Characterization of Virus-Like Particles Produced by the Expression of Rotavirus Capsid Proteins in Insect Cells

SUE E. CRAWFORD,[1] MARIE LABBÉ,[2] JEAN COHEN,[2] MICHAEL H. BURROUGHS,[1]
YONG-JIE ZHOU,[1] AND MARY K. ESTES[1]*

*Division of Molecular Virology, Baylor College of Medicine, Houston, Texas 77030,[1] and Institut National de la
Recherché Agronomique, Station de Recherché de Virologie et d'Immunologie Moleculaires,
C. R. J. Domaine de Vilvert, 78350 Jouy-en-Josas, France[2]*

Rotaviruses are triple-layered particles that contain four major capsid proteins, VP2, VP4, VP6, and VP7, and two minor proteins, VP1 and VP3. We have cloned each of the rotavirus genes coding for a major capsid protein into the baculovirus expression system and expressed each protein in insect cells. Coexpression of different combinations of the rotavirus major structural proteins resulted in the formation of stable virus-like particles (VLPs). The coexpression of VP2 and VP6 alone or with VP4 resulted in the production of VP2/6 or VP2/4/6 VLPs, which were similar to double-layered rotavirus particles. Coexpression of VP2, VP6, and VP7, with or without VP4, produced triple-layered VP2/6/7 or VP2/4/6/7 VLPs, which were similar to native infectious rotavirus particles. The VLPs maintained the structural and functional characteristics of native particles, as determined by electron microscopic examination of the particles, the presence of nonneutralizing and neutralizing epitopes on VP4 and VP7, and hemagglutination activity of the VP2/4/6/7 VLPs. The production of VP2/4/6 particles indicated that VP4 interacts with VP6. Cell binding assays performed with each of the VLPs indicated that VP4 is the viral attachment protein. Chimeric particles containing VP7 from two different G serotypes also were obtained. The ability to express individual proteins or to coexpress different subsets of proteins provides a system with which to examine the interactions of the rotavirus structural proteins, the role of individual proteins in virus morphogenesis, and the feasibility of a subunit vaccine.

The importance of rotaviruses as etiologic agents of infantile diarrhea in humans and animals is now clearly established (15, 25). The recognition that rotaviruses are the major cause of life-threatening diarrheal disease and of significant morbidity in young children has focused efforts on disease prevention and control against these viruses. Studies on rotavirus gene structure and function are helping these efforts. For example, the cloning and subsequent expression of individual protein products have helped to elucidate the function of each protein in virus structure, replication, and assembly (12, 34). Analysis of the antigenic, functional, and molecular properties of expressed gene products, alone or in conjunction with other viral proteins, is allowing intrinsic properties of each gene product to be determined. Finally, the availability of large amounts of immunogenic structural proteins is facilitating vaccine testing and the production of inexpensive diagnostic tests.

The genome of rotaviruses consists of 11 segments of double-stranded RNA. The double-stranded RNA is enclosed in the viral capsid, which consists of three layers of proteins (12). The rotavirus outer layer is composed of the glycoprotein VP7 and dimeric spikes of VP4 (12, 37, 42, 46). Both outer capsid proteins induce neutralizing antibodies (6, 12, 22, 36). VP4 is the viral hemagglutinin (24, 31). The major capsid protein VP6, which comprises >80% of the protein mass of the particle, is located on the inner capsid and contains the subgroup antigen (15). The presence of VP6 on virus particles has been associated with viral RNA polymerase activity (5). Baculovirus-expressed VP6 spontaneously forms into the native trimeric conformation and can further assemble into

heterogeneous particle-like structures (17, 38, 39). The core particle contains VP1, VP2, VP3, and the 11 RNA segments. VP2, which comprises about 90% of the core protein mass, forms particles when expressed alone and is one protein that binds to viral RNA (27, 47).

Since expressed VP2 assembles into core-like particles and coexpression of VP2 and VP6 results in the formation of double-layered virus-like particles (VLPs) (27), we expressed different combinations of VP2, VP4, VP6, and VP7, using the baculovirus expression system to determine what other rotavirus-like particles could be formed. We report the production, purification, and characterization of VP2/6, VP2/4/6, VP2/6/7, and VP2/4/6/7 VLPs. VLPs containing a VP7 from two different serotypes also were characterized.

MATERIALS AND METHODS

Cells and virus. The serotype G3 simian rotavirus SA11 clone 3 (16) or serotype G1 human rotavirus Wa was cultivated in fetal rhesus monkey kidney (MA104) cells in the presence of trypsin as previously described (14). Triple-layered SA11 particles were purified by CsCl density gradient centrifugation. The baculovirus recombinants used in this study were BacRf2A (26), pVL941/SA11-4, pAc461/SA11-6 (15), pVL941/SA11-9 (serotype G3), and pVL1392/HRV8697-9 (serotype G1). The constructions of pVL941/SA11-4, pVL941/SA11-9, and pVL1392/HRV8697-9 are described below. *Spodoptera frugiperda* 9 (Sf9) cells were grown and maintained in TNM-FH medium containing 10% fetal bovine serum (FBS) (43).

Cloning and construction of baculovirus recombinants. Cloned DNAs (cDNAs) for SA11 gene segment 4, which codes for VP4, and for SA11 gene segment 9, which codes for VP7, were synthesized from mRNA as previously described (17).

* Corresponding author. Mailing address: Division of Molecular Virology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Phone: (713) 798-3585. Fax: (713) 798-3586. Electronic mail address: mestes@bcm.tmc.edu.

