eight HA units of expressed VP4 or RRV were reacted with human erythrocytes in the presence of MAbs or guinea pig hyperimmune anti-RRV serum. N-MAbs directed toward the VP8 and VP5 portions of VP4 inhibited hemagglutination of both RRV and the expressed VP4 protein (Table 1). VP5-specific N-MAb, M2, and M7 have a low HI titer against RRV but have considerable activity against expressed VP4 (Table 1).

Glycophorin inhibits HA. Hemagglutinating proteins from other viral systems (1, 7, 17, 24, 35, 39) bind erythrocyte proteins that are posttranslationally modified by N-acetyl neuraminic (sialic acid) containing glycosyl residues. In addition, rotavirus attachment to erythrocytes has been shown to involve carbohydrates on the surface of erythrocytes (4), and sialoglycoproteins inhibit in vitro and in vivo rotavirus replication (53). To determine if VP4 binds by general or specific interactions with sialoglycoproteins, several glycoproteins were used as inhibitors of HA. The only protein to inhibit RRV or VP4 protein agglutination of erythrocytes was the human erythrocyte protein, glycophorin (Fig. 4). Addition of glycophorin inhibited HA by VP4 and RRV at concentrations as low as 1.0 μg/ml. However, neuraminidase treatment of glycophorin prior to testing completely abolished its inhibitory activity (Fig. 4). Glycoproteins tested which did not affect HA assays include submaxillary gland mucin (data not shown), fetal calf fetuin, chicken ovalbumin, turkey ovalbumin, and bovine serum albumin. Addition of N-acetyl neuraminic acid alone also failed to inhibit HA (Fig. 4). Although the glycosylated form of glycophorin could have competed for the VP4 erythrocyte-binding site in order to inhibit HA, this binding event is unlikely and these results suggest that glycophorin is the erythrocyte receptor for rotavirus.

DISCUSSION

The construction of a RRV gene 4 recombinant baculovirus and the expression of the encoded VP4 product are presented in this study. Approximately 5% of the total cellular protein of VP4-AcNPV–infected cells was in an

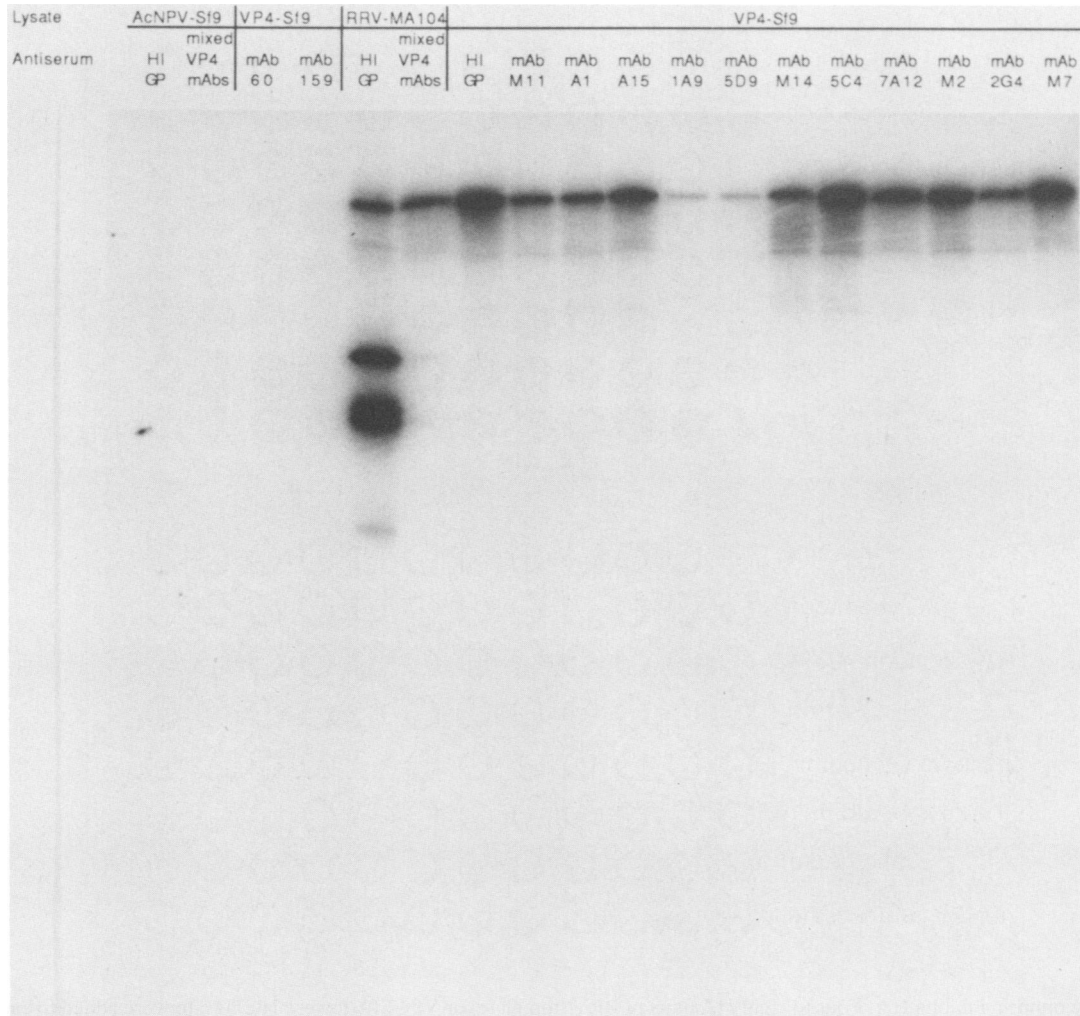| Lysate	AcNPV-Sf9		VP4-Sf9		RRV-MA104		VP4-Sf9											
Antiserum	HI GP	mixed VP4 mAbs	mAb 60	mAb 159	HI GP	mixed VP4 mAbs	HI GP	mAb M11	mAb A1	mAb A15	mAb 1A9	mAb 5D9	mAb M14	mAb 5C4	mAb 7A12	mAb M2	mAb 2G4	mAb M7

