The VP5 Domain of VP4 Can Mediate Attachment of Rotaviruses to Cells

SELENE ZÁRATE, RAFAELA ESPINOSA, PEDRO ROMERO, ERNESTO MÉNDEZ, CARLOS F. ARIAS, AND SUSANA LOPEZ *

Departamento de Genética y Fisiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos 62250, México

Received 23 July 1999/Accepted 7 October 1999

Some animal rotaviruses require the presence of sialic acid (SA) on the cell surface to infect the cell. We have isolated variants of rhesus rotavirus (RRV) whose infectivity no longer depends on SA. Both the SA-dependent and -independent interactions of these viruses with the cell are mediated by the virus spike protein VP4, which is cleaved by trypsin into two domains, VP5 and VP8. In this work we have compared the binding characteristics of wild-type RRV and its variant nar3 to MA104 cells. In a direct nonradioactive binding assay, both viruses bound to the cells in a saturable and specific manner. When neutralizing monoclonal antibodies directed to both the VP8 and VP5 domains of VP4 were used to block virus binding, antibodies to VP8 blocked the cell attachment of wild-type RRV but not that of the variant nar3. Conversely, an antibody to VP5 inhibited the binding of nar3 but not that of RRV. These results suggest that while RRV binds to the cell through VP8, the variant does so through the VP5 domain of VP4. This observation was further sustained by the fact that recombinant VP8 and VP5 proteins, produced in bacteria as fusion products with glutathione -transferase, were found to bind to MA104 cells in a specific and saturable manner and, when preincubated with the cell, were capable of inhibiting the binding of wild-type and variant viruses, respectively. In addition, the VP5 and VP8 recombinant proteins inhibited the infectivity of RRV, suggesting that RRV could bind to the cell through two sequential steps mediated by the interaction of VP8 and VP5 with SA-containing and SA-independent cell surface receptors, respectively.

The initial interaction of a virus with its host cell involves the recognition of, and a stable binding to, an appropriate receptor on the surface of the cell. Even though a great amount of work has been invested in the study of rotaviruses, little is known about the initial interactions of these viruses with their host cells.

Rotaviruses are the leading cause of morbidity and mortality due to acute gastroenteritis in children younger than 2 years (23). These viruses belong to the Reoviridae family and are composed of a genome of 11 segments of double-stranded RNA surrounded by three concentric layers of protein. The outermost layer is formed by VP7, a 37-kDa glycoprotein, which forms a smooth layer, and by VP4, an 88-kDa protein, which forms the spikes that extend from the surface of the particle (11).

It has been shown that VP4 has essential functions in the early virus-host interactions, including receptor binding and cell penetration (1, 5, 28, 31, 36). The infectivity of rotavirus is greatly enhanced by and apparently is dependent on the trypsin treatment of the viral particle; this proteolytic treatment results in the specific cleavage of VP4 into polypeptides VP5 and VP8 (10, 12, 27). The cleavage of VP4 does not affect cell binding but has been associated with the entry of the virus into the cell (3, 15, 22).

In vivo, rotavirus infection is highly restricted to the mature tip cells of the small intestine (23). The infection in vitro is also restricted, being most permissive in a variety of epithelial cell lines of renal and intestinal origin (11). The high selectivity of these viruses suggests the presence of specific receptors in the surface of susceptible cells, which might be at least one of the factors responsible for determining their selective tropism.

Some rotaviruses of animal origin bind to the cell surface through a sialic acid (SA)-containing cell receptor (2, 14, 24, 31). Human rotaviruses, in contrast, do not require SA to infect the cells (14). Recently, we isolated variants of a SA-dependent rhesus rotavirus (RRV) which no longer depend on the presence of SA to bind and thus to infect the cell (31). The characterization of these variants indicated that binding to SA is not an essential step in infection of cells by animal rotaviruses. It also showed that the initial interaction with SA, which is probably nonspecific, can be superseded by an interaction with a secondary receptor (SA independent), which might be responsible at least in part, for the tropism of these viruses. We have also shown that the SA-independent interaction of the RRV variants is mediated by VP4, through a site in the viral protein different from the SA-binding domain, located in VP8 (32).

