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Arginine-Glycine-Aspartic Acid-Specific Binding by Foot-and-Mouth Disease Viruses to the Purified Integrin αvβ3 In Vitro

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The integrin αvβ3 has been shown to act as the receptor for internalization of foot-and-mouth disease virus (FMDV) (A12), with attachment being through a highly conserved RGD motif located on the G-H loop of viral capsid protein VP1. In addition, however, we have recently shown that efficient infection of culture-grown cells by FMDV (O1BFS) requires binding to cell surface heparan sulfate. In this study, we have used a solid-phase receptor binding assay to characterize the binding by FMDV to purified αvβ3 in the absence of heparan sulfate and other cell surface components. In this assay, FMDV (O1BFS) successfully replicated authentic ligand binding by cellular αvβ3 in terms of its high affinity, dependence on divalent cations, and activation by manganese ions. Virus binding to this preparation of αvβ3 was exquisitely sensitive to competition by short RGD-containing peptides (50% inhibition at $<10^{-8}$ M peptide), and this inhibition was highly sequence specific, with the equivalent RGE peptide being at least 10^4 fold less effective as a competitor. Representative viruses of the other six serotypes of FMDV bound to αvβ3 in a similar RGD-specific manner, although significant differences in sensitivity to RGD peptides suggest that the affinity of the different FMDV serotypes for αvβ3 is influenced, in part, by the variable amino acid residues in the VP1 G-H loop on either side of the RGD.

Foot-and-mouth disease virus (FMDV), the highly infectious agent of foot-and-mouth disease, is an economically important animal virus that infects cloven-hooved animals. The seven serotypes of FMDV (types O, A, C, Asia-1, and the South African Territories [SAT] types 1, 2, and 3) constitute the *Aphthovirus* genus of the family *Picornaviridae*. FMDV are small, nonenveloped, icosahedral viruses with a single-stranded, positive-sense RNA genome of approximately 8,400 nucleotides (3). The virus capsid is made up from 60 copies each of four virus-encoded proteins, VP1 to VP4; VP1 to VP3 form the outer capsid shell, and VP4 lines the interior surface (1). An unusual structural feature of the outer capsid surface is a long, conformationally flexible loop of VP1. This loop (the G-H loop) forms a major antigenic site on the virus and includes at its apex an Arg-Gly-Asp (RGD) motif (43, 46, 49).

FMDV enters cells by receptor-mediated endocytosis (9, 11), in a process that we have recently shown to be greatly enhanced by the initial attachment of the virus to cell surface heparan sulfate (22). Once the virus is inside an endosome, the low pH of this compartment triggers uncoating of the viral genome, which is then translocated across the endosomal membrane into the cytosol. On primate cells, the RGD-binding integrin αvβ3 has been shown to act as a receptor for the internalization of FMDV (A12) (6). Mutational studies with a cloned infectious cDNA of the same virus have shown that the interaction with the integrin is mediated by the G-H loop RGD and that this motif is essential for entry, since mutations in this sequence, including the conservative change to RGE, resulted in noninfectious virus particles that failed to enter cells (29). Recently, similar results were obtained with a cloned infectious cDNA of FMDV (O1K), since most mutations introduced into the RGD motif resulted in virus particles that were deficient only in their ability to enter cells (27). However, in contrast to strain A12, changing the RGD to RGE produced viable virus particles (27). Nevertheless, the G-H loop RGD is highly conserved among FMDV serotypes, indicating that they will enter cells by means of an RGD-binding integrin.

Integrins serve as cellular receptors for several other viruses including adenovirus type 2 (48), echoviruses 1 and 8, (4, 5), and coxsackievirus A9 (37), and they have been implicated in cell entry of echoviruses 22 and 23 (45) and human papillomaviruses (15). Integrins are a large family of heterodimeric, transmembrane glycoproteins containing two large chains, α and β (21). The family is composed of 15 α and 8 β subunits, which associate to form over 20 different αβ combinations (21). Integrins bind a diverse array of ligands including other cell surface adhesion receptors, serum proteins, and components of the extracellular matrix in mediating numerous processes involving cell-cell and cell-matrix adhesion. In fulfilling these roles, integrins act as bidirectional signalling receptors that transmit signals from both inside the cell outward and from outside in (12, 13, 40). The ligand-binding specificity is determined by the αβ combination, and many integrins are able to bind more than one ligand. Many integrins, including αvβ3, bind to their ligands by recognizing the tripeptide RGD (21). Although originally identified as a receptor for vitronectin, αvβ3 is now known to recognize the RGD tripeptide in up to nine different protein ligands (16, 31). Binding to these ligands is differentially regulated by divalent cations. Divalent cations are essential for integrin-ligand interactions (21, 32, 42). They regulate the affinity and specificity of ligand binding most probably by influencing the shape of the binding pocket (14, 26). Binding of ligands to αvβ3 is usually supported by Mg^{2+} and can be enhanced by Mn^{2+} (42). Ca^{2+} supports the binding of many ligands such as vitronectin and fibronectin but

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inhibits the binding of others like fibrinogen and osteopontin (20, 42).

