Integrin $\alpha_v\beta_3$ mediates rotavirus cell entry

Carlos A. Guerrero*, Ernesto Méndez, Selene Zárate, Pavel Isa, Susana López, and Carlos F. Arias†

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

Edited by Wolfgang K. Joklik, Duke University Medical Center, Durham, NC, and approved October 4, 2000 (received for review June 28, 2000)

Rotavirus strains differ in their need for sialic acid (SA) for initial binding to the cell surface; however, the existence of a postattachment cell receptor, common to most, if not all, rotavirus strains, has been proposed. In the present study, antibodies to the α_v and β_3 integrin subunits, and the $\alpha_v\beta_3$ ligand, vitronectin, efficiently blocked the infectivity of the SA-dependent rhesus rotavirus RRV, its SA-independent variant nar3, and the neuraminidase-resistant human rotavirus strain Wa. Vitronectin and anti-β_3 antibodies, however, did not block the binding of virus to cells, indicating that rotaviruses interact with $\alpha_v\beta_3$ at a postbinding step, probably penetration. This interaction was shown to be independent of the tripeptide motif arginine-glycine-aspartic acid present in the natural ligands of this integrin. Transfection of CHO cells with $\alpha_v\beta_3$ genes significantly increased their permissiveness to all three rotavirus strains, and the increment of virus infectivity was reverted by incubation of these cells either with antibodies to β_3 or with vitronectin. These findings implicate $\alpha_v\beta_3$ integrin as a cellular receptor common to neuraminidase-sensitive and neuraminidase-resistant rotaviruses, and support the hypothesis that this integrin could determine, at least in part, the cellular susceptibility to rotaviruses.

Rotaviruses, the leading cause of severe dehydrating diarrhea in infants and young children worldwide, are nonenveloped viruses that posses a genome of 11 segments of double-stranded RNA contained in a triple-layered protein capsid. The outermost layer is composed of two proteins, VP4 and VP7. VP4 forms spikes that extend from the surface of the virus, and it has been associated with a variety of functions, including initial attachment of the virus to the cell membrane and the penetration of the virion into the cell (1).

Rotaviruses have very specific cell tropism, infecting only enterocytes on the tip of intestinal villi (2), which suggests that specific host receptors must exist. *In vitro*, they also display restricted tropism, binding to a variety of cell lines, but efficiently infecting only those of renal or intestinal epithelium origin (3). Despite advances in knowledge regarding the molecular and structural biology of the virus, little is known about rotavirus cell receptors. It is known that some animal rotavirus strains attach to sialic acid (SA) on cell surfaces, and this interaction has been shown to be required for the efficient infection of virus to susceptible cells, both *in vitro* and *in vivo* (4). However, the binding of animal rotaviruses to an SA-containing cell receptor has been shown to be nonessential, because variants whose infectivity is no longer dependent on the binding to these acid sugars have been isolated (5). The secondary importance of SA as the attachment site for rotaviruses is also demonstrated by the fact that the infectivity of most, if not all, human rotavirus (HRV) strains is not affected by neuraminidase (NA) treatment of cells (6–8).

Integrins are a family of α/β heterodimers of cell adhesion receptors that mediate cell–extracellular matrix and cell–cell interactions, and are known to function as signaling receptors for a variety of cellular processes, including spreading, migration, proliferation, differentiation, and survival (9–11). These cell molecules are commonly used as receptors for many different viruses, including echoviruses 1, 8, 9, and 22 (12–15), coxsackievirus A9 (16), foot-and-mouth disease virus (17, 18), papillomavirus (19), adenovirus (20), adeno-associated virus type 2 (21), and hantaviruses (22), with integrin $\alpha_v\beta_3$ being, so far, the most frequently used as virus receptor (14, 16, 17, 20, 22).

Recently, it was found that rotavirus surface proteins contain sequence binding motifs for $\alpha_2\beta_1$, $\alpha_4\beta_1$, and $\alpha_x\beta_2$ integrins. Antibodies to these integrins, and peptides containing these sequence motifs, were shown to block the infectivity of simian rotavirus strain SA11 and the HRV strain RV5 (23). In addition, $\alpha_2\beta_1$ and $\alpha_4\beta_1$ integrins have been shown to mediate the attachment and entry of rotavirus SA11 into the human myelogenous leukemic cell line K562 (24).

We recently reported that proteins from MA104 cells, extracted with the nonionic detergent octyl β-glucoside under noncytolytic conditions, have the capacity to inhibit the infectivity of rotaviruses when preincubated with the virus before cell infection (25). In the present study, we have identified one of these proteins as the β_3 integrin subunit, and we demonstrate that $\alpha_v\beta_3$ integrin interacts with NA-sensitive and -resistant strains at a postattachment step and is capable of promoting rotavirus infection of the poorly permissive CHO (Chinese hamster ovary) cells.

Materials and Methods

Cells and Viruses. MA104 cells were cultured in Eagle's minimal essential medium (MEM) supplemented with 10% (vol/vol) FCS. CHO cells were grown in DMEM with 10% (vol/vol) FCS. CHO cells transfected with $\alpha_{IIb}\beta_3$ (CHO-A5) and $\alpha_v\beta_3$ (CHO-VNRC) integrins (26) were grown in DMEM/10% FCS, in the presence of 400 μg/ml G418 (GIBCO). Rotavirus strains RRV, Wa, and nar3 (5, 8) were propagated in MA104 cells (8). Reovirus serotype 1 was obtained from C. Ramos (Instituto Nacional de Salud Pública, Cuernavaca, Morelos, Mexico) and was grown in L929 cells as previously described (27). Poliovirus type 3 was obtained from R. M. del Angel (Centro de Investigación y Estudios Avanzados del Instituto Politécnico Nacional, Mexico D.F.) and grown in MA104 cells. Rabbit polyclonal antibody against reovirus type 1 was kindly provided by P. Lee (Univ. of Calgary, Alberta, Canada).

