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The pH Stability of Foot-and-Mouth Disease Virus Particles Is Modulated by Residues Located at the Pentameric Interface and in the N Terminus of VP1

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ABSTRACT

The picornavirus foot-and-mouth disease virus (FMDV) is the etiological agent of a highly contagious disease that affects important livestock species. The FMDV capsid is highly acid labile, and viral particles lose infectivity due to their disassembly at pH values slightly below neutrality. This acid sensitivity is related to the mechanism of viral uncoating and genome penetration from endosomes. In this study, we have analyzed the molecular basis of FMDV acid-induced disassembly by isolating and characterizing a panel of novel FMDV mutants differing in acid sensitivity. Amino acid replacements altering virion stability were preferentially distributed in two different regions of the capsid: the N terminus of VP1 and the pentameric interface. Even more, the acid labile phenotype induced by a mutation located at the pentameric interface in VP3 could be compensated by introduction of an amino acid substitution in the N terminus of VP1. These results indicate that the acid sensitivity of FMDV can be considered a multifactorial trait and that virion stability is the fine-tuned product of the interaction between residues from different capsid proteins, in particular those located within the N terminus of VP1 or close to the pentameric interface.

IMPORTANCE

The viral capsid protects the viral genome from environmental factors and contributes to virus dissemination and infection. Thus, understanding of the molecular mechanisms that modulate capsid stability is of interest for the basic knowledge of the biology of viruses and as a tool to improve the stability of conventional vaccines based on inactivated virions or empty capsids. Using foot-and-mouth disease virus (FMDV), which displays a capsid with extreme acid sensitivity, we have performed a genetic study to identify the molecular determinants involved in capsid stability. A panel of FMDV mutants with differential sensitivity to acidic pH was generated and characterized, and the results showed that two different regions of FMDV capsid contribute to modulating viral particle stability. These results provide new insights into the molecular mechanisms of acid-mediated FMDV uncoating.

Foot-and-mouth disease virus (FMDV) is the etiological agent of a highly contagious disease that affects important livestock species such as swine, cattle, sheep, and goats (1, 2). This virus is the type species of the *Aphthovirus* genus within the *Picornaviridae* family. The FMDV genome consists of a single-stranded positive-sense RNA molecule of about 8.5 kb in length (3). Like those of other RNA viruses, FMDV populations are complex and dynamic distributions of variants termed quasispecies that exhibit a high potential for variation and adaptation, which is reflected in the seven serotypes and the multiple antigenic variants identified (4, 5).

The viral capsid contains 60 copies of each of the four structural proteins (VP1 to VP4) arranged into 12 pentameric subunits that constitute intermediates of capsid assembly and disassembly (6, 7). An intriguing feature of the FMDV capsid is the extreme sensitivity to acidic pH, which results in virus inactivation after exposure of virions to mildly acidic pH (8). At pH slightly below neutrality, FMDV capsid disassembles into pentameric subunits (9) and there is good correlation between loss of infectivity by *in vitro* exposure to acidic pH and capsid dissociation (10–12). The acid sensitivity of FMDV has been related to the mechanism of virus penetration in host cells (13–15); the current model supports the hypothesis that the acidification of endosomes, where FMDV virions are sorted, triggers viral uncoating and genome release (16–18). Consistently, blockage of endosome acidification using the lysosomotropic weak base ammonium chloride (NH_4Cl), which acts as a proton sink within the endosomes (19, 20), inhibited FMDV infection by impairing virus uncoating without affecting virus binding or entry (10, 13, 21). We previously reported that under the conditions of impaired acidification of endosomes in cells treated with NH_4Cl, FMDV variants with enhanced acid sensitivity could be selected (10). The analysis of these mutants revealed that the mechanism to escape the inhibitory effect of NH_4Cl was based on the elevation of the uncoating pH (9, 13). On the other hand, point mutations in the viral capsid can enhance acid resistance by lowering the pH required for uncoating (11, 12, 22–24). One such amino acid substitution, N17D,

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which is located at the N terminus of VP1, has been selected in three different serotypes (11, 23, 24).

In this study, we have further analyzed the genetic determinants of the pH stability of the FMDV capsid by isolating and characterizing a novel panel of acid-labile mutants. Amino acid replacements altering virion stability were preferentially located in two well-defined regions of the capsid: the N terminus of VP1 and the pentameric interface. Furthermore, the acid-labile phenotype induced by a mutation in VP3 (A116V) located at the pentameric interface could be compensated by the rational introduction of an amino acid substitution in a different capsid region: the internal N terminus of VP1 (N17D). These results indicate that these two capsid regions modulate the acid stability of FMDV particles.

MATERIALS AND METHODS

Cells, viruses, animal samples, infections, and virus titrations. The origin of BHK-21 cells and culture procedures have been described previously (18, 25). The C-S8c1 isolate is a biological clone from type C FMDV (26). Mutants m6 and c2 are two C-S8c1 variants with increased acid resistance and enhanced acid sensitivity, respectively (10, 11). O1K is a type O FMDV recovered from the infectious clone pO1K (27). Vesicular fluid from a lesion (aphtha) developed in a pig experimentally infected with 10^4 PFU of FMDV C-S8c1 and collected at 3 days postinfection was used (28). Procedures for infections in liquid medium and virus titration in semisolid agar medium were as described previously (10, 18).

Acid-induced inactivation assays. A previously published protocol was followed (10). Briefly, equal amounts of viral samples (PFU) were mixed with phosphate-buffered saline (PBS) solutions of different pHs for 30 min at room temperature, pH was neutralized with 1 M Tris (pH 7.6), and the remaining infectivity in each sample was determined by titration in BHK-21 cells.

Inhibition of endosomal acidification with NH_4Cl. Cell monolayers were treated 1 h prior to infection with 25 mM NH_4Cl in culture medium supplemented with 25 mM HEPES at pH 7.4 to buffer extracellular pH, and the drug was maintained throughout the rest of the assay (10).

