Echovirus 22 (EV22) is a picornavirus forming a distinct molecular cluster together with echovirus 23. EV22 has an Arg-Gly-Asp (RGD) peptide motif in its capsid protein VP1; similar motifs are known to mediate many cell-cell and microbe-host interactions. To identify peptide sequences that specifically bind to EV22 and potentially play a role in receptor recognition, we have used here peptide libraries displayed in filamentous phage. We isolated an EV22-binding motif CLRSG(R/F)GC. The synthetic CLRSGRGGC peptide was able to inhibit EV22 infection. The infection was also inhibited by an RGD-containing peptide representing the C terminus of the EV22 capsid protein VP1 and CWDGWLC (an RGD-binding peptide; Pasqualini, R., Koivunen, E., and Ruoslahti, E. (1995) J. Cell Biol. 130, 1189–1196). As the EV22-recognizing sequence LRSQ is found in the integrin β1 chain and the entire LRSQGRG hexapeptide occurs in the matrix metalloproteinase 9 (MMP-9), we carried out blocking experiments with anti-integrin and anti-MMP-9 antibodies. EV22 infection could be blocked in cell cultures with anti-αv, β1, and, to a lesser extent, with anti-MMP-9 antibodies. These results imply that EV22 recognizes preferentially αvβ1-integrin as a cellular receptor and MMP-9 may also play a role in the cell-surface interactions of the virus.

Echovirus 22 (EV22) was originally classified as an enterovirus in the family Picornaviridae, but recent molecular data suggest that it is a representative of an independent picornavirus genus (1). Picornaviruses are small non-enveloped RNA viruses that include several pathogens of man and animals, and their medical and economic importance has stimulated considerable research activity. These viruses have a single-stranded mRNA genome, between about 7 and 8.5 kilobases in length, surrounded by an icosahedral capsid consisting of 60 copies of four structural proteins VP1–VP4 (for a review, see Ref. 2); however, in EV22 the maturation cleavage between VP2 and VP4 does not seem to occur (3). EV22 is known to cause both diarrhea and respiratory infections, and occasionally the infection is complicated by the involvement of the central nervous system (4). EV22 infection is common in childhood and more than 85% of young adults have EV22 antibodies.

Interestingly, the sequence of EV22 revealed that it carries a tripeptide motif arginine-glycine-aspartic acid (RGD) in its capsid protein VP1 (1). Such an RGD motif is involved in multiple biological recognition reactions; in particular, the cellular matrix proteins vitronectin and fibronectin use their RGD sequence in binding to cell-surface receptors known as integrins (for reviews, see Refs. 5 and 6; Table I). Integrins that recognize the RGD motif include α2β1, α5β1, α6β1, αvβ5, and αvβ3 subunit combinations. A number of microbes are also able to utilize this motif in binding to their host cell (Table I). For instance, during the initiation of adenovirus 2 (7), coxsackievirus A9 (CVA9; Refs. 8–10), echovirus 22 (EV22; Ref. 3), and foot-and-mouth disease virus (11) infections, cellular interactions are known to be mediated by the RGD motifs found in viral proteins. It has also been reported that the bacteria species Yersinia (12) and Bordetella (13) can recognize integrins on the cell surface.

During the past few years, several other picornavirus receptors, in addition to those recognized by CVA9, foot-and-mouth disease virus, and EV22, have been identified. The cellular receptor of the major group of rhinoviruses is the intercellular adhesion molecule-1 (14, 15). Intercellular adhesion molecule-1 and the poliovirus receptor (16) are members of the immunoglobulin superfamily. Furthermore, it has been reported that the integrin αvβ3 (VLA-2) acts as a cellular receptor for EV1 (17, 18), whereas several other EVs interact with the decay accelerating factor (CD55; Refs. 19, 20). At least six EV serotypes (subtypes 6, 7, 11, 12, 20, and 21) and three coxsackie-B viruses (subtypes 1, 3, and 5; Refs. 21 and 22) can use decay accelerating factor as their receptor. A polypeptide belonging to the nucleolin family is also involved in coxsackie-B viruses cell-surface interactions (23).

It has been reported previously that CVA9 can compete with the binding of EV22 to green monkey kidney cells (9). This suggests that EV22 and CVA9 may share a common cellular receptor. The aim of the present study was to illustrate further the interactions of these RGD-containing human picornaviruses with the host-cell membrane proteins and to identify receptors interacting with the viruses. For this purpose, the technique of affinity selection of virus-binding peptides from a phage display random peptide library was applied. We isolated peptide motifs that suggest the involvement of integrins and also, quite unexpectedly, of a proteolytic enzyme MMP-9 in the cellular entry of EV22.

