JOURNAL OF VIROLOGY, Mar. 2011, p. 2733–2740 Vol. 85, No. 6
0022-538X/11/$12.00 doi:10.1128/JVI.02245-10

A Single Amino Acid Substitution in the Capsid of Foot-and-Mouth Disease Virus Can Increase Acid Resistance▽†

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Received 26 October 2010/Accepted 14 December 2010

Foot-and-mouth disease virus (FMDV) particles lose infectivity due to their disassembly at pH values slightly below neutrality. This acid-dependent disassembly process is required for viral RNA release inside endosomes. To study the molecular determinants of viral resistance to acid-induced disassembly, six FMDV variants with increased resistance to acid inactivation were isolated. Infection by these mutants was more sensitive to drugs that raise the endosomal pH (NH_4Cl and concanamycin A) than was infection by the parental C-S8c1 virus, confirming that the increase in acid resistance is related to a lower pH requirement for productive uncoating. Amino acid replacement N17D at the N terminus of VP1 capsid protein was found in all six mutants. This single substitution was shown to be responsible for increased acid resistance when introduced into an infectious FMDV clone. The increased resistance of this mutant against acid-induced inactivation was shown to be due to its increased resistance against capsid dissociation into pentameric subunits. Interestingly, the N17D mutation was located close to but not at the interpentamer interfaces. The mutants described here extend the panel of FMDV variants exhibiting different pH sensitivities and illustrate the adaptive flexibility of viral quasispecies to pH variations.

Foot-and-mouth disease virus (FMDV) is the causative agent of a highly contagious disease of cloven-hoofed animals (23) that poses important restrictions for international trading (11, 45, 49, 51). FMDV is the type species of the *Aphthovirus* genus within the family *Picornaviridae* (20). Its genome is composed of a single RNA molecule of positive polarity and about 8.5 kb in length. Like other RNA viruses, FMDV populations consist of complex and dynamic distributions of variants termed quasispecies (17) and exhibit a high potential for variation and adaptation, reflected in seven serotypes and multiple antigenic variants (18, 50).

FMDV RNA is protected by a capsid that comprises 60 copies of each of the four structural proteins (VP1 to VP4) arranged in an icosahedral lattice of 12 pentameric subunits, which constitute intermediates of capsid assembly and disassembly (56). After attachment to the host cell using a variety of receptors, such as different $\alpha_v\beta$ integrins, heparan sulfate glycosaminoglycans (for some tissue culture-adapted variants), or other not-well-characterized molecules (2, 3, 5, 25–28, 46), FMDV particles are internalized by endocytosis mediated by clathrin (for viruses using integrin receptor) or caveolae (in the case of variants using heparan sulfate). In both cases, FMDV particles are delivered to early endosomes for capsid disassembly and viral genome release (7, 29, 33, 43, 44).

FMDV particles display extreme acid lability, being inactivated at pH values slightly below neutrality (12, 35, 41, 55). The pH sensitivity of FMDV is required for capsid disassembly triggered by acidification inside endosomes, allowing release of the RNA genome within infected cells (4, 9, 10). An acid-labile capsid is not a general feature of picornaviruses; in fact, it is only shared by other aphthoviruses, cardiovirus, and rhinovirus (41). Histidine residues located close to the interpentameric interface act as pH sensors, triggering FMDV capsid dissociation after their protonation at the acidic pH inside the endosome and the establishment of electrostatic repulsions between capsid subunits (1, 12, 19, 55). Capsid disassembly of FMDV into pentameric intermediates is accomplished with the release of the internal VP4 protein. Since VP4 is a highly hydrophobic and myristoylated protein, its exposure could facilitate endosomal membrane permeabilization and viral RNA release from the endosome (6, 14, 15, 30).

Although the role of endosomal acidification was related to FMDV uncoating more than 20 years ago (4, 9, 10), the molecular determinants that mediate FMDV uncoating at acidic pH have not been fully elucidated. The isolation and characterization of mutants displaying alterations in the uncoating mechanism should provide a useful tool for the study of this process. Along this line, FMDV variants with increased acid lability have been previously characterized (35); an FMDV variant with increased resistance to acid inactivation was also isolated (54), although the molecular basis for this phenotype was not elucidated. In the present study, we have addressed the study of the molecular determinants associated with viral resistance to acid inactivation as an approach to provide a deeper insight into the structural bases for FMDV uncoating. To this end, six FMDV variants with increased resistance to acid inactivation were isolated. Infection by these mutants displayed increased sensitivity to drugs that raise endosomal pH, con-

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▽ Published ahead of print on 22 December 2010.