Nucleotide sequencing and site-directed mutagenesis of infectious cDNA clones. Viral RNA extraction, cDNA synthesis, amplification by reverse transcription-PCR (RT-PCR), and DNA sequencing were performed as described previously (10). Nucleotide positions correspond to those in the sequences of FMDV C-S8c1 (GenBank accession number AJ133357.1) or O1K (GenBank accession number X00871.1) (29, 30). Selected nucleotide substitutions were introduced into infectious cDNA clone pMT28, which contains the complete coding sequence of C-S8c1 (31). pMT28-VP3 A116V was constructed by introducing the DNA fragment containing nucleotides located between the SfiI and AvrII restriction sites of mutant av1 into pMT28. To this end, cDNA was amplified from av1 RNA by RT-PCR, digested with SfiI and AvrII (New England BioLabs), ligated into pMT28 digested with the same enzymes, and used to transform *Escherichia coli* DH5α as described previously (10). To construct the double mutant pMT28-VP3 A116V + VP1 N17D, the region encompassing the DNA fragment located between nucleotides 1804 and 3946 of the C-S8c1 genome was amplified by PCR from plasmid pMT28-VP1 N17D (11) and subcloned into pGEM-T easy vector (Promega) by following the instructions provided by the manufacturer. This intermediate vector, termed pGEM-VP1 N17D, was used as a template to conduct site-directed mutagenesis using the QuikChange II kit (Agilent) and oligonucleotide primers GGGCCGACCGACG*T*GAAAGCTCGGTAC ATGG and CCATGTACCGAGCTTT*C*ACGTCGGTCGGCCC. The nucleotide substitution introduced is in italics. Plasmid pGEM-VP1 N17D + VP3 A116V resulting from site-directed mutagenesis was digested with SfiI and AvrII, ligated into pMT28 digested with the same enzymes, and used to transform *E. coli* DH5α. Infectious clones pMT28-VP1 V11I, pMT28-VP1 T12A + VP1 N17D, pMT28-VP3 A116T + VP3 A118V + VP1 N17D, and pMT28-VP3 A116V + VP1 N17D + VP1 T22N were engineered by placing into pMT28 the corresponding DNA fragments located between SfiI and AvrII sites that were amplified by RT-PCR from the viral populations carrying these mutations. Viral RNA was synthesized from infectious cDNA clones and used to transfect BHK-21 cells (10). The nucleotide sequences of engineered cDNA clones and viruses were confirmed by DNA sequencing.

Molecular graphics and structure analysis. The atomic coordinates of FMDV C-S8c1 (32) and O1 (33) were used. Structures were visualized using PyMol Molecular Graphics System version 1.5.0.4 (Schrödinger, LLC). Contact information of selected residues was obtained using VIPERdb (34). Amino acid residues were numbered according to FMDV C-S8c1 or O1K sequences, as described above.

Data analysis. Data are presented as means ± standard deviations (SD). Analysis of variance (ANOVA) was performed using SPSS 15 (SPSS, Inc.). Bonferroni's correction was applied for multiple comparisons. Statistically significant differences are indicated in figures by one asterisk for a P value of <0.05 or two asterisks for a P value of <0.005.

RESULTS

A novel FMDV mutant with increased acid sensitivity directly isolated from an animal sample. Treatment with NH_4Cl has been successfully used to isolate FMDV mutants with enhanced acid sensitivity from cell culture-passaged viral populations (10). Going one step further, we explored the possibility to isolate FMDV mutants with increased acid sensitivity directly from animal lesions. To this end, vesicular fluid collected from an aphtha developed in a pig experimentally infected with type C FMDV C-S8c1 was used. The consensus sequence of the capsid coding region of the virus contained in this sample showed no differences from the sequence of the parental C-S8c1. Tenfold serial dilutions of this vesicular fluid were plated in parallel in BHK-21 cells treated or not with 25 mM NH_4Cl. Lysis plaques were developed in both control and NH_4Cl-treated cells, although the amount of PFU recovered from vesicular fluid was about 2 orders of magnitude lower in cells treated with NH_4Cl than in control cells (Fig. 1A). Five viral plaques developed in NH_4Cl-treated cells (clones av1 to av5) were successfully amplified by infection in liquid medium containing NH_4Cl. The nucleotide sequence of the capsid coding region of these viruses was determined and compared to that of the parental C-S8c1 virus. The single nucleotide substitution C2897T leading to amino acid replacement VP3 A116V was found in clones av1 to av5; this substitution was not detected in the consensus sequence of the parental population of the vesicular fluid. Interestingly, replacement VP3 A116V was located very close to replacement VP3 A118V previously found in the acid-labile mutant c2 (10). Since av1 to av5 carried the same capsid sequence, virus av1 was selected for subsequent analyses and its acid sensitivity was compared with that of the parental C-S8c1 in inactivation assays (Fig. 1B). In these experiments, two derivatives of C-S8c1, one with increased acid resistance (m6, which carries replacement VP1 N17D [11]) and the other with enhanced acid sensitivity (c2, which carries replacement VP3 A118V [10]), were included as controls. The infectivity of av1 was significantly reduced by treatment with pH 6.7 compared to that of C-S8c1, thus confirming that this mutant, directly isolated from an animal lesion, displayed enhanced acid sensitivity.

Amino acid replacement VP3 A116V confers an increase in acid sensitivity that can be compensated by the introduction of amino acid replacement VP1 N17D. Amino acid replacement VP3 A116V, responsible for the increase in acid lability of mutant av1, was located close to the pentameric interface (Fig. 2A). On the

