

Arginine-Glycine-Aspartic Acid Motif Is Critical for Human Parechovirus 1 Entry

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The human parechovirus 1 RGD motif in VP1 was studied by mutagenesis. An RGD-to-RGE change gave only revertant viruses with a restored RGD, while deletion of GD was lethal and nonrevertable. Mutations at the +1 and +2 positions had some effect on growth properties and a +1 M-to-P change was lethal. These studies indicate that the RGD motif plays a critical role in infectivity, presumably by interacting with integrins, and that downstream amino acids can have an influence on function.

Human parechovirus 1 (HPeV1) is a common pathogen that usually causes mild disease, typically in children (21, 43, 44). It belongs to the human parechovirus species of the recently recognized *Parechovirus* genus of *Picornaviridae* (24). Picornaviruses have a single-stranded RNA genome of positive polarity, 7,100 to 8,500 nucleotides long and surrounded by an icosahedral capsid usually consisting of four structural proteins, VP1 to VP4 (41). However, in HPeV1 the maturation cleavage of VP0 to VP2 and VP4 appears not to occur (16, 42). A characteristic form of the 2A protein is among other distinctive features of this genus (14, 39).

Several types of molecules, including integrins, immunoglobulin superfamily members, and complement regulatory proteins, have been recruited as picornavirus receptors (7). These molecules interact with structures on the virus surface, particularly the canyon, but in most cases the precise details of these interactions are not known. Sequence analysis of HPeV1 indicated a possible determinant of virus-host cell interaction, since the VP1 C terminus contains an arginine-glycine-aspartic acid motif (RGD) (16, 22, 42). This also occurs in the closely related parechovirus, HPeV2 (12, 33). RGD motifs are known to participate in cell-cell and cell-matrix interactions and to play a role in host cell recognition by several viruses through interactions with cell surface integrins (15, 38). The picornaviruses coxsackievirus A9 (CAV9) and echovirus 9 (EV9) also contain RGD motifs, located in precisely the same position in VP1 as that in HPeV1 (4, 5, 40, 50–52). Another picornavirus, foot-and-mouth-disease virus (FMDV), contains an RGD motif, but it is in the prominent VP1 G-H loop (6, 8, 45, 46). Biochemical and genetic approaches have established a role for the RGD motif in FMDV, CAV9, and EV9 internalization (3, 9, 13, 25–28, 32, 35, 36, 52). In the latter two viruses, this

role is not critical for infectivity in cultured cells, as viable RGD-less mutants exist (13, 50).

In order to investigate the significance of the RGD motif in HPeV1 replication, several mutations were introduced within and downstream of this sequence. This was achieved by producing a cassette vector, pHPeV-YB, from the complete cDNA clone pHPeV1 (29). This allowed any of the amino acids GDMANL (HPeV1 nucleotide positions 3002 to 3017) to be changed by ligating annealed pairs of oligonucleotides, giving the appropriate sticky ends, into pHPeV-YB cut with *SacII*/*BsmI*. Linear cDNA templates were prepared by digestion at a *MluI* site immediately downstream of the HPeV1 poly(A) tract. cRNAs obtained by transcription were transfected into tissue culture cells (green monkey kidney [GMK], A549 [human lung carcinoma], and human rhabdomyosarcoma [RD] cells) by using Lipofectin as previously described (13). Transfected cells were incubated at 37°C for 17 h and overlaid with 0.5% carboxymethyl cellulose–0.5% agarose in culture medium. Individual virus plaques were picked 3 days after the transfection and were propagated once. When a cytopathic effect was seen, infected cells were freeze-thawed twice to release viruses. Infected cell lysates were kept as stock viruses at –70°C. Plaque titrations were performed in duplicate by adsorbing virus for 1 h to confluent cell monolayers and then overlaying with carboxymethyl cellulose-agarose. After 2 to 3 days of incubation, viral plaques were visualized by staining with 0.1% crystal violet in 1% ethanol.

Table 1 shows the sequences of the oligonucleotides used to construct mutants, the mutations introduced, and their effects. The names of the mutant cDNAs reflect the virus protein and specific amino acid position mutated (e.g., pD1224E is a D [aspartic acid]-to-E [glutamic acid] change in VP1 at position 224). The presence of the mutations in viruses recovered from transfections was confirmed by reverse transcription-PCR and sequencing across the RGD region (11).

