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NOTES

The Coxsackievirus A9 RGD Motif Is Not Essential for Virus Viability†

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An RGD (arginine-glycine-aspartic acid) motif in coxsackievirus A9 has been implicated in internalization through an interaction with the integrin $\alpha_v\beta_3$. We have produced a number of virus mutants, lacking the motif, which have a small-plaque phenotype in LLC-Mk_2 and A-Vero cells and are phenotypically normal in RD cells. Substitution of flanking amino acids also affected plaque size. The results suggest that interaction between the RGD motif and $\alpha_v\beta_3$ is not critical for virus viability in the cell lines tested and therefore that alternative regions of the CAV-9 capsid are involved in internalization.

Significant advances have been made in our understanding of receptor-virus interactions in the picornaviruses (14, 16, 20), and a number of cellular receptors have been identified (6, 7, 15, 24, 31, 35). In the case of the major receptor group of human rhinoviruses (1, 41) it is known that the receptor ICAM-1 (13, 37, 40) interacts with a deep canyon on the surface of the virus particle (10, 11, 23, 25, 30). Key determinants of receptor binding are thus thought to be protected from immune intervention, allowing antigenic diversity. In contrast, surface residues are involved in binding of the aphthovirus, foot-and-mouth disease virus (FMDV), since an RGD (arginine-glycine-aspartic acid) motif, located in an exposed position, has been implicated by peptide-blocking studies (2, 4, 12, 38) and mutagenesis (22, 27). The enterovirus coxsackievirus A9 (CAV-9) also contains an RGD motif (8, 9). This is in a surface-accessible location within an apparent extension, relative to other enteroviruses, at the C terminus of VP1 (8, 9, 26, 28). The RGD motif is unique among sequenced representatives of this picornavirus genus, with the exception of echoviruses 22 and 23, which share few typical enterovirus features and which constitute a distinct genetic group of picornaviruses (18, 36). The RGD motif and some aspects of its immediate context are conserved in CAV-9 strains isolated over a 25-year period, despite considerable variation of flanking residues, suggesting that it plays a functional role in virus replication (9). Moreover, it has been shown that RGD-containing peptides block CAV-9 infectivity, and the molecule recognized by the CAV-9 RGD motif has been shown to be the integrin $\alpha_v\beta_3$ (28, 29). In an attempt to gain a more detailed understanding of virus-cell interactions in CAV-9, we have constructed a number of mutant cDNAs containing either deletions or substitutions involving the RGD motif.

In order to allow specific mutagenesis of CAV-9, a full-length infectious cDNA clone (pCAV-9) was produced from the previously described overlapping subgenomic clones generated by cDNA cloning (data not shown) (8). From this, PCR mutagenesis was used to construct a subclone cassette containing the restriction enzyme sites *Bbs*I and *Bst*XI. These flank the region encoding the RGD motif and adjacent residues and allow the mutagenesis of up to 14 codons in this region by the insertion of a complementary pair of specific oligonucleotides. The integrity of the cassette was confirmed by nucleotide sequencing. The oligonucleotides used to produce the mutant cDNAs are shown in Fig. 1. After the introduction of appropriate oligonucleotides into the cassette, the restriction enzymes *Bss*HII and *Bst*BI were used to transfer the mutated fragment into the full-length cDNA. The sequence of mutant cDNAs was confirmed at both the subclone and full-length cDNA stages. The use of this cassette system ensures that all of the cDNAs produced differ only in the intended region.

The characteristics of the mutant sequences introduced into pCAV-9 cDNA are summarized in Table 1 and include four deletions and three substitutions. In the former, the region deleted included the RGD motif alone (pCAV-9d4) or the RGD motif and an additional two (pCAV-9d5), five (pCAV-9d16), and eight (pCAV-9d12) amino acids. One of the substitution mutants (pCAV-9RGE) was designed so that the RGD motif would be destroyed by replacement of the codon for aspartic acid (D) with a codon for the chemically similar residue, glutamic acid (E). In the others, the codon for the residue immediately downstream of the RGD region was changed from a methionine to a leucine (pCAV-9L) or a histidine (pCAV-9H). In addition, in one construct (pCAV-9M), the wild-type sequence was incorporated into the manipulated cDNA.

