NS35 and Not vp7 Is the Soluble Rotavirus Protein Which Binds to Target Cells

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Recent studies using radiolabeled rotavirus lysates have demonstrated a 35-kilodalton viral protein that binds specifically to the surface of MA104 cells (N. Fukuhara, O. Yoshie, S. Kitakoa, and T. Konno, *J. Virol.* 62:2209-2218, 1988; M. Sabara, J. Gilchrist, G. R. Hudson, and L. A. Babiuk, *J. Virol.* 53:58-66, 1985). The binding protein was identified as vp7, an outer capsid glycoprotein and the product of rotavirus gene 9. These studies concluded that vp7 mediated viral attachment to MA104 cells and that the binding of a soluble viral protein to a cell monolayer mirrored the attachment of infectious rotavirus to permissive tissue culture cells. In the process of determining which viral protein adheres to the *in vivo* target cell in rotavirus infection, the mammalian enterocyte, we found that a similar 35-kilodalton rhesus rotavirus (RRV) protein bound to both MA104 cells and murine enterocytes. However, further analysis of this protein by immunoprecipitation, inhibition of glycosylation, and partial proteolysis showed that it was not the RRV gene 9 product, vp7, but the gene 8 product, NS35. Similar results were obtained by using porcine rotavirus (OSU) and bovine rotavirus (NCDV) strains. Binding studies using the *in vitro*-expressed products of RRV genes 8 and 9 confirmed these results. Since double-shelled virions inhibited the binding of NS35 to cells, we looked for the presence of this protein in preparations of purified virus. Examination of density gradient-purified virus preparations revealed biochemical and immunological evidence that NS35 copurifies in small amounts with double-shelled virions. Thus, these studies clearly demonstrated that when rotavirus proteins are prepared in a soluble form from infected cells, NS35, and not vp7, binds to the surfaces of MA104 cells and murine enterocytes. The observations do not confirm previous experimental results which supported the hypothesis that vp7 was the viral attachment protein. They are consistent with but do not prove the hypothesis that NS35 functions as the rotavirus attachment protein.

Rotaviruses are important etiologic agents of gastroenteritis in humans and a variety of other mammalian species (11). Rotavirus exhibits specific tropisms for host range, host age, and tissue. Viruses from heterologous species are far less virulent than homologous strains (12, 15, 24). In several species, only the young develop symptomatic infections (35). Rotaviruses infect only mature villus tip enterocytes in the host small intestine and in most cases do not disseminate. These phenomena could be related to the earliest step in the target cell-virus interaction, viral attachment, as has been shown for reoviruses (34). Furthermore, because inhibition of attachment is one mechanism by which the host immune response can prevent disease, understanding the mechanism of viral binding to susceptible host cells is of interest.

Rotaviruses are members of the family *Reoviridae* and consist of a nonenveloped double capsid containing 11 segments of double-stranded RNA. Although there has been some dispute over which viral proteins are part of the mature virion structure, vp4, the gene 4 product, and vp7, usually the gene 9 product, are generally accepted as major outer capsid components (8, 9, 21). Both of these viral proteins are neutralizing antigens, and vp4 has been shown to mediate hemagglutination (16), *in vitro* growth restriction (14), and *in vivo* virulence (24).

Previous studies designed to determine which rotavirus protein mediates attachment to cell surfaces have examined binding to cell monolayers of metabolically radiolabeled

soluble viral proteins. In experiments similar to those used by Lee et al. (20) to demonstrate that sigma 1 is the reovirus binding protein, Fukuhara et al. (13) and Sabara et al. (28) have found 35- and 38.2-kilodalton (kDa) proteins, respectively, which specifically adhered to MA104 cells. The adherent proteins were identified as the glycoprotein vp7 by their molecular weight, reactivity with monoclonal antibodies (MAbs), and a reported shift in molecular weight when the lysates were prepared in the presence of tunicamycin. These results appeared plausible because vp7 is an outer capsid protein and a major neutralization antigen. In attempting to extend these studies to the *in vivo* target cell, the enterocyte, we found a similar 35-kDa rotavirus protein which binds to murine enterocytes and MA104 cells. However, detailed analysis of this protein indicated that it is not a glycoprotein, that it can only be immunoprecipitated by a MAb directed against NS35 and not by anti-vp7 MAbs, and that it has a partial protease digestion pattern identical to that of NS35 but unlike that of vp7. Reticulocyte lysate-expressed products of gene 9 (vp7) and gene 8 (NS35) were studied; it was found that the expressed gene 8 product was able to bind to cells, whereas the gene 9 product was not able to do so. In addition, the gene 8 product had an identical partial protease digestion pattern to that of the protein in metabolically labeled lysates which adhered to cells. Because purified virions inhibited the binding of NS35 to cells, we sought to determine whether NS35 was present on the virus. Evaluation of CsCl-purified rhesus rotavirus (RRV) by solid-phase immunoassay with MAb to NS35 indicated that the adherent protein (NS35) copurified with double-shelled

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virions. For RRV, the presence of NS35 on the virus was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and partial protease digestion of the peptide associated with purified virion.

