Tolerance to mutations in the foot-and-mouth disease virus integrin-binding RGD region is different in cultured cells and *in vivo* and depends on the capsid sequence context

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Engineered RNAs carrying substitutions in the integrin receptor-binding Arg-Gly-Asp (RGD) region of foot-and-mouth disease virus (FMDV) were constructed (aa 141–147 of VP1 capsid protein) and their infectivity was assayed in cultured cells and suckling mice. The effect of these changes was studied in the capsid proteins of two FMDVs, C-S8c1, which enters cells through integrins, and 213hs$^-$, a derivative highly adapted to cell culture whose ability to infect cells using the glycosaminoglycan heparan sulfate (HS) as receptor, acquired by multiple passage on BHK-21 cells, has been abolished. The capsid sequence context determined infectivity in cultured cells and directed the selection of additional replacements in structural proteins. Interestingly, a viral population derived from a C-S8c1/L144A mutant, carrying only three substitutions in the capsid, was able to expand tropism to wild-type (wt) and mutant (mt) glycosaminoglycan-deficient CHO cells. In contrast, the 213hs$^-$ capsid tolerated all substitutions analysed with no additional mutations, and the viruses recovered maintained the ability of the 213hs$^-$ parental virus to infect wt and mt CHO cells. Viruses derived from C-S8c1 with atypical RGD regions were virulent and transmissible for mice with no other changes in the capsid. Substitution of Asp143 for Ala in the C-S8c1 capsid eliminated infectivity in cultured cells and mice. Co-inoculation with a neutralizing monoclonal antibody directed against the type C FMDV RGD region abolished infectivity of C-S8c1 virus on suckling mice, suggesting that FMDV can infect mice using integrins. Sequence requirements imposed for viral entry *in vitro* and *in vivo* are discussed.

INTRODUCTION

Foot-and-mouth disease virus (FMDV) is a member of the family *Picornaviridae* and is the aetiological agent of a highly contagious and severe disease of cloven-hoofed animals (Domingo *et al.*, 1990; Pereira, 1981; Sáiz *et al.*, 2002). The mature virus particle consists of a non-enveloped icosahedral capsid enclosing a single-stranded, positive-sense RNA genome of about 8.5 kb. The capsid is composed of 60 copies of four virus-encoded structural proteins, VP1–VP4. A major structural feature of the outer capsid surface of the virion is a long and exposed flexible loop, the G–H loop of VP1, which includes a widely conserved Arg-Gly-Asp (RGD) motif (Acharya *et al.*, 1989; Fry *et al.*, 2005; Logan *et al.*, 1993). This loop is highly immunogenic and constitutes one of the major antigenic sites of FMDV involved in neutralization of viral infectivity (Mateu & Verdaguer, 2004).

The FMDV RGD motif mediates interaction with RGD-dependent integrins including $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_6$ and $\alpha_v\beta_8$ used as receptors to initiate infection on cultured cells (Berinstein *et al.*, 1995; Jackson *et al.*, 2000b, 2002, 2004; Neff *et al.*, 2000). Genetically engineered viruses unable to infect cultured cells or cause disease in susceptible animals, carrying deletions or mutations in the RGD motif, have been reported extensively (Leippert *et al.*, 1997; Mason *et al.*, 1994; McKenna *et al.*, 1995; Rieder *et al.*, 1996). Differences in the integrin-binding specificity have been found for FMDV serotypes (Duque & Baxt, 2003), and the involvement of the G–H loop residues in infectivity has been analysed (Mateu *et al.*, 1996). The sequence requirements for interaction with different integrins have been

studied using purified human and bovine integrins (Duque *et al.*, 2004; Jackson *et al.*, 1997, 2000a), as well as transfected cell lines expressing specific integrins (Burman *et al.*, 2006; Dicara *et al.*, 2008; Duque & Baxt, 2003; Jackson *et al.*, 2000b; Rieder *et al.*, 2005).

The high potential of FMDV for variation and adaptation may lead to modifications affecting receptor usage, tropism and antigenic diversity (Domingo *et al.*, 2003; Jackson *et al.*, 2003; Mason *et al.*, 2003; Ruiz-Jarabo *et al.*, 2004). Adaptation of FMDV to cell culture can result in selection of variants that use the glycosaminoglycan heparan sulfate (HS) as receptor (Jackson *et al.*, 1996; Sa-Carvalho *et al.*, 1997). RGD replacements, such as RGG, RED and GGG, or the exchange of 8 aa spanning the RGD motif for the FLAG peptide sequence DYKDDDDK, yielded infectious viruses only in the context of variants highly passaged on cell culture (Baranowski *et al.*, 2000, 2001; Martínez *et al.*, 1997; Ruiz-Jarabo *et al.*, 1999). Additionally, after multiple cytolytic passages, FMDV isolate C-S8c1 acquired substitutions in the capsid that increased infectivity on BHK-21 cells and allowed infection of mutant glycosaminoglycan-deficient CHO cells in an HS-independent pathway. RGD replacements in these viruses did not affect infectivity on BHK-21 and CHO cells, suggesting the involvement of an alternative mechanism of entry independent of RGD and HS (Baranowski *et al.*, 1998, 2000).