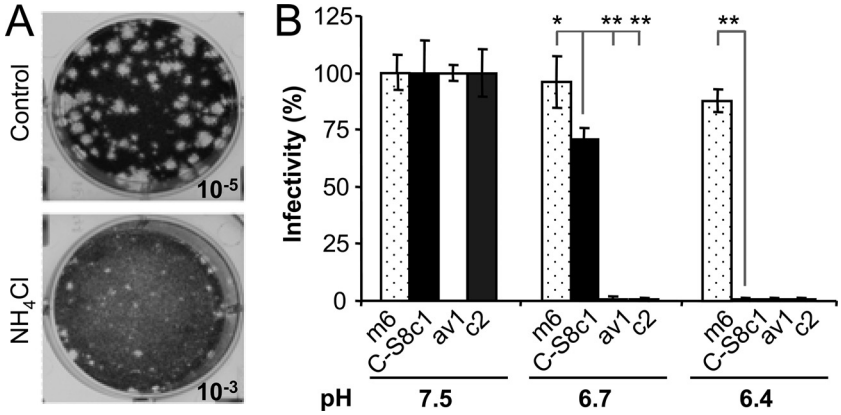

FIG 1 Characterization of FMDV mutants isolated in the presence of NH_4Cl from vesicular fluid of an FMDV-infected pig. (A) Lysis plaques developed by viruses recovered in the absence or in the presence of NH_4Cl. BHK-21 cells were treated or not with 25 mM NH_4Cl, and 10-fold serial dilutions of vesicular fluid from a pig experimentally infected with FMDV C-S8c1 were plated. Cell monolayers were fixed and stained with crystal violet at 30 h postinfection to visualize lysis plaques. The dilution of the viral sample is indicated in each case. (B) Acid inactivation assays of mutant viruses isolated from vesicular lesions in the presence of NH_4Cl. Equal amounts of the different viruses (C-S8c1, av1, c2, or m6) were treated with PBS at pH 7.5, 6.7, or 6.4, neutralized, and plated on BHK-21 cells. Infectivity was calculated as the percentage of PFU recovered at each different pH relative to that obtained at pH 7.5. av1 is an individual biological clone recovered from vesicular fluid. m6 and c2 are two C-S8c1 variants with increased acid resistance and enhanced acid lability, respectively (10, 11). Statistically significant differences between C-S8c1 and mutant viruses are indicated by one asterisk for a *P* value of <0.05 or two asterisks for a *P* value of <0.005.

other hand, we had previously reported that replacement VP1 N17D found in C-S8c1 variant m6 increased the acid resistance of virions (11). VP1 N17D was located about 25 Å from VP3 A116V in an internal part of the capsid (Fig. 2A and B). To study whether VP1 N17D replacement could compensate the acid-labile phenotype conferred by replacement VP3 A116V, the nucleotide substitution responsible for VP3 A116V was introduced either alone or combined with that of VP1 N17D into the infectious clone pMT28, which contains the complete coding sequence of FMDV C-S8c1 (29). In this way, infectious clones pMT28-VP3 A116V and pMT28-VP3 A116V + VP1 N17D were engineered, and the corresponding viruses were recovered by transfection of BHK-21 cells with *in vitro*-synthesized viral RNAs. The VP3 A116V + VP1 N17D double mutant and VP3 A116V single mutant were viable, both developed lysis plaques in BHK-21 cells (Fig. 2C), and both were genetically stable after 10 passages in BHK-21 cells without accumulating any additional replacement. In this experiment, the parental virus recovered from infectious clone pMT28 (C-S8c1) and the VP3 A118V (10) and VP1 N17D (11) single mutants were included as controls. No significant differences in plaque size were found among mutant viruses and C-S8c1, except for a significant, albeit slight, reduction in the plaque size of the virus encoding amino acid replacement VP3 A118V, which is consistent with previous results showing that introduction of this replacement resulted in a slight reduction of plaque size (10). In addition, no major differences in the viral growth curves of these viruses were noticed (Fig. 2D). The acid resistance of the mutant viruses was analyzed, and the pH_{50} values (defined as the pH that causes a loss of 50% of the infectivity), which constitute a useful indication of acid resistance (10–12), were calculated and compared to that of C-S8c1 (Fig. 2E). As expected, virus carrying VP1 N17D at the N terminus of VP1 was the most acid-resistant virus (pH_{50}, 6.13 [11]). In contrast, the most acid-labile viruses were those carrying amino acid replacements VP3 A116V and VP3 A118V (pH_{50}s, 6.87 and 6.93 [10], respectively). The VP3 A116V + VP1 N17D double mutant (pH_{50}, 6.67) displayed an intermediate phenotype between acid-resistant and acid-labile viruses, with an inactivation profile similar to that of C-S8c1 virus (pH_{50}, 6.65 [10, 11]). Therefore, these results indicated that the introduction of replacement VP1 N17D into a highly acid-labile capsid carrying replacement VP3 A116V increased virion acid resistance to a level similar to that of the parental C-S8c1 virus, with minor effects on viral growth.

C-S8c1 variants passaged in the presence of NH_4Cl select different amino acid replacements that increase acid lability of virions. The genetic determinants of capsid destabilization that mediate adaptation of FMDV to grow under conditions of impaired endosome acidification were further addressed. C-S8c1 and mutant viruses carrying amino acid replacements VP1 N17D or VP3 A116V + VP1 N17D were recovered from the corresponding infectious clones and subjected to serial passages in BHK-21 cells in the presence of NH_4Cl until a cytopathic effect was detectable (usually about 18 h postinfection). The virus harboring the single replacement VP3 A116V was not included in these assays since this mutant already displayed a highly acid-labile capsid (Fig. 2E). Viral RNA was extracted and the consensus nucleotide sequence of the capsid coding region of each viral population was analyzed at passage 10 (Table 1). When no nucleotide substitution was found imposed in the viral population, the corresponding viruses were subjected to 10 additional passages (Table 1, asterisks). These series of experiments were performed in triplicate for each virus. All viruses analyzed retained the initial mutations and acquired upon passaging replacements at different capsid residues, except the VP3 A116V + VP1 N17D mutant in experiment 2, in which a pseudoreversion of one of the amino acid replacements engineered in this virus (VP1 D17G) was found. Amino acid replacements were accumulated in VP3 or VP1, except for VP2 G193C in experiment 2 with the VP1 N17D mutant. Interestingly, two replacements involving the same VP1 residue (T12A and T12I) were found in two different populations (experiment 1 with the VP1 N17D mutant and experiment 3 with the VP3 A116V + VP1 N17D mutant, respectively). It is remarkable that the viral population resulting from experiment 3 with the VP1 N17D mutant accumulated two additional nucleotide substitutions responsible

