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RGD sequence of foot-and-mouth disease virus is essential for infecting cells via the natural receptor but can be bypassed by an antibody-dependent enhancement pathway

(picornavirus/infectious cDNA/virus-like particles/virus adsorption/electroporation)

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ABSTRACT **Foot-and-mouth disease virus appears to initiate infection by binding to cells at an Arg-Gly-Asp (RGD) sequence found in the flexible βG–βH loop of the viral capsid protein VP1. The role of the RGD sequence in attachment of virus to cells was tested by using synthetic full-length viral RNAs mutated within or near the RGD sequence. Baby hamster kidney (BHK) cells transfected with three different RNAs carrying mutations bordering the RGD sequence produced infectious viruses with wild-type plaque morphology; however, one of these mutant viruses bound to cells less efficiently than wild type. BHK cells transfected with RNAs containing changes within the RGD sequence produced noninfectious particles indistinguishable from wild-type virus in terms of sedimentation coefficient, binding to monoclonal antibodies, and protein composition. These virus-like particles are defined as *ads*⁻ viruses, since they were unable to adsorb to and infect BHK cells. These mutants were defective only in cell binding, since antibody-complexed *ads*⁻ viruses were able to infect Chinese hamster ovary cells expressing an immunoglobulin Fc receptor. These results confirm the essential role of the RGD sequence in binding of foot-and-mouth disease virus to susceptible cells and demonstrate that the natural cellular receptor for the virus serves only to bind virus to the cell.**

Foot-and-mouth disease virus (FMDV), which comprises the aphthovirus genus of Picornaviridae, is an important pathogen of livestock (1, 2). The virus particle contains a loop on its surface between β-strands G and H of the capsid protein VP1 (G–H loop) (3), which is often mutated in antigenic variants (4–8). The G–H loop also contains a highly conserved three-amino-acid sequence, Arg-Gly-Asp (RGD), which has been implicated in cell binding by competition studies using synthetic peptides (9, 10). Since RGD is important in binding of extracellular matrix proteins to several integrins (11), an integrin could be the cell surface receptor for FMDV. X-ray diffraction studies of the virus have provided further evidence for an integrin as the receptor for FMDV, since the RGD residues in the G–H loop mimic those of γII-crystallin, a known ligand for integrins (12). Despite this suggestive evidence, the RGD sequence has not been confirmed as the viral attachment site on FMDV. Moreover, Roivainen *et al.* (13) have shown that another picornavirus, coxsackievirus A9, can infect cells by both RGD-dependent and RGD-independent pathways.

In addition to directly binding to cellular receptors, some viruses can infect cells through the immunoglobulin Fc receptor (FcR) in the presence of virus-specific antibodies (14–18). This pathway, defined as antibody-dependent enhancement (ADE) of infection, may play a role in pathogenesis, most notably in the case of the flavivirus that causes dengue (19). We have recently demonstrated that FMDV can infect cells in tissue culture by this pathway, suggesting that FMDV can enter cells in an RGD-independent manner (20). ADE may also play some role in natural foot-and-mouth disease, since macrophages can be infected with antibody-complexed FMDV, although these cells produce low levels of infectious virus (P.W.M. and B.B., unpublished data).

Construction of a highly infectious cDNA of FMDV (21) has allowed us to examine the importance of the RGD sequence in cellular attachment and infection. Genome-length RNAs transcribed from cDNAs with mutations near or within the RGD sequence were transfected into cells to produce either infectious or noninfectious viruses, respectively. The latter viruses have been defined as *ads*⁻, since they contain all normal viral components except the intact RGD sequence, do not adsorb to cells, but can infect FcR-expressing cells in the presence of FMDV-specific antibodies.

MATERIALS AND METHODS

Cell Lines, Viruses, and Plasmids. Baby hamster kidney cells (BHK-21, clone 13) and the Chinese hamster ovary cell line expressing the murine FcRII-B2 receptor (CHO-B2) (obtained from Karl Matter and Ira Mellman, Department of Cell Biology, Yale University School of Medicine, New Haven, CT) were maintained as described (20). $pRMC_{35}$, a full-length infectious cDNA clone of FMDV type A_{12}, has been described (21); all plasmids used to produce mutant viruses are derivatives of $pRMC_{35}$. The virus derived from $pRMC_{35}$ is designated $A_{12}IC$; this and all other virus stocks were prepared and titrated in BHK cells (21).

