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Cleavages at the three junctions within the foot-and-mouth disease virus capsid precursor (P1–2A) by the 3C protease are mutually independent

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ABSTRACT

The foot-and-mouth disease virus capsid precursor, P1–2A, is cleaved by the 3C protease (3C^{pro}) to VP0, VP3, VP1 and 2A. The P1–2A precursor (wt or mutant) was expressed alone or with 3C^{pro} and processing of P1–2A was determined. The VP2 K217R and VP3 I2P substitutions (near the VP0/VP3 junction) strongly reduced the processing at this junction by 3C^{pro} while the substitution VP2 K217E blocked cleavage. At the VP3/VP1 junction, the substitutions VP3 Q2221P and VP1 T1P each severely inhibited processing at this site. Blocking cleavage at either junction did not prevent processing elsewhere in P1–2A. These modifications were also introduced into full-length FMDV RNA; only wt and the VP2 K217R mutant were viable. Uncleaved VP0–VP3 and the processed products were observed within cells infected with the mutant virus. The VP0–VP3 was not incorporated into empty capsids or virus particles. The three junctions within P1–2A are processed by 3C^{pro} independently.

1. Introduction

Foot-and-mouth disease (FMD) is a highly contagious disease of cloven-hoofed animals including cattle, pigs and sheep. The disease can cause enormous economic losses during an outbreak because of severe restrictions on international trade. FMD has been successfully eradicated from Europe but it is still endemic in large areas of Africa and Asia (Alexandersen et al., 2003). FMD sometimes causes epidemics in previously disease-free areas, as for instance the outbreak in the UK in 2001, which resulted in the loss of several million animals with total costs estimated at about £ 8 billion (Alexandersen et al., 2003).

FMD is caused by infection with foot-and-mouth disease virus (FMDV), which is a small, non-enveloped, RNA virus belonging to the genus *Aphthovirus* within the large family of picornaviruses. Each FMDV particle contains a single-stranded positive-sense RNA genome of around 8400 nucleotides (nt) surrounded by a protein shell (capsid), which is roughly spherical and about 25–30 nm in diameter. The capsid is composed of 60 copies of 4 different structural proteins, termed VP1, VP2, VP3 and VP4. There are seven different serotypes of FMDV, namely O, A, C, SAT1, SAT2, SAT3 and Asia-1. There is no cross-protection between these serotypes and there is significant nucleotide sequence diversity between them, especially in the capsid protein coding regions.

The FMDV genome contains a single, long open reading frame

(ORF) of around 7000 nt. Translation initiation on the viral RNA to produce the encoded polyprotein is achieved by the internal ribosomal entry site (IRES), located within the 5'-untranslated region (UTR). The polyprotein is processed largely by virus-encoded proteases. Processing into the primary products, namely the Leader protease (L^{pro}), P1–2A, P2 and P3 occurs rapidly, during and after, synthesis. The L^{pro} is responsible for the cleavage at its own C-terminus, i.e. at the L/P1–2A junction, while the 2A peptide (only 18 amino acids long) is required for the “cleavage” at the 2A junction; this is a protease independent break in the polypeptide chain and is termed “ribosomal skipping” (Donnelly et al., 2001) or “StopGo” (Atkins et al., 2007). After this initial processing, all other cleavages are catalyzed by the 3C protease (3C^{pro}), except for the maturation of VP0 into VP2 and VP4. The P1–2A precursor is subsequently processed to VP0, VP3 and VP1 and the 2A peptide. The capsid proteins VP0, VP3 and VP1 remain associated with each other (in a protomer) and assemble into pentamers. Twelve of these pentamers can then assemble around a single RNA molecule to form a virus particle, but they are also able to assemble into empty capsid particles without any RNA (Abrams et al., 1995; Gullberg et al., 2013a). During the assembly of the pentamers into a virus particle, the VP0 is cleaved to generate VP2 and VP4 by a process that is currently not understood. The VP4 is entirely internal within the virus particle, whereas VP1, VP2 and VP3 are exposed on the capsid surface, contributing to the antigenic properties of the virus. Moreover, these

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The 3C^{pro} from different picornaviruses cleave the protein junctions with different specificities, i.e. with different amino acids upstream and downstream of the cleavage site. The amino acid residues flanking the cleavage sites are designated as P4 P3 P2 P1 / P1' P2' P3' P4' respectively. In many picornaviruses (e.g. the enteroviruses, like poliovirus) the 3C^{pro} cleavage sites always contain a glutamine residue (Q) at the P1 position, however FMDV 3C^{pro} shows greater diversity in its recognition sequence and is able to process junctions containing either a glutamate (E) or a glutamine (Q) at the P1 position. The VP0/VP3 junction, in most FMDVs, belongs to the E/x (where x can be a variety of residues) group of sites (with the exception of most of the SAT serotypes, which have a Q). The amino acid sequence for this junction in A22 Iraq FMDV is PSKE/GIVP, whereas the consensus sequence for the VP0/VP3 junction for both serotype O and A FMDVs is PSKE/GIFF. However, comparison of the different FMDV junctions from different serotypes indicates that 3C^{pro} is able to process the E/x junction without being very constrained by the nature of the P2 residue, as it can be lysine (K), arginine (R), threonine (T), histidine (H) or glutamine (Q) (Curry et al., 2007). The P1' residue seems to have a strong influence on the junction since it is generally a glycine (G) but sometimes serine (S). At residue P2', the VP0/VP3 junction in A22 Iraq FMDV has an isoleucine (I) residue, interestingly in the E/x group of sites this residue is most often a proline (P), but can also be a G, leucine (L), valine (V) or I (as in the A22 Iraq strain) (Curry et al., 2007). However, for the VP0/VP3 junction it is commonly an isoleucine and only 10 out of 133 sequences have a valine (Carrillo et al., 2005). The VP3/VP1 junction generally belongs to the Q/x group of sites and thus usually has a Q at

We have earlier investigated the characteristics of the cleavage at the VP1/2A junction (Gullberg et al., 2013b; Gullberg et al., 2014), which belongs to the Q/x group of sites, and determined important properties of the amino acid residues at the P2 and P2' positions at this junction (Gullberg et al., 2014; Kristensen et al., 2016). Surprisingly it was demonstrated that cleavage at the VP1/2A junction is not required for virus viability (Gullberg et al., 2013b; Kristensen et al., 2016). We have now expanded the analysis of FMDV capsid protein processing to investigate the junctions between VP0/VP3 and VP3/VP1 that belong to the E/x and Q/x groups respectively. These junction cleavages were investigated by expressing the P1–2A precursor alone or together with the 3C^{pro} in a transient expression assay and also using full-length FMDV RNA transcripts.