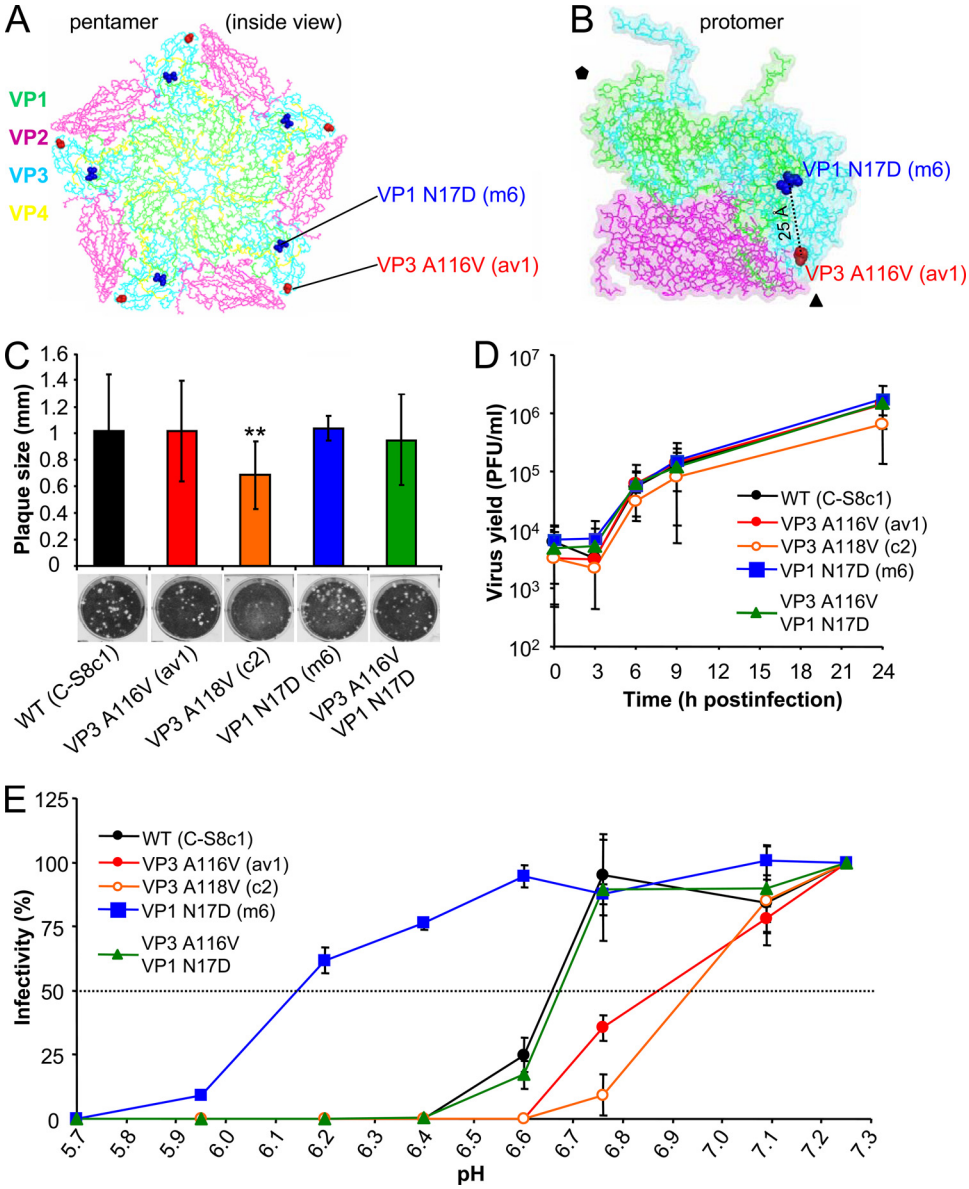

FIG 2 Amino acid substitution VP1 N17D compensates the acid-labile phenotype conferred by amino acid replacement VP3 A116V. (A) Location on the structure of C-S8c1 capsid of amino acid substitutions VP3 A116V and VP1 N17D. An inside schematic view of a pentameric subunit showing only amino acid main chains is shown. VP1 is green, VP2 is magenta, VP3 is cyan, and VP4 is yellow. (B) Inside schematic view of a biological protomer of C-S8c1 showing the location of amino acid substitutions in panel A. VP4 is not shown, for clarity. The distance between VP1 N17 and VP3 A116 is shown. The positions of 5-fold and 3-fold symmetry axes are indicated by a pentagon and a triangle, respectively. (C) Analysis of the plaque size of viruses recovered from infectious clone pMT28 (wild type [WT]) and its derivatives encoding amino acid substitutions VP3 A116V, VP3 A118V, VP1 N17D, and VP3 A116V + VP1 N17D. BHK-21 cells were infected in semisolid agar medium, and plaques were visualized by staining with crystal violet. About 100 viral plaques were analyzed for each virus. Double asterisks indicate statistically significant differences between the mutants and the WT (ANOVA, $P < 0.005$). (D) Single-step growth curve analysis of FMDVs recovered from the infectious clones indicated in panel C. BHK-21 cells were infected (multiplicity of infection [MOI] of 1 PFU/cell), and the virus titer in the supernatants was determined by plaque assay at different times postinfection. (E) Acid sensitivity of viruses recovered from infectious clone pMT28 and its derivatives shown in panel C. Equal amounts (PFU) of the different viruses were treated with PBS at different pHs for 30 min and the pH was neutralized and the remaining infectivity was titrated using BHK-21 cells and semisolid agar medium. Infectivity was calculated as the percentage of PFU recovered at each different pH relative to that obtained at pH 7.3. The intersection between the inactivation curves and the dashed lines indicates pH_{50} values; see the text for details.

for the introduction of replacements at residues important for the acid stability of the viral capsid (reference 10 and this report). One of them, VP3 A118V, had been previously found in acid-labile mutant c2 (10), while VP3 A116T was different from that found in mutant av1 (A116V). When the selection of the nucleotide substitutions responsible for replacements VP3 A116T and VP3 A118V was traced along passages, the two mutations were simultaneously detected in passage 3 and became dominant from passage 4 (data not shown).