Construction of Mutated cDNAs. cDNA molecules with specific changes in codons 143–147 of VP1 (Fig. 1) were created by standard polymerase chain reaction (PCR) techniques (22). Some mutations were introduced into a *Pst* I–*Pvu* II fragment comprising most of VP1, by use of an existing *Nru* I site. Other mutations were introduced into a plasmid containing a 4.26-kb *Eco*RI fragment by using a *Bam*HI site added by the addition of silent mutations at codon 148 of VP1 (Fig. 1). After mutagenesis, all plasmids were sequenced through the entire amplified region with Sequenase (United States Biochemicals). Mutated fragments were then introduced into the full-length infectious clone, $pRMC_{35}$, by standard techniques, and mutations were resequenced. All amino acid numbers are based on sequence data of Robertson *et al.* (23).

***In Vitro* Transcription and Transfection.** *In vitro* RNAs were transcribed from *Not* I-linearized plasmids by using the

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Abbreviations: FMDV, foot-and-mouth disease virus; FcR, Fc receptor; ADE, antibody-dependent enhancement; CPE, cytopathic effect.

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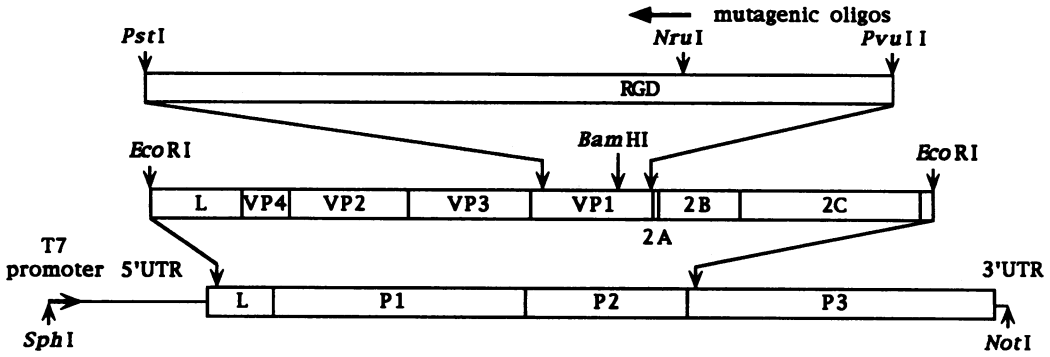

FIG. 1. Position of mutated sequences on the FMDV genome. cDNA fragments used to engineer mutant viruses are shown above the FMDV portion of $pRMC_{35}$. UTR, untranslated region; oligos, oligonucleotides.

Megaprep T7 kit (Ambion, Austin, TX) or the method of van der Werf *et al.* (24). Synthetic RNAs were introduced into BHK cells by using Lipofectin (GIBCO/BRL; ref. 21); virus stocks derived from these primary transfectant viruses were used in all experiments. Selected RNAs were transfected into BHK cells by a modification of the electroporation method of Liljeström *et al.* (25). Cells (0.8 ml at 1–3 × 10^7 cells per ml in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline) were mixed with 10–20 μg of RNA in a 0.4-cm cuvette, pulsed twice at 1500 V and 14 μF in an IBI GeneZapper (IBI), diluted with growth medium, and incubated in culture plates for 2–4 hr prior to removal of unattached cells and incubation overnight at 37°C.

Radiolabeling and Sucrose Density Gradient Analysis. Infected cells or cells transfected by electroporation were labeled for 12–16 hr with [^{35}S]methionine, and culture fluids were harvested, clarified by low-speed centrifugation, and resolved on 10–50% (wt/vol) sucrose density gradients (20).

Sequence Analysis of Recovered Viruses. RNA extracted from Lipofectin-derived virus stocks (see above) or from viruses derived from revertant populations was used as a template for cDNA synthesis with Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL) and random primers, and the VP1 region was amplified by PCR (21). PCR products were cleaved with *Pvu* II (to expose a 5′ phosphate), digested with λ exonuclease (GIBCO/BRL; ref. 26), and sequenced as described above.

Cell Binding Studies. FMDV and FMDV-like particles (from electroporated cells), biosynthetically labeled with [^{3}H]uridine or [^{35}S]methionine, were purified and used for virus-binding assays as described (27). Values shown are the percent of labeled virus bound to BHK cells in 30 min at 22°C.

Infection of FcR-Expressing Cells. Culture fluids harvested from electroporated cells were treated with monoclonal antibody 2PD11 and allowed to attach to CHO-B2 cells as described by Mason *et al.* (20). Two hours after infection, cells were radiolabeled as described above.

