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# The Ability of Integrin $\alpha_v\beta_3$ To Function as a Receptor for Foot-and-Mouth Disease Virus Is Not Dependent on the Presence of Complete Subunit Cytoplasmic Domains

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**The integrin $\alpha_v\beta_3$ has been shown to function as one of the integrin receptors on cultured cells for foot-and-mouth disease virus (FMDV), and high-efficiency utilization of the bovine homolog of this integrin is dependent on the cysteine-rich repeat region of the bovine $\beta_3$ subunit. In this study we have examined the role of the cytoplasmic domains of the $\alpha_v$ and $\beta_3$ subunits in FMDV infection. We have found that truncations or extensions of these domains of either subunit, including deletions removing almost all of the cytoplasmic domains, had little or no effect on the ability of the integrin to function as a receptor for FMDV. The lysosomotropic agent monensin inhibited viral replication in cells transfected with either intact or cytoplasmic domain-truncated $\alpha_v\beta_3$. In addition, viral replication in transfected cells was inhibited by an $\alpha_v\beta_3$ function-blocking antibody but not by function-blocking antibodies to three other RGD-directed integrins, suggesting that these integrins are not involved in the infectious process. These results indicate that alterations to the cytoplasmic domains of either subunit, which lead to the inability of the integrin receptor to function normally, do not abolish the ability of the integrin to bind and internalize this viral ligand.**

Integrins are heterodimeric cell surface receptors consisting of α and β subunits that are involved in binding extracellular matrix proteins, cell-cell interactions, and signal transduction (20, 23). The two subunits interact noncovalently at the cell surface to bind their natural ligands via a ligand-binding region which is made up of elements of both subunits (16). Integrin subunits are type I membrane proteins consisting of a large N-terminal extracellular domain and smaller transmembrane and cytoplasmic domains. The cytoplasmic domains of the α and β integrin subunits, specifically certain sequence motifs within those domains, have been shown to be involved in inside-out and outside-in signal transduction, integrin activation, and conformational changes leading to alterations in ligand-binding affinities and connections to the cytoskeleton (6, 7, 18, 22, 27, 37, 38, 41, 44).

Foot-and-mouth disease virus (FMDV), an aphthovirus in the family *Picornaviridae*, utilizes the integrin $\alpha_v\beta_3$ as a receptor in cultured cells (4, 35, 36). We have recently molecularly cloned the bovine homolog of this integrin and shown that the high-efficiency utilization of the bovine integrin as a receptor for FMDV is dependent on sequences found within the cysteine-rich repeat region of the bovine $\beta_3$ subunit extracellular domain (35). As part of an ongoing study of the roles that the various functional domains within the integrin subunits play in FMDV infection, we have examined subunits with altered cytoplasmic domains for their ability to retain viral receptor function.

**Generation of bovine $\alpha_v$ and $\beta_3$ subunits with altered cytoplasmic domains.** We generated two truncation mutants and one extension mutant for each of the subunits, as shown in Fig. 1, utilizing the plasmids pBov$\alpha_v$ZEO and pBov$\beta_3$ZEO, which encode the bovine $\alpha_v$ and $\beta_3$ integrin subunits, respectively (35). pBov$\alpha_v$ZEO was used as the template for a 20-cycle PCR with the N-terminal PCR primer 5′GGAAGGTGCCTACGAAGCTGAG3′ and the following C-terminal primers: for $\alpha_v\Delta30$, 5′GGAATTCCTTACATCCTGTACATTACAA3′; for $\alpha_v\Delta20$, 5′GGAATTCCTTATTGAGGTGGCCGTACACG3′; and for $\alpha_v$X29, 5′GGAATTCCTTAGTTTCAGAGTTTCCTTCGCC3′. Plasmids encoding the altered $\alpha_v$ subunits were created utilizing *Bst*EII and *Eco*RI sites shared by pBov$\alpha_v$ZEO and the PCR products. pBov$\beta_3$ZEO was also used as the template for a 20-cycle PCR using the N-terminal PCR primer 5′CCACGCGTGGTGTGAGCTCCTG3′ and the following C-terminal primers: for $\beta_3\Delta39$, 5′CGGGATCCTTAGTCATGGATGGTGATGAG3′; for $\beta_3\Delta31$, 5′CGGGATCCTTAGGCTCTGGCTCTCTCTTC3′; and for $\beta_3$X32, 5′CGGGATCCTTAAGTGCCCCGGTACGTGATATTG3′. Plasmids encoding the altered $\beta_3$ subunits were created utilizing *Mlu*I and *Bam*HI sites shared by pBov$\beta_3$ZEO and the PCR products. All of the plasmid constructs were sequenced through the region that was subcloned.