The mutant populations passaged in the presence of NH_4Cl displayed significantly increased acid sensitivities relative to those of the parental populations (Fig. 3). These results support the

TABLE 1 Mutations selected in the capsid coding regions of type C FMDV variants after serial passage in the presence of NH_4Cl

Parental virus	Expt[b]	Mutation(s) selected in viral capsid protein[a]			
		VP4	VP2	VP3	VP1
Wild type (C-S8c1)	1			C2895A **D115E***	
	2				T3259C **Y18H***
	3				G3238A **V11I**
VP1 N17D mutant	1				A3241G **T12A**
	2		G2473T **G193C**		
	3			G2896T **A116T**	
				C2903T **A118V**	
VP3 A116V + VP1 N17D mutant	1				C3272A **T22N**
	2				A3257G **D17G***
	3				C3242T **T12I***

[a] In each case the nucleotide substitution and corresponding amino acid replacement (in bold) found after serial passages in the presence of 25 mM NH_4Cl are indicated relative to the corresponding parental population. Numbering is as for the C-S8c1 genome (29). Nucleotide substitutions were found in viral populations after 10 serial passages or after 10 additional passages (a total of 20 passages). An asterisk indicates a nucleotide substitution detected upon 20 serial passages not found dominant at passage 10.
[b] Numbers in the experiment column are individual passage series.

notion that despite the initial genetic background, the amino acid replacements selected upon the passages are responsible for acid-labile phenotypes to cope with the impaired endosomal acidification exerted by NH_4Cl.

Amino acid replacements selected in C-S8c1 variants passaged in the presence of NH_4Cl cluster in the pentameric interface or in the N terminus of VP1. The amino acid replacements acquired by the viruses passaged in the presence of NH_4Cl (Table 1) were mapped to the C-S8c1 structure (Fig. 4A). Four amino acid replacements, one in VP2 (G193C) and three in VP3 (D115E, A116T, and A118V), were located close to the pentameric interface, a region involved in capsid stability (10, 35). On the other hand, all amino acid replacements found in VP1 (V11I, T12A, T12I, D17G, Y18H, and T22N) were located within the internal part of the capsid at the N terminus of this protein, emphasizing the involvement of this region in the acid stability of the FMDV capsid. To demonstrate that the mutations found in NH_4Cl-passaged viruses were responsible for the increase in acid sensitivity, four amino acid combinations found in these naturally selected mutants were introduced into plasmid pMT28. Thus, infectious clones pMT28-VP1 V11I, pMT28-VP1 T12A +VP1 N17D, pMT28-VP3 A116T + A118V + VP1 N17D, and pMT28-VP3 A116V + VP1 N17D + VP1 T22N were constructed, and the corresponding viruses were recovered after transfection of *in vitro*-synthesized viral RNAs. The acid sensitivities of these viruses were analyzed and compared in each case with that of the parental virus (Fig. 4B to D). The virus carrying replacement VP1 V11I displayed an acid-labile profile (pH_{50}, 6.92) compared to C-S8c1 (pH_{50}, 6.65) (Fig. 4B), confirming that VP1 V11I was responsible for its acid-labile phenotype. Likewise, introduction of replacement VP1 T12A or VP3 A116T + A118V into the genetic background of the acid-resistant VP1 N17D virus resulted in an elevation of the pH_{50} from 6.13 (VP1 N17D) to 6.9 and 6.93 for VP3 A116T + A118V + VP1 N17D and VP1 T12A + N17D viruses, respectively (Fig. 4C). In addition, the introduction of replacement VP1 T22N in the genetic background of VP3 A116V + VP1 N17D virus also increased its acid sensitivity, raising the pH_{50} from 6.67 for the VP3 A116V + VP1 N17D mutant to 6.9 for the VP3 A116V + VP1 N17D + T22N mutant (Fig. 4D). Taken together, these results confirm that the pentameric interface and the VP1 N terminus define two capsid regions key for the control of acid stability of FMDV capsid, and that different amino acid substitutions in these regions can modulate this phenotype.

Mutations in VP3 or at the N terminus of VP1 can modulate the acid resistance of type O FMDV. To study if the results obtained with type C could be extrapolated to other FMDV serotypes, we isolated and characterized type O FMDV mutants with increased acid sensitivity. Tenfold serial dilutions of O1K FMDV were plated in parallel in cells treated or not with 25 mM NH_4Cl. As for type C FMDV, lysis plaques were developed by FMDV O1K in both control and treated cell monolayers; the amount of PFU recovered in cells treated with NH_4Cl was about 2 orders of magnitude lower than that of control cells. Complete sequencing of the capsid coding region from four FMDV clones selected from NH_4Cl-treated cells (fc4, fc9, fc11, and fc13) showed that they carried nucleotide substitutions leading to amino acid replacements in either the VP3 or VP1 N terminus as the only mutations present in their capsids (Table 2). Two of them displayed nucleotide substitutions introducing amino acid replacements into the N terminus of VP1 (T12N in fc4 and T2A in fc13). The other two mutants (fc9 and fc11) carried the same nucleotide substitution leading to replacement VP3 T156A, so only the fc9 mutant was included in further experiments. The replacements were shown to be located very close to each other in the structure of type O FMDV capsid (Fig. 5A). The acid sensitivity of these mutants was compared with that of the parental O1K virus (Fig. 5B). The three mutants displayed an increase in acid sensitivity (pH_{50}s, 6.76, 6.81, and 6.97 for viruses carrying amino acid replacements VP3 T156A, VP1 T12N, and VP1 T2A, respectively) compared with O1K (pH_{50}, 6.53), confirming that the introduction of these replacements in the viral capsid increased the acid lability of type O FMDV.

DISCUSSION

Understanding the molecular basis that control capsid stability is relevant not only for the knowledge of a basic aspect of FMDV biology, but also to gain information to improve stability of conventional vaccines based on inactivated virions (12, 36) or future vaccines based on empty capsids (37). In this study, we have isolated a panel of FMDV mutants with increased sensitivity to acidic pH by performing infections in the presence of NH_4Cl. A mutant isolated from vesicular fluid of an infected animal carried amino