Briefly, polyadenylated mRNA was cloned by using the cDNA Synthesis System Plus kit (Amersham International, Amersham, England). The synthesized cDNA was ligated into SmaI-cut, dephosphorylated pUC13 (gene 4) or into PstI-cleaved, dG-tailed pBR322 (gene 9). The 5' end of gene 4 was cloned by using an internal primer to extend the cDNA to the end of the gene, using SA11 mRNA as the template. A second primer to the 5' end was annealed to the first strand of cDNA, and the second strand of cDNA was synthesized by using the Klenow fragment of DNA polymerase (Promega, Madison, Wis.). A full-length cDNA for gene 4 was obtained by ligation of two overlapping clones into pGEM 3Zf+ (Promega).

The gene segment 9, coding for a serotype G1 VP7 from a human rotavirus isolate (Houston 8697, 1991), was cloned by reverse transcription-PCR (1). Briefly, the first-strand cDNA was synthesized by using a primer to the 3' end (GGTCACAT bpCATACAATTCTAATCTAAG) and reverse transcriptase. Double-stranded cDNA was synthesized by using a primer to the 5' end (GGCTTTAAAAGAGAGAATTTCCGTCTGG) by PCR with Taq polymerase (Life Science Inc., St. Petersburg, Fla.). The double-stranded cDNA was cloned into the pCR 1000 vector (TA Cloning System; Invitrogen Co., San Diego, Calif.) and designated pCR1000/HRV8697/G9.

For construction of baculovirus recombinants, the full-length SA11 gene 4 cDNA was subcloned by blunt-end ligation into the Klenow filled-in BamHI site of pVL941. The SA11 gene 9 cDNA from bp 92 to the end of the SA11 gene segment was inserted into pVL941 by blunt-end ligation. This resulted in the utilization of the second in-phase initiation codon for the expression of VP7. The serotype G1 gene 9-containing plasmid, pCR1000/HRV8697/G9, was digested with NotI and SpeI and cloned into the NotI-XbaI sites of pVL1392. Baculovirus recombinants for SA11 genes 4 and 9 were obtained by calcium phosphate precipitation using wild-type baculovirus DNA and the rotavirus gene-containing baculovirus transfer vector as previously described (13). The baculovirus recombinant for the serotype G1 gene 9 was obtained by the Lipofectin transfection method. Lipofectin (Gibco-BRL, Gaithersburg, Md.) was diluted (2 parts Lipofectin to 1 part water), and 20 μl of diluted Lipofectin was mixed with 20 μl of water containing 100 ng of Baculogold linearized baculovirus DNA (PharMingen, San Diego, Calif.) and 500 ng of the recombinant plasmid. The Lipofectin-DNA mixture, incubated at room temperature for 15 min, was added to the medium (1.5 ml of Grace's insect medium containing 0.5% FBS) covering 2×10^6 Sf9 cells plated on T25 flasks and incubated at 27°C for 4 h. Then 2.5 ml of TNM-FH medium containing 10% FBS was added, and the transfection culture was harvested at 4 days posttransfection. Stocks of triple plaque-purified recombinant virus were prepared from each of the transfections. Visualization of plaques was facilitated by addition of 0.01% neutral red to the standard plaque assay overlay (43).

Purification of VLPs synthesized in infected Sf9 cells. To produce each of the different VLPs or chimeric particles, Sf9 cells were coinfected with different combinations of the baculovirus recombinants BacRf2A, pVL941/SA11-4, pAc461/SA11-6, and pVL941/SA11-9 or pVL1392/HRV8697-9, which code for the rotavirus proteins VP2, VP4, VP6, and VP7, respectively, at a multiplicity of infection of 10 PFU per cell. The infection was done in TNM-FH medium containing 10% FBS or in Grace's insect medium containing 0.5% FBS. The VLPs were radiolabeled in vivo by the addition of 20 μCi of ^{35}S-trans (ICN, Irvine, Calif.) 28 h postinfection. The cells and medium were harvested at 144 h postinfection, and the medium was clarified by centrifugation for 10 min at 1,000 rpm in a Damon IEC centrifuge. The clarified medium was extracted

with an equal volume of 1,1,2-trichloro-1,2,2-trifluoroethane (Dupont, Wilmington, Del.) and centrifuged for 15 min at 1,500 rpm in a Damon IEC centrifuge. The aqueous phase was layered over a 35% sucrose cushion and centrifuged for 1 h at 25,000 rpm in an SW28 rotor. The resulting pellet was suspended in TNC (10 mM Tris [pH 7.4], 140 mM NaCl, 10 mM $CaCl_2$), then CsCl was added to obtain a refractive index of 1.3640, and the mixture was centrifuged 18 h at 35,000 rpm in an SW50.1 rotor. The gradients were fractionated, and fractions which contained the VLPs were pooled and centrifuged through a second CsCl gradient. The second gradient was fractionated, and fractions which contained the triple-layered VLPs were pooled and then concentrated and desalted by centrifugation in a 100,000 NMWL filter unit (Millipore Products Division, Bedford, Mass.) or pelleted by centrifugation for 2 h at 35,000 rpm in an SW41 rotor and suspended in TNC buffer. Particle integrity was determined by negative-stain electron microscopy (EM) and by biochemical analysis (see below).

Characterization of VLPs. The VLPs were analyzed by polyacrylamide gel electrophoresis (PAGE), using a modification of the method of Laemmli with 12% separating and 4% stacking gels as previously described (32). Samples were dissociated by boiling for 3 min in sample buffer containing 1% sodium dodecyl sulfate (SDS), 10% 2-mercaptoethanol, 0.5 M urea, 0.05 M Tris-HCl (pH 6.8), 10% glycerol, and 0.0025% phenol red. Radiolabeled proteins were monitored on gels following fluorography as previously described (32). Western blot (immunoblot) analysis using a mouse hyperimmune serum prepared against triple-layered SA11 virus was performed as previously described (6). The Western blots were scanned and analyzed on a UVP System 5000 gel documentation and analysis system (Ultra Violet Products, Science Park, Cambridge, England).