The amino acid sequences of the cytoplasmic domains of the wild-type and altered subunits are shown in Fig. 1. There is some question as to where the exact borders between the transmembrane and cytoplasmic domains occur in the integrin subunits. We have marked the border as lying at the YR junction for the $\alpha_v$ subunit (Fig. 1a) and the WK junction for the $\beta_3$ subunit (Fig. 1b). A recent study suggests that the first five residues of the $\alpha_v$ subunit (RMGFF) and the first six residues of the $\beta_3$ subunit (KLLITI) cytoplasmic domains, as we have represented them, are located within the cell membrane in the absence of interactions with intracellular proteins and become exposed to the cytoplasm upon binding to intra-

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FIG. 1. Predicted amino acid sequences of wild-type and altered integrin subunit cytoplasmic domains. The sequences of the cytoplasmic domains are in uppercase letters. The last four residues of the transmembrane domains are in lowercase letters. Functionally important sequence motifs, as noted in the text, are italicized and underlined.

cellular proteins, resulting in conformational changes leading to integrin activation and signaling (1). The mutant $\alpha_v$ subunits are shown in Fig. 1a. The first, $\alpha_v\Delta30$, retains only two cytoplasmic domain amino acids and is truncated before the GFFKR motif, which is conserved among all α subunits. This motif maintains integrins in a low-affinity state (8, 37, 49) and has been reported to be necessary for stabilization of the integrin αβ complex (48). This mutant is also lacking the PPQ(L)EE(DD) motif, which defines a β-turn in the α subunit cytoplasmic domain and is found in seven other α subunits. A human $\alpha_v\beta_3$ heterodimer with a truncated α subunit cytoplasmic domain lacking this motif cannot bind to either vitronectin or fibronectin (18). The second mutant, $\alpha_v\Delta20$, contains the GFFKR motif and the five amino acids downstream of it but is truncated at the last residue of the PPQEE motif. The final mutant, $\alpha_vX29$, contains the complete cytoplasmic domain of the $\alpha_v$ subunit with an additional 29 amino acids added to the C terminus.

The mutant $\beta_3$ subunits are shown in Fig. 1b. The first of these, $\beta_3\Delta39$, retains only 8 amino acids while $\beta_3\Delta31$ retains 16 amino acids of the $\beta_3$ cytoplasmic domain. Neither of these two constructs contain the NPL(X)Y or the NI(X)T(X)Y motifs that have been shown to be important for signal transduction, integrin affinity states, and interaction with cytoplasmic integrin-associated proteins (7, 9, 10, 13, 15, 17, 25, 28, 31, 32, 44, 46, 54). Both of these truncations, however, still retain the membrane-proximal region of the subunit cytoplasmic domain, which has also been reported to control ligand-binding affinity and to regulate signal transduction (21, 22). The third $\beta_3$ subunit mutant, $\beta_3X32$, retains both the NPLY and NITY motifs along with an additional 32 amino acids added to the C terminus of the cytoplasmic domain. Additions to the cytoplasmic domains of both the α and β subunits were made because the specific conformations of these domains appear to play a role in the way they control ligand affinity and signal transduction (21).

**Analysis of integrins with altered cytoplasmic domains.** Coupled in vitro transcription-translations were performed to check that the mutant-encoding plasmids that were generated were encoding proteins of the expected sizes. In all cases, the relative sizes of the resulting altered integrin subunits compared to the corresponding wild-type subunits were as expected (not shown).

