Sequence adaptations affecting cleavage of the VP1/2A junction by the 3C protease in foot-and-mouth disease virus-infected cells

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The foot-and-mouth disease virus (FMDV) capsid protein precursor P1-2A is cleaved by the virus-encoded 3C protease to VP0, VP3, VP1 and 2A. It was shown previously that modification of a single amino acid residue (K210E) within the VP1 protein and close to the VP1/2A cleavage site, inhibited cleavage of this junction and produced ‘self-tagged’ virus particles. A second site substitution (E83K) within VP1 was also observed within the rescued virus [Gullberg et al. (2013). J Virol 87, 11591–11603]. It was shown here that introduction of this E83K change alone into a serotype O virus resulted in the rapid accumulation of a second site substitution within the 2A sequence (L2P), which also blocked VP1/2A cleavage. This suggests a linkage between the E83K change in VP1 and cleavage of the VP1/2A junction. Cells infected with viruses containing the VP1 K210E or the 2A L2P substitutions contained the uncleaved VP1-2A protein. The 2A L2P substitution resulted in the VP1/2A junction being highly resistant to cleavage by the 3C protease, hence it may be a preferred route for ‘tagging’ virus particles.

INTRODUCTION

Foot-and-mouth disease virus (FMDV) is the prototypic member of the genus Aphthovirus within the family Picornaviridae. The virus exists in seven distinct serotypes (O, A, C, SAT1–3 and Asia-1), and a large number of different FMDV genome sequences have been determined (e.g. Carrillo et al., 2005). The positive sense RNA genome (~8400 nt) includes a single large ORF (~7000 nt) encoding a large polyprotein (Belsham, 2005). During and after synthesis of the viral polyprotein, it is processed by virus-encoded proteases to generate some 15 distinct mature products plus multiple precursors. The FMDV polyprotein includes two trans-acting proteases, the Leader (L) protease (produced in two forms termed Lab and Lb) and the 3C protease (3C<sup>pro</sup>). The L protease is only responsible for cleaving the L/P1-2A junction within the FMDV polyprotein (Strebel & Beck, 1986; Medina et al., 1993). However, it also induces cleavage of the translation initiation factor eIF4G, which results in the inhibition of host cell cap-dependent, protein synthesis (Belsham, 2005). The 3C<sup>pro</sup> is responsible for cleaving all other junctions within the FMDV polyprotein except for the 2A/2B junction and the VP4/VP2 junction. Cleavage at the 2A/2B junction is dependent on the 2A peptide and is thought to be due to ‘ribsosomal skipping’ (Donnelly et al., 2001). Cleavage of VP0 to VP4 and VP2 occurs on encapsidation of the viral RNA and also within assembled empty capsid particles (Curry et al., 1995; Gullberg et al., 2013a, b) by an unknown mechanism.

The FMDV capsid protein precursor, P1-2A, is cleaved by the 3C<sup>pro</sup> to VP0, VP3, VP1 and 2A. The cleavage of the VP1/2A junction appears to be the slowest of these within cell-free translation systems (Ryan et al., 1989) and within mammalian cells (e.g. see Gullberg et al., 2013a, b) as VP1-2A can be detected under some conditions (e.g. with reduced 3C<sup>pro</sup> expression) when all other junctions are fully cleaved. However, in peptide cleavage assays using short synthetic substrates including eight residues on either side of the various 3C<sup>pro</sup> cleavage sites, it was found that the VP1/2A cleavage site was the most rapidly processed protein junction (Birtley et al., 2005).