Ligands, Peptides, and Antibodies. Laminin, glycophorin A, chondroitin sulfate A, BSA, and collagen type I were obtained from Sigma, fibronectin was obtained from GIBCO, and vitronectin was either purchased from Sigma or purified from human plasma as described previously (28). All proteins were used at 10 μg/ml, unless otherwise indicated. Peptides GRGDSP and GRGESP (hereafter called RGD and RGE, respectively) were obtained from GIBCO and used at 400 μg/ml. Polyclonal goat IgG

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: SA, sialic acid; NA, neuraminidase; HRV, human rotavirus; RRV, rhesus rotavirus.

*Permanent address: Departamento de Bioquímica, Facultad de Medicina, Universidad Nacional de Colombia, Bogotá, Colombia.

†To whom reprint requests should be addressed at: Instituto de Biotecnología/UNAM, A.P. 510–3, Colonia Miraval, Cuernavaca, Morelos 62250, Mexico. E-mail: arias@ibt.unam.mx.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.250299897. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.250299897

antibodies directed against an epitope located at the amino terminus of integrin subunits α_2, α_3, α_4, α_v, α_x, α_{IIb}, β_2, and β_3, and mAb 4B7R to subunit β_1, were obtained from Santa Cruz Biotechnology and used at 20 μg/ml. mAbs to integrins α_1 (FB12), α_2 (P1E6), α_3 (P1B5), α_4 (P1H4), α_5 (P1D6), α_6 (NK1-GoH3), α_v (P3G8), α_{IIb} (CA3), $\alpha_v\beta_3$ (LM609), β_1 (P4G11), β_2 (P4H9), β_3 (25E11), and β_4 (ASC-9), purchased from Chemicon, were used at 10 μg/ml. mAbs to integrins α_2 (P1E6, 3.2 μg/ml), β_2 (MHM23, 41 μg/ml), and α_4 (P4G9, 8.1 μg/ml) were purchased from Dako and used at the concentrations indicated. mAb B5-IVF2 to β_5 (Upstate Biotechnology) was used at 10 μg/ml. mAb 26 to β_3 (Transduction Laboratories) was used at 5 μg/ml.

Infectivity Assay. MA104 or CHO cells in 96-well plates were washed twice with MEM, and then about 1,000 focus-forming units (ffu) of RRV, nar3, or Wa rotavirus, or of control virus, reovirus or poliovirus, were adsorbed to the cells for 45 min at 4°C (for 1 h at 37°C in the case of CHO cells). After the adsorption period, the virus inoculum was removed, the cells were washed twice with MEM, and cultures were maintained for 14 h at 37°C. Infected cell cultures were fixed and tested with an immunoperoxidase focus detection assay, as described previously (29). The ffu were counted by using a Visiolab 1000 station (Biocom, Paris; ref. 25).

Blocking Assays. To evaluate the blocking activity of integrin ligands and antibodies, and of RGD and RGE peptides, MA104 or CHO cells were washed twice with MEM and incubated with indicated concentrations of the reagents in MEM, for 60 min (90 min for antibodies) at 37°C. For all incubations with vitronectin, MEM containing 400 μM Mn^{2+} instead of Ca^{2+} was used (30), whereas for all other procedures, including washings, regular MEM with Ca^{2+} was used. After the incubation step, reagents were removed and the cells were infected as described above. To evaluate whether antibodies to β_3 and vitronectin were able to inhibit rotavirus infectivity if added after the virus had been adsorbed, MA104 cells in 96-well plates were washed twice with MEM and chilled on ice for 5 min, and the virus was adsorbed at 4°C for 60 min. The cells were then washed twice with ice-cold MEM, and either vitronectin (1.5 μg/ml) or anti-β_3 antibodies (Santa Cruz Technology, 20 μg/ml) were added, and the mixture was incubated for 1 h at 4°C. The cells were washed once with MEM and maintained for 14 h at 37°C before immunostaining for virus. As control for these experiments, vitronectin or anti-β_3 antibodies were added for 1 h at 4°C before addition of the viruses for 1 h at 4°C, or were added to the cells after the virus had been adsorbed for 1 h at 37°C.

Binding Assay. The binding assay was carried out as described by Zárate *et al.* (31). Briefly, a suspension of 5×10^4 cells, preincubated either with 20 μg/ml of a goat polyclonal antibody to the β_3 integrin subunit (Santa Cruz Technologies) or with 1.5 μg/ml of vitronectin for 1 h at 4°C, were mixed with 300 ng of purified virus in MEM/1% BSA in a final volume of 200 μl and incubated for 1 h at 4°C with gentle mixing. The cell–virus complexes were washed three times with ice-cold PBS containing 0.5% BSA. In the final wash, the cells were transferred to a fresh tube, and then treated with 50 μl of lysis buffer (50 mM Tris, pH 7.5/150 mM NaCl/0.1% Triton X-100). The virus present in the lysates was quantified by an ELISA (31). In all binding assays, a binding control with no cells was performed.