TABLE 1. Mutations found in the capsid coding regions of FMDV mutants with increased resistance to acid induced inactivation selected after two serial acid treatments

Genomic region	Mutation(s) found in viral population[a]						
	m1	m2	m3	m4	m5	m6	C-S8c1
VP4							
VP2	T1996C **F34L**						
VP3	A2576T* **D9V**	A2576T* **D9V**	A2576T* **D9V**	A2576T* **D9V**	A2576T* **D9V** G3000A		
	T3117C	T3117C	T3117C	T3117C	T3117C	T3117C	T3117C
VP1	A3256G **N17D**	A3256G **N17D** C3750T	A3256G **N17D**	A3256G **N17D**	A3256G **N17D** C3390*	A3256G **N17D**	

[a] The nucleotide position in the C-S8c1 genome (52) and the substitution found are indicated. For nonsynonymous substitutions, amino acid replacements are indicated in boldface. *, nucleotide mixture at this position between mutant and base in C-S8c1. See the text for details.

firming that the increase in acid resistance was related to a lower pH requirement for productive uncoating within host cells. A single amino acid substitution located at the N terminus of VP1 protein, common in all mutants isolated, was found to be responsible for the increased resistance to acid inactivation, which correlated with an increased resistance to acid-induced dissociation into pentameric subunits. These results extend the range of pH sensitivities of FMDV mutants (35) and illustrate the adaptive flexibility of viral quasispecies to environmental changes, in this case related to pH variations.

MATERIALS AND METHODS

Cells, viruses, infections, and virus titrations. The origin and culture procedures for BHK-21 cells have been described (32, 33). C-S8c1 is a biological clone of a type C FMDV isolate (48). c2 is a FMDV variant with increased resistance to endosomal acidification blockage (35). Procedures for infections and virus titration in semisolid agar medium were as described previously (33–35, 48).

Radioactive labeling and purification of virions. A previously published procedure (37, 39) was followed. Briefly, FMDV virions (C-S8c1 and mutants) were metabolically labeled with [^{35}S]methionine (EasyTag Express protein labeling mix; Perkin-Elmer) during infection of BHK-21 cells. The total virus produced was centrifuged through a cushion of 20% sucrose in TNE buffer (10 mM Tris-HCl [pH 7.5], 0.1 M NaCl, 1 mM EDTA) in an AH-625 rotor (Sorvall) at 25,000 rpm for 2.5 h at 4°C. The viral pellet was resuspended in TNE buffer and centrifuged in 7.5 to 30% sucrose density gradients in the same buffer at 37,000 rpm for 1 h at 4°C in an SW40 rotor (Beckman). The fractions containing full virions (sedimentation coefficient, 140S) were pooled and extensively dialyzed against phosphate-buffered saline (PBS). The integrity of virions was analyzed in 7.5 to 45% sucrose density gradients at 18,000 rpm for 18 h at 4°C in an SW40 rotor (Beckman Instruments).

Acid-induced inactivation assays. A modification of a previously reported procedure was followed (30, 35). Briefly, equal amounts (10 μl containing ~10^6 PFU) of the virus tested were mixed with 300 μl of PBS solutions (50 mM $NaPO_4$ and 140 mM NaCl) of different pHs for 30 min at room temperature. The solution was neutralized by adding 100 μl of 1 M Tris (pH 7.6), and the remaining PFU counts in each sample were determined by plaque assay on BHK-21 cells. The number of PFU developed was determined and is expressed as the percentage of infectivity compared to that obtained using PBS at pH 7.4 or 7.6.

Acid-induced disassembly assays. Aliquots (100 μl) of ^{35}S-labeled purified virions were mixed with 300 μl of PBS solutions (50 mM $NaPO_4$ and 140 mM NaCl) of different pHs (i.e., pH 5.6 to 7.2) for 30 min at room temperature. The solution was neutralized by adding 100 μl of 1 M Tris (pH 7.6). Subsequently, the solution was loaded in 7.5 to 45% sucrose density gradients and centrifuged at 4°C in an SW40 rotor (Beckman Instruments) at 18,000 rpm for 18 h. The gradients were fractionated in 0.5-ml aliquots, and the radioactivity was determined by using a liquid scintillation counter. Values were expressed as a percentage of the amount of full virions (sedimentation coefficient, 140S) obtained at different pHs, relative to the amount obtained at pH 7.2.

Thermal inactivation assays. A previously published procedure was followed (37). Briefly, equal amounts of infectious virions (~10^7 PFU) were incubated at 42 or 50°C during 0, 30, 60, or 120 min. At these times, virus samples were frozen, and the remaining virus titer was determined by plaque assay.

Endosomal acidification blockage. Inhibition of endosomal acidification with NH_4Cl was performed as described previously (4, 33). Briefly, cells were treated for 1 h prior to infection with 25 mM NH_4Cl in culture medium supplemented with 25 mM HEPES at pH 7.4, and the drug was maintained throughout the rest of the assay. Alternatively, cells were pretreated with 1 μM concanamicin A (ConA) 30 min prior to infection, and the drug was only maintained during the first infection hour (7, 33). Control samples were treated in parallel with the same amount of drug solvent (dimethyl sulfoxide).

Viral RNA extraction, cDNA synthesis, and DNA sequencing. Viral RNA was extracted from supernatants of infected cell cultures by using TRI reagent (Sigma). cDNA synthesis by reverse transcription, amplification by PCR, and DNA sequencing have been previously described (35). DNA sequences were confirmed by at least two independent sequencing reactions. Nucleotide positions correspond to those of the FMDV C-S8c1 isolate (52).