EXPERIMENTAL PROCEDURES

Cells and Viruses—A549, a human lung carcinoma cell line (obtained from the American Type Culture Collection, ATCC), was used for EV22 (1 propagation and plaque tests, and LLC-Mk2, cells (ATCC) were used to grow CVA9 (strain Griggs, ATCC). The viruses were purified in...
Phage Display Peptide Libraries—We used a combination of phage libraries containing 5–9 amino acid long cyclic peptides inserted in the pH 8 capsid protein of the M13 phage. The libraries were constructed using the fusion vector (25) as described earlier (26–28). The phage solution was then transferred to the virus-coated wells, and after incubation for 1 h at 4 °C, the presence of 1 mg/ml BSA was neutralized with pH 7.5 Tris-HCl. The eluted phages were amplified using K91kan bacteria and purified by precipitation with polyethylene glycol as described earlier (25). After amplification, the phage procedure was repeated twice as described above with the exception that the wells were coated with 1 μg of the purified virus. Phage DNA was purified (25) and sequenced using the Sequenase 2.0 kit (U. S. Biochemical Corp.). Peptide sequences found in the phages were compared against protein sequences in the SwissProt data base using the FASTA program (29).

Peptides and Antibodies—The synthesized peptides were purified by high performance liquid chromatography. CLRSGRGC was reduced and alkylated essentially as described (30) and subsequently purified by high performance liquid chromatography. The following antibodies were used in the virus blocking experiments: the monoclonal antibodies included anti-α, L230 (ATCC), anti-α, P1D6 (Life Technologies, Inc.), and anti-β, 90B1B10 B7 (kindly provided by Prof. Ismo Virtanen, Department of Anatomy, University of Helsinki). Anti-MMP-9 (Ref. 31; kindly provided by Dr. Timo Sorsa, Department of Periodontology, University of Helsinki) and anti-β, R322 (a gift from Dr. Jyrki Heino, MediCity Research Laboratory, University of Turku) were polyclonal rabbit antisera.

Table of the Infectivity with the Peptides and Antibodies—The A549 cells were grown as a monolayer in 3.5-cm diameter wells (8). The cells were washed once with Hank’s balanced salt solution supplemented with 20 m Hepes, pH 7.4 (h-Hanks), and 50 μl of antibodies or peptides (dilutions made in h-Hanks, containing 0.6% fetal calf serum) was added on the cells and incubated for 45 min at room temperature. Fifty μl (approximately 100 plaque-forming units) of EV22 or CAV9 dilution (made in h-Hanks, 0.6% fetal calf serum, containing the corresponding antibody or peptide) was added onto the cells and incubated for 15 min at room temperature. The virus solution in the plates was replaced with 0.5% carboxymethyl cellulose in the culture medium, and the incubation was continued for 48 h in a CO₂-humidified incubator at 37 °C. Prior to counting the number of the virus plaques, the cells were stained for 5 min with crystal violet solution (0.25% crystal violet, 2% formaline, 10% ethanol, 0.5% CaCl₂, 35 mm Tris).

RESULTS

Identification of EV22- and CAV9-binding Peptides from the Phage Display Library and Their Comparison with Known Sequences—EV22- and CAV9-binding peptides were selected from phage display peptide libraries containing random peptides from five to nine amino acids, each peptide flanked by cysteine residues. Microtiter wells were coated with the purified viruses, and the phages exhibiting binding activity to the virus were selected by a biopanning protocol.

After three cycles of panning in EV22-coated wells, 170 times more phages could be eluted from the virus-coated wells than from those coated with BSA (control wells). Seventeen phage plaques were selected for further sequence analysis. Twelve of them carried the cyclic peptide CLRSGRGC, and five peptides sharing the same amino acid motif with the EV22-binding phages

| Table I | Alignment of regions of selected viral and cellular polypeptides containing an RGD sequence |
| --- | --- |
| EV, echovirus; CAV, coxsackie A virus; FMDV, foot and mouth disease virus. |
| **EV22** | RCPNPPFFLPAPKVTSSRALKGDMANLTVNG |
| **EV23** | RCPNFPFLAPAPKVTSSRALKGDMANLTVNG |
| **CAV9** | FIDTDRKDINTTVAQSRKMGDMSTLTH |
| **FMDV** | VLATVNYCGRSNNVAPNLRKDQLVMAQ |
| Adenovirus 2 | NSNAAAMQFVSEDNRHDARFPATRAE |
| Fibronectin | TISGLKPGVDYTITVYAVTG |
| Vitronectin | CSYQYSCSTTYAACEKCPQTVGDFVTMPED |
| Fibrinogen | PSRKSKSSSYKPTTSTSYGRDSTFKS |
| Osteopontin | DLAFETEVTFFTVPTDYGDRGDSVGLR |
| Bone sialoprotein | GELEYTGNYEYNQYFGRRSHYRAXED |

