Antibody and host cell recognition of foot-and-mouth disease virus (serotype C) cleaved at the Arg-Gly-Asp (RGD) motif: a structural interpretation

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Foot-and-mouth disease virus (FMDV) of serotype C (isolate C-S8c1) was cleaved in situ by trypsin at the Arg-Gly-Asp (RGD) motif, which is involved both in attachment of FMDV to cells and in recognition of a major antigenic site (site A) by antibodies. Though 99.4% of the RGD moieties were cleaved, the virus remained infectious. A synthetic peptide which represented the sequence of the VP1 G–H loop of C-S8c1, including the RGD motif, greatly inhibited FMDV attachment to cells. The same peptide inhibited, very effectively and to the same extent (50% inhibition at about 1 μM), the infectivity of both intact and trypsin-treated virus. Replacement of Asp with Glu at the RGD motif abolished the inhibitory effects of the peptide. Thus, the RGD motif is involved in the infectivity of both intact and RGD-cleaved serotype C FMDV.

Trypsin treatment did not affect the reactivity of the virus with some monoclonal antibodies (MAbs) directed to site A whose epitopes involve mainly residues contiguous to the cleaved bond, but diminished the reactivity with site A MAbs whose epitopes include the RGD sequence and flanking residues. However, high concentrations of any site A MAb tested neutralized close to 100% of the infectious trypsin-treated virus. We propose that, in spite of covalent cleavage, the high number of intramolecular non-covalent interactions observed within the G–H loop of FMDV C-S8c1 (complexed to antibody) may hold the RGD in a nearly correct conformation and allow—a albeit with reduced affinity—antibody and cell receptor recognition of RGD-cleaved FMDV.

Introduction

Foot-and-mouth disease virus (FMDV) is a picornavirus which causes an economically important disease of cattle and other cloven-hoofed animals (reviews in Pereira, 1981; Domingo et al., 1990). FMDV is highly diverse antigenically and includes seven serotypes (A, O, C, Asia1, SAT1, SAT2 and SAT3) and multiple variants. Early experiments showed that trypsin treatment of FMDV of serotype O dramatically decreased the infectivity and immunogenicity of this virus (Wild & Brown, 1967; Wild et al., 1969). The trypsin-sensitive, immunogenic site involves residues around positions 135–160 of capsid protein VP1 (Strohmaier et al., 1982; Pfaff et al., 1982; Bittie et al., 1982; reviewed in Domingo et al., 1990). This site (antigenic site A) is located within an exposed and flexible loop (the G–H loop of VP1) (Acharya et al., 1989; Logan et al., 1993) which includes a highly conserved RGD (Arg-Gly-Asp) motif. The RGD sequence is involved in attachment of FMDV to cells, as recently documented by site-directed mutagenesis of an infectious clone of FMDV of serotype A (Mason et al., 1994). In addition, short RGD-containing synthetic peptides inhibited, albeit to a low extent, the attachment of FMDV serotypes O (Fox et al., 1989) and A (Baxt & Becker, 1990) to cells. The RGD motif is also part of site A epitopes recognized by anti-FMDV neutralizing antibodies (Novella et al., 1993; Verdaguier et al., 1995). The C terminus of VP1 includes a minor antigenic site (site C) which in serotype O forms with site A a discontinuous antigenic domain (Xie et al., 1987; Parry et al., 1989). In this serotype, the VP1 C terminus may also be involved in cell attachment (Fox et al., 1989). In contrast to serotype O, in FMDV of serotype C sites A and C appear to be topologically and functionally independent (Lea et al., 1994). Trypsin-
insensitive antigenic sites – which are independent of sites A and C – have been characterized in FMDV serotypes O (Xie et al., 1987; Kitson et al., 1990), A (Thomas et al., 1988; Baxt et al., 1989; Bolwell et al., 1989; Saiz et al., 1991) and C (Lea et al., 1994).