Information currently available indicates that FMDV utilizes integrins for entry in the natural host, and there is no evidence of the use of alternative receptors *in vivo* (McKenna *et al.*, 1995; Neff *et al.*, 1998). However, the role of the different integrin receptors on viral tissue tropism and pathogenesis has not yet been established, and changes in receptor specificity may also occur during FMDV replication *in vivo*. Amino acid substitutions affecting the RGD motif (R141G) or positions +1 and +4 (L144P and L147P) have been selected in viruses escaping an immune response to synthetic peptides in vaccinated cattle (Taboga *et al.*, 1997; Tami *et al.*, 2003), and in a C-S8c1 virus adapted to guinea pig (Núñez *et al.*, 2001). Moreover, an engineered chimeric virus carrying KGE instead of RGD on a type O capsid sequence was able to replicate and cause mild disease in pigs (Zhao *et al.*, 2003).

Adult mice are not among the natural hosts of FMDV, but during the first weeks of life, mice are susceptible to inoculation of virus particles and viral RNA, inducing a rapid disease with tremors, ataxia, paralysis of the hind limbs and death (Baranowski *et al.*, 2003; Skinner, 1951). The murine model has been characterized and used extensively for FMDV vaccine development and immunization studies, showing that the virus can replicate upon experimental inoculation for up to 12–72 h post-inoculation and is subsequently cleared by the adaptive immune response (Fernández *et al.*, 1986). Recently, we have shown that the RGD mutant L147P is unable to grow in cell culture. However, it can be lethal and transmissible in suckling mice when inoculated as *in vitro*-transcribed RNA derived from an FMDV infectious clone (Baranowski *et al.*, 2003). In this study, we analysed the effect of amino acid substitutions in the RGD region within two different capsid contexts: the C-S8c1 isolate and a second isolate corresponding to virus recovered after multiple passages of C-S8c1 in BHK-21 cells, which was negatively selected for binding to heparin (isolate 213hs$^-$). The viability of the resultant mutants was assayed *in vitro* on cultured cells, as well as in suckling mice by *in vivo* transfection of the corresponding RNA transcripts. Our work provides new data on how sequence changes in the RGD region can affect virulence in suckling mice and infection of cultured cells in two different FMDV capsid sequence contexts resembling field isolates and virus adapted to cell culture, respectively.

METHODS

Mutagenesis and construction of plasmids. Amino acid substitutions in the RGD region were introduced into the VP1 capsid proteins of two different FMDV viruses (Fig. 1). C-S8c1 is a plaque-purified clone from natural isolate C-Sta Pau Sp/70 (Sobrino *et al.*, 1983). The 213hs$^-$ capsid sequence corresponds to MARLShs$^-$ virus, a monoclonal antibody (mAb)-resistant mutant isolated with mAb SD6 from C-S8c1 virus after 213 passages on BHK-21 cells and following negative heparin-binding selection (Baranowski *et al.*, 1998). Site-directed mutagenesis was carried out using p3242/C-S8c1 (Baranowski *et al.*, 1998) as template for the PCR. Supplementary Table S1 (available in JGV Online) shows the oligonucleotides used to introduce specific mutations. PCR fragments were digested with *Bss*HII and *Avr*II (hatched boxes in Fig. 1) and ligated into p3242/C-S8c1 and p3242/213hs$^-$, previously digested with the same enzymes. Plasmid p3242/213hs$^-$ was generated by exchange of the *Sfi*I–*Bss*HII fragment (nt 2827–3395) carrying mutation K173M in the VP3 protein, conferring the negative heparin-binding phenotype in MARLS virus (Baranowski *et al.*, 1998), with the corresponding fragment in plasmid p3242/213 (Baranowski *et al.*, 1998, 2000); to generate this construct, PCR was performed using p3242/213 as template and the primers listed in Supplementary Table S1. Finally, full-length cDNAs carrying mutations in VP1 were constructed by inserting the corresponding *Ngo*MI fragments from derivatives p3242/C-S8c1 and p3242/213hs$^-$ into the *Ngo*MI site of plasmid pO1KΔ3242 (Baranowski *et al.*, 1998).

