Systematic Replacement of Amino Acid Residues within an Arg-Gly-Asp-containing Loop of Foot-and-Mouth Disease Virus and Effect on Cell Recognition*

(Received for publication, January 11, 1996, and in revised form, March 1, 1996)

Mauricio G. Mateu‡, M. Luz Valero§, David Andreuš, and Esteban Domingo¶

From the Centro de Biología Molecular “Severo Ochoa” (CSIC-UAM), Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid and the Departament de Química Orgànica, Universitat de Barcelona, 08028 Barcelona, Spain

The conserved Arg-Gly-Asp (RGD) motif found in a hypervariable, mobile antigenic loop of foot-and-mouth disease virus (FMDV) is critically involved in virus attachment to cells by binding to an integrin, probably related to αvβ3. Here we describe (i) the synthesis of 241 15-mer peptides, which represent this loop of FMDV (isolate C-S8c1) and single variants in which each amino acid residue was replaced by 16 others and (ii) the inhibitory activity of these peptides on the ability of FMDV C-S8c1 to recognize and infect susceptible cells. This approach has allowed a first detailed evaluation of the specificity of each residue within a RGD-containing protein loop on cell recognition. The results indicate that, in addition to the exquisitely specific RGD triplet, two highly conserved Leu residues located at positions +1 and +4 downstream of the RGD and, to a lesser extent, the residue at position +2 are the only critical and specific determinants within the loop in promoting cell recognition of a viral ligand. The results support the proposal that, in spite of their involvement in antibody recognition, RGD and other FMDV loop residues are remarkably conserved because of their essential role in cell recognition.

The Arg-Gly-Asp (RGD) triplet mediates cell adhesion by being a major determinant in the interaction of a number of protein ligands with cell surface receptors of the integrin superfamily (reviews in Refs. 1–5). In addition, the RGD motif mediates recognition by cells and infectivity of a variety of pathogens, including adenovirus 2 (6, 7), coxsackievirus A9 (8, 9), echovirus 22 (10), foot-and-mouth disease virus (FMDV) (11–16), and others. The use of RGD-containing peptides (e.g. Refs. 1, 2, 17, 18 and references therein), protein ligand mutants (19–22), and phage display peptide libraries (23–26) unequivocally established the primary role and high specificity of the RGD motif in ligand binding to several integrins. NMR spectroscopy and x-ray crystallography have revealed that functional RGD motifs are located at the tip of solvent-exposed, highly mobile protein loops. The motif generally adopts a turn conformation with the Arg and Asp side chains oriented outward and in opposite directions (4, 5, 27–29).

FMDV, a highly variable RNA virus of the Picornaviridae family, causes one of the economically most important diseases of farm animals (reviews in Refs. 30 and 31). FMDV is able to infect cells via a conserved RGD motif, which is recognized by one or several integrins probably related to αvβ3, the vitronec- tin receptor (15). This RGD triplet is also involved in antibody recognition of FMDV by being a critical part of a major, hypervariable antigenic site (site A) (32, 33). Site A is located in a long, solvent-exposed, and flexible loop (the G-H loop of capsid protein VP1), which could not be positioned in the structures determined for native virions (34, 35). A structure for the G-H loop was, however, elucidated upon chemical reduction of a serotype O virion (36). A similar structure has been determined for the G-H loop of a serotype C (isolate C-S8c1) virus, as reproduced by a 15-mer peptide (A15) complexed with the Fab fragment of an antivirus neutralizing antibody (33) (see Fig. 1). In both structures, the RGD motif adopts a very similar conformation, which resembles those found in other integrin ligands. Short RGD-containing peptides inhibited weakly (IC50 in the micromolar range) the infectivity and cell attachment of FMDV (12, 13, 16). Remarkably, long peptides (16–19 amino acids), which reproduced the G-H loop of serotype C viruses, inhibited very effectively FMDV infectivity (IC50 about 0.8 µM) and attachment to cells (16). The relevance of the RGD motif in cell recognition of FMDV has been confirmed using site-directed mutagenesis (14).