The structurally conservative change of RGD→RGE (clone pD1224E*) was initially introduced, as RGE is known not to function in integrin binding (38). A cDNA clone derived from pHPeV-YB but containing the wild-type sequence (pD1224D) was also produced. RNA from pD1224D gave plaques (3×10^4 plaques/ μ g of RNA) 3 days after transfection, which is typical

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TABLE 1. Oligonucleotides used to produce mutant HPeV1 cDNA and the phenotypes of viruses recovered

pHPeV1 cDNA	Oligonucleotides used to produce mutant cDNAs ^a	Amino acid sequence encoded ^b	Effect of mutation
Wild type (WT)	5' GGTGATATGGCCAACC 3' 3' CGCCACTATACCGGTT 5'	RGDMAN	WT properties
pD1224E*	5' GG GAA ATGGCCAACC 3' 3' CGCCT CTT TACCGGTT 5'	RG E MAN	Lethal ^c
pΔGD	5' ATGGCCAACC 3' 3' CGTACCGGTT 5'	R--MAN	Lethal
pM1225L/R	5' GGTGAC CNC GCAAACC 3' 3' CGCCACTG GNG CGTTT 5'	RGD L AN RGD R AN	Viable (WT properties) Viable (WT properties)
pM1225H	5' GGTGAC CAT GCTAACC 3' 3' CGCCACTG GTAC GATT 5'	RGD H AN	Viable (small plaque)
pM1225P	5' GGTGAC CCAG CCAAACC 3' 3' CGCCACTG GGT CGGTT 5'	RGD P AN	Lethal ^d
pA1226S/C/Y	5' GGTGACAT TNT AACC 3' 3' CGCCACTGTAC AN ATT 5'	RGD M SN RGD M CN RGD M YN	Viable (WT properties) Viable (small plaque) Viable (small plaque)

^a When annealed, the oligonucleotides give the overhangs shown, which are compatible to those generated by *Sac*II and *Bsm*I, sites for which are located in the pHPeV-YB cassette vector. Mutated codons are marked by bold type.

^b Mutated amino acids are marked by bold type.

^c Only revertant viruses (E→D) were obtained.

^d Only pseudorevertant viruses (P→L) were obtained.

of wild-type HPeV1. Following transfection of pD1224E*, a small number of plaques (5 plaques/μg of RNA) were obtained after 3 days. Several of the viruses were propagated and then sequenced in the RGD region. All the sequences showed the presence of an aspartic acid (D) codon (GAU), representing a reversion from the original glutamic acid (E) codon (GAA) (Table 2). These were genuine revertants, as all exhibited a unique, silent mutation introduced into the preceding glycine codon (G1223) of pD1224E*. This indicates that HPeV1 carrying an RGE sequence is not viable, but that reversion to RGD gives a virus with wild-type growth properties. All revertant viruses contained the same codon (GAU) for aspartic acid, possibly reflecting the codon preference of HPeV1, as most codons end in A or U in the genome of this virus (12).

To further analyze the RGD motif, a GD deletion was introduced into cDNA. RNA transfected from this cDNA clone (pΔGD) was not infectious and no virus was recovered from the liquid overlay, even after multiple blind passages. Thus, deletion of the GD dipeptide of the RGD motif is a lethal, nonrevertable mutation. As RGD-less CAV9 mutants show no growth impairment on RD cells (13), transfections of pD1224E and pΔGD RNA were also performed using these cells. However, in neither case was an RGD-less HPeV1 recovered. Similarly, these constructs did not yield viable viruses in A549 cells. These results indicate that in GMK, RD, and A549 cells, a functional RGD is required for HPeV1 replication. This contrasts with the ability to produce viable CAV9 RGE or RGD deletion mutants (13) and the lack of an RGD motif in the nonpathogenic EV9 strain Hill (50). However, in FMDV A12, mutation of RGD is lethal and it has been shown that RGD-deleted viruses are unable to bind to cells (26, 28).