***In vitro* transcription and transfection.** Plasmids were linearized by digestion with *Hpa*I and transcripts were generated using SP6 RNA polymerase (Promega). After transcription, reaction mixtures were treated with 1 U RQ1 DNase (μg RNA)$^{-1}$ (Promega). The RNA concentration was estimated by agarose gel electrophoresis and ethidium bromide staining, and transcripts were used for infectivity assays in mice and cell culture. Semi-confluent monolayers of BHK-21 cells ($\sim 5 \times 10^6$), grown in Dulbecco's modified Eagle's medium (Gibco) supplemented with 5 % fetal calf serum (Gibco), were transfected with 5–10 μg RNA using Lipofectin (Invitrogen) (Sáiz *et al.*, 2001). After transfection, cells were incubated at 37 °C for up to 6 days until cytopathic effect (CPE) development. Aliquots of 100 μl of each transfection medium were used to infect approximately 2.5×10^6 fresh BHK-21 cells and wild-type (wt) and mutant (mt) glycosaminoglycan-deficient pgsA-745 CHO cells. Total RNA was phenol/chloroform extracted from supernatants of transfected or infected cells for RT-PCR amplification and subsequent sequencing of

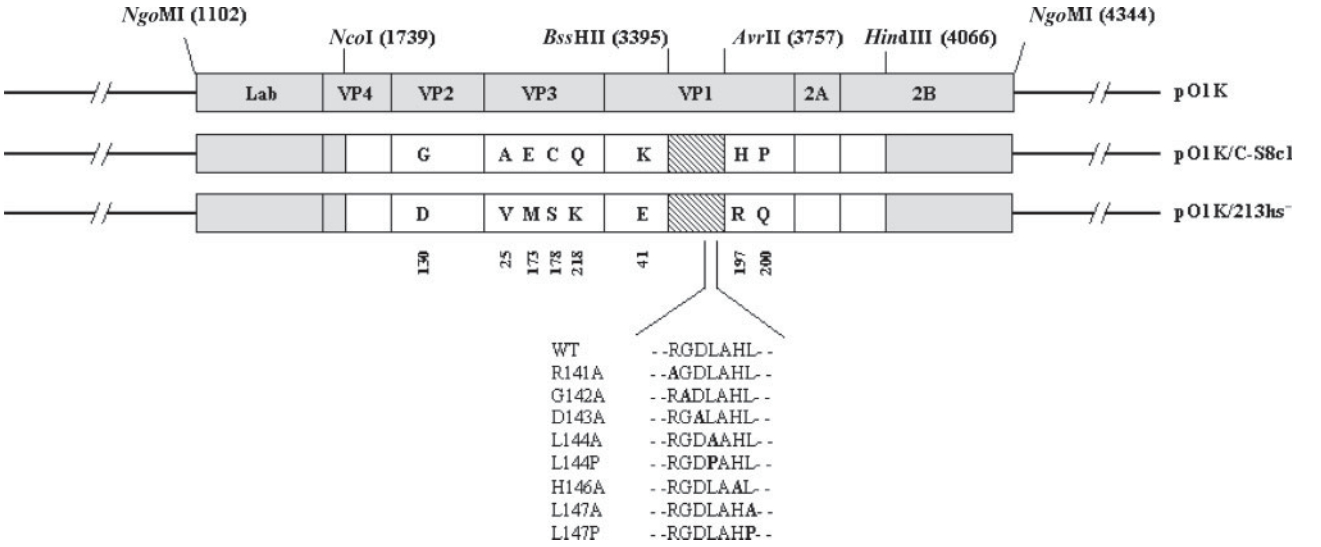

Fig. 1. Schematic representation of FMDV full-length clones carrying amino acid substitutions in the RGD region. Restriction sites used for mutagenesis are indicated on the pO1K plasmid. Oligonucleotides used to introduce the specific mutations are listed in Supplementary Table S1. C-S8c1 genomic regions are represented by open boxes. Hatched boxes indicate the recombinant PCR fragments inserted, with the mutations listed below. Differences in the amino acid sequences of the chimeric genomes containing capsids from C-S8c1 or 213hs$^-$ virus, respectively, are indicated. Nucleotide numbering is as given by Escarmís *et al.* (1996).

the 2067 bp cDNA fragment corresponding to the capsid genes VP2–VP1 (Baranowski *et al.*, 2003).

Heparin-binding assay. Binding to heparin was determined using Sepharose CL-6B beads (Pharmacia Biotech) as described previously (Baranowski *et al.*, 1998). Briefly, binding was estimated as the ratio of p.f.u. remaining in the supernatant of serial dilutions of a viral suspension after incubation with control beads relative to heparin–Sepharose beads where a ratio of 2 was taken as the limit of positive detection and ~1 indicated no detectable binding.

Virulence assay on suckling mice. Groups of suckling Swiss mice of about 1 week old were inoculated intraperitoneally with 100 µl of different amounts of RNA diluted in PBS containing 20 µg Lipofectin (Gibco), as described previously (Baranowski *et al.*, 2003). The death of animals was scored for up to 7 days after inoculation and survivors were then euthanized. For infectivity transmission assays, crude homogenates were prepared from dead or euthanized animals previously inoculated with RNA (Baranowski *et al.*, 2003) and used at a 1 : 100 dilution to inoculate suckling mice. Total RNA was phenol/chloroform extracted from mice homogenates for RT-PCR amplification and subsequent sequencing of the VP2–VP1 region, as above.