2.1. Optimisation of the transient expression assay

The FMDV A22 Iraq P1–2A capsid precursor has been expressed, within BHK cells, both alone and in the presence of the FMDV 3C^{pro} using a transient expression system. The plasmid expressing the P1–2A product is a derivative of the full-length plasmids used for virus rescue and thus contains the IRES and the coding regions for the Leader protease (L^{pro}) as well as the P1–2A capsid precursor but has had the coding regions for the non-structural proteins (including 3C^{pro}) removed, see [Fig. 1](#). The L^{pro} can have an adverse effect on the level of product generated from the expression system ([Polacek et al., 2013](#); [Guan and Belsham, 2017](#)). Therefore, a mutation which results in an amino acid substitution (W52A) within L^{pro} ([Guan and Belsham, 2017](#)) was introduced into the cDNA. The modified L^{pro} retains the L/P1 cleavage activity but does not efficiently induce cleavage of the translation initiation factor eIF4G; this factor is required for the initiation of cap-dependent protein synthesis. The presence of this modification greatly enhanced the expression of the P1–2A capsid precursor from such plasmids in the transient expression assay and, as expected, no loss of the intact eIF4G was detected within transfected cells (c.f. the constructs with the wt L^{pro}, see [Supplementary material Fig. S1](#)). Thus, for all the subsequent analyses in the transient expression system the W52A derivatives of the plasmids expressing the L-P1–2A region of the genome were employed. Furthermore, to get the highest levels of processed capsid protein expression, the amounts of the plasmids encoding the P1–2A precursor and the 3C^{pro} were optimized (see Methods).

Fig. 2. Effect of substitutions at the VP0/VP3 junction on capsid precursor processing. BHK cells (infected with vTF7-3 (Fuerst et al., 1986)) were transfected with plasmids that express the P1–2A (wt or mutant having a substitution at the VP0–VP3 junction) alone (odd numbered lanes) or with the plasmid pSKRH3C (Belsham et al., 2000) encoding the 3C^{pro} (even numbered lanes). A negative control (No DNA) was included (lane 9). Cell lysates were prepared and analysed by immunoblotting. Molecular mass markers (kDa) are indicated on the left. The structural proteins corresponding to the different bands are indicated on the right of the figure and were detected using the following: (a) guinea pig anti-FMDV O-Man antisera, (b) murine monoclonal anti-VP2 antibodies (4B2) and (c) guinea pig anti-FMDV A-Iraq antisera. Bound antibodies were visualized using the appropriate HRP-conjugated secondary antibodies (Dako) and a chemiluminescence detection kit (Pierce & Warriner, ECL Western Blotting Substrate, Thermo Fisher Scientific). The results are representative of at least 3 separate transfections that yielded similar results.

2.2. Processing of the P1–2A precursor at the VP0/VP3 junction

To investigate the effect of the P2 residue in the E/x junction (VP2/VP3) we introduced two different mutations individually into the cDNA that result in the VP2 K217R substitution and the VP2 K217E substitution, these were designed as fairly conservative (retaining the positive charge) or fairly drastic (positive to negative charge) changes, respectively. In addition, the VP3 I2P substitution was introduced to see whether this would have a similar effect on the E/x junction, as observed previously at the Q/x site (at the VP1/2A junction (Gullberg et al., 2014)). The different substitutions that were analysed are shown in Fig. 1.

Expression of the P1–2A coding region for the wt and the three different mutants (VP2 K217R, VP2 K217E and VP3 I2P), in the absence of 3C^{pro}, led to the synthesis of products corresponding to the P1–2A precursor (approximately 85 kDa) (Fig. 2a, b and c, lanes 1, 3, 5 and 7), as expected. In the presence of 3C^{pro}, the wt P1–2A was efficiently processed to generate VP0 (ca. 37 kDa) and VP1 (ca. 28 kDa) (note that the specific antibodies used do not detect VP3 (Gullberg et al., 2013a) but presumably this was also made) (Fig. 2a, b and c, lane 2). The processing of the VP3/VP1 junction in the wt P1–2A seems to happen a little more slowly than the processing of the VP0/VP3 junction, since the VP3–VP1 product (ca. 49 kDa) was readily detected but only very little of the VP0–VP3 product (ca. 58 kDa) was observed (Fig. 2a, lane 2).

When the VP0/VP3 junction was modified (as in the VP2 K217R, VP2 K217E and VP3 I2P mutants), products corresponding to the unprocessed VP0–VP3 were readily detected (see Fig. 2a and Fig. 2b, lanes 4, 6 and 8). Some cleavage of the modified junction still occurred with the VP2 K217R and VP3 I2P mutants since VP0 was also produced (Fig. 2a and b, lanes 4 and 8) but this was not the case with the VP2 K217E mutant (Fig. 2a and b, lane 6) suggesting a severe block on processing at this site as a result of this substitution. The production of VP1 (following cleavage of the unmodified VP3/VP1 junction) was detected from the wt precursor and also from each of the mutant precursors (Fig. 2c, lanes 2, 4, 6, 8). It is concluded that amino acid substitutions at the P2 and P2' positions adversely affect the processing at the VP0/VP3 junction (an E/x site) but this change does not block processing at the VP3/VP1 junction.

2.3. Processing of P1–2A capsid precursor at the VP3/VP1 junction

Using similar analyses, the production and processing of the P1–2A precursor from the wt plasmid or one of the mutants encoding an amino acid substitution close to the VP3/VP1 junction, were analysed in transient expression assays by immunoblotting and the results are shown in Fig. 3.

The VP3/VP1 junction belongs to the Q/x group of sites, like the VP1/2A junction. To investigate the effect of changes near this junction we introduced substitutions at the P1 and the P1' residues, namely VP3 Q221P and VP1 T1P, these were both expected to be highly deleterious to cleavage. The two different substitutions are indicated in Fig. 1.

As expected, expression of the plasmids encoding P1–2A (wt) and the mutants VP3 Q221P and VP1 T1P in the absence of 3C^{pro} led to the synthesis of products corresponding to the P1–2A precursor (Fig. 3a, lanes 1, 3 and 5). In the presence of 3C^{pro}, the P1–2A wt was again efficiently processed to generate VP0 and VP1 (Fig. 3a, b and c, lane 2) as in Fig. 2. Processing of the VP3 Q221P and VP1 T1P P1–2A mutants also produced VP0 (Fig. 3a, b, lanes 4 and 6). However, in contrast to the P1–2A (wt) (lane 2), no VP1 was detected (Fig. 3c, lanes 4 and 6) but a major product corresponding to the uncleaved VP3–VP1 (ca. 49 kDa) was observed (Fig. 3c, lanes 4 and 6). This indicates that processing of the VP3/VP1 junction in the P1–2A precursor by 3C^{pro} was severely inhibited by these single amino acid substitutions. However, abrogating cleavage of this junction did not block processing of the VP0/VP3 junction within the P1–2A.