Previous studies of FMDV binding to its integrin receptor have been complicated by the abundance of its heparan sulfate coreceptor on the surface of susceptible cells. This paper reports the study of the binding of FMDV to purified preparation of human integrin αvβ3 in an enzyme-linked immunosorbent assay. We show that viruses representative of all FMDV serotypes bind to αvβ3 in an RGD-dependent interaction. We also show that for type O FMDV, binding to the integrin occurs through a high-affinity interaction that can be inhibited by an RGD-containing peptide derived from the G-H loop of VP1.

MATERIALS AND METHODS

Protein purification. (i) Viruses. Purification on sucrose gradients of viruses from infected BHK cells has been described in detail previously (11).

(ii) ^{35}S labelling of FMDV (O1KB64). BHK cells were infected at a multiplicity of 10 pfu/cell for 30 min with FMDV (O1KB64) (17), washed twice with Eagle's methionine- and cysteine-free medium, and incubated in the same medium for a further 1.5 h with frequent changes. At this point, ^{35}S-Express (40 MBq; specific activity, >37.0 TBq/mmol [NEW Life Sciences]) was added, and the infection was continued until advanced signs of cytopathic effects were visible. The cells were chilled on ice for 20 min, Nonidet P-40 was added to a final concentration of 0.5%, and incubation was continued for a further 15 min. The lysate was centrifuged at 2,000 × *g* and 4°C for 10 min. The supernatant was adjusted to 1% Sarkosyl and 10 mM EDTA, incubated on ice for 15 min, and centrifuged on a 30% sucrose cushion at 100,000 × *g* and 12°C for 2.5 h. The labelled virus was purified on a sucrose gradient.

(iii) Integrin αvβ3. Integrin was purified from human placenta by affinity chromatography with monoclonal antibody 23C6 (41). Briefly, placenta was homogenized in 50 mM Tris (pH 7.5)–150 mM NaCl–1 mM $CaCl_2$–1 mM $MgCl_2$–1 mM $MnCl_2$–1% Triton X-100–1 mM phenylmethylsulfonyl fluoride–1 μg of each of leupeptin, pepstatin, and antipain per ml. The clarified lysate was applied to the antibody column. The column was washed with the same buffer, and the integrin was eluted with 30 mM β-octyl glycoside in 20 mM sodium acetate (pH 3.1) and immediately neutralized with 2 M Tris-HCl (pH 8.0). The purity of the integrin preparation was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and shown to be devoid of other integrin chains (α1 to α6 or β1 and β5) by enzyme-linked immunosorbent assay with a panel of specific antibodies (41).

Antibodies and peptides. The GRGDSP and GRGESP peptides were purchased from Life Technologies. The O1BFS VP1 G-H loop peptide (142-PNL RGDLQVLAQ-153) was synthesized on the peptide synthesis facility at the Oxford Centre for Molecular Science, New Chemistry Laboratory, Oxford, United Kingdom. The antibodies used in these studies were LM609 (anti-αvβ3) (obtained from David Cheresh, Scripps Research Institute, La Jolla, Calif.) and L230 (anti-αv) (obtained from John Marshall. Imperial Cancer Research Fund, St. Thomas' Hospital. London, United Kingdom).

Solid-phase binding assay. Plastic 96-well plates were coated with integrin (1 μg/ml) in coating buffer (20 mM Tris [pH 7.4], 150 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$) for 16 h at 4°C. The plates were washed with coating buffer and blocked with binding buffer (BBB; 50 mM Tris [pH 7.4], 100 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2% bovine serum albumin [radioimmunoassay grade]) at room temperature for 2 h. The wells were washed twice with binding buffer. Virus, in binding buffer, was added to the wells in 100 μl for 1 h at room temperature. The plates were then washed with binding buffer without bovine serum albumin, and bound virus was detected with a guinea pig anti-FMDV polyclonal antiserum (raised against the homologous virus) followed by a rabbit anti-guinea pig alkaline phosphate conjugate (Sigma). For competition with peptides, 50 μl of virus (5 μg/ml) was mixed with an equal volume of 2× peptide immediately before addition to the wells. For inhibition by antibodies, integrin-coated plates were preincubated with antibody (50 μl) for 30 min before the addition of 50 μl of virus (5 μg/ml). Bound virus was detected as above.