MATERIALS AND METHODS

Cell culture. MA104 cells were grown in medium 199 with 10% fetal calf serum and L-glutamine, penicillin G, and streptomycin as previously described (30).

Virus cultivation and purification. RRV, NCDV, and OSU strains of rotavirus were grown on MA104 cell monolayers after trypsin activation as previously described (30). For purified virus, infected-cell cultures were freeze-thawed twice, trichlorotrifluoroethane extracted, pelleted through a 30% (wt/vol) sucrose cushion, and run on CsCl (1.37 g/ml) gradients at 28,000 rpm on a rotor (SW40; Beckman Instruments, Inc., Fullerton, Calif.) for 16 to 20 h. The resultant gradient was fractionated by bottom puncture. Bands containing single-shelled, double-shelled, and empty particles were either pooled (see Fig. 8), resuspended in CsCl (1.37 g/ml), or individually resuspended (data not shown), and recentrifuged for an additional 16 to 20 h. Double- and single-shelled particles were identified by enzyme-linked immunosorbent assay (ELISA) using vp6- and vp7- specific MAbs, by buoyant density, and by hemagglutination. When single-shelled virions were desired for competition studies, virus-containing fractions were incubated for 1 h with 10 mM EDTA at 37°C, followed by equilibrium centrifugation with CsCl. Fractions recovered from CsCl gradients were routinely dialyzed exhaustively against TNC buffer (10 mM Tris, 50 mM NaCl, 2 mM CaCl). Purified virus used for competition studies was dialyzed against phosphate-buffered saline (PBS), pH 7.4.

Radiolabeled virus. To prepare ^{35}S -labeled virus, monolayers were inoculated with 5 to 10 PFU per cell of trypsin-activated virus in serum-free medium. After 3 to 4 h, the medium was replaced with methionine-free minimal essential medium containing 50 to 100 $\mu\text{Ci/ml}$ of ^{35}S -labeled methionine (Trans-label; ICN Radiochemicals). Virus was harvested 24 h postinoculation and purified as described above. Purified virus contained approximately 10^{10} PFU per ml and 2,000 cpm/ μl .

Adsorption studies. Radiolabeled lysates were prepared as described previously (30). Two hundred microliters of lysate per well was added to washed MA104 cell monolayers in 24-well plates or to 10^5 intestinal epithelial cells in 15-ml polystyrene centrifuge tubes. Incubation with rocking proceeded for 1.5 h at 4°C. The cells were then washed at least three times with cold PBS and lysed with Laemmli sample buffer for analysis on SDS polyacrylamide gels. When immunoprecipitation of the rotavirus protein attached to cells was to be performed, the monolayers were lysed in PBS with 1% Nonidet P-40 for 30 min at 4°C and then centrifuged for 10 min in a microfuge. The supernatant was used for immunoprecipitation.

Isolation of intestinal epithelium. A modification of the method of Bjerknes and Chang (1) was used to isolate intestinal cells. Briefly, mice were killed by cervical dislocation and their small intestines were rapidly removed, everted over a glass capillary tube, and washed in cold Ca-Mg-free PBS (CMF). The everted intestine was then incubated in 30 mM EDTA in CMF at 37°C for 15 min. Cells were released by moderate agitation, washed twice in medium 199 containing 5% fetal calf serum, and counted on a hemacytometer.

Immunoprecipitation. Radiolabeled lysates were incubated at 4°C with monoclonal antibodies or sera for 2 to 24 h. Twenty microliters of Sepharose CL-4 protein A beads (Sigma Chemical Co., St. Louis, Mo.) was added, and 30 min later, the beads were washed three times with wash buffer A (50 mM NaCl, 10 mM Tris, 1% Nonidet P-40) and once with 50 mM Tris (pH 6.8). Laemmli sample buffer was added and samples were boiled for 2 min and subjected to SDS-PAGE by the method of Laemmli (19) with 10% resolving gels. Gels were fixed in 50% methanol–10% acetic acid, washed in water, and soaked in 17.5% salicylic acid before drying and fluorography.

