JOURNAL OF VIROLOGY, Sept. 1991, p. 4735–4740
0022-538X/91/094735-06$02.00/0

Vol. 65, No. 9

RGD-Dependent Entry of Coxsackievirus A9 into Host Cells and Its Bypass after Cleavage of VP1 Protein by Intestinal Proteases

MERJA ROIVAINEN,[1]* TIMO HYYPIÄ,[2] LIISA PIIRAINEN,[1] NISSE KALKKINEN,[3] GLYN STANWAY,[4] AND TAPANI HOVI[1]

Enterovirus Laboratory and Molecular Biology Unit, National Public Health Institute,[1] and Institute of Biotechnology, University of Helsinki,[3] Helsinki, and Department of Virology, University of Turku, Turku,[2] Finland, and Department of Biology, University of Essex, Colchester, United Kingdom[4]

Received 21 March 1991/Accepted 15 June 1991

The recently reported nucleotide sequence of coxsackievirus A9 (CAV-9) showed that unlike other enteroviruses, CAV-9 has an insertion of about 17 amino acids at the C-terminal end of VP1 (K. H. Chang, P. Auvinen, T. Hyypiä, and G. Stanway, J. Gen. Virol. 70:3269–3280, 1989). This sequence includes the RGD (arginine-glycine-aspartic acid) motif which is known to be important in certain protein-protein interactions. We studied the inhibitory effect of RGD-containing peptides in the attachment of CAV-9 to African green monkey kidney cells. A peptide corresponding to the RRGDM sequence derived from the inserted segment of CAV-9 was found to block virus attachment effectively, and the inhibition was dose dependent. Substitution of glutamic acid for the homologous aspartic acid completely abolished the inhibitory effect, indicating great specificity of the action. During replication in the gut, all enteroviruses are exposed to host proteolytic enzymes. Exposure of CAV-9 to purified trypsin or human intestinal fluid resulted in selective cleavage of the VP1 capsid protein. Intact and trypsin-cleaved VP1 proteins gave identical N-terminal sequences, indicating that cleavage of VP1 takes place near the C terminus. Attachment of proteolytically cleaved infectious CAV-9 to green monkey kidney cells was not prevented by RGD-containing peptides, indicating that cleaved CAV-9 is able to bypass RGD-dependent entry. The altered receptor specificity of proteolytically cleaved viruses may have important consequences in the pathogenesis of enteric infections.

Attachment of a virus to its cellular receptor is a prerequisite for viral replication, and different viruses can use different cell surface structures as their receptors. Receptor molecules of some viruses, including members of the *Picornaviridae* family (coxsackie B viruses (CBV); rhinoviruses, and polioviruses) have already been identified (6, 15, 17, 27). The receptors of rhino- and polioviruses are known to belong to the immunoglobulin superfamily of glycoproteins (6, 17, 27). However, some viruses (Semliki Forest virus and adenovirus type 2) are known to be able to utilize several cell surface proteins as receptors (7, 19, 28). A mutant of coxsackievirus B3 (CBV-3), an enterovirus, has been described (20) that, unlike the parental strain, is able to bind to and replicate in human rhabdomyosarcoma cells, reflecting the flexibility of virus-host cell interactions.

The complete genomic RNA sequence of coxsackievirus A9 (CAV-9), another enterovirus, was recently determined and found to have a high degree of homology with CBV (2) but much less with CAV-21, which is, in turn, more closely related to polioviruses (8). The most striking feature of CAV-9 in comparison with other enteroviruses was, however, an insertion of about 17 amino acids at the C-terminal end of VP1 (2). This segment includes the RGD (arginine-glycine-aspartic acid) tripeptide, which is also present in wild-type CAV-9 strains isolated over a 25-year period (3). The RGD motif is important in many proteins of the extracellular matrix and blood that promote cell adhesion (25). Recently it was shown that another picornavirus, foot-and-mouth disease virus, has an RGD sequence in its VP1 protein and the sequence is involved in binding of the virus to target cells (4).

We and others have shown that host proteolytic enzymes specifically cleave the VP1 proteins of certain strains of polioviruses (5, 11, 18, 21a–23). After cleavage, the polioviruses remain fully infectious but molecular and antigenic modifications can be seen at the virion surface (5, 11, 18, 21a–23). Proteolytic modification of viruses by host enzymes is most likely not restricted to polioviruses, because other enteroviruses are also similarly exposed to these enzymes during their replication in the alimentary tract.

Here we describe experiments designed to test the hypothesis that the RGD sequence has a role in virus-host cell interaction. RGD-containing synthetic peptides were found to interfere with attachment of the virus to host cells. However, infection of cells by CAV-9 modified by proteolytic cleavage could not be prevented by the peptides.

MATERIALS AND METHODS

Cell cultures. GMK cells, a continuous cell line of African green monkey kidney origin, were used for virus propagation. Eagle's minimal essential medium supplemented with 10% (cell growth) or 1% (virus propagation) fetal calf serum was used. Virus propagation medium also contained 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.4) and 20 mM $MgCl_2$.