Infectious clone manipulation, *in vitro* transcription, and transfection of BHK-21 cells. Plasmid pMT28 (21), which contains the full-length cDNA of FMDV C-S8c1, was used to construct plasmid pMT28-VP1 N17D bearing the nucleotide substitution A3256G found in the capsid coding region of the six FMDV mutants with increased resistance to acid induced inactivation (Table) 1. To this end, cDNA obtained from reverse transcription of RNA extracted from virus m6 was digested with SfiI and AvrII (New England Biolabs), and the resultant fragment purified by agarose gel electrophoresis was ligated into pMT28 vector digested with the same restriction endonucleases using T4 DNA ligase (Promega). Amplification of plasmid DNA was performed in *Escherichia coli* DH5α. Nucleotide sequences of infectious DNA clones were confirmed by DNA sequencing as described above. Transcription of viral RNA from infectious clones and transfection of cells with *in vitro*-synthesized viral RNA was performed as reported elsewhere (35).

Competition experiments. A modification of a previously published procedure (40) was followed. Briefly, a mixture containing equal amounts of each pair of competing viruses derived from infectious clones—initial multiplicity of infection (MOI) of 0.1 (0.05 for each virus)—was treated with pH 7.6 or pH 6.0 buffers, neutralized, and used to infect a 35-mm-diameter tissue culture dish. When a complete cytopathic effect was observed, the viruses were harvested. Viral suspensions (50 μl) were incubated at pH 7.6 or 6.0, neutralized, and used to infect fresh cell monolayers. These cycles of pH treatment, infection, and harvest were repeated up to four times in triplicate. Viral RNA was extracted, and the cDNA encoding VP1 protein was synthesized and sequenced. The proportions of the competing genomes were estimated from the chromatograms as a ratio of the integrated areas of each nucleotide under each peak of mutated position (40).

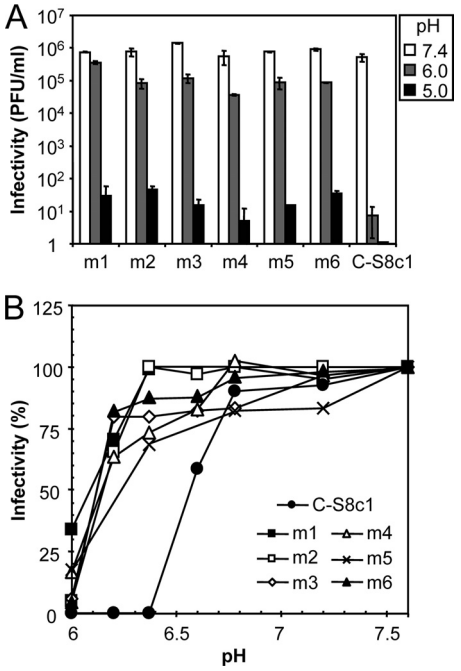

FIG. 1. Resistance to acid-induced inactivation of FMDV mutants selected after pH 6 treatment. (A) Equal PFU amounts of the different viruses were treated with acid buffers (pH 6.0 and 5.0) or pH 7.4 as a control. The samples were then neutralized and plated on BHK-21 monolayers. (B) Acid sensitivity profiles of FMDV mutants selected after pH 6 treatment. Infectivity was calculated from experiments performed as in panel A as the percentage of PFU recovered at each different pH relative to that obtained at pH 7.5.

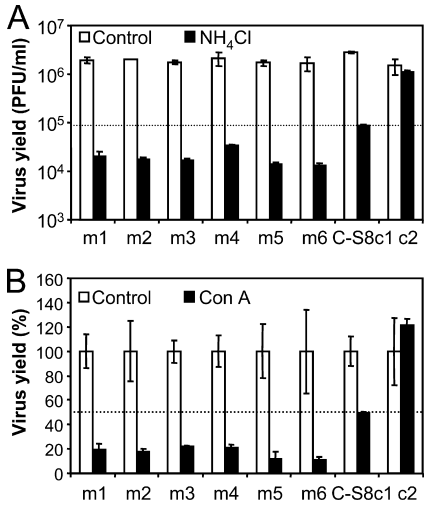

FIG. 2. Sensitivity to endosomal acidification blockage of FMDV mutants resistant to acid pH inactivation. (A) Sensitivity to NH_4Cl treatment. Monolayers of BHK-21 cells treated or not treated with 25 mM NH_4Cl were infected with the mutants and the parental virus C-S8c1 (MOI of 0.5). FMDV mutant c2 with increased resistance to endosomal acidification blockage (35) was also included. Virus yield was determined 8 h postinfection. (B) Sensitivity to ConA treatment. Monolayers treated or not with ConA (1 μM) were infected as described for panel A.

Molecular graphics. The atomic coordinates of FMDV C-S8c1 (31) and the programs RasMol (47) and Pymol (DeLano Scientific, Inc.) were used to locate the mutated amino acid residues on the capsid structure (35).