ELISA to analyze epitope integrity. The anti-VP7 monoclonal antibodies (MAbs) used in the enzyme-linked immunosorbent assay (ELISA) were two serotype G3 neutralizing MAbs, YO-1E2, kindly provided by K. Taniguchi (44), and 159, and 60, a nonneutralizing MAb kindly provided by H. B. Greenberg (11). The anti-VP4 MAbs used in the ELISA were two nonneutralizing MAbs, 3D8 and 5E4 (6), and 2G4, a neutralizing MAb, provided by H. B. Greenberg. Polyvinyl 96-well microtiter plates were coated with 100 μl (2 μg/ml) of high-pressure liquid chromatography-purified MAb in carbonate-bicarbonate buffer (pH 9.6). The plates were incubated overnight at room temperature and then were blocked with 200 μl of BLOTTO (5% [wt/vol] Carnation nonfat dry milk) per well for 2 h at 37°C and washed five times. All washes were performed with 0.01 M phosphate-buffered saline (PBS) containing 0.05% Tween 20 (Sigma Chemical Co., St. Louis, Mo.). The different types of VLPs or CsCl-purified triple-layered SA11 were diluted 1:4 and 1:8 in 0.5% BLOTTO, then 50 μl was added to the blocked wells of the microtiter plates, and the mixtures were incubated for 2 h at 37°C. After the plates were washed five times, 50 μl of hyperimmune guinea pig antirotavirus serum was added to each well, and the plates were incubated for 2 h at 37°C. After an additional five washes, horseradish peroxidase-conjugated goat anti-guinea pig immunoglobulin G antibody (HyClone Laboratories, Logan, Utah) was added, and the plates were incubated for 1 h at 37°C. The plates were washed again five times, and ABTS [2,2'-azinobis(3-ethylbenzthiazoline sulfonic acid); Sigma) was added. After a 30-min incubation at room temperature, optical densities were determined at 414 nm with a Titertek Multiskan plate reader (Flow Laboratories, Inc., McLean, Va.).

HA assays. Hemagglutination (HA) assays were performed

in round-bottom 96-well microtiter plates by preparing serial twofold dilutions of the different VLPs or triple-layered SA11 in PBS containing 0.3% bovine-serum albumin (BSA) in a final volume of 50 μl. An equal volume of 0.5% chicken erythrocytes (RBCs) in PBS was added to each well and to control wells containing PBS alone. Results of the test were read after 1 h at room temperature.

Cell binding. Monolayers of MA104 cells were trypsinized, washed, and suspended in TNC buffer containing 1% BSA. [^{35}S]methionine-labeled VLPs or triple-layered SA11, quantitated by the Bio-Rad protein assay, were diluted in TNC containing 1% BSA, and then 0.5×10^6 MA104 cells were added for a total volume of 200 μl. The mixture was allowed to shake for 1 h on ice, and then the cells were washed three times with ice cold TNC buffer and lysed in 200 μl of PBS containing 1% SDS. The cell lysate was counted in 5 ml of Econo-Safe (Research Products International Corp., Mount Prospect, Ill.) by using a Beckman LS 3801 scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). The amount of particles bound was plotted as a function of the amount of particles that had been mixed with the MA104 cells. Each assay with the VLPs was performed in duplicate and repeated at least twice with different preparations of VLPs. The specific activities of the various preparations of VLPs ranged from 1,000 to 16,000 cpm/μg.

MAb typing assay for VP7. The serotype G1 and G3 VLPs were tested in the typing assay, a sandwich ELISA, using MAbs specific for serotypes G1 (KU-4), G2 (S2-2G10), G3 (YO-1E2), and G4 (ST-2G7) and the VP7 common MAb (60-F2D4) as previously described (33).

Immunoelectron microscopy. The chimeric VLPs were labeled with MAb-gold probes. In this procedure, purified G1 and G3 MAbs were labeled with 5- and 15-nm colloidal gold AuroBeads (Amersham, Arlington Heights, Ill.), respectively, as described by the manufacturer. Briefly, AuroBeads (adjusted to pH 9) were mixed with the minimal protecting amount of the MAb (dialyzed against 2 mM borax buffer, pH 9). After the addition of BSA (1%, final concentration), the MAb-gold probe was pelleted, purified on a 10 to 30% glycerol gradient, and then dialyzed against 20 mM Tris (pH 8.2)–150 mM NaCl–1% BSA. The VLPs containing serotype G1, G3, or G1 and G3 VP7s were put onto a carbon-coated 400-mesh copper grid with a 2% Parlodion support film. The grids were placed onto 5 μl of serotype G1 (KU-4)–5-nm gold or serotype G3 (YO-1E2)–15-nm gold probes diluted 1:2 with TNC buffer or onto a 1:1 mixture of serotype G1- and G3-gold probes overnight at 4°C. The grids were negatively stained with 1% ammonium molybdate (pH 5.5) and examined in a Phillips CM-10 electron microscope.

Production of antisera to VLPs containing serotype G1 or serotype G1 and G3 VP7s. Female CD-1 mice (Charles River Breeding Laboratories, Portage, Mich.) were confirmed to be seronegative by ELISA testing of serum obtained at time of arrival. Groups of three seronegative mice were inoculated intramuscularly with 2 μg of purified VLPs containing the G3 VP7 or 5 μg of VLPs containing both the G1 and G3 VP7s in Freund's complete adjuvant. A second inoculation of VLPs in Freund's incomplete adjuvant was administered 14 days later. Serum was collected at 0 and 28 days postinoculation from each of the mice.