Viruses. CAV-9, strain Griggs, obtained from the American Type Culture Collection (Rockville, Md.), was plaque purified and propagated in GMK cells in disposable plastic bottles. For preparation of radioactively labeled CAV-9, the virus was metabolically labeled with [^{35}S]methionine. When the virus-induced cytopathic effect was complete, both the culture medium and the cells were harvested and the cells were disrupted with 0.5% Nonidet P-40 in phosphate-buffered saline. The cell extract was cleared by centrifugation

* Corresponding author.

for 10 min at 1,000 × *g* and mixed with the culture medium. Sodium dodecyl sulfate was added to a 0.5% concentration, and the virus was pelleted through a 20% (wt/wt) sucrose cushion (Beckman SW 28 rotor, 27,000 rpm, 4 h, 15°C). The virus pellet was thoroughly suspended in TNE buffer (20 mM Tris-HCl buffer (pH 7.4), 0.15 mM NaCl, 1 mM EDTA) and further purified by velocity sedimentation on a 15 to 30% (wt/wt) sucrose gradient (SW 40 rotor, 38,000 rpm, 1.75 h, 5°C). Fractions (0.7 ml) were collected from the gradient, and the radioactivity in 5-μl aliquots was determined. Virus was collected by pelleting from the peak fractions and stored at −70°C. Infectivity was measured by the plaque assay.

Enzyme treatment. Samples of virus preparations were treated with purified trypsin (1 μg/μg of virion protein) or human intestinal fluid (4 μl/μg of virion protein) for 1 h at 37°C (21a–23). Enzyme reactions were stopped by adding fetal calf serum to a 5% concentration.

Analysis of protein composition. Proteins of purified virus preparations were separated by electrophoresis on 10 to 20% polyacrylamide gradient gels or on 17% minigels under reducing conditions (14), electrically transferred to nitrocellulose sheets (Schleicher & Schuell), and analyzed by autoradiography or for immunoreactivity with rabbit antiserum to CAV-9.

Plaque assay. Confluent GMK cell monolayers were grown in six-well plates (diameter, 3 cm) to a density of approximately 0.8 × 10^6 cells per well. The cells were washed once with Hanks balanced salt solution supplemented with 20 mM HEPES, pH 7.4 (h-Hanks). The peptides were administered to the dry cell monolayers in 50 μl of h-Hanks and incubated at room temperature (RT) for 45 min. The peptide solution was then removed and replaced by 100 PFU of purified virus diluted in 70 μl of h-Hanks supplemented with 0.6% fetal calf serum and the corresponding peptide. After 15 min of incubation at RT, the unadsorbed inoculum virus was thoroughly removed and 2 ml of plaquing overlay (0.5% carboxymethyl cellulose in the culture medium) was added. The amount of adsorbed infectious virus was scored by counting the plaques after 46 h of incubation at 36°C.

Attachment assay for radioactively labeled virus. Microwell cultures of GMK cells were washed once with h-Hanks before addition of 5 μl of the peptides. After 45 min of incubation at RT, the supernatant was removed and new aliquots of peptides were added, together with purified radioactively labeled virus (10^7 PFU, about 9,000 cpm) diluted in h-Hanks containing with 0.6% fetal calf serum. The virus was allowed to attach for 45 min at RT. The unadsorbed inoculate virus was removed, the cells were washed twice with h-Hanks before solubilization in 0.3 N NaOH, and the cell-associated radioactivity was assessed by liquid scintillation.

N-terminal sequence analysis. The structural proteins from trypsin-treated and untreated virus were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5% gel) as described by Laemmli (14). The gel was aged for 3 days at +4°C prior to electrophoresis (27). Separated proteins were electrophoretically transferred onto a polyvinylidene difluoride membrane and visualized by staining with Coomassie brilliant blue (27). The bands corresponding to the VP1 protein were cut out and applied to the reaction cartridge of a gas–pulsed-liquid sequencer equipped with an on-line phenylthiohydantoin amino acid analyzer (12). Edman degradation was performed with the 01CPVD program (27).

Chemicals. [^{35}S]methionine (SJ 1015) was from Amersham (Little Chalfont, United Kingdom). Trypsin was a tosylsulfonyl phenylalanyl chloromethyl ketone-treated preparation (catalog no. T 8642) from Sigma Chemical Co. Human intestinal fluid was kindly provided by E. Savilahti, Children's Hospital, University of Helsinki. The oligopeptide VESSK (catalog no. V 6128) was from Sigma. Oligopeptides RRGDM, LRGDM, and RGD were synthesized by J. Hermonen at the Department of Medical Biochemistry, University of Turku. Before use, the authenticity of the latter peptides was checked by amino acid sequencing (see above).