The monkey kidney cell-lines LLC-Mk_2, A-Vero, and Vero were maintained in minimal essential medium containing 1% amino acids, 10% heat-inactivated fetal bovine serum, and 100 mg of gentamicin per liter. The human rhabdomyosarcoma cell line RD was maintained in minimal essential medium containing 2% amino acids, 2% vitamins, and 10% fetal bovine serum (not heat inactivated). For the production of virus plaques, a plaquing overlay was used, which consisted of the appropriate medium to which 0.5% carboxymethylcellulose were added. In all experiments, cells were incubated at 37°C in 25-cm^2 tissue culture flasks. Virus plaques were visualized by staining with 0.2% crystal violet in 1% ethanol.

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† This work is dedicated to the memory of Ki Ha Chang.

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                    XbaI
pCAV-9RGE/    GCGCAATCTAGACGTCGGGGTGA(C,A)ATGTCCACCCTTAACAC
pCAV-9M   GCACCGCGTTAGATCTGCAGCCCCACT(G,T)TACAGGTGGGAATTGTGCGTA

                    XbaI
pCAV-9H/L     GCGCAATCTAGACGTCGGGGTGACC(T,A)CTCCACCCTTAACAC
          GCACCGCGTTAGATCTGCAGCCCCACTGG(A,T)GAGGTGGGAATTGTGCGTA

                                ScaI
pCAV-9d4        GCGCAAAGTCGGCGTATGAGTACTCTTAACAC
              GCACCGCGTTTCAGCCGCATACTCATGAGAATTGTGCGTA

                             ScaI
pCAV-9d5        GCGCAAAGTATGAGTACTCTTAACAC
              GCACCGCGTTTCATACTCATGAGAATTGTGCGTA

                    ScaI
pCAV-9d16       ATGAGTACTCTTAACAC
              GCACTACTCATGAGAATTGTGCGTA


pCAV-9d12       CTTAACAC
              GCACGAATTGTGCGTA
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FIG. 1. Oligonucleotides used to produce mutant cDNAs. Regions in boldface type are identical to the wild-type CAV-9 sequence, and restriction enzyme sites introduced to facilitate identification of mutant cDNAs are underlined.

DNA constructs were linearized downstream of the CAV-9 cDNA and transcribed by the Promega large-yield transcription method (39). RNA (0.1 μg) was mixed with 190 μl of OPTI-MEM medium (Gibco) and 10 μl of lipofectin reagent (Gibco), incubated at room temperature for 15 min, and overlaid onto LLC-Mk_2 cells prewashed twice with OPTI-MEM. Cells were incubated for 16 h at 37°C, the supernatant was removed, and plaquing overlay was applied. A further incubation at 37°C for 96 h was performed to allow the development of plaques. Well-separated plaques arising from the transfections were passaged once on monolayers of LLC-Mk_2 cells by being grown for 72 h. The sequence of progeny viruses was confirmed in the region of interest by RNA isolation, reverse transcription, PCR, and cycle sequencing. The tissue culture supernatants were utilized, after plaque titration, for subsequent experiments. The titers given represent PFU measured by absorption to cells for 90 min at 37°C. Plaque phenotypes were confirmed by incubation of infected monolayers for 72 h before being stained with crystal violet. The plaque sizes given represent the average of 30 plaque diameters.

All of the mutant constructs described above yielded viable viruses upon transfection of RNA, and in each case, the infectivity was broadly the same (approximately 3.0×10^3 PFU/μg of RNA (Fig. 2). The plaque phenotypes of recovered viruses in LLC-Mk_2 cells are summarized in Table 1. It can be seen

TABLE 1. CAV-9 mutants and observed plaque sizes on LLC-Mk_2 cells[a]

Recovered virus	Sequence[b]	Plaque diameter (mm)[c]
Native sequence	TTVAQSRRRGDMSTLNTH	
CAV-9M		2.2 ± 0.7
CAV-9d4	***	1.1 ± 0.4
CAV-9d5	*****	1.0 ± 0.4
CAV-9d12	**********	1.0 ± 0.4
CAV-9d16	********	1.0 ± 0.4
CAV-9RGE	E	1.4 ± 0.6
CAV-9L	L	2.6 ± 0.7
CAV-9H	H	1.3 ± 0.6

[a] The viruses were recovered from the full-length cDNA constructs pCAV-9M, pCAV-9d4, pCAV-9d5, pCAV-9d12, pCAV-9d16, pCAV-9RGE, pCAV-9L, and pCAV-9H respectively.
[b] Deleted amino acids are represented by asterisks.
[c] Values are means ± standard deviations.