RESULTS

Isolation of FMDV mutants with increased resistance to acid inactivation. Infectious FMDV mutants with increased acid resistance were isolated by biological cloning after acid treatment. About 10^6 PFU of C-S8c1 were incubated 30 min at room temperature in PBS at pH 6.0 or 7.4 (control) and then neutralized. These samples were used to infect BHK-21 cell monolayers in semisolid agar medium. After 48 h, cells infected with samples treated at pH 6.0 showed approximately $0.0029 \pm 0.0005\%$ (selection frequency of 2.9×10^{-5}) of the number of plaques observed in control cells. The viruses recovered from six lysis plaques were amplified by infection in liquid medium (first passage) and subjected to a second treatment with pH 6.0 and subsequent amplification in liquid medium.

The viral populations obtained, termed mutants m1 to m6, were used for further experiments. The six mutants analyzed showed a decreased acid sensitivity compared to that of the parental virus C-S8c1 (Fig. 1A). Thus, relative to the drastic loss of infectivity observed (about 5 orders of magnitude) upon pH 6.0 treatment of C-S8c1, mutants m2 to m6 showed an infectivity reduction of only about 1 order of magnitude, which was even lower for mutant m1. When incubation was performed at pH 5.0, an infectivity reduction of about 5 orders of magnitude was observed for mutants m1 to m6, whereas no C-S8c1 PFU were recovered (Fig. 1A), confirming the increased resistance of these mutants to acid-induced inactivation of infectivity.

A detailed analysis of the sensitivity to pH values from 6 to 7.5 (Fig. 1B) confirmed that mutants m1 to m6 displayed inactivation profiles that are shifted to lower pH values relative to that exhibited by C-S8c1. When pH_{50} values (defined as the pH value leading to a 50% loss of infectivity [35]) were calculated, viruses m1 to m6 displayed very similar values (6.07, 6.15, 6.11, 6.15, 6.22, and 6.11, respectively). In the case of C-S8c1 the pH_{50} value was 6.58, as previously reported (35).

Increased acid sensitivity correlates with higher sensitivity to endosomal acidification blockage. FMDV uncoating leading to productive infection is triggered by acidification inside endosomes where viral particles are delivered (4, 7, 29, 33). Thus, infection by FMDV particles with increased acid lability displays higher resistance to the endosomal acidification blockage induced by different drugs such as NH_4Cl and ConA (35). These drugs were used to study whether an opposite effect was observed with the acid-resistant mutants. Compared to C-S8c1, mutants m1 to m6 showed, as expected, an increased sensitivity to NH_4Cl (Fig. 2A) and ConA treatment (Fig. 2B). In these experiments, mutant c2, which encodes a capsid with increased acid lability that confers resistance to endosomal acidification inhibition (35), was included as a control. These results indicate that the higher resistance to acid inactivation shown by these mutants is related to an increased sensitivity to drugs that raise the endosomal pH.

Analysis of mutations in the capsid-coding regions of acid-resistant FMDV mutants. To identify the genotypic changes responsible for the increased resistance to acid inactivation, the complete capsid coding regions of mutants m1 to m6 and