RESULTS

Production of Mutated Full-Length cDNAs. Full-length cDNA molecules containing mutations within the G–H loop of VP1 were generated as shown in Fig. 1; sequences inserted in these cDNA molecules are shown in Fig. 2. These sequences were selected on the following criteria: nonconservative mutations selected for the regions surrounding the RGD sequence were based on comparisons of naturally occurring variants of FMDV, which revealed no proline (P) residues bordering RGD, no negatively charged residues preceding the RGD, and no charged residues of any type following the RGD sequence (7). Mutations within the RGD sequence (KGE, KGD, and RGE) were selected to conserve the "positive–glycine–negative" charge motif, since it seemed likely that RGD or a closely related sequence would be essential for adsorption and infection.

Identification and Characterization of Viruses with Mutations Bordering the RGD. T7 transcripts derived from plasmids listed in Fig. 2 were checked for their ability to cause cytopathic effect (CPE) and produce plaques following transfection into BHK cells with Lipofectin. Transcripts derived from plasmids with mutations bordering the RGD sequence (pRM-DRGD, pRM-RGDK, and pRM-PRGD) yielded CPE, plaques, and specific infectivities similar to transcripts from the parental infectious clone (Table 1), indicating that mutations encoded by these RNAs had no effect on viability. Genomic sequences of these viruses were identical to the plasmid templates, proving that these sequences had been successfully incorporated into viable genomes.

The antigenic properties of mutant viruses were evaluated by using a panel of monoclonal antibodies to FMDV type A_{12} which recognize epitopes either within or outside the G–H loop (27). All three mutant viruses reacted with these antibodies, including those that recognize epitopes within the G–H loop (Table 2). In the case of A_{12}RGDK, reduced binding to antibodies 6FF5 and 7SF3 was expected since this virus was also mutated at codon 152, the site altered in monoclonal-antibody escape variants isolated with these two antibodies (27).

Cell binding assays were undertaken to identify any subtle differences in binding properties of these three viruses, even though stationary-phase growth titers and plaque morphologies of the A_{12}PRGD, A_{12}DRGD, and A_{12}RGDK viruses were indistinguishable from wild type (results not shown). These studies showed that A_{12}RGDK and A_{12}PRGD viruses bound to cells nearly as well as the wild type, whereas A_{12}DRGD binding was reduced by about 50% (Table 2). The difference in binding may not confer a significant disadvantage for this mutant in tissue culture, since four additional passages in BHK cells at low multiplicities of infection (<0.05) did not select viruses with changes in the G–H loop sequences.

	Amino acid residue in VP1			
	130	140	150	160
	*	*	*	*
$pRMC_{35}$	Y NGTNKYSASG	SGVRGDFGSL	APRVARQLPA	
pRM-DRGD	- ----------	--D-------	----------	
pRM-PRGD	- ----------	--P-------	----------	
pRM-RGDK	- ----------	------K---	-L--------	
pRM-KGD	- ----------	---K------	----------	
pRM-RGE	- ----------	-----E----	----------	
pRM-KGE	- ----------	---K-E----	----------	

FIG. 2. Amino acid sequences of the G–H loops of the wild-type virus and mutant genomes. Dashes designate identity with wild type.

Table 1. Properties of synthetic RNAs with mutations near the RGD sequence

cDNA clone	Sequence*	Codon change†	Specific infectivity‡
$pRMC_{35}$	VRGDF	—	4.2×10^3
pRM-DRGD	DRGDF	GUG → GAC	6.6×10^3
pRM-PRGD	PRGDF	GUG → CCC	2.0×10^3
pRM-RGDK§	VRGDK	UUU → AAA	1.4×10^3

*Amino acids 143–147 of VP1.
†Codon change required to produce desired mutation.
‡Specific infectivity of transcripts (plaque-forming units/μg) determined using Lipofectin.
§pRM-RGDK was derived from an oligonucleotide preparation randomized at codons 147 and 152; as a result, this cDNA encodes a lysine at 147 and a leucine substituted for a proline at position 152.

Identification and Characterization of *ads*⁻ Viruses. Transcripts derived from pRM-KGD, pRM-RGE, and pRM-KGE did not produce plaques when transfected into BHK cells by Lipofectin (Table 3). Therefore, electroporation was employed to better characterize these RNAs, since it provides a highly efficient method for transfection that can be used to study the biosynthetic capacity of RNAs in the absence of a complete viral replication cycle (25, 28). Essentially 100% of cells that survived the electric pulses showed virus-like CPE 12–16 hr following transfection with each of the three mutant RNAs. CPE appeared to be identical among cells transfected with viral RNA, RNA derived from $pRMC_{35}$, or the three mutant plasmids.