2.4. Processing of P1–2A capsid precursor at the VP1/2A junction

The VP1/2A junction also belongs to the Q/x group and was not modified in any of the mutants described above. However, the processing of the VP1/2A junction is known to be the slowest within the context of the P1–2A precursor (Gullberg et al., 2013a, 2013b; Ryan et al., 1989), and thus we investigated whether modifying the processing of one of the other junctions between the structural proteins affected the VP1/2A cleavage site.

Processing of the VP1/2A junction from the P1–2A precursor from the plasmids encoding the wt P1–2A or mutant P1–2As with single

Fig. 3. Effect of substitutions at the VP3/VP1 junction on capsid precursor processing. The P1-2A precursor (wt or mutant having a substitution at the VP3-VP1 junction) was expressed alone or with 3C^{pro} in transient expression assays in BHK cells as in Fig. 2. Cell lysates were prepared and analysed by immunoblotting. The odd numbered lanes shows the P1-2A precursors expressed alone and the even numbered lanes shows the precursor co-expressed with the 3C^{pro}. A negative control (No DNA) is included in lane 9. Molecular mass markers (kDa) are indicated on the left. The structural proteins corresponding to the different bands are indicated on the right of the figure and were detected using the following antibodies: (a) guinea pig anti-FMDV O-Man antiserum, (b) murine anti-VP2 antibody and (c) guinea pig anti-FMDV A-Iraq antiserum. Bound antibodies were visualized using the appropriate HRP-linked secondary antibodies (Dako) and a chemiluminescence detection kit (Pierce® ECL Western Blotting Substrate, Thermo Fisher Scientific). The results are representative of 3 separate transfections that yielded similar results.

amino acid substitutions at either the VP0/VP3 or the VP3/VP1 junction, was determined in transient expression assays by immunoblotting using an anti-2A antibody and the results are shown in Fig. 4. A P1-2A mutant with the VP1 K210E substitution (Gullberg et al., 2013b; Kristensen et al., 2016), which inhibits cleavage at the VP1/2A junction, served as positive control (Fig. 4, lanes 8 and 9). Expression of the plasmids encoding the wt and mutant P1-2A products in the absence of 3C^{pro} led to the expected synthesis of the P1-2A precursor in each case

Fig. 4. Cleavage of the VP1/2A junction is unaffected by blocking cleavages elsewhere in P1-2A. BHK cells were transfected with the P1-2A (wt), P1-2A (VP2 K217E), P1-2A (VP1 T1P) and a positive control P1-2A (VP1 K210E) either alone or together with the plasmid encoding the 3C^{pro} as described for Fig. 2. Cell lysates were prepared and analysed by immunoblotting using a rabbit anti-2A antibody (ABS31 Merck Millipore). Bound antibodies were visualized using HRP-linked anti-rabbit secondary antibodies (Dako) and a chemiluminescence detection kit (Pierce® ECL Western Blotting Substrate, Thermo Fisher Scientific). Molecular mass markers (kDa) are indicated on the left. The structural proteins containing 2A corresponding to the different bands are indicated on the right. A negative control (No DNA) (lane 7), and positive controls (where VP1-2A processing is blocked) (lanes 8 and 9) are shown. These analyses were performed following each of the transfections used to generate the results in Fig. 2 and Fig. 3 with very similar results.

(Fig. 4, lanes 1, 3, 5 and 8). When co-expressed with 3C^{pro}, processing of the P1-2A occurred. The VP1 K210E mutant (positive control) yielded a clear product that is detected by the anti-2A antibody at around 28 kDa that is VP1-2A (Fig. 4, lane 9) as expected. In contrast, no VP1-2A could be detected from the wt or from the other mutants indicating that cleavage at the VP1/2A junction had occurred in each case (Fig. 4, lane 2, 4 and 6). Similarly, no product was detected in the negative control (No DNA, Fig. 4, lane 7). Taken together, these results indicate that blocking the cleavage at either the VP0/VP3 junction or the VP3/VP1 junction had no adverse effect on processing at the VP1/2A junction.

2.5. Substitutions at the VP0/VP3 or VP3/VP1 junctions in the full-length virus

The effect of modifying the VP0/VP3 and VP3/VP1 junctions on virus infectivity was also examined. The modifications resulting in the different amino acid substitutions were tested in conjunction with specific silent (synonymous) mutations (see Methods) within a full-length FMDV cDNA based on the backbone of a chimeric O1 Kaufbeuren (O1K) virus containing the coding region for the VP2-VP3-VP1-2A of the A22 Iraq strain of FMDV (as described previously, (Polacek et al., 2013; Kristensen et al., 2016; Porta et al., 2013)). The amino acid substitutions are indicated in Fig. 1. Full-length RNA transcripts were produced in vitro from the linearized plasmids and were introduced into BHK cells by electroporation. After 2 passages in cells, high titre virus stocks were obtained from the parental chimeric FMDV (wt) and from the FMDV (VP2 K217R) mutant. Three passages were performed for the other mutants, but none of them produced CPE and no virus could be rescued (Table 1). To determine whether the introduced mutations were retained in the FMDV (VP2 K217R) mutant, RNA was extracted from the virus harvests and following RT-PCRs, the region encoding the P1-2A precursor was sequenced at passage two, three and four. It was found that the rescued virus from the FMDV (VP2 K217R) mutant had retained both the encoded amino acid substitution and the silent mutation (tag). Moreover, no second site mutations were

Table 1
Rescued viruses.

Name	VP0/VP3 junction	VP3/VP1 junction	Rescued virus
pO1K/A22 wild-type (wt)	ELPSKE/GIVPVA	IDPRSQ/TTTTGE	+
pO1K/A22 (VP2 K217R)	ELPSRE/GIVPVA		+
pO1K/A22 (VP2 K217E)	ELPSEE/GIVPVA		–
pO1K/A22 (VP3 I2P)	ELPSKE/GVPVVA		–
pO1K/A22 (VP3 Q221P)		IDPRSP/TTTTGE	–
pO1K/A22 (VP1 T1P)		IDPRSQ/PTTTTGE	–

observed within the sequence encoding the P1–2A precursor (data not shown).