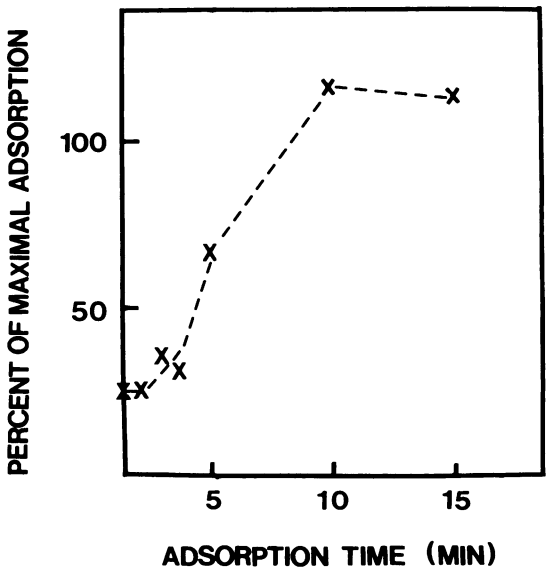

FIG. 1. Kinetics of CAV-9 adsorption to GMK cells under plaquing conditions. GMK cell monolayers were infected with CAV-9 (100 PFU). After different adsorption periods at RT, the unadsorbed inoculum virus was removed and the plaquing overlay was added. The amount of adsorbed infectious virus was scored by counting the plaques after 46 h of incubation at 36°C. The results shown are means of six parallel wells. The 100% value stands for a result obtained after 1 h of adsorption at 36°C.

RESULTS

Kinetics of adsorption. First we defined the optimal conditions for adsorption of CAV-9 to GMK cells. RT was used rather than 0 or 4°C to improve the rate of attachment (1) while still not allowing virus entry (16). We also used plaquing conditions, i.e., relatively low concentration of virus competing for the binding sites with the peptides.

A standard amount of purified CAV-9 virus (100 PFU) was allowed to attach to the GMK cell monolayers for various periods at RT, and the amount of infectious virus adsorbed was determined by counting plaques after 2 days. Adsorption of CAV-9 to the cells was relatively rapid under the conditions used, and 10 min of incubation at RT was sufficient for maximum infectivity, i.e., that found after 60 min of adsorption at 36°C (Fig. 1). Therefore, in subsequent experiments the adsorption time for CAV-9 was 15 min.

Effect of RGD-containing oligopeptides on CAV-9 attachment to GMK cells. Three different oligopeptides containing the RGD sequence were tested for efficacy in preventing CAV-9 attachment and subsequent infection. In these experiments, GMK cell monolayers preincubated with the peptides for 45 min at RT were infected with purified CAV-9 in the presence of the peptides.

An oligopeptide with the sequence RRGDM, based on

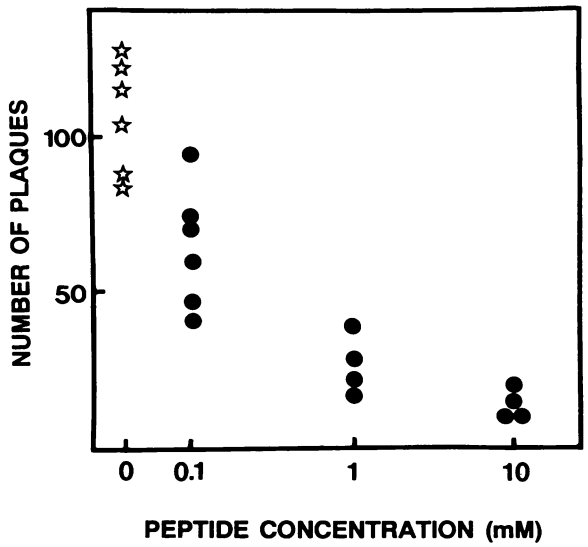

FIG. 2. Dose-dependent inhibitory effect of RRGDM on attachment of CAV-9 to GMK cells. GMK cell monolayers were incubated with h-Hanks or the RRGDM peptide for 45 min at RT. After removal of the peptide solution, the cells were infected with purified CAV-9 in the absence (☆) or presence of the RRGDM peptide (●). The virus was allowed to attach to the cells for 15 min at RT, and the unadsorbed inoculum virus was removed. The amount of adsorbed infectious virus was scored by counting plaques after 46 h of incubation at 36°C. Each incubation was done in four to six parallel cell cultures, and the results from them are shown by individual symbols.

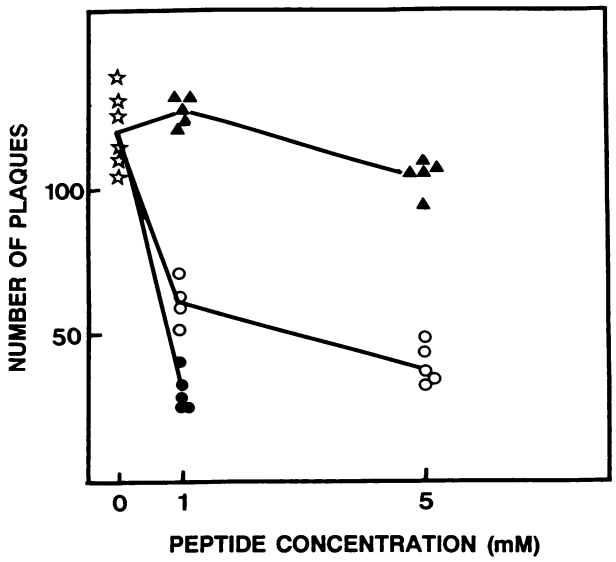

FIG. 3. Specificity of inhibition of CAV-9 attachment by RGD-containing peptides. A standard amount of purified CAV-9 was allowed to attach to GMK cells preincubated with h-Hanks (☆) or different oligopeptides (●, RRGDM; ○, LRGDM; ▲, RRGEM). For details, see the legend to Fig. 2.