FIG. 3. Comparison of RRV- and baculovirus-expressed VP4 protein by immunoprecipitation. Sf9 cells were infected with wild-type AcNPV or VP4-baculovirus recombinants and labeled with 100 μCi of [^{35}S]methionine 24 to 36 h postinfection. MA104 cells were infected with RRV and labeled as described above from 4 to 10 h postinfection. Monolayers were lysed with RIPA buffer, and lysates were centrifuged at 100,000 × g for 40 min and then used for immunoprecipitation (19). VP4-recombinant lysates were precipitated with ascites from VP4-specific MAbs M11, A1, A15, M14, 5C4, 7A12, M2, 2G4, and M7 and with low-titer hybridoma cell supernatants from 1A9 and 5D9 secreting cells. Guinea pig hyperimmune anti-RRV serum (HI GP) was used to precipitate wild-type AcNPV, VP4-AcNPV lysates, or RRV lysates as indicated. Immunoprecipitations of VP4-AcNPV lysates by VP7-specific MAbs 159 and 60 were run as controls. RRV or AcNPV cell lysates precipitated with a mixture of all of the VP4-specific MAbs are labeled above as mixed VP4 MAbs.

expressed VP4 protein band (86 kDa). Since the VP4 gene was inserted into the polyhedrin gene by homologous recombination, we compared the VP4 expression level to the level of native polyhedrin protein (29-kDa) expression (Fig. 1). The polyhedrin protein was expressed at a level of 15% of total cellular protein. It was previously reported that the pACYM1 vector was able to synthesize 50% of the lymphocytic choriomeningitis virus N protein, but the expression level was determined by the incorporation of radioactive precursors 24 h postinfection and did not account for preexisting unlabeled protein (34). Additionally, high levels of overall recombinant protein expression may be obtained later in the infectious cycle (48 to 99 h postinfection) (41). Expression levels were in the range previously reported for the expression of the SA11 inner capsid protein, VP6, in baculovirus (11). Considering the size of the VP4 protein and the variable expression levels of foreign genes by recombinant AcNPV (41), the 5% expression level was remarkable

(Fig. 2). The high level of protein synthesized should make it possible to purify VP4 and study its antigenicity, immunogenicity, and functions. Because VP4 is made in large quantities and remains antigenic, it is reasonable to consider studies directed at determining the three-dimensional structure of the VP4 protein.