To characterize the domains of the VP4 protein that interact with the surface of the host cell which ultimately lead to penetration of the virus into the cell, we have compared the binding characteristics of RRV and one of its SA-independent variants, nar3, to MA104 cells. We found that while wild-type (wt) RRV initially binds to the cell through VP8 (13, 21, 36), the SA-independent variant interacts with the cell through VP5. This finding supports our previous suggestion that the interaction of animal rotaviruses with the cell surface might involve at least two sites on the VP4 protein and directly assigns a novel cell interaction role to VP5.
Tris-HCl [pH 7.5], 140 mM NaCl, 10 mM CaCl₂), extracted with Freon, and the virus was pelleted by centrifugation for 60 min at 25,000 rpm at 4°C in an ultracentrifuge.

Cells, viruses and monoclonal antibodies. MA104 cells were cultured in Eagle’s minimal essential medium (MEM) supplemented with 10% fetal bovine serum. RRV was obtained from H. B. Greenberg, Stanford University, Stanford, Calif., and rotavirus variant nar3 has been described previously (31). RRV and nar3 were propagated in MA104 cells as previously described (9).

To prepare purified virus, virus-infected cells were harvested after complete cytopathic effect was attained, the cell lysate was frozen and thawed twice, and the virus was pelleted by centrifugation for 60 min at 25,000 rpm at 4°C in an SW28 rotor (Beckman). The virus pellet was resuspended in TNC buffer (10 mM Tris-HCl [pH 7.5], 140 mM NaCl, 10 mM CaCl₂), extracted with Freon, and subjected to isopycnic centrifugation in CsCl as previously described (10). The protein content of the purified triple-layered particles was determined by the Bradford protein assay (Bio-Rad).

The infectious titer of the trypsin-activated (10 μg of trypsin per ml for 30 min at 37°C) viral preparations was determined by an immunoperoxidase focus assay with MA104 cells grown in 96-well tissue culture plates, as previously described (21). The DNA fragment encoding VP5 was obtained from the vector polylinker, and 529 amino acid residues of the VP4 protein (from amino acid 248 to 776), resulting in a fusion protein of approximately 84 kDa. The expression and purification of the GST-fusion proteins were performed essentially as described by Isa et al. (21).

Cloning, expression, and purification of GST fusion proteins. The cloning and expression of the RRV VP8 protein as a fusion protein with GST has already been described (21). The DNA fragment encoding VP8 was obtained from the cdNA clone of RRV VP8 (6) by digestion of the gene with BamH I and XhoI; the ends were made blunt with T4 DNA polymerase and the Klenow fragment, and the DNA fragment containing nucleotides 749 to 2347 of the RRV VP4 gene was cloned into the Smal site of the pGEX 4T-2 vector (Pharmacia). The resultant fusion protein, GST-VP8, contained 226 amino acids from the GST protein, the thrombin recognition site, 3 amino acids resulting from translation of part of the vector polylinker, and 529 amino acid residues of the VP4 protein (from amino acid 248 to 776), resulting in a fusion protein of approximately 84 kDa. The expression and purification of the GST-fusion proteins were performed essentially as described by Isa et al. (21).

RESULTS

Binding characteristics of RRV and nar3 to MA104 cells. The binding of RRV and nar3 to MA104 cells was determined by a direct, nonradioactive assay, in which increasing amounts of CsCl gradient-purified viruses were incubated with a constant number of MA104 cells in suspension for 1 h at 4°C. The cell-bound virus was separated from free virus by three washes with PBS–0.5% BSA and detected in the final cell pellet by an ELISA with a goat polyclonal anti-rotavirus serum as the capture antibody and a rabbit polyclonal anti-rotavirus serum as the detecting antibody. Similar nonradioactive binding assays have been described previously (17, 19, 20).