Flow Cytometry. MA104 and CHO cells grown to 80% confluence were washed and brought into a single-cell suspension by incubation with 0.5 mM EDTA in PBS at 37°C and dispersed by gentle pipetting. Cells were collected by low-speed centrifugation ($200 \times g$) and resuspended in ice-cold MEM without serum, and the cell concentration was determined with a hemocytometer. In each experiment, 2.5×10^5 cells were incubated with either mAb LM609 or IgG1 control antibody (5 μg/ml) for 1 h at 4°C, washed twice with 2% (vol/vol) FCS in PBS, and then incubated with fluorescein-conjugated anti-mouse IgG antibodies (12 μg/ml; Zymed) for 1 h at 4°C. Antibody binding was analyzed by using a FACScan flow cytometer and CELLQUEST software (Becton Dickinson) with appropriate gating parameters.

Results

Antibodies to $\alpha_v\beta_3$ Integrin Inhibit Rotavirus Infectivity. Several protein bands with the ability to block rotavirus infection were isolated by preparative gel electrophoresis from MA104 cell extracts obtained with the nonionic detergent octyl β-glucoside (25). Tryptic peptides from one of these bands, with an apparent molecular mass of 110 kDa, were sequenced; one of them was found to be identical to amino acids 266–279 of the human β_3 integrin subunit, whereas two other peptides were derived from filamin and spectrin proteins. Given this finding, antibodies to β_3 were tested for their ability to block the infectivity of the SA-dependent simian rotavirus RRV, its NA-resistant variant nar3, and the natural NA-resistant HRV strain Wa. A monoclonal antibody (mAb 26) to this integrin inhibited the infectivity of all three rotavirus strains by 40–45%, depending on the virus strain (Fig. 1*A*). Because β_3 is known to associate with integrin subunits α_v and α_{IIb} (9), we tested the blocking activity of antibodies to these integrin subunits. A polyclonal antibody to α_v, or a mAb (LM609) that recognizes both α_v and β_3 subunits, inhibited the infectivity of rotaviruses (Fig. 1*B*), whereas a mAb to subunit α_{IIb} had no effect (not shown).

Because $\alpha_2\beta_1$, $\alpha_4\beta_1$, and $\alpha_x\beta_2$ integrins have been suggested to play a role during rotavirus infection (25), the blocking activity of antibodies directed against each subunit of these integrins was compared with the activity of antibodies to α_v and β_3 (Fig. 1*B*). Antibodies to α_2, α_4, and β_2 inhibited the infectivity of all three rotavirus strains by 22–44%, depending on the antibody and the virus strain tested, whereas mAbs to α_x and β_1 had low or no inhibitory capacity, depending on the virus strain. On the other hand, antibodies to either α_v or β_3 inhibited all strains by 44–50%, with the exception of nar3, which was reduced by 27% by the α_v antibody. Antibodies to integrin subunits α_1, α_3, α_5, α_6, β_4, β_5, and α_{IIb} did not block the infectivity of any of the three viruses by more than 9% (not shown).

The Block in Infectivity by mAbs to α_2 and β_3 Integrins Is Additive. When antibodies directed against each subunit of a given integrin heterodimer were mixed, no additive inhibition of infectivity was observed (not shown). However, when combinations of antibodies directed against different integrins were tested, antibodies to $\alpha_2\beta_1$ and $\alpha_v\beta_3$ had a clear additive blocking effect (Student's t test, $P < 0.001$), suggesting that these integrins are involved in different stages of rotavirus infection. None of the other integrin antibody combinations blocked the infectivity of the viruses additively (Fig. 2).

Inhibition of Rotavirus Infectivity by Integrin Ligands. The incubation of cells with various integrin ligands showed that vitronectin, which is known to interact with $\alpha_v\beta_3$, blocked rotavirus infectivity by 60–70% at 0.5 μg/ml (Fig. 3). Fibronectin, which is also an $\alpha_v\beta_3$ ligand, inhibited infectivity by 30–50% when used at 20 times the above concentration, whereas collagen type I, which binds to $\alpha_2\beta_1$, blocked virus infectivity by 20% at 10 μg/ml. Other integrin ligands and glycoproteins, such as laminin, chondroitin sulfate, glycophorin A, and BSA, had no effect on rotavirus infectivity when incubated with cells before virus infection (Fig. 3).