For *in vivo* neutralization assays, mice were injected intraperitoneally with a mixture containing 50 µl virus at different dilutions and 50 µl tenfold dilution of supernatant of hybridoma culture of mAb SD6 (Mateu *et al.*, 1987). Neutralizing mAb SD6 was raised against C-S8c1 FMDV and recognizes a non-conserved epitope (aa 138–156) in the immunogenic region of VP1 including the RGD motif (Mateu *et al.*, 1987, 1990). After inoculation, dead mice were scored as above. When mAb SD6 was used under the same conditions for *in vitro* neutralization assays on IBRS-2 cells, after incubation at 37 °C for 48 h, 100 % neutralization of infectivity was observed for C-S8c1 virus at an m.o.i. of 1 (the highest tested), whilst no neutralizing effect could be detected for O1K virus, even at the lowest m.o.i. assayed yielding CPE (m.o.i. of 0.01).

RESULTS AND DISCUSSION

High tolerance to substitutions in the FMDV RGD region may generate viruses with expanded tropism on cell culture, independent of the capsid sequence context

The amino acid substitutions shown in Fig. 1 were introduced into full-length cDNA clones containing the VP1 protein from FMDV C-S8c1 or 213hs$^-$, respectively. C-S8c1 derives from a pig isolate (Sobrino *et al.*, 1983) and uses integrins as receptors (Baranowski *et al.*, 1998; Jackson *et al.*, 2000b). Its derivative, 213, was modified to abolish the ability to infect cells using HS, acquired after multiple passage on BHK-21 cells (Baranowski *et al.*, 1998), by introducing mutation K173M into the VP3 protein to produce 213hs$^-$. Mutant RNAs derived from full-length clones (Fig. 1) were transfected into BHK-21 cells, in all cases generating infectious virus. On average, a complete CPE was observed at around 24 h post-transfection (p.t.) for 213hs$^-$ and 4 days for C-S8c1 mutants. When fresh BHK-21 monolayers were inoculated with aliquots of transfection medium, CPE was detected within 3 days post-infection (p.i.).

Sequencing of capsids from the viruses recovered (Table 1) showed that, in the C-S8c1 sequence context, the D143A and L144P mutations were not viable, as only the corresponding revertants were recovered from transfections. In contrast, H146A replacement was fully tolerated and complete CPE was observed at around 24–48 h p.t. with no additional changes in the capsid. Previous data

Table 1. Capsid sequences of viruses recovered from transfection of mutant RNAs derived from C-S8c1 and 213hs$^-$ clones and subsequent passage in cell culture

Amino acid residues at the mutated position and additional substitutions found in the capsid proteins are indicated. The transfected mutant sequence is underlined. Substitutions found that are not in VP1 are indicated with the corresponding capsid protein and position. Sequence mixtures at single positions are indicated with a solidus. Amino acids corresponding to the C-S8c1 or 213hs$^-$ parental sequence are indicated in bold. When viral populations recovered from independent experiments showed differences in capsid sequence, these are indicated as (i) and (ii), respectively.

RNA	Transfection	Passage in cell culture*		
	BHK-21	BHK-21	CHO	
			Wild-type	pgsA-745
C-S8c1	C-S8c1 wt	+	−	−
C-S8c1/R141A	A141, **S**/R139, **T**/M148, **H**/R†154, VP2 **I**/F†132	A141, **S**139R, **T**148M	−	ND
C-S8c1/G142A	T142	T142	−	ND
C-S8c1/D143A	**D**143	+	−	ND
C-S8c1/L144A	(i) A/V144	(i) M†/V144	(i) −	(i) ND
	(ii) A/V†144, **T**/K148‡	(ii) V144, **T**/K148	(ii) A144, **A**145V, VP2 **K**198E‡	(ii) A144, **A**145V, VP2 **K**198E
C-S8c1/L144P	**L**144	+	−	ND
C-S8c1/H146A	A146	+	−	ND
C-S8c1/L147A	(i) A147	(i) A147, VP3 **D**9V	(i) ND	(i) ND
	(ii) A147, **T**/A†148	(ii) A147, **T**/A†148	(ii) −	(ii) ND
213hs$^-$	213hs$^-$ wt‡	+	**D**/G†59	**D**/G†59
213hs$^-$/R141A	A141	+	A141, **D**/G†59, VP3 **M**/K†173	A141, **D**/G†59
213hs$^-$/G142A	A142	+	A142, **D**/G†59	A142, **D**/G†59
213hs$^-$/D143A	A143‡	+	A143	A143
213hs$^-$/L144A	A144	+	A144, **D**/G59, **Q**/R†/K200	A144, **D**/G59, **Q**/R†/K200
213hs$^-$/L144P	P144	+	P144, VP3 **M**/K†173	P144, VP3 **M**/K†173
213hs$^-$/H146A	A146	+	A146, VP3 **M**/K†173	A146, VP3 **M**/K†173
213hs$^-$/L147A	A147	+	A147, **D**/G†59, **Q**/R†200	A147, **D**/G†59, **Q**/R200
213hs$^-$/L147P	P147	+	P147, **D**59G	P147, **D**59G

*Results of at least two independent infections. ND, Not done; +, infection yielding cytolytic viruses whose capsid sequences were not determined; −, no CPE after infection and three blind passages.