part of the 17-amino-acid VP1 insertion of CAV-9, was most effective in blocking CAV-9 attachment. The inhibitory effect was evident at a 0.1 mM concentration, and inhibition was dose dependent. A 10 mM concentration of this peptide inhibited attachment of CAV-9 to GMK cells by about 85% (Fig. 2). Two other RGD-containing oligopeptides, LRGDM and RGD, were also capable of preventing CAV-9 attachment. In both cases, however, no inhibition was observed at the 0.1 mM concentration, indicating less effective blocking than that observed with RRGDM. The concentrations of LRGDM (Fig. 3) and RGD (data not shown) needed to prevent CAV-9 attachment maximally were 5 to 10 times higher than those of RRGDM.

Two control peptides were used in these experiments. In one of them, RRGEM, the aspartic acid of RRGDM was replaced by glutamic acid while the other was the commercially available unrelated pentapeptide VESSK. Both control peptides behaved similarly and showed no inhibitory effect on CAV-9 attachment. The results obtained with RRGEM are shown in Fig. 3.

Two other enteroviruses, poliovirus type 3 and CBV-3, which do not have the corresponding RGD sequence, were also tested for attachment to GMK cells in the presence of oligopeptides RRGDM and RRGEM, but no inhibitory effect was seen (Fig. 4).

The blocking effect of RGD-containing oligopeptides was also demonstrated in experiments in which cell attachment of radioactively labeled CAV-9 was studied. In these experiments, a much greater amount of infectious virus (10^7 PFU) was used than in the plaque assays (10^2 PFU). After adsorption for 45 min at RT, the cells were washed twice and the cell-associated radioactivity was assessed. The RRGDM oligopeptide, which was most effective under plaquing conditions, interfered with the virus binding in this test system as well but reached a maximum of only 50% inhibition at a 10 mM concentration (Table 1).

Cleavage of VP1 of CAV-9 by host proteolytic enzymes. Previously, it has been shown that host proteolytic enzymes can significantly modify molecular and antigenic structures of certain strains of poliovirus (5, 11, 18, 21a–23). To study

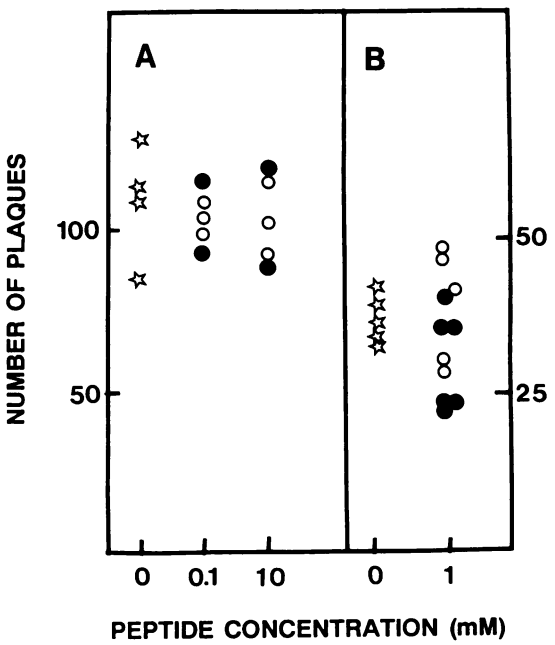

FIG. 4. GMK cell monolayers preincubated with h-Hanks (☆), RRGDM (●), or RRGEM (○) were infected with 100 PFU of poliovirus type 3 (A) or 40 PFU of CBV-3 (B) in the presence of corresponding peptides. For details, see the legend to Fig. 2.

TABLE 1. Blocking effect of RRGDM on attachment of purified [^{35}S]methionine-labeled CAV-9 to GMK cells[a]

RRGDM concn (mM)	Cell-bound radioactivity	
	Mean cpm (range)	% Inhibition
0	483 (467–500)	0
0.1	347 (282–383)	28
1	306 (276–324)	37
5	284 (273–295)	41
10	250 (198–289)	48

[a] GMK cell monolayers in microwell cultures were incubated with the indicated concentrations of the RRGDM oligopeptide for 45 min at RT, and 9,000 cpm in 10 μl of metabolically labeled CAV-9 per well was allowed to attach to the cells for 45 min at RT. Cell-bound virus was determined as described in Materials and Methods.

the cleavability of CAV-9, metabolically labeled, purified CAV-9 was incubated with purified trypsin or human intestinal fluid. After incubation, the protein composition was analyzed in polyacrylamide gel electrophoresis. The results obtained with trypsin- and human intestinal fluid-treated viruses were identical. In both cases, the VP1 protein band disappeared and a new band appeared (Fig. 5). This band migrated only slightly faster than the original one, suggesting that a small fragment (about 2 kDa) had been cleaved from VP1. The cleavage was also seen in immunoblots. Suboptimal enzyme concentrations led to incomplete cleavage of VP1, indicating that the cleavage was concentration dependent (Fig. 6).