The binding of purified RRV and nar3 to MA104 cells as measured by this direct assay was dose dependent and saturable (Fig. 1A). To show that the saturation observed in the
binding of either nar3 or RRV, the MAbs directed to VP8 or VP6 and VP7, respectively, did not affect significantly the above. Figure 2 shows that while MAbs 255 and 159, directed the cell-bound virus was quantitated by ELISA, as described temperature, this mixture was added to cells in suspension, and incubated for 1 h at 4 °C with gentle shaking. The amount of cell-bound virus was preincubated with PBS as a control (w/o MAb). The arithmetic means and expressed as the percentage of the virus binding obtained when the virus particles were preincubated with PBS as a control (w/o MAb). The arithmetic means and standard deviations for two independent experiments performed in duplicate are shown. The concentrations of MAbs used were as follows: 255, 159, 2G4, 1A9, and 7A12, 75 μg/ml; M11, 50 μg/ml; and M14, 100 μg/ml.

MAbs to VP5 and VP8 differentially prevent the binding of nar3 and RRV. We have previously shown that in contrast to wt RRV, the SA-independent variant nar3 is not neutralized by MAbs directed to the VP8 domain of VP4 (MAbs 7A12, 1A9, M11, and M14), even though these MAbs bind to the variant as efficiently as to the wt virus, as judged by HA inhibition and ELISA (31). On the other hand, MAb 2G4, which is directed to the VP5 domain of VP4 (29), inhibits the infectivity of both wt and variant viruses (31). It has been shown that MAbs directed to VP8 are able to neutralize the infectivity of RRV by preventing the initial attachment of the virus to the cell (36) while MAb 2G4 inhibits its infectivity at a not yet defined postbinding step.

Since RRV and the variant nar3 apparently have distinct requirements for cell binding, we characterized the effect of MAbs directed to the outer-shell proteins VP7, VP8, and VP5, on the attachment of these viruses to MA104 cells. We also used, as control, a MAb directed to the inner-shell protein, VP6. In these assays, a fixed amount of purified virus (300 ng) was preincubated with the different MAbs for 1 h at room temperature, this mixture was added to cells in suspension, and the cell-bound virus was quantitated by ELISA, as described above. Figure 2 shows that while MAbs 255 and 159, directed to VP6 and VP7, respectively, did not affect significantly the binding of either nar3 or RRV, the MAbs directed to VP8 or VP5 did have a differential effect, depending on the virus tested. While the VP8 MAbs reduced the attachment of RRV to cells, as previously reported (36), the binding of the SA-

binding curves for both viruses was due to saturation of the attachment sites in the cell surface and was not the result of saturation of the detection system, a direct ELISA of the purified viruses was performed in parallel to the binding assay; the optical density (OD) readings of the direct ELISA kept increasing up to 1.2 (Fig. 1B). No virus was detected in the ELISAs of control experiments where no cells were added during the binding assay (data not shown).

MAbs to VP5 and VP8 differentially prevent the binding of nar3 and RRV. We have previously shown that in contrast to wt RRV, the SA-independent variant nar3 is not neutralized by MAbs directed to the VP8 domain of VP4 (MAbs 7A12, 1A9, M11, and M14), even though these MAbs bind to the variant as efficiently as to the wt virus, as judged by HA inhibition and ELISA (31). On the other hand, MAb 2G4, which is directed to the VP5 domain of VP4 (29), inhibits the infectivity of both wt and variant viruses (31). It has been shown that MAbs directed to VP8 are able to neutralize the infectivity of RRV by preventing the initial attachment of the virus to the cell (36) while MAb 2G4 inhibits its infectivity at a not yet defined postbinding step.

Since RRV and the variant nar3 apparently have distinct requirements for cell binding, we characterized the effect of MAbs directed to the outer-shell proteins VP7, VP8, and VP5, on the attachment of these viruses to MA104 cells. We also used, as control, a MAb directed to the inner-shell protein, VP6. In these assays, a fixed amount of purified virus (300 ng) was preincubated with the different MAbs for 1 h at room temperature, this mixture was added to cells in suspension, and the cell-bound virus was quantitated by ELISA, as described above. Figure 2 shows that while MAbs 255 and 159, directed to VP6 and VP7, respectively, did not affect significantly the binding of either nar3 or RRV, the MAbs directed to VP8 or VP5 did have a differential effect, depending on the virus tested. While the VP8 MAbs reduced the attachment of RRV to cells, as previously reported (36), the binding of the SA-

binding curves for both viruses was due to saturation of the attachment sites in the cell surface and was not the result of saturation of the detection system, a direct ELISA of the purified viruses was performed in parallel to the binding assay; the optical density (OD) readings of the direct ELISA kept increasing up to 1.2 (Fig. 1B). No virus was detected in the ELISAs of control experiments where no cells were added during the binding assay (data not shown).