†Residue in a proportion of approximately $\geqslant$50 %.

‡Viruses with hs$^-$ phenotype confirmed by a heparin–Sepharose binding assay (ratio ~1) as described in Methods.

have shown the relative insensitivity of this position to peptide inhibition of infectivity (Mateu *et al.*, 1996). Interestingly, a Thr substitution at position 142 was observed in viruses obtained after transfection with G142A RNA as well as following infection, whilst reversion to G was not detected. Conversion from Ala (GCG) to Thr (ACG) involves transition G→A, whilst reversion to Gly (GGG) would need the transversion C→G. Mateu *et al.* (1996) demonstrated the high inhibitory effect of peptides carrying T142 on viral infectivity, suggesting their binding to cell integrins. Mutants R141A and L147A maintained the corresponding substitutions, but additional changes were acquired in the G–H loop and other capsid proteins after transfection or passage on BHK-21 cells. For mutant R141A, CPE was complete at 6 days p.t. Sequence mixtures were detected at three VP1 positions within the G–H loop and one in the VP2 E–F loop (Acharya *et al.*, 1989); R139 has been found in viruses resistant to SD6 neutralizing mAb (Martínez *et al.*, 1997). After passage in BHK cells, only the S139R and T148M substitutions were detected in the capsid. For L147A, in the initial experiment the substitution was fully tolerated with no other changes until passage on BHK-21 cells, and cytopathic manifestations were observed at 2 days p.t. In a second experiment, CPE was delayed (4 days p.t.) and the sequence mixture found at position 148 was maintained after infection of BHK-21 cells; A148 has been reported for a 50-passage virus carrying a GGG motif (Ruiz-Jarabo *et al.*, 1999). Replacement L147P was not included in this study, as Núñez *et al.* (2001) previously found it to be deleterious in the C-S8c1 capsid. Substitution L144A was initially tolerated as a mixture with Val and, in one case, with an additional change at position 148. This last virus population induced complete CPE at 6 days p.t. After

infection, Met and/or Val was found at this position. V144 has been reported in a virus resistant to SD6 mAb (Martínez *et al.*, 1997), and Met is a residue normally found at this position in other FMDV serotypes (Burman *et al.*, 2006). Reversion from Ala (GCG) to Leu (TTG) would require 2 nt substitutions (a transversion and a transition, respectively). However, the replacements Ala (GCG) to Val (GTG) and Val (GTG) to Met (ATG) would only need a single change (transition). As a mixture of Ala/Val was firstly detected in transfection supernatants and then after passage Val/Met mixture was selected, it seems likely that sequence changes occurred in that order. L144 has been predicted to be a relevant residue for integrin binding in previous studies using RGD peptides (Burman *et al.*, 2006; Dicara *et al.*, 2008; Mateu *et al.*, 1996). Our results showed the relevance of L144 for integrin binding in the context of the virus. The viability of C-S8c1 virus with changes in the RGD region has not been reported previously in cell culture, as only substitutions affecting integrin recognition domains have been found after multiple passage in cells (Baranowski *et al.*, 1998; Martínez *et al.*, 1997).

When the capsid sequence of viruses recovered from 213hs$^-$ mutants was determined, significant differences were found relative to their C-S8c1 counterparts. Indeed, the corresponding Ala or Pro mutations were recovered in all cases from BHK-21 cells with no other substitutions in the capsid (Table 1). Interestingly, 213hs$^-$ capsid tolerated the mutations D143A (recovered at 48 h p.t.), L144P and L147P, all of which were deleterious in the C-S8c1 capsid. Residues at the RGD +1 and +4 sites have been shown to be major contributors to integrin-binding specificity (Burman *et al.*, 2006).