In contrast to some other picornavirus 3C proteases, the FMDV enzyme is able to cleave a variety of different junction sequences (the amino acid residues at these junctions are indicated as P4P3P2P1/P1-Q/P3’P4’). The cleavage sites recognized by the FMDV 3C<sup>pro</sup> have either glutamine (Gln, Q) or glutamate (Glu, E) at the P1 position (i.e. P1-Q or P1-E) of the junction to be cleaved (Curry et al., 2007). The VP1/2A junction for serotype O FMDVs is generally PxKQ/xLNF. The residue P1-Q together with the P4-Pro (P), P2-Lys (K) and P4’-Phe (F) residues, represent the most important determinants of 3C<sup>pro</sup> specificity at this site (Birtley et al., 2005; Curry et al., 2007; Zunszain et al., 2010). Studies of aligned FMDV 3C<sup>pro</sup> cleavage sequences from over 100 strains of the virus (including representatives of all seven serotypes) revealed that sites with P1-Q have a strong selectivity for P2 K,
indicating that recognition of the P1 residue by 3Cpro is influenced by the P2 residue (Curry et al., 2007).

Recently, we showed that modification of the P2 K residue (to E) at the VP1/2A junction, in a serotype O P1-2A pre-cursor, resulted in the formation of empty capsid particles containing the uncleaved VP1-2A product when the pre-cursor was co-expressed with the FMDV 3Cpro (Gullberg et al., 2013b). Furthermore, substitution of this residue within a serotype O virus, by modification of a full-length cDNA that produces a chimeric virus termed O1K/O1 Manisa VP1K210E (Gullberg et al., 2013b), also inhibited cleavage at the VP1/2A junction and resulted in the formation of infectious virus particles containing the uncleaved VP1-2A. The ‘self-tagged’ viruses containing this modification also acquired, during the virus rescue procedure, a second amino acid substitution within VP1 (E83K). This could have been a cell culture adaptation, as has been detected previously (Zhao et al., 2003; Maree et al., 2010). However, it is now shown here that there is an apparent linkage between the E83K substitution within VP1 and the cleavage of the VP1/2A junction since introduction of the E83K substitution generated an alternative adaptation at the VP1/2A junction generating a stable VP1-2A.

![Diagram](image)

**Fig. 1.** (a) Schematic structure of FMDV O1K B64 cDNA and its derivatives. The structure of the pT7S3 plasmid (Ellard et al., 1999) containing the full-length cDNA of the O1K B64 strain and previously published modifications to this plasmid (Bøtner et al., 2011) are shown at the top. The Nheli and Apal restriction enzyme sites (as indicated) were used as described in Methods to introduce cDNA fragments corresponding to the coding region for the proteins VP2-VP3-VP1-2A. The amino acid sequences at the VP1/2A junctions are shown with the P2 and P2′ positions indicated in bold text. The FMDV O1K/O1M wt and the VP1 K210E mutant derivative have been described elsewhere (Gullberg et al., 2013b), and the FMDV O1K/O1M VP1 E83K mutant was produced as described here (see Methods). The full-length plasmids were linearized using HpaI prior to *in vitro* transcription and virus rescue. The locations of restriction sites used are marked as Apal, HpaI, Nheli and Xbal. Sequence changes in the rescued viruses are indicated. (b) Growth kinetics of FMDVs. Baby hamster kidney (BHK) cells were infected (m.o.i. of 0.1 TCID₅₀ per cell) with O1K/O1M wt, mutants [VP1 K210E (E83K) or VP1 E83K (2A L2P)] or O1M (Lindholm). At the indicated post-infection (p.i.) times, samples were frozen and the virus yields (as TCID₅₀) were determined.
RESULTS