Plaque-reduction neutralization assay. Plaque reduction neutralization assays were carried out with approximately 50 PFU per well in six-well plates (Costar, Cambridge, Mass.) as previously described (14).

RESULTS

Production of VLPs. The cDNA coding sequences for the SA11 gene segments 4 and 9 were cloned into the baculovirus transfer vector pVL941 or pVL1392 for the human rotavirus gene 9. Individual recombinants obtained, designated pVL941/SA11-4, pVL941/SA11-9, and pVL1392/HRV8697-9, respectively, were tested and confirmed for their ability to produce VP4 and VP7 (see below). To produce VLPs, Sf9 cells were coinfected with different combinations of the baculovirus recombinants BacRf2A, pVL941/SA11-4, pAc461/SA11-6, and pVL941/SA11-9 or pVL1392/HRV8697-9. Particles were purified from the media of cells infected with the different combinations of recombinant baculoviruses and analyzed by EM. Coexpression of BacRf2A and pAc461/SA11-6 resulted in the formation of VP2/6 particles (Fig. 1A), which had structural features similar to those of double-layered virions (Fig. 1B) as previously described (26). Coexpression of BacRf2A, pAc461/SA11-6, and pVL941/SA11-4 resulted in the formation of VP2/4/6 particles (Fig. 1C). Formation of these VP2/4/6 particles indicated that VP4 interacted directly with VP6 in the absence of the second outer capsid protein, VP7. Comparison of native double-layered rotavirus VP2/6 and VP2/4/6 particles by EM revealed similar structures indicating that the addition of VP4 to the VP2/4/6 particles does not perceptively change the structure or the diameter of these particles. The presence of VP4 on the VP2/4/6 particles was confirmed by both biochemical and biological studies (see below).

Coexpression of BacRf2A, pAc461/SA11-6, and pVL941/SA11-9 and of BacRf2A, pVL941/SA11-4 pAc461/SA11-6, and pVL941/SA11-9 resulted in the formation of VP2/6/7 (Fig. 1D) and VP2/4/6/7 (Fig. 1E) particles, respectively, with structures similar to those of triple-layered virions (Fig. 1F). VP2/6/7 and VP2/4/6/7 VLPs of the bovine Rf strain also were produced and had properties similar to those of the VLPs containing SA11 VP6 and VP7 (data not shown). VP2/4/6/7 VLPs containing VP7 from either serotype G1, G3, or G6 showed no differences in negative-stain electron micrographs (data not shown). EM analysis of purified VP2/4/6/7 particles, stored at 4°C for at least 1 year, revealed that the particles remained intact and thus are very stable.

Composition of the recombinant VLPs. The different types of particles were analyzed by SDS-PAGE and Western blot analysis to confirm the presence of each of the structural proteins in the expressed particles. Comparison of the different ^{35}S-labeled VLPs analyzed by SDS-PAGE (Fig. 2A) or Western blotting (Fig. 2B) showed that each of the genes used to infect the insect cells was expressed and incorporated into each of the respective particle types.

The proteins in the VP2/4/6/7 and triple-layered SA11 particles were detected by Western blotting with a polyclonal antiserum made against triple-layered SA11 particles in hyperimmunized mice (Fig. 3). In some preparations of VP2/4/6/7 VLPs, the detected VP4 migrated at the same position as uncleaved VP4 of authentic triple-layered SA11 particles grown in the absence of trypsin (lanes 2 and 3). In other preparations of VP2/4/6/7 VLPs, a protein which migrated with VP5*, a trypsin cleavage product of VP4, was detected (lanes 1 and 2) which migrated with VP5* from virus grown in the presence of trypsin (lane 4).

Although EM of these VLPs revealed that each preparation contained 90 to 95% triple-layered particles, variation was seen in the amount of the different proteins incorporated into the VLPs according to the method used for quantitation (data not shown). Quantitation of the relative amounts of the proteins by scanning of Western blots and analyzing the results with a Gelbase analysis
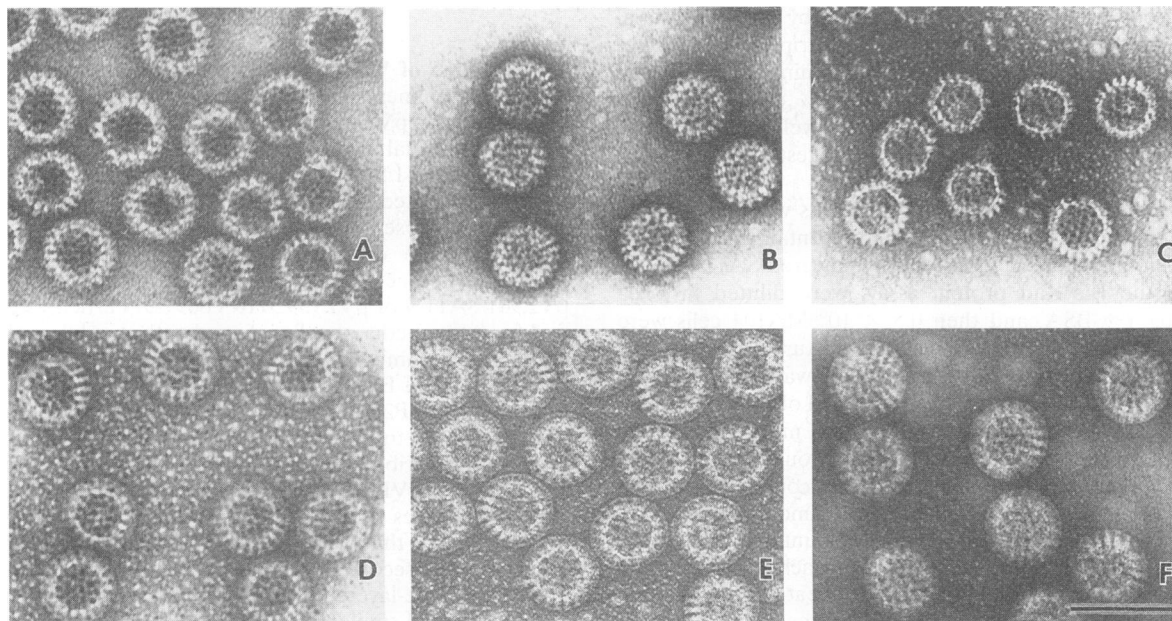
FIG. 1. Electron micrographs of different formulations of VLPs produced by coexpression of baculovirus recombinants containing rotavirus genes. (A) VP2/6 particles; (B) native double-layered SA11 virus; (C) VP2/4/6 particles; (D) VP2/6/7 particles; (E) VP2/4/6/7 particles; (F) native triple-layered SA11 virus. Magnification bar equals 100 nm.