ELISA. To examine reactivity of fractions from RRV CsCl density gradients to MAbs 191 and 129, 50- μl portions of 1:5 dilutions of the fractions were allowed to adsorb to immulon 96-well plates overnight. After being washed with PBS and blocked for 1 h with 10% fetal calf serum in PBS, 50 μl of 1:5 dilution of MAb cell culture supernatant was added and allowed to incubate for 2 h at 37°C. After being washed again, 50 μl of 1:1,000 goat anti-mouse alkaline phosphatase conjugate (Kirkegaard and Perry) was added for 1 h. After being washed, the plates were developed with *p*-nitrophenyl phosphate (Sigma) in 1% diethenalamine (pH 9.8).

MAbs. MAbs 129, 60, and 191 have been previously described (26, 31). MAbs 129 and 60 were isolated after immunization of mice with the human Wa rotavirus strain. They immunoprecipitate vp7 from a large variety of rotavirus isolates but do not neutralize or inhibit hemagglutination. MAb 191, derived from a mouse immunized with RRV, recognizes the NS35 protein from a variety of rotavirus isolates but neither neutralizes nor inhibits hemagglutination (H. Greenberg, unpublished data). It immunoprecipitates the rabbit reticulocyte lysate-expressed product of gene 8 but not that of gene 9 (D. M. Bass, unpublished results).

Partial proteolysis (Cleveland digest). Partial proteolytic digestion of ^{35}S -labeled proteins excised from preparative gels was performed as previously described (6). Briefly, bands of interest were excised from preparative SDS–10% polyacrylamide gels, trimmed, and placed in wells of a 4% stacking gel (5 cm long). The separating gel was 15% acrylamide. The gel slices were allowed to equilibrate for 1 h with 25 μl of soak buffer (0.125 M Tris [pH 6.8], 0.1% SDS, 2% beta-mercaptoethanol). An additional 10 μl of soak buffer containing 10% glycerol was added, followed by 20 μl of Laemmli sample buffer containing the desired concentration of protease. Electrophoresis was then carried out with 50 V (constant voltage) for 14 h. Gels were fixed in 50% methanol–10% acetic acid, soaked in 17.5% salicylic acid, dried, and exposed to film for 2 to 8 weeks at -70°C .

Expression of cDNA of RRV genes 8 and 9. Plus-stranded RNA was transcribed from full-length clones of genes 8 and 9 in Bluescript plasmids by using T3 RNA polymerase system (Stratagene, Inc.). RNA transcripts were translated in a rabbit reticulocyte lysate (Stratagene, Inc.) in the presence of [^{35}S]methionine (60 μCi). The translation products were diluted 1:100 in medium 199 for cell binding studies and immunoprecipitations.

RESULTS

NS35, not vp7, is the viral protein in metabolically labeled lysates which binds to target cells. Our initial experiments were directed at confirming and extending the results of Sabara et al. (28) and Fukuhara et al. (13). Isolated intestinal epithelium and MA104 cells were incubated with radiolabeled RRV lysate at 4°C, washed extensively, and subjected

FIG. 1. Binding of proteins from ^{35}S -labeled RRV-infected and mock-infected MA104 cell lysates to MA104 cells, isolated suckling mouse gut epithelial cells, and isolated adult mouse gut epithelial cells. Lysates prepared as described in Materials and Methods were incubated with cells for 1.5 h at 4°C . The cells were then washed three times with PBS, lysed with Laemmli sample buffer, and subjected to SDS-PAGE on 10% acrylamide gels. Lanes: M lys, lysate from mock-infected MA104 cells; R lys, lysate from RRV-infected MA104 cells; MM, mock-infected lysate incubated with MA104 cells; RM, RRV lysate incubated with MA104 cells; MS, mock lysate incubated with 10^5 suckling mouse gut epithelial cells; RS, RRV lysate incubated with 10^5 suckling mouse gut epithelial cells; MA, mock lysate incubated with 10^5 adult mouse gut epithelial cells; RA, RRV lysate incubated with 10^5 adult mouse gut epithelial cells; GP, immunoprecipitation with hyperimmune guinea pig serum; RRV, ^{35}S -labeled purified RRV; 129, immunoprecipitation with MAb 129 (anti-vp7); 191, immunoprecipitation with MAb 191 (anti-NS35).

to SDS-PAGE. The results (Fig. 1) show a 35-kDa viral protein which adhered to both MA104 cells and murine enterocytes, while no labeled protein from mock-infected lysates adhered. Occasionally, in other experiments when the 35-kDa band was overexposed, a faint band corresponding to the nonstructural viral glycoprotein, NS28, was also observed binding to cells (see Fig. 6 for example). It remains unclear whether NS28 was binding independently or as part of a heterodimer with the 35-kDa protein. In several experiments such as that shown in Fig. 1, there appeared to be less binding of the 35-kDa protein to adult murine enterocytes than to equal numbers of suckling murine enterocytes. This could be relevant to the decreased susceptibility of adult mice to symptomatic rotavirus infection.