Expressing a large amount of VP4 protein has currently allowed us to approach antigenic and functional questions about VP4 using direct biological assays. We have evaluated the expressed VP4 protein in tests as a first step in studying the VP4 protein function. The recombinant VP4 protein agglutinates erythrocytes in tests normally used to assay viral HA. In erythrocyte-binding tests, approximately 4 μg of total VP4-Sf9 cell lysate (0.2 μg of VP4 protein) was equivalent to 8 HA units or about 5 × 10^6 PFU of RRV. Control AcNPV-Sf9 cell lysates had no HA activity and did not enhance or inhibit the ability of RRV to hemagglutinate. The ability of VP4 to hemagglutinate strongly suggests that
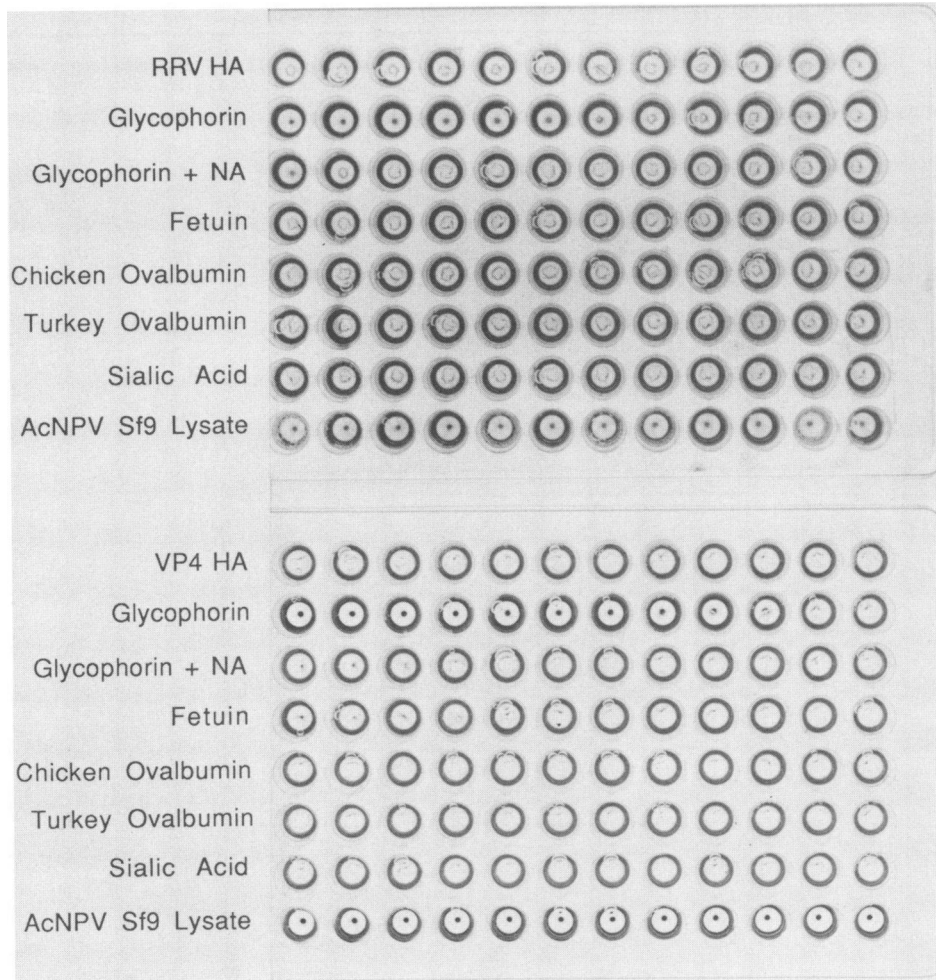
FIG. 4. Glycophorin inhibits HA. Four to eight HA units of RRV (top plate) or VP4-Sf9 (lower plate) lysate were added to each well along with a 1-to-100 dilution of human erythrocytes. The VP4 HA or RRV HA lanes contain no exogenous proteins and serve as positive HA controls. The AcNPV-Sf9 lysate control lanes represents the activity of AcNPV-Sf9 lysate alone in HA. The proteins shown were added at 100 μg/ml in well one and diluted twofold in the wells from left to right. All dilutions were done with PBS (pH 7.4) and 1% bovine serum albumin fraction V. Glycophorin + NA is glycophorin (approximately 85% type A and 15% type B) that was treated with 0.1 U of neuraminidase (NA) in 80 μl at 37°C for 1 h (1.0 μg/μl in PBS). Neuraminidase activity was inhibited by the addition of 20 mM EDTA (39), and the samples were diluted and used in the assay. The first row of the AcNPV-Sf9 lysate control contains 40 times the amount of equivalent VP4-Sf9 cell lysate used in the HA assay and was diluted in the same manner as the other proteins.

the protein maintains its native conformation when expressed in insect cells and that it functions as a hemagglutinin in the absence of other outer or inner capsid rotavirus components. This observation extends previous genetic studies that associated gene 4 with the ability of viruses to hemagglutinate (25).

The ability of the VP4 protein to hemagglutinate allowed us to study how the VP4-erythrocyte interaction was affected by MAbs that are directed at VP4. Previously characterized N-MAbs specific for the VP8 portion of VP4 were all shown to efficiently inhibit HA of VP4 protein and RRV. In a comparison of VP4 versus RRV HI effects, two MAbs (M2 and M7) directed at the VP5 portion of VP4 had substantially higher HI titers against expressed VP4 than against virion, while one MAb directed at VP5 (2G4) reacted equally well with both proteins (Table 1). Interestingly, all three of these MAbs select for VP5 variants with mutations in a similar location (32). The reason for these relative differences in titer are not obvious, but they may be related

to subtle changes in conformation of expressed VP4 relative to VP4 attached to the outer capsid of the virion as well as to the location of the HA-binding domain on the protein.