In addition to the RGD motif, some flanking residues play a role, either directly or most probably through modulation of the conformation of the RGD triplet, in the specific recognition of some ligands by different integrins (1, 2, 18, 37, 38). Recently, Rieder et al. (39) constructed four site-directed mutants of FMDV of serotype A, which differed in one or several VP1 residues located close to the C terminus of the RGD triplet. Some of these mutants showed differences in attachment to cells and infectivity. However, systematic amino acid replacement studies, aimed at establishing the specificity of each residue around the RGD motif on cell recognition of FMDV or any other RGD-containing ligand, are lacking. The high efficiency of type C long peptides in inhibiting cell recognition of FMDV renders them useful reagents for such studies. In this report we describe (i) the synthesis of 241 15-mer peptides, which reproduce site A of FMDV C-S8c1 and single variants of this sequence, and (ii) the systematic evaluation of the inhibitory effect of each variant peptide on FMDV infectivity. The results indicate that, in addition to the RGD triplet, two highly conserved Leu residues located at positions +1 and +4 downstream of the RGD motif and the residue at position +2 are the

*Work in Madrid was supported by Dirección General de Investigación Científica y Técnica (DGICYT) Grants PB94-0034-C02-01 and PB94-0845 and Fundación Ramón Areces; work in Barcelona was supported by DGICYT Grants PB91-0266 and PB94-0845 and Fundación Ramón Areces. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Predoctoral fellow of the Ministerio de Educación y Ciencia, Spain.

¶ To whom correspondence should be addressed. Tel.: 34-1-3978485; Fax: 34-1-3974799.

1 The abbreviations used are: FMDV, foot-and-mouth disease virus; PBS, phosphate-buffered saline.
only critical and specific determinants within site A in promoting cell recognition of a type C FMDV.

EXPERIMENTAL PROCEDURES

Virus and Cell Lines—FMDV C-S8c1 is a plaque-purified virus clone derived from C-S8 (C1 Santa Pau-Sp/70), a representative isolate of serotype C viruses from Europe (40). The BHK-21 cell line was obtained from the ATCC.

Peptide Synthesis—A group of 240 analogs of the reference peptide A15 (YTASARGDLAHLTTT) was prepared by systematic single-residue replacements at every position with 16 of the genetically coded amino acids (all except Cys, Met, and Trp (37)). The peptides were synthesized as C-terminal carboxamides by simultaneous solid-phase procedures on PAL-MBHA resins (41) using Fmoc (N-9-fluorenylmethoxycarbonyl) chemistry in an Abimed AMS242 instrument. Peptide resins were deprotected and cleaved by treatment with trifluoroacetic acid/triethylamine/water (95:2.5:2.5, 2 h, room temperature). The peptides were deprotected and cleaved by treatment with trifluoroacetic acid/triethylamine/water (95:2.5:2.5, 2 h, room temperature). The peptides were then taken up in 0.1M acetic acid and lyophilized. The peptides used showed contents >80% in the target sequence, as judged by analytical high pressure liquid chromatography. The identity of the synthetic peptides was further confirmed by amino acid analysis and electrospray mass spectrometry, which were consistent with the expected compositions. Stock solutions of peptides in phosphate-buffered saline (PBS, complete) and soluble peptide concentration were rechecked by amino acid analysis. The pH of the stock solutions was also checked and, if necessary, adjusted to neutrality.

Cyclization of FMDV Peptides—The head-to-tail cyclic version of the reference peptide, cyclo-(YTASARGDLAHLTTT), was synthesized by solution cyclization of a suitable, fully protected precursor (42). The synthesis of peptides A21 (YTASARGDLAHLTTTHARHLP) and A22Cys (TTCYASARGDLAHLTTTCHL) and cyclization via a disulfide bond of the latter peptide have been described (43).