High titers of infectious virus were released from cells transfected with $A_{12}IC$ virion RNA or $pRMC_{35}$ transcript RNA. Although radiolabeled virus peaks were readily detectable for both samples (see Fig. 3), cells transfected with virion RNA produced 10–20 times more infectious virus than cells transfected with the $pRMC_{35}$ transcript (Table 4). This difference in yield of infectious virus was probably due to differences in lengths of the poly(C) tracts in these genomes. In particular, production of infectious virus by cells transfected with *in vitro* RNAs with shorter-than-virion-length poly(C) tracts has been shown to be slow, presumably due to the time required for elongation of the poly(C) tracts (21). The pRM-KGD and pRM-RGE RNA-transfected cells produced 10,000-fold less infectious virus than cells transfected with $pRMC_{35}$ RNA (Table 4), consistent with the production of revertants at the frequency expected for a single base mutation (29, 30). Sequence analysis of selected plaques harvested from transfected cells confirmed that the infectious viruses had regained the RGD coding sequence. The KGD revertants contained an alternative arginine codon (AGA) at position 144, whereas all of the RGE revertants regained the wild-type aspartic codon (GAU) at position 146. As expected from predicted reversion frequencies, no plaque-forming units were recovered from cells transfected with the double-mutant RNAs derived from pRM-KGE (Table 4).

Table 2. Properties of viruses with mutations near the RGD sequence

Virus	Antibody reactivity* 2FF11, 6EE2, 2PD11	Antibody reactivity* 6FF5, 7SF3	Cell binding†, %
$A_{12}IC$	+	+	70
$A_{12}DRGD$	+	+	28
$A_{12}PRGD$	+	+	61
$A_{12}RGDK$‡	+	±	63

*Determined by radioimmunoprecipitation: +, strong reaction; ±, weak reaction, similar to reaction with monoclonal-antibody escape mutants altered at position 152. Monoclonal antibodies are grouped by reactivity: 6FF5 and 7SF3 bind to the G–H loop, and the others bind elsewhere (6).
†Determined at a constant virus/cell ratio (1000:1).
‡This virus also contains a leucine substituted for a proline at position 152 (see Table 1).

Table 3. Properties of synthetic RNAs with mutations within the RGD sequence

cDNA clone	Sequence*	Codon change(s)†	Specific infectivity‡
$pRMC_{35}$	VRGDF	—	4.2×10^3
pRM-KGD	VKGDF	CGA → AAA	<1
pRM-RGE	VRGEF	GAU → GAG	<1
pRM-KGE	VKGEF	CGA → AAA GAU → GAG	<1

*Sequence of amino acids 143–147 of VP1.
†Codon change required to produce desired mutation.
‡Specific infectivity of transcripts (plaque-forming units/μg) determined using Lipofectin.

Sucrose density gradient analysis of culture fluid recovered from radiolabeled electroporated cells revealed that each of the mutant RNAs produced virus-like particles. In the case of transfections with the wild-type RNAs (which produced infectious virus), both virions (140 S) and empty capsids (70 S) were identified in the gradients (Fig. 3). Peaks at 140 S and 70 S were also recovered from cells transfected with transcripts of pRM-KGD (Fig. 3), pRM-KGE (Fig. 3), and pRM-RGE (results not shown). SDS/polyacrylamide gel electrophoresis revealed VP1, -2, -3, and -4 in each of the "virus" peaks, and VP0, VP1, and VP3 in each of the "empty capsid" peaks (results not shown).

Cell binding studies demonstrated that these virus-like particles were not able to bind to BHK cells (Table 4), indicating that they were adsorption-defective (*ads*⁻) viruses. All of the KGD and RGE revertants produced wild-type plaques, and a detailed examination of cell binding by one of these revertants revealed that it bound to cells as well as the wild-type virus (results not shown), conclusively showing that the RGD sequence is required for adsorption to and infection of BHK cells. Interestingly, all *ads*⁻ viruses reacted with a panel of monoclonal antibodies (Table 4), including some which have been mapped to the G–H loop by using neutralization escape mutants (27), indicating that conservative changes inserted into the G–H loop did not induce major changes in the antigenic structure of the virion.