software package indicated the stoichiometry of proteins in VP2/4/6/7 VLPs was similar to that in particles analyzed simultaneously and probed with a polyclonal anti-SA11 serum. However, this quantitation is not precise, as the amount of VP2 detected in the VLPs is underestimated because the polyclonal antiserum made to SA11 was used to detect the proteins and the expressed VP2 is from the Rf bovine strain of rotavirus. Also, Labbé et al. (27) reported that VP2 core-like particles purified in the absence of protease inhibitors contained a second VP2-specific polypeptide which migrated slightly lower than authentic VP2. Zeng et al. (47) also found other VP2-specific polypeptides which migrate either lower or higher than VP2. The anti-SA11 serum used in this report does not detect all of these forms of VP2. Stoichiom-

etry based on Western blots or polyacrylamide gels stained with either silver nitrate or Coomassie blue are rough estimates since the data obtained are dependent on the amount of protein detected by an antibody or stained by the dye and the ability of the densitometer to quantitate the intensity of stained protein. Electron cryomicroscopy will be used to more accurately assess the stoichiometry of the proteins in these particles.

Antigenic and HA properties of the recombinant VLPs. The different particles were screened by using a MAb-based ELISA for reactivity with anti-VP4 and anti-VP7 MAbs. In the ELISA, the different VLPs and triple-layered SA11 were captured by the VP7 MAbs YO-1E2 and 159, two serotype G3, neutralizing MAbs, or 60, a nonneutralizing MAb. The VP4
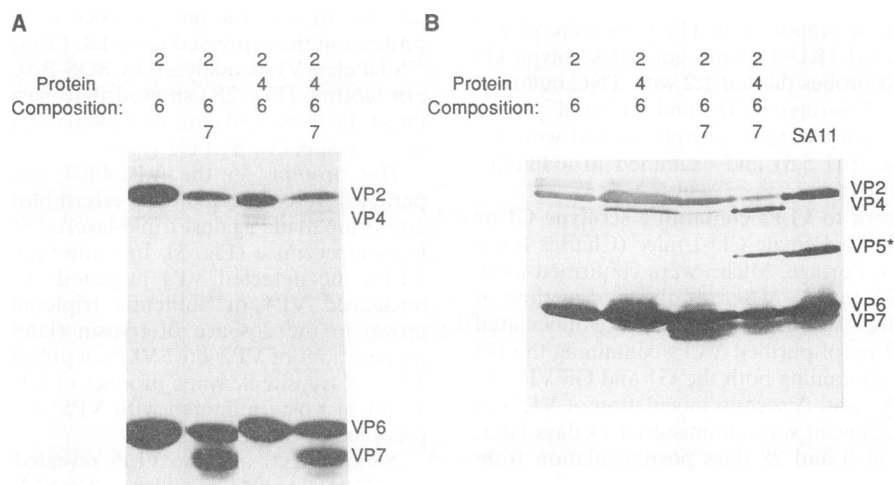

FIG. 2. SDS-PAGE and Western blot analysis of VLP preparations. VLPs made in infected insect cells were purified and separated by SDS-PAGE. ^{35}S-trans-labeled particles were detected by autoradiography (A), or unlabeled VLPs were transferred to nitrocellulose and detected with a hyperimmune anti-SA11 mouse serum (B). The protein composition of each particle is indicated above each lane. The locations of the individual proteins are indicated on the right of each panel.
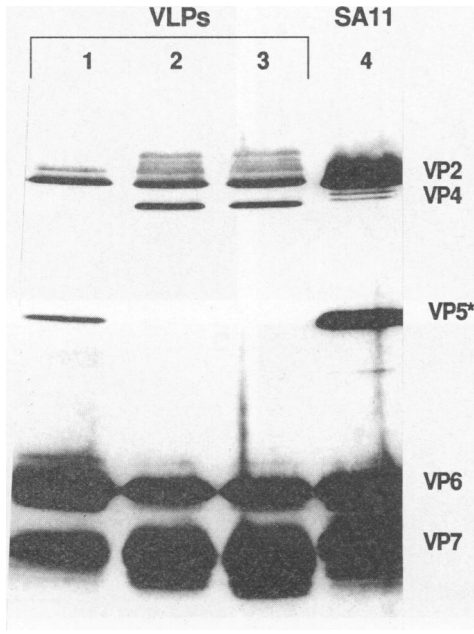
FIG. 3. Comparison of native triple-layered SA11 and VP2/4/6/7 particles by Western blot analysis. Purified VP2/4/6/7 VLPs (lanes 1 to 3), from three different preparations, or SA11 (lane 4) were separated by SDS-PAGE and transferred onto nitrocellulose. Rotavirus proteins were detected by a hyperimmune anti-SA11 mouse serum. The locations of the individual proteins are indicated on the right.