2.6. Growth curve of the FMDV (wt) and FMDV (VP2 K217R)

The growth of the FMDV (wt) and the FMDV (VP2 K217R) mutant was determined by infecting BHK cells, using a MOI of 0.05 TCID₅₀/cell, and harvesting the cells at different time points post infection. From each time point, samples were titrated to determine the virus yield (TCID₅₀/ml). The results showed that there was no major difference between the growth rates of the two viruses (see [Supplementary material Fig. S2](#)).

2.7. Delayed processing of the VP0-VP3 junction within cells infected by the FMDV (VP2 K217R) mutant

The production of the viral capsid proteins within cells infected with the rescued viruses, FMDV (wt) and the FMDV (VP2 K217R) mutant was visualized from cell extracts by immunoblotting using antibodies directed against the different FMDV capsid proteins. BHK cells were infected with the FMDV (wt) and the FMDV (VP2 K217R), and cell lysates were prepared at different time points (as indicated in [Fig. 5](#)). The FMDV (wt) P1–2A precursor was efficiently processed to generate the structural proteins (VP0, VP2, VP1, see [Fig. 5](#), lanes 3, 5 and 7) and, also presumably VP3 and VP4 (not detected by these antibodies). From the FMDV (VP2 K217R) P1–2A, each of the mature structural proteins was also detected, however the processing intermediate, VP0-VP3 (around 58 kDa) could also be detected at all time points ([Fig. 5a and b](#), lanes 4, 6 and 8) indicating a significant delay in the processing of the modified VP0/VP3 junction (as observed in the transient expression assays, see [Fig. 2a and b](#)). This was particularly pronounced at the early stages of infection, when the level of the 3C^{pro} is relatively low.

2.8. The VP0-VP3 intermediate is not incorporated into either mature FMDV particles or empty capsids

To determine whether the uncleaved VP0-VP3 intermediate was incorporated into either empty capsids or virus particles, clarified lysates from cells infected with either FMDV (wt) or the FMDV (VP2 K217R) mutant were analysed by sucrose gradient ultra-centrifugation. The fractions were analysed by ELISA to determine the fractions containing either protomers/pentamers (fraction 2), empty capsids (fraction 9) or complete virus particles (fraction 20), see [Fig. 6a](#). These three fractions were analysed by immunoblotting. Fraction 2 comprised un-assembled material ([Fig. 6b](#) lanes 1 and 2) and included VP0 and also, for the mutant, VP0-VP3. Fraction 9 contained empty capsids and both VP0 and VP2 were present ([Fig. 6b](#), lanes 3 and 4). Fraction 20 included intact virus particles; almost no VP0 was detected, but a high level of VP2 was observed ([Fig. 6b](#), lanes 5 and 6). The VP0-VP3 intermediate could only be detected in fraction 2 for the FMDV (VP2 K217R) mutant, indicating that this intermediate was not incorporated into either the assembled empty capsids or the complete virus particles. Interestingly,

Fig. 5. Production of uncleaved VP0-VP3 within FMDV-infected cells. BHK cells were infected with FMDV (wt) and FMDV (VP2 K217R). Cell lysates were prepared at 3 h p.i. (lane 3 and 4), 6 h p.i. (lane 5 and 6) and 22 h p.i. (lane 7 and 8) and analysed by immunoblotting. Molecular mass makers (kDa) are indicated on the left. Cell extracts containing the wt P1–2A expressed alone and together with 3C^{pro} from a transient expression assay were included to act as markers for the structural proteins. Uninfected cells (negative control) were also included (lane 9). The structural proteins are indicated on the right of the figure. The antibodies used to detect the structural proteins were: (a) guinea pig anti-FMDV O-Man antisera, (b) murine anti-VP2 antibody and (c) guinea pig anti-FMDV A-Iraq antisera. Bound antibodies were visualized using appropriate HRP-linked secondary antibodies (Dako) and a chemiluminescence detection kit (Pierce® ECL Western Blotting Substrate, Thermo Fisher Scientific). Similar results were obtained in 4 independent virus-infection experiments.

the intact P1–2A precursor could also be detected in fraction 2 for the FMDV (VP2 K217R) mutant, see [Fig. 6b](#) lane 2. The three different fractions were also analysed with the anti-FMDV O-Man antibody; this confirmed the presence of the unprocessed VP0-VP3 intermediate only being present in FMDV (VP2 K217R) fraction 2 (data not shown).

3. Discussion

The FMDV P1–2A precursor is processed by 3C^{pro} into VP0, VP3, VP1 and 2A. The cleavage occurs either at Q/x or E/x junctions, and for both of these types of junction the nature of the residues flanking the junctions is of high importance for optimal cleavage. In the present study we have examined the consequences of either blocking or

Fig. 6. Analysis of VP0-VP3 within assembled and un-assembled virus components within FMDV-infected cells. (a) BHK cells were infected with FMDV wt (solid line) and FMDV VP2 K217R (dotted line), using an MOI of 1, and harvested at 18 h p.i. The clarified lysates were loaded on 15–30% sucrose gradients and separated into separate fractions following centrifugation. FMDV proteins in the different fractions were detected using a serotype specific antigen ELISA. The absorbances were plotted for each fraction. The background (mock infected BHK cells) OD was subtracted. The locations of protomers/pentamers, empty capsids and virus particles are indicated. Fraction 1 is the top of the gradient. (b) Proteins from fractions corresponding to the protomers/pentamers (fraction 2), the empty capsids (fraction 9) and the virus particles (fraction 20) were analysed by immunoblotting as in Figs. 2–4, using the monoclonal anti-VP2 antibody. Molecular mass makers (kDa) are indicated on the left and identification of the structural proteins are shown on the right. The results presented were generated from a single, optimized, experiment with the presence of the VP0-VP3 precursor being consistent with the analyses presented in Fig. 5.

delaying the processing of individual junctions within the FMDV P1–2A precursor by the 3C^{pro} by substituting specific amino acids flanking the junctions.

The structure of FMDV 3C^{pro} closely resembles the conformation of chymotrypsin (Allaire et al., 1994), and indeed, it belongs to the family of chymotrypsin-like serine proteases. The active site is responsible for the substrate binding and is located between two six-stranded β -barrels (Matthews et al., 1999). Normally, the serine proteases contain a catalytic triad composed of the amino acids Ser-His-Asp at the active site, but the FMDV 3C^{pro} has Cys-His-Asp/Glu at the active site (Sweeney et al., 2007; Gosert et al., 1997).