Affinity measurement. A virus binding isotherm was constructed with radiolabelled O1KB64 (specific activity = 2.9 pg/cpm). Serial dilutions of virus (50 μl) in BBB were added to triplicate wells of an integrin-coated plate (prepared and blocked as described above) for 10 h at 4°C. Nonspecific binding was measured by adding a 25-fold excess of unlabelled virus to the control wells. Unbound virus was removed, and the wells washed three times with 50 μl of BBB. Washes and the unbound fraction were pooled to give the total unbound virus. Bound virus was removed from the plate with three washes with 2 N NaOH at 80°C. Integrin-bound and unbound virus were counted on a Packard Tri-Carb 460 CD liquid scintillation system.

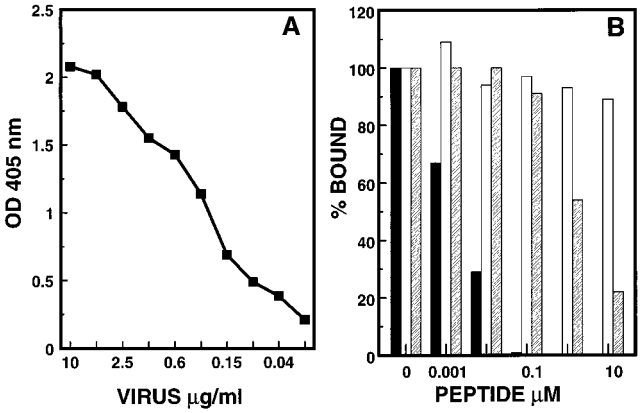

FIG. 1. (A) O1BFS binding to αvβ3 in a solid-phase assay. The amount of virus bound (expressed as the OD_{405}) is plotted against the concentration of virus added/well. Integrin was immobilized and bound virus was detected as described in Materials and Methods. Nonspecific binding was measured in the presence of 5 mM EDTA and was 0.2 OD unit. Each datum point is the average for duplicate wells. (B) Peptide competition of O1BFS binding to αvβ3. The percentage of virus bound is plotted against the concentration of competitor peptide. Solid boxes, GRGDSP; open boxes, GRGESP; hatched boxes, the O1BFS G-H loop peptide (PNLRGDLQVLAQ). The 100% binding level is the amount of virus bound in the absence of peptide.

RESULTS

Binding of O1BFS to purified αvβ3. FMDV (A12) has been shown to use the RGD-binding integrin αvβ3 as a receptor for internalization on primate cells (6). In this study, we have used purified integrin to determine whether viruses representative of other FMDV serotypes bind αvβ3, since some of these viruses also bind to other components of cellular membranes (8, 22, 44). Figure 1A shows that O1BFS binds to purified αvβ3 in a concentration-dependent and saturable manner. The binding of natural ligands to αvβ3 is inhibited by short RGD-containing peptides (33). Figure 1B shows that the peptide GRGDSP efficiently competed the binding of O1BFS to αvβ3. The concentration of peptide required to compete binding by 50% (IC_{50}) is 5 nM. The effect of this peptide is specific, since an RGE version (GRGESP) at up to 10 μM had no effect on virus binding (Fig. 1B). A longer RGD-containing peptide with a sequence of the G-H loop of VP1 (141-VPNLRGDLQVLA-152) also competed binding, although less efficiently (IC_{50} = 1 μM). The binding of O1BFS was also specifically inhibited by antibodies to either αvβ3 or αv, which are known to inhibit the binding of natural ligands to αvβ3 (Fig. 2A). It was of interest to determine the cation dependence of FMDV binding to αvβ3, since the binding of natural ligands is differentially regulated by divalent cations. In general, Mn^{2+} enhances ligand binding to αvβ3 whereas Ca^{2+} can either support or inhibit. We found that O1BFS binding to αvβ3 was also dependent on divalent cations, since it did not occur in 5 mM EDTA (data not shown). FMDV O1BFS bound to αvβ3 when both Ca^{2+} and Mg^{2+} ions were present, and this binding was enhanced by the addition of Mn^{2+} (Fig. 2B). The concentration of virus at 50% binding in the absence of Mn^{2+} was eight times greater than that required to achieve the same level of binding when this cation was present (Fig. 2B). The maximum optical density (OD) reading achieved for virus binding to αvβ3 was the same in the absence and presence of Mn^{2+}, indicating that the total number of available virus binding sites is not altered by the absence of Mn^{2+} from the coating buffer (Fig. 2B). Manganese is known to enhance ligand binding to integrins, including αvβ3 (14, 42). The details of how Mn^{2+} acts to enhance ligand