Modification of the VP1/2A cleavage site

Plasmids containing the full-length FMDV cDNA based on the backbone of O1 Kaufbeuren, but containing the capsid coding region of O1 Manisa, have been described previously (Gullberg et al., 2013b) and are referred to here as pO1K/O1M (see Fig. 1). These plasmids can be used to produce RNA transcripts in vitro that initiate infection when introduced into cells. The earlier studies showed that modification of the VP1 residue K210 to E210, close to the VP1/2A junction in this chimeric virus, inhibited cleavage at this junction and resulted in the production of ‘self-tagged’ virus particles containing the 2A peptide (Gullberg et al., 2013b). This rescued virus also acquired a second site substitution (E83K in VP1) and so is referred to as O1K/O1M K210E (E83K); this second modification could have been a cell culture adaptation, as described previously for other FMDVs (Zhao et al., 2003; Maree et al., 2010). Interestingly, a cell culture-grown strain of O1M, termed O1M (Lindholm), also contained both of these substitutions, in contrast to the O1M field strain (Gullberg et al., 2013b). This virus had clearly arisen independently from the rescued O1K/O1M K210E (E83K) virus. To examine the relationship between these two changes in more detail, the change conferring the E83K substitution in VP1 was introduced alone into the O1K/O1M cDNA and viable virus was rescued (see Fig. 1a). The mutant viruses each grew (Fig. 1b) at least as well as the parental virus (O1K/O1M). Sequence analysis of the VP2-2A coding region (i.e. for the whole capsid precursor except for the internal VP4 protein) from the rescued virus (O1K/O1M VP1 E83K) surprisingly showed that the rescued virus (at passage 2) had acquired a second site mutation in close proximity to the VP1/2A junction. This mutation produced an L-to-P substitution at residue 2 of the 2A peptide, i.e. at position P2’ in the VP1/2A cleavage site, here referred to as 2A L2P (see Table 1). The effect of the 2A L2P substitution on the production of the FMDV capsid proteins, and specifically on the cleavage of the VP1/2A junction, was analysed in extracts from FMDV-infected cells using immunoblotting (see Fig. 2). As expected, the capsid proteins VP0 and VP2 were readily detected, using an anti-VP2 antibody, kindly

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Table 1. Amino acid sequence differences within the VP2-2A region of the capsid precursor between O1K/O1 Manisa wt, O1K/O1 Manisa VP1K210E and O1K/O1 Manisa VP1 E83K

The substitutions were observed at passage 2 (P2) and 3 (P3) as indicated.

### RESULTS

**Modification of the VP1/2A cleavage site**

Plasmids containing the full-length FMDV cDNA based on the backbone of O1 Kaufbeuren, but containing the capsid coding region of O1 Manisa, have been described previously (Gullberg et al., 2013b) and are referred to here as pO1K/O1M (see Fig. 1). These plasmids can be used to produce RNA transcripts in vitro that initiate infection when introduced into cells. The earlier studies showed that modification of the VP1 residue K210 to E210, close to the VP1/2A junction in this chimeric virus, inhibited cleavage at this junction and resulted in the production of ‘self-tagged’ virus particles containing the 2A peptide (Gullberg et al., 2013b). This rescued virus also acquired a second site substitution (E83K in VP1) and so is referred to as O1K/O1M K210E (E83K); this second modification could have been a cell culture adaptation, as described previously for other FMDVs (Zhao et al., 2003; Maree et al., 2010). Interestingly, a cell culture-grown strain of O1M, termed O1M (Lindholm), also contained both of these substitutions, in contrast to the O1M field strain (Gullberg et al., 2013b). This virus had clearly arisen independently from the rescued O1K/O1M K210E (E83K) virus. To examine the relationship between these two changes in more detail, the change conferring the E83K substitution in VP1 was introduced alone into the O1K/O1M cDNA and viable virus was rescued (see Fig. 1a). The mutant viruses each grew (Fig. 1b) at least as well as the parental virus (O1K/O1M). Sequence analysis of the VP2-2A coding region (i.e. for the whole capsid precursor except for the internal VP4 protein) from the rescued virus (O1K/O1M VP1 E83K) surprisingly showed that the rescued virus (at passage 2) had acquired a second site mutation in close proximity to the VP1/2A junction. This mutation produced an L-to-P substitution at residue 2 of the 2A peptide, i.e. at position P2’ in the VP1/2A cleavage site, here referred to as 2A L2P (see Table 1). The effect of the 2A L2P substitution on the production of the FMDV capsid proteins, and specifically on the cleavage of the VP1/2A junction, was analysed in extracts from FMDV-infected cells using immunoblotting (see Fig. 2). As expected, the capsid proteins VP0 and VP2 were readily detected, using an anti-VP2 antibody, kindly