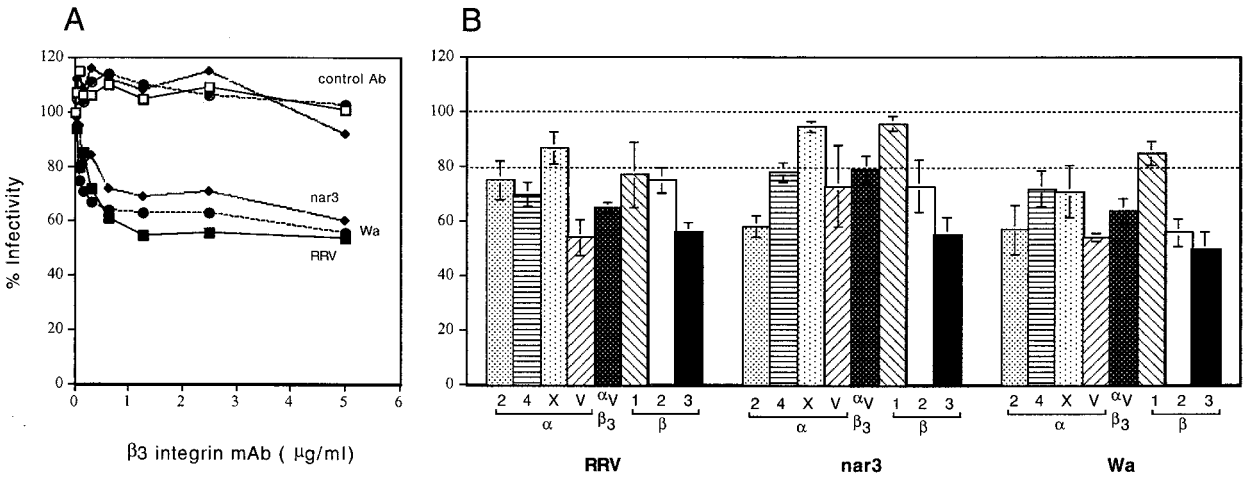

Fig. 1. Rotavirus infectivity is inhibited by antibodies to $\alpha_v\beta_3$ integrin. Antibodies to β_3 integrin (*A*) or to different integrin subunits (*B*) were added to monolayers of MA104 cells for 90 min at 37°C. After incubation with antibody, the cells were washed twice with MEM, and then RRV, nar3, or Wa viruses were adsorbed for 45 min at 4°C, the viral inoculum was removed, and the cultures were maintained for 14 h at 37°C. Cells were then fixed and immunostained. Data are expressed as percentage of the virus infectivity obtained when the cells were preincubated with MEM as control. The average numbers of foci representing 100% infectivity were 134, 122, and 139 in *A*, and 83, 98, and 86 in *B*, for RRV, nar3, and Wa, respectively. The bars represent the standard error of at least three independent experiments performed in duplicate. In *B*, the dotted lines at 80% and 100% infectivity are shown for reference. The antibody used in *A* was mAb 26. The antibodies used in *B* were as follows: polyclonal goat antibodies to α_4, α_x, α_v, and β_3 (20 μg/ml); mAb 4B7R to β_1 (20 μg/ml); mAb LM609 to $\alpha_v\beta_3$ (10 μg/ml); mAb P1E6 to α_2 (3.2 μg/ml); and MHM23 to β_2 (41 μg/ml).

Rotaviruses Interact with a Region of $\alpha_v\beta_3$ Different from Its RGD-Binding Site. Typically, $\alpha_v\beta_3$ integrin recognizes its ligands through the tripeptide RGD (9); however, neither VP4 nor VP7 proteins of any of the tested rotavirus strains have this consensus sequence. To evaluate whether rotaviruses were interacting with this integrin by an RGD sequence formed in the three-dimensional structure of the viral proteins, or by an RGD-independent binding site, an RGD peptide was used to block viral infectivity. Incubation of the cells with this peptide inhibited infectivity of all three rotavirus strains by 20%, as compared with 70% inhibition caused by vitronectin (Fig. 4). Incubation of the cells with RGD before the addition of vitronectin relieved the blocking capacity of this protein, indicating that RGD efficiently blocked the attachment of vitronectin to $\alpha_v\beta_3$. A control peptide, RGE, neither blocked rotavirus infectivity nor relieved the blocking effect of vitronectin. These results indicate that rotaviruses bind to $\alpha_v\beta_3$ through an alternative region, different from the RGD-binding site, and suggest that vitronectin might be blocking rotavirus infectivity through steric hindrance. The fact that the RGD peptide was able to block rotavirus infectivity at a low level suggests that the virus binds to $\alpha_v\beta_3$ through a site proximal to the RGD-recognition domain.

Rotaviruses Interact with $\alpha_v\beta_3$ at a Postattachment Step. To determine whether the interaction of rotaviruses with $\alpha_v\beta_3$ occurred during attachment or at a postattachment step, rotavirus binding inhibition experiments with vitronectin and antibodies to β_3 were carried out. Neither vitronectin nor antibodies to the β_3 subunit inhibited the binding of any of the rotavirus strains tested (Fig. 5*A*). These results suggest that $\alpha_v\beta_3$ is not used by rotaviruses for

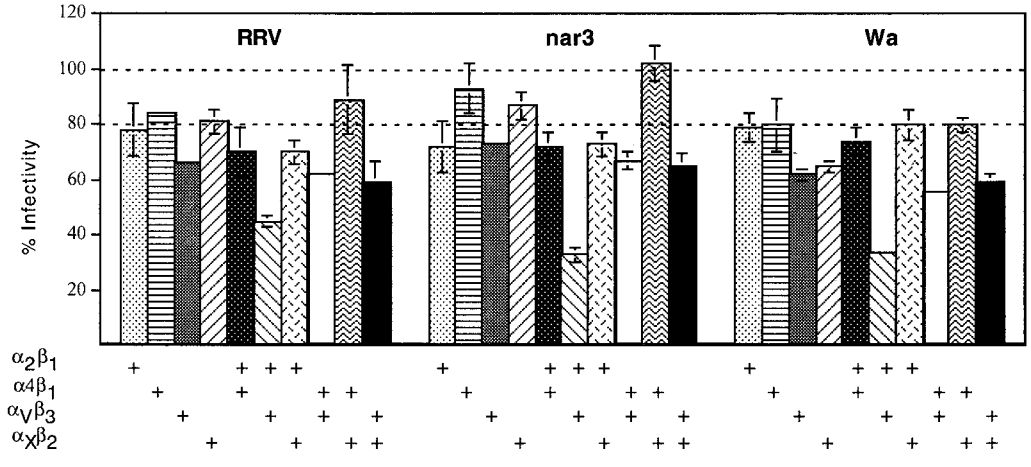

Fig. 2. Antibodies to $\alpha_v\beta_3$ and $\alpha_2\beta_1$ integrins inhibit rotavirus infectivity additively. Combinations of antibodies directed against different integrins were tested for their ability to block rotavirus infectivity in MA104 cells, as described in the legend for Fig. 1. Data are expressed as percentage of the virus infectivity obtained when the cells were preincubated with MEM as control. The average numbers of foci representing 100% infectivity were 128, 123, and 141 for RRV, nar3, and Wa, respectively. The bars represent the standard error of at least three independent experiments performed in duplicate. The dotted lines at 80% and 100% infectivity are shown for reference. The antibodies used were the same as described in the legend for Fig. 1, except for mAb LM609, which was not used.