In order to explore further the mechanism of viral entry used by the mutants to infect cultured cells, supernatants from BHK-21 monolayers transfected with the different mutants were inoculated onto CHO cells. FMDV isolates adapted to grow in cell culture can infect wt CHO cells using HS as a receptor, whilst C-S8c1 virus is unable to infect this cell line due to restriction of its entry through integrins. Mutant glycosaminoglycan-deficient pgsA-745 CHO cells were inoculated in the same way to address whether any of the mutants had acquired the alternative uncharacterized entry route described for C-S8c1 virus after 100 passages carrying an RGG mutation and hs$^-$ (Baranowski *et al.*, 1998, 2000). O1K virus derived from the pO1K full-length clone (able to use HS as a receptor) at an m.o.i. of 0.1 induced complete CPE in wt CHO at 24–48 h p.i., whilst no CPE could be detected in mt CHO cells (data not shown). Interestingly, CPE was detected in wt and mt CHO cells at 48–72 h p.i. with viruses carrying the VP1 sequence A/V144 and T/K148 recovered from BHK-21 cells transfected with C-S8c1/L144A. Sequencing of the viruses generated in CHO cells revealed the presence of a substitution from Ala to Val at position 145 of the G–H loop, as reported in a virus resistant to SD6 mAb (Martínez *et al.*, 1997), and an additional change at VP2 position 198 from Lys to Glu (Table 1). With the aim of ruling out the use of HS as a receptor by these viruses, their hs$^-$ phenotype was confirmed by heparin-binding assays (Table 1). These results showed that C-S8c1 can modify its receptor usage and expand its tropism to CHO cells with as few as three changes in the capsid protein. It is worth noticing that viral populations carrying the A/V144 sequence but lacking the T/K148 mixture were unable to infect CHO cells, as were the remaining viruses recovered from BHK-21 transfections (Table 1). Lys at position 198 of the VP2 protein is involved in non-covalent interactions between pentamer subunits in the C-S8c1 capsid (Mateo *et al.*, 2003). Substitution of Lys for the negatively charged Glu may be inducing structural changes in the capsid affecting receptor interaction.

In contrast, all 213hs$^-$ mutants generated from transfections induced CPE in wt and mt CHO cells at 24–48 h p.i. Although maintaining the corresponding mutations in the RGD region, sequence mixtures or substitutions were detected at positions 59 and 200 of VP1 and position 173 of VP3 throughout the mutants capsids (Table 1). Position 59 is part of the B–C loop at the amino terminus of VP1 (Acharya *et al.*, 1989), in which mutations perturbing the stability of the G–H loop have been reported (Parry *et al.*, 1990). Q200R substitution, in antigenic site C (Mateu & Verdaguer, 2004), was selected in viruses carrying a FLAG peptide sequence after passage in BHK-21 (Baranowski *et al.*, 2001). D/G59 and Q/R200 mixtures were also found in IBRS-2 cells transfected with some of the 213hs$^-$ mutants (data not shown), suggesting that these substitutions are not linked specifically to replication in CHO cells. In some of the viruses recovered from wt and mt CHO cells, partial reversions to Lys at position 173 in the VP3 protein were detected (Table 1). As pgsA-745 CHO cells are HS deficient, no relevant contribution to viral entry through HS can be expected for this sequence mixture in these viruses.

Viruses recovered from transfection of 213hs$^-$/D143A RNA infected wt and mt CHO cells with no other changes in the capsid. This mutation was lethal in the C-S8c1 context, presumably abolishing interaction with integrins. The hs$^-$ phenotype of 213hs$^-$/A143 viruses was confirmed by a heparin–Sepharose binding assay (Table 1). This result demonstrated that these viruses can infect CHO cells using an RGD- and HS-independent route. Binding to heparin in viruses recovered from transfections with 213hs$^-$/wt RNA was also not detected (Table 1). Interestingly, unlike 213hs$^-$ viruses, a mAb-resistant viral population generated after passage 213 (MARLS) lost infectivity in wt and mt CHO cells after selection by 10 rounds of heparin–Sepharose binding (MARLShs$^-$) (Baranowski *et al.*, 1998). The contribution of other sequences in the viral genome or present in the capsid at amounts below detection levels for this difference between 213hs$^-$ and MARLShs$^-$ viruses cannot be ruled out.

Our results showed that FMDV parental genomes can accommodate substitutions in the RGD region, whilst additional changes in flanking residues are, in some cases,

Table 2. Infectivity of RNA transcripts in suckling mice and virulence of resultant viruses

Amount of RNA inoculated	No. dead animals/no. inoculated	
	RNA infectivity*	Virulence†
C-S8c1/R141A		
50 μg	0/2	
10 μg	1/11 (4)	5/6
5 μg	0/6 (1)	
1 μg	0/10	
500 ng	0/4	
200 ng	0/2	
100 ng	0/2	
C-S8c1/G142A		
20 μg	5/5	3/3
10 μg	4/5	
5 μg	1/1	
1 μg	0/5	
500 ng	0/4	
200 ng	0/2	
100 ng	0/2	
C-S8c1/D143A		
100 μg	0/2 (1)	0/3
50 μg	0/2	0/3
10 μg	0/1	
1 μg	0/5	
500 ng	0/4	
200 ng	0/2	
100 ng	0/2	
C-S8c1/L144A		
50 μg	2/2	
10 μg	4/5	3/3
5 μg	4/4	
1 μg	3/10 (1)	
500 ng	0/9 (2)	
200 ng	0/5	
100 ng	0/4	
C-S8c1/L144P		
50 μg	1/2 (1)	
10 μg	2/5	1/3
5 μg	1/5 (1)	
1 μg	4/6 (1)	
500 ng	1/5	
200 ng	0/2	
100 ng	0/2	
C-S8c1/H146A		
10 μg	1/4 (2)	1/3
5 μg	2/5	
1 μg	0/5	
C-S8c1/L147A		
10 μg	2/2	3/3
5 μg	3/3	
1 μg	2/5	