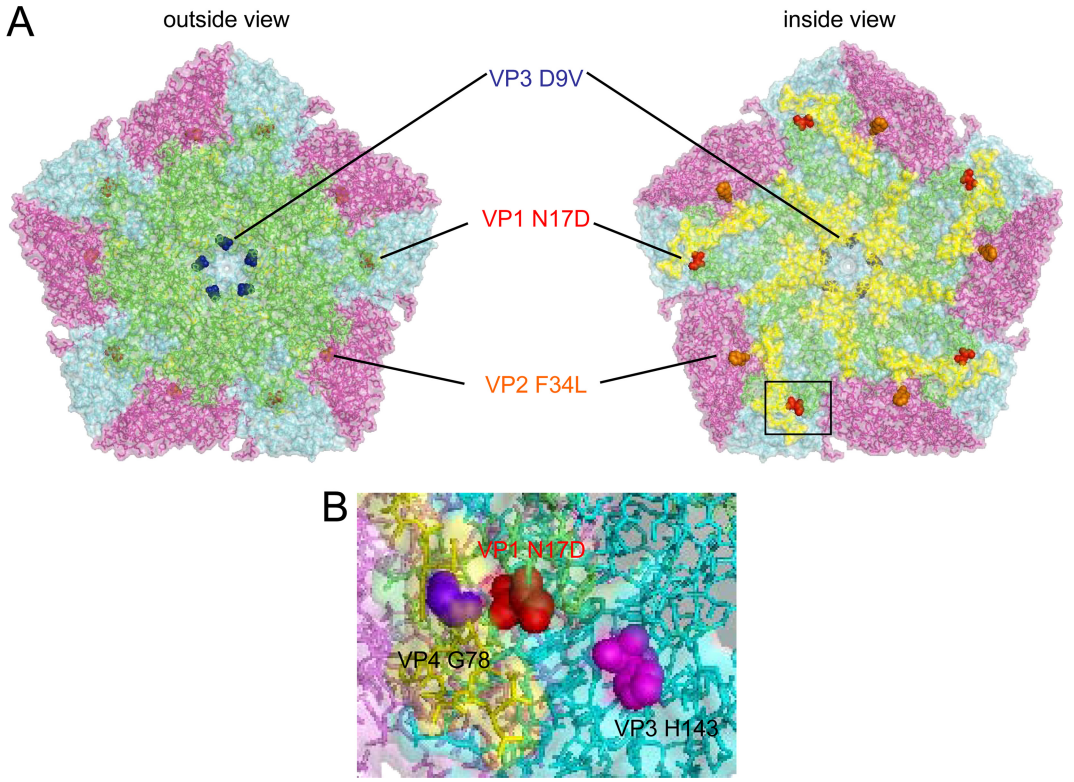

FIG. 3. Location on the structure of the C-S8c1 capsid (31) of amino acid residues found to be substituted in FMDV mutants with increased resistance to acid inactivation. (A) Outside and inside schematic views of a pentameric subunit in the capsid are shown. VP1 is green, VP2 is magenta, VP3 is cyan, and VP4 is yellow. (B) Close-up view of the region boxed in panel A showing relevant neighbor amino acids to the VP1 N17D position (red). VP4 G78 establishes a hydrogen bond with N17 which should be disrupted upon N17D amino acid replacement, and VP3 H143 is implicated on an acid pH-sensing mechanism for FMDV uncoating. See the text for details.

of the parental population C-S8c1 were sequenced and compared. Nucleotide and amino acid replacements found in viruses selected after two cycles of acid treatment and selection are shown in Table 1. Remarkably, all mutants showed a common nucleotide substitution A3256G leading to amino acid replacement VP1 N17D. Viruses m1 to m5 displayed also various degrees of nucleotide mixture at position 2576 between A (the nucleotide present in C-S8c1 sequence) and T (leading to amino acid replacement VP3 D9V); the A/T % values were as follows: m1, 53/47; m2, 28/72; m3, 68/32; m4, 78/21; and m5, 81/19. Virus m1 also exhibited an additional dominant nonsynonymous nucleotide substitution T1996C responsible for VP2 F34L amino acid replacement. The rest of the nucleotide substitutions found were synonymous.

The nucleotide substitution leading to replacement VP1 N17D was shown already imposed in all six viral progenies from cells infected with the virus recovered after the first cycle of acid treatment and selection (first passage). Such imposition was not observed for substitutions responsible for replacements VP2 F34L in virus m1 (present at ca. 60%) and VP3 D9V, for which only traces (lower than 5%) were detected in viruses m1 to m5.

None of the amino acid replacements selected involved invariant residues in the FMDV capsid (8). Replacement VP1 N17D found in viruses m1 to m6 affected a residue located at the N terminus of VP1 protein, within an internal region of the capsid, not far from the interpentamer interfaces (Fig. 3A). This amino acid replacement produced a disruption of a hydrogen bond between VP1 N17 and VP4 G78 and was located close to VP3 H143, which has been implicated in acid-induced capsid disassembly (1, 19, 55) (Fig. 3B). Residue VP3 9 was located at the outer surface of the capsid close to the capsid pore (5-fold symmetry axis), and residue VP2 34 was located at the internal surface of the capsid. Taken together, these results suggest that an internal capsid amino acid replacement (VP1 N17D) was responsible for the acid-resistant phenotype of viruses m1 to m6.

Amino acid substitution VP1 N17D confers increased resistance to acid-induced inactivation of FMDV. To test whether amino acid replacement VP1 N17D is sufficient to confer resistance to acid induced inactivation, a plasmid carrying the mutation responsible for this replacement was derived from the infectious clone pMT28, which encodes the complete genomic sequence of FMDV C-S8c1. The consensus nucleotide sequence of the capsid-coding region of the virus derived from the modified infectious clone (mutant VP1 17D) was determined and compared to that of the virus derived from the nonmutated, parental infectious clone pMT28 (C-S8c1). The only nonsynonymous nucleotide replacement found was that leading to replacement VP1 N17D (data not shown). Viruses derived from the mutated and the parental infectious clones were tested in acid-induced inactivation assays (Fig. 4). A significant increase in the acid resistance was observed for mutant VP1 N17D compared to C-S8c1. Virus VP1 N17D displayed a

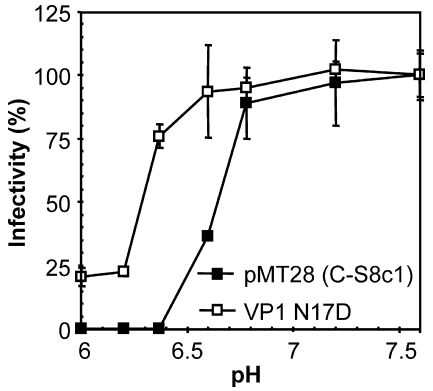

FIG. 4. The single amino acid substitution N17D in VP1 is responsible for increased acid resistance in FMDV mutants. Acid sensitivity of viruses C-S8c1 (derived from infectious clone pMT28) or VP1 D17 (a derivative carrying the mutation responsible for replacement VP1 N17D). Equal PFU amounts of the different viruses were treated with acid buffers, and samples were neutralized and plated. Infectivity was calculated as the percentage of PFU recovered in BHK-21 cells relative to the obtained at pH 7.6.