To ensure that the observed adherence was not a nonspecific interaction with membrane lipid, we performed similar binding studies using human type O erythrocytes. Results demonstrated no binding of the 35-kDa protein, although a protein of the same molecular weight as vp4 did adhere to erythrocytes (data not shown).

To characterize the specificity of the binding of the 35-kDa protein to cells, we examined the ability of double-shelled and single-shelled rotavirus particles to inhibit this interaction. Purified infectious double-shelled RRV was able to

FIG. 2. SDS-PAGE analysis of binding competition studies using double-shelled virions, single-shelled particles, and radiolabeled RRV lysates. (a) Competition with double-shelled RRV virions. Lane RRV contains a small sample of ^{35}S -labeled RRV. For lanes 2 through 6, the indicated amounts (in micrograms) of nonlabeled double-shelled particles were added to radiolabeled RRV lysate and allowed to adsorb to MA104 monolayers at 4°C for 1.5 h. Monolayers were then processed as described in the legend to Fig. 1. (b) Competition with single-shelled RRV virions. Lane LYS contains ^{35}S -labeled RRV lysate. Lanes 2 through 6 as described above, but single-shelled virions were used as the unlabeled competitor.

compete with the 35-kDa protein for binding sites on MA104 cells in a dose-dependent manner (Fig. 2a), as has been previously shown by Sabara et al. (28) and Fukuhara et al. (13). The specificity of binding was further confirmed by the failure of equal amounts of single-shelled virions to effectively compete (Fig. 2b).

In our preliminary studies (Fig. 1), the precise origin of the adherent protein could not be determined by migration alone, although the band (lanes RM, RS, and RA) did appear to comigrate with protein immunoprecipitated by MAb 191 and not with the proteins immunoprecipitated by MAb 129. To determine whether this putative viral attachment protein was indeed glycoprotein vp7, we prepared metabolically labeled lysates from tunicamycin-treated cells infected with RRV. The results of binding studies with MA104 cells and of immunoprecipitation studies used to identify specific viral proteins are shown in Fig. 3. The RRV protein which bound to MA104 cells and the protein precipitated by MAb 191 (Fig. 3, lanes 3 through 6) comigrated and showed no shift in molecular mass with tunicamycin treatment. Identical observations were made by using NCDV lysates made with and without tunicamycin (data not shown). RRV vp7 immunoprecipitated by MAb 129 in its glycosylated form (Fig. 3, lane 7) migrated as two slightly higher molecular mass forms of approximately 37 and 39 kDa. RRV vp7 immunoprecipitated from lysates made in the presence of tunicamycin migrated at a reduced molecular mass of 35 kDa and comigrated with the protein which adheres to MA104 cells. (Fig. 3, lane 8).

Since nonglycosylated vp7 and NS35 comigrate, we attempted to directly determine the origin of the adherent

FIG. 3. Effect of tunicamycin on RRV proteins in ^{35}S -labeled lysates of RRV-infected MA104 cells. Lanes 1 and 2, lysates immunoprecipitated with hyperimmune guinea pig anti-RRV serum; lanes 3 and 4, lysates adsorbed to MA104 cells; lanes 5 and 6, lysates immunoprecipitated with MAb 191 directed against NS35; and lanes 7 and 8, lysates immunoprecipitated with MAb 129 directed against vp7. Lanes 2, 4, 6, and 8 were performed with lysate generated in the presence of 2 μg of tunicamycin per ml. Lane 8 required a longer exposure time.

protein. In separate experiments, after absorbing lysates from RRV-, NCDV-, and OSU-infected cells to MA104 monolayers and washing, cells were solubilized and clarified, and the adherent protein was immunoprecipitated with selected MAb. The results depicted in Fig. 4 demonstrate that with all three virus strains, MAb 191 directed at NS35 (lanes 4, 7, and 10) could precipitate the cell-bound peptide, while MAb 129 directed at vp7 (lanes 3, 6, and 9) could not.