The critical requirement for an RGD motif can be circumvented in some FMDV strains. Viable virus particles were produced after changing the RGD to RGE in FMDV strain O1K (25). It is therefore probable that FMDV O1K can use more than one receptor and/or that other regions, in addition to RGD, may be responsible for cell binding. One study showed that an FMDV O1K strain binds to heparan sulfate on

the cell surface, enabling efficient infection (18). High-efficiency binding is due to a mutation (histidine to arginine) at residue 56 in VP3, selected by adaptation to tissue culture cells (10). FMDV mutants lacking both an RGD motif and heparan sulfate binding ability have been isolated, suggesting that there is at least one more potential receptor-antireceptor interaction open to this virus (1). Possible HPeV1 interactions with heparan sulfate were investigated by using heparin-Sepharose. HPeV1 was incubated with heparin-Sepharose and the amount of unbound virus was determined by plaque titration assay and compared to that of cells treated with the control (Sepharose). This experiment indicated there was no HPeV1 binding to heparin (data not shown), making it unlikely that heparan sulfate plays a role in HPeV1 infection. HPeV1 infection is also not blocked by heparin (data not shown). Furthermore, multiple blind passage of GD-deleted HPeV1 did not give a virus able to infect by an alternative route.

Another molecule implicated in entry of RGD-containing picornaviruses is β₂ microglobulin, as infection with CAV9 and EV9 is blocked by antibodies to this protein by interference with a postbinding step (48, 49). However, an antibody which efficiently blocked CAV9 infection of RD cells had no effect on

TABLE 2. Comparison of revertant virus sequences obtained from pD1224E* with those from parental cDNA, pD1224D (pseudo-wild type), and wild type (HPeV1 wt)

Virus or cDNA	Nucleotide and amino acid sequences ^a				
HPeV1 wt ^b	R	G	D	M	A
	C G G G G U G A U A U G G C A				
pD1224D.....	R	G	D	M	A
	C G C G G T G A T A T G G C C				
pD1224E*.....	R	G	E	M	A
	C G C G G <u>A</u> G A A A T G G C C				
Revertants from pD1224E*.....	R	G	D	M	A
	C G C G G <u>A</u> G A U A U G G C C				

^a Nucleotides different from the wild-type cDNA are shown in bold. The silent nucleotide change used to confirm that the revertant viruses were obtained from pD1224E* is underlined.

^b wt, wild type.

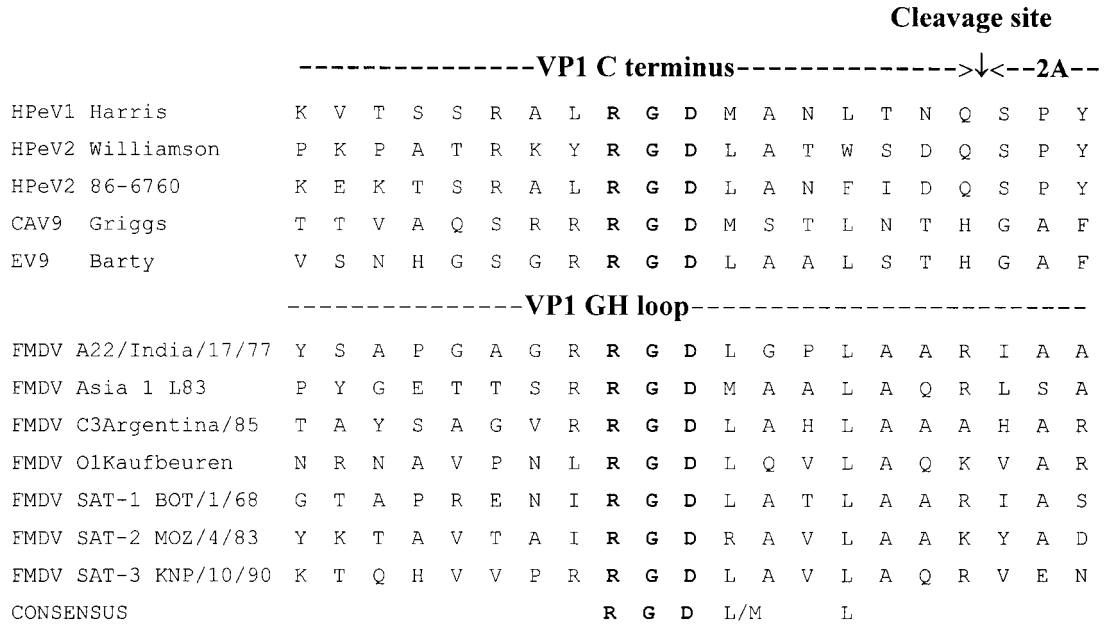


FIG. 1. Amino acid sequence comparison of the VP1 C-terminal region of representative strains of HPeV1, HPeV2, and the enteroviruses CAV9 and EV9 (4, 12, 16, 33, 51). The sequences of the RGD-containing G-H loop regions of representatives of each FMDV serotype are also shown (1, 6, 8, 45, 46).