The drastic effect of trypsin treatment on the infectivity and immunogenicity of type O virus was explained as being due to cleavage at several exposed basic residues, leading to the loss of a 16 amino acid segment within the G–H loop (residues 138–154, including the RGD motif) and of the C terminus (residues 201–213) of VP1 (Strohmaier et al., 1982). Thus, trypsin treatment entirely removed antigenic sites A and C, including the cell attachment site. Trypsin treatment drastically reduced the infectivity of FMDV serotype A also, but not the infectivity or immunogenicity of FMDV serotype C (Rowlands et al., 1971). Recently, a structure for the G–H loop (residues 136–150, Fig. 1a) of a serotype C FMDV (isolate C-S8c1) complexed to a neutralizing monoclonal antibody (MAb SD6) has been elucidated (Verdaguer et al., 1995). The present study provides a molecular interpretation of the preserved antigenicity and infectivity of FMDV type C cleaved by trypsin at the critical RGD motif.

Methods

**Viruses and cells.** Plaque-purified FMDV isolate C-S8c1 has been described previously (Sobrino et al., 1983). Encephalomyocarditis virus (EMCV) was kindly provided by L. Carrasco (CBMSO, Madrid).

**MAbs and synthetic peptides.** The MAbs used in this work are directed to antigenic sites A, C or D of type C FMDVs and the corresponding epitopes, except that of MAb 7LA5-1, have been previously characterized (Mateu et al., 1987, 1989, 1990, 1992, 1994; Novella et al., 1993; Lea et al., 1994). MAb 7LA5-1 was provided by A. Alonso from the Pan American Foot-and-Mouth Disease Center (CPFA, Rio de Janeiro, Brazil). This MAb belongs to the IgG1 isotype and reacts very weakly with VP1 in enzyme-linked immuno-electrotransfer blot (EITB) assays. The epitope recognized by MAb 7LA5-1 has been located within site A by using synthetic peptides and MAAb-resistant mutants (J. Hernández and others, unpublished results).

A former hybridoma from the CPFA, designated 7LA5 and assigned to the IgG3 isotype by the CPFA (da Silva et al., 1993), was found to be unreactive both with VP1 and with synthetic peptides (Mateu et al., 1988, 1990). This former preparation from CPFA could correspond to a mixed hybridoma population which included 7LA5-1. Peptides were synthesized by solid-phase procedures as previously described (Carreño et al., 1992; Novella et al., 1993), and were at least 95% pure, as determined by HPLC. The peptides used in the present study are listed in Fig. 1b.

**Trypsin treatment of virus.** FMDV C-S8c1 virions were purified by sucrose gradient centrifugation as described (Díez et al., 1990). Purified virions were incubated for 1 h at 37 °C in sucrose–TNE buffer (50 mM-Tris–HCl pH 7.5, 5 mM-EDTA, 100 mM-NaCl) in the presence of either active trypsin (Sigma) (trypsin-treated virus, TT) or of soybean trypsin inhibitor (Sigma)-inactivated trypsin (mock-treated virus, MT) at a virus:enzyme ratio of 50:1 (w/w). Then, a twofold excess of soybean trypsin inhibitor was added to the mixtures and further incubated for 5 min at 37 °C. MT and TT virus were dialysed against TNE buffer and purified by centrifugation in a sucrose gradient as above. Viruses were analysed by SDS–PAGE using 8 M-urea–15% or 17% polyacrylamide gels and protein bands were visualized by Coomassie blue or silver staining. The amounts of viral proteins and of fragments were quantified by densitometry of the stained gels.

**Peptide sequencing.** Purified TT virions (50 µg) were electrophoresed in a 8 M-urea–17% polyacrylamide gel, and the proteins were electrotransferred to ProBlott membranes (Applied Biosystems) according to the manufacturer’s instructions. The VP1 fragments generated by trypsin digestion were visualized by Coomassie blue staining. The bands of interest were excised from the membrane and the N-terminal sequences determined by automated Edman degradation in a gas-phase protein sequencer (Applied Biosystems).