To analyze whether integrin subunits with truncated or extended cytoplasmic domains could still function as receptors for FMDV, we utilized a previously described transient-expression assay system in COS-1 cells (35). Cells were plated at a density of $10^5$ cells/well on six-well plates the day prior to transfection. Transfections were performed with 2.0 μg of each integrin-encoding plasmid utilizing the transfection reagent FuGENE 6 (Roche Molecular Biochemicals) according to the manufacturer's instructions. Twenty-four hours after transfection, the cells in each well were trypsinized and replated onto two wells of a 24-well plate. After a further 18 h of incubation, one well for each transfected condition was infected with FMDV type $A_{12}$, strain 119ab, at a multiplicity of infection (MOI) of 10 PFU/cell and labeled between 4 and 18 h after infection with [$^{35}$S]methionine. The other well was fixed with acetone-methanol (50:50) and analyzed by immunohistochemistry for integrin expression using the anti-$\alpha_v\beta_3$ monoclonal antibody (MAb) LM609 (MAB1976; Chemicon International) as previously described (35). Only transfections in which equal amounts of immunostaining were detected in all experimental conditions were used to analyze the results of viral infection (35). To evaluate FMDV replication in the infected-radiolabeled cultures, cell lysates were prepared in 1% Triton X-100. Equal amounts of trichloroacetic acid-precipitable counts per minute were immunoprecipitated (IP), using a virus-specific MAb, and proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% polyacrylamide gel as described (35).

The results in Fig. 2 are representative of one such trans-

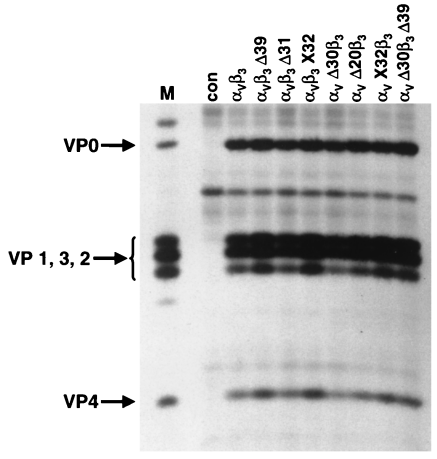


FIG. 2. Analysis of viral protein synthesis in COS-1 cells transfected with integrin subunit cDNAs. Cells were transfected with plasmids encoding integrin subunits as shown. Transfected cells were infected with FMDV type $A_{12}$ at an MOI of 10 PFU/cell and labeled between 4 and 18 h with [$^{35}$S]methionine. Cell extracts were analyzed by IP and SDS–10% PAGE as described in the text. The locations of viral structural proteins IP from infected and labeled BHK-21 cells (lane M) are shown on the left. con, nontransfected-infected cells (control).

fection. Viral replication is evident in cells transfected with both wild-type bovine $\alpha_v$- and $\beta_3$-expressing plasmids, as evidenced by the synthesis of viral proteins which are not present in nontransfected-infected cells. When cells are transfected with a wild-type $\alpha_v$ subunit and any of the altered $\beta_3$ subunits, viral replication appears to be unaffected. Similarly, when any of the three altered $\alpha_v$ subunits are transfected with the wild-type $\beta_3$ subunit, levels of infection reach those seen when both wild-type subunits are present. Viral replication was also unaffected when the two subunits with the shortest cytoplasmic domains ($\alpha_v\Delta30$ and $\beta_3\Delta39$) were expressed together.

**Viral replication mediated by integrins with truncated cytoplasmic domains in the presence of monensin.** We have previously shown that the lysosomotropic ionophore monensin inhibited the replication of representative strains of all seven serotypes of FMDV (2). Monensin interferes with proton and pH gradients and raises the pH of endocytic vesicles (33, 40). In the presence of monensin, the virus adsorbed to cells normally; however, it was unable to undergo the initial alteration of the 140S virion to 12S pentameric subunits, which probably occurs within acidified endocytic vesicles and results in the release of the genome RNA (2, 3). To examine whether virus utilizing expressed integrins with truncated cytoplasmic domains as receptors infected cells through an eclipse mechanism similar to that of virus utilizing intact receptors, we transfected cells with intact and cytoplasmic domain-truncated integrin subunits, followed by infection in the presence or absence of monensin.