The active site of the enzyme typically recognizes the different side chains of the amino acids flanking the cleavage site. The side chains serve as features for binding and directing the peptide/polypeptide backbone into the correct orientation in the active site. Hydrogen bonds are formed between the substrate and the protease and these contacts are further strengthened by non-polar contacts between the peptide side chains and the protease. The S1 pocket of the FMDV 3C^{pro} is responsible for binding to the side chain of the P1 residue of the substrate (Birtley et al., 2005). The P1-E side chain binds in almost the same manner as the alternative P1-Q side chain, thus making the FMDV 3C^{pro} able to process junctions containing either of these amino acids at this position. The hydrogen bonds from the P1 residue to the side chains of His¹⁸¹ and Thr¹⁵⁸ in the 3C^{pro} are important, as an earlier study showed that substitutions to other amino acids, within synthetic peptide substrates, that contained either uncharged (norleucine), longer (adipic acid) or shorter (aspartate) side chains, blocked the processing of these substrates (Zunszain et al., 2010).

3.1. Processing of the P1–2A precursor in the transient expression assay

At the VP0/VP3 junction (see Fig. 1), which has a glutamate (E) at the P1 position and a lysine (K) at the P2 position, we introduced different substitutions both upstream and downstream of the junction. Interestingly, the fairly conservative VP2 K217R substitution (each has a positively charged side chain) at residue 2 strongly affected the processing of this VP0/VP3 junction (E/x group). VP0 could be detected, but the elevated level of the processing intermediate VP0-VP3 indicated that the processing rate at the VP0/VP3 junction had been reduced. We previously reported that the VP1 K210R substitution at the VP1/2A junction (the P2 position in a Q/x group site) did not affect cleavage at this junction (Kristensen et al., 2016). However, it should be noted that the P2 position in the Q/x group is, in some FMDVs, an R whereas R is never present at the P2 position in the E/x sites (Curry et al., 2007).

Substituting the positively charged lysine (K) residue for the negatively charged glutamate (E) at the P2 residue in the VP0/VP3 junction (VP2 K217E) severely impeded the cleavage. This is consistent with earlier findings on the VP1/2A junction where the VP1 K210E substitution greatly reduced the processing of the VP1/2A junction (Gullberg et al., 2013b; Kristensen et al., 2016). These results revealed that the P2 residue is of high importance in the E/x group and that processing can be reduced to different degrees according to how conservative the substitution is.

The VP0/VP3 junction was also modified by changing one amino acid downstream of the cleavage site, at the P2' position, in the VP3 I2P mutant. This substitution adversely affected the processing of the junction as seen with the VP2 K217R substitution. This is interesting as the introduction of a proline residue at P2' may be expected to induce a more drastic change in structure compared to the arginine substitution

at the P2 position. It is clear that 3C^{pro} was able to process the modified junction since VP0 still could be detected (Fig. 2), but a product corresponding to VP0-VP3 was also detected at a similar level as with the K217R, indicating that the 3C^{pro} cleavage at this site was slowed. An earlier study has reported that a substitution from leucine to alanine at the P2' residue in a synthetic peptide corresponding to a Q/x group site only caused a minor effect on the processing (Birtley et al., 2005). Interestingly, we have shown earlier that substituting the leucine for a proline at the P2' residue in the VP1/2A junction in full length FMDV, completely blocked the processing of this junction (Gullberg et al., 2014). However proline is actually one of the most abundant amino acids at the P2' position in the E/x group of sites within FMDVs, whereas it is never seen at the P2' position in the Q/x group (Curry et al., 2007). This result indicates that even though changing an isoleucine to a proline is a fairly drastic change and the cleavage of this junction is clearly reduced, the 3C^{pro} has a broad tolerance for different amino acids at this position and a variety of residues are found in other FMDV junctions at this specific position in the E/x sites.

The VP3/VP1 junction has a glutamine (Q) at the P1 position. As mentioned above, 3C^{pro} is able to cleave junctions containing either a glutamate or a glutamine residue at the P1 position as the side chains of these amino acids are able to interact with the S1 pocket of the 3C^{pro}. It was therefore not surprising that cleavage by 3C^{pro} was completely prevented when the P1 residue was changed to a proline (VP3 Q221P).

At the P1' position, large hydrophobic side chains are required for optimal cleavage of the Q/x-group. The P1' residue is very often either leucine, isoleucine or threonine (Curry et al., 2007). Leucine and isoleucine are very similar, but threonine seems to stand out from the group since it has an -OH side chain. An earlier study showed that substituting a leucine to a glycine at the P1' position in a synthetic peptide representing the VP1/2A sequence caused a ten-fold reduction in cleavage rate. In contrast, substitutions to valine or cysteine, which both have non-polar side chains only reduced the cleavage rate by two-fold (Zunszain et al., 2010). In the VP3/VP1 junction a threonine is present at the P1' position in all FMDVs (Carrillo et al., 2005). Threonine, does not have a large hydrophobic side chain, as leucine and isoleucine do, indicating that other properties of these amino acids must also affect the processing. Substituting the P1' residue in the Q/x group (VP1 T1P) at the VP3/VP1 junction severely inhibited the processing of this junction. Introducing a proline is known to be very disruptive in the polypeptide chain and therefore was expected to have a negative effect. However, it is interesting that although for the Q/x group substitution to a proline at either P1' in the VP3/VP1 junction (as shown here) or substitution to a proline at P2' in the VP1/2A junction (Gullberg et al., 2014) almost completely blocked cleavage, in contrast changing the P2' residue to a proline in the VP0/VP3 junction (E/x group) only caused a moderate reduction in processing by 3C^{pro}.

For all of the mutants having a substitution near the VP0/VP3 junction, the production of VP1 from the modified P1–2A could be detected in the presence of 3C^{pro}, and for all of the mutants having a substitution in the VP3/VP1 junction, VP0 could be detected following processing. Furthermore, the VP1/2A junction was also fully processed in all of these mutants. These results indicate that 3C^{pro} is able to process these 3 junctions independently and that processing of these junctions does not need to occur in a fixed order. However, it is noteworthy that in the wt protein, processing of the VP3/VP1 junction seems to occur more slowly than processing of the VP0/VP3 junction. This contrasts with earlier studies that indicated that initial processing of the P1–2A precursor happens at the VP3/VP1 junction, followed by processing of the VP0/VP3 junction with the final processing at the VP1/2A junction (Bablanian and Grubman, 1993). For this earlier study, the rabbit reticulocyte lysate *in vitro* translation system was used to produce the P1–2A precursor. The use of this cell-free analysis versus the cell-based analysis used here might explain the differences observed. Blocking processing at either the VP0/VP3 or the VP3/VP1 junctions did not affect the processing of the VP1/2A junction, as

shown in Fig. 4 (and indeed, we have shown earlier that blocking cleavage of the VP1/2A junction does not impede any of the other cleavages as viable virus can still be obtained with the 2A L2P mutant (Gullberg et al., 2014)).