MAbs to VP5 and VP8 differentially prevent the binding of nar3 and RRV. We have previously shown that in contrast to wt RRV, the SA-independent variant nar3 is not neutralized by MAbs directed to the VP8 domain of VP4 (MAbs 7A12, 1A9, M11, and M14), even though these MAbs bind to the variant as efficiently as to the wt virus, as judged by HA inhibition and ELISA (31). On the other hand, MAb 2G4, which is directed to the VP5 domain of VP4 (29), inhibits the infectivity of both wt and variant viruses (31). It has been shown that MAbs directed to VP8 are able to neutralize the infectivity of RRV by preventing the initial attachment of the virus to the cell (36) while MAb 2G4 inhibits its infectivity at a not yet defined postbinding step.

Since RRV and the variant nar3 apparently have distinct requirements for cell binding, we characterized the effect of MAbs directed to the outer-shell proteins VP7, VP8, and VP5, on the attachment of these viruses to MA104 cells. We also used, as control, a MAb directed to the inner-shell protein, VP6. In these assays, a fixed amount of purified virus (300 ng) was preincubated with the different MAbs for 1 h at room temperature, this mixture was added to cells in suspension, and the cell-bound virus was quantitated by ELISA, as described above. Figure 2 shows that while MAbs 255 and 159, directed to VP6 and VP7, respectively, did not affect significantly the binding of either nar3 or RRV, the MAbs directed to VP8 or VP5 did have a differential effect, depending on the virus tested. While the VP8 MAbs reduced the attachment of RRV to cells, as previously reported (36), the binding of the SA-

binding curves for both viruses was due to saturation of the attachment sites in the cell surface and was not the result of saturation of the detection system, a direct ELISA of the purified viruses was performed in parallel to the binding assay; the optical density (OD) readings of the direct ELISA kept increasing up to 1.2 (Fig. 1B). No virus was detected in the ELISAs of control experiments where no cells were added during the binding assay (data not shown).

MAbs to VP5 and VP8 differentially prevent the binding of nar3 and RRV. We have previously shown that in contrast to wt RRV, the SA-independent variant nar3 is not neutralized by MAbs directed to the VP8 domain of VP4 (MAbs 7A12, 1A9, M11, and M14), even though these MAbs bind to the variant as efficiently as to the wt virus, as judged by HA inhibition and ELISA (31). On the other hand, MAb 2G4, which is directed to the VP5 domain of VP4 (29), inhibits the infectivity of both wt and variant viruses (31). It has been shown that MAbs directed to VP8 are able to neutralize the infectivity of RRV by preventing the initial attachment of the virus to the cell (36) while MAb 2G4 inhibits its infectivity at a not yet defined postbinding step.

Since RRV and the variant nar3 apparently have distinct requirements for cell binding, we characterized the effect of MAbs directed to the outer-shell proteins VP7, VP8, and VP5, on the attachment of these viruses to MA104 cells. We also used, as control, a MAb directed to the inner-shell protein, VP6. In these assays, a fixed amount of purified virus (300 ng) was preincubated with the different MAbs for 1 h at room temperature, this mixture was added to cells in suspension, and the cell-bound virus was quantitated by ELISA, as described above. Figure 2 shows that while MAbs 255 and 159, directed to VP6 and VP7, respectively, did not affect significantly the binding of either nar3 or RRV, the MAbs directed to VP8 or VP5 did have a differential effect, depending on the virus tested. While the VP8 MAbs reduced the attachment of RRV to cells, as previously reported (36), the binding of the SA-
the detection antibody was a rabbit anti-GST antibody was performed (data not shown).

