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Virus-Like Particle-Induced Fusion from Without in Tissue Culture Cells: Role of Outer-Layer Proteins VP4 and VP7

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We recently described an assay that measures fusion from without induced in tissue culture cells by rotavirus, a nonenveloped, triple-protein-layered member of the *Reoviridae* family (M. M. Falconer, J. M. Gilbert, A. M. Roper, H. B. Greenberg, and J. S. Gavora, J. Virol. 69:5582–5591, 1995). The conditions required for syncytium formation are similar to those for viral penetration of the plasma membrane during the course of viral infection of host cells, as the presence of the outer-layer proteins VP4 and VP7 and the cleavage of VP4 are required. Here we present evidence that virus-like particles (VLPs) produced in *Spodoptera frugiperda* Sf-9 cells from recombinant baculoviruses expressing the four structural proteins of rotavirus can induce cell-cell fusion to the same extent as native rotavirus. This VLP-mediated fusion activity was dependent on trypsinization of VP4, and the strain-specific phenotype of individual VP4 molecules was retained in the syncytium assay similar to what has been seen with reassortant rotaviruses. We show that intact rotavirus and VLPs induce syncytia with cells that are permissive to rotavirus infection whereas nonpermissive cells are refractory to syncytium formation. This finding further supports our hypothesis that the syncytium assay accurately reflects very early events involved in viral infection and specifically the events related to viral entry into the cell. Our results also demonstrate that neither viral replication nor rotavirus proteins other than VP2, VP6, VP4, and VP7 are required for fusion and that both VP4 and VP7 are essential. The combination of a cell-cell fusion assay and the availability of recombinant VLPs will permit us to dissect the mechanisms of rotavirus penetration into host cells.

Group A rotaviruses are the leading causes of severe dehydrating gastroenteritis in young children and cause substantial mortality and morbidity worldwide (18, 24). Rotavirus, a member of the *Reoviridae*, is a nonenveloped icosahedral virus consisting of a segmented, double-stranded RNA genome surrounded by three concentric protein layers. Previous studies have established that the rotavirus outer layer proteins, viral protein 4 (VP4; 88 kDa) and viral protein 7 (VP7; 34 kDa), are required for viral infectivity (3, 12). VP7, a glycoprotein, is the major component of the outer layer, whereas VP4 is much less abundant and forms dimeric spikes that project out from the VP7-coated viral surface (38, 42). Although the exact function of VP7 is not clear, VP4 has been shown to be a determinant of host range and virulence and is directly involved in cell attachment and rotavirus entry into cells (20, 22, 34, 40).

For infectivity to occur, proteolytic cleavage of the precursor VP4 to two noncovalently associated subunits, VP8* (28 kDa) and VP5* (60 kDa), is required (10, 11, 26). In vivo, this event occurs in the lumen of the intestine, while in vitro this proteolysis is mimicked by incubation of the virus with the protease trypsin. VP8* is presumably the subunit involved viral attachment to specific cell surface receptors (14, 22, 40). The VP8* of many nonhuman animal rotaviruses binds to sialic acid residues on either proteins or lipids on the cell surface (2, 15, 25, 27). Whether sialic acid is the primary rotavirus receptor for these nonhuman rotaviral strains or whether it is necessary to induce a specific conformation of VP8*, VP5*, or VP7 required for either binding a second receptor or for direct entry is not known. To date, attempts to definitely identify a functional extracellular receptor for rotavirus, other than sialic acid, have not been successful. VP5* contains two provocative sequence motifs that are hypothesized to be involved in virus penetration of host cells (30). One of these motifs is a region that has sequence similarity to the putative internal fusion peptides of Sindbis virus and Semliki Forest virus. The other is a putative alpha-helical coiled-coil domain that is conserved among viruses as diverse retroviruses, alphaviruses, and paramyxoviruses (4).

The mechanism of rotavirus entry into cells is not well understood. To infect a susceptible cell, rotavirus must first bind and then initiate steps to penetrate that cell and enter the cytoplasm. It is theorized that specific binding between a cellular receptor and VP4 must occur in order to initiate infection. This binding to the receptor is hypothesized to trigger an entry-related conformational change(s) in VP4 and/or VP7 required for membrane penetration and subsequent delivery of the viral genome to the host cell cytoplasm for replication. These conformational changes are dependent on the cleavage of VP4 to VP8* and VP5*, since virus containing uncleaved VP4 can bind to cells as well as virus containing the cleaved proteins, but it cannot successfully enter the cytoplasm and replicate (1, 7, 19, 23).

Although experiments have demonstrated that rotavirus enters cells in a pH-independent fashion, it is not clear whether rotavirus penetrates cells directly through the plasma membrane or whether the virus enters the cell through the early endosomal membrane after receptor-mediated endocytosis (16, 23, 28, 39, 44). We described previously an assay that measures the ability of virus to induce syncytia when added to cells (13). We hypothesized that the ability of rotavirus to induce fusion from without is related to its ability to penetrate the plasma membrane, thus providing an assay to identify

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factors involved in viral entry into cells. In this assay, MA104 cells, a tissue culture cell line permissive for rotavirus infection, can form polykaryons when incubated with rotavirus. This fusion from without occurs only if the cells have been preincubated with cholesterol, a compound which increases membrane fluidity and enhances virus-induced syncytia with several types of enveloped viruses (9, 36, 37). Syncytium production requires cleavage of VP4 by the protease trypsin, and this parallels the requirement for the proteolytic activation of VP4 seen for infectivity in vitro and in vivo. Syncytium formation also depends on the presence of VP4 and/or VP7, as particles depleted of both outer layer proteins (double-layered particles [DLPs]) could not induce polykaryon formation (13).

Detailed analysis of the role of the outer layer proteins VP4 and VP7 in entry of rotavirus into cells has been impeded by the fact that a method to alter a specific rotavirus gene product and recover it in infectious virus is not yet available. To circumvent this difficulty, we have used virus-like particles (VLPs) (8) as an alternative to intact rotavirus particles. VLPs are formed by coinfecting *Spodoptera frugiperda* 9 (Sf-9) cells with four different recombinant baculoviruses, each expressing one of the four main structural proteins of rotavirus (VP2, VP4, VP6, or VP7). The coexpressed rotavirus proteins assemble into particles that are similar to intact rotavirus particles by electron microscopic, biochemical, and immunological criteria (8). The VLPs have also been shown to bind specifically to MA104 cells (8). In this study, we demonstrate that VLPs are able to induce cell-cell fusion between cholesterol-supplemented MA104 cells. We also now show that cells that are permissive for rotavirus infection are capable of forming syncytia whereas cells that are nonpermissive to rotavirus infection cannot form syncytia. Polykaryon formation requires the presence of both VP4 and VP7 and the cleavage of VP4 to VP8* and VP5*. With the ability of these recombinant VLPs to induce syncytia, we will now be able to examine the role of VP4 and VP7 in rotavirus entry on a molecular level.