Cells were cotransfected with either $\alpha_v$ and $\beta_3$ subunits or $\alpha_v\Delta30$ and $\beta_3\Delta39$ subunits. Forty-eight hours after transfection, cultures were incubated in the presence or absence of 50 μM monensin for 30 min prior to infection with FMDV type $A_{12}$. Viral replication was determined by pulse-labeling cells with [$^{35}$S]methionine between 5 and 6 h postinfection and analyzing cell extracts by IP and SDS-PAGE. The results are shown in Fig. 3. Cells cotransfected with intact integrin subunits and infected in the presence of monensin failed to synthesize viral proteins, indicating that the drug interfered with viral replication. Interestingly, cells cotransfected with cytoplasmic domain-truncated integrin subunits and infected in the presence of monensin also failed to synthesize viral proteins. In contrast, cells cotransfected with either intact or truncated integrins synthesized normal amounts of viral proteins when monensin was added at 2 h after infection, indicating that monensin inhibited an early event in viral replication, probably at the eclipse phase, as we have shown previously (2). Therefore, complete integrin subunit cytoplasmic domains are not necessary for internalization of virions into endocytic vesicles.

Thus, bovine $\alpha_v\beta_3$ is able to function as a receptor for FMDV in the absence of motifs that are known to be required for the normal function of integrins in the context of their natural ligands. The truncations of the cytoplasmic domains of either the $\alpha_v$ or $\beta_3$ subunits and the addition of random amino acids to the subunits' cytoplasmic domains did not affect the ability of integrins to serve as receptors for FMDV, and the results show that when utilized as receptors, integrins altered in this manner were utilized as well as intact integrins.

**Effect of integrin function-blocking antibodies on viral infection in transfected cells.** The internalization of natural integrin ligands has not been extensively studied. The NPXY motif, found in the cytoplasmic domains of all β subunits and

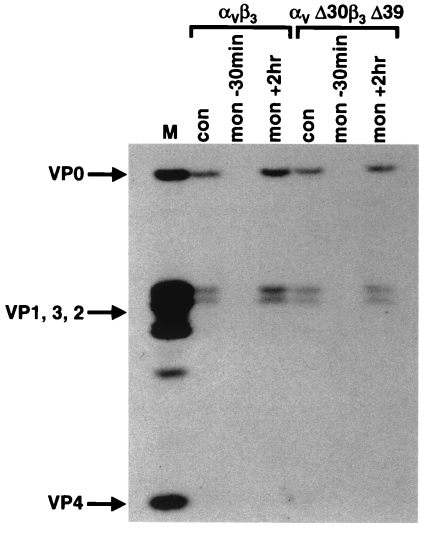


FIG. 3. Analysis of transfected COS-1 cells infected in the presence of monensin. Cells were cotransfected with integrin subunits containing intact cytoplasmic domains or $\alpha_v\Delta30$ and $\beta_3\Delta39$ as shown. Thirty minutes prior to infection, the medium was removed from some wells and replaced with medium containing 50 μM monensin (lanes mon −30min). Cells were infected in either the presence or absence (con) of monensin as noted. At 2 h after infection, the medium was removed from other wells and replaced with medium containing 50 μM monensin (lanes mon +2hr). At 4.5 h after infection, all cultures were incubated in minimal essential medium without L-methionine, with or without monensin, for 30 min. [$^{35}$S]methionine (75 μCi) was added, and cells were labeled for 1 h. Cell extracts were prepared, and analysis of viral proteins by IP and SDS–10% PAGE was done as described in the text. The locations of marker FMDV structural proteins (lane M) are shown on the left.

other transmembrane receptors, has been reported to be required for internalization of bacteria mediated by $\beta_1$ integrins (47), internalization mediated by the nonintegrin low-density lipoprotein receptor (12), and a signal for clathrin complex assembly (11). More recent results have shown that sequences surrounding the NPXY motif are important in association of the $\alpha_v\beta_5$ receptor with clathrin-coated pits via the $\beta_5$ cytoplasmic domain (13). The internalization of vitronectin by $\alpha_v\beta_3$, however, appears to require a signal as a result of the ligation of the $\alpha_5\beta_1$ integrin (39). This cross talk between the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins requires the $\beta_3$ cytoplasmic domain (5). Our results would rule out a cross talk mechanism of viral internalization, since important cytoplasmic domain motifs have been deleted from some of the subunit constructs. The results, however, might indicate that other cell surface molecules are acting as coreceptors for viral infection or that the expression of $\alpha_v\beta_3$ in COS cells is activating another receptor and the integrin itself is not involved in viral binding or internalization. Although, at this time, we have no evidence that a coreceptor, either integrin or nonintegrin, is required for infection, we examined the possibility that other RGD-directed integrins might be involved in the infectious process in $\alpha_v\beta_3$-transfected COS cells.