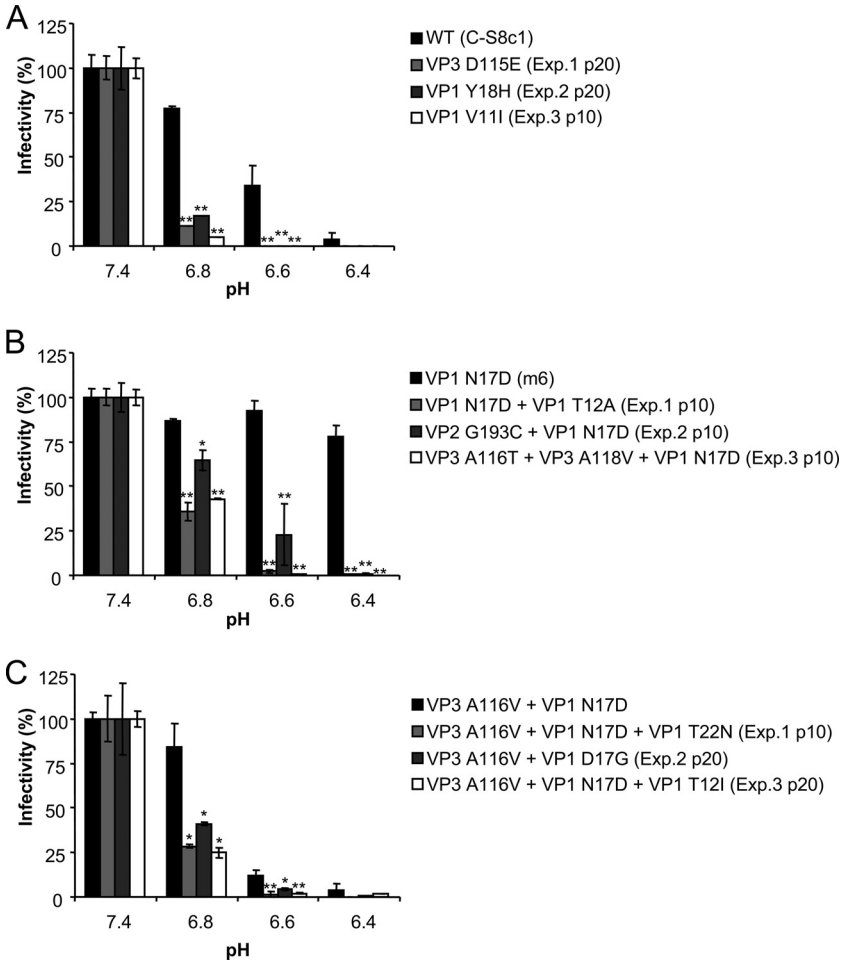

FIG 3 Viral populations selected after serial passage in the presence of NH_4Cl display acid-labile virions. Shown is a comparison of acid sensitivities of parental viruses (C-S8c1 in panel A, the VP1 N17D mutant in panel B, and the VP3 A116V + VP1 N17D mutant in panel C) with mutant viral populations selected after serial passage in the presence of NH_4Cl. Equal amounts of the different viruses (PFU) recovered from infectious clones were treated with PBS at pH 7.5, 6.8, 6.6, and 6.4, neutralized, and plated on BHK-21 cells. Infectivity was calculated as the percentage of PFU recovered at each different pH relative to that obtained at pH 7.5. "Exp." (experiment) indicates an individual passage series. The passage analyzed and the amino acid replacements displayed by the capsid (Table 1) are indicated in each case. Statistically significant differences between each parental virus and mutant populations are indicated by one asterisk for a *P* value of <0.05 or by two asterisks for a *P* value of <0.005.

acid replacement VP3 A116V, which mapped close to replacements VP3 A118V and VP3 A123T, which were found in two acid-labile mutants previously isolated from a cell culture-amplified FMDV stock (10). These three amino acid replacements were located along β-sheet E of VP3 very close to the pentameric interface, suggesting a common mechanism for their modes of action. As in the case of VP3 A118V and A123T, the small side chain of VP3 A116 is completely buried, and the substitution introduces a bulkier side chain whose accommodation into VP3 probably requires some deformation of the protein that alters pentamer-pentamer interactions, thus increasing the acid lability of the capsid (10).

Amino acid replacement VP1 N17D confers acid resistance to type C (11) and, as recently reported, to type O (23) and Asia1 (24) FMDVs, which makes VP1 N17 an interesting determinant of the acid stability of the FMDV capsid. Considering the functional relationships between distant amino acid residues described for picornavirus uncoating (38), we analyzed whether VP1 N17D replacement could compensate the acid labile phenotype. Indeed, introduction of VP1 N17D restored the highly acid-labile virion of mutant VP3 A116V (pH_{50}, 6.87) to a level (pH_{50}, 6.67) very similar to that of the parental C-S8c1 virus (pH_{50}, 6.65). Therefore, these results suggest that residues placed close to the pentameric interface, where A116V is located, and the internal VP1 N terminus, where VP1 N17D is placed, modulate the acid sensitivity of the FMDV capsid.

The analysis of compensatory mutations selected in FMDV capsids provides insights on the regions that regulate capsid stability (39). Consistent with this view, serial passage of the different FMDV variants in the presence of NH_4Cl promoted the selection of diverse mutations that were mostly located at the pentameric interface (VP2 G193C; VP3 D115E, A116T, and A118V) or within the N terminus of VP1 (VP1 V11I, T12I, T12A, D17G, Y18H, and T22N). Interestingly, in one of these mutants (VP3 A116T), residue VP3 A116 was found to be replaced by a threonine instead of by the valine observed in the VP3 A116V mutant isolated from vesicular fluid of an infected animal. This alternative replacement introduces a bulkier side chain that, as noted above, could alter