| Table II | Selection of peptides binding to EV22 and CAV9 from the phage display library |
| --- | --- |
| EV22 binding peptides | CAV9 binding peptides |
| CLRSGRGC (12) | CVDNGRC (11) |
| CLRSGRGC (5) | CVDLGRG (2) |
| CVDVGRC (2) |  |

| Table III | Examples of receptors and other extracellular proteins containing the identical amino acid motifs with those found in EV22- and CAV9-binding phages (see Table II) |
| --- | --- |
| The sequence source was the SwissProt database, FASTA program was used for the sequence homology analysis. The amino acids identical to those found in the phage sequences are shown in parentheses. |

Proteins sharing the same amino acid motif with the EV22-binding phages

- Matrix metalloproteinase 9
- Lysozyme activation gene 3 protein
- ACTβ5 antigen
- Fibrinogen-like protein
- Integrin β₁
- Leukocyte antigen-related protein
- Urothelin

Proteins sharing the same amino acid motif with the CAV9-binding phages

- Integrin β₁ subunit
- Villin
- Kallmann syndrome protein
- Tyrosine-protein kinase receptor FLT4
- Lymphocyte differentiation antigen CD38
- Macrophage colony-stimulating factor 1 receptor
- Transferrin receptor protein CD71
- Vasointestinal polypeptide receptor 2
- Vasopressin V1A receptor
- Von Willebrand factor

Proteins sharing the same amino acid motif with the CAV9-binding phages

- KVELSVDVQEDL
- VFLLOVDVQEDV
- VTVYVDVQEDLP
- DQGQVDVQEDRM
- VDQCSVWDVQDAKG
- AQLVQVDVQEDYF
- ALSDGWVDVQEDNE
- RCACGVDWQDNITCW
- IQMWSWDVQEDMSVW
- KALSVDVQEDHLSI
had the CLRSGFGC sequence (Table II). After three rounds of panning in CA9-coated wells, approximately five times more phage were eluted from the wells containing the virus than from the control wells. Fifteen of the CA9-binding phages were sequenced. Eleven had a sequence CVWDQGIC, two contained CVWDLGRC, and two CVWDLGGIC sequence (Table II). Although the phage libraries used in the study contained peptides of different length, all the sequenced phage carried a hexapeptide. This suggests that the cyclic hexapeptide motifs bind more tightly to the viruses than other peptides in the libraries.

The consensus sequences LRSG (EV22) and VWD (CAV9) were compared against the sequences in the SwissProt data bank (Table III). Since it is known that integrins α2β1 and α5β1 recognize the RGD sequence in fibronectin (32), it is notable that, for instance, the β1 integrin subunit also shares the sequence LRSG in its extracellular domain (33). A complete identity with the LRSGR peptide was found in the matrix metallopeptinase 9 (MMP-9; Ref. 34). It has been recently shown that MMP-2 is colocalized with integrin α2β1 on the cell surface of invasive cells (35).

Among the VWD-containing proteins is integrin β1 subunit which contains a VWDQ sequence in its extracellular domain; integrin α2β1 recognizes the RGD sequence in vitronectin (32).

**EV22-binding Peptides Block Infectivity**—To study further the functional role of selected virus-binding peptides in the cell-surface interactions of EV22 and CA9, the capability of synthetic peptides to block the infection was assayed. An RGD-containing peptide representing the C terminus of EV22 VP1 polypeptide (SRALRGDMANLTQN) and an RGD-binding peptide (CWDDGWLC; Ref. 36) were also used in the blocking experiments. The results of these experiments, obtained by the plaque tests, are shown in Fig. 1. The C-terminal EV22 VP1 peptide and the cyclic CLRSGRFC peptide blocked EV22 infection at a concentration of 0.1 mM, whereas the control peptide (NGKKNWKKIM, the N terminus of EV22 VP3) did not interfere with the initiation of infection at this concentration. The activity of CLRSGRFC was lost after reduction and alkylation of the cysteines (not shown). At a concentration of 1 mM, the RGD-recognizing CWDDGWLC peptide blocked the infection, whereas at lower concentrations the effect disappeared.