MATERIALS AND METHODS

Cells and viruses. The fetal rhesus monkey kidney cell line MA104 was obtained from Biowhittaker (Walkersville, Md.), CV-1 cells were obtained from American Type Culture Culture (Rockville, Md.), and L cells and HEp2 cells are as described by Bass et al. (1). All cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Biowhittaker) with glucose (4.5 g/liter) and containing 10% fetal bovine serum (FBS; Hyclone, Logan, Utah), 100 IU of penicillin per ml, 100 IU of streptomycin per ml, 0.29 mg of L-glutamine (Irvine Scientific, Santa Ana, Calif.) per ml, and 0.25 mg of amphotericin B (GibcoBRL; Gaithersburg, Md.) per ml in a 5% CO_2 incubator at 37°C. Both strains of rotavirus, a simian strain (rhesus rotavirus [RRV]) and a murine strain (EW), were prepared as follows. MA104 monolayers were infected (10 PFU/cell) with trypsin-activated RRV or EW in DMEM, in the absence of FBS and trypsin. Virus was harvested 9 to 12 h postinfection, by freezing and thawing three times. The samples were first cleared of debris by centrifugation at 1,000 × *g* at 4°C for 15 min followed by centrifugation over a 35% (wt/vol) sucrose cushion in 10 mM Tris–100 mM NaCl–2 mM $CaCl_2$ (TNC) at 4°C at 125,000 × *g* for 2 h. The resulting virus pellet was resuspended in DMEM and stored at −70°C. Virus was titered by a focus forming assay (27) and was found to have 10^9 focus-forming units (FFU)/ml for RRV and 5×10^7 FFU/ml for EW. Purified, concentrated RRV (2.68 mg/ml; 1.7×10^{11} PFU/ml) was a gift of S. Cholodofsky (Wyeth-Ayerst, Radnor, Pa.).

Sf-9 cells were grown in Insect Xpress protein-free medium (Biowhittaker) supplemented with 100 IU of penicillin per ml, 100 IU of streptomycin per ml, 0.29 mg of L-glutamine per ml, and 2.5% FBS in monolayer culture at 27°C. Recombinant virus stocks (VP2 and VP6 of the RF virus strain [gift of J. Cohen], VP4 of either the RRV or EW strain, and VP7 of the EW strain) were grown in Sf-9 cells in Insect Xpress medium supplemented with 60 IU of penicillin and streptomycin per ml and 0.17 mg of L-glutamine per ml.

Antibodies and reagents. The VP4 antibodies used were the neutralizing monoclonal antibody (MAb) M2 and the nonneutralizing MAb HS-2 (40, 43). The VP7 antibody used was the nonneutralizing MAb 60 (43). Rabbit hyperimmune serum was also used (5). For immunoprecipitation, antibodies were precoupled to protein A-Sepharose (Sigma) for 2 h at 4°C. The antibody-bead complexes were washed extensively and added to the ^{35}S-labeled VLPs, and the samples were immunoprecipitated overnight at 4°C. Samples were washed and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described below. Protein concentrations were determined by using a bicinchoninic acid protein reagent kit (Pierce Chemical Company, Rockford, Ill.).

VLP production. VLPs composed of either VP2/6, VP2/4/6, VP2/6/7 (referred to as partial VLPs), or VP2/4/6/7 (referred to as complete VLPs; VLPs containing the RRV strain VP4 are called RRV VLPs, and those containing the EW strain VP4 are called EW VLPs) were prepared as described previously (8). Briefly, monolayers of cells were infected with the desired combination of recombinant baculoviruses at a multiplicity of infection (MOI) of 5 in Insect Xpress media. After incubation for 24 to 36 h at 27°C, the inoculum was removed by aspiration, and medium containing the protease inhibitors pepstatin, aprotinin, and leupeptin (0.5 μg/ml; Sigma Chemical Company, St. Louis, Mo.) was added. These protease inhibitors were added daily until the cells were harvested at 6 to 7 days postinfection. For ^{35}S-radiolabeled VLP production, 25 μCi of [^{35}S]methionine (Amersham, Arlington Heights, Ill.) in methionine-free media (Ex-Cell 401; JRH Biosciences, Lenexa, Kans.) was added. Cells were pelleted at 3,300 × *g* for 15 min at 4°C. The sample was recentrifuged, first at 12,000 × *g* in a JA-14 rotor (Beckman Instruments, Inc., Fullerton, Calif.) for 30 min at 4°C and then at 200,000 × *g* in a Ti 45 rotor (Beckman) for 2 h at 4°C. The pellet was resuspended in TNC containing aprotinin, leupeptin, and pepstatin and then repelleted through a 35% (wt/vol) sucrose in TNC at 300,000 × *g* for 90 min at 4°C. The VLP pellets were resuspended in CsCl (1.36 g/ml) in TNC and banded by isopycnic centrifugation at 250,000 × *g* for 24 to 36 h at 4°C in an SW-40 rotor (Beckman). The bands were collected and rebanded as described above. These bands were collected, dialyzed extensively against TNC, and stored at 4°C.

VP4 and VP4/VP7 production. Recombinant, baculovirus-expressed VP4 was produced as described by Mackow et al. (29). The amount of protein was determined, and the amount of VP4 present was determined by Western blot assay (17) and compared with the amount of VP4 in a known concentration of purified RRV. Soluble VP4 and VP7 were prepared from purified RRV as follows. One hundred micrograms of virus was treated or not with 10 μg of trypsin (type XIII, *N*-tosyl L-phenylalanine chloromethyl ketone [TPCK] treated; Sigma) per ml at 37°C for 60 min. After digestion, trypsin was inactivated by adding equimolar amounts of *N*-α-p-tosyl-L-lysine chloromethyl ketone (TLCK; Sigma). The virus samples were then treated with 10 mM EDTA at 37°C for 60 min, and the soluble proteins were separated from the DLPs by centrifugation over a 35% (wt/vol) sucrose-TNE cushion at 300,000 × *g* for 90 min at 4°C. The material that did not pellet was dialyzed extensively against TNC and concentrated in a Centricon 10 concentrator (Amicon, Beverly, Mass.), and the amount of VP4 and VP7 present was determined by both Western blotting and protein assay as described below.