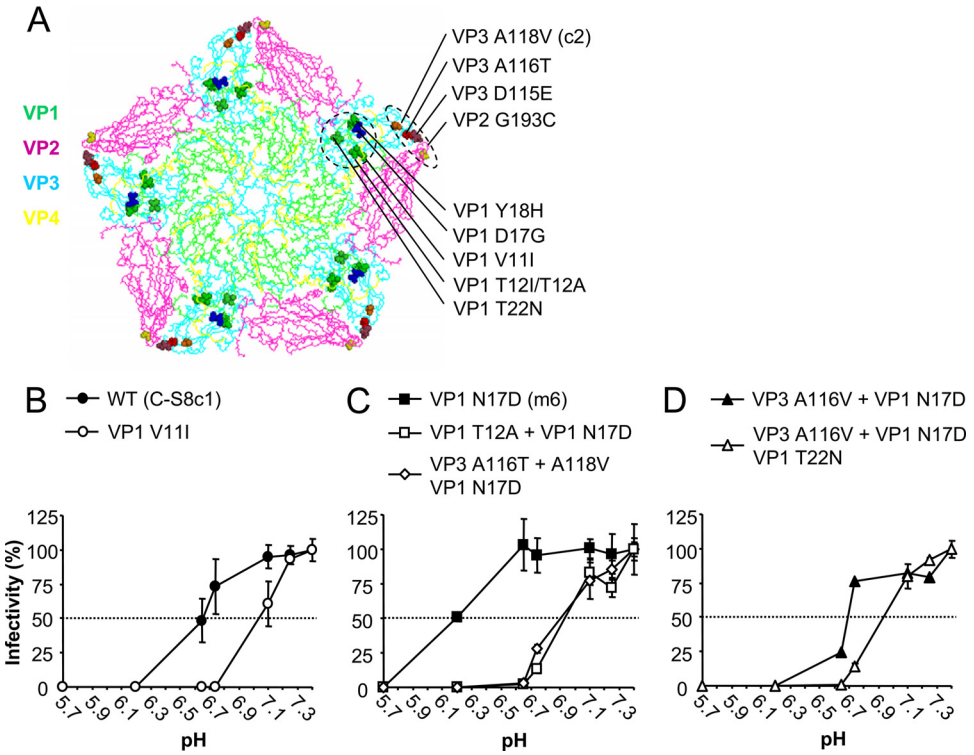

FIG 4 Analysis of amino acid substitutions selected in C-S8c1 variants passaged in the presence of NH_4Cl. (A) Inside schematic view of a pentameric subunit in the capsid of C-S8c1 displaying the location of amino acid residues found to be substituted in FMDV populations passaged in the presence of NH_4Cl. The groups of substitutions located at the VP1 N terminus or at the pentameric interface are encircled. Only amino acid main chains are shown, for clarity. VP1 is green, VP2 is magenta, VP3 is cyan, and VP4 is yellow. (B to D) Acid sensitivity profiles of viruses recovered from infectious clone pMT28 and its derivatives engineered to carry nucleotide substitutions found in FMDV variants passaged in the presence of NH_4Cl. Each engineered virus is compared with its parental virus. Acid inactivation assays were carried out as described in Materials and Methods and in the legend to Fig. 2. Infectivity was calculated as the percentage of PFU recovered at each different pH relative to that obtained at pH 7.4. The intersection between the inactivation curves and the dashed line indicates pH_{50} values; see the text for details.

pentamer-pentamer interactions and increase the acid lability of the capsid. A similar alteration could also explain the effect of the other neighbor substitutions found near the pentameric interface (VP2 G193C and VP3 D115E).

The selection of the mutant virus carrying replacements VP3 A116T + VP3 A118V + VP1 N17D as a result of the passage of the VP1 N17D mutant in the presence of NH_4Cl merits special consideration. This naturally engineered mutant exploits the combination of amino acid replacements in the pentameric interface and in the N terminus of VP1 that was originally behind the rationale followed to design the VP3 A116V + VP1 N17D double mutant. Hence, this mutant reinforces the mechanistic connection between these regions of the capsid to modulate pH stability. The complete series of mutants related to this virus goes from the acid-resistant VP1 N17D mutant (pH_{50}, 6.13) to the acid-labile VP3 A116T + VP3 A118V + VP1 N17D mutant (pH_{50}, 6.9), whose pH_{50} was similar to those of the acid-labile VP3 A116V and VP3 A118V mutants (6.87 and 6.93, respectively). Overall, these results support the hypothesis that the N terminus of VP1 and the pentameric capsid interface modulate FMDV acid sensitivity. These findings are also consistent with previous observations showing that the combination of different amino acid replacements in FMDV capsid can induce additive effects on pH stability (12).

TABLE 2 Mutations selected in the capsid coding region of type O FMDV mutants isolated in the presence of NH_4Cl

Clone	Mutation(s) found in viral capsid protein[a]			
	VP4	VP2	VP3	VP1
fc4				C2924A **T12N**
fc9			A2695G **T156A**	
fc11			A2695G **T156A**	
fc13				A2893G **T2A**

[a] The nucleotide position in the O1K genome (30) and the substitution found in individual viral clones obtained after virus plating in the presence of 25 mM NH_4Cl are displayed. For nonsynonymous substitutions, amino acid replacements are shown in bold.

Regarding the N terminus of VP1, different amino acid replacements associated with an increase in acid resistance of the capsid have been described for FMDVs from type A (22), C (10), O (23), or Asia1 (24). However, to our knowledge, no evidence for amino acid replacements in this region that increase acid lability is available. In addition to a pseudorevertant virus, VP1 D17G virus, selected as a result of passage of the VP1 N17D mutant in the presence of NH_4Cl, we found a series of amino acid replacements involving five positions within the N terminus of VP1 (V11, T12, Y18, and T22 in type C FMDV and T12 and T2 in type O FMDV). Two of these positions, T2 and V11, have been described as invariant among FMDV serotypes (3). Interestingly, three different sub-