Inhibition of Virus Infectivity by Synthetic Peptides—The assays were carried out essentially as described (16). Briefly, cell monolayers were washed with cell culture medium (Dulbecco’s modified Eagle’s medium) and incubated for 45 min at room temperature with PBS as a control or with several 10-fold dilutions of A15 or variant peptides in PBS. The final concentrations of peptide ranged from 0.1 μM to 1 mM, depending on the variant tested. A defined amount of FMDV C-S8c1 diluted in PBS was then added (about 40–100 plaque-forming units), and the cells were further incubated for 45 min at room temperature. After washing with Dulbecco’s modified Eagle’s medium, the cells were covered with an agar overlay and incubated for 36 h at 37 °C. The monolayers were fixed and stained and the virus plaques counted. The IC50 value is the concentration of peptide that produces a 50% inhibition of virus infectivity (Fig. 2A). The IC50 was determined for each variant peptide by dividing its IC50 value by the IC50 of the homologous (A15) peptide. Each experiment was carried out in duplicate. Because the different concentrations of each peptide were tested in entirely independent experiments, the dose-response curves obtained for the peptides further confirmed that no gross experimental errors occurred. Reproducibility of the assay was checked by including in each experiment several concentrations of peptide A15 as a positive control and a high concentration (100 μM) of an inactive variant (A15 with Glu substituted for Asp-143) (16) as a negative control. To provide another independent confirmation of the values obtained, an additional series of experiments was carried out in duplicate using the approximate IC50 of each peptide obtained in the first series.

RESULTS

Inhibition of FMDV Infectivity by Linear and Cyclic Synthetic Peptides, Which Represent Antigenic Site A—Peptide A15 precisely reproduces antigenic site A (VP1 residues 136–150) of FMDV C-S8c1 with regard to reactivity with monoclonal and polyclonal anti-virus antibodies (Refs. 44 and 45, and references therein). Because of the availability of the three-dimensional structure of peptide A15 complexed to an antibody (Fig. 1), we have chosen this peptide as a model of the G-H loop to study the role of amino acid residues within this segment in determining the ability of FMDV to recognize and infect susceptible cells. Consistently with our previous results using related peptides of similar length (16), A15 very efficiently inhibited the infectivity of the homologous FMDV C-S8c1 (IC50 = 0.5 μM) (Fig. 2A).

The ability of short RGD-containing synthetic peptides to inhibit binding of protein ligands to integrins is sometimes significantly modulated by cyclization (e.g. Refs. 37 and 46). We have tested the effect of cyclization via an amide bond linking the N- and C-terminal residues of A15 on the inhibitory capacity of the peptide in virus infectivity assays (Fig. 2B). Interestingly, such head-to-tail cyclization led to complete loss of in-

![Fig. 1. Stereo view of the three-dimensional structure of peptide A15 (YTASARGDLAHLTTT; residues 136–150 of FMDV capsid protein VP1) as determined using a complex with an antivirus neutralizing antibody (33). The figure was produced from the atomic coordinates of the complex using the RasMol program (64). The RGD motif (residues 141–143), Leu-144, and Leu-147 are labeled.](http://www.jbc.org/)

![Fig. 2. Inhibition of FMDV C-S8c1 infectivity by linear and cyclic homologous peptides. A, inhibition by peptide A15. Values indicated at concentrations of 0.1, 1, 10, and 100 μM peptide are the average of 8, 10, 17, and 2 determinations, respectively, obtained in independent experiments, each carried out in duplicate. Standard deviations are indicated. B, inhibition by linear and cyclic peptides. The experiment was carried out in duplicate. Duplicates differed 0–15% in plaque reduction values. ●, linear A15; ○, head-to-tail cyclic A15; ▲, linear A21; Δ, linear A22Cys; △, cyclic disulfide A22Cys (see "Experimental Procedures").](http://www.jbc.org/)
hibitory activity. This result suggests that an adequate conformation of the RGD motif is critical for cell recognition of the FMDV loop and that this particular cyclization prevented adoption of such conformation by the peptide. The same cyclization also abolished reactivity of the peptide with anti-FMDV antibodies.\textsuperscript{2} In contrast, disulfide cyclization of a related peptide (peptide A22Cys) had no effect on its antigenicity (43) and on its inhibitory activity in infectivity assays (Fig. 2B).

\textsuperscript{2} M. L. Valero and M. G. Mateu, unpublished results.
The capacity of every possible singly substituted peptide representing variant forms of the G-H loop (residues 136–150, including the RGD) to inhibit FMDV C-S8c1 infectivity was compared with that of the homologous A15 peptide. The only exceptions were Cys, Met, and Trp, which were not included to avoid ambiguity in interpreting the results due to oxidation (37). The results obtained using the complete replacement set are summarized in Figs. 3 and 4 and described below.