Fusion assay. For fusion assays, 5×10^5 cells were plated in 25-cm^2 flasks in 7.5 ml of DMEM containing 10% FBS. After 24 h, medium was replaced with 7.5 ml of DMEM containing 7.5% FBS and 100 μM cholesterol (Sigma) and incubated for 16 h at 37°C in a CO_2 incubator. Virus and VLPs were pretreated or not with 10 μg of trypsin per ml at 37°C for 30 min, and then trypsin was inactivated by the addition of TLCK. Sonicated VP4 was treated with 100 μg of trypsin per ml at 37°C for 30 min to achieve cleavage of VP4 to VP5* and VP8*. Cholesterol-treated cells were washed twice with phosphate-buffered saline (PBS), removed from the flask by trypsinization, and resuspended in DMEM without serum at 10^6 cells per ml. Then 1×10^5 to 1.25×10^5 cells (as indicated in the Figure legends) were added to each microcentrifuge tube and incubated on ice for 5 min. Cells were pelleted at 500 × *g* and then resuspended in 150 μl (final volume) of cold DMEM containing either virus, at an MOI of 75, or VLPs or soluble proteins at the indicated concentrations. For comparison of native virus and VLPs, the protein concentration of a CsCl-purified, titered stock of RRV triple-layered particles (TLPs) was determined to be approximately 2.5×10^9 FFU per mg of protein. Therefore, the addition of 3.75 μg of VLP protein to cells was approximately equivalent to an MOI of 75 FFU per cell. For VP4 and VP7, the amount of protein added was determined by comparison with known amounts VP4 and VP7 in titered virus by Western blot assay with the MAbs HS-2 and 60, respectively. To achieve the equivalent amount of VP4 protein present in RRV TLPs, 9 μg of protein for soluble VP4 and 1.5 μg of protein for soluble VP4 and VP7 are required to approximate an MOI of 75 FFU per cell. Both the MOI and the amount of VLP or soluble protein used in each assay are indicated in the Figure legends. Samples were incubated on ice for 15 min and then briefly microcentrifuged at 500 × *g*. After incubation at 37°C for 15 min, 400 μl of DMEM was added, and the samples were resuspended, plated into six-well dishes containing 1 ml of DMEM supplemented with 15% FBS, and incubated for 2 h at 37°C in a CO_2 incubator. For experiments involving CV-1, HEp2, and L cells, cells were examined for syncytia after 3 h at 37°C in a CO_2 incubator to facilitate cell plating. Cells were then examined for syncytium formation by phase-contrast microscopy with an Olympus CK2 inverted microscope (Olympus America, Inc., Melville, N.Y.). Cells containing two or more nuclei were considered to have undergone fusion events. Samples were tested in duplicate; a minimum of 500 nuclei were counted per sample (five sets of approximately 100 nuclei each), and the number of nuclei in polykaryons was noted. Data are averaged and presented as number of nuclei in syncytia per 100 total nuclei counted.

SDS-PAGE. Samples were processed for SDS-PAGE as described previously (17) and electrophoresed at the indicated acrylamide concentrations (Novex, San Diego, Calif.). For Western blot assays following electrophoresis, proteins were transferred to nitrocellulose (Schleicher & Schuell, Keene, N.H.) and probed with either HS-2 alone or HS-2 and 60. The bound antibodies were detected with goat anti-mouse immunoglobulin G coupled to alkaline phosphatase (Kirkegaard & Perry, Gaithersburg, Md.). The alkaline phosphatase signal was detected by a using a 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium (BCIP-NBT) phosphatase substrate system kit (Kirkegaard & Perry). For autoradiography, the gels were fixed and enhanced with Entensify (Dupont, Boston, Mass.), dried, and then exposed to film (X-Omat, Eastman-Kodak, Rochester, N.Y.). All figures were scanned with a UMAX model UC1260 scanner. The figures were prepared by using Adobe Photoshop, Adobe Illustrator, and Canvas programs.

Trypsinization. ^{35}S-labeled complete RRV and EW VLPs and the partial VLPs were treated or not with 10 μg of TPCK-treated trypsin per ml for 30 min at 37°C. The reactions were quenched by treatment with an equimolar amount of TLCK. Samples were then diluted into lysis buffer containing additional protease inhibitors and immunoprecipitated with rabbit hyperimmune serum overnight at 4°C. Samples were washed and then subjected to SDS-PAGE as described above. Unlabeled VP4 and soluble VP4 and VP7 were trypsinized as described above, and the proteins were detected by Western blot assay as described above.

Neuraminidase treatment. Neuraminidase (sialidase from *Vibrio cholerae*) was purchased from Boehringer Mannheim (Indianapolis, Ind.). For treatment, approximately 2×10^6 cells were washed with PBS and treated with or without 60 mU of neuraminidase at 37°C for 1 h. Cells were then rinsed with DMEM, trypsinized as described above, and used in the syncytium assay.

Hemagglutination assay. Type O blood was drawn from healthy donors, washed three times in Alsever's solution, and diluted to 0.5% in PBS containing 1% bovine serum albumin (BSA; fraction V; Sigma) (PBS-BSA). Virus, VLP, or protein samples (at equivalent VP4 concentrations) were serially diluted twofold into the wells of a 96-well dish with PBS-BSA. Then 50 μl of 0.5% erythrocytes (RBCs) in PBS-BSA was added to each well and incubated at room temperature for 2 h. Hemagglutination units were calculated by the method of Hirst (21).

RESULTS

Production and composition of VLPs containing RRV VP4 (RRV VLPs). VLPs containing RF VP2, RRV VP4, RF VP6, and EW VP7 were produced as described in Materials and Methods. The protein composition of the RRV VLPs was examined by SDS-PAGE and fluorography (Fig. 1A, lane 6). The presence of VP4 is difficult to detect in Fig. 1A because (i) there is significantly less VP4 than the other viral proteins on particles and (ii) there is some proteolysis of VP2 which comigrates with the VP4 protein (13, 45). To ensure that the RRV VLPs retained the antigenic integrity of native rotavirus particles, the RRV VLPs were examined by immunoprecipitation with MAbs directed against epitopes on VP4 and VP7 (Fig. 1B, lanes 6 and 12, respectively). The immunoprecipitation of the RRV VLPs with these MAbs indicated that RRV VLPs were appropriately assembled by this criteria. To ensure that the VP4 in the particle could be cleaved by trypsin to VP8* and VP5*, RRV VLPs were treated with or without trypsin, and the products of digestion were examined by immunoprecipitation and SDS-PAGE (Fig. 2A, lanes 11 and 12). Prior to digestion with trypsin, little or no VP5* was detected, but after incubation with 10 μg of trypsin per ml for 30 min at 37°C, VP4 was cleaved to VP5* and presumably VP8*. These results indicated that the RRV VLPs contained the expected proteins and that at least some of the appropriate epitopes presented on mature virions were preserved. Additionally, RRV VLP VP4 was properly cleaved to VP8* and VP5* in the presence of trypsin.