The recombinant proteins were found to compete the binding of RRV and nar3 in a selective manner. GST-VP8 decreased the binding of RRV by 75% compared to the binding of the virus in the absence of the recombinant protein, whereas it did not alter the attachment of the nar3 variant. Conversely, GST-VP5 was able to displace the binding of nar3 but had no effect on the binding of RRV. Preincubation of the cells with a mixture of GST-VP5 and GST-VP8 fusion proteins did not increase the inhibitory effect observed with the individual proteins. The GST protein, used as a control, did not

FIG. 3. Binding of the recombinant proteins GST-VP8, GST-VP5, and GST to MA104 cells. (A) The indicated amounts of affinity-purified GST-VP5 (a), GST-VP8 (b), and GST (c) were incubated with $5 \times 10^4$ MA104 cells in suspension for 1 h at 4°C. The amount of cell-bound protein was determined by an ELISA as described in Materials and Methods. The total amount of recombinant protein added to each assay mixture is plotted against the OD$_{405}$ reading obtained in the ELISA plate. (B) OD readings obtained when the indicated amounts of recombinant proteins were directly assayed in an ELISA. The inset shows the SDS-PAGE analysis of the affinity-purified fusion proteins used in the ELISA and binding assays. MW, molecular weight in thousands.

FIG. 4. Binding of the recombinant proteins GST-VP5, GST-VP8, and GST in the presence of anti-rotavirus MAbs. Affinity-purified GST-VP5, GST-VP8, or GST alone (1.5 µg of each) were preincubated for 1 h at room temperature with the indicated MAbs (75 µg/ml) in a final volume of 100 µl. After incubation with the MAbs, the fusion protein-antibody mixture was added to a suspension of MA104 cells ($5 \times 10^4$ cells/binding assay) and incubated for 1 h at 4°C with gentle shaking. The amount of cell-bound protein was determined by an ELISA as described in Materials and Methods. Controls of protein binding without cells were used in each experiment (results not shown). Data are expressed as the percentage of the recombinant protein binding obtained when the fusion proteins were preincubated with PBS as a control (w/o MAb). The arithmetic means and standard deviations for two independent experiments performed in duplicate are shown. The specificity of the MAbs used is shown on the left.

FIG. 5. Effect of the recombinant proteins GST-VP8, GST-VP5, and GST on the binding of RRV and nar3 viral particles to MA104 cells. Affinity-purified fusion proteins (1.5 µg of each) were preincubated with $5 \times 10^4$ MA104 cells in suspension for 1 h at 4°C. The excess unbound protein was removed, and then 300 ng of either RRV or nar3 viral particles was added, and the mixture was further incubated for 1 h at 4°C. The amount of virus or fusion protein bound to cells was determined by an ELISA as described in Materials and Methods. Data are expressed as the percentage of the virus binding obtained when the cells were preincubated with PBS as a control. The arithmetic means and standard deviations for two independent experiments performed in duplicate are shown. In the ELISAs, where the fusion proteins were detected, no significant differences in the binding of the recombinant proteins to the cells were found (results not shown).
modify the binding of either virus. Taken together, these results indicate that nar3 and RRV bind to MA104 cells through two different domains of VP4. The fact that the mixture of the two fusion proteins did not further decrease the binding of either virus suggests that wt RRV binds initially to the cell mainly through VP8 while nar3 does so mainly through VP5.

The infectivity of nar3 and RRV is decreased by the recombinant VP5 and VP8 proteins. Since the GST fusion proteins specifically and selectively inhibited the binding of nar3 and RRV, we evaluated how these recombinant proteins influenced the infectivity of these viruses. To do this, MA104 cells in 96-well plates were preincubated with the recombinant proteins or PBS as control and then a fixed amount of the virus was added. After 1 h of binding at 4°C, the nonbound virus was removed and the infection was left to proceed for 16 h, at which time the cells were fixed and stained for the presence of viral antigen.