**Immunoassays.** Enzyme immunodot (EID) and EITB assays were done as described (Mateu et al., 1987, 1988). A modified quantitative EITB assay was carried out to determine residual uncleaved VP1 in TT virus. Different amounts (up to 10 µg VP1) of MT and TT virus were incubated with MAbs 6F6, 7LA5 (Mateu et al., 1987) and 7CA11 (Mateu et al., 1988) to quantify VP3 and VP1, respectively. After incubation with a second antibody conjugated to peroxidase (goat anti-mouse IgG-peroxidase, Bio-Rad), the reaction was developed by luminescence using ECL Western blotting detection reagents (Amersham), according to the manufacturer’s instructions.

**Virus neutralization assays and inhibition of infectivity.** Plaque-reduction neutralization assays were carried out as described (Mateu et al., 1987). Inhibition of virus infectivity by synthetic peptides was analysed by plaque-reduction assays using a general experimental design similar to that of Roivainen et al. (1991). The concentration of a peptide required to inhibit 50% of the viral infectivity was given as its IC50 value.

**Inhibition of FMDV attachment to cells.** FMDV C-S8c1 virions were metabolically labelled with [35S]methionine (Amersham) as described (De la Torre et al., 1988), and purified by sucrose gradient centrifugation. Attachment of [35S]methionine-labelled FMDV to BHK-21 cells and inhibition of attachment by synthetic peptides was carried out essentially as described by Fox et al. (1989). The concentration of a peptide required to inhibit attachment to 50% was given as its IC50 value.

Results

**Inhibition of attachment of a serotype C FMDV to cells by RGD-containing synthetic peptides.** A peptide which mimicked the amino acid sequence of the VP1 G–H loop (including the RGD motif) of C-S8c1 (peptide 3, Fig. 1) very effectively inhibited attachment of FMDV serotype C to cells (IC50 = 8 µM) (Fig. 2). The same effect was observed with a peptide of comparable length which mimicked the sequence of a different type C isolate (C4 Tierra del Fuego-Arg/66; data not shown). Shorter peptides which contained RGD either alone (peptide 1) or in the sequence context of FMDV serotype C (peptide 2) (Fig. 1) had much less effect (Fig. 2). As expected, a control peptide without the RGD sequence (peptide 5, Fig. 1) did not inhibit attachment (Fig. 2). A
Fig. 1. (a) VP1 G-H loop of FMDV C-S8c1. The numbering refers to amino acid positions in VP1. The RGD motif is boxed. The underlined segment corresponds to antigenic site A. The approximate locations of continuous epitopes within site A are also indicated. Each epitope is designated by the name of the corresponding neutralizing MAb. A thick line delimits its minimal location as deduced by the use of synthetic peptides and variant viruses (Mateu et al., 1989, 1990, 1992, 1995b; Carreño et al., 1992; Novella et al., 1993). A thin line indicates some effect on binding of the corresponding residues. (b) Synthetic peptides used in the present study. The C-terminal cysteine in peptides 3, 4 and 5 is not present in the authentic VP1 sequence.

Fig. 2. Inhibition of attachment of FMDV C-S8c1 to BHK-21 cells by synthetic peptides. The amount of 35S-labelled virus attached to cells is plotted against the concentration of competitor peptide: •, peptide 1; O, peptide 2; △, peptide 3; ◊, peptide 4; □, peptide 5 (Fig. 1b). Standard deviations are indicated. The amount of 35S-labelled virus attached in the absence of any competitor peptide corresponded to 880 c.p.m.

substitution at the RGD (Asp to Glu) in peptide 3 (peptide 4, Fig. 1) completely abolished the inhibitory effect (compare peptides 3 and 4 in Fig. 2). Thus, the RGD motif is involved in the attachment of serotype C

FMDVs to susceptible cells, as previously shown for serotypes O and A.