RRV VLPs induce syncytia with MA104 cells in a trypsin-dependent and concentration-dependent fashion. Since RRV VLPs appeared virus-like by the above biochemical and serologic criteria, we next examined whether these particles could induce syncytia in cholesterol-supplemented MA104 cells in a manner similar to native RRV virus particles (13). Trypsinized or mock-treated RRV VLPs and RRV were incubated with MA104 cells as described in Materials and Methods. Cells were examined microscopically, and nuclei were counted. Cells

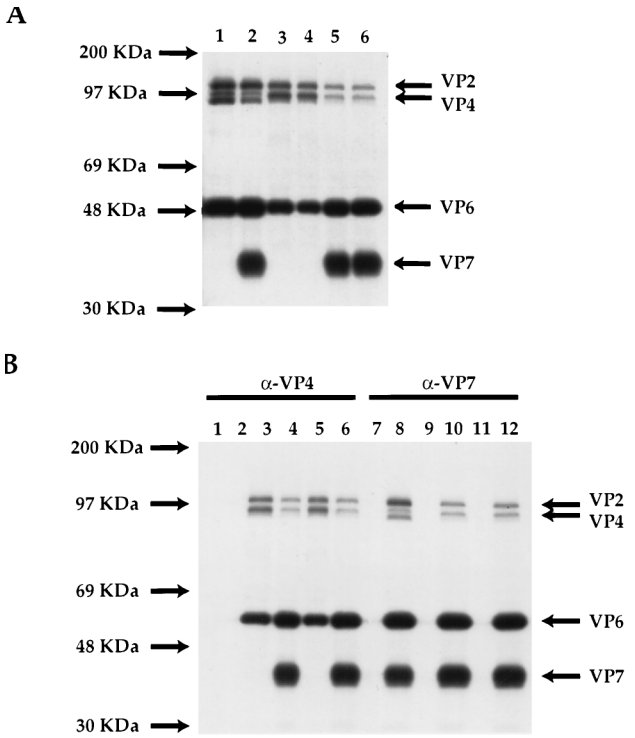

FIG. 1. (A) ^{35}S-labeled VLPs. ^{35}S-labeled RRV and EW VLPs and the partial VLPs were examined by SDS-PAGE on a 10% acrylamide gel (Novex). The gel was fixed, fluorographed, and exposed to X-ray film (X-Omat; Kodak). Lane 1, VP2/6 VLPs; lane 2, VP2/6/7 VLPs; lane 3, EW VP2/4/6 VLPs; lane 4, RRV VP2/4/6 VLPs; lane 5, EW VP2/4/6/7 VLPs; lane 6, RRV VP2/4/6/7 VLPs. Molecular weight markers are indicated on the left. Migration positions of the viral proteins are indicated on the right. (B) Immunoprecipitation of ^{35}S-labeled VLPs. ^{35}S-labeled RRV and EW VLPs and the partial VLPs were immunoprecipitated with a MAb to either VP4 (MAb M2; lanes 1 to 6) or VP7 (MAb 60; lanes 7 to 12) and examined by SDS-PAGE on a 10% acrylamide gel. The gel was fixed, fluorographed, and exposed to X-ray film. Lanes 1 and 7, VP2/6 VLPs; lanes 2 and 8, VP2/6/7 VLPs; lanes 3 and 9, EW VP2/4/6 VLPs; lanes 4 and 10, EW VP2/4/6/7 VLPs; lanes 5 and 11, RRV VP2/4/6 VLPs; lanes 6 and 12, RRV VP2/4/6/7 VLPs. Molecular weight markers are indicated on the left. Migration positions of the viral proteins are indicated on the right.

that were not incubated with either virus or VLPs had very few polykaryons, as did cells that were incubated with RRV or RRV VLPs that had not been treated with trypsin (Fig. 3). As expected, RRV that had been treated with trypsin induced cell cell fusion at a level three- to fourfold higher than that for virus that was not trypsin activated. Two different concentrations of RRV VLPs were tested to see if they could induced syncytia in MA104 cells. Since VLPs cannot infect cells, the protein concentration of a purified and titered amount of RRV was used to standardize the quantity of VLPs added to the cells (see Materials and Methods). Amounts of RRV VLP protein equivalent to 50 and 100 FFU per cell were treated or not treated with trypsin prior to incubation with MA104 cells. Only the VLPs that had been pretreated with trypsin induced syncytia in MA104 cells; untrypsinized VLPs and native RRV induced the same number of syncytia as in cells that were untreated (Fig. 3). The number of nuclei seen in polykaryons was dependent on the amount of RRV VLPs added per cell. RRV VLPs added at a concentration equivalent to 50 FFU per cell resulted in only a 2.5-fold increase in the number of polykaryons formed, whereas doubling the number of RRV VLPs bound per cell increased the number of syncytia approximately 5-fold. This concentration-dependent increase in RRV