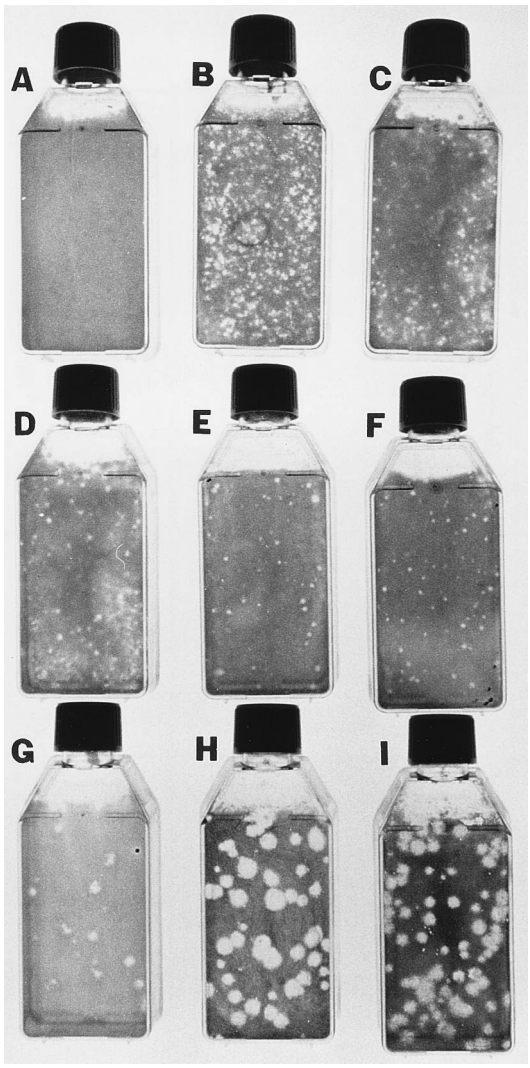

FIG. 2. Plaque sizes of the recovered CAV-9 strains after one passage. Virus was absorbed to LLC-Mk_2 monolayers, which were incubated under an agarose-carboxymethylcellulose overlay for 72 h. Bottles: A, uninfected cells; B, CAV-9 RGE; C, CAV-9d4; D, CAV-9d5; E, CAV-9d16; F, CAV-9d12; G, CAV-9H; H, CAV-9L; I, CAV-9M (in which the wild-type sequence was introduced).

that all of the strains in which the RGD motif was destroyed (CAV-9d4, CAV-9d5, CAV-9d12, CAV-9d16, and CAV-9RGE) have a small-plaque phenotype compared with the wild type (CAV-9M). Thus, although not critical to the viability of the virus, the loss of the RGD motif impairs its growth properties in LLC-Mk_2 cells. In view of our previous observations about the pattern of conservation of the amino acid immediately downstream of the RGD motif in CAV-9 isolates and in echoviruses 22 and 23, mutations were also introduced at this site (9, 19, 35, 36). Substitution from methionine to leucine, a residue found in some natural isolates of CAV-9, gives a virus (CAV-9L) with a normal- or even enhanced-plaque-size phenotype (2.6 ± 0.7 mm plaque diameter compared with 2.1 ± 0.7 mm plaque diameter for the wild type, CAV-9M) (Fig. 2 and Table 1). In contrast, the mutant CAV-9H exhibits a small-plaque phenotype (1.3 ± 0.6 mm in diameter). Substitutions at this downstream position thus clearly have the ability to change, either positively or negatively, the growth properties of CAV-9 in LLC-Mk_2 cells.