![Fig. 2. Expression of FMDV capsid proteins in BHK cells infected with O1K/O1M wt, VP1 K210E (E83K) or VP1 E83K (2A L2P) mutants and O1M (Lindholm) (m.o.i. of 0.1 TCID₅₀ per cell). Cell lysates were prepared at the indicated hours p.i. Aliquots were analysed for the presence of (a) FMDV VP2, (b) VP1 or (c) 2A by immunoblotting. Uninfected BHK cells were used as a negative control. Molecular mass markers (kDa) are shown on the left side.](image)
provided by Li Yu (Yu et al., 2011), in lysates from cells infected with each of the viruses analysed, and the pattern of these bands was identical in each case (Fig. 2a). In contrast, using an anti-VP1 monoclonal antibody also kindly provided by Li Yu (Yang et al., 2011), two different forms of this capsid protein were detected (Fig. 2b). The wt O1K/O1M virus only made a single VP1 product, as expected (Fig. 2b, lanes 2 and 3). However, the O1K/O1M VP1K210E (E83K) virus produced both VP1-2A (as observed previously by Gullberg et al., 2013b) and VP1 itself (see Fig. 2b, lanes 4 and 5). The generation of VP1 was especially apparent at the later stage of infection, suggesting that some cleavage of this mutant VP1/2A junction is possible when high levels of the 3Cpro are present. The O1K/O1M VP1 E83K (2A L2P) mutant virus produced VP1-2A with little or no detectable VP1. Thus this mutant product, containing the 2A L2P substitution, seemed to be very stable and hence highly resistant to the action of 3Cpro (Fig. 2b, lanes 6 and 7). The O1M (Lindholm) virus, described previously by Gullberg et al. (2013a, b), which also contains both the E83K and K210E substitutions in VP1, produced a mixture of VP1 and VP1-2A (Fig. 2b, lanes 8 and 9). This is consistent with the results for the O1K/O1M VP1 K210E (E83K) virus. Confirmation that the products identified as VP1-2A indeed contained the 2A peptide sequence was achieved using an anti-2A antibody (Fig. 2c). Consistent with the apparent stability of the VP1-2A product containing the 2A L2P substitution, the strongest signal with the anti-2A antibody was obtained in samples derived using this mutant virus (Fig. 2c, lanes 6 and 7).

Additional evidence for the effect of the 2A L2P substitution on the VP1/2A junction cleavage was obtained using immunofluorescence staining of cells using the anti-2A antibodies. As seen previously (Gullberg et al., 2013b), no signal was obtained with the anti-2A antibodies in uninfected cells or those infected with the parental O1K/O1M virus (Fig. 3e, f); probably the free 2A peptide, produced by the wt virus, is rapidly degraded in the infected cells. In contrast, cells infected with the O1K/O1M K210E (E83K) or with the O1K/O1M E83K (2A L2P) mutant viruses were efficiently stained by the anti-2A antibody (Fig. 3g, h). Cells infected with the wt and mutant viruses were stained with an anti-FMDV antiserum (Fig. 3b–d) as expected.

To demonstrate that the 2A peptide remained attached to the VP1 protein in assembled virus particles, harvests from virus-infected cells were fractionated on sucrose gradients and the fractions were assayed using ELISAs specific for the FMDV capsid proteins or the FMDV 2A peptide (Fig. 4). The wt O1K/O1M virus produced a clear peak of FMD virions migrating near the bottom of the gradient, but these virus particles were not detected by the anti-2A ELISA (Fig. 4a). However, the virions obtained from cells infected with the O1K/O1M VP1 K210E (E83K) virus were clearly recognized by the anti-2A ELISA (Fig. 4c) as well as by the anti-FMDV ELISA, thus indicating that these mutant FMD virions contained the 2A peptide.