Proteins of intact and trypsin-cleaved CAV-9 were separated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto a polyvinylidene difluoride membrane, and the bands representing intact and cleaved VP1, respectively, were cut out for amino acid sequencing. The result, Gly-Asp-Val-Glu-Glu-Ala-Ile-Glu, was the same for both of the VP1 proteins, suggesting that the observed

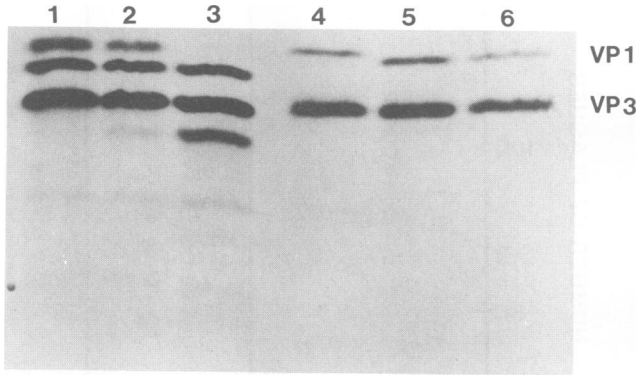

FIG. 5. Cleavage of CAV-9 by host proteolytic enzymes. A 0.45-μg sample of [^{35}S]methionine-labeled CAV-9 and a 0.01-μg sample of [^{35}S]methionine-labeled poliovirus type 3 strain Saukett were incubated with purified trypsin or human intestinal fluid in 15 μl of buffer as described in Materials and Methods. The resulting cleavages in polypeptide composition were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Purified type 3 polioviruses (lanes 1 to 3) and CAV-9 (lanes 4 to 6) were analyzed as such (lanes 1 and 4) or after treatment with trypsin (lanes 2 and 5) or intestinal fluid (lanes 3 and 6). Note that type 3 poliovirus was only partially cleaved with trypsin in this experiment (lane 2).

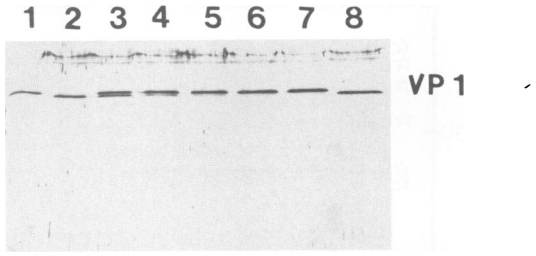

FIG. 6. Dose dependence of the cleavage of CAV-9 by purified trypsin. A 0.01-μg sample of [^{35}S]methionine-labeled CAV-9 was incubated with various concentrations of trypsin in 10 μl of buffer for 60 min at 36°C. The proteins were separated by 17% polyacrylamide gel electrophoresis under reducing conditions, electrically transferred to a nitrocellulose filter, and analyzed for immunoreactivity with rabbit antiserum to CAV-9. Lanes: 1, untreated control; 2 and 8, 10 ng of trypsin; 3 to 7, 1, 0.5, 0.25, 0.1, and 0.05 ng of trypsin, respectively. Note the gradual appearance of intact VP1 in lanes 2 through 7.

change in the mobility of VP1 after trypsin treatment of the virus is due to C-terminal processing. Simultaneously, the result confirms the suggested polyprotein cleavage site which generates VP1 during virus maturation.

The exact trypsin cleavage site is not known, but there are three potential cleavage sites (R) located in tandem in the 17-amino-acid C-terminal insertion. Cleavage at any of these sites would result in a product corresponding closely in size to the molecular weight seen in our gels. It would also result in loss of the RGD sequence. After proteolytic cleavage, CAV-9 was, however, found to be infectious, although with slightly reduced infectivity.

In subsequent experiments, attachment of cleaved CAV-9 to GMK cells was studied under plaquing conditions in the presence of the RRGDM oligopeptide. The results presented in Fig. 7 show that RRGDM was unable to block attachment

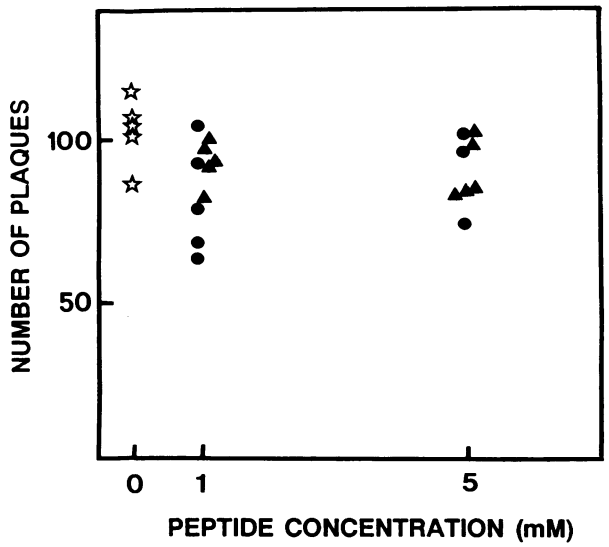

FIG. 7. Attachment of proteolytically cleaved CAV-9 to GMK cells in the presence of the RRGDM oligopeptide. GMK cell monolayers preincubated with h-Hanks (☆) or peptides (●, RRGDM; ▲, RRGEM) were infected with trypsin-cleaved CAV-9 (100 PFU) in the absence or presence of the peptides. For details, see the legend to Fig. 2.