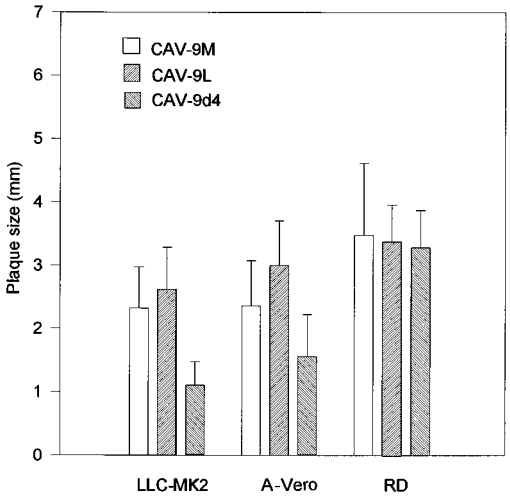

FIG. 3. Plaque sizes generated by CAV-9M, CAV-9L, and CAV-9d4 on monolayers of LLC-Mk_2, A-Vero, and RD cells after 72 h of incubation. An average of 30 individual plaque diameters is given in each case, together with their standard deviations.

Three of the virus strains generated (CAV-9d4, CAV-9L, and CAV-9M) were also tested in three other cell-lines, A-Vero, Vero, and RD. Vero cells did not support the growth of any of the strains, while in A-Vero cells, the relative differences in plaque size were maintained. Overall, all the strains grew more efficiently in A-Vero cells than in LLC-Mk_2 cells (Fig. 3). In contrast, all of the strains generated wild-type-size plaques in RD cells. To further compare the characteristics of mutant and wild-type strains, single-cycle growth experiments were performed with CAV-9d4, CAV-9L, and CAV-9M in LLC-Mk_2 and RD cells (Fig. 4). Several flasks containing cell monolayers were infected with CAV-9 strains at a multiplicity of infection of 1. At intervals of 2 h, duplicate flasks were freeze-thawed twice to liberate infectious virus and the supernatant was subjected to plaque assay. It can be seen (Fig. 4A) that for strains CAV-9M and CAV-9L, most infectious virus was produced by 6 or 8 h in LLC-Mk_2 cells. In contrast, in cells infected with CAV-9d4, the production of infectious virus appeared to be substantially delayed; significant levels were observed only after 12 h, and the yield of infectious virus particles per cell was much lower than that seen with CAV-9M or CAV-9L. In RD cells, there was little difference in the appearance of the growth curves of the strains, with virus production in each case being essentially complete by 10 h (Fig. 4B).

To further investigate the RGD motif, the binding kinetics of two of the strains generated, CAV-9M and CAV-9d4, were investigated. Equal amounts of infectious virus (500 PFU with the titers determined on RD cells) in 1 ml of growth medium were added to several monolayers of RD, LLC-Mk_2, or A-Vero cells, which were then incubated at 37°C. At given times after addition of the virus, the monolayers were washed twice with growth medium, to remove unbound virus, overlaid with plaquing medium, and then incubated at 37°C to allow plaques to develop. This experiment gives an indication of the rate of binding to cells of virus particles capable of producing infectious foci. It can be seen that there is little difference in the attachment of CAV-9M to the three cell-lines tested (Fig. 5). In contrast, the RGD-less mutant, CAV-9d4, interacts efficiently with RD cells but interacts markedly less efficiently with LLC-Mk_2 cells (Fig. 5A and B). Under the conditions of this assay, it is our consistent observation that CAV-9d4 binds extremely poorly to A-Vero cells (Fig. 5C). This experiment indicates that the reduced growth efficiency of CAV-9d4 in LLC-Mk_2 and A-Vero cells is likely to be related to inefficient binding to cells, resulting from the absence of the RGD motif.