For comparison, empty capsid particles were also generated in transient expression assays and analysed in the same way. Consistent with earlier results (Gullberg et al., 2013b), empty capsid particles produced from the wt O1M capsid sequences were weakly recognized in the anti-2A ELISA (Fig. 4b), while the empty capsid particles from the O1M

![Fig. 3. Immunostaining of BHK cells infected with wt and mutant serotype O FMDVs. (a, e) Uninfected cells or (b, f) cells infected (m.o.i. of 0.1 TCID\(_{50}\) per cell) with O1K/O1M wt, (c, g) O1K/O1M VP1 K210E (E83K) or (d, h) O1K/O1M VP1 E83K (2A L2P) were fixed at 4 h p.i. Virus proteins were detected with (a–d) an anti-FMDV O1M antibody or (e–h) an anti-2A antibody and a secondary antibody labelled with Alexa Fluor 568 (red). The cell nuclei were visualized with DAPI (blue). Bar, 50 μm.](http://vir.sgmjournals.org)
VP1 K210E cassettes (without the E83K second site mutation) were more efficiently recognized in this assay (Fig. 4d). It should be noted that in the transient expression system used, the level of 3Cpro was deliberately reduced relative to the P1-2A capsid precursor (Gullberg et al., 2013a, b), and thus higher levels of residual VP1-2A can be expected to be present within the wt O1M empty capsids than in the wt virions generated within FMDV-infected cells, which contain high levels of the 3Cpro.

The status of the capsid proteins within the sucrose gradient purified empty capsids (fractions 7, Fig. 4b, d) and virions (fractions 13, Fig. 4a, c) was also determined using immunoblotting. The empty capsids produced from the O1M capsid precursor (OP1-2A-mIRES-3C) contained both VP0 and VP2 (Fig. 5b, lane 1) together with both VP1 and some VP1-2A (Fig. 5a, c, lane 1); these results are fully consistent with the ELISA results (see above and Fig. 4). The empty capsids produced from the O1M VP1 K210E mutant contained a similar pattern of VP0 and VP2 as OP1-2A-mIRES-3C (Fig. 5b, lane 2) but contained only VP1-2A and no detectable VP1 (Fig. 5a, lane 2). As expected, the wt O1K/O1M virions contained VP2, but little or no VP0 (Fig. 5b, lane 3), and VP1, but no detectable VP1-2A (Fig. 5a, c, lane 3). The O1K/O1M VP1 K210E (E83K) mutant virus particles also contained VP2, with no apparent VP0 (Fig. 5b, lane 4), but only VP1-2A, without any VP1, was observed (Fig. 5a, c, lane 4).

**DISCUSSION**

The studies presented here provide evidence for a linkage between the substitution E83K within the VP1 capsid protein of serotype O FMDV and changes that inhibit the cleavage of the VP1/2A junction. The association between these events occurred in at least three independent virus preparations. In the cell culture-passaged O1M (Lindholm) strain, the capsid precursor sequence was found to differ at three sites relative to the field strain (Gullberg et al., 2013b). These were each in the VP1 protein, and the substitutions were E83K, S134C and K210E. Rescue of the O1K/O1M K210E virus from a plasmid-derived RNA transcript also resulted in the rapid accumulation of the E83K substitution (by passage 2) in addition to the K210E substitution. Most remarkably, the introduction of the
E83K substitution alone into the O1K/O1M cDNA resulted in the rapid generation (again by passage 2) of a second site substitution (L2P) within the 2A amino acid sequence of the rescued virus (Table 1). Both the VP1 K210E and the 2A L2P modifications within the serotype O virus (O1K/O1M) strongly inhibited cleavage of the VP1/2A junction (see Figs 2 and 3).

As shown previously (Gullberg et al., 2013b), the presence of the K210E substitution generates ‘self-tagged’ virus particles that contain the 2A peptide. Evidence presented here suggests that some cleavage of the K210E mutant VP1/2A junction can occur in virus-infected cells, although the purified virions only appeared to contain the uncleaved VP1-2A protein (Fig. 5). However, the 2A L2P mutant junction appeared more resistant to the high level of 3Cpro generated within FMDV-infected cells as little or no cleavage of this mutant VP1-2A protein was apparent (Fig. 2). Thus, ‘self-tagging’ using this modification may be preferable to the K210E substitution.