Another possible explanation accounting for the relative differences in HI titer between selected MAbs arises from the manner in which a single expressed VP4 protein agglutinates erythrocytes. Normally with a multivalent virus, at least two erythrocytes are bound by each virion to produce agglutination. However, unless VP4 is at least bivalent (i.e., a dimer or trimer) or multiple VP4s are attached to some intracellular component, the HA reaction should not be expected to proceed with the expressed VP4 protein. When analyzed by polyacrylamide gel electrophoresis in the absence of 2-mercaptoethanol, boiling, or sodium dodecyl sulfate, there is no apparent multimerization of the VP4 protein for RRV lysates or from the baculovirus-expressed VP4 protein (data not shown). However, hemagglutinating activity is associated with the cell membrane fraction of VP4-infected Sf9 cell lysates (unpublished observations).

This type of VP4 subcellular attachment could enhance the HI response of MAbs to the VP5 region but not alter MAb recognition by ELISA (Table 1). It is interesting to note that the reovirus sigma 1 protein expressed in *E. coli* also maintains the ability to agglutinate erythrocytes (35).

The question of whether HA represents a nonspecific interaction between VP4 and a variety of sialic acid-modified proteins or a specific VP4-erythrocyte interaction was addressed by measuring the ability of selected glycoproteins or sialic acid to inhibit HA. Several sialic acid-containing proteins have previously been demonstrated to inhibit the replication of rotaviruses in MA104 cells, presumably by inhibiting viral attachment to cells (53). Proteins that reportedly caused greater than a 50% reduction in rotavirus replication in MA104 cells at 10 to 100 µg/ml (53) (turkey ovalbumin, chicken ovalbumin, fetuin, or mucin) were not found to have an effect on HA by expressed VP4 or RRV, even at 100 µg/ml (Fig. 4). In contrast, the erythrocyte sialoglycoprotein, glycophorin, inhibited HA by RRV and VP4 protein at 1 µg/ml. The specificity of this reaction was documented by demonstrating that glycophorin treated with neuraminidase had no HI activity (Fig. 4). In addition, preliminary experiments have demonstrated that glycophorin bound to a solid-phase matrix specifically bound to VP4 protein. This data suggests that glycophorin is the specific erythrocyte protein recognized by VP4 during HA and provides the basis for further detailed analyses of VP4 erythrocyte interactions. Glycophorin has recently been shown by Paul and Lee (39) to be the reovirus erythrocyte receptor. However, the reovirus hemagglutinin, sigma 1 (49 kDa), has no significant amino acid homology with the rotavirus hemagglutinin, VP4 (86.5 kDa). If glycophorin is the receptor for both rotavirus and reovirus, then these viruses have approached erythrocyte binding by using distinct protein sequences.

Aside from a functional analysis of VP4 HA, we have studied the antigenicity of the VP4 protein by ELISA, immunoprecipitation, and Western blot analysis. All three methods demonstrate the integrity of the neutralizing epitopes on the expressed VP4 protein. MAbs that recognize six discrete neutralization epitopes on RRV VP4 all bind to the baculovirus-expressed VP4 protein in ELISA. These neutralization sites are involved in both heterotypic and homotypic neutralization and are located in the VP8 and VP5 portions of the VP4 protein (32). In addition, passive-transfer studies have demonstrated that antibodies directed at regions on both VP5 and VP8 are capable of mediating protective immunity (38; Matsui et al., submitted). Expressed VP4 protein was also recognized by hyperimmune anti-RRV serum on Western blots and by N-Mabs under reduced and nonreduced conditions, suggesting that the VP4 neutralization epitopes are not conformationally determined and are conserved on the expressed VP4 polypeptide (data not shown). In light of these findings, the baculovirus-expressed VP4 protein may be useful for studying immune responses to a single rotavirus outer capsid polypeptide and as a synthetic rotavirus vaccine.

In this study, we have constructed a baculovirus-VP4 recombinant virus that expressed high levels of VP4 protein. The expressed polypeptide functions as a hemagglutinin and maintains viral neutralization sites in the absence of other rotavirus outer or inner capsid components. This indicates that the expressed VP4 protein is conformationally conserved and antigenically indistinguishable from VP4 on the rotavirus outer capsid. Current studies are aimed at purifying large amounts of expressed VP4 protein for crystalliza-

tion and toward analyzing the immunogenicity and binding properties of the RRV hemagglutinin.

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