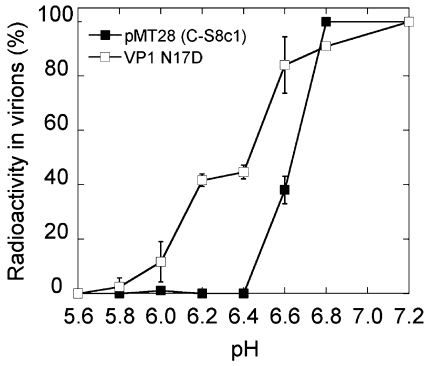

FIG. 5. pH-induced disassembly of FMDV virions. The remaining percentage of intact virions (sedimentation coefficient, 140S) is represented as a function of the pH of the buffer, relative to the amount of intact virion at a pH of 7.2 (taken as 100%). Mutant virions (VP1 N17D, □) and the nonmutated control virions (pMT28 [C-S8c1], ■) were radioactively labeled, purified, and assayed in parallel as described in Materials and Methods. The average values and error bars corresponding to two independent measurements are indicated. Dissociation of the virus was correlated with an increase in the amount of pentameric capsid subunits (sedimentation coefficient, 12S) (39).

pH_{50} value of 6.25 (very close to the pH_{50} values for viruses m2 to m6), whereas the pH_{50} value determined for C-S8c1 virus derived from the parental infectious clone was 6.62. These results confirm that amino acid replacement VP1 N17D is responsible for the increased resistance to acid inactivation phenotype.

The increased resistance of mutant VP1 N17D against acid-induced inactivation is due to its increased resistance against capsid dissociation. To investigate whether the increased resistance of mutant VP1 N17D to biological inactivation is related to an impaired dissociation into subunits, virions of C-S8c1 and the VP1 N17D mutant were radiolabeled and purified through sucrose gradients. Similar amount of virions (sedimentation coefficient, 140S) were obtained in both cases. The viruses were then incubated at different pHs for a defined amount of time and subjected to analytical sedimentation analysis to determine the amount of intact virions remaining after the treatment. The results (Fig. 5) show that the VP1 N17D mutant virion is substantially more resistant than the parental C-S8c1 virion to acid-induced dissociation of the capsid into pentameric subunits. Their respective pH_{50} values for dissociation (about 6.3 and 6.6, respectively) were very similar to their pH_{50} values for inactivation of infectivity (see above), and their dissociation and inactivation curves overlapped to a large extent. Thus, the increased resistance of mutant VP1 N17D to acid-induced inactivation is due to its increased resistance to capsid dissociation into pentameric subunits.

Biological fitness of an acid-resistant FMDV virion. Viruses recovered from infectious clones were tested in competition experiments performed at different pHs (7.6 and 6.0). Equal PFU amounts of C-S8c1 and VP1 N17D viruses were mixed (initial MOIs of 0.1 [0.05 for each virus]), incubated at pH 7.6 (control) or 6.0, and used to infect BHK-21 cells. Viruses recovered from these infections were harvested, incubated again at pH 7.6 or 6.0, and further passaged. Approximate percentages of competing genomes during the four serial passages analyzed were determined (Fig. 6). In three independent experiments performed at pH 7.6, a similar proportion of the competing genomes (ca. 50%) was observed along the four passages. Conversely, in each of the three independent experiments performed at pH 6.0, mutant VP1 N17D was already dominant in the first passage (ca. 90%), and its proportion progressively increased during passages up to values close to 100%. These results show that replacement VP1 N17D, responsible for increased resistance to acid inactivation, confers a selective advantage in serial passages after incubation at low pH (6.0), whereas it did not drastically alter viral fitness when incubations were performed at a neutral pH. (pH 7.6).

Amino acid substitution VP1 N17D also confers increased resistance to heat inactivation of FMDV. To continue the characterization of the physicochemical alterations caused by replacement VP1 N17D, the effect of high temperature on the infectivity of C-S8c1 and VP1 17D viruses was analyzed. Rel-

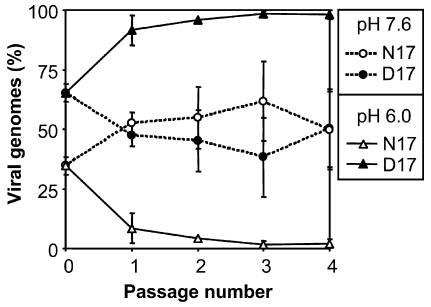

FIG. 6. Competition experiments between viruses C-S8c1 (N17) or VP1 D17 (D17) during serial passages of a virus mixture, in which viruses were incubated at pH 7.6 or pH 6.0 prior to each infection. An initial MOI of 0.1 (0.05 for each virus) was used for the each infection. Approximate percentages of competing genomes during serial passages are represented. Passage 0 denotes the initial mixture of viruses. The proportions of A (in VP1 N17) and G (in VP1 D17) at position 3256 were determined by nucleotide sequencing as described in Materials and Methods. The average proportions of viral genomes found in three independent competition experiments are represented.