The RGD sequence of a serotype C FMDV is cleaved in situ by trypsin without loss of any peptide segment

FMDV C-S8c1 was treated with trypsin as described in Methods. As a control, an aliquot of the same virus was subjected to the same treatment, except that inactivated trypsin was used. Both the mock-treated (MT) and the trypsin-treated (TT) viruses were purified and analysed by SDS–PAGE. Silver staining of gels showed that only VP1 (apparent M, 27000) of TT virus had been cleaved (more than 99%), yielding two fragments (L and S)
Table 1. Infectivity of trypsin-treated FMDV C-S8c1

<table>
<thead>
<tr>
<th>Experiment*</th>
<th>MT virus titre (p.f.u./ml)</th>
<th>TT virus titre (p.f.u./ml)</th>
<th>Percentage infectivity† (TT/MT virus)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>$1.9 \pm 0.3 \times 10^9$</td>
<td>$1.2 \pm 0.2 \times 10^8$</td>
<td>6.2%</td>
</tr>
<tr>
<td>2</td>
<td>$4.4 \pm 0.2 \times 10^8$</td>
<td>$4.4 \pm 0.1 \times 10^7$</td>
<td>10.0%</td>
</tr>
<tr>
<td>3</td>
<td>$5.1 \pm 0.2 \times 10^8$</td>
<td>$4.8 \pm 0.1 \times 10^7$</td>
<td>9.4%</td>
</tr>
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* Different virus preparations were used in each experiment. Titres correspond to an average of four (experiment 1) or three (experiments 2 and 3) determinations. The corresponding standard deviations are also indicated. Titrations were carried out using the same amount (in protein weight) of purified MT and TT viruses.
† Percentage infectivity of the TT virus relative to the MT virus.

of apparent $M_r$ about 17000 and 10000 (Fig. 3). Quantitative EITB assays showed that about 99.4% of the VP1 in TT virus was cleaved in this preparation. A significantly more extensive digestion could not be achieved, even with a 10-fold higher concentration of trypsin and longer incubation times (data not shown).

To determine the VP1 site cleaved by trypsin, the amino-terminal sequences of the long and the short polypeptide fragments derived from VP1 were determined. The sequences obtained were XTTTG and GDLAHLT, respectively (in which X corresponds to an undefined amino acid). Alignment with the amino acid sequence of VP1 showed that the two fragments obtained corresponded to the N- and C-terminal parts, respectively, of VP1, and that trypsin cleavage occurred between the arginine and the glycine residues of the RGD motif (compare Fig. 1 a). The sequencing result is consistent with the apparent size of the fragments. The Arg residue closest to the RGD on the N-side is located 17 residues away in the sequence, and is not exposed on the capsid of C-S8c1; the last exposed Arg, and the last Lys to the N-side of the RGD, occur 27 and 32 residues, respectively, from the cleavage site detected (Lea et al., 1994; Mateu et al., 1994). In addition, the short fragment of VP1 reacted in EITB assays with MAb 7JA1, which recognizes a continuous epitope within the C-terminal residues (195–209) of VP1 (results not shown). These observations indicate that, in contrast to FMDV type O, trypsin cleavage of FMDV type C occurred exclusively at the RGD motif, without loss of any peptide fragment.

Cleavage of the RGD motif does not abolish infectivity of a serotype C FMDV

Trypsin treatment decreased the infectivity of the TT virus population to about 10% of the control MT virus (Table 1). However, as documented above (Fig. 3), only about 0.6% of VP1 remained uncleaved in the same preparations used for infectivity measurements. Thus, type C virion populations with most (or all) RGD moieties cleaved retained a substantial amount of infectivity, in agreement with early results (Rowlands et al., 1971; see also Discussion).

Inhibition of infectivity of intact and trypsin-treated serotype C FMDV by RGD-containing synthetic peptides

To test whether the infectivity of TT virus C-S8c1 cleaved at the RGD sequence was due to an entry mechanism different from that mediated by RGD, we quantified the inhibition of the infectivity of TT and MT virus by synthetic peptides containing the RGD sequence. The results with intact virus (Fig. 4a) were similar to those obtained with the same virus in cell attachment inhibition assays: 50% inhibition of C-S8c1 infectivity was achieved at concentrations of peptide 3 (Fig. 1b) of only 0.8 μM, and replacement of Asp by Glu at the RGD completely abolished the inhibitory effect. The effect was specific also in that preincubation of FMDV with RGD-containing peptide, followed by dialysis to eliminate the peptide, did not diminish the