MAbs used to capture the particles were 3D8 and 5E4, two nonneutralizing MAbs, and 2G4, a neutralizing MAb. All particles which contained VP7, i.e., native triple-layered SA11, and the VP2/6/7 and VP2/4/6/7 VLPs showed a positive reaction with the VP7 MAbs (Table 1). Conversely, the particles which contained VP4, native triple-layered SA11, and the VP2/4/6 and VP2/4/6/7 VLPs all showed a positive reaction with the VP4 MAbs. Therefore, the VLPs retained epitope integrity to each of the three VP4 and three VP7 MAbs used in the ELISA.

We also determined if the different virus-like particles could hemagglutinate chicken RBCs. Chicken RBCs were hemagglutinated by triple-layered SA11 and the VP2/4/6/7 VLPs (Table 1). In contrast, VP2/6, VP2/4/6, and VP2/6/7 VLPs did not hemagglutinate the chicken RBCs. Since VP4 has been shown to be the hemagglutinin and baculovirus-expressed VP4 alone
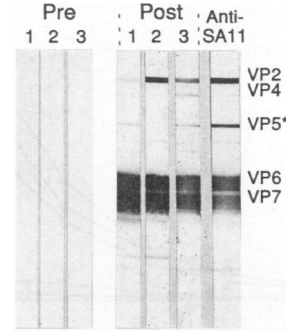

FIG. 4. Immune response to individual rotavirus proteins following immunization of mice with VP2/4/6/7 particles. SA11-infected cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and detected with preimmune sera (pre, mice 1 to 3) or sera from mice immunized with VP2/4/6/7 particles (post, mice 1 to 3). A control blot was detected by a hyperimmune anti-SA11 mouse serum, and the locations of the detected proteins are indicated on the right.

has HA activity (data not shown) (31), the lack of reactivity of VP2/4/6 particles to hemagglutinate chicken RBCs was unexpected. Several preparations of VP2/4/6 particles, which were shown to contain VP4 or VP5* by Western blotting, were screened for their HA ability; none of the preparations hemagglutinated chicken RBCs.

Antiserum produced in mice against the recombinant VP2/4/6/7 particles also detected the viral proteins VP2, VP4, VP6, and VP7 by Western blot analysis (Fig. 4), although the amount of antibody produced to each protein differed for each mouse. These results confirmed that each of the proteins in the VLPs is antigenic.

Cell binding properties of the VLPs. We also used these different VLPs to address the question of which rotavirus protein is involved in cell binding. The VP2/4/6, VP2/4/6/7, and triple-layered particles were able to bind to MA104 cells (Fig. 5), whereas the VP2/6 and VP2/6/7 particles did not bind to MA104 cells. This result indicates that only those particles which contain VP4 bind to MA104 cells.

Characterization of the serotype G1 and G3 VLPs. To determine if chimeric particles could be obtained, VLPs were produced by coinfecting Sf9 cells with the baculovirus recombinants for genes 2, 4, and 6 and either the SA11 (G3) gene 9 or both the SA11 (G3) and the HRV8697 (G1) gene 9. These VLPs were characterized by using a MAb ELISA typing assay for VP7. The VLPs containing the G3 VP7 reacted with the MAb for serotype G3, and the VLPs containing the G1 and G3

TABLE 1. Epitope and HA activities of VLPs

Protein	MAb	ELISA reactivity (OD$_{414}$ × 1,000)[a]										BLOTTO
		VP2/6		VP2/4/6		VP2/6/7		VP2/4/6/7		SA11		
		1:4	1:8	1:4	1:8	1:4	1:8	1:4	1:8	1:4	1:8	
VP7	YO-1E2	2	14	57	36	**137**	39	**421**	**177**	**294**	**107**	0
	159	52	34	97	74	**908**	**517**	**1,757**	**1,645**	**1,231**	**887**	15
	60	28	11	99	53	**212**	94	**557**	**451**	**173**	**136**	23
VP4	3D8	0	26	**313**	**180**	0	27	**776**	**451**	**310**	38	5
	5E4	0	26	**611**	**268**	0	26	**624**	**316**	**125**	53	5
	2G4	23	23	**1,021**	**545**	75	38	**1,544**	**1,381**	**253**	45	0
HA[b]		−		−		−		+		+		NT[c]

[a] Positive ELISA readings are shown in boldface. OD$_{414}$, optical density at 414 nm.
[b] Chicken RBCs were used in the HA assays.
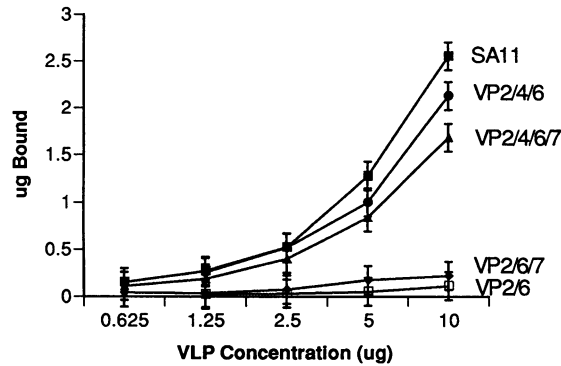[c] NT, not tested.

FIG. 5. Cell binding properties of the different VLPs. The binding of increasing amounts of ^{35}S-trans-labeled triple-layered SA11, VP2/6, VP2/4/6, VP2/6/7, or VP2/4/6/7 VLPs were tested with a constant amount of MA104 cells in a standard cell binding assay.