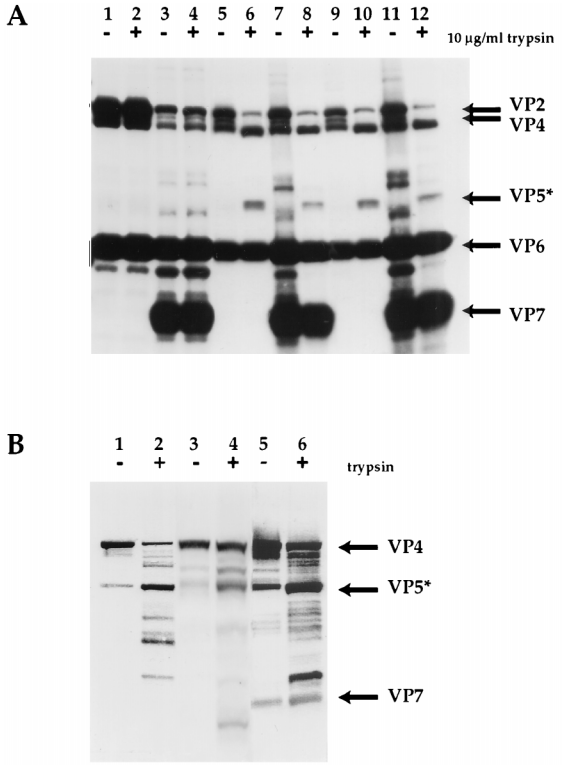

FIG. 2. (A) Trypsinization of ^{35}S-labeled VLPs. ^{35}S-labeled RRV and EW VLPs and the partial VLPs were treated (+) or not (−) with trypsin (10 μg/ml) for 30 min at 37°C. The reactions were quenched with TLCK, and the samples were immunoprecipitated with rabbit hyperimmune serum and examined by SDS-PAGE on a 10% acrylamide gel. The gel was fixed, fluorographed, and exposed to X-ray film. Lanes 1 and 2, VP2/6 VLPs; lanes 3 and 4, VP2/6/7 VLPs; lanes 5 and 6, EW VP2/4/6 VLPs; lanes 7 and 8, EW VP2/4/6/7 VLPs; lanes 9 and 10, RRV VP2/4/6 VLPs; lanes 11 and 12, RRV VP2/4/6/7 VLPs. Molecular weight markers are indicated on the left. Migration positions of the viral proteins are indicated on the right. (B) Trypsinization of VP4 and VP4/VP7. RRV virus (1.5 μg; lanes 1 and 2), VP4 (4 μg of total protein; lanes 3 and 4), and VP4/VP7 (0.5 μg of total protein; lanes 5 and 6) were treated (+) or not (−) with trypsin for 30 min at 37°C and examined by SDS-PAGE on a 10% acrylamide gel as described in Materials and Methods. The electrophoresed proteins were transferred to nitrocellulose, and the VP4 protein was detected with MAb HS-2 (a carboxyl-terminal specific VP4 MAb) (lanes 1 to 6). The VP7 protein in the soluble VP4/VP7 samples (lanes 5 and 6) was detected with MAb 60 (a VP7-specific MAb). The bound antibodies were detected with goat anti-mouse immunoglobulin G coupled to alkaline phosphatase. The alkaline phosphatase signal was detected by a BCIP-NBT phosphatase substrate system kit.

VLP-mediated fusion from without is similar to the behavior of RRV virions reported previously (13). These results demonstrate that the RRV VLPs induce syncytia as efficiently as trypsinized, native RRV viral particles. This cell-cell fusion is dependent on trypsinization of VP4 and increases proportionally with increasing numbers of particles added per cell, similar to what is observed with infectious rotavirus in cell culture. In addition, the ability of VLPs, which lack the viral genome and are therefore noninfectious, to induce syncytia confirms our previous observation that fusion from without is not dependent on viral replication (13).

Syncytium formation occurs only with cells permissive to rotavirus infection. To further demonstrate that the conditions required for syncytium formation in our assay are similar to those required for viral infectivity, we next examined polykaryon formation in another permissive (CV-1) and two nonpermissive (L cells and HEp2) cell lines. Prior studies had indicated that growth restriction in L cells and HEp2 cells was at the level of viral entry, not binding (1). Cells were preincubated overnight with cholesterol as previously described. Cells were then incubated with either trypsinized or mock-treated RRV or RRV VLPs. Cells were examined microscopically and nuclei were counted as described above. As before, only trypsin-treated RRV and RRV VLPs could induce syncytia and only in the permissive cell lines, MA104 and CV-1 (Fig. 4). No specific syncytia were seen with either L cells or HEp2 cells, although HEp2 cells had a slightly higher basal level of syncytia than the other cell lines (Fig. 4). The infectivity titers of RRV on all cells were tested and determined to be the same regardless of whether cells were preincubated with cholesterol (1×10^9 and 1.5×10^8 for MA104 and CV-1 cells; 2×10^4 and 1×10^5 for HEp2 and L cells). These data indicate that only cells that are permissive to rotavirus penetration are susceptible to RRV or RRV VLP-induced syncytium formation.

VP4 strain specificities are reproduced with VLPs. The VP4 of many animal rotaviruses binds to sialic acid residues on the cell surface (2, 15, 25). Whether sialic acid is the primary rotavirus receptor for these strains is not known, but it is clear that if cells are treated with neuraminidase to remove cell surface sialic acid residues, the infectivity of these sialic acid-dependent strains is significantly reduced (27). To examine the role of sialic acid dependence in syncytium formation, a neuraminidase-insensitive strain of rotavirus, EW, and RRV, a neuraminidase-sensitive strain of rotavirus, were grown and purified. VLPs differing only in the strain serving as the source of VP4 used, either EW or RRV, were also prepared. The protein composition of the EW VLPs was analyzed by SDS-PAGE and fluorography (Fig. 1A, lane 5), immunoprecipitation (Fig. 1B, lanes 4 and 10), and trypsinization (Fig. 2A, lanes 7 and 8). The EW VLPs appeared to be intact by biochemical and serological criteria, similar to the RRV VLPs. The ability of these VLPs, as well as EW and RRV virus particles, to hemagglutinate type O RBCs was also assessed (Table 1). As previously demonstrated, RRV, the sialic-dependent strain, can hemagglutinate RBCs, whereas EW does not hemagglutinate. We observed that the ability of VLPs to hemagglutinate RBCs depends on the origin of the VP4. VLPs containing EW VP4 cannot hemagglutinate RBCs, but VLPs containing RRV VP4 can. These findings confirm previous genetic and biochemical studies which identified VP4 as the viral hemagglutinin (14, 22, 27, 29).

To determine whether the sialic acid binding phenotype is conserved in the MA104 syncytium assay, viruses and VLPs were treated with trypsin and then bound to MA104 cells that were pretreated with neuraminidase in PBS or with PBS alone. As would be predicted from previous binding and infectivity studies, RRV required the presence of sialic acid residues on the cell surface for syncytium formation. Cells that were pretreated with neuraminidase had a three- to fourfold decrease in syncytium formation (Fig. 5). The EW strain was completely insensitive to neuraminidase treatment of cells, forming polykaryons equally with treated and untreated MA104 cells. Accordingly, RRV VLPs were sensitive to neuraminidase treatment (a fourfold decrease in syncytium production), whereas EW VLPs were not. These data indicate that the requirement for binding of VLPs for cell surface sialic acid in the syncytium assay duplicates the requirement for sialic acid among the different strains of rotavirus (2, 15, 27). Since the RRV and EW VLPs differ only in the strain of VP4 that comprises the particle, this finding confirms the infectivity data implicating VP4 in the sialic acid dependence phenotype. This observation further demonstrates the similarity between VLP and virion functions and confirms the original observation that the parameters affecting syncytium formation closely resemble the parameters of the early stages of viral infection.