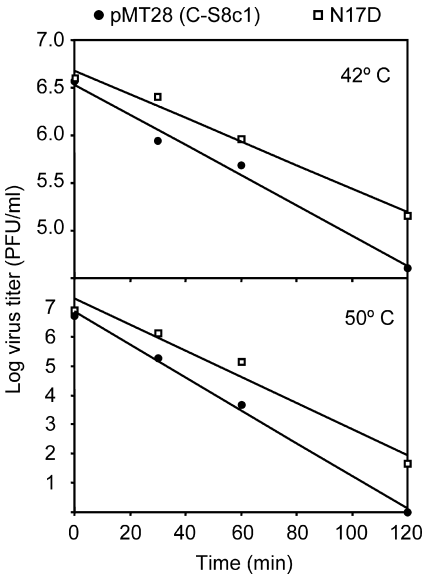

FIG. 7. The single amino acid replacement N17D in VP1 increases FMDV resistance to thermal inactivation. Equal PFU amounts of the different viruses were incubated at 42 or 50°C, and the remaining infectivity in viral samples was determined by plaque assay. Each point corresponds to the average of three independent experiments.

ative to C-S8c1, a moderate increase in resistance to virus inactivation at 42 or 50°C was observed for mutant VP1 N17D (Fig. 7). The t_{50} values (calculated as the time in minutes value leading to a 50% loss of infectivity) derived from these data were 20 min for C-S8c1 and 41 min for VP1 17D at 42°C and 17.7 min for C-S8c1 and 24.7 min for VP1 17D at 50°C. These experiments confirmed that amino acid substitution VP1 N17D also confers some increase in the resistance of FMDV to thermal inactivation.

DISCUSSION

A few FMDV mutants with altered acid sensitivity have been described. We recently showed that mutations in structural proteins can reduce FMDV stability at acidic pH (35). An FMDV mutant with increased resistance against acid inactivation has also been described (54) but not fully characterized. In addition, the rational introduction of a charge reversal mutation in the FMDV capsid led to impaired dissociation of the nucleic acid-free capsid under acidic conditions (19). In the present study, type C FMDV mutants (named m1 to m6) with increased resistance to acid-induced inactivation were isolated from a viral population after *in vitro* incubation at pH 6.0. The selection frequency of these mutants—2.9×10^{-5}—was similar to that reported for FMDV monoclonal antibody resistant mutants (36), but it was 4 orders of magnitude lower than that reported for FMDV mutants with increased capsid lability selected by NH_4Cl treatment ($\sim 10^{-1}$) (35). This lower frequency of acid-resistant mutants could in principle be due to a fitness loss related to modifications of the acid-dependent uncoating mechanism, as suggested by the small plaque phenotype reported for type A acid resistant FMDVs (54). However, viruses m1 to m6 displayed plaque size and growth kinetics similar to those of the C-S8c1 parental virus (data not shown). All mutants with increased resistance to acid inactivation isolated in the present study carried a common nucleotide substitution responsible for amino acid replacement VP1 N17D. Introduction of this replacement into an infectious clone was sufficient to confer an increase in the resistance to acid-induced inactivation in the viruses recovered. Replacement in mutant VP1 N17D also conferred a selective advantage relative to the parental C-S8c1 virus in mixed-serial infections in which viruses were incubated at pH 6.0 prior to infection. When this experiment was performed at pH 7.6, no alteration in the proportion of the parental and the mutant residues was observed. This result indicates that the relatively low mutant frequency of acid-resistant variants in FMDV populations may not be due to a substantially reduced biological fitness.

Amino acid replacement VP1 N17D is located at the N terminus of VP1 on the inner surface of FMDV capsid. This is a nearly isosteric replacement (since the amide and carboxylate groups have similar volumes and geometries) but introduces an additional negative charge per capsid protomer (60 additional charges in the virion). No positively charged groups suitable to form a short-range coulombic interaction with this newly introduced negatively charged carboxylate are close enough in the capsid structure. In addition, residue 17 in VP1 is relatively close to the interpentameric interface, but it neither forms part of the interpentamer or interprotomer interfaces nor establishes any short-range interpentameric or interprotomeric interaction that could result in capsid stabilization. The amide group in VP1 N17 only establishes a hydrogen bond with the main chain oxygen of VP4 G78 residue of the same protomer that would disappear upon VP1 N17D mutation, which could destabilize the interaction between VP1 and the C terminus of VP4 within the same protomer. In natural FMDV empty particles, which display an increased resistance to acid disassembly compared to virions (12), both the N terminus of VP1 and the C terminus of VP4 are more disordered than in the corresponding virions (13). In the case of type A acid-resistant FMDV, this mutant displayed four amino acid replacements (54), one of which was also located at the N terminus of VP1, but the role of each of these mutations in the acid-resistant phenotype was not determined. Conformational rearrangements in the N terminus of VP1 have been recently implicated on the uncoating mechanism of equine rhinitis A virus (ERAV) (53), an *Aphthovirus* that shares physicochemical properties with FMDV such as buoyant density and acid lability (41, 42). As described for FMDV (see the introduction), ERAV is internalized within host cell following a clathrin-dependent pathway, and its uncoating is supposed to occur within early endosomes (22), where low pH should trigger capsid disassembly into pentameric subunits via a transient 80S intermediate particle which has lost the genomic RNA (53). However, besides similarities between ERAV and FMDV, this uncoating intermediate has not yet been described for FMDV.