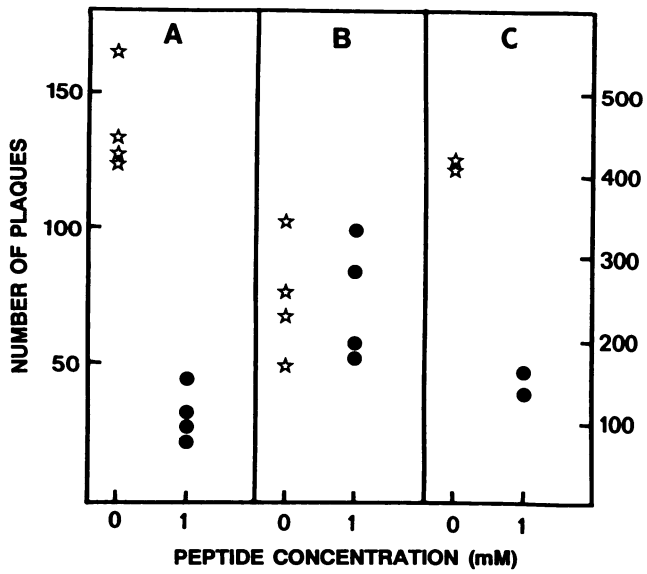

FIG. 8. Inhibition of progeny virus of trypsin-cleaved CAV-9 by RRGDM. GMK cell monolayers were infected with 130 PFU of intact CAV-9 (A), 90 PFU of trypsin-cleaved CAV-9 (B), or 400 PFU of progeny of trypsin-cleaved CAV-9 (C) in the absence (☆) or presence (●) of the RRGDM peptide. For details, see the legend to Fig. 2.

of cleaved CAV-9 to cells, even at a concentration of 5 mM. Since trypsin treatment also caused some reduction of CAV-9 infectivity, we then tested the possibility that a subpopulation of the virus was both trypsin resistant and insensitive to RGD-containing oligopeptides. However, progeny of trypsin-cleaved CAV-9, which has intact VP1, was readily inhibited by RRGDM (Fig. 8).

DISCUSSION

The present series of experiments was planned to analyze whether the RGD sequence found in the extended C terminus of VP1 of CAV-9 (2) would be needed for attachment of the virus to its cellular receptor, analogously to that reported for foot-and-mouth disease virus, another picornavirus, which also has this sequence in the VP1 protein (4). Five different oligopeptides were tested for the capacity to prevent attachment of CAV-9 to GMK cells. Three of the peptides contained the RGD sequence, and two others were used as controls. The results showed that the oligopeptides containing the RGD sequence were effective in preventing CAV-9 attachment under conditions in which the control peptides showed no inhibitory effect.

The specificity of the interaction was reflected in the observation that the most effective of the RGD-containing peptides was RRGDM, which is directly derived from the sequence of the insertion of CAV-9 VP1. The tripeptide RGD was effective to a lesser extent, indicating that the length of the peptide is critical for binding efficacy. A third peptide, LRGDM, derived from the sequence of echovirus 22 (9), also inhibited CAV-9 attachment less efficiently than RRGDM, indicating that the presence of the RGD motif as such was not sufficient for optimal binding. In agreement with these results, Fox et al. observed in studies on foot-and-mouth disease virus that, in addition to the number of amino acids, the nature of the adjacent amino acids can be critical (4). In one of the control peptides (RRGEM), substitution of glutamic acid (E) for the homologous aspartic acid (D) completely abolished the inhibitory activity of the peptide, indicating the great specificity of the action. The specificity of inhibition of CAV-9 attachment by the RGD-containing oligopeptides was further demonstrated by showing that the peptides did not affect the cell surface binding of two other enteroviruses, poliovirus type 3 and CBV-3, which do not have the RGD-containing extended VP1.

The present results are based on a plaque assay to assess the degree of virus attachment to cells, while this is very often measured by using radioactively labeled virus preparations. Application of the latter method demonstrated a similar inhibitory effect of RGD-containing peptides. However, by using a 10 mM concentration of RRGDM, only 50% inhibition of CAV-9 attachment was observed, which was much less than that obtained under plaquing conditions. It is unlikely that the longer adsorption time used in the radioactive test would have reduced the inhibition, since under plaquing conditions the results remained similar in experiments in which longer adsorption times were used (data not shown). The most likely explanation for the different results is a less optimal concentration ratio between peptide and virus in the radioactive test system, in which 10^7 PFU of labeled CAV-9 was allowed to attach to the cells, instead of only 100 PFU, as in the plaque assay.