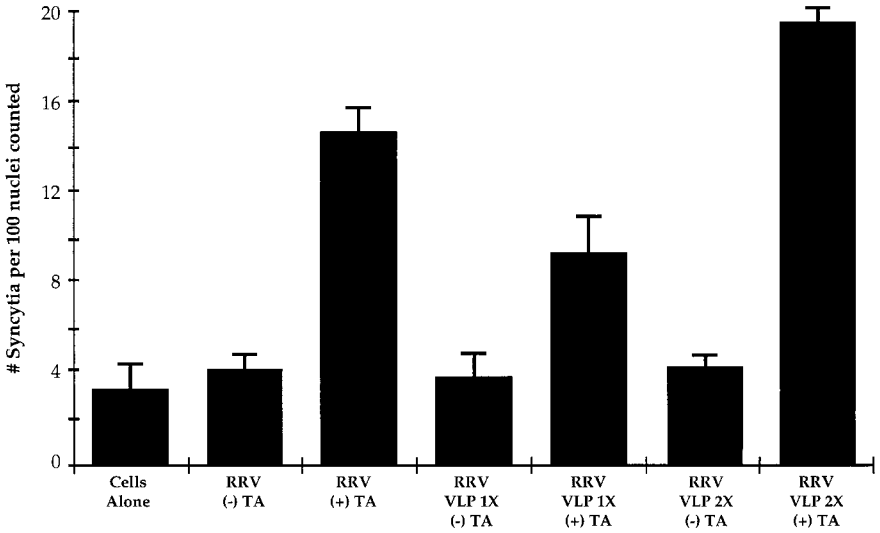

FIG. 3. Syncytium formation of RRV and RRV VLPs. RRV (75 FFU per cell) and RRV VLPs (at the protein equivalent of 50 FFU per cell [1×] or 100 FFU per cell [2×]; see Materials and Methods) were treated [(+) TA] or mock-treated [(−) TA] with trypsin for 30 min at 37°C. Samples were incubated with MA104 cells, at 4°C for 15 min and at 37°C for 15 min, plated into six-well tissue culture dishes, and incubated for 2 h at 37°C in a CO_2 incubator. Cells were examined microscopically, and the number of syncytia per 100 nuclei was determined as described in Materials and Methods.

Polykaryon formation by VLPs requires both outer layer proteins, VP4 and VP7. Previous work investigating the basic requirements for rotavirus entry into cells determined that TLPs but not DLPs could efficiently infect cells (3, 12) or induce syncytia (13). Since the outer layer of rotavirus contains both VP4 and VP7, it was not clear if either or both proteins are required for virus penetration and/or syncytium formation. To examine this question, we prepared VLPs containing different combinations of the rotavirus structural proteins. VP2/6 VLPs consisting of only VP2 and VP6 are analogous to DLPs in that they lack both outer layer proteins. VP2/4/6 VLPs, containing VP2, VP6, and either RRV or EW VP4, and VP2/6/7 VLPs, containing VP2, VP6, and VP7, have only one of the two outer coat proteins. The protein composition of these particles was analyzed by SDS-PAGE and fluorography (Fig. 1A) as well as immunoprecipitation with VP4- and VP7-specific MAbs (Fig. 1B). These data indicated that the partial VLPs contain only the desired proteins. In addition, in particles that contain VP4 but not VP7, cleavage to VP5* is demonstrated to be similar to what is seen with complete VLPs (Fig. 2A, lanes 6 and 10 versus lanes 8 and 12), indicating that the presence of VP7 is not required for the proper trypsinization of VP4.

To examine the ability of VP4 to induce syncytia, Sf-9 cells infected with recombinant baculovirus expressing RRV VP4 were sonicated on ice. The sample was examined for VP4

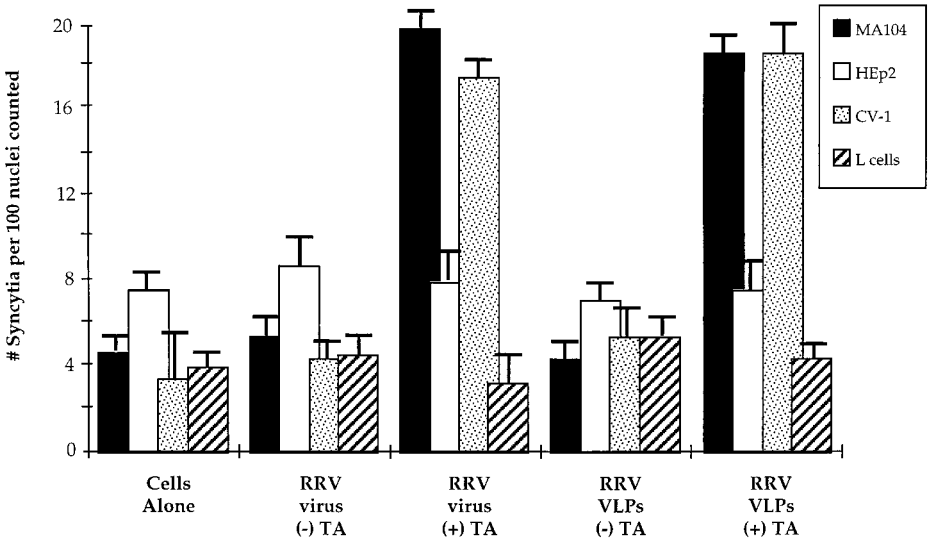

FIG. 4. Syncytium formation in permissive and nonpermissive cell lines. RRV (at 75 FFU per cell) and RRV VLPs (at the protein equivalent of 75 FFU per cell) were treated with treated [(+) TA] or mock-treated [(−) TA] with trypsin and then incubated with cholesterol-pretreated MA104, CV-1, HEp2, or L cells, at 4°C for 15 min and at 37°C for 15 min, plated into six-well tissue culture dishes, and incubated for 3 h at 37°C in a CO_2 incubator. Cells were examined microscopically, and the number of syncytia per 100 nuclei was determined as described in Materials and Methods.

TABLE 1. Hemagglutination[a]

Virus strain, VLPs, or protein	HA units/ml
RRV	1,280
EW	0
RRV VLPs	640
EW VLPs	0
Recombinant VP4	640
Soluble VP4/VP7	0
2/6 VLPs	0
RRV 2/4/6 VLPs	0
EW 2/4/6 VLPs	0
2/6/7 VLPs	0

[a] RRV, RRV and EW VLPs, partial VLPs, and soluble protein at equivalent protein concentrations in PBS-BSA were serially diluted into twofold into the wells of a 96-well dish containing human type O RBCs at a concentration of 0.5% in PBS-BSA. Samples were incubated at room temperature for 2 h, and hemagglutination (HA) was assessed.

protein and for cleavage of VP4 by trypsin in a Western blot assay and compared with a known amount of TLP RRV TLPs (Fig. 2B, lane 3 versus lane 1 and lane 4 versus lane 2, respectively). Although there is slightly more aberrant cleavage with the recombinant, baculovirus-expressed VP4 than with VP4 in the intact viral particle, there appears to be a significant amount of protein migrating at the position of VP5* as recognized by MAb HS-2, indicating that a substantial portion of the VP4 is cleaved to VP5* by trypsin. The ability of the soluble VP4 to hemagglutinate RBCs was also assessed by hemagglutination. The amount of recombinant RRV VP4 protein added into the first well of the hemagglutination titration was approximately equivalent by Western blot to the amount of RRV virus and RRV VLPs in their first wells. The recombinant VP4 hemagglutinated RBCs as efficiently as RRV and RRV VLPs (Table 1). Of note, most of the recombinant VP4 that hemagglutinated RBCs sedimented with the insoluble fraction of the Sf-9 cell sonic extract (data not shown). Soluble, sucrose-gradient purified, recombinant VP4 can neither hemagglutinate RBCs nor induce cell-cell fusion (data not shown).