The GST-VP8 protein was found to reduce the infectivity of RRV by 60%, while it did not affect that of nar3, as compared to control wells where no protein was added (taken as 100% infectivity) (Fig. 6A). When GST-VP5 was tested in this assay, the reverse was found; the infectivity of the SA-independent variant was reduced by 50% whereas that of RRV was not significantly affected. Preincubation of the cells with a mixture of the two recombinant proteins resulted in an inhibition of infectivity similar to that observed when either the GST-VP5 for nar3 or the GST-VP8 for RRV was individually tested. Preincubation of the cells with GST did not affect the infectivity of either virus. These results suggest that VP8 and VP5 block the infectivity of RRV and nar3, respectively, by blocking their binding to the cell surface.

The recombinant VP5 protein decreases the infectivity of RRV in NA-treated cells. We also characterized the effect of the recombinant proteins on the infectivity of RRV and nar3 in MA104 cells that had been treated with NA. Since the infectivity of RRV decreases up to 80% in NA-treated cells compared to that in untreated cells (31), the amount of RRV used in these experiments was increased sixfold to maintain a similar number of FFU per well (~2,000 FFU/well) under both conditions. MA104 cells in 96-well plates were treated with NA as described in Materials and Methods and then preincubated with the recombinant proteins. Under these conditions, GST-VP5 reduced the infectivity of both nar3 and RRV by 75% (Fig. 6B). The GST-VP8 protein had a less pronounced effect on RRV, reducing its infectivity to 65% of that observed in controls, whereas it did not significantly affect the infectivity of nar3 compared to the GST control protein. When a mixture of GST-VP5 and GST-VP8 was added to the cells, the infectivity of RRV was reduced to 15% while that of the SA-independent variant nar3 was reduced to about 50%. Findings not significantly different from those obtained when the individual proteins were tested. Taken together, these results indicate that on cells treated or not treated with NA, the SA-independent variant binds preferentially through the VP5 domain of VP4 while RRV binds to untreated cells through VP8 and to NA-treated cells mainly through VP5.

DISCUSSION

The attachment of a virus to its cellular receptor is the first step in infection and may control the efficiency of virus entry. A detailed understanding of the molecular interactions between the viral attachment proteins and the cellular proteins involved is a prerequisite for understanding the translocation of the virus into the cytoplasm of the cell.

In this work we have studied by a direct, nonradioactive binding assay the cell attachment characteristics of the SA-dependent rotavirus RRV and of its variant nar3, which no longer requires SA to infect the cell. The binding of these viruses to NA-treated cells could not be evaluated by this kind of assay, since the treated cells in suspension tend to aggregate, giving unreliable results.

We found that these two viruses initially attach to the cell surface of untreated MA104 cells through different domains of VP4. It has been previously shown (13, 21, 26) that RRV binds to a SA-containing molecule through VP8, while we report that the SA-independent variant binds to an asialo receptor through VP5. We have previously shown that although this variant does not need SA to infect the cell, it retains its ability to agglutinate erythrocytes in a SA-dependent manner (31). However, apparently the variant binds to a SA-independent cell surface molecule even in normal, untreated cells, since the attachment of these viruses to untreated cells is blocked by preincubation of the cells with GST-VP5 and not by preincubation with GST-VP8. In addition, MAbs to VP8, which efficiently block the RRV cell attachment, do not block nar3 binding, while the reverse was observed with the VP5 MAb 2G4. These results also suggest that the binding to SA on the

FIG. 6. Effect of the recombinant proteins GST-VP5, GST-VP8, and GST on the infectivity of RRV and nar3 viral particles. Affinity-purified fusion proteins (35 µl of a 50 ng/µl solution) were added to monolayers of MA104 cells in 96-well plates, either treated (B) or not treated (A) with NA, for 30 min at 4°C. Then, 2 x 10^4 FFU of RRV or nar3 (A) or 1.2 x 10^4 FFU of RRV, or 2 x 10^3 FFU of nar3 (B) was added per well, and after a 30-min adsorption period at 4°C, the inoculum was removed and the infection was left to proceed for 16 h at 37°C. At this time, the wells were fixed and immunostained as described in Materials and Methods. Data are expressed as the percentage of the virus infectivity obtained when the cells were preincubated with PBS as a control. The arithmetic means and standard deviations for three independent experiments performed in duplicate are shown.
surface of erythrocytes and the surface of MA104 cells might not represent the same type of interaction.