An alternative, nonexclusive model for explaining acid resistance in mutant VP1 N17D is based on the fact that residue 17 of VP1 is located only about 10 Å from the interpentamer interface and about 15 and 8 Å, respectively, from H140 and H143 in VP3 of the same protomer. In FMDV, conserved histidines at these positions (equivalent to residues 141 and 144 in type O or 142 and 145 in type A) may provide a pH-dependent switch for capsid dissociation in the acidic environ-

ment of the endosome; the protonated histidines could establish an electrostatic repulsion with the dipole of an alpha helix in the neighboring pentamer, facilitating capsid dissociation (1, 19, 55). The introduction of an additional negative charge in mutant VP1 N17D could partially neutralize the positive charge of the protonated H143, reducing the interpentamer repulsion at acidic pH and leading to increased resistance against acid-induced dissociation into pentamers, as observed here.

Along this line, it is interesting that the N17D mutation also led to a somewhat increased resistance against thermal inactivation of the FMDV virion at neutral pH. A previously engineered neutral-to-charged A65H substitution in VP2 at the interpentameric interfaces in the FMDV capsid led to a similar, moderate increase in resistance against thermal inactivation at neutral pH (39). However, while mutants with a substantially shifted pH-dependent stability (35, 54; the present study) can be readily isolated from FMDV populations, mutants with a highly increased stability against thermal inactivation do not appear to be generally present in FMDV quasispecies (38). In addition, whereas acid inactivation of the FMDV virion is directly caused by dissociation of the capsid into pentameric subunits (19; the present study), its thermal inactivation at moderate temperatures appears to be due to a conformational change without capsid dissociation, with dissociation occurring only at much longer incubation times (39). Thus, the mechanisms for the increased resistance against acid inactivation or thermal inactivation of mutant N17D and other FMDV mutants may be quite different.

The additional amino acid replacement VP2 F34L found in mutant m1 (but not in m2 to m6) is located at an internal capsid position. Mutant m1 showed slightly higher resistance to acid inactivation than those of the rest of mutants isolated (Fig. 1A), suggesting that this replacement could be responsible for its slightly increased acid resistance. For ERAV, conformational rearrangements in the N terminus of VP2 have been also described in the uncoating intermediate (53).

Five of the six mutants (m1 to m5) displayed an additional nucleotide mixture at position 2576 responsible for amino acid replacement VP3 D9V. This position has been involved in interprotomer interactions and a salt bridge with VP1 K109 (40), which should be disrupted upon substitution. Amino acid replacements at this position have been also described in viruses recovered from persistent cell culture infections (16, 24, 52). The proportion of the nucleotide substitution leading to VP3 D9V was increased after a second acid treatment (data not shown), suggesting that this additional replacement could be related to an increase in acid resistance. However, virus m6, which lacks VP3 D9V substitution (Table 1), showed a degree of resistance to acid inactivation similar to that of mutants m2 to m5, which carried this mutation at different proportions (Fig. 1). Even when further experiments are required to determine the biological effect of replacement VP3 D9V, an alternative possibility is that this mutation might compensate for some detrimental effect of the replacement VP1 N17D. Indeed, the selection of compensatory mutations in response to amino acid replacements that alter FMDV capsid stability has been previously documented (40).

Enhanced resistance to acid inactivation correlated with an increase in the sensitivity of these mutants to drugs that raise endosomal pH, such as NH_4Cl and ConA. This effect is opposite to that previously shown for mutants with increased capsid lability that were resistant to these drugs (35). These observations confirm that resistance to *in vitro* acid inactivation is related to a lower pH requirement for productive uncoating within host cells. In this way, the acid-resistant mutants described here extend the spectrum of FMDV variants, including pH_{50} values ranging from 6.95 for mutant c2 (35) to 6.07 for mutant m1. These results further illustrate the adaptive flexibility of viral quasispecies to external changes, in this case alterations associated with pH variations.

ACKNOWLEDGMENTS

We thank E. Domingo and C. Escarmís for infectious clone pMT28.

Work at the F.S. laboratory was supported by grants from Ministerio de Ciencia e Innovación (MICINN) BIO2008-0447-C03-01 and CSD2006-0007; work at the M.G.M. laboratory was supported by grants from MICINN (BIO2009-10092) and Comunidad de Madrid (S-2009/MAT/1467). An institutional grant from Fundación Ramón Areces is also acknowledged.

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