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Fig. 4. Inhibition of infectivity of FMDV C-S8c1 virions (a) and of MT and TT C-S8c1 virions (b) by synthetic peptides in a plaque assay. The percentage reduction of virus plaques is plotted against the peptide concentration. Symbols for the different peptides are: x, peptide 1; o, peptide 2; △, peptide 3; ▽, peptide 4; □, peptide 5 (Fig. 1b). In (b), open and closed symbols correspond to MT and TT viruses, respectively. Each value is an average of three determinations; standard deviations are indicated.
Cleavage at the RGD sequence does not abolish reactivity with MAbs directed against antigenic site A of a serotype C FMDV

The reactivity of MT and TT viruses with 21 MAbs was studied by immunodot and neutralization assays. The MAbs used recognize either continuous epitopes within either antigenic site A (compare Fig. 1a) or site C, or discontinuous epitopes within antigenic site D (which involves residues of VP1, VP2 and VP3 near the capsid 3-fold axis). Such epitopes have been previously characterized (Mateu et al., 1990; Lea et al., 1994). As expected, any MAb directed to site C or D reacted to a similar extent with MT and TT viruses in immunodot assays (Fig. 5). In contrast, MAbs SD6, 5A2, 7FC12, 4C4 and 6D11, directed against site A epitopes which involve the RGD sequence and residues at both sides of this triplet (Mateu et al., 1989, 1990, 1992 and unpublished results; Novella et al., 1993; Verdaguerr et al., 1995) had no detectable reactivity with TT virus (Fig. 5). Interestingly, MAbs 7JD1, 7CA11 and 4G3, which recognize site A epitopes which essentially involve residues at the C terminus of the cleavage point (Mateu et al., 1990, 1992 and unpublished results; Novella et al., 1993), and MAbs 7AH1 and 7LA5, which are also directed to site A, reacted strongly and to the same extent with MT and TT viruses (Fig. 5). Results of virus neutralization assays (Fig. 6) were similar to those of EID assays. However, the more sensitive neutralization assay showed that infectious TT virus can be neutralized close to 100 % by high concentrations of any tested site A MAb (Fig. 6a).

Thus, cleavage at RGD within antigenic site A of C-S8c1 affects only a part of the site A epitopes tested and decreases, but does not abolish, recognition of such epitopes by MAbs.

Discussion

The strong inhibition of FMDV serotype C infectivity and cell attachment by RGD-containing peptides confirms that the RGD motif participates in recognition of this FMDV serotype by the cell receptor(s). Remarkably, 50 % inhibition was achieved at concentrations in the micromolar range, using long RGD peptides (16–19 amino acids) which mimicked the VP1 G–H loop of type C viruses. In contrast, long RGD peptides which infectivity. Also, no RGD peptide inhibited EMCV infectivity (results not shown).

The RGD-containing peptides inhibited TT and MT virus infectivity to similar extents (Fig. 4b). Thus, the results suggest that FMDV virions with their RGD moieties extensively cleaved (but not deleted) are able to infect cells via the RGD motif, as do intact virions.
Fig. 6. Neutralization of infectivity of MT and TT viruses by MAbs directed to antigenic sites A and D. (a) Neutralization by three representative MAbs which recognize sites A (SD6 and 7JD1) or D (2E5). For each MAb, the percentage reduction in the number of plaques obtained in a plaque assay is plotted against the log of the inverse of the dilution of supernatant of hybridoma culture used in the assays. ○, MT virus; ●, TT virus. Equal amounts of infectious MT and TT viruses were used. (b) Summary of neutralization by all tested MAbs. Values for TT virus are relative to those of intact (MT) virus and were derived from assays similar to those in (a). A concentration of MAb which neutralized 90% of the MT virus plaques neutralized more than 70% (■), more than 30% (□) or less than 30% (□) of the TT virus plaques. MAb 7JA1, which recognizes site C, is very weakly neutralizing (Mateu et al., 1988) and was not tested.

represented the sequence of a serotype O FMDV did not inhibit virus attachment to cells (Fox et al., 1989). Also, short RGD peptides, including those derived from type O (Fox et al., 1989), A (Baxt et al., 1990) or C (this work) were about 100- to 1000-fold less efficient than type C long peptides in inhibiting FMDV recognition by host cells. Such differences may be due to a sequence-dependent tendency of long type C peptides to adopt a biologically relevant conformation. It is remarkable that such type C peptides accurately mimic the antigenic specificity of the corresponding variant viruses (Mateu et al., 1989, 1992).