While in VP8 the amino acids that might be in contact with the SA moiety of the receptor have been located (21), the region of VP5 that interacts with the cell surface has not been determined. The putative fusion region between amino acids 384 and 404 (29) and the integrin binding site located between amino acids 308 and 310 (4) might be good candidates to play this role.

Sequence analysis of the VP4 gene of the SA-independent variants showed that the gene product had three amino acid changes, at positions 37 (Leu-Pro), 187 (Lys-Arg), and 267 (Tyr-Cys), with respect to the parental RRV gene product (32). Recently, we reported that the new Cys at position 267 is involved in the formation of an alternate disulfide bridge with the Cys at position 318 in the VP4 of a SA-independent variant, and we proposed that this alternate disulfide bond, by altering the conformation of the VP5 protein, might allow the variant virus to directly interact with an asialo molecule in the cell membrane, superseding the initial interaction with the SA-containing receptor (6). In the assays reported here, we used a VP5 fusion protein derived from the wt virus; nevertheless, this fusion protein was able to efficiently attach to the cell surface, and block the binding and infectivity of the variant virus. These results suggest that the recombinant wt VP5 protein, when expressed out of the context of the virus structure, can adopt the proper conformation needed to interact with the SA-independent receptor on the surface of MA104 cells.

Since the recombinant GST-VP8 and GST-VP5 proteins were able to compete for the attachment of RRV and nar3, respectively, we investigated whether these fusion proteins were able to block their infectivity; we found that in untreated cells, GST-VP8 was able to decrease the infectivity of RRV while the GST-VP5 protein decreased the infectivity of nar3, consistent with our findings in the binding assays. On the other hand, in NA-treated cells, the recombinant proteins had a different effect; GST-VP5, which in untreated cells inhibited the attachment and infectivity only of nar3, was now able to reduce by 75% the residual (20% of that observed in untreated cells) infectivity of RRV (Fig. 6B). These results suggest that RRV is able to interact through VP5, with the asialo receptor in NA-treated cells, albeit with lower efficiency than that of the variant nar3. The inhibition of GST-VP5 on the variant was consistent, although slightly more pronounced than in untreated cells. The GST-VP8 fusion protein blocked about 35% of the infectivity of RRV, indicating that the treatment of the cells with NA might leave a small amount of SA on the surface of the cells that might be resistant to this treatment and that this SA can still be used by the SA-dependent strain to attach and infect the cell. As expected, GST-VP8 did not inhibit significantly the infectivity of nar3. Taken together, these results suggest that the residual infectivity observed for SA-dependent strains in NA-treated cells (2, 31) might be the consequence of the interaction of the viruses with residual SA left on the cell surface, in addition to a direct interaction of VP5 with an asialo receptor, which would seem to be less efficient for these strains than the VP8-SA interaction.

The observation that the MAb 2G4, which binds to VP5, blocks the binding of the variant without altering the binding of wt RRV, while it is able to neutralize the infectivity of both viruses, together with the finding that GST-VP5 blocks the infectivity of both viruses in NA-treated cells, gives further support to the idea that there are at least two sequential interactions of animal rotaviruses with cell surface molecules. The first, which involves the initial binding of the virus, through the VP8 domain of VP4, to a SA-containing compound, followed by a second virus-cell interaction step which involves VP5 and a SA-independent molecule. The SA-independent variant nar3 apparently does not require the first interaction, since it is able to efficiently interact directly through VP5 with the second asialo molecule in the cell membrane (Fig. 7).