Cells were transfected with $\alpha_v\beta_3$ cDNAs, as described, and 30 min prior to infection with FMDV type $A_{12}$, they were incubated with function-blocking antibodies to $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_5$, or $\beta_1$. All of these integrins interact with their natural ligands through an RGD sequence (42), and the $\alpha_v\beta_6$ integrin has recently been shown to be a receptor for FMDV (24), a result we have confirmed in our laboratory (not shown). In this experiment, we measured productive viral replication by determining the plaque titer of infectious virus immediately following a 45-min adsorption period and after 24 h of incubation. As a control, to determine the level of FMDV replication in COS cell cultures, a nontransfected culture was infected with an FMDV type $O_1$ Campos variant containing a heparin-binding site in VP3 (43), which we have previously shown to require only the presence of cell surface heparan sulfate (HS), and not $\alpha_v\beta_3$, to infect cells (36). The results of this experiment are shown in Fig. 4.

In nontransfected COS cells, there was no increase in titer of type $A_{12}$ at 24 h, indicating that viral replication did not take place in these cells, as we have previously shown in experiments utilizing detection of radioactively labeled viral proteins (35) (Fig. 2). In contrast, the heparin-binding type $O_1$ variant showed an increase in titer of about 10-fold after 24 h in nontransfected cells, also confirming previously reported results (36). In $\alpha_v\beta_3$-transfected COS cells, the type $A_{12}$ virus titer increased about 10-fold, again confirming results obtained by detection of viral proteins in infected cells (35) (Fig. 2). When transfected cells were treated with integrin function-blocking antibodies, only the antibody to $\alpha_v\beta_3$ and not those against any of the other integrins was capable of inhibiting viral replication (Fig. 4). The same experiment performed in cells transfected with the $\alpha_v\Delta30$ and $\beta_3\Delta39$ cDNAs yielded nearly identical results (not shown). These results indicate that, in this transfection system, the $\alpha_v\beta_3$ integrin is absolutely required for productive viral infection and at least three other RGD-directed integrins ($\alpha_v\beta_5$, $\alpha_v\beta_6$, and $\alpha_5\beta_1$) do not appear to be involved in this process. In addition, since the anti-$\beta_1$ antibody has been shown to inhibit the function of at least two other $\beta_1$ integrins ($\alpha_2\beta_1$ and $\alpha_4\beta_1$) (19), other integrins of this subclass are also probably not involved in viral infection. At this time, however, we cannot rule out a role for other cell surface molecules as coreceptors for either FMDV adsorption or internalization. We can, however, rule out HS as a coreceptor for type $A_{12}$, as we have previously shown that this virus can replicate in $\alpha_v\beta_3$ cDNA-transfected HS-deficient CHO cells (36).