To independently investigate the origin of the RRV protein that binds to cells and to confirm the specificity of MAb 191 and 129 for NS35 and vp7, respectively, partial protease digestions were carried out on selected RRV proteins. The results shown in Fig. 5 demonstrate that the MAb 191 immunoprecipitate, the *in vitro*-expressed product of cloned gene 8, and the 35-kDa protein which adheres to cells (lanes C8, 191, and MA) share identical and clearly distinct digestion patterns from several forms of vp7, including vp7 immunoprecipitated with MAb 129, vp7 isolated from virions, and nonglycosylated vp7 expressed from cDNA in a rabbit reticulocyte system (lanes 129, vp7, C91, and C92).

To ensure that these results were not peculiar to the RRV strain, similar binding studies and immunoprecipitations were carried out with several additional rotavirus strains. Representative results for NCDV, a bovine isolate, and OSU, a porcine isolate, are shown in Fig. 6. In each of these strains, a peptide which comigrated with the MAb 191 immunoprecipitation product adhered to MA104 monolayers. Both of these rotaviruses differ from RRV in that their vp7 proteins comigrated with NS35 by SDS-PAGE. A similar comigration could be observed by using RRV lysates if SDS-PAGE were carried out under nonreducing conditions (data not shown). Thus it is possible that the actual identity of the adherent peptide could be easily mistaken for vp7, especially in strains in which NS35 and vp7 comigrate.

Finally, to test our results directly, we performed binding studies using *in vitro*-expressed products of cDNA clones of

FIG. 4. Immunoprecipitation of the rotavirus peptide attached to MA104 cells. Radiolabeled lysates of RRV, NCDV, and OSU (as indicated) were adsorbed to MA104 cells at 4°C for 1 h. Monolayers were subsequently washed, solubilized with 1% Nonidet P-40 and immunoprecipitated with selected MAb. Lane 1 shows purified radiolabeled RRV; lanes 2, 5, and 8 show the MA104 adherent rotavirus peptide before solubilization; lanes 3, 6, and 9 show immunoprecipitations of the adherent protein after solubilization with MAb 129 (anti-vp7); and lanes 4, 7, and 9 show immunoprecipitations of the adherent protein after solubilization with MAb 191 (anti-NS35).

RRV genes 8 and 9. Figure 7 shows that the 35-kDa product of gene 8 adhered to MA104 monolayers, while the product of gene 9 did not adhere (data not shown). The *in vitro*-expressed product of gene 9 could be immunoprecipitated by MAb 60 and 129, but not by MAb 191, while the product of gene 8 could be immunoprecipitated by MAb 191, but not by MAb 129 and 60 (data not shown). Both expressed gene 8 and 9 products had partial protease digestion patterns essentially identical to those of NS35 and vp7 (Fig. 5).

NS35 is present on double-shelled virions. Having established that the rotavirus protein in cell lysates which adheres to susceptible cell surfaces is NS35, the gene 8 product, and not vp7, the gene 9 product, we next sought to determine whether NS35 might be present on intact purified infectious viral particles. As its name implies, many previous investigators have felt that NS35 is nonstructural (8, 9), although at least one investigator found it to be present on virions (23). We pursued these studies because intact double-shelled virus, but not single-shelled virus, was able to specifically compete with NS35 for binding sites on MA104 cells (Fig. 2), strongly suggesting that NS35 was present in purified virus preparations (13, 28).

A solid-phase ELISA was developed to detect the presence of NS35 in fractions of purified RRV doubly banded on CsCl gradients. This ELISA used NS35-specific MAb 191 as the detection antibody. Figure 8 shows typical results of such an assay. Reactivity with MAb 191 paralleled reactivity of MAb 129 and hemagglutination titer with peaks at the double-shelled particle fraction (fraction 9) and the hemagglutinating empty capsid fraction (fraction 13). Similar results were obtained when viral particles were separated by size on a rate zonal sucrose gradient or rebanded as double-shelled particles alone in CsCl gradients and tested by ELISA (data not shown). In those studies, the single- and

FIG. 5. Partial proteolysis (Cleveland digest) of RRV proteins. Radiolabeled proteins were cut from preparative 10% gels and placed in lanes of a 4% stacking gel and a 15% separating gel with 100 μ g of chymotrypsin per lane in the sample buffer. Lane vp6, digest of vp6 from purified RRV; lane C8, digest of rabbit reticulocyte lysate-expressed product of cloned gene 8; lane 191, digest of peptide immunoprecipitated from RRV lysate with MAb 191; lane MA, digest of RRV peptide adherant to MA104 cells; lane 129, digest of each of the peptide immunoprecipitated by MAb 129; lanes C91 and C92, digests of rabbit reticulocyte-expressed product of two clones of RRV gene 9; lane vp7, vp7 isolated from purified radiolabeled RRV.

double-shelled particles had the expected densities of 1.38 and 1.36, respectively.