The results presented in this work also suggest that the two contacts of the virus with the cell surface are sequential, such that in wt RRV the initial contact with the SA-containing molecule might "facilitate" the interaction with the second, SA-independent molecule. In the variant, the amino acid changes in VP4 probably induce a conformational change in the protein, such that its VP5 domain can interact directly with the SA-independent molecule, surpassing the initial contact of VP8 with SA. Therefore, one would expect that GST-VP5, which binds to the asialo receptor, should be able to compete the secondary interaction of RRV with this molecule and thus block its infectivity. This inhibition was not observed; however, it could be explained if RRV, once attached to the cell through VP8, were able to efficiently displace the already bound recombinant VP5. This would not be the case for the nar3 variant (or for RRV in NA-treated cells), since the initial interaction of this virus is with the asialo receptor, and so when GST-VP5 is blocking this molecule, the variant cannot bind. In further support of the sequential interactions of RRV with two cell molecules is the finding that a MAb directed to the cell surface of MA104 cells, which blocks the cell binding of nar3 but not that of RRV, is able to inhibit the infectivity of both viruses (S. Lopez, R. Espinosa, P. Isa, S. Zarate, E. Mendez, and C. F. Arias, unpublished results).

Although the present data strongly support the existence of two different interactions between wt RRV and the cell surface, at this point it is not possible to establish whether two sites in the same cell surface molecule or two cell molecules are involved in the interactions with VP8 and VP5. The fact that in infection competition assays the wt and variant viruses com-

![FIG. 7. Model for the early interactions of animal rotaviruses with MA104 cells. wt RRV interacts primarily with a SA-containing cell receptor through the VP8 domain of VP4 (thick arrow). After this initial interaction, which might induce a conformational change in VP4, the virus interacts with a second, SA-independent cell receptor, through the VP5 domain of VP4. This interaction might (?) facilitate the entry of the virus into the cell. A small proportion of the wt RRV virus can interact directly through VP5 with the SA-independent molecule (dashed arrow; see the text). The SA-independent variant nar3, due to the amino acid changes in its VP4 protein, interacts directly through VP5 with the asialo receptor. For the sake of clarity, the SA-containing and asialo cellular receptors are depicted in this model as two separate entities; however, they could be two domains of the same receptor molecule; this point remains to be clarified (see the text).]
pete with each other reciprocally (33) suggests that if it is not the same cellular entity, the two cellular molecules might be in close proximity.

Recently, it was reported that a recombinant RRV-VP5 protein produced in bacteria is able to specifically permeabilize liposomes (8). The GST-VP5 protein used in our assays was not able to promote the coentry of α-sarcin into MA104 cells, which has been shown to correlate with virus entry (7, 25); thus, the two activities that have been reported for VP5 (reference 8 and this work) might represent two different functions of this protein and might reside in two different domains of this S29 amino acid polypeptide. This issue is currently under investigation.

The list of examples of viruses that have more than one interaction with the surface of the host cell is accumulating (16, 18, 34, 35), suggesting that virus attachment is a multistep process, more complex than the bimolecular virus-cell interaction previously envisioned. Multiple viral attachment proteins can bind to different cell receptors, or different binding sites in either the viral protein or the cell receptor, may act together to modulate each other or to contribute in complementary functions. The cell receptors that bind to different virus ligands might act sequentially; thus, binding of the virus to the first cellular component could cause conformational changes in the virus or in the host cell that are necessary before the second interaction can take place.

For rotaviruses, recent competition experiments with strains of human and animal origin together with the SA-independent variants suggest that there might be at least one other interaction, in addition to the two described in this work, between the virus and the cell surface (33). Further studies to characterize the cellular molecules and the viral protein domains involved in these interactions should provide insight into the highly selective tropism of rotaviruses.

ACKNOWLEDGMENTS

We are grateful to Gabriel Corkidi for developing the system for the semiautomatic cell counter and to Leticia Vega for technical support with this system.

This work was partially supported by grants 75197/572106 from the Howard Hughes Medical Institute, G0012-N9607 from the National Council for Science and Technology—Mexico, and IN207496 from the National Council for Science and Technology—Mexico, and IN207496 from Howard Hughes Medical Institute, G0012-N9607 from the National Council for Science and Technology—Mexico, and IN207496 from Howard Hughes Medical Institute, G0012-N9607 from the National Council for Science and Technology—Mexico.