VP7s were positive with both G1 and G3 MAbs (data not shown). Antiserum produced to the VLPs containing the G3 or the G1 and G3 VP7s was assayed for the ability to neutralize either SA11 (serotype G3) or Wa (serotype G1) (Table 2). Antiserum produced to the VLPs containing the G3 VP7 was able to neutralize SA11 but not Wa. In contrast, antiserum to the VLPs containing the G1 and G3 VP7s was able to neutralize SA11 and Wa, indicating that both VP7s were immunogenic and able to induce neutralizing antibodies.

KU-4–5-nm gold (serotype G1) or YO–1E2-15-nm gold (serotype G3) probes were used to determine if the VLPs made with both G1 and G3 VP7s contained both VP7s on a single particle. The KU-4–5-nm gold probe surrounded the VLPs containing the G1 VP7 (Fig. 6A), and the YO-1E2–15-nm gold probe surrounded the VLPs containing the G3 VP7 (Fig. 6C). In contrast, the KU-4–5-nm gold probe did not react with the VLPs containing the G3 VP7 (Fig. 6B), and the YO-1E2–15-nm gold probe did not react with the VLPs containing the G1 VP7 (data not shown). The VLPs made with both G1 and G3 VP7s were surrounded by both gold probes used (Fig. 6D), indicating that chimeric particles that contained VP7 from both serotypes G1 and G3 were produced.

DISCUSSION

The production of single or combinations of viral proteins in high yields from expression vector systems offers new ways to study viral protein functions in virus structure, replication, and

TABLE 2. Reciprocal of titer of antiserum made to different serotypes of VLPs with indicated test virus (serotype)[a]

Mouse	VLP G serotype	Dose (μg)	Reciprocal of titer[b]			
			Wa (G1)		SA11 (G3)	
			Pre	28 DPV	Pre	28 DPV
1	3	2	<50	<50	<50	800
2	3	2	<50	<50	<50	1,600
3	3	2	<50	<50	<50	1,600
1	1 and 3	5	<50	100	<50	6,400
2	1 and 3	5	<50	100	<50	6,400
3	1 and 3	5	<50	200	<50	3,200

[a] Serum neutralizing antibody response in mice immunized with serotype G3 or G1 and G3 VLPs, using Freund's adjuvant. Plaque reduction neutralization assays were performed against Wa and SA11.

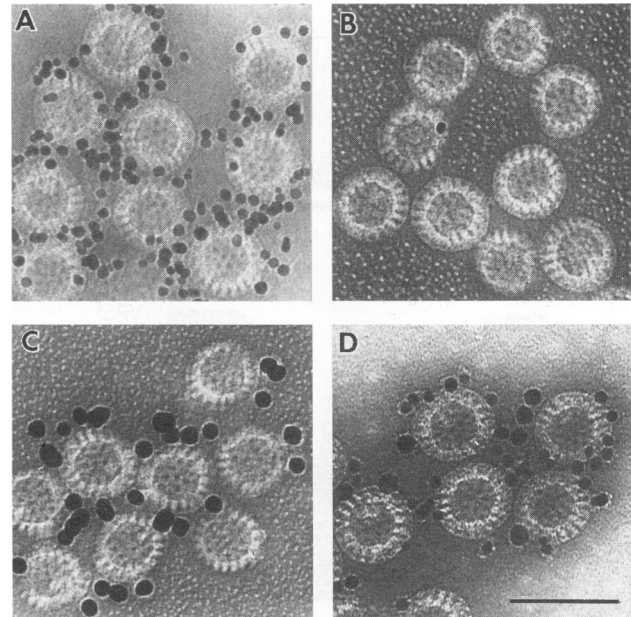[b] Pre, preinfection; 28 DPV, 28 days after immunization.

FIG. 6. Immunogold labeling of VLPs containing G1, G3, or G1 and G3 VP7s. The KU–4-5-nm gold probe was reacted with VLPs containing a G1 VP7 (A) or a G3 VP7 (B). The YO-1E2–15-nm gold probe was reacted with VLPs containing a G3 VP7 (C). The KU-4–5-nm and YO-1E2–15-nm gold probes were reacted with VLPs containing both G1 and G3 VP7s (D). Magnification bar equals 100 nm.

assembly. Redmond (39) reported that spherical particles which resembled smooth, double-shelled rotavirus could be assembled from baculovirus recombinants expressing the bovine rotavirus strain C486 VP6 and VP7. Our experience in coexpressing SA11 VP6 and VP7 in insect cells or mixing these proteins in vitro confirmed that VP6/VP7 particle-like structures can be formed but that these structures are heterogeneous and not stable (data not shown). In contrast, use of VP2 as a scaffolding protein permitted VLPs of different protein compositions (i.e., VP2/6, VP2/4/6, VP2/6/7, and VP2/4/6/7) to be formed by the expression of different combinations of VP2, VP4, VP6, and VP7. The recombinant VLPs were harvested from the media or cells 5 days postinfection, and they shared many similarities to native rotavirus particles, including the ability to be processed by routine purification procedures, excellent stability, and similar structural features. The VP2/6/7 and VP2/4/6/7 particles are 90 to 95% complete double-layered and triple-layered particles, respectively, as estimated by negative-stain EM, and VP2/4/6/7 particles stored at 4°C for at least 1 year remained intact by EM and biochemical criteria.

Western blot analysis revealed that different preparations may contain VP4, VP5*, or a mixture of VP4 and VP5*. It has not been determined when the cleavage event of VP4 occurs, whether particles are formed with a full-length VP4 which subsequently is cleaved, or if particles can be formed following insertion of a cleaved VP4. However, this specific cleavage probably occurs after VP4 is inserted in the VLP because it probably requires the dimeric conformation of the VP4 spike. Since the VLP particles with VP5* were not produced in the presence of trypsin, endogenous proteases from the media or cells must be responsible for this cleavage. This hypothesis is consistent with the observation that VLPs containing full-length VP4 can be obtained from Sf9 cells coinfected and maintained in media containing serine protease inhibitors (data not shown).