HPeV1 infection of the same cells (data not shown). Similarly, the failure of an antibody to β_2 microglobulin to block HPeV1 infection of A549 cells has been reported (23). Thus, if molecules other than α_v -containing integrins are involved in the binding or internalization of HPeV1, they probably do not include heparan sulfate or β_2 microglobulin.

There are some similarities in the amino acids which tend to occur downstream of the RGD motif in FMDVs, CAV9, and EV9 (5, 17, 40), and the consensus sequence RGD(M/L)XXL can be derived (Fig. 1). HPeV1 (and to a lesser extent HPeV2) conforms to this consensus (12, 22, 33, 42). A +1 L is found in two completely sequenced HPeV2 strains and in a wild-type HPeV1 strain, while M is found in three wild-type HPeV1 strains (12, 22, 33). In the majority of more than 30 wild-type CAV9 isolates, together with most FMDV strains and EV9, L is present (4, 5, 40, 51). In FMDV, some SAT-2 strains show arginine (R) at position +1 (2). The +2 position (RGDM4, [A1226]) is one of the few RGD-flanking amino acids conserved between HPeV1 and HPeV2 (12). Alanine at position +2 was also found in many of the viruses compared (Fig. 1).

To investigate the significance of this conservation, the mutant cDNAs pM1225L, pM1225R, pM1225H, pM1225P, pA1226S, pA1226C, and pA1226Y were produced. Mutant RNAs (except from pM1225P) were infectious (Table 1), and sequencing revealed retention of the mutation in the progeny virus. However, after changing M1225 to P, no M1225P mutant virus was recovered and only a pseudorevertant virus, containing leucine (L) at position 225 instead of proline (P), was obtained (Table 3). This was a genuine pseudorevertant, as it contained a silent change at the A1226 codon (GCC), introduced only into pM1225P.

The plaque size of each mutant virus is shown in Table 4. Plaque sizes of M1225L, M1225R, and A1226S are similar to those of wild-type HPeV1, while a small plaque phenotype was

observed for M1225H, A1226Y, and A1226C. This largely, but not entirely, correlates with the titer achieved upon propagation, as M1225L, M1225R, and A1226Y grew to wild-type levels, while M1225H, A1226S, and A1226C achieved a lower titer upon passage (data not shown). The reason for the lack of correlation shown by A1226Y and A1226S is not clear but probably resides in the relatively minor nature of the effect on RGD function and resulting growth defects. Growth curves of the mutants were similar to those of wild-type HPeV1 but appeared to have a slightly delayed time course (data not shown). The results show that none of these mutations (except M1225P) destroy the viability of HPeV1, and so the RGD motif can function with a number of different downstream amino acids. However, the nature of both the +1 and +2 amino acids can have some effect on RGD function as shown, for instance, by the plaque size of mutants and by lethality of the M1225P change. These results are similar to those seen in CAV9, where a position +1 M-to-H change gave a small plaque mutant (13), while an equivalent mutation in FMDV

TABLE 3. Comparison of partial sequence of pseudorevertant virus obtained from pM1225P with those of parental cDNA and the wild-type sequence

Virus or cDNA	Nucleotide and amino acid sequence ^a				
HPeV1 wt ^b	R	G	D	M	A
	C	G	G	G	U
	G	A	U	A	U
	G	G	C	A	C
	A	G	C	C	A
pM1225P	R	G	D	P	A
	C	G	C	G	T
	G	A	C	C	A
	<u>C</u>	<u>C</u>	<u>A</u>	<u>G</u>	<u>C</u>
Pseudorevertant from pM1225P	R	G	D	L	A
	C	G	C	G	U
	G	A	C	C	U
	<u>A</u>	<u>G</u>	<u>C</u>	<u>C</u>	<u>A</u>

^a Nucleotides different from the wild-type cDNA are shown in bold. A unique, silent nucleotide change introduced into pM1225P and used to confirm that pseudorevertant (P→L) viruses were obtained in this construct is underlined.