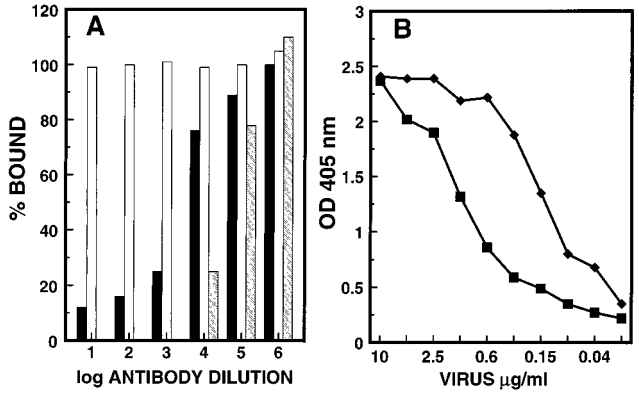

FIG. 2. (A) Antibody competition of O1BFS binding to αvβ3. The percentage of virus bound is plotted against the log dilution of competitor antibodies at the starting concentrations given below. The antibodies used were ascites of LM609 (anti-αvβ3) (solid boxes) (starting dilution 1 = 1/1,000), a control ascites of a similar content of a matched isotype antibody (open boxes) (dilution 1 = 1/1,000), and L230 (anti-αv) (hatched boxes) (starting dilution 4 = 18 μg/ml). Each datum point is the average for duplicate wells. The 100% binding level is the amount of virus bound in the absence of antibody. (B) Manganese enhances the binding of O1BFS to αvβ3. The amount of virus bound (expressed as the OD_{405}) was plotted against the concentration of virus added/well. Integrin was immobilized in coating buffer or in coating buffer without Mn^{2+}. Virus binding was determined in binding buffer or in binding buffer without Mn^{2+}. Solid squares show virus binding when the integrin was immobilized and virus bound in the absence of Mn^{2+}. Solid diamonds show virus binding when the integrin was immobilized and virus bound in the presence of Mn^{2+}. Each datum point represents the mean for duplicate wells.

binding is unclear, but evidence suggests that it alters the conformation of the integrin in a manner that favors ligand binding (14, 26).

To quantify the binding affinity, the immobilized integrin was titrated with a solution of radiolabelled virus. Figure 3A shows a binding isotherm for the interaction between O1KB64 and αvβ3, and Fig. 3B shows a Scatchard plot of the data in Fig. 3A, corrected for nonspecific binding. This yields an apparent equilibrium dissociation constant (K_d) of 3×10^{-11} M.

Binding of non-type O FMDV to purified αvβ3. Figure 4 shows that viruses representative of serotypes other than type O also bind αvβ3 in a concentration-dependent and saturable manner. Since these heterotypic strains differ considerably in sequence around the RGD motif of the VP1 G-H loop (Table 1), we looked for quantitative differences in their susceptibility to competition by the GRGDSP peptide. In all cases, virus binding was specifically inhibited by the RGD peptide. The RGE peptide had a minimal effect at 10 μM (data not shown). The IC_{50} of the RGD peptide was higher for the SAT-2 and SAT-3 strains (Table 1).

DISCUSSION

The integrin αvβ3 has been shown to act on primate cells as a receptor for internalization of FMDV (A12) (6). We have recently shown that infection of cells grown in culture by type O FMDV (O1BFS) and (O1KB64) requires initial attachment of the virus to cell surface heparan sulfate (22), a low-affinity receptor that is abundantly expressed in the extracellular matrix (23). We have sought here to study the formation of the virus-integrin complex in isolation from other cellular components, especially heparan sulfate and other RGD-binding integrins. The solid-phase assay used in this study successfully replicates authentic ligand binding by cellular αvβ3 in its dependence on divalent cations, activation by manganese ions, and sequence-specific competition by RGD-containing peptides. The affinity (apparent $K_d \approx 10^{-11}$) of FMDV (O1KB64) for αvβ3 is high; assuming that it relates to monovalent binding (see below), this provides additional evidence that this preparation of immobilized human αvβ3 is capable of binding FMDV as a functional, native receptor. The K_d is higher than for natural ligands of αvβ3 (where this has been measured) (20) and should be high enough to promote efficient cellular uptake (47).