3.2. Processing of the P1–2A precursor in infectious virus

Using the transient expression assay, we showed that the VP2 K217R and the VP3 I2P substitutions markedly reduced cleavage of the junction by the 3C^{pro}, whereas the other substitutions tested essentially blocked the cleavage. Interestingly, the full-length FMDV RNA encoding the VP2 K217R substitution was the only one of the mutants that was able to form infectious virus particles and replicate. It is surprising that the mutant containing the VP3 I2P substitution did not replicate, since cleavage seemed to be reduced to about the same level as for the VP2 K217R substitution in the transient expression assay. Comparing the proteins expressed by the wt virus with the mutant virus containing the VP2 K217R substitution within infected cells clearly showed reduced processing of the VP0/VP3 junction, however this modification does not seem to affect the level of the mature structural proteins to a high degree, as similar amounts of the fully processed proteins were detected in cells infected with the wt and mutant virus containing the VP2 K217R substitution (Fig. 5). These results are consistent with our earlier findings for the Q/x group sites, showing that processing of the VP1/2A junction was not completely blocked by the substitution VP1 K210R (Kristensen et al., 2016).

Separation of protomers/pentamers, empty capsids and virus particles was achieved using sucrose gradient centrifugation (Fig. 6a). By immunoblotting, it was apparent that the VP0-VP3 intermediate was only present in unassembled material (Fig. 6b). This contrasts with the fact that FMDV is able to incorporate uncleaved VP1–2A into the virus particle (Gullberg et al., 2013b). However, based on the structure of the pentamer from an assembled A22 Iraq virus particle it is clear that the C-terminus of VP2 and the N-terminus of VP3 are located very far apart (Fig. 7) (Curry et al., 1996), (although adjacent in the uncleaved P1–2A) and this presumably explains why the assembled virus particles are not able to incorporate the VP0-VP3 intermediate. There was little difference in the growth rate of the FMDV (wt) and the FMDV (VP2 K217R) viruses (Fig. S2). High amounts of empty capsids are produced in both the wt and the mutant, thus suggesting that the structural proteins are produced in excess. Moreover, it seems that the majority of the VP0-VP3 intermediate gets cleaved, especially later in infection (Fig. 5). Both of these features may explain why a small amount of non-functional VP0-VP3 intermediate can be tolerated and not cause a selective disadvantage to the mutant FMDV.

Overall, the results presented here provide important information about the two different groups of junctions within the capsid precursor that are cleaved by the FMDV 3C^{pro}. The results show that there is no strict order of cleavage of the structural proteins, as blocking of one junction did not prevent cleavage of the other junctions.

The 3C^{pro} is responsible for cleaving 10 out of the 13 cleavage sites in the polyprotein, thus making the enzyme an attractive drug target to interfere with the lifecycle of the virus (Curry et al., 2007). Moreover, 3C^{pro} is functionally conserved among different serotypes of FMDV, which provides a possibility of targeting multiple serotypes instead of just one serotype, as is the case with current vaccines.

4. Methods

4.1. Plasmid construction

The plasmid pO1K/A22 contains a full-length cDNA corresponding to a chimeric FMDV genome as previously described (Polacek et al., 2013; Kristensen et al., 2016). It includes the capsid coding sequence from FMDV A22 Iraq and the rest of the genome from FMDV O1K. Variants of this plasmid encoding amino acid substitutions at either the

Fig. 7. Separation of the C-terminus of VP2 and the N-terminus of VP3 during capsid assembly. The structure of the FMDV A22 Iraq capsid pentamer (PDB 4GH4) (Curry et al., 1996) was visualized using pdb-viewer. An enlargement of the 5-fold axis of the pentamer is shown in the red box where the N-termini of the VP3s are marked with red symbols. The C-termini of the VP2s are marked with blue circles. The cleavage site in the uncleaved P1–2A precursor is also indicated.

Table 2
Primers used for site-directed mutagenesis.

Primers for megaprimers	Changes	Name
Plasmids for the Transient expression assay, introducing Leader (W52A) substitution		
SG31_Fw: CCACGACAACCTGCGGTTGAACGCC	Leader (W52A) TGG- > GCG	P1–2A (wt)
14TPN6_Rev: CAAACAGGTGCTTCTTGAAAAATCTTTC		
Plasmids for the Transient expression assay, introducing substitutions in the VP2/VP3 and VP3/VP1 junctions, note that all constructs contains the Leader (W52A) substitution		
14TPN5_Fw: GAGTGTGGGAGTCACGTACG	VP2 K217R (AAA- > CGT)	P1–2A (VP2 K217R)
1PTK18_Rev: GACCGGAACAATCCCTCAGCGAGGGGAG	VP3 V3V (GTA- > GTT)	
13LPN48_Fw: CCCCTCGGAGGAGGGGATTGTACCG	VP2 K217E (AAA- > GAG)	P1–2A (VP2 K217E)
13LPN50_Rev: GCACGCCTCAGCCACATCCAAC		
13LPN49_Fw: CCCCTCGAAGGAGGGGCGTGTACCG	VP3 I2P (ATT- > CCT)	P1–2A (VP3 I2P)
13LPN50_Rev: GCACGCCTCAGCCACATCCAAC		
13LPN22_Fw: GCTCACCACCACTACCAACCGGG	VP3 Q221P (CAA- > CCC)	P1–2A (VP3 Q221P)
13LPN30_Rev: GAGGTCAATGACATGTGTGG		
13LPN23_Fw: GCTCACAACCACTACCAACCGGG	VP1 T1P (ACC- > CCC)	P1–2A (VP1 T1P)
13LPN30_Rev: GAGGTCAATGACATGTGTGG		
Plasmids for production of full-length FMDV RNAs		
14TPN5_Fw: GAGTGTGGGAGTCACGTACG	VP2 K217R (AAA- > CGT)	pO1K/A22 (VP2 K217R)
1PTK18_Rev: GACCGGAACAATCCCTCAGCGAGGGGAG	VP3 V3V (GTA- > GTT)	
14TPN5_Fw: GAGTGTGGGAGTCACGTACG	VP2 K217E (AAA- > GAG)	pO1K/A22 (VP2 K217E)
1PTK22_Rev: AGCGACCGGTACGATCCCTCCTCCGAGGG	VP3 I2I (ATT- > ATC)	
14TPN5_Fw: GAGTGTGGGAGTCACGTACG	VP3 I2P (ATT- > CCT)	pO1K/A22 (VP3 I2P)
1PTK20_Rev: GTACAGGCCCTCCTCGAGGGGAGCTCAC	VP2 K217K (AAA- > AAG)	
14TPN7_Fw: GACAAATCAAGGTTTATGCCAACATTGC	VP3 Q221P (CAA- > CCC)	pO1K/A22 (VP3 Q221P)
1PTK24_Rev: CGGTGGTAGTAGTGGGTGAGCGGGGTCA	VP1 T1T (ACC- > ACT)	
14TPN7_Fw: GACAAATCAAGGTTTATGCCAACATTGC	VP1 T1P (ACC- > CCC)	pO1K/A22 (VP1 T1P)
1PTK26_Rev: CCGTGGTAGTGGGCTGTGAGCGGGGTCA	VP3 Q221Q (CAA- > CAG)	

Bold = Nucleotide changes generating amino acid changes. Underlined = silent mutations.