Several previous studies have sought to determine which rotavirus protein mediates rotavirus attachment to cell surfaces. Initial studies which examined binding of metabolically radiolabeled soluble viral proteins to cell monolayers identified VP7 to be the rotavirus protein which binds specifically to MA104 cells (19, 41). Bass et al. (3) reported that NS35 bound murine enterocytes and MA104 cells. The relevance of these latter results is unclear because the binding of soluble viral proteins to cells may not accurately reflect events that mediate binding of infectious virions to cells and expressed NS35 alone did not bind to cells (3). Subsequent studies have implicated VP4 as the cell-binding protein. Baculovirus-expressed VP4 was shown to compete with rotavirus particles for binding sites on murine enterocytes (4). In addition, Ruggeri and Greenberg (40) reported that antibodies directed at the VP8* fragment of VP4 inhibit binding of virus to MA104 cells, although Méndez et al. (35) observed that the interaction of animal rotavirus variants with cells occurred at a site different from the sialic acid-binding site located on VP8*. We used VLPs containing either or both VP4 and VP7 to address this issue of the rotaviral attachment protein. Since the VP2/4/6 and VP2/4/6/7 VLPs, as well as native triple-layered SA11, bound to MA104 cells, our data support the idea that VP4 is the viral attachment protein for these cells.

Testing of the VLPs for HA yielded less definitive results. While VP2/4/6/7 VLPs showed HA, VP2/4/6 VLPs did not. This result was unexpected because VP4 expressed alone has HA activity (this report and reference 31). A deficiency of VP4 on the VP2/4/6 particles is probably not responsible for the lack of HA since all preparations of VP2/4/6 particles screened for HA activity were shown to contain VP4 or VP5* by Western blotting. Thus, the inability of the VP2/4/6 particles to hemagglutinate indicates that VP7 may be required for the display of HA activity on particles. VP4 may interact with VP7 near the HA domain (mapped between amino acids 47 and 247 [18, 29]) of VP4, based on the observations that (i) anti-VP7 MAbs can inhibit the HA activity of VP4 (20), (ii) trypsin cleavage of VP4 abolishes reactivity of the virus with certain nonneutralizing VP7-specific MAbs (10), (iii) VP7 epitopes are altered but HA activity is retained in NP1b MAb escape mutants in which the NP1b MAbs mapped (amino acids 180 and 183) in the HA domain of VP4, (iv) the NP1b MAbs bind to a critical site for virus stability since triple-layered particles disassemble as a result of NP1b MAb binding (48), and (v) combinations of heterologous VP4 and VP7 in reassortants affected the expression of a VP4 epitope (9). The conformation of VP4, without the support of VP7, may change when interacting with the VP6 layer so that the HA domain is inaccessible. Since the cell binding domain is still functional in the VP2/4/6 particles, this would support the suggestion that there is a cell binding domain in the VP5* region of VP4 (35).

Our ability to produce VLPs with different protein compositions indicates several noteworthy properties about rotavirus protein and particle structure, function, and assembly. VLP formation indicates that (i) each of the expressed proteins sustains basic intrinsic properties related to folding, oligomeric formation, and protein-protein interactions, (ii) these capsid proteins provide the structural integrity of the different particles, and (iii) the formation of these particles does not require any of the nonstructural proteins or nucleic acid for stability. Our ability to produce VP2/6/7 VLPs indicates that VP4 insertion is not a necessary requirement for particle formation, and the assembly of VP2/4/6 VLPs indicates that VP4 interacts directly with VP6 for particle formation. These data support conclusions of recent structural studies of rotavirus obtained by electron cryomicroscopy and computer imaging processing of native rotavirus. Shaw

et al. (42) observed that VP4 interacts with two molecules of VP7 on the outer surface of the particle, and on the inside, the large globular domain of VP4 interacted with all six of the VP6 molecules surrounding the type II channels. Shaw et al. (42) also noted that it was possible that there were more specific interactions between the VP4 dimer and two of the six VP6 molecules. Electron cryomicroscopy of the VP2/4/6 VLPs as well as of mutations of specific contact sites in VP4 and VP6 are in progress to further enhance our understanding of this interaction. Taken together, these results support the idea that VP4 is attached to VP6 on subviral particles prior to particle budding into the endoplasmic reticulum. This idea also is consistent with recent data indicating that NSP4 possesses a binding domain for VP4 as well as for VP6 (2).

Finally, we also have shown that chimeric VLPs containing either serotype G1 or G3 VP7s or VLPs which contained both G1 and G3 VP7s can be obtained. This finding was not unexpected since we are able to use the Rf bovine VP2 as a scaffolding protein on which to build the VP6 and the VP7 and VP4 layers and reassortants containing the outer capsid proteins from different serotypes can be obtained during replication in mammalian cells (8). In addition, others have suggested that mosaic capsids containing two different VP7 specificities can be formed in cells infected with two viruses of different serotype specificity (45). However, it was not confirmed that VLPs containing the outer capsid protein from different serotypes could be made from coexpression of proteins in the absence of active rotavirus replication. Our ability to use individual baculovirus recombinants to form particles allows VLPs composed of VP2/6 and either or both VP4 and VP7 from different P and G types to be formed and evaluated as subunit vaccines and also to determine the role that VP4 or VP7 alone plays in neutralization and protection. Our results also suggest that a polyclonal serum to VP2/4/6 particles containing different P types can be prepared, and this may aid in the development of P-typing reagents.

These results add to an increasing literature on the success of producing VLPs by using baculovirus (7, 21, 23, 26). Thus, rotavirus VLPs exemplify complex particle formation with some similarities to orbiviruses (28, 30).

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