RGD-containing peptides also inhibit coxsackie A9 virus infectivity, which is mediated by a RGD motif recognized by integrin αvβ3 (Roivainen et al., 1991, 1994). This same integrin has been involved in cell recognition of FMDV (Berinstein et al., 1995). RGD peptides had no effect on the infectivity of coxsackie A9 virus which had been cleaved at the RGD, which suggests that such cleaved viruses are able to infect cells via a second, RGD-independent mechanism (Roivainen et al., 1991). In contrast, our results indicate that FMDV type C extensively cleaved at RGD still uses a RGD-dependent mechanism to infect cells. One possibility to explain the substantial infectivity of the TT FMDV type C population is the presence of one or very few uncleaved RGD moieties in a fraction of the virions. Though we do not exclude this explanation, we consider it unlikely because (i) entry of picornaviruses to cells may require binding to several receptor molecules (Rueckert, 1990); (ii) uncleaved RGD moieties were resistant to extensive trypsin treatment, and they might also be inaccessible to the cell receptor (e.g., due to attached membrane fragments; see Racaniello, 1990); (iii) a similar treatment with trypsin drastically decreased the infectivity of type O virus (Rowlands et al., 1971). An alternative explanation may be provided by the conformation adopted by the FMDV loop. The structure of a complex between the Fab fragment of anti-FMDV MAb SD6 and the 15-mer peptide which represents the G–H loop of C-SS1 (positions 136–150, Fig. 1a) has been determined (Verdaguer et al., 1995). In the peptide the RGD adopts an open turn conformation, similar to that observed in the G–H loop of chemically reduced type O FMDV and in integrin-binding proteins (Logan et al., 1993). The conformation adopted in the G–H loop by the RGD and flanking residues may be restricted by the high number of hydrogen bonds and other non-covalent intramolecular interactions observed within the loop (Verdaguer et al., 1995; Mateu, 1995). Induced fit of a cleaved RGD to achieve the relevant conformation would entail a higher entropy cost; however, the non-covalent intraloop
interactions formed may hold the cleaved attachment site in a near-correct conformation and allow cell recognition without a high energetic cost. This may be reflected in the substantial infectivity of the RGD-cleaved virus population.

Though it is now clear that a substantial fraction of antibodies elicited by FMDV in natural hosts is not directed to site A (Feigelstock et al., 1992; Mateu et al., 1994, 1995a), about half of the neutralizing activity in sera of swine infected or vaccinated with FMDV C-S8c1, on average, corresponds to antibodies against site A (Mateu et al., 1995a). Such antibodies, and the site A MAbs used in the present study, generally recognize similar antigenic features (Mateu et al., 1995b). Thus, it could be expected that cleavage of the RGD would affect virus antigenicity and immunogenicity. However, in spite of covalent cleavage at the RGD, FMDV of serotype C conserves, in addition to sites C and D, part of the antigenicity of site A. Noncovalent interactions within the loop, as discussed above, may be responsible for such antigenic preservation.

It has been shown that some poliovirus variants can be cleaved at a major antigenic site by intestinal proteases, with a complete alteration of the immunodominance of different sites (Roivainen et al., 1990a, b). We have observed trace amounts of cleaved VP1 in FMDV grown in cell culture. Thus, cleavage of RGD might also occur in vivo and be biologically relevant by altering recognition of infectious FMDV by a subset of antibodies. This point is under investigation. The observations reported here validate further studies on the interaction between FMDV variants and the cell using substituted synthetic peptides, and may also facilitate isolation of the FMDV receptor(s) – probably one or several integrins (Bernstein et al., 1995) – by affinity chromatography (Roivainen et al., 1994).

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