The peptide found in the CA9-binding phages (CVWDQGIC) had no effect on the EV22 growth. For comparison, blocking experiments with the peptides were also studied in CA9 infection. Only the RGD-containing peptide blocked the infectivity of this virus. The blocking effect at a peptide concentration of 0.1 mM was even 40% higher, when compared with the EV22 blocking activity. None of the other peptides significantly inhibited CA9 infection.

**Anti-integrin and Anti-MMP-9 Antibodies Block EV22 Infection**—Since the consensus sequence LRSG (found in the EV22-binding phage) is present in the integrin β1 subunit and in MMP-9, we carried out blocking experiments using anti-integrin and anti-MMP-9 antibodies (Fig. 2A). The monoclonal anti-αv antibody and polyclonal serum recognizing the β1 integrin subunit exhibited blocking of the EV22 infection at high dilutions (1:1000), whereas the monoclonal anti-β3 antibody had some effect on the virus growth only at a dilution of 1:10. The polyclonal anti-MMP-9 antibody inhibited EV22 infection but to a lesser extent than the anti-αv and -β3 antibodies. The monoclonal anti-αv antibody used had no effect on the virus growth at any dilution.

The inhibitory effect of the antibodies was also studied in CA9 infection (Fig. 2B). CA9 infection was blocked with the anti-αv and -β3 antibodies (30 and 60% inhibition, respectively) at dilutions of 1:100. The anti-β3 antibody also blocked the virus infection at the same dilution, although the effect was significantly lower. Some blocking of CA9 infection was also seen with the anti-MMP-9 antibody, but the effect vanished at the antibody dilution of 1:100. As in the case of EV22, the anti-αv antibody had no effect on the initiation of the growth cycle of CA9.

**DISCUSSION**

In this study we have applied an approach based on phage display peptide libraries to elucidate cell-surface interactions of EV22. We demonstrate that EV22 infection can be blocked with (i) the CLRSGRFC peptide found in the EV22-binding phages, (ii) an RGD-containing peptide representing the C terminus of EV22 capsid protein VP1, and (iii) an RGD-binding peptide (36). The LRSG consensus sequence, found in the EV22 binding phages, is present in the extracellular domain of integrin β1 subunit. Integrins α2β1 and α5β1 are known to bind to the RGD sequence (32). The amino acid sequence found in the EV22-
binding phages is also identical to the sequence in the matrix metalloproteinase 9 (MMP-9).

To illuminate further the role of integrins and MMP-9 in the cell-surface interactions of EV22, we carried out blocking experiments with anti-integrin and anti-MMP-9 antibodies. The monoclonal \(\alpha_v\) antibody and polyclonal \(\beta_3\) antiserum clearly inhibited the infection, whereas the anti-\(\beta_3\) antibody had a blocking effect only at high concentrations. Furthermore, anti-MMP-9 antibody also inhibited EV22 infection, although less efficiently. These results suggest that integrin \(\alpha_v\beta_3\) acts preferentially as a cellular receptor for EV22 and MMP-9 may also be involved in the receptor interactions of the virus.

CAV9-binding peptides were also selected from the phage display library, and blocking experiments were performed. Only the RGD-containing peptide clearly blocked the virus infection. Because the sequence (VWDQ) found in the CAV9 binding phage is present in the integrin \(\beta_3\) chain, we carried out virus blocking tests with a monoclonal anti-\(\alpha_v\beta_3\) antibody, but it had no effect on the virus growth (data not shown). However, the CAV9 infection was blocked by the anti-\(\alpha_v\) monoclonal antibody and rabbit antiserum recognizing the \(\beta_3\) subunit. It is possible that the peptide identified by the phage display library and the RGD-recognizing peptide both bind the RGD-containing motif in CAV9, but this interaction does not necessarily block infectivity. This phenomenon has also been observed in experiments where the motif has been deleted by trypsin treatment (8) or by mutation (10). The anti-\(\beta_3\) integrin subunit as well as the anti-MMP-9 antibody also had a blocking effect but only when used at the higher concentrations. Previously published data support the idea that at least \(\alpha_v\beta_3\) integrin plays a role in the cell-surface interactions of CAV9 (9).