In addition to the greater sensitivity of the plaque assay, it was also preferred because it directly assessed the attachment of infectious virus that is capable of initiating a productive replication cycle. A recent publication (13) described a set of poliovirus mutants that appeared to attach poorly, if at all, to cellular membranes in binding tests but still grew well in cell cultures. The replication of the mutants was blocked by a monoclonal antibody directed against the HeLa cell receptor of polioviruses, indicating that these mutants still use the same receptor. These results question the relevance of conventional virus binding tests (13).

Previously it has been shown that several poliovirus strains are sensitive to host proteolytic enzymes (human intestinal fluid, trypsin, and plasmin) in such a way that the immunodominant BC loop of VP1 is selectively cleaved (5, 11, 18, 21a–23). Although this cleavage resulted in molecular and antigenic modifications of the virion surface, the virus remained fully infectious. There is no reason to suppose that proteolytic modification of viruses by host enzymes is restricted to polioviruses, since during replication in the gut all enteric viruses are exposed to host proteolytic enzymes. Indeed, both purified trypsin and human intestinal fluid cleaved a small fragment from CAV-9 VP1. The target sites of trypsin are known to be the peptide bonds following arginine or lysine. The exact cleavage site in the VP1 of CAV-9 is not known, but it must be near the C terminus because the N-terminal amino acid sequences of both intact and trypsin-cleaved VP1 proteins were found to be identical. Since the cleavage rendered the virus insensitive to RGD peptides, the most plausible candidates for cleavage sites are three arginines in the inserted segment of VP1. Cleavage at any of the C-terminal arginines would result in a product with the molecular weight observed but also loss of the RGD sequence.

Trypsin-cleaved CAV-9 was still infectious. If the virus has lost the RGD sequence but is infectious, it has to be internalized by a mechanism for which the RGD sequence is not needed. In experiments in which the ability of RGD-containing peptides to block the attachment of cleaved CAV-9 was studied, no inhibitory effect was seen, indicating no role for the RGD sequence in attachment of cleaved

CAV-9. These results imply that CAV-9 is capable of attaching to host cells by using at least two mechanisms—one an RGD-mediated interaction and the other(s) by using unknown determinants. Usually CAV-9 must bind to the RGD-independent binding site with a low affinity compared with the RGD-mediated interaction, since RGD-containing peptides have such a marked effect. Alternatively, a second receptor-binding site may be blocked in some way by the C-terminal extension and be available only when proteolytic cleavage has removed this. One possibility is that the extension obstructs the "canyon," known to be involved in receptor binding in some picornaviruses (25). Modification of virus-host cell interactions by selective cleavage of a capsid protein is not specific for CAV-9, since our recent results obtained with poliovirus type 3 indicate that both the binding affinity to host cells and the rate of uncoating are altered after proteolytic cleavage by intestinal trypsin (21). CAV-9 shows extensive amino acid sequence homology with CBV-3, which is able to bind to two different receptors (20) and also has a wider tissue tropism than CAV-9 in experimentally infected mice (10). It is possible that CAV-9, after trypsin cleavage, acquires a capacity to recognize one of the CBV-3 receptors.

In conclusion, the results presented here implicate the RGD sequence in the attachment of CAV-9 to GMK cells. There is probably more than one receptor or potential receptor site for CAV-9 in these cells, because attachment of protease-cleaved CAV-9 was not inhibited by oligopeptides containing the RGD sequence. This is the first documentation of an altered receptor specificity of a virus proteolytically cleaved by host enzymes. The phenomenon may be relevant for other enteric viruses and may have important consequences in the pathogenesis of infection.

ACKNOWLEDGMENTS

This work was supported by grants from the Academy of Finland and the Sigrid Jusélius Foundation, Helsinki, Finland.