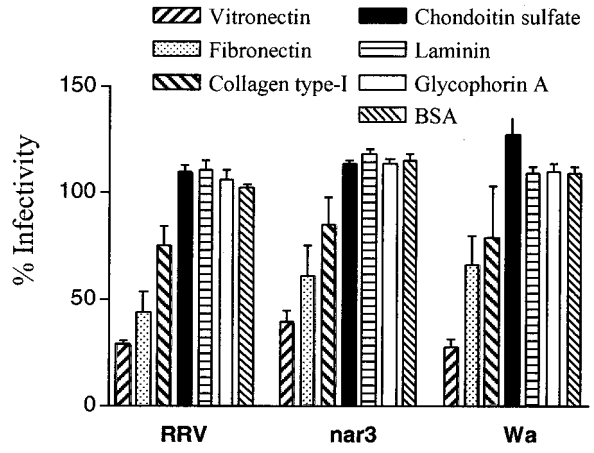

Fig. 3. Inhibition of rotavirus infectivity by integrin ligands. MA104 cells were incubated with either 0.5 μg/ml of vitronectin or 10 μg/ml of fibronectin, collagen type I, chondroitin sulfate, laminin, glycophorin A, or BSA for 60 min at 37°C, washed, and infected with rotaviruses as described in the legend for Fig. 1. Data are expressed as percentage of the virus infectivity obtained when the cells were preincubated with MEM as control. The average numbers of foci representing 100% infectivity were 128, 123, and 141 for RRV, nar3, and Wa, respectively. The bars represent the standard error of at least three independent experiments performed in duplicate.

their initial attachment to the cell surface. In addition, if vitronectin or the anti-β_3 antibody was added to the cells after the viruses had been adsorbed at 4°C, infectivity inhibition still occurred (Fig. 5*B*). Of interest, the inhibitory effect of the antibody was greater when added after adsorption of the virus than when added before the virus. On the other hand, if the virus was adsorbed for 60 min at 37°C (a temperature that allows the internalization of the virus into the cell) before vitronectin or the anti-β_3 antibody was added, no inhibitory effect was observed (not shown).

Recombinant β_3 Integrin Promotes Rotavirus Infection of CHO Cells. CHO cells, which are about 1000-fold less susceptible to viral infection than MA104 cells, and the stable transfected CHO variant cell lines VNRC and A5, which express the $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ integrins, respectively (26), were used to determine whether β_3 integrin expression facilitates rotavirus infectivity. Both VNRC and A5 cells were 3 to 4 times more susceptible to rotavirus infection than parental CHO cells. This increase in infectivity was shown to be blocked by incubation with either an antibody to β_3 or vitronectin (shown in Fig. 6*A* for VNRC cells), indicating that the augmented infectivity observed in these cells is due to the expression of β_3 integrin. The level of $\alpha_v\beta_3$ cell surface expression in VNRC cells as compared with parental CHO and MA104 cells is shown in Fig. 6*B*.

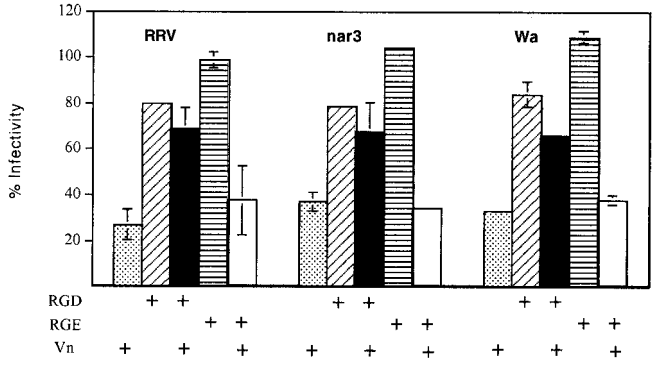

Fig. 4. Rotaviruses attach to a site on $\alpha_v\beta_3$ different from the integrin RGD-recognition domain. MA104 cells were incubated with MEM or peptides GRGDSP (RGD) or GRGESP (RGE) (400 μg/ml) for 60 min at 37°C. The cells were washed and vitronectin (Vn, 1.5 μg/ml) was subsequently added to control (MEM) or peptide-incubated cells for 60 min at 37°C. The cells were then washed and infected with rotaviruses as described in the legend for Fig. 1. Data are expressed as percentage of the virus infectivity obtained when the cells were preincubated with MEM as control. The average numbers of foci representing 100% infectivity were 109, 161, and 114 for RRV, nar3, and Wa, respectively. The bars represent the standard error of at least two independent experiments performed in duplicate.

Discussion

Comparative characterization of three rotavirus strains, the SA-dependent simian rotavirus RRV, its NA-resistant variant nar3, and the HRV strain Wa, which is naturally resistant to NA treatment of cells, has been used to understand the early events of rotavirus infection. At least three cell surface sites seem to be involved in the rotavirus–MA104 cell interaction during the early steps of infection with these three viruses (8, 32, 33). In the present study, $\alpha_v\beta_3$ integrin has been identified as a postbinding receptor for rotavirus in these cells.