The blocking experiments suggest that EV22 may bind to the \(\alpha_v\beta_3\) integrin with the RGD-containing C terminus of capsid protein VP1. In addition, our results indicate that at least one of the virus-binding sites is evidently located in the region of amino acids 103–106 (LRSG) in the \(\beta_3\) chain. This site is located near the I domain, a region in the \(\beta_3\) subunit that is known to participate in the binding to the ligand (37). However, the results do not reveal how the \(\alpha_v\) chain is involved in the virus-receptor interactions. One possibility is that in the experimental conditions the anti-\(\alpha_v\) antibody binds to the \(\alpha_v\) subunit in such a manner that sterically blocks the binding of EV22 to the \(\beta_3\) chain.

Our results raise the possibility that matrix metalloproteinase 9 (MMP-9) is involved in the cell-surface interactions of EV22 because the infection could be inhibited by the CLRSGRGCG peptide and by anti-MMP-9 antibody. The LRSGRG sequence is present in the C-terminal hemopexin domain of MMP-9 which has been shown to be important for the ability of the protein to dimerize (38). We have also independently isolated the LRSGXG motif (where X is preferably arginine) during phanning on purified MMP-9 using the same phage libraries described here. These results suggest that the LRSGGR sequence could be involved in the dimerization of MMP-9, and EV22 may be able to bind MMP-9 through the dimer interface. It has been shown recently that matrix metalloproteinase 2 interacts with integrin \(\alpha_v\beta_3\) on the cell surface (35), and the integrin can simultaneously bind to proteolyzed collagen fragments. The \(\alpha_v\beta_3\) integrin could also interact with MMP-9, and the complex might then be involved in the internalization of EV22. Alternatively, EV22 could separately interact with either \(\alpha_v\beta_1\) or MMP-9.

Three human picornaviruses (CAV9, EV22, and EV23) have a functional RGD motif; in addition, foot-and-mouth disease virus interacts with the \(\alpha_v\beta_3\) integrin using the viral RGD sequence (11, 39). In the three human viruses, the motif is located at the C terminus of the capsid polypeptide VP1. In CAV9, this region can be deleted by trypsin treatment (8) or the RGD motif mutated (10) without complete loss of infectivity indicating that the virus can use alternative pathways in its entry into the host cell. The processing of the C-terminal extension of CAV9 VP1 by proteolytic enzymes may have implications in the pathogenicity because during the infection in the gut the virus is prone to the action of intestinal proteases. Whether destruction of the RGD motif in EV22 abolishes the infectivity is, however, currently unknown.

EV22 and CAV9 compete for cell-surface binding, and the latter is known to interact with the vitronectin receptor (\(\alpha_v\beta_3\)-integrin; Ref. 9). This observation, suggesting that EV22 would also utilize an RGD-recognizing integrin on the cell surface, is strongly supported by our findings that peptides interacting specifically with the virus share sequence homology with the integrin \(\beta_3\)-subunit, and the infection is blocked by \(\beta_3\)-antibodies. Although CAV9 and EV22 exhibit similarities in the cell-surface recognition mechanisms, there are also clear differences. A monoclonal antibody that is able to block the infection

\[^3\] E. Koivunen, H. Valtanen, A. Rainsalo, C. Kantor, C. G. Gahmberg, T. Salo, Y. T. Konttinen, and T. Sorsa, submitted for publication.
caused by typical echoviruses (excluding serotypes 22 and 23) and CAV9 is unable to inhibit EV22 infectivity (40). This suggests that additional receptor activities can also be important and may be crucial in the determination of tissue tropism and pathogenesis of these viruses.

Due to the possibility of rapidly selecting large numbers of peptides that recognize target molecules, the phage display technique blocked the infectivity of the virus, the peptides identified in a similar manner by using CAV9 as a target did not inhibit the growth cycle of CAV9. This can be explained either by inefficient binding of the latter peptides or by the differences in cell-surface recognition mechanisms of these two viruses; the RGD-mediated attachment may be the major mechanism in EV22, whereas CAV9 is able to use alternative pathways in its entry as already shown by mutation analysis of the RGD-containing region in CAV9 (10). Our results clarify previously reported receptor specificities of EV22 and propose new, perhaps more complex, mechanisms for EV22 entry.

Acknowledgments—We thank Drs. Jyrki Heino, Merja Roivainen, Timo Sorsa, and Prof. Ismo Virtanen for providing the antibodies. Drs. Jyrki Heino, Merja Roivainen, Timo Sorsa, Glyn Stanway, and Prof. Ismo Virtanen for providing the antibodies. Drs. Jyrki Heino, Merja Roivainen, Timo Sorsa, Glyn Stanway, and Prof. Ismo Virtanen for providing the antibodies. Drs. Jyrki Heino, Merja Roivainen, Timo Sorsa, Glyn Stanway, and Prof. Ismo Virtanen for providing the antibodies.

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