The previous identification of a 17-amino-acid extension to the VP1 protein of CAV-9, relative to other enteroviruses, was highly striking in view of the fact that this virus is otherwise a typical member of this genus (8, 26). This extension contains an RGD motif which has an immediate context similar to that seen in the transforming growth factor β1 precursor, in which a role for RGD has not been established, and also to the RGD of tenascin, which interacts with the integrin $\alpha_v\beta_3$ (19, 33, 34). A number of lines of evidence suggest that the RGD motif contained within the CAV-9 extension is functionally significant. These include its conservation in strains isolated over a 25-year period, the ability of RGD-containing synthetic peptides to interfere with CAV-9 replication in GMK cells, and the demonstration of an interaction with the integrin molecule $\alpha_v\beta_3$, a molecule which commonly recognizes RGD motifs in cellular adhesion systems as well as in the internalization of

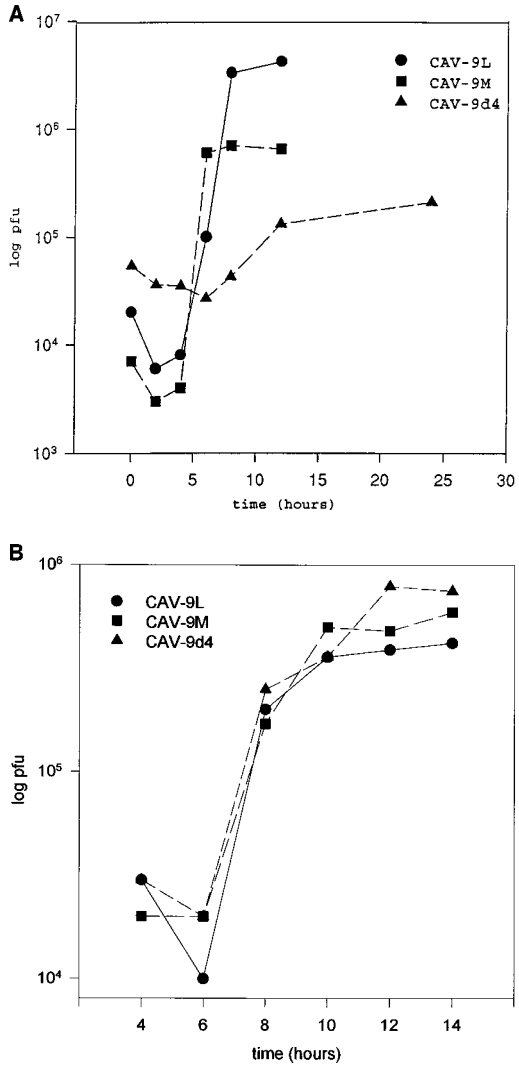

FIG. 4. Growth curves of CAV-9M, CAV-9L, and CAV-9d4 in monolayers of LLC-Mk_2 (A) and RD (B) cells. Cells were infected at a multiplicity of infection of 1, and at intervals, flasks were subjected to freeze-thawing to liberate infectious virus. Virus titers were measured by plaque assay.