Replication of *ads*⁻ Viruses Following FcR-Mediated Adsorption. Studies were undertaken to test whether *ads*⁻ virions could infect cells via an alternative pathway. We infected CHO-B2 cells with antibody-treated *ads*⁻ virions to determine whether these mutant FMDVs could infect cells via the FcR. Sucrose density gradient profiles showed that CHO-B2 cells infected with antibody-complexed *ads*⁻ virions produced radiolabeled particles that migrated at 140 S and 70 S (Fig. 4), and radioimmunoprecipitates prepared from

Table 4. Properties of *ads*⁻ viruses recovered from electroporated cells

RNA*	Virus	pfu/ml†	Antibody reactivity‡	Cell binding, %
$A_{12}IC$	$A_{12}IC$	4×10^6	+	53
$pRMC_{35}$	$A_{12}IC$	3×10^5	+	65
pRM-KGD	$A_{12}KGD$	5×10^1	+	2
pRM-RGE	$A_{12}RGE$	7×10^1	+	2
pRM-KGE	$A_{12}KGE$	0§	+	2

*cDNA clone or virus used as source of RNA.
†Amount of infectious virus recovered from electroporated cells; pfu, plaque-forming units.
‡Determined by radioimmunoprecipitation; +, strong reactions with all five monoclonal antibodies listed in Table 2.
§None detected in a 200-μl sample.

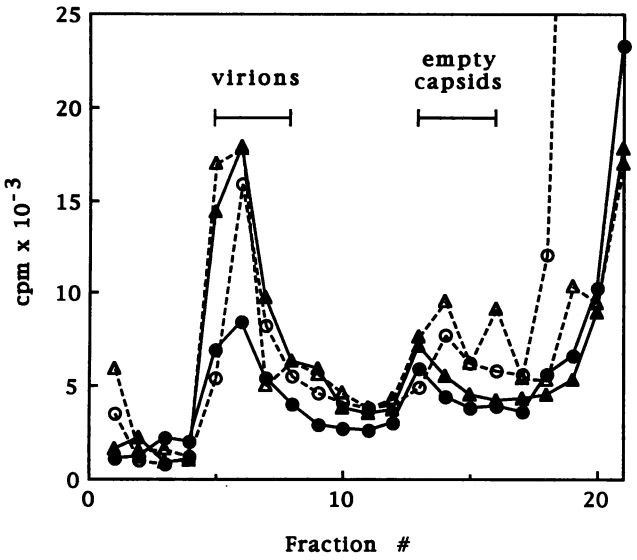

FIG. 3. Analysis of particles released from electroporated BHK cells. Cells were electroporated with the indicated RNAs and labeled overnight with [^{35}S]methionine. Culture fluids were harvested and resolved on sucrose gradients, and 25 μl of each fraction was assayed for radioactivity in a liquid scintillation counter. ●, Viral RNA; ○, $pRMC_{35}$ transcript; ▲, pRM-KGD transcript; △, pRM-KGE transcript.

peaks harvested from these gradients confirmed the presence of the expected FMDV proteins (results not shown).

DISCUSSION

The G–H loop of VP1 of FMDV is responsible for many interesting biological characteristics of the virus (see Introduction). This study involved a mutational analysis of the G–H loop to determine which amino acid residues are responsible for attachment of FMDV to susceptible cells. By using an infectious cDNA clone of FMDV (21), infectious virions were produced with mutations at amino acid residues bordering the RGD sequence reported to be necessary for cell

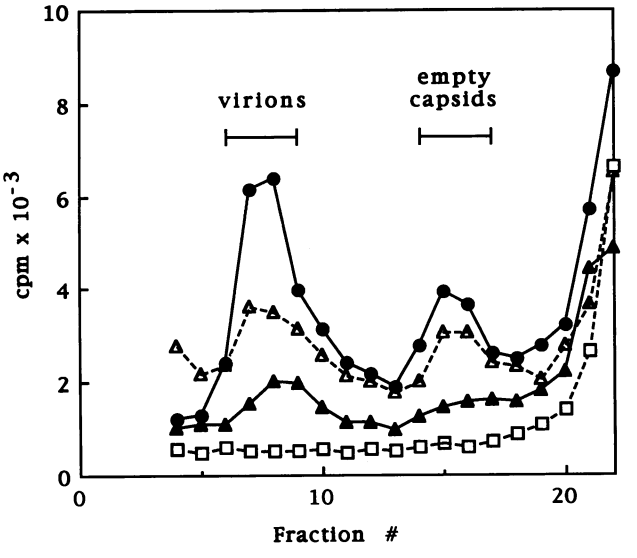

FIG. 4. Analysis of particles released from CHO-B2 cells after antibody-mediated infection. Cells were infected with antibody-complexed viruses or *ads*$^-$ mutants and labeled overnight with [^{35}S]methionine, and culture fluids were analyzed as described in Fig. 3. ●, Antibody-treated A_{12}IC; ▲, antibody-treated A_{12}KGD; △, antibody-treated A_{12}KGE; □, no virus.