To attempt to visualize NS35 in purified preparations of 35 S-labeled double-shelled virus by SDS-PAGE, we loaded radiolabeled virus on long separating gels (18 to 20 cm) under reducing conditions (Fig. 9). In nonreducing gels, the NS35 band comigrated with vp7. Visualization of the 35-kDa band required loading large amounts (200,000 cpm) of radiolabeled virus (compare lane RRV in Fig. 2a and lane RRV in Fig. 9). Using this approach, we were able to clearly resolve a virion-associated protein band of appropriate molecular mass (35 kDa) by SDS-PAGE and fluorography (Fig. 9). This band was clearly distinct from vp7 and comigrated with NS35 by SDS-PAGE. To confirm the identity of this band, the 35-kDa band from the purified virus preparation was excised and subjected to partial proteolysis as described by Cleveland et al. (6) and compared with the digestion profile of vp7, NS35 isolated by immunoprecipitation and the in vitro-expressed product of gene 8. The results in Fig. 10 confirm that the band seen in highly purified RRV virion preparations is identical by partial proteolysis to NS35.

We quantitated the amount of NS35 on the virion relative to vp7 by densitometry of autoradiograms of 35 S-labeled

FIG. 6. Immunoprecipitations of RRV, OSU, and NCDV proteins from 35 S-labeled lysates. Lysates were prepared as described in Materials and Methods and immunoprecipitated with hyperimmune guinea pig anti-RRV serum (lanes designated gp), MAb (MA) 191, MAb 60, MAb 129, or bound to MA104 cells as indicated under lanes.

purified RRV and found the NS35 band equivalent to a maximum of 5% of the amount of protein as vp7. Thus NS35 is present in very low copy number in virions.

DISCUSSION

We began our work on rotavirus attachment to cells by attempting to confirm and extend the studies of Sabara et al. (28) and Fukuhara et al. (13) by determining whether vp7 also mediated attachment to the murine enterocyte. By using

FIG. 7. Binding of rabbit reticulocyte lysate-expressed cDNA gene 8 clone to MA104 cells. In lane C8, the product of in vitro translation of RRV gene 8 was incubated with a monolayer of MA104 cells in medium 199 at 4°C for 1.5 h, washed, and solubilized with Laemmli sample buffer before SDS-PAGE on 10% acrylamide gels. RRV lanes contain purified 35 S-labeled RRV.

FIG. 8. RRV was purified twice on CsCl gradients as described in Materials and Methods. Fractions (300 μ l) from the second purification were collected by bottom puncture and assayed by hemagglutination ($\square$) and ELISA ($\blacklozenge$). (a) ELISA for NS35 antigen using MAb 191 as the detection antibody. (b) ELISA for vp7 antigen using MAb 129 as the detection antibody. HA, Hemagglutination; OD₄₀₅, optical density at 405 nm.

essentially identical methodology, our initial results appeared similar to their previously published findings. A 35-kDa viral protein was found to bind to MA104 cells and murine enterocytes. Further experiments, however, led us to a quite different interpretation of the experimental results, namely, that the viral protein which attaches to MA104 cell monolayers and murine enterocytes in NS35 and not vp7.

The failure of tunicamycin to cause a decrease in the molecular weight of the cell-binding protein was the first

evidence that the cell-binding protein was not the glycosylated form of vp7. Further studies showed that the putative attachment protein from RRV, NCDV, and OSU rotavirus strains isolated by binding to cells could not be immunoprecipitated with well-characterized MAb directed against vp7, yet could be precipitated by a MAb directed against NS35. Furthermore, partial protease mapping showed that the 35-kDa protein which bound to cells was unrelated to a variety of forms of vp7 isolated from infected-cell lysates, from purified virions, and expressed *in vitro* without glycosylation from full-length cDNA clones. It was identical to the translation product of gene 8 and to the protein immunoprecipitated by MAb 191. Finally, we confirmed these results by demonstrating that the *in vitro*-expressed product of gene 8

FIG. 9. Polypeptide analysis by SDS-PAGE of purified ³⁵S-labeled RRV (RRV) and NS35 (MA) isolated by adherence of ³⁵S-labeled RRV lysate to MA104 cells. The lower band in the MA lane corresponds to RRV NS28, a nonstructural glycoprotein.

