Assembly and characterization of foot-and-mouth disease virus empty capsid particles expressed within mammalian cells

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The foot-and-mouth disease virus (FMDV) structural protein precursor, P1-2A, is cleaved by the virus-encoded 3C protease (3Cpro) into the capsid proteins VP0, VP1 and VP3 (and 2A). In some systems, it is difficult to produce large amounts of these processed capsid proteins since 3Cpro can be toxic for cells. The expression level of 3Cpro activity has now been reduced relative to the P1-2A, and the effect on the yield of processed capsid proteins and their assembly into empty capsid particles within mammalian cells has been determined. Using a vaccinia-virus-based transient expression system, P1-2A (from serotypes O and A) and 3Cpro were expressed from monocistronic cDNA cassettes as P1-2A-3C, or from dicistronic cassettes with the 3Cpro expression dependent on a mutant FMDV internal ribosome entry site (IRES) (designated P1-2A-mIRES-3C). The effects of using a mutant 3Cpro with reduced catalytic activity or using two different mutant IRES elements (the wt GNRA tetraloop sequence GCGA converted, in the cDNA, to GAGA or GTTA) were analysed. For both serotypes, the P1-2A-mIRES-3C construct containing the inefficient GTTA mutant IRES produced the highest amount of processed capsid proteins. These products self-assembled to form FMDV empty capsid particles, which have a related, but distinct, morphology (as determined by electron microscopy and reconstruction) from that determined previously by X-ray crystallography. The assembled empty capsids bind, in a divalent cation-dependent manner, to the RGD-dependent integrin αvβ6, a cellular receptor for FMDV, and are recognized appropriately in serotype-specific antigen ELISAs.

INTRODUCTION

Foot-and-mouth disease virus (FMDV) is the prototypic member of the genus Aphthovirus within the family Picornaviridae. The virus is responsible for one of the most economically important diseases of livestock including cattle, pigs and sheep plus many different cloven-hoofed wildlife species (Alexandersen et al., 2003; Grubman & Baxt, 2004). As with other picornaviruses, the FMDV particle consists of a single copy of the positive-sense RNA genome (~8300 nt) within a protein capsid (Belsham, 2005). This capsid comprises 60 copies of four different virus proteins VP4 (1A), VP2 (1B), VP3 (1C) and VP1 (1D); the VP4 is internal but each of the other proteins is surface exposed (Acharya et al., 1989). The FMDV proteins are produced from a single large polyprotein that is co-translationally cleaved into precursor proteins (L, P1-2A, P2 and P3) and subsequently into some 15 different mature proteins. The capsid proteins are produced from the P1-2A precursor through the action of the 3C protease (3Cpro) which processes it to VP0 (1AB), VP3 and VP1 plus the 2A peptide (18 aa). Cleavage of VP0 (to VP4 and VP2) occurs on encapsidation of the viral RNA but can also occur more slowly, independently from this process (Curry et al., 1997). The P1-2A precursor is modified, by the cellular myristylation system, on its N-terminal gly (G) residue (Chow et al., 1987), following cleavage of the L/P1 junction by the L protease (Medina et al., 1993). The processed FMDV capsid proteins can self-assemble into empty capsid particles (Abrams et al., 1995). Empty capsid particles, generated in FMDV-infected cells, share the same antigenic and immunogenic characteristics.
as the intact virus but they are not infectious (Rowlands et al., 1975).

Seven distinct serotypes of FMDV are known, namely O, A, C, Asia-1, SAT1, SAT2 and SAT3. There is little or no cross-protection between the different serotypes and sometimes even within a serotype, thus there has to be careful matching between vaccine strains and the viruses involved in disease outbreaks. Serotype O is the most frequently reported virus but some countries have concurrent infections with multiple serotypes (e.g. within Eurasia, serotypes O, A and Asia-1 have circulated recently, see http://www.wrlfmd.org/fmd_genotyping/). The SAT serotype viruses are usually confined to sub-Saharan Africa while serotype C has not been identified from outbreaks for several years.

In addition to determining the antigenic properties of the virus, the surface-exposed capsid proteins of FMDV are also critical for binding of the virus to cells. The major cellular receptor for FMDV is the integrin αvβ6 that is expressed on epithelial cells and binds to an RGDLXXL motif within the VP1 protein (Jackson et al., 2000; Monaghan et al., 2005). During growth in cell culture [e.g. in baby hamster kidney (BHK) cells], virus adaptation can occur, for example cell-culture-adapted serotype O viruses can use heparan sulfate as an alternative receptor due to amino acid substitutions within VP3 (Jackson et al., 1996). However, such cell-culture-adapted viruses can be attenuated in animals (Botner et al., 2011; Lohse et al., 2012; Sa-Carvalho et al., 1997).

Vaccines against foot-and-mouth disease (FMD) are widely used where the disease is endemic (e.g. within Africa and southern Asia) and were successful for combating the disease within Europe in the 1960s/1970s. However, now vaccination against FMD is not permitted within the European Union except during a disease outbreak and was used in The Netherlands in 2001 (Bouma et al., 2003). Currently, the production of FMDV vaccines requires the growth of large amounts of infectious virus which is then inactivated before use; thus vaccine production plants represent a potential risk for the escape of infectious FMDV (Rodrigue & Grubman, 2009).