VP0/VP3 or VP3/VP1 cleavage sites were produced using site-directed mutagenesis (Chen et al., 2000). Phusion® High-Fidelity DNA Polymerase (Thermo Fisher Scientific) was used in all of the PCR amplifications according to the manufacturer's instructions unless otherwise noted. Briefly, fragments were amplified in PCRs, using the pO1K/A22 plasmid as template, together with primers specifying the desired mutations, see Table 2. The PCR products (580 bp for the VP0/VP3 junction and 160 bp for the VP3/VP1 junction) were gel purified using the GeneJET Gel purification kit (Thermo Fisher Scientific) and used as megaprimers for a second round of PCR, again using the wt pO1K/A22 as template to make full length plasmids of approximately 11,000 bp. After the PCR and subsequent *DpnI* digestion of the template DNA, the products were purified using the GeneJET PCR purification kit (Thermo Fisher Scientific) and transformed into chemically competent *Escherichia coli* (*E. coli*) cells. Plasmids were amplified from individual

colonies, purified using the GeneJet Plasmid Miniprep Kit (Thermo Fisher Scientific) and sequenced by Sanger Sequencing using the BigDye Terminator v.3.1 Cycle Sequencing kit and a 3500 Genetic Analyzer (Applied Biosystems). To prepare constructs (lacking the 3C^{pro} coding region) suitable for the transient expression assays, plasmids were digested with *ApaI* to remove most of the cDNA encoding the non-structural proteins downstream of the 2A peptide (Fig. 1). The digested plasmids were analysed using agarose gel electrophoresis and the largest fragments purified using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific). The fragments were self-ligated using Ready To Go T4 DNA ligase (Amersham Biosciences) and transformed into *E. coli*. From the resulting colonies, plasmids were purified using a Plasmid Midi Kit (Qiagen) and sequenced to confirm the absence of the FMDV sequence downstream of the 2A. These constructs contained the cDNA region encoding the wt

Leader (L) protease, which adversely affects the expression level of the P1–2A precursor in the transient expression assay (see [Supplementary material Fig. S1](#)). The W52A mutant form of the L^{pro} retains the L/P1 cleavage activity but is unable to induce cleavage of the translation initiation factor eIF4G (Guan and Belsham, 2017). This modification was introduced into each of the constructs by site-directed mutagenesis, as described above, see [Table 2](#) for primers. The PCR products were *DpnI* digested, transformed into *E. coli*, purified using a Plasmid Midi Kit (Qiagen) and sequenced to confirm the presence of expected mutations and the absence of other changes in the P1–2A region. All of these constructs contained the Leader (W52A) substitution and lacked the coding region for the non-structural proteins. These constructs were called: P1–2A (wt), P1–2A (VP2 K217R), P1–2A (VP2 K217E), P1–2A (VP3 I2P), P1–2A (VP3 Q221P) and P1–2A (VP1 T1P).

The five mutant plasmids (derived from pO1K/A22) containing the chimeric full-length FMDV coding sequence with different substitutions (VP2 K217R, VP2 K217E, VP3 I2P, VP3 Q221P or VP1 T1P) were further modified to introduce different silent mutations near the modified junctions enabling tracking of each mutant in case of potential sequence reversions. The silent changes introduced and primers used to make them are listed in [Table 2](#). Briefly, forward and reverse primers were used in PCRs with each of the five plasmids to create megaprimers (580 bp for the VP0/VP3 junction and 160 bp for the VP3/VP1 junction), which were subsequently used in a long PCR with the same template to introduce the silent mutations. The PCR products were *DpnI* digested, transformed into *E. coli* and the presence of the desired silent mutation in each plasmid was verified by sequencing. These constructs were termed: pO1K/A22 (VP2 K217R), pO1K/A22 (VP2 K217E), pO1K/A22 (VP3 I2P), pO1K/A22 (VP3 Q221P) and pO1K/A22 (VP1 T1P). See [Fig. 1](#) for an overview of the different substitutions (note that the silent mutations are not shown in [Fig. 1](#), but can be seen in [Table 2](#)).

4.2. Transient expression assays

For the transient expression assays, baby hamster kidney (BHK) cells were seeded into 35-mm wells approximately 24 h before starting the assay. The BHK cells (around 90% confluent) were infected with a recombinant vaccinia virus, termed vTF7-3 (Fuerst et al., 1986), that expresses the T7 RNA polymerase. After one hour, this virus was removed and the infected cells were transfected with the indicated plasmid DNA using FuGENE 6 (Promega) essentially as described previously (Belsham et al., 2008). For the co-transfections, 1 µg of the plasmid containing the FMDV P1–2A coding region and 10 ng of the plasmid (pSKRH3C) (Belsham et al., 2000) encoding the FMDV 3C^{pro} were mixed prior to transfection. The cells were incubated in 5% CO₂ at 37 °C overnight. The cells were lysed with 500 µL Buffer C (20 mM Tris-HCl (pH 8.0), 125 mM NaCl and 0.5% NP-40) and the cell extracts were clarified by centrifugation at 18,000 × g for 10 min at 4 °C.