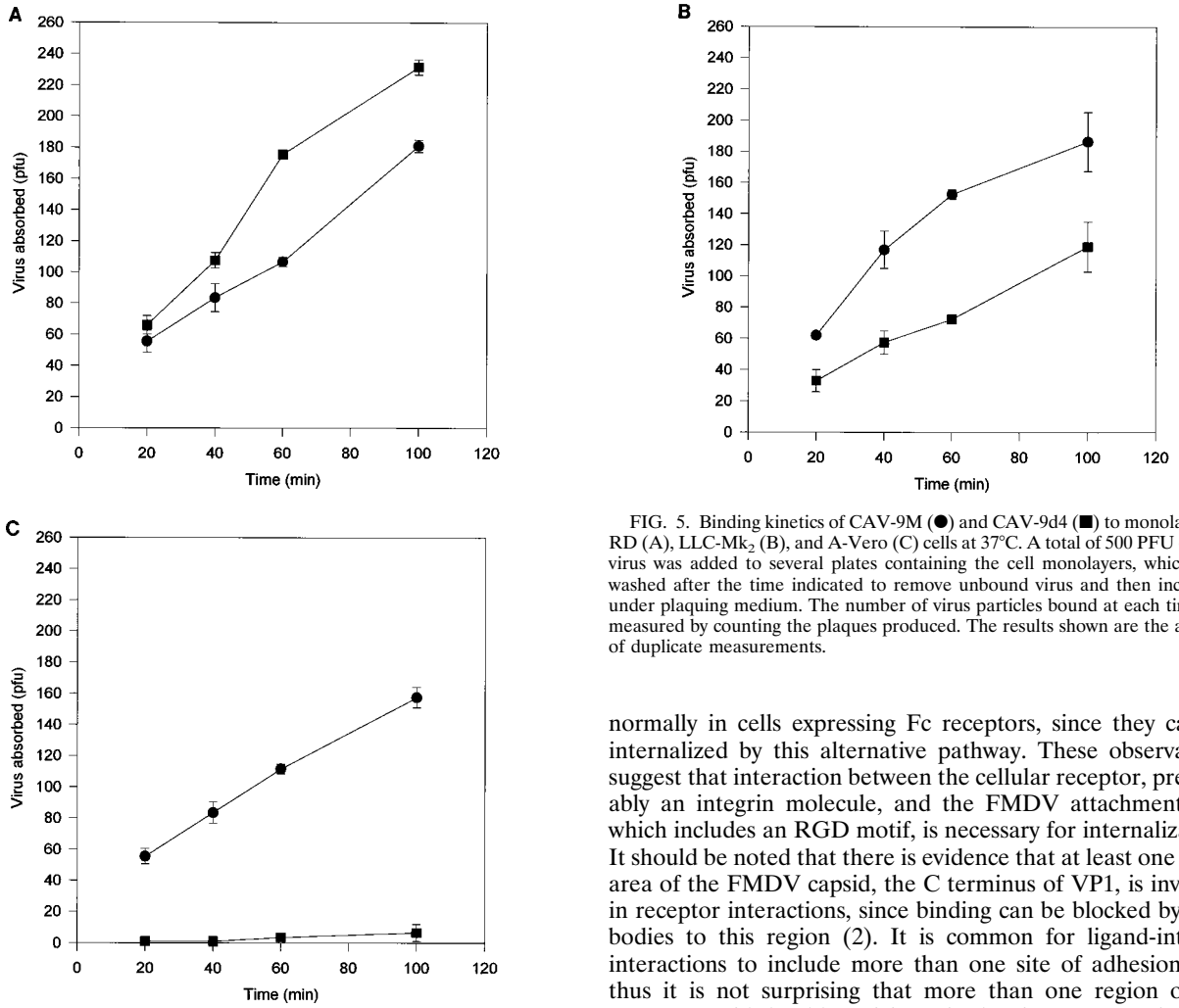

FIG. 5. Binding kinetics of CAV-9M (●) and CAV-9d4 (■) to monolayers of RD (A), LLC-Mk_2 (B), and A-Vero (C) cells at 37°C. A total of 500 PFU of each virus was added to several plates containing the cell monolayers, which were washed after the time indicated to remove unbound virus and then incubated under plaquing medium. The number of virus particles bound at each time was measured by counting the plaques produced. The results shown are the average of duplicate measurements.

bacterial and viral pathogens (9, 17, 21, 28, 29, 32). Despite this strong direct and circumstantial evidence, the viability of all of the mutants demonstrates that any interaction between RGD and $\alpha_v\beta_3$ is not essential for infectivity in any of the cell lines tested. Although not playing an obligatory role in virus infectivity, the small-plaque phenotype of CAV-9d4 in LLC-Mk_2 and A-Vero cells suggests that the RGD motif is of significance in the interaction between CAV-9 and these cells. In contrast, the lack of a phenotypic difference between CAV-9M, CAV-9L, and CAV-9d4 in RD cells indicates that the RGD motif plays little or no role in the infection of these cells. This result also demonstrates that any growth difference seen between the wild-type virus and mutant virus in LLC-Mk_2 and A-Vero cells is not due to an inherent structural defect arising from the mutation, since this would presumably also be manifested in viruses infecting RD cells.