FMDV binds the human form of αvβ3 specifically and avidly, although primates are not natural hosts for the virus. However, integrins are highly conserved among mammals (21), and some laboratory strains of FMDV do have a limited ability to grow in primate cells (reference 6 and our unpublished

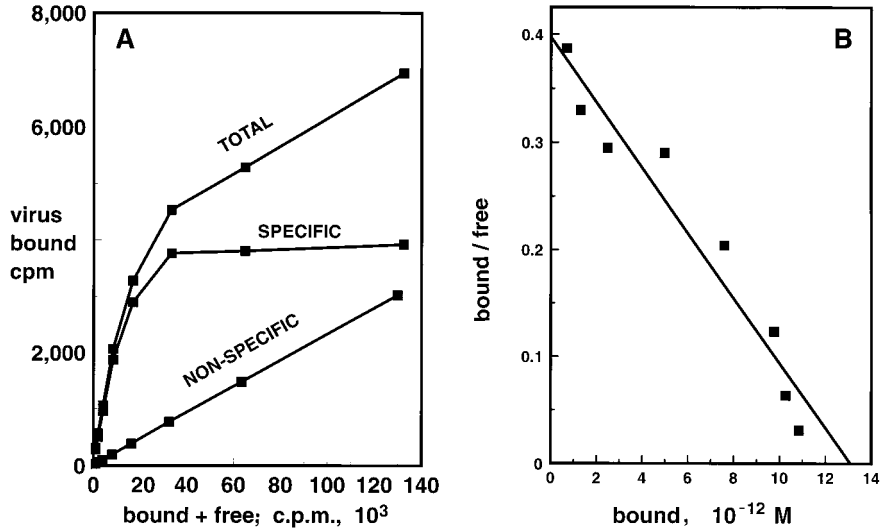

FIG. 3. Affinity of ^{35}S-labelled O1KB64 for αvβ3. (A) Virus binding isotherm. The amount of virus bound to integrin is plotted against the input virus (bound + free). Nonspecific binding was measured by adding a 25-fold excess of unlabelled virus to control wells. The nonspecific binding was subtracted from the total binding to give the specific component. Each datum point is the average for triplicate wells. (B) Scatchard plot of the specific binding data shown in panel A. The K_d (3×10^{-11}) was calculated from the slope of the line.

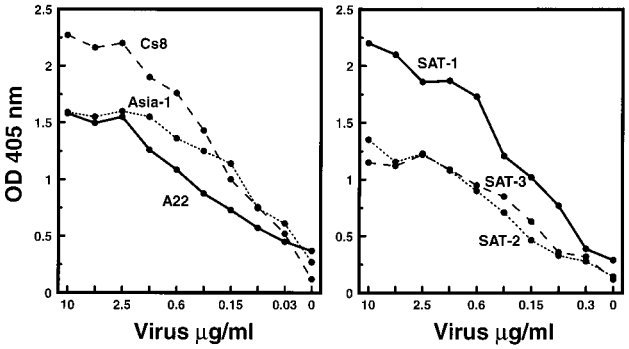

FIG. 4. Non-type O viruses bind αvβ3. The amount of virus bound (expressed as the OD_{405}) is plotted against the concentration of virus added/well. Nonspecific binding (approximately 0.2 OD unit) was measured by omitting virus from control wells. Each datum point is the average for duplicate wells. The viruses used are indicated in the figure. A22, A22 Iraq (7); Asia-1, Asia-1/IND/63/72 (35); C-S8c1 (43); SAT-1, SAT-1 Bot-1 (24); SAT-2, SAT-2 Rho 1/48 (38); SAT-3, SAT-3 Zim 4/81 (10).

observations). This suggests that human αvβ3 is competent to not only bind FMDV but also mediate viral infection. Indeed, Mason et al. (28) have shown that integrins can be replaced altogether by a surrogate cell surface receptor capable of carrying FMDV into the endocytic pathway. It seems likely, therefore, that the inability of FMDV to infect primate hosts is caused by factors other than the presence of competent receptors for internalization.