REFERENCES

1. **Baxt, B., and H. L. Bachrach.** 1980. Early interactions of foot-and-mouth disease virus with cultured cells. Virology **104:** 42–55.
2. **Chang, K. H., P. Auvinen, T. Hyypiä, and G. Stanway.** 1989. The nucleotide sequence of coxsackievirus A9; implications for receptor binding and enterovirus classification. J. Gen. Virol. **70:**3269–3280.
3. **Chang, K. H., et al.** Unpublished data.
4. **Fox, G., N. R. Parry, P. V. Barnett, B. McGinn, D. J. Rowlands, and F. Brown.** 1989. The cell attachment site on foot-and-mouth disease virus includes the amino acid sequence RGD (arginine-glycine-aspartic acid). J. Gen. Virol. **70:**625–637.
5. **Fricks, C. E., J. P. Icenogle, and J. M. Hogle.** 1985. Trypsin sensitivity of the Sabin strain of type 1 poliovirus: cleavage sites in virions and related particles. J. Virol. **54:**856–859.
6. **Greve, J. M., G. Davis, A. M. Meyer, C. P. Forte, S. C. Yost, C. W. Marlor, M. E. Kamarck, and A. McClelland.** 1989. The major human rhinovirus receptor is ICAM-1. Cell **56:**839–847.
7. **Helenius, A., B. Morein, E. Fricks, K. Simons, P. Robinson, V. Schirrmacher, C. Terhorst, and J. L. Strominger.** 1978. Human (HLA-A and HLA-B) and murine (H-2K and H-2D) histocompatibility antigens are cell surface receptors for Semliki Forest virus. Proc. Natl. Acad. Sci. USA **75:**3846–3850.
8. **Hughes, P. J., C. North, P. D. Minor, and G. Stanway.** 1989. The nucleotide sequence of coxsackievirus A21. J. Gen. Virol. **70:**2943–2952.
9. **Hyypiä, T., C. Horsnell, M. Maaronen, M. Khan, N. Kalkkinen, P. Auvinen, L. Kinnunen, and G. Stanway.** Submitted for publication.
10. **Hyypiä, T., M. Kallajoki, M. Maaronen, G. Stanway, R. Kandolf, P. Auvinen, and H. Kalimo.** Submitted for publication.
11. **Icenogle, J. P., P. D. Minor, M. Ferguson, and J. M. Hogle.** 1986. Modulation of humoral response to a 12-amino-acid site on the poliovirus virion. J. Virol. **60:**297–301.
12. **Kalkkinen, N., and C. Tilgmann.** 1988. A gas/pulsed-liquid sequencer constructed from a Beckman 890D instrument by using Applied Biosystems delivery and cartridge blocks. J. Protein Chem. **7:**242–243.
13. **Kaplan, G., D. Peters, and V. R. Racaniello.** 1991. Poliovirus mutants resistant to neutralization with soluble cell receptors. Science **250:**1596–1599.
14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
15. **Mapoles, J. E., D. L. Krah, and R. L. Crowell.** 1985. Purification of a HeLa cell receptor protein for group B coxsackieviruses. J. Virol. **55:**560–566.
16. **Marsh, M., and A. Helenius.** 1989. Virus entry into animal cells. Adv. Virus Res. **36:**107–151.
17. **Mendelsohn, C., E. Wimmer, and V. R. Racaniello.** 1989. Cellular receptor for poliovirus: molecular cloning, nucleotide sequence, and expression of a new member of the immunoglobulin superfamily. Cell **56:**855–865.
18. **Minor, P. D., M. Ferguson, A. Phillips, D. I. Magrath, A. Huovilainen, and T. Hovi.** 1987. Conservation in vivo of protease cleavage sites in antigenic sites of polioviruses. J. Gen. Virol. **68:**1857–1865.
19. **Oldstone, M. B. A., A. Tishon, F. J. Dutko, S. I. T. Kennedy, J. J. Holland, and P. W. Lambert.** 1980. Does the major histocompatibility complex serve as a specific receptor for Semliki Forest virus? J. Virol. **34:**256–265.
20. **Reagan, K. J., B. Goldberg, and R. L. Crowell.** 1984. Altered receptor specificity of coxsackievirus B3 after growth in rhabdomyosarcoma cells. J. Virol. **49:**635–640.
21. **Roivainen, M., A. Airakslnen, L. Piirainen, and T. Hovi.** Submitted for publication.

21a. **Roivainen, M., and T. Hovi.** 1987. Intestinal trypsin can significantly modify antigenic properties of polioviruses: implications for the use of inactivated poliovirus vaccine. J. Virol. **61:**3749–3753.

22. **Roivainen, M., and T. Hovi.** 1988. Cleavage of VP1 and modification of antigenic site 1 of type 2 poliovirus by intestinal trypsin. J. Virol. **62:**3536–3539.
23. **Roivainen, M., A. Huovilainen, and T. Hovi.** 1990. Antigenic modification of polioviruses by host proteolytic enzymes. Arch. Virol. **111:**115–125.
24. **Rossmann, M. G., E. Arnold, J. W. Erickson, E. W. Frankenberger, P. J. Griffith, H. Hecht, J. E. Johnsson, G. Kamer, M. Luo, A. G. Mosser, R. R. Rueckert, B. Sherry, and G. Vriend.** 1985. Structure of a common cold virus and functional relationship to other picornaviruses. Nature (London) **317:**145–153.
25. **Ruoslahti, E., and M. D. Pierschbacher.** 1987. New perspectives in cell adhesion: RGD and integrins. Science **238:**491–497.
26. **Speicher, D. W.** 1989. Microsequencing with PVDF membranes: efficient electroblotting, direct protein adsorption and sequencer program modifications, p. 24–35. *In* T. E. Hugli (ed.), Techniques in protein chemistry. Academic Press, Inc., San Diego.
27. **Staunton, D. E., V. T. Merluzzi, R. Rothlein, R. Barton, S. D. Marlin, and T. A. Springer.** 1989. A cell adhesion molecule, ICAM-1, is the major surface receptor for rhinoviruses. Cell **56:**849–853.
28. **Svensson, U.** 1985. Role of vesicles during adenovirus 2 internalization into HeLa cells. J. Virol. **55:**442–449.