*No. of dead/no. of inoculated animals after inoculation with the corresponding RNAs. Transcripts derived from parental full-length clone 213hs$^-$ and the 213hs$^-$/D143A mutant up to 20 μg failed to kill any mice. The number of animals showing early signs of disease (transient tremors and ataxia) and subsequent healing is shown in parentheses.

†No. of dead/no. of inoculated animals after inoculation with homogenates from single animals from the corresponding RNA-inoculated mice. In groups including survivors after RNA inoculation, homogenates were prepared from a dead mouse (Baranowski *et al.*, 2003). For the C-S8c1/D143A mutant, two mice were euthanized, including one that showed early signs of disease.

selected. However, aa 143 remained resistant to changes, probably due to structural constraints essential for integrin binding in the wt capsid sequence. In contrast, viral genomes highly adapted to cell culture were able to incorporate these mutations with no other changes in the capsid, maintaining their capacity to enter cells using the non-RGD, non-HS route. In fact, this still uncharacterized route would be the only possible way known so far to enter BHK-21 cells for D143A, L144P and L147P mutants. Replacements selected in C-S8c1 and 213hs$^-$ mutant capsids in cultured cells were different, showing substantial flexibility to changes that are accommodated differently depending on the capsid context. The concept of tolerance to a given mutation is therefore relative and depends on the capsid sequence.

Virulence on suckling mice is only abolished by replacements in position D143 of the RGD region

To address the effect of substitutions in the RGD region on FMDV infectivity and transmission in suckling mice, the different mutant RNAs were assayed by intraperitoneal injection, as described previously (Baranowski *et al.*, 2003) (Table 2). In the C-S8c1 context, all of the RNAs except mutant D143A were infectious, although with different levels of lethality. Transcripts derived from the parental full-length clone pO1K/C-S8c1 (Fig. 1), used to construct all of the C-S8c1 mutants assayed here, were fully infectious at RNA amounts of approximately 1 μg (Baranowski *et al.*, 2003). D143A transcripts up to 100 μg failed to kill any mice. Several inoculations with homogenates from either an animal that showed early signs of disease after RNA injection or an asymptomatic mouse also failed to kill. Thus, the presence of D143 seems to be a strict requirement for C-S8c1 infectivity in mice, as observed in BHK-21 cells (Table 1). This is in agreement with the high level of conservation of residue D143 among natural FMDV isolates (Carrillo *et al.*, 2005) and supports its relevance in binding to integrin receptors. Very low levels of infectivity were exhibited by R141A RNA. Only one animal died after inoculation with 10 μg of transcript, but homogenates from this mouse were lethal in a new round of inoculation, indicating the presence of infectious virus. This suggests that R141 can occasionally become dispensable and is consistent

with previous data *in vivo* showing that viruses carrying SGD or GGD could be isolated from cattle infected with FMDV type A and C isolates, respectively (Rieder *et al.*, 2005; Taboga *et al.*, 1997; Tami *et al.*, 2003). H146A RNA killed mice with 5 μg of transcript, and homogenates from dead mice killed only one of the three animals inoculated. This result contrasted with the absolute tolerance to the H146A mutation in cultured cells (Table 1), suggesting that this replacement affects fitness differently in mice compared with cell culture. The substitutions G142A, L144A and L147A were well tolerated with RNA amounts $\leqslant$5 μg able to kill approximately 50 % of the inoculated animals. L144P RNA was lethal from 0.5 μg but none of the doses assayed could kill 100 % of the inoculated mice. We previously described the infectivity of L147P transcripts in mice (Baranowski *et al.*, 2003). As mentioned above, and again consistent with the lethality patterns obtained in mice, L144P and L147P have both been found in viable viruses replicating in target species (Taboga *et al.*, 1997; Tami *et al.*, 2003).

The capsid sequence of viruses generated after RNA inoculation was determined as above from the mice homogenates used for virulence assays. In all cases, the capsid sequence was maintained. Only for the L144P mutant was an additional replacement of L201I detected in the VP1 carboxyl terminus within antigenic site C (Mateu & Verdaguer, 2004). Substitution R141A was confirmed, with no additional changes, in viruses infecting one of the five mice that died in the virulence assay. For D143A, viral RNA could not be amplified by RT-PCR, confirming that no infectious virus was generated.