FIG. 10. Partial proteolysis (Cleveland digest) of RRV proteins. Radiolabeled proteins were cut from preparative 10% gels and placed in lanes of a 4% stacking gel and a 15% separating gel with 100 μ g of chymotrypsin per lane in the sample buffer. Lanes: 1, vp7 from purified radiolabeled virus; 2, NS35 isolated by immunoprecipitation with MAb 191; 3, NS35 expressed *in vitro* with a rabbit reticulocyte system from gene 8 cDNA; 4, NS35 isolated from purified radiolabeled RRV in a gel similar to Fig. 9.

bound to cells in the original binding assay, whereas the product of gene 9 did not bind.

In previously published studies using the same methods (13, 28) Sabara and Fukuhara have found a single protein of similar molecular weight which bound to MA104 cells and could be inhibited from cells by an excess of unlabeled virus. The adherent peptide was identified as vp7 by several techniques, including molecular weight determination, MAb and monospecific polyclonal antibodies, serotype-specific serum, and an observed shift in molecular weight when the viral proteins were produced in the presence of tunicamycin.

Although the lysates in both previous studies contained multiple molecular weight forms of vp7, only one form adhered to the monolayer. In report of Sabara et al. (28), the two protein forms were said to represent reduced and nonreduced vp7. Only the lower-molecular-weight nonreduced (and putatively nonreducible) form bound to cells. We would conclude that the nonreduced form actually represented NS35. In nonreducing conditions, RRV NS35 comigrates with vp7 and might easily be misinterpreted as nonreduced vp7.

Fukuhara et al. (13) reported that only the lower-molecular-weight, less glycosylated of two forms of strain KUN vp7 bound to MA104 cells. An alternative explanation of these findings is that, as in the NCDV and OSU strains, KUN strain NS35 comigrates with one form of vp7. Previously published descriptions of both the C486 bovine strain used by Sabara et al. (27) and the KUN human strain used by Sato et al. (29) show NS35 migrating between the highest-molecular-weight form of the glycoprotein vp7 and its nonglycosylated form which is produced in the presence of tunicamycin. A similar profile of viral proteins is seen with the NCDV and OSU strains (Fig. 6).

The molecular basis of multiple forms of vp7 in a single stock of virus remains somewhat enigmatic. Earlier sequence data has shown that gene 9 contains two in frame start codons which can both be utilized, but this accounts for relatively minor differences in molecular weight (5). The multiple forms of vp7 observed in our studies as well as other studies (8, 13, 17, 28) may represent genetically diverse subpopulations of virus within a single stock as previously shown (10) but could represent incomplete glycosylation of vp7 during posttranslational processing or partial deglycosylation during harvesting or purification (17, 18).

Fukuhara et al. (13) used two MAbs putatively directed against vp7 to indirectly suggest that the adherent 35-kDa protein was vp7. In one experiment, they used a neutralizing MAb, S2-2G10, to inhibit the competition of unlabeled virions with the cell-binding protein (13). These results could be alternatively explained by simple aggregation of the competing virus by the MAb, in which case the specificity of the MAb is not relevant to the identification of the attachment protein. Another MAb, AH6, which is said to immunoprecipitate vp7 but not to neutralize it, was used to adsorb the radiolabeled rotavirus lysate before incubation with the monolayer and decreased binding of the 35-kDa protein dramatically. Neither of our nonneutralizing vp7 MAbs (60 and 129) was able to inhibit binding, while MAb 191 was able to inhibit binding (data not shown). The differences in interpretation of these observations could be explained if the protein specificity assigned to AH6 were incorrect. Monospecific polyclonal and monoclonal antibody specificities could be incorrectly assigned if a comigrating viral peptide were not taken into consideration.

Our results differ strikingly from those of both Sabara et

al. (28) and Fukuhara et al. (13) in that we observed no molecular weight shift in the putative attachment protein of several rotavirus strains in the presence of tunicamycin (Fig. 3; also data not shown). In our experiments, tunicamycin was shown to reduce the molecular weight of vp7 immunoprecipitated by MAb 129. We are unable to explain this discrepancy between our results and those of the previous studies.

Notwithstanding this major difference in results, our proteolytic peptide mapping data and binding studies with *in vitro*-expressed proteins establish conclusively that the rotaviral protein which adheres to MA104 cells and enterocytes in the binding assay described is NS35 and not vp7. To ensure that our prototype rotavirus strain, RRV, is not anomalous in this regard, we have shown that NS35 from several serotypically unrelated strains adheres to target cells in this assay. We therefore think it unlikely that the differences in these studies can be explained by the fact that different viral strains were employed.