Previous studies have demonstrated the presence of an E83K substitution within the VP1 protein of cell culture-adapted serotype O and SAT2 FMD viruses (Zhao et al., 2003; Maree et al., 2010). However, in neither case was the sequence of the 2A coding region examined nor the status of the VP1 protein determined, thus the possible association with a change in sequence close to the VP1/2A junction in these viruses is not known. The amino acid residue 83 in VP1 is located on the surface of the virion and is located around the fivefold axis of symmetry as seen in Fig. 6 (Logan et al., 1993). This residue is well separated from the C terminus of the VP1 protein that is also present on the virion surface (Logan et al., 1993). Thus, it is not clear how the apparent connection between the substitutions at these sites can occur within the context of the mature virus particle (but see below). However, it is striking that the coupling of the changes at E83K and at the VP1/2A junction occurred in three independent serotype O viruses.

An earlier example of an amino acid substitution (M54I) within the serotype C FMDV VP1 affecting 3Cpro mediated processing of the capsid precursor has been reported (Escarmís et al., 2009). This modification of residue 54 was observed during sequential passaging of serotype C FMDV in baby hamster kidney (BHK) cells. Analysis of this substitution in isolation indicated that it resulted in less efficient processing of the VP3/VP1 junction and hence accumulation of VP1 precursor forms; however, their analyses did not determine the presence or absence of the 2A peptide in the VP1 precursors (see Escarmís et al., 2009). Interestingly, residue 54 within VP1 is also surface exposed on the virion (Fig. 6c) and it is also separated from the location of the C terminus of VP3 within the virion (see Fig. 6c). Indeed residue 54 of VP1 is located close to the C terminus of VP1. However, as indicated previously (Escarmís et al., 2009), the locations of these sites within the capsid precursor proteins, prior to 3Cpro cleavage, are not known. Thus the interaction between residues such as M54 and E83 within VP1 and the processing of the P1-2A capsid precursor by 3Cpro, could either reflect a direct effect on the recognition of P1-2A by 3Cpro or could be mediated through the action of a protein affecting the structure of the precursor, e.g. the cellular chaperone Hsp90 (Geller et al., 2007). It is noteworthy that residue M54 is highly conserved in serotype O, A, C and Asia-1 FMD viruses but not in a limited number of SAT strains (Carrillo et al., 2005). The E83 residue is also highly conserved in serotype O but is more variable within other serotypes. Knowledge of the structure of the uncleaved FMDV P1-2A precursor could help to understand the effect of these VP1 substitutions on the capsid precursor processing.

Fig. 5. Characterization of serotype O FMDV empty capsids and O1K/O1M virions. Selected sucrose gradient fractions (from analysis shown in Fig. 4) containing empty capsids (fr. 7) and virions (fr. 13) were analysed by SDS-PAGE and immunoblotting using antibodies specific for (a) all FMDV capsid proteins, (b) FMDV VP2 alone or (c) FMDV 2A alone. Molecular mass markers (kDa) are indicated on the left.
METHODS