There has, therefore, been considerable interest in producing empty capsid particles for use as potential vaccines against FMD (Abrams et al., 1995; Cao et al., 2009; Porta et al., 2013a, b). In addition, defective human adenovirus vectors which express the components required for FMDV empty-capsid-particle formation have been developed (Moraes et al., 2002, 2011; Pacheco et al., 2005).

There has been some success in using different systems for the production of the FMDV empty capsid particles but there have also been a number of challenges. For example, it has not been possible to isolate single recombinant vaccinia viruses which co-express the P1-2A precursor with the 3Cpro (Abrams et al., 1995). Furthermore, in the baculovirus expression system, the 3Cpro has proved to have adverse effects on protein expression (Porta et al., 2013a). This may result from the fact that the FMDV 3Cpro has a number of cellular targets including certain translation initiation factors (eIF4G and eIF4A; Belsham et al., 2000), cytoskeleton components (Armer et al., 2008) and histone H3 (Falk et al., 1990). Indeed, constitutive expression of FMDV 3Cpro can only be achieved at low levels in mammalian cells (Martinez-Salas & Domingo, 1995). This has led to attempts to decrease the level of 3Cpro activity that is co-expressed with the P1-2A precursor, and it has been shown that equimolar amounts of the 3Cpro are not required to achieve efficient processing of the P1-2A within both insect and mammalian cells (Polacek et al., 2013; Porta et al., 2013a, b).

One strategy to reduce the level of 3Cpro activity is to use mutant forms of this protease with reduced enzymic activity; such mutants have been described previously (Sweeney et al., 2007). An alternative system relies on reducing the amount of 3Cpro expression relative to the P1-2A. This can be achieved by differential levels of transcription, for example using different promoters to drive the expression of two different cDNA cassettes (as used with enterovirus 71; Chung et al., 2010). Alternatively, a single cDNA cassette containing the coding sequences for FMDV P1-2A and 3Cpro has been used in insect cells with the two coding sequences being separated by a frame-shift signal (Porta et al., 2013a, b). In addition, two separate ORFs can be expressed from a single dicistronic mRNA with an inefficient (mutant) internal ribosome entry site (IRES) located between them. The use of a mutant IRES (designated mIRES) can result in relatively low level translation of the downstream ORF (here 3Cpro). In the current study, alternative systems for the expression and processing of FMDV capsid proteins (from serotypes O and A) within mammalian cells have been analysed. Using the optimal system, efficient assembly of empty capsid particles which bind to the αvβ6 integrin and appropriately to serotype-specific anti-FMDV antibodies has been demonstrated.

RESULTS

Structure of FMDV cDNA cassettes
To analyse the expression, processing and assembly of FMDV capsid proteins, FMDV cDNA cassettes derived from serotype O and serotype A viruses were constructed. Schematic representations of these cassettes, all under the control of the bacteriophage T7 promoter, are shown in Fig. 1. The plasmids encode the FMDV structural protein precursor P1-2A alone or with 3Cpro as either a single ORF (in P1-2A-3C cassettes), or as two separate ORFs within a single RNA transcript with the 3Cpro expression being dependent on the FMDV IRES (in P1-2A-IRES-3C cassettes). To reduce the level of 3Cpro activity generated and to determine the optimal system for the co-expression of this protease with the capsid protein precursor, two different strategies were used. Either (i) a mutant 3Cpro
(3C\textsuperscript{pro} C142S), with reduced protease activity (Birtley et al., 2005; Sweeney et al., 2007), was included in the cassettes or (ii) mIRES elements, with reduced ability to direct internal initiation of protein synthesis, were employed to produce the 3C\textsuperscript{pro}. Previously, it has been shown that the 3C\textsuperscript{pro} initiation of protein synthesis, were employed to produce mIRES elements, with reduced ability to direct internal initiation of protein synthesis, were employed to produce the 3C\textsuperscript{pro}. Previously, it has been shown that the 3C\textsuperscript{pro} C142S substitution reduces the activity of 3C\textsuperscript{pro} \textit{in vitro} to <1% of the wt protease activity (Sweeney et al., 2007). Two different mIRES elements were designed and constructed based on previous results with the closely related encephalomyocarditis virus (EMCV) IRES (Robertson et al., 1999). This type of IRES contains a highly conserved GNRA tetraloop motif (GCGA in FMDV), which is essential for maximum activity (Fernandez-Miragall & Martinez-Salas, 2003; Lopez de Quinto & Martinez-Salas, 1997; Roberts & Belsham, 1997; Robertson et al., 1999). This motif was modified in the cDNA to either GAGA or GTTA, as such mutants in the EMCV IRES have less than 10% of wt IRES activity (Robertson et al., 1999).

Reduced level of 3C\textsuperscript{pro} activity enhances accumulation of processed FMDV capsid proteins

In the following experiments, plasmids which produce monocistronic (P1-2A and P1-2A-3C) and dicistronic RNA transcripts (P1-2A-mIRES-3