Evaluation of the Effect of Single Amino Acid Replacements within the RGD Triplet on the Capacity of Peptide A15 to Inhibit FMDV Infectivity—Systematic replacements at the RGD triplet (Figs. 3 and 4) clearly showed that almost any single substitution, either of Arg-141 or Asp-143, increased the IC50 by much more than 2000-fold relative to that of the homologous peptide. Exceptions included replacement of Arg-141 by Ser, Gly, or Gln and of Asp-143 by Asn (relative IC50 about 200) on the inhibitory capacity of the peptide.

In contrast to the high tolerance for replacements within the hypervariable stretches, three of the four residues following the RGD triplet (LAHL, positions 144–147; Fig. 5), were not generally replaceable in this assay (Figs. 3 and 4). In particular, most replacements of residues Leu-144 or Leu-147, which are highly conserved in most FMDV serotypes and also in other RGD-containing viruses (Fig. 5; see “Discussion”), drastically decreased the inhibitory effect of the A15 peptide. Replacements of Leu-144 by most residues led to relative IC50 values from about 40 to higher than 2200. Some chemically conservatively substituted (Ile, Val), and also Gln had less of an effect, with a relative IC50 of about 15. The only replacements accepted at this position were the basic residues Lys and Arg. Leu-147 was not replaceable by most residues (relative IC50 from about 30 to 900). Ala and Tyr and, again, Arg and Lys had a significant but not dramatic negative effect at this position (relative IC50 from 10 to 25). Only some chemically conservative substitutions (Ile, Val, and Phe) were acceptable. Half of the replacements of Ala-145 also led to substantial negative effects, which were, however, generally milder (relative IC50 from 6 to 65) than those at positions 144 and 147. In contrast, replacements of His-146, located within the same stretch, had either a minor effect or no significant effect at all.

**DISCUSSION**

The efficient, specific inhibitory effect on FMDV infectivity of A15 and related peptides is primarily due to inhibition of virus binding to cells (16). The use of long peptides permitted the systematic probing of the effect of replacements along most of the length of the RGD-containing protein loop under study. This FMDV loop is considered to act as an essentially self-contained unit, which is loosely connected to the rest of the viral capsid (36, 45, 47). For example, single and multiple substituted site A peptides faithfully mimic reactivity with monoclonal antibodies and antigenic specificity of the homologous type C viruses (44, 48).3 Thus, functional studies with variant peptides representing this loop receive further validation. However, some caution is needed in interpreting the results; the effect of multiple substitutions on FMDV site A antigenicity is not necessarily additive, and it depends on the sequence context (49); thus, double or multiple replacements may have unexpected effects also on cell recognition.

Any replacement within the RGD triplet, especially of Arg and Asp, decreased dramatically recognition of peptide A15 by the FMDV receptor. The protruding Arg and Asp side chains of this RGD motif have been shown to directly interact with residues in the concave paratope of an antivirus neutralizing antibody (33), and they may similarly interact with the cell receptor. The presence of any side chain at the central position of this triplet could sterically interfere with receptor binding and/or impair adoption of an adequate RGD conformation. The much greater effect of the methyl group in RAD, relative to those of the larger side chains in RSD, RTD, RND, and RYD, and the most drastic effect of Pro in RD supports the latter possibility. A role of the conformation adopted by this RGD motif in receptor recognition is supported by the drastic effect of cyclization of peptide A15 on its inhibitory capacity. It may be noticed also that the functionality of SGD, GGD, KGD, and RGN, though very low, was higher than that of any other peptide substituted at Arg or Asp. This may have some signif-

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3 M. G. Mateu, unpublished results.
4 These observations may favor an indirect role on receptor charge between the parent and the substituted amino acid (Fig. 4), relate well with similarities in hydrophobicity, size, and/or replaceability did not correlate with the possibility that some FMDV variants could be recognized by different integrins and that immune pressure may occasionally lead to a switch in the putative integrin recognized by the virus. Early experiments indeed suggested that some FMDV of different serotypes could bind to different receptors (63).

Recent modeling of peptide variants and circular dichroism experiments has suggested a role for these residues in the recognition of an RGD-containing virus.

Acknowledgments—We gratefully acknowledge J. Camargo for supplying the cyclic disulfide peptide A22Cys and I. Fita, E. Giralt, J. Hernandez, and N. Verdaguer for helpful discussions.

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J. Biol. Chem. 1996, 271:12814-12819.
doi: 10.1074/jbc.271.22.12814

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