Studies in which RGD-containing peptides were used to inhibit cell attachment have been reported for FMDV serotypes O, A, and C (2, 18, 19, 30). Compared with these studies, which were done on whole cells, the most remarkable feature of virus binding by purified αvβ3 is its exquisite sensitivity to peptide competition. Thus, short RGD-containing peptides inhibit the binding to cells only weakly, and concentrations up to 10 mM are required to obtain significant inhibition (2, 18, 19, 30). The effects on cell attachment by longer RGD-containing peptides (with their sequence derived from the G-H loop of the virus under study) appear to depend on the virus. For O1BFS, such a peptide failed to inhibit cell attachment at 15 mM (18), whereas for C-S8c1, a G-H loop peptide was found to be more effective than short peptides at inhibiting cell attachment (IC_{50} = 8 μM) (19, 30). In our study, by contrast, we found that binding of O1BFS to purified αvβ3 was efficiently inhibited by both types of peptide, with the short peptide, GRGDSP (IC_{50} = 5 nM), being more potent than the longer G-H loop peptide (IC_{50} = 1 μM). Moreover, that sensitivity was absolutely specific for the D residue, since the RGE analog was $>10^4$-fold less potent as an inhibitor. Results obtained in different laboratories are not directly comparable, since the types of integrin expressed on the cells, which were uncharacterized, may have been different and the peptides used also varied in length and sequence. Nevertheless, the vast difference between the in vitro and in vivo data is very striking. One possible explanation is that each virion may be able to bind only a single molecule of immobilized αvβ3, whereas on the plasma membrane virus particles might be able to recruit several integrin molecules, thereby binding more strongly and hence requiring more competitor to dislodge them. However, where extremely high peptide concentrations were found to be necessary to inhibit virus binding to cells, we suspect that the effect of the peptide may have been to interfere with the predominantly electrostatic contacts between the virus and heparan sulfate. This effect may also account for the low sequence specificity reported for the inhibition of cell attachment of types O and A by short RGD-containing peptides (2, 18). The ability of FMDV (C-S8c1) to infect cells is much more sensitive to peptide competition than the ability to bind to them (19), which suggests that the peptide is blocking virus attachment to binding sites on the cell membrane other than those used for internalization.

TABLE 1. Sequence around the RGD motif and IC_{50} of the GRGDSP peptide for FMDV strain

Virus strain	G-H loop sequence[a]	IC_{50} (nM)
O1BFS	YNRNAVPNL**RGD**LQVLAQKVA	5
A22	YSAGGTGR**RGD**LGPLAARVA	5
CS-8c	YTASA**RGD**LAHLTTT	8
Asia-1	YGTQPTR**RGD**LAVLAQRVS	17
SAT-1	YKPTGTAPRENI**RGD**LATLAAR	26
SAT-2	YKQEAKAI**RGD**RAVLAAK	100
SAT-3	YSNTQHVTPR**RGD**MAVLAQRVA	120

[a] Sequence data for the VP1 G-H loop is from the references given in the legend to Fig. 4.

The amount of GRGDSP peptide required to inhibit the binding to αvβ3 by 50% (IC_{50}) was found to be similar for most viruses except for SAT-2 and SAT-3, which required more peptide to achieve this level of inhibition (Table 1). The observed difference in IC_{50}s may reflect a difference in the binding affinity of the viruses for αvβ3. The amino acid sequence flanking the RGD is different in each of the viruses used in this study (Table 1). The amino acid sequence immediately adjacent to the RGD and its conformational environment are involved in determining both the binding specificity and affinity of integrins for their ligands (25, 34, 39). Indeed, amino acid changes in residues flanking the G-H loop RGD have been reported to influence the ability of several FMDV serotypes to bind and infect cells in culture (27, 29, 30, 36). One possible explanation for the observed difference in IC_{50}s could be that the conformational and sequence environments of the RGD in SAT 2 and SAT-3 viruses are thermodynamically better suited to binding αvβ3. Alternatively, the binding of SAT-2 and SAT-3 viruses may be strengthened by a secondary stabilizing interaction, as is the case for the interaction between αvβ3 and vitronectin (33).

Little is known of the sterochemistry of RGD-dependent ligand binding to integrins. The crystal structures of several FMDV serotype strains have been determined, but the long integrin binding loop is invariably disordered under physiological conditions, implying a requirement for ligand flexibility. The ability of purified αvβ3 to bind FMDV in a biologically authentic manner may pave the way forward to a structural analysis of virus-integrin binding.

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