Soluble VP4 and VP7 were recovered from EDTA-treated RRV TLPs, previously treated or not with trypsin, by centrifugation over a 35% sucrose–TNE cushion as described in Materials and Methods. These proteins were examined by Western blot assay (Fig. 2B, lanes 6 and 5, respectively) and compared with intact RRV TLPs. The VP4 was efficiently cleaved to VP5* in the trypsin-treated samples, whereas in the nontreated samples, much less VP5* was seen. These samples were also examined for the ability to hemagglutinate RBCs, but no hemagglutination was detected (Table 1). As indicated in Materials and Methods, in this preparation, both VP4 and VP7 are soluble, as defined by their migration during ultracentrifugation.

RRV virions, complete RRV VLPs, the partial VLPs lacking VP4, VP7, or both, and the soluble proteins were treated with trypsin and then incubated with MA104 cells in the syncytium assay. Although both RRV and the complete RRV VLPs resulted in cell-cell fusion, neither the partial VLPs, recombinant VP4, nor the soluble VP4/VP7 proteins could induce syncytia even though they were present at amounts equivalent to those of the complete RRV VLPs (Fig. 6). These data indicate that particles lacking either VP4 or VP7 are not competent to induce polykaryon formation and that recombinant VP4 or soluble VP4 and VP7 alone are also unable to induce syncytia. This result indicates that both VP4 and VP7 are required as part of a particle structure to achieve the proper conformation for rotavirus-induced fusion from without of MA104 cells.

DISCUSSION

In this report, we have demonstrated that we have produced baculovirus-expressed VLPs that are similar to intact rotavirus by biochemical and immunological criteria. These particles contain the appropriate rotavirus structural proteins, they are immunoprecipitated by conformation-dependent MAbs to VP4 and VP7, and the VP4 protein in the particles is proteo-

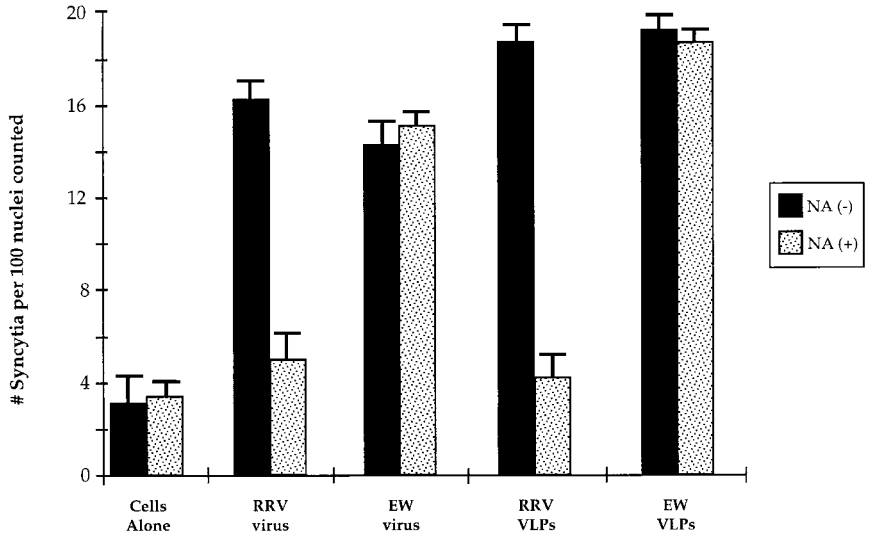

FIG. 5. Syncytium formation of RRV and EW and RRV and EW VLPs: effect of neuraminidase treatment. MA104 cells were pretreated with 60 mU of neuraminidase (NA) in PBS (solid bars) or PBS alone (dappled bars) for 1 h. RRV and EW and RRV and EW VLPs were treated with trypsin. EW and RRV (at 60 FFU per cell) and VLP samples (at the protein equivalent of 75 FFU per cell) were incubated with MA104 cells, at 4°C for 15 min and at 37°C for 15 min, plated into six-well tissue culture dishes, and incubated for 2 h at 37°C in a CO_2 incubator. Cells were examined microscopically, and the number of syncytia per 100 nuclei was determined as described in Materials and Methods.

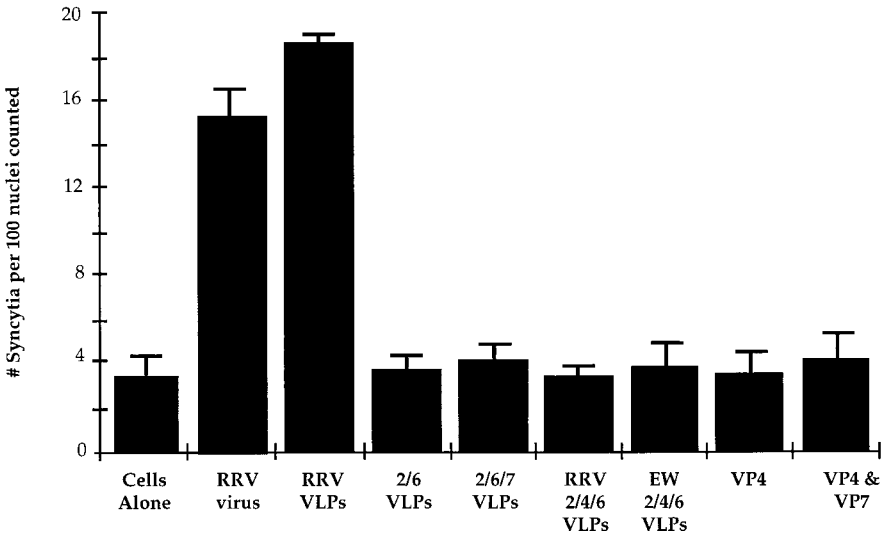

FIG. 6. Syncytium formation of RRV, RRV VLPs, and incomplete VLPs. RRV (at 75 FFU per cell), RRV VLPs, incomplete VLPs, or soluble proteins (at the protein or VP4 equivalent of 75 FFU per cell; see Materials and Methods) were treated with trypsin and then incubated with MA104 cells, at 4°C for 15 min and at 37°C for 15 min, plated into six-well tissue culture dishes, and incubated for 2 h at 37°C in a CO_2 incubator. Cells were examined microscopically, and the number of syncytia per 100 nuclei was determined as described in Materials and Methods.

lytically processed to VP5* and VP8* by the action of trypsin. Using a previously described assay system that examines the interaction of rotavirus with the host cell surface by measuring rotavirus-induced polykaryon formation of MA104 cells (13), we have now demonstrated that baculovirus-expressed VLPs can induce polykaryon formation as well as intact, native rotavirus virions. Using these VLPs, we have made several findings important to understanding the mechanism of rotavirus entry into cells. We show not only that VLPs are structurally similar to rotavirus but also that they appear to be functionally similar to rotavirus, since trypsin-activated VLPs induce syncytia of MA104 cells to the same extent as trypsin-activated, wild-type rotavirus. We also confirm our previous data (13) that viral replication is not an essential requirement for syncytium formation since VLPs can form polykaryons as efficiently as infectious virus.