Other workers have used antisera raised against rotavirus peptides isolated by SDS-PAGE to determine the specific rotavirus protein responsible for cell attachment. Matsuno and Inouye (22) used such an approach to show that antisera against nonreduced vp7 inhibited binding of radiolabeled virus to cell monolayers, while antisera against the reduced form of vp7 did not inhibit binding. It is perhaps significant that RRV NS35 and vp7 can not be resolved by SDS-PAGE under nonreducing conditions. Sabara et al. (28) and Greenberg (unpublished data) have shown that antibodies directed against either vp7 or vp4 can inhibit viral binding. Furthermore, antibodies may inhibit viral binding indirectly, either by steric interference or by inducing conformational changes in the outer capsid.

Having demonstrated that soluble NS35 binds to target cells and that CsCl-purified RRV can compete for the NS35 cellular binding sites, we wondered whether NS35 might be a structural rotavirus protein. Most previous investigators have reported that NS35 is nonstructural (8, 9, 21, 32), although at least one study found it present on purified virions (23), and bands visible by SDS-PAGE which could represent NS35 are seen in several studies of purified virus (3, 4, 8).

NS35 is a nonglycosylated rotaviral protein which is found in relative abundance in lysates of rotavirus-infected cells. It appears to be relatively immunogenic, since antibodies against it are found in both hyperimmune and postinfection sera (33; H. Greenberg, unpublished data). It is encoded by one of three double-stranded RNA segments (segments 7, 8, or 9) which often comigrate in electrophoresis. Sequence data is available for the protein (2), and preliminary evidence indicates that it is highly conserved among isolates (7). Previous studies using immunocytology have localized NS35 with MAb 191 to large aggregates of viroplasm in infected cells (26). The function of NS35 in rotavirus replication is unknown, although one study has suggested that it may be an important component of the rotavirus replicase particle (25).

We found both immunological and direct biochemical evidence for its presence on double-shelled virus in very small amounts. First, we showed by ELISA that a peak of immunoreactivity to NS35, as measured with MAb 191, was coincident on CsCl gradients with double-shelled particles detected by both hemagglutination and reactivity to vp7 with MAb 129 protein by SDS-PAGE of highly purified double-shelled preparations of RRV and confirmed its identity by partial proteolysis (Fig. 9 and 10).

Further evidence for the presence of NS35 in purified virus preparations is provided by competition experiments in which purified virus competes with NS35 for binding sites on susceptible cells (Fig. 2). Similar experiments by both Sabara et al. (28) and Fukuhara et al. (13) also demonstrated inhibition of attachment of the putative binding protein by excess purified virus. In light of the failure of single-shelled virions to inhibit NS35 binding, it would seem likely that the double-shelled virion preparation contains unlabeled NS35 that is able to compete with the labeled protein in the lysate.

It is possible that NS35 contaminated or was nonspecifically bound to our purified virus. Purified RRV used in our experiments was banded twice on isopycnic gradients and contained minimal evidence by SDS-PAGE for contamination with other cellular or nonstructural proteins. In addition, purified single-shelled particles from the same preparation did not contain significant nonspecifically bound NS35. It is also possible that the NS35 detected in these preparations was present in only a small subpopulation of the purified virus. Further studies will be required to determine definitively whether NS35 is a structural protein.

Although we have demonstrated that NS35 adheres to enterocyte and MA104 cells in our assay and that NS35 copurifies with infectious double-shelled virus, our studies do not unequivocally establish NS35 as the rotavirus cell attachment protein. It should be emphasized that the binding assay employed, the binding of metabolically labeled soluble viral proteins to cells, may not accurately reflect events that mediated binding of infectious virions to cells. Hence, this type of assay cannot by itself prove which viral protein(s) functions in mediating cell attachment. They do however raise questions as to the strength of the evidence indicating that vp7 serves such a function and suggest a plausible alternative binding scheme. At the moment we do not feel there is sufficient evidence to conclude which rotavirus protein(s) serves as the cell attachment protein. If NS35 were indeed the viral attachment protein and if the lysate binding assay accurately reflects virion attachment, an excess of NS35 should be able to compete with rotavirus particles for cell binding sites. In addition, antibodies against purified expressed NS35 might also reduce viral binding to cells. Further studies along these lines are currently in progress.

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ADDENDUM IN PROOF

We have recently expressed NS35 in a baculovirus system analogous to that used to express vp4 (E. R. Mackow, J. W. Barnett, H. Chan, and H. B. Greenberg, *J. Virol.* 63: 1661-1668, 1989). The expressed protein failed to compete with virions in an infectivity assay. Monospecific antisera raised against expressed NS35 had neither detectable neutralization activity nor hemagglutination inhibition activity.

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