Plasmid construction. The full-length FMDV cDNAs used in this study are shown in Fig. 1. The strain ‘O1 Manisa’ is abbreviated throughout as O1M. Plasmids O1K/O1M wt (pO1K/O1M wt) and O1K/O1M VP1K210E (pO1K/O1M VP1K210E) have been described previously (Gullberg et al., 2013b). The pO1K/O1M VP1E83K plasmid was generated as described for the construction of pO1K/O1M VP1K210E. Briefly, the cDNA corresponding to the O1M VP2-2A coding region was amplified from pGEM-3Z-O-P1-2A-mIRES-3C VP1E83K using primers FMDVO_NheIVP4VP2_Fw and FMDVO_ApaI2A2B_Re as described by Gullberg et al. (2013b). The VP2-2A amplicon (~2000 bp) encoding the VP1 E83K substitution was digested with NheI and ApaI and inserted into a similarly digested intermediate plasmid containing the ~5 kbp XbaI fragment from pT7S3, a plasmid containing the full-length FMDV O1Kaufbeuren cDNA (Ellard et al., 1999), as described previously (Bøtner et al., 2011). The cDNA for the VP3 coding region of FMDV O1M includes an NheI site; thus, this insertion was performed in two steps. The resulting plasmid was digested with XbaI, and the fragment was ligated into the XbaI-digested backbone of pT7S3 (Ellard et al., 1999) to generate pO1K/O1M VP1E83K. Plasmids were amplified in Escherichia coli Top10 cells (Invitrogen), purified (Midiprep kit; Fermentas) and verified by sequencing.

Rescue of modified viruses from cDNA. Plasmids containing the full-length FMDV cDNA sequences were linearized with HpaI, purified (Qiagen PCR purification kit; Qiagen) and transcribed in vitro using T7 RNA polymerase (Megascript kit; Ambion) as described by the manufacturer. The RNAs were analysed using agarose gel electrophoresis and then introduced into BHK cells by electroporation as described previously (Bøtner et al., 2011; Nayak et al., 2006). At 1–3 days post-electroporation, the viruses were harvested and amplified in one or two subsequent passages (P2 and P3) in BHK cells. After these passages, viral RNA was extracted (QiAmp RNA blood mini kit; Qiagen), reverse transcribed using ready-to-go you-prime first-strand beads (GE Healthcare Life Sciences), and the FMDV cDNA corresponding to the VP2-2A

Fig. 6. Location of surface-exposed residues E83K, M54 and K210 in the serotype O FMDV virus. (a) A surface rendered view of a pentameric subunit of the virus capsid of the FMDV O1 BFS (1FOD.pdb) (Logan et al., 1993) showing the location of the exposed FMDV O1M amino acid residues. VP1, VP2, VP3 and VP4 are shown in blue, green, red and yellow, respectively. The pentamer shows the location of the surface-exposed amino acid substitutions in FMDV O1M VP1 E83K (cyan) and VP1 K210E (orange) based on a multiple sequence alignment (CLUSTAL W) and the C terminus of VP1 (grey) (Thompson et al., 1994). (b) Schematic illustration of coupled substitutions within the capsid protein precursor (P1-2A). The proteins are colour coded as in (a) and individual residues are also highlighted. (c) The pentamer shows the location of the surface-exposed VP1 M54 (pink) in FMDV O1M based on a multiple sequence alignment. The enlarged rectangle, viewed from a different angle, highlights the location of this residue relative to the C terminus of VP1 (grey) and the C terminus of VP3 (black). The N terminus of VP1 is not surface exposed.
coding region was amplified in a PCR (Expand high-fidelity PCR system; Roche). Control reactions, lacking reverse transcriptase, were used to show that the PCR products obtained were derived from the viral RNA template and not from residual DNA template. The amplicons (~2000 bp) including the entire VP2-2A coding region were visualized in agarose gels, then purified (GeneJET gel extraction kit, Fermentas) and sequenced, on both strands, with an ABI Genetic Analyzer 3500 (Applied Biosystems) using the Big Dye Chemistry (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit, version 1.1, Applied Biosystems). Sequences were analysed using Vector NTI software (Invitrogen).

Virus titres were determined as TCID₅₀ ml⁻¹ by titration in BHK cells according to standard procedures (Reed & Muench, 1938).

Virus infection of BHK cells. Monolayers of BHK cells, grown in 35 mm wells, were inoculated with the rescued viruses or O1M (Lindholm) (a cell culture-grown strain, see Gullberg et al., 2013b) at an m.o.i. of 0.1 TCID₅₀ per cell. At the indicated times post-infection (p.i.), the infected cells were harvested either by freezing (at ~80 °C) to determine virus yield (as TCID₅₀) as described by Reed & Muench (1938) or by preparation of cell lysates. The cell monolayers were lysed in 20 mM Tris/HCl (pH 8.0), 125 mM NaCl and 0.5 % NP-40 and the resultant lysates were clarified (at 18 000 g for 10 min at 4 °C).