It has recently been demonstrated that in another picornavirus, FMDV, an RGD motif located in the prominent GH loop region of VP1 is critical for virus infectivity (22, 27). When this sequence is removed, RNA transfected into cells yields viruses which are capable of replication and assemble normally but which cannot initiate further cycles of infection since they cannot interact with the receptor (22). Furthermore, in the presence of anti-FMDV antibodies, the virus can replicate normally in cells expressing Fc receptors, since they can be internalized by this alternative pathway. These observations suggest that interaction between the cellular receptor, presumably an integrin molecule, and the FMDV attachment site, which includes an RGD motif, is necessary for internalization. It should be noted that there is evidence that at least one other area of the FMDV capsid, the C terminus of VP1, is involved in receptor interactions, since binding can be blocked by antibodies to this region (2). It is common for ligand-integrin interactions to include more than one site of adhesion, and thus it is not surprising that more than one region of the FMDV capsid should participate in the virus-receptor interaction (17). The FMDV results suggest, however, that the other regions, in the absence of the RGD motif, cannot bring about binding (22).

Our observations with CAV-9 contrast with the FMDV situation, since removal of the RGD motif yields mutants which are viable, although they have a reduced capacity to infect LLC-Mk_2 and A-Vero cells, as evidenced by growth curves and/or plaque sizes (Fig. 2 to 4). In RD cells, the removal of RGD has little effect on infectivity, and this motif presumably plays a less significant role in infection. This difference between CAV-9 and FMDV is unexpected, since the RGD flanking sequences in CAV-9 are highly reminiscent of those seen in FMDV, with the same limited range of amino acids being found at the +1 and +4 positions (RGD$\underline{X}XX\underline{X}$) (9, 19). The mutants CAV-9L and CAV-9H demonstrate that mutations in the +1 position have an effect on growth efficiency. In the case of CAV-9L, in which the +1 position is changed to a leucine residue observed in some natural isolates of CAV-9, efficiency in terms of plaque size appears to increase relative to that of CAV-9M (9). In contrast, CAV-9H, with the more radical change to a histidine residue at this position, has a reduced plaque size. Differences in the properties of strains of FMDV with RGD flanking mutations have also been observed (27). Similar patterns of conservation in FMDV and CAV-9, in residues which presumably affect RGD function, suggest that the integrin recognized by FMDV is identical or highly similar

to $\alpha_v\beta_3$, shown to interact with CAV-9. It might therefore seem probable that a similar entry mechanism would be employed, but the results suggest that this is not necessarily the case. The results are, however, consistent with the likely evolutionary history of CAV-9. The degree of identity between CAV-9, the coxsackie B viruses, and typical echoviruses is high, implying that the acquisition of the VP1 C-terminal extension is a relatively recent event. In this case, it is likely that CAV-9 shares the ability to be internalized in a non-RGD-dependent manner with these close relatives, all of which lack the RGD motif. The viability of the RGD-less mutants of CAV-9 suggests that if $\alpha_v\beta_3$ is utilized it is not the sole receptor for CAV-9 and that at least one other molecule can be utilized for internalization into LLC-Mk_2 and A-Vero cells. In the case of RD cells, it is likely that this or another receptor is of major importance in internalization, since CAV-9d4 does not have impaired growth properties relative to the wild type, CAV-9M. The impairment of growth properties seen in LLC-Mk_2 and A-Vero cells may then be due to factors such as lower abundance or lower affinity of the virus for this receptor relative to $\alpha_v\beta_3$. This impairment of growth potential is consistent with the observed ability of RGD-containing peptides to largely block infectivity in some cell lines (28). Since removal of the RGD motif reduces growth efficiency markedly in LLC-Mk_2 and A-Vero cells, it is to be expected that pretreatment with peptide, which similarly prevents interaction with $\alpha_v\beta_3$, would have an apparent blocking effect on virus infectivity in the plaque reduction assay used.

It has been shown that in adenovirus 2, binding to cells occurs via an interaction between an unknown cellular receptor and the fiber protein, but that internalization requires a second step, involving the virus penton base and another cell surface protein (3, 5, 42, 43). Interestingly, the key component of the penton base is an RGD motif, while the cell surface molecule is $\alpha_v\beta_3$, with some involvement by $\alpha_v\beta_5$. Mutation of the RGD motif gives an adenovirus which can still be internalized, but in which the efficiency of the process is much reduced (3, 43). It is therefore possible that rather than being the primary receptor for CAV-9, $\alpha_v\beta_3$ acts as part of a stepwise entry process, facilitating but being nonessential in this process. The mutants described here should enable these possibilities to be explored and should enable further dissection of the role of the RGD motif in CAV-9 infectivity.

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