When transcripts derived from parental full-length clone 213hs$^-$ and the 213hs$^-$/D143A mutant were assayed, amounts up to 20 μg were innocuous for mice (data not shown). The high level of attenuation found for these genotypes in mice is in agreement with previous reports showing the attenuated phenotype *in vivo* conferred by mutations selected during serial passage in cultured cells (Neff *et al.*, 1998; Sa-Carvalho *et al.*, 1997). Indeed, we have shown previously the lack of infectivity in suckling mice of FMDV O1K transcripts bearing an H56R substitution in the VP3 protein, selected in type O isolates upon passage in cell culture (Baranowski *et al.*, 2003). Interestingly, supernatant from BHK-21 cells transfected with 213hs$^-$ transcripts inoculated into mice resulted in 100 % lethality at a tenfold dilution (data not shown). The viruses recovered from dead mice had amino acid mixtures of VP1 R197 with Ser and VP3 K218 with Arg and Gln throughout the capsid, suggesting the relevance of these positions to regaining infectivity *in vivo*. However, several trials to infect mice with supernatants from 213hs$^-$/D143A-transfected cells failed to kill any mice (not shown).

Although additional effects on the viral cycle of the replacements analysed here cannot be ruled out, our results show that substitutions in the RGD region are differentially tolerated in cultured cells and animals, suggesting different sequence requirements for viral entry *in vitro* and *in vivo*. These constraints can be modulated efficiently in cultured cells by the surrounding capsid sequence, and, in some cases, the viral population rapidly selects additional changes in the capsid (see C-S8c1 mutants R141A, L144A and L147A). However, mutant viruses with non-canonical RGDs were infectious for mice with no or few changes in their capsids (Table 2 and L144P mutant, respectively). Some of these viruses showed patterns of lethality not directly dependent on the inoculation dose, probably due to suboptimal binding affecting integrin affinity and/or specificity.

The integrin receptor $\alpha_v\beta_6$ is expressed constitutively on epithelial cells targeted by FMDV in cattle and sheep (Brown *et al.*, 2006; Monaghan *et al.*, 2005). Similarly, $\alpha_v\beta_6$ in mouse epithelia is highly expressed in the lung, skin and kidney during organogenesis, although barely detectable in adult animals, but is rapidly and transiently induced in response to local injury or inflammation (Huang *et al.*, 1996). Activation *in vivo* of different integrin species during infection has been suggested as a mechanism to switch on receptors for viral entry (Luo *et al.*, 2007). Although there is no direct experimental evidence on receptors used by FMDV in mice, and no extrapolation regarding virulence or pathology can be made to natural viral hosts at present, our results for the susceptibility of suckling mice to FMDV RNAs were in all cases consistent with information available on their viability in target species (see the L144P and L147P mutants). In fact, infectivity in primary bovine thyroid cell cultures of guinea pig-adapted FMDV, carrying the L147P mutation, can be specifically inhibited with anti-$\alpha_v\beta_6$ mAb, strongly suggesting that this virus, infectious for pigs and suckling mice, is using this integrin as a receptor in the animal host (Núñez *et al.*, 2007). With the aim of testing the involvement of the RGD region in FMDV virulence in mice, we tried *in vivo* neutralization assays with virus preparations co-inoculated with mAb SD6 (Table 3). Neutralizing mAb SD6 recognizes the immunogenic region of VP1 spanning aa 138–156 in the G–H loop enclosing the RGD (Mateu *et al.*, 1987, 1990). The effect of blockage of infectivity was dramatic for

Table 3. Infectivity of C-S8c1 in mice is abolished by incubation with specific neutralizing mAb SD6 binding to the RGD region

No. of dead/no. of inoculated animals at 4 days p.i. with dilutions of O1K or C-S8c1 virus (5.4×10^7 and 2.7×10^6 p.f.u. ml^{-1}, respectively) in the presence or absence of SD6 mAb.

SD6	O1K		C-S8c1			
	10^{-3}	10^{-5}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
−	2/5	0/5	5/5	5/5	5/5	5/5
+	3/5	2/5	0/5	0/5	0/5	0/5

C-S8c1 virus against which the mAb had been elicited. However, the virulence of O1K virus was not affected by the presence of the mAb, which is unable to bind heterologous type O isolates. As monovalent steric inhibition of receptor binding is the major mechanism by which antibody SD6 neutralizes FMDV infectivity (Verdaguer *et al.*, 1997), these results indicate that viral entry in mice is determined by capsid sequences overlapping the binding area of SD6, mainly the RGD region, supporting the use of integrins for FMDV infection in suckling mice. In agreement with this, the patterns of lethality observed in mice for the different transcripts were, in all cases, consistent with previous data *in vivo* regarding the corresponding altered positions. Therefore, we believe that the RNA virulence assay in suckling mice can be widely used for screening of FMDV genotypes that are viable *in vivo*. However, further characterization of the integrins expressed in mice will help to correlate receptor usage in mice compared with the natural host species.

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