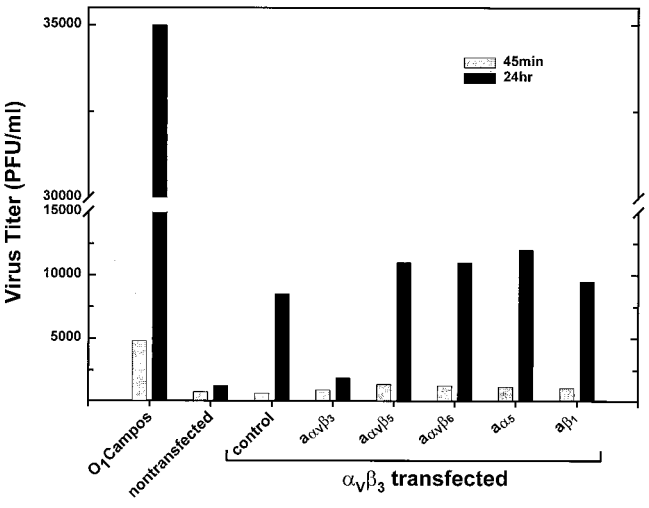


FIG. 4. Effect of anti-integrin function-blocking MAbs on viral replication in transfected COS-1 cells. Cells were cotransfected with $\alpha_v\beta_3$-encoding plasmids as described in the text. Thirty minutes prior to infection, paired transfected cell cultures were incubated at room temperature with the following function-blocking anti-integrin MAbs at a concentration of 25 μg/ml (all antibodies were from Chemicon International Inc): anti-$\alpha_v\beta_3$ (clone LM609; MAB1976), anti-$\alpha_v\beta_5$ (clone P1F6; MAB1961), anti-$\alpha_v\beta_6$ (clone 10D5; MAB2077Z), anti-$\alpha_5$ (clone CLB-705; MAB1986), and anti-$\beta_1$ (clone 6S6; MAB2253). Transfected and nontransfected cultures were infected in pairs with type $A_{12}$ at an MOI of 1 PFU/cell in the presence of the antibodies. One nontransfected culture pair was infected with the HS-binding $O_1$ Campos variant vCRM4 (43) at an MOI of 1 PFU/cell. After an adsorption period of 45 min at 37°C, all cultures were washed with a low-pH buffer (25 mM MES [morpholineethanesulfonic acid, pH 5.5], 140 mM NaCl) to inactivate any nonadsorbed or noninternalized virus. After the addition of medium, one of the infected pairs was immediately frozen at −70°C to determine viral infectivity remaining at the end of the adsorption period (shaded bars). The other pair was incubated for 24 h at 37°C (solid bars) and then placed at −70°C. After thawing, cell debris was removed by centrifugation, and plaque titer was determined on BHK-21 cells.

These results are similar to those reported for three other picornaviruses (poliovirus, rhinovirus 14, and coxsackievirus B3), all of whose single-subunit receptors appear to function normally in the absence of cytoplasmic domains (26, 45, 52). Human adenovirus (Ad) requires interaction with the integrin $\alpha_v\beta_5$ or $\alpha_v\beta_3$ for internalization into cells through a clathrin-coated pit pathway requiring dynamin (51, 53). We have not yet examined the role of dynamin in FMDV internalization, but in studies with two other picornaviruses, human rhinovirus 14 required dynamin for productive infection while poliovirus did not (14). Recently it has been shown that Ad internalization requires signaling through the focal adhesion kinase pathway involving phosphoinositide-3-OH kinase and GTP-binding proteins, all of which are activated following binding of inte-

grins to their natural ligands (29, 30). In addition, the cytoplasmic domain of the $\beta_5$ subunit of the $\alpha_v\beta_5$ integrin is essential for Ad-mediated gene delivery via host cell membrane penetration from endosomes (50). However, truncations of the $\beta_5$ cytoplasmic domain, which still retains the NPXY motif, and do not allow Ad-mediated gene delivery do not abolish $\alpha_v\beta_5$-mediated Ad internalization (50). At least two picornavirus receptors, the coxsackievirus and adenovirus receptors, and ICAM-1 also do not require their transmembrane domains for receptor function (45, 52). Soluble human $\alpha_v\beta_3$ lacking both the transmembrane and cytoplasmic domains of both subunits can still bind to its natural ligands with high affinity (34); however, we have not examined either soluble or glycosylphosphatidylinositol-anchored $\alpha_v\beta_3$ for virus binding or the ability to act as a functional receptor.

The results we have presented, however, indicate that deletions of any of the important cytoplasmic domain motifs had little or no effect on receptor utilization by FMDV. In fact, the results showing that monensin still inhibited infection mediated by cytoplasmic domain-truncated $\alpha_v\beta_3$ suggest that these receptors are internalizing FMDV through the same mechanism as complete integrins. In addition, we have also shown that three other RGD-directed integrins do not appear to play any role in productive viral infection. Further studies will be necessary to delineate the exact mechanism by which intact and altered integrin subunits internalize virus.

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