^b wt, wild type.

TABLE 4. Plaque sizes of HPeV1 mutants on GMK cells

Virus	Amino acid sequence of recovered viruses	Plaque diam (mm) ^a
HPeV1 wt ^b	RALRGDMANLTK	4.2 ± 1.0
M1225L	-----L-----	3.9 ± 0.7
M1225R	-----R-----	4.0 ± 1.0
M1225H	-----H-----	2.0 ± 0.8
A1226S	-----S-----	3.8 ± 0.9
A1226Y	-----Y-----	2.8 ± 0.7
A1226C	-----C-----	2.2 ± 0.8

^a Mean ± standard deviation.^b wt, wild type.

O1K gave a virus incapable of inducing a cytopathic effect (25). Other workers have also reported a significant effect of flanking residues on RGD function (27, 35).

Extensive work has shown that the RGD motifs of FMDV, CAV9, and EV9 are involved in cell binding and entry. The HPeV1 RGD motif is in a similar sequence context to these functional RGDs and is located in an analogous position to those of CAV9 and EV9. In view of these similarities, the HPeV1 RGD motif may also be involved in the early stages of replication, presumably in cell attachment. Indeed, HPeV1 was demonstrated to compete for cell surface binding with CAV9 (37). Blocking of infectivity with RGD peptides has also been reported (42). Studies using phage display libraries showed that HPeV1 can bind to phage expressing an amino acid sequence found in the integrin β_1 subunit (34). Antibody blocking experiments suggested a role for integrins $\alpha_v\beta_1$ and anti- $\alpha_v\beta_3$ (34), and recent work supports this conclusion (23, 47). α_v integrins are known to bind RGD motifs, and the data suggest that HPeV1 may use its RGD region to recognize one or both of these molecules as cellular receptors. However, the work described here does not exclude a role in other postentry steps or in assembly.

The reason for the conserved pattern of amino acids downstream of the RGD region in diverse picornaviruses is not clear, but these amino acids may influence the efficiency of binding or even the integrin selected. For instance, it has been shown that FMDV isolates with a +1 leucine bind efficiently to integrin $\alpha_5\beta_1$, while a +1 arginine abolishes binding to $\alpha_5\beta_1$ but is part of an efficient ligand for $\alpha_v\beta_3$ (20). This conservation may therefore be the basis of the common recognition of integrin $\alpha_v\beta_3$ reported for all of these viruses (23, 30–32, 37, 47), although $\alpha_v\beta_1$, $\alpha_5\beta_1$, and $\alpha_v\beta_6$ may also be involved (19, 20, 23).

This is the first reported study to evaluate the HPeV1 RGD motif using site-directed mutagenesis. Previous blocking experiments using integrin antibodies and peptides indicated the importance of the RGD region, and so these results are not unexpected (23, 34, 42, 47). However, blocking experiments on CAV9 revealed an important role for its RGD but mutational analysis showed that this role is not absolutely necessary, indicating that there may be alternative pathways (13, 36, 37). Thus, the mutational analysis of HPeV1 is valuable and the results indicate that the RGD motif, although present in a similar sequence context, functions in somewhat different ways in picornaviruses. In HPeV1, as in FMDV, it appears to have a critical role, which can only be circumvented in the case of FMDV under some conditions when heparan sulfate and pos-

sibly other molecules can serve as an alternative receptor. In contrast, CAV9 and EV9 can exist in RGD-less forms, and in some cells these mutants grow efficiently (13, 50, 52). Thus, in these viruses it is possible that an RGD-dependent step is a component of one entry pathway among two or more alternatives. However, it should be noted that all CAV9 clinical isolates studied to date possess an RGD motif (5, 40), while the presence of an RGD motif correlates with EV9 pathogenesis in mice (52). In addition, FMDV strains virulent for cattle utilize the RGD-binding integrin $\alpha_v\beta_3$ as a receptor (30). These observations imply that the RGD motif plays a key role in normal host infections in FMDV, CAV9, EV9, and HPeV1.

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