binding (9, 10). Characterization of these mutant viruses revealed that nonconservative substitutions at positions 143 and 147 of VP1 had no detectable effect on virus growth or plaque appearance; however, one mutant, A_{12}DRGD, exhibited a 50% decrease in binding to BHK cells. Since our assay cannot detect subtle differences in binding, it is possible that the other two mutants adsorb to cells slightly differently from the wild-type virus. In contrast, mutations within the RGD sequence had a profound effect on viral replication. Cells transfected with these mutant full-length transcripts produced virus-like particles that were unable to bind to and initiate infection of BHK cells. Revertant viruses isolated from cells transfected with pRM-KGD or pRM-RGE RNAs were fully infectious and had regained the RGD sequence. These results establish unambiguously that this sequence is essential for the attachment of FMDV to its normal receptor.

While conservation of the RGD sequence in naturally occurring isolates of FMDV is consistent with the essential requirement for binding, this three-amino acid sequence could participate in other aspects of replication. However, the ability of *ads*$^-$ virions to infect CHO-B2 cells by ADE indicates that the RGD sequence is not required for any steps in replication subsequent to adsorption. Furthermore, the ability of *ads*$^-$ viruses to infect CHO-B2 cells by ADE provides strong evidence that the cellular receptor for FMDV functions only in docking the virus to the cell, in contrast to the receptor for poliovirus which is needed for events subsequent to adsorption (20, 31, 32).

Many cell adhesion-related protein ligands of integrins contain an RGD sequence (11), and three-dimensional structures of two of these proteins, fibronectin (33) and the snake venom disintegrin kistrin (34), have revealed that their RGD sequences are at the apex of extended loops similar to the G–H loop of FMDV (12). Mutation studies on these two proteins have shown that a mutated fibronectin containing an RGD-to-KGD mutation was unable to promote cell spreading (35), whereas the same substitution in kistrin had only a minor effect (36). However, the RGD sequence is required for kistrin function, since nonconservative changes at either the arginine or aspartic residue abrogated function (36).

Although this study has clearly demonstrated that the RGD sequence is essential for binding FMDV to cells, RGD may not be all that is required. Specifically, although integrins which recognize RGD are expressed on CHO cells (37), FMDV binds wild-type CHO cells poorly and cannot infect these cells (20). Inability of these cells to be infected by FMDV appears to be related to the absence of the normal viral receptor, since CHO cells expressing the FcR can be infected by antibody-complexed FMDV (20). The concept of participation of other virion surface features in binding of FMDV to cells is supported by (*i*) proteolytic cleavage studies showing that the removal of the C terminus of VP1 reduces binding to cells (9), (*ii*) differences in binding among serotypes of FMDV (38), and (*iii*) the binding characteristics of intertypic chimeras of FMDV, which are intermediate between their parents (unpublished data).

All mutant viruses with changes at amino acid residues 143–146 of VP1 bound to our panel of monoclonal antibodies, suggesting that mutations within or near the RGD sequence did not greatly affect virus structure. The finding that all three *ads*$^-$ virions were recognized by antibodies 6FF5 and 7SF3 (which react with the G–H loop) suggests that conservative changes in the RGD sequence may not significantly alter the antigenic structure of the loop. These data are particularly interesting since changes at the residue following RGD (residue 147) has been implicated in production of antigenic variants of serotype A virus (4). The proximity of residue 147 to the RGD sequence suggests that the RGD sequence itself could be part of the neutralizing domain, consistent with studies using monoclonal antibodies (39, 40). Although our

data on monoclonal-antibody reactivity suggest that a portion of the major neutralizing site is outside the RGD sequence, antibodies binding to these sites could block infection by sterically hindering cell binding at the RGD sequence.

To our knowledge, this is the first report that genetically engineered viruses lacking normal cell binding sites can be propagated in cells using an alternative receptor. Further development of genetically engineered FMDVs and cell surface receptors will have applications in studying reception and early events in virus entry and may provide a method for producing safer vaccine candidates for foot-and-mouth disease.

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