Immunoblot analysis. Immunoblotting was performed using cell lysates according to standard methods as described previously (Polacek et al., 2013). Briefly, aliquots were mixed with Laemmli sample buffer (with DTT); the proteins were separated by SDS-PAGE (12.5 % or 15 % polyacrylamide) and transferred to PVDF membranes (Millipore). Specific proteins were detected, as indicated in the figure legends, with primary antibodies recognizing FMDV capsid proteins (anti-FMDV O1 Manisa guinea pig serum), serotype O FMDV VP1 [monoclonal antibody 8E8, kindly provided by L. Yu, Harbin, China (Yang et al., 2011), FMDV VP2 [monoclonal antibody 4B2 (Yu et al., 2011), also from L. Yu] and FMDV 2A (ABS31; Millipore). Bound proteins were visualized using appropriate secondary, HRP-conjugated, antibodies (Dako) and a chemiluminescence detection kit (ECL Prime, Amersham) with a Chemi-Doc XRS system (Bio-Rad).

Immunofluorescence assays. Monolayers of BHK cells, grown on glass coverslips in 35 mm well plates, were infected with O1K/O1M viruses (m.o.i. of 0.1 TCID₅₀ per cell). At the indicated times p.i., the cells were fixed, stained and mounted as previously described using anti-FMDV O1Manisa and anti-FMDV 2A (as above) (Gullberg et al., 2013b). Images were captured using an epifluorescence microscope.

Antigen ELISAs. The serotype-specific FMDV antigen ELISA for serotype O was performed as described (OIE, 2009; Roeder & Le Blanc Smith, 1987). The ELISA used to detect the FMDV 2A peptide has been described previously (Gullberg et al., 2013b).

Sucrose gradient analysis. At 30 h p.i. (with complete cytopathic effect), samples prepared from BHK cells (one T75 flask) infected with FMDV O1K/O1M wt or VP1 K210E (m.o.i. of 0.1 TCID₅₀ per cell) were clarified at 18 000 g for 5 min at 10 °C. The viruses were then concentrated by centrifugation (245 000 g for 1.5 h at 10 °C using a Beckman Coulter SW 55 Ti rotor) through a 30 % (w/v) sucrose cushion in 40 mM sodium phosphate buffer (pH 7.6) with 100 mM NaCl (buffer P) before being sedimented through a 15–30 % (w/v) sucrose gradient in buffer P at 245 000 g in the SW 55 Ti rotor for 1 h at 10 °C.

Cell extracts (400 μl of lysate from one 35 mm well per gradient), from BHK cells that had been infected with the vaccinia virus vTF7-3 (which expresses T7 RNA polymerase) and transfection with pGEM-3Z-O-P1-2A-mIRES-3C or pGEM-3Z-O-P1-2A-mIRES-3C VP1 K210E, as previously described (Gullberg et al., 2013a, b), were loaded onto 15–30 % (w/v) sucrose gradients and centrifuged as described for the FMDV-infected cell lysates. Viral proteins were detected in collected fractions using the serotype-specific FMDV antigen ELISA and FMDV 2A-specific ELISA as described above.

Location of amino acid substitutions in the VP1 protein. The locations of the FMDV serotype O1M amino acid changes, VP1 E83K and VP1 K210E substitutions were mapped, based on a multiple sequence alignment (CLUSTAL W) (Thompson et al., 1994), onto the structure of the closely related FMDV O1 BFS virus [Protein Data Bank accession number 1FOD.pdb (Logan et al., 1993)]. The X-ray crystal structure of the FMDV O1 BFS capsid was then modelled and visualized using Chimera (Pettersen et al., 2004; Sanner et al., 1996).

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