4.3. Immunoblot analysis of samples from transient expression assay

Immunoblotting was performed using cell lysates mixed with either 2 × Laemmli sample buffer (Bio-Rad) or red loading buffer Pack (New England BioLabs) (containing 25 mM DTT). The proteins were separated by SDS-PAGE (4–20%, 12% or a 7.5% Bis-Tris gels (Bio-Rad)) and transferred to either PVDF membranes (Millipore) or nitrocellulose membranes (Bio-Rad). PBS containing bovine serum albumin (BSA) (5%), (for the anti-VP2 antibody and the anti-FMDV O-Man antibody) or skimmed milk powder (5%) (for the anti-2A antibody, the anti-eIF4G antibody and the anti-FMDV A-Iraq antibody) and Tween (0.1%) was used as blocking buffer and dilution buffer for the primary and secondary antibodies. The proteins were detected using guinea pig anti-FMDV O-Manisa antisera (1:1000), or anti-VP2 antibodies (1:2000) (mouse monoclonal antibody 4B2 (Yu et al., 2011), kindly provided by L. Yu, Harbin, P. R. China), guinea pig anti-FMDV A-Iraq antisera (1:1000), FMDV anti-2A-peptide antibody (1:1000) (Rabbit, ABS31

Merck Millipore) or anti-eIF4G antibody (1:1000) (Goat, SC-9602 Santa Cruz Biotechnology) as primary antibodies. The bound proteins were visualized using appropriate HRP-conjugated secondary antibodies (Dako) and a chemiluminescence detection kit (Pierce * ECL Western Blotting Substrate, Thermo Fisher Scientific). Images were captured using either a Chem-Doc XRS system (Bio-Rad) or a G:BOX Chemi XX6 (Syngene).

4.4. Rescue of infectious virus from modified FMDV cDNA

The plasmids, containing the full-length wt or mutant FMDV cDNAs, i.e. pO1K/A22 (VP2 K217R), pO1K/A22 (VP2 K217E), pO1K/A22 (VP3 I2P), pO1K/A22 (VP3 Q221P) and pO1K/A22 (VP1 T1P) were linearized by digestion with *HpaI* and purified using the GeneJET PCR purification kit (Thermo Fisher Scientific). The linearized plasmids were transcribed in vitro using the MEGAscript® T7 Transcription Kit (Thermo Fisher Scientific). An aliquot (1 µL) of each RNA sample was visualized following agarose gel electrophoresis to check yield and integrity and the rest (19 µL) was introduced into BHK cells by electroporation as described previously (Nayak et al., 2006).

The cells were transferred to Falcon flasks and Eagle's medium containing 5% calf serum was added. The cells were incubated overnight at 37 °C. The rescued viruses were amplified using three passages in BHK cells. Cytopathic effect (CPE) was only detected with the FMDV (wt) and the mutant virus containing the VP2 K217R substitution, termed FMDV (VP2 K217R). After each passage, viral RNA, from the wt and the mutant virus was isolated using the RNeasy Mini Kit (Qiagen) and reverse transcribed using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare Life Sciences) together with random primers. The cDNA corresponding to the P1–2A region was amplified as four overlapping fragments of around 1000 bp by AmpliTaq Gold DNA Polymerase (Thermo Fisher Scientific), visualized on agarose gels and purified using the GeneJET PCR purification kit (Thermo Fisher Scientific) (primers listed in [Table S1](#), see [Supplementary material](#)). For each RNA, a negative control, lacking the reverse transcriptase, was included in the RT-PCRs to verify that the PCR products were obtained from viral RNA and not from residual plasmid template. The PCR products were sequenced (primers are listed in [Table S1](#), see [Supplementary material](#)) using the BigDye Terminator v.3.1 Cycle Sequencing kit and a 3500 Genetic Analyzer (Applied Biosystems). Sequences were analysed using Geneious 9.0.2 (Biomatters).

4.5. Titration of FMDV (wt) and FMDV (VP2 K217R)

After the three passages in BHK cells, both the rescued FMDV (wt) and the FMDV (VP2 K217R) stocks were titrated. Briefly, 50 µL BHK suspension (400,000 cells/ml in Eagles medium with 20% fetal bovine serum (FBS)) were loaded in each well of a 96-well plate and 50 µL Eagles medium was added. Lastly, 50 µL of virus dilutions from 10^{−2} to 10^{−9} were added to the wells. Each dilution was tested in quintuplicate together with two negative controls. The plates were incubated at 37 °C in 5% CO₂ and read each day for three days after infection, and the titres (TCID₅₀ /ml) were calculated.

4.6. Immunoblot analysis of FMDV-infected cells

Monolayers of BHK cells, grown in 35 mm wells (approximately 200,000 cells per well), were infected with either the FMDV (wt) or the FMDV (VP2 K217R) at a MOI of 1 TCID₅₀/cell. Cells were lysed using Buffer C (as described above) at 3, 6 and 22 h post-infection. The samples were analysed by immunoblotting as described above.

4.7. Growth curve of FMDV (wt) and FMDV (VP2 K217R)

BHK cells were infected with the FMDV (wt) and the FMDV (VP2 K217R) using a MOI = 0.05. Samples were harvested at 0, 2, 5, 10, 15,

24 and 30 h p.i. and then titrated as described above.

4.8. Sucrose gradient analysis

BHK cells (1 well in a 6-well plate) were infected with O1K/A22 FMDV (wt) or O1K/A22 FMDV (VP2 K217R) (MOI of 1 TCID₅₀ per cell). At 18 h p.i., the medium was removed and the cells were lysed with 250 µL buffer C containing protease inhibitor (cOmplete Mini, EDTA-free protease inhibitor (Roche)). Samples were clarified by centrifugation for 18,000 g for 10 min and 200 µL of the supernatant was loaded directly onto each 15–30% sucrose gradient. For making the gradients, sucrose was dissolved in PBS and the 15% sucrose solution was added to the tube. The 30% sucrose solution was applied afterwards in the bottom by putting a needle through the 15% sucrose solution. Lastly the gradients were finished using a gradient maker (Gradient Master 108 (BIOCOMP)). The samples were centrifuged at 245,000 × g in a SW 55 Ti rotor (Beckman Coulter) for 1 h at 10 °C. Fractions of 240 µL were collected from the top of the gradient by using a Piston Gradient Fractionator™ (BIOCOMP) and analysed by antigen ELISA (see below) and immunoblotting (as above).

4.9. ELISA

An antigen detection enzyme-linked immunosorbent assay (ELISA) against FMDV serotype A, was performed as described previously (Gullberg et al., 2013b; Roeder et al., 1987). Briefly plates were coated with FMDV A22 Iraq rabbit antisera overnight, the plate was washed and blocked with dilution buffer (2% BSA diluted in PBS buffer with 1% Tween) and the gradient fractions were added. Detection of the bound FMDV proteins was achieved using polyclonal guinea pig anti-FMDV A22 Iraq sera plus rabbit anti-guinea pig Ig conjugated to horseradish peroxidase (Dako). A dissolved SIGMAFAST™ OPD tablet (Sigma-Aldrich) was added, and after seeing colour develop, Stop-solution (1 mM H₂SO₄) was added. The plates were read in a SpectraMax® i3 plate reader (Molecular Devices).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2018.07.010.

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