Rotavirus binding to $\alpha_v\beta_3$ has been shown to be RGD-independent, consistent with the fact that neither of the surface proteins, VP4 or VP7, nor the protein that forms the intermediate layer of the virus, VP6, has an RGD sequence motif. Hantavirus cell entry has also been shown to be mediated by β_3 integrins (22). Like that of rotaviruses, their entry is not blocked by RGD peptides and is still mediated by $\alpha_{IIb}\beta_3$-integrin mutants defective in ligand binding, indicating that their interaction with β_3 is independent of the integrin binding to physiologic ligands. The binding of $\alpha_v\beta_3$ to sequences other than RGD is not without precedent, since such an interaction has been reported for other proteins, including matrix metalloproteinase 2, basic fibroblast growth factor, and ADAM 23/MDC3 human disintegrin (34).

Rotavirus nar3 binds to MA104 cells through the VP5 domain of VP4 (31), and more recently we have found that this attachment is mediated by $\alpha_2\beta_1$ integrin (32). We also found that RRV interacts with this integrin after initially binding to a SA-containing compound through the VP8 domain of VP4 (32). The fact that antibodies to $\alpha_2\beta_1$ and $\alpha_v\beta_3$ blocked rotavirus infection in an additive manner suggests that these integrins play a role at different stages of virus entry, an observation consistent with the idea that regardless of the primary cell molecule recognized by these three rotavirus strains, they all engage in a postattachment interaction with integrin $\alpha_v\beta_3$. It is of interest to note that rotaviruses bind to essentially every cell line that has been tested, although they efficiently infect only cells of intestinal or renal origin (3). Thus, the interaction with $\alpha_v\beta_3$ could be responsible, at least in part, for the restricted cell range of these viruses.

By flow cytometry $\alpha_v\beta_3$ integrin has been found in MA104, COS7, and Caco-2 cells (Fig. 6*B* and ref. 35), which are all susceptible to rotavirus infection. On the other hand, this integrin was not detected, or was barely detected, in CHO, BHK, and K562 cells (Fig. 6*B* and ref. 36), which are 100- to 1000-fold less susceptible to infection by these viruses. Thus, there seems to be a correlation between the susceptibility to rotavirus infection and the presence of $\alpha_v\beta_3$ integrin. Analysis of a larger panel of permissive and semipermissive cell lines is, however, needed to confirm this observation.

Rotavirus infection was initially reported to be mediated by integrins by Coulson's group (23, 24). They demonstrated that rotavirus SA11 binding to the human myelogenous leukemic cell line K562 increases with integrins $\alpha_2\beta_1$ and $\alpha_4\beta_1$ expression via transfection (24), and that the binding increase resulted in an augmented infection of the transfected cells. It was also shown that treatment of K562 cells with the phorbol ester phorbol 12-myristate 13-acetate (PMA) significantly increases cell infec-

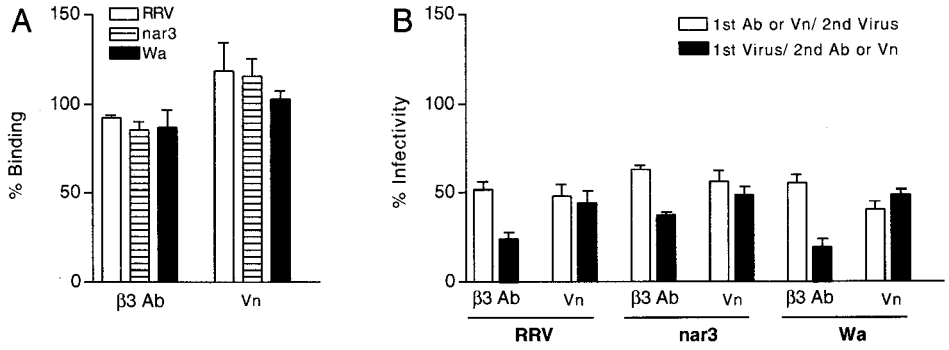

Fig. 5. Rotaviruses interact with $\alpha_v\beta_3$ at a postattachment step. (*A*) MA104 cells in suspension were incubated with a goat polyclonal antibody (20 μg/ml) against β_3 integrin, or with vitronectin (Vn, 1.5 μg/ml) for 1 h at 4°C. The cells were washed once with PBS and subsequently mixed with purified RRV, nar3, or Wa rotaviruses for 1 h at 4°C. After washing, the cells were lysed and the attached viruses were quantified by an ELISA (31). (*B*) MA104 cells were incubated in 96-well plates with rotaviruses for 1 h at 4°C, and then either β_3 antibodies or vitronectin (at the same concentrations used in *A*), were added for 1 h at 4°C (1st virus/2nd Ab or Vn). In the control experiment (1st Ab or Vn/2nd virus), vitronectin and antibodies were added before the viruses, using the same incubation conditions. The cells were then further incubated for 14 h at 37°C and immunostained for the virus. Data are expressed as percentage of the virus binding, or infectivity, when the cells were preincubated with MEM as control. The average numbers of foci representing 100% infectivity were 130, 99, and 114 for RRV, nar3, and Wa, respectively. The bars represent the standard error of at least two independent experiments performed in duplicate.

tion, without increasing virus binding levels, leading to the conclusion that the induction of the endogenous $\alpha_2\beta_1$ gene expression was the most likely basis for the augmented infectivity. It is known, however, that in addition to the induction of $\alpha_2\beta_1$, PMA also induces high expression levels of $\alpha_v\beta_3$ integrin in K562 cells (36). The fact that Hewish *et al.* (24) did not detect an enhancement in SA11 virus binding to PMA-treated cells suggests that the infectivity increase was the result of a postbinding interaction. Thus, it is likely that at least a fraction of the infectivity increase in PMA-treated K562 cells could have been the result of the induced $\alpha_v\beta_3$ expression. Because CHO cells do not express $\alpha_2\beta_1$ on their surface (unpublished observation), the rotavirus infectivity enhancement observed in the VNRC and A5 β_3-expressing cells is likely to be due only to the enhanced expression of this integrin.