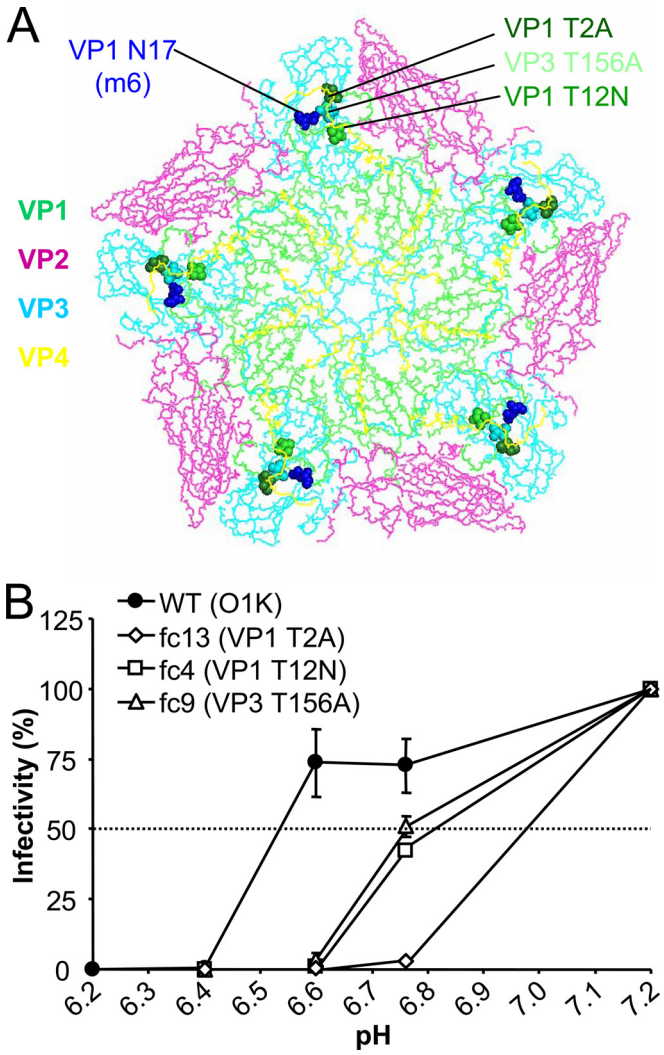

FIG 5 Characterization of type O FMDV mutants isolated in the presence of NH_4Cl. (A) Inside schematic view of a pentameric subunit in the capsid of type O FMDV displaying the location of amino acid residues found to be substituted in mutants isolated in the presence of 25 mM NH_4Cl. Only amino acid main chains are shown for clarity. The location of VP1 N17 is also indicated. VP1 is green, VP2 is magenta, VP3 is cyan, and VP4 is yellow. (B) Acid sensitivity profiles of type O mutant viruses isolated in the presence of NH_4Cl. Acid inactivation assays were carried as described in Materials and Methods and in the legend to Fig. 2. Infectivity was calculated as the percentage of PFU recovered at each different pH relative to that obtained at pH 7.2. The intersection between the inactivation curves and the dashed line indicates pH_{50} values; see the text for details.

stitutions at residue T12 (alanine and isoleucine in type C and asparagine in type O) were found in acid-labile mutants. The N terminus of VP1 is important for picornavirus uncoating, including the acid-resistant enteroviruses (40). In acid-labile picornaviruses such as rhinoviruses, this region is involved in capsid-RNA interactions and is externalized from the capsid during uncoating (41, 42). Indeed, in the aphthovirus equine rhinitis A virus, which is phylogenetically closely related to FMDV, the N terminus of VP1 also undergoes structural rearrangements when viral particles are exposed to acidic pH (43). However, one must be cautious with these comparisons because both rhinoviruses and equine rhinitis A virus undergo conformational rearrangements during capsid dissociation, changes that have not been reported for FMDV. Indeed, the current model for FMDV disassembly points to a direct dissociation of the capsid into pentameric subunits without the occurrence of any uncoating intermediate. In any case, a role of the VP1 N terminus in capsid stability and FMDV uncoating could resemble evolutionarily shared features in the picornaviruses for capsid disassembly. This general feature beneath a unified mechanism for picornavirus cell entry should be shared from acid-resistant enteroviruses to acid-labile picornaviruses such as rhinoviruses or FMDV (43). Additional evidence of the involvement of the N terminus of VP1 in capsid stability is provided by the observation that FMDV empty capsids display a disordered VP1 N terminus, which may be related to the different acid sensitivities of empty capsids and virions (9). Amino acid substitution VP3 T156A, observed in two type O acid-labile mutants, was mapped very close to the VP1 N terminus. According to the contact information available at VIPERdb (34), residue VP3 T156 can establish contacts with VP1 V11 and T13, suggesting that mutations in this residue can affect the function of the VP1 N terminus. Such potential interaction is also observed for type C FMDV, in which VP1 V11, which maps very close to residue VP3 T155, the position equivalent to T156 in type O FMDV, was replaced by isoleucine in an acid-labile mutant. Overall, the results obtained with type C FMDV were reinforced by the detection of mutants within the VP1 N terminus of type O FMDV, supporting the notion that capsid stability is regulated by common mechanisms in these two serotypes (10, 23). Nevertheless, the contribution to the acid stability of FMDV virions of capsid regions other that those described here remains to be addressed.

Mechanistically, the current model for FMDV uncoating proposes that protonation of two histidine residues located in VP3 (H140 and H143 or H141 and 144 in type C and O FMDV, respectively) provides the pH-dependent switch for capsid dissociation (7, 9, 44, 45). The protonation of these residues introduces an electrostatic repulsion with the dipole of an alpha helix in the neighboring pentamer, facilitating capsid dissociation. The mutations in the VP1 N terminus or at the pentameric interface identified in this study could modulate this mechanism, altering the uncoating pH threshold. In addition, amino acid residues of VP1 N terminus T2, V11, T12, and N17, which were replaced in these mutants, interact with different amino acid residues of VP4. Since FMDV dissociates into pentameric subunits releasing RNA and VP4 (46), the amino acid replacement here described could also modulate this process, contributing to the alterations of the acid lability of viral capsid observed. It is remarkable that different amino acid substitutions located at the N terminus of VP1 could result in diverse phenotypes, which could be explained by the residues involved and the replacements selected. Thus, N17D was the only replacement that resulted in an increase in acid resistance. This is a nearly isosteric replacement (11) leading to the introduction of a negatively charged amino acid, which could partially neutralize the positive charge of the protonated H143, resulting in an increase in acid resistance (11). Supporting this hypothesis, the pseudorevertant virus carrying D17G restored the uncharged nature of the residue and rendered a virus more acid labile than its parental virus carrying N17D. Conversely, replacement Y18H, found in an acid-labile mutant, introduced a protonable residue that could result in an increase of the positive charge of VP1 N terminus region at acidic pH. Nevertheless, no alteration in the charge balance of the N terminus was observed in the remaining

mutants isolated. Thus, while replacements T2A and T12A resulted in a loss of lateral side chains, substitutions V11I, T12I, T12N, and T22N introduced amino acid residues with bulkier side chains. It should be noted that all the replacements found in the N terminus of VP1 affected amino acid residues that interact with multiple residues located in VP4 and also in VP2 and VP3. Thus, the complexity of the interactions established by the residues located at the N terminus of VP1 could explain why different amino acid replacements can result in opposed phenotypes.

In summary, our results have revealed a functional relationship between two distant capsid regions that modulate the acid stability of the FMDV capsid. These findings suggest that capsid stability behaves as a multifactorial trait that is the fine-tuned product of a complex interaction between different capsid proteins.

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