By demonstrating that trypsin-activated rotavirus could induce syncytia between the plasma membranes of MA104 cells, we hypothesized that our assay was similar to the processes involved in rotavirus entry into susceptible cells. The syncytium assay is quantitative and is performed under conditions similar to those used for rotavirus infectivity assays. Specifically, this assay occurs at physiologic calcium conditions and neutral pH and requires the cleavage of VP4 and the presence of the outer-layer proteins VP4 and VP7. To further illustrate that this assay is biologically relevant, we examined the ability of RRV to form syncytia with cells that are either permissive or nonpermissive to rotavirus infection. We now demonstrate that only cells that are permissive to rotavirus infection are capable of forming syncytia when incubated with trypsin-activated intact rotavirus. Nonpermissive cells, which have been shown to be blocked for rotavirus infection at the level of membrane penetration (1), do not form syncytia with trypsin-treated rotavirus. In addition, the VLPs also show this same selectivity in our syncytium assay, as they form polykaryons with permissive cells but not with nonpermissive cells. We feel that the specificity of this assay for permissive cells and the fact that the syncytium assay is dependent on conditions required for rotavirus infection indicate that this assay is a good model with which to dissect what occurs as rotavirus penetrates the cellular membranes of susceptible cells.

In the interest of further characterizing the VLP-induced fusion-from-without assay, we examined the role of the specific VP4 molecule in this entry assay. Infectivity assays have shown that there is rotavirus strain-specific dependence on the presence of cell surface sialic acid residues (15). Using VLPs identical except that they contained VP4 from either the RRV strain (sialic acid dependent) or the EW strain (sialic acid independent), we demonstrated that the strain-specific phenotype of the individual VP4 molecules is retained in the fusion assay similar to what has been observed with rotavirus reassortants (6, 35). This indicates that our assay will be amenable to examining different VP4 proteins, including VP4 from other strains and recombinant VP4 molecules, and their role in syncytium formation.

Using baculovirus-expressed VLPs, we have employed a defined, recombinant system to examine the rotavirus proteins involved in syncytium formation. Rather than relying on various biochemically prepared forms of rotavirus (TLPs, DLPs, or EDTA-treated virus, which by necessity contain VP1 and VP3), this baculovirus expression system allows the expression of specific combinations of rotavirus proteins. Our results examining intact and partial VLPs illustrate that only rotavirus proteins VP2, VP6, VP4, and VP7 are required for fusion and that both VP4 and VP7 are essential for fusion. Additionally, our data indicate that neither VP4 alone nor soluble VP4 and VP7 can function to induce polykaryon formation. The inability of soluble VP4 and VP7 to initiate fusion contrasts with the results of Ruiz et al. (41). This discrepancy may reflect the different conditions and methodologies of the assay systems. Their assay measures fluorophore release from plasma membrane vesicles induced by soluble VP4/VP7 permeabilization of the membrane, in the absence of Ca^{2+}, whereas our assay examines polykaryon formation of MA104 cells induced by soluble VP4/VP7 in the presence of Ca^{2+}. In addition, our assay is dependent on the use of cells that are susceptible to rotavirus infection, while their assay is not (41).

Previous work by Crawford et al. (8) indicated that the only

requirement for VLP binding to MA104 cells is the presence of VP4 on the particles. We have also demonstrated that only VP4-containing particles can bind to MA104 cells (data not shown). Therefore, the inability of the VP2/6 and VP2/6/7 VLPs to induce fusion from without can be attributed to the inability of these particles to bind to the cell surface. VP4 is apparently essential for binding to the cell surface, which is a prerequisite for viral entry and presumably syncytium formation. In contrast, partial VLPs lacking VP7 (VP2/4/6 particles) bind to MA104 cells (data not shown and reference 8) but still fail to induce syncytia. Therefore, VP7 is also necessary for fusion. Further experiments allowed us to address the contribution of VP7 in greater detail. Clearly VP7 is not required for VP4 to bind to the surface of cells because VLPs lacking VP7 still bind MA104 cells. VP7 is not required for the correct proteolytic processing of VP4 because VP4 in VP2/4/6 particles is cleaved by trypsin, to a similar extent as in the complete VLP counterparts (Fig. 2A). VP4 in VP2/4/6 particles is also recognized as efficiently as complete VLPs by conformation-specific MAbs (Fig. 1B; data not shown; and reference 8). The only discernible difference between the RRV VP2/4/6 and RRV VP2/4/6/7 particles, other than their failure to induce syncytia, is that the RRV VP2/4/6 VLPs cannot efficiently bind sialic acid residues on the surface of RBCs, as indicated by their failure to hemagglutinate (Table 1 [RRV VLPs versus RRV VP2/4/6 VLPs] and reference 8). Therefore, we conclude that VP7 is required for RRV VP4-containing particles to bind sialic acid. Two possible models explain the failure of VP7-lacking particles to bind sialic acid and to induce syncytia. One model is that VP7 is required for VP4 to adopt a conformation necessary for sialic acid binding. If VP4 is unable to bind sialic acid, by this model, it cannot proceed to catalyze entry-related functions. The other model, however, contends that VP7 is required for VP4 to adopt a conformation necessary for membrane interaction and that this conformation happens to also be required for sialic acid binding.

The VP7-dependent VP4 requirement for syncytium formation is not merely related to sialic acid binding, as evidenced by experiments with the sialic acid-independent strain, EW. For the EW rotavirus strain, binding to cell surface sialic acid residues is not a prerequisite for viral infectivity (27, 31). Complete VLPs containing EW VP4 are competent to induce syncytia (Fig. 5) even though they cannot hemagglutinate RBCs (Table 1). Clearly, these VLPs recapitulate the properties of the EW strain, a virus where sialic acid binding is not necessary for polykaryon formation. Interestingly, we observe that VLPs with EW VP4 but lacking VP7 are unable to induce syncytia. We conclude that VP7 is required for membrane fusion even when the ability of VP4 to bind sialic acid is dispensable. Therefore, although VP4 of either strain may be present on a particle and these particles can bind to MA104 cells, only in the presence of VP7 will VP4 achieve the conformation(s) required either for VP4 to efficiently bind to the cell or for VP4 to interact with cellular membranes and induce syncytia. This influence by VP7 on the conformation of VP4 has also been suggested by other workers (32).

We propose that the combination of a cell-cell fusion assay and recombinant VLPs will permit us to examine the molecular mechanism by which rotavirus interacts with cellular membranes. In particular, we will now be able to begin to investigate how VP4 and VP7 are involved in the early events in rotavirus penetration of host cells by engineering specific mutations in these proteins and then assessing their activity of the resultant VLPs in syncytium assays.

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