Integrin subunit β_3, as well as $\alpha_2\beta_1$ integrin, has been reported to be present in murine and human enterocytes, primarily associated with the basolateral cell surface (37–39). Thus, if these integrins are involved in facilitating rotavirus cell entry in a natural infection, they might be initially available for virus interaction in limited amounts, unless the cell–cell contact regions are disrupted to expose their basolateral surface. In this regard, in a recent study rotaviruses were shown to induce structural and functional alterations in tight junctions of polarized intestinal Caco-2 cell monolayers (40). In addition, in polarized MDCK cells, $\alpha_2\beta_1$ integrin is exposed apically to the tight junctions (41). Finally, it is also relevant that enteroviruses such as coxsackievirus A9 and echovirus 9 can employ $\alpha_v\beta_3$ integrin as cellular receptor (14, 16), and that adenovirus-mediated gene delivery to the intestinal epithelium is dependent on, or is significantly increased by, the presence of $\alpha_v\beta_3$, among other integrins (35, 38).

Rotavirus cell infection involves a delicate cell and tissue tropism which may require the specific presence of several cell molecules organized in a precise manner. This idea is consistent with the fact that although expression of $\alpha_v\beta_3$ integrin in CHO cells (present study) or $\alpha_2\beta_1$ integrin in K562 cells (24) enhances the susceptibility of these cells to rotavirus infection, the level of permissiveness achieved does not attain that of MA104 cells, indicating that other cell surface molecules important for rotavirus entry are absent from the former cell lines. It remains to be established, among other things, whether any of the receptor molecules described so far are irreplaceable, and if, in fact, there exists a unique infectivity pathway for rotaviruses, with distinct entry points for different virus strains.

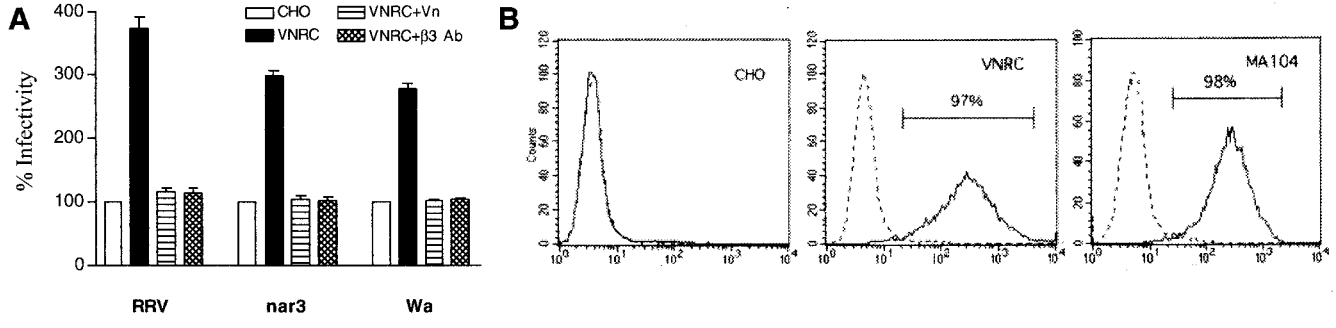

Fig. 6. (*A*) Recombinant β_3 integrin promotes CHO cell infection. Parental CHO cells (CHO) or stably transfected CHO cells expressing the α_v and β_3 integrin subunit genes (VNRC) (26) were infected in 96-well plates with rotaviruses. VNRC cells were also infected after they had been preincubated with either vitronectin (1.5 μg/ml; VNRC+Vn) or β_3 goat polyclonal antibodies (20 μg/ml; VNRC+β_3 Ab). Data are expressed as percentage of the virus infectivity obtained when the cells were preincubated with MEM as control. The average numbers of foci representing 100% infectivity were 72, 157, and 43 for RRV, nar3, and Wa, respectively. CHO cells are about 1000-fold less infectable than MA104 cells by the three viruses tested, and therefore dilutions of virus stocks were adjusted accordingly, to count the above-indicated number of infected cells in control wells. The bars represent the standard error of at least four independent experiments performed in duplicate. (*B*) Flow cytometric analysis of $\alpha_v\beta_3$ integrin surface expression in parental CHO, VNRC, and MA104 cells. mAb LM609 (solid lines), which recognizes the $\alpha_v\beta_3$ heterodimer, and the isotype IgG1 control antibody (dashed lines) were used at 5 μg/ml.

We are grateful to Rafaela Espinosa and Pedro Romero for their excellent technical assistance, and to the computer staff, specially to Alma Martínez Valle, for their technical support. This work was partially supported by Grants 75197-527106 from the Howard Hughes Medical Institute, G0012-N9607 from the National Council for Science and Technology–Mexico, and IN201399 from the Dirección General de Apoyo al Personal Académico, Universidad Nacional Autónoma de México.

1. Estes, M. K. (1996) in *Virology*, eds. Fields, B. N., Knipe, D. M. & Howley, P. M. (Raven, New York), Vol. 2, pp. 1625–1655.
2. Kapikian, A. Z. & Chanock, R. M. (1996) in *Virology*, eds. Fields, B. N., Knipe, D. M. & Howley, P. M. (Raven, New York), Vol. 2, pp. 1657–1708.
3. Estes, M. K. & Cohen, J. (1989) *Microbiol. Rev.* **53,** 410–449.
4. Ludert, J. E., Feng, N., Yu, J. H., Broome, R. L., Hoshino, Y. & Greenberg, H. B. (1996) *J. Virol.* **70,** 487–493.
5. Méndez, E., Arias, C. F. & López, S. (1993) *J. Virol.* **67,** 5253–5259.
6. Ciarlet, M. & Estes, M. K. (1999) *J. Gen. Virol.* **80,** 943–948.
7. Fukudome, K., Yoshie, O. & Konno, T. (1989) *Virology* **172,** 196–205.
8. Méndez, E., López, S., Cuadras, M. A., Romero, P. & Arias, C. F. (1999) *Virology* **263,** 450–459.
9. Hynes, R. O. (1992) *Cell* **69,** 11–25.
10. Juliano, R. L. & Haskill, S. (1993) *J. Cell Biol.* **120,** 577–585.
11. Ruoslahti, E. & Reed, J. C. (1994) *Cell* **77,** 477–478.
12. Bergelson, J. M., Shepley, M. P., Chan, B. M. C., Hemler, M. E. & Finberg, R. W. (1992) *Science* **255,** 1718–1720.
13. Bergelson, J. M., St. John, N., Kawaguchi, S., Chan, M., Stubdal, H., Modlin, J. & Finberg, R. W. (1993) *J. Virol.* **67,** 6847–6852.
14. Nelsen-Salz, B., Eggers, H. J. & Zimmermann, H. (1999) *J. Gen. Virol.* **80,** 2311–2313.
15. Pulli, T., Koivunen, E. & Hyypia, T. (1997) *J. Biol. Chem.* **272,** 21176–21180.
16. Roivainen, M., Piirainen, L., Hovi, T., Virtanen, I., Riikonen, T., Heino, J. & Hyypia, T. (1994) *Virology* **203,** 357–365.
17. Jackson, T., Sharma, A., Ghazaleh, R. A., Blakemore, W. E., Ellard, F. M., Simmons, D. L., Newman, J. W., Stuart, D. I. & King, A. M. (1997) *J. Virol.* **71,** 8357–8361.
18. Jackson, T., Sheppard, D., Denyer, M., Blakemore, W. & King, A. M. (2000) *J. Virol.* **74,** 4949–4956.
19. Evander, M., Frazer, I. H., Payne, E., Qi, Y. M., Hengst, K. & McMillan, N. A. (1997) *J. Virol.* **71,** 2449–2456.
20. Wickham, T. J., Mathias, P., Cheresh, D. A. & Nemerow, G. R. (1993) *Cell* **73,** 309–319.
21. Summerford, C., Bartlett, J. & Samulski, R. J. (1999) *Nat. Biotechnol.* **5,** 78–82.
22. Gavrilovskaya, I. N., Shepley, M., Shaw, R., Ginsberg, M. H. & Mackow, E. R. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 7074–7079.
23. Coulson, B. S., Londrigan, S. L. & Lee, D. J. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 5389–5394.
24. Hewish, M. J., Takada, Y. & Coulson, B. S. (2000) *J. Virol.* **74,** 228–236.
25. Guerrero, C. A., Zárate, S., Corkidi, G., López, S. & Arias, C. F. (2000) *J. Virol.* **74,** 9362–9371.
26. Díaz-González, F., Forsyth, J., Steiner, B. & Ginsberg, M. H. (1996) *Mol. Biol. Cell.* **7,** 1939–1951.
27. Cuadras, M. A., Arias, C. F. & López, S. (1997) *J. Virol.* **71,** 9065–9074.
28. Yatohgo, T., Izumi, M., Kashiwagi, H. & Hayashi, M. (1988) *Cell Struct. Funct.* **13,** 281–292.
29. Lizano, M., López, S. & Arias, C. F. (1991) *J. Virol.* **65,** 1383–1391.
30. Hu, D. D., Barbas, C. F. & Smith, J. W. (1996) *J. Biol. Chem.* **271,** 21745–21751.
31. Zárate, S., Espinosa, R., Romero, P., Méndez, E., Arias, C. F. & López, S. (2000) *J. Virol.* **74,** 593–599.
32. Zárate, S., Espinosa, R., Romero, P., Guerrero, C. A., Arias, C. F. & López, S. (2000) *Virology* **277,** in press.
33. López, S., Espinosa, R., Isa, P., Merchant, M. T., Zárate, S., Méndez, E. & Arias, C. F. (2000) *Virology* **273,** 160–168.
34. Cal, S., Freije, J. M., López, J. M., Takada, Y. & López-Otín, C. (2000) *Mol. Biol. Cell.* **11,** 1457–1469.
35. Croyle, M. A., Walter, E., Janich, S., Roessler, B. J. & Amidon, G. L. (1998) *Hum. Gene Ther.* **9,** 561–573.
36. Burger, S. R., Zutter, M. M., Sturgill-Koszycki, S. & Santoro, S. A. (1992) *Exp. Cell Res.* **202,** 28–35.
37. Beaulieu, J. F. (1992) *J. Cell Sci.* **102,** 427–436.
38. Hamilton, T. E., McClane, S. J., Baldwin, S., Burke, C., Patel, H., Rombeau, J. L. & Raper, S. E. (1997) *J. Pediatr. Surg.* **32,** 1695–1703.
39. Martín-Villa, J. M., Ferre-López, S., López-Suárez, J. C., Corell, A., Pérez-Blas, M. & Arnaiz-Villena, A. (1997) *Tissue Antigens* **50,** 586–592.
40. Obert, G., Pfeiffer, I. & Aervin, A. L. (2000) *J. Virol.* **74,** 4645–4651.
41. Tafazoli, F., Holmström, A., Forsberg, A. & Magnusson, K. E. (2000) *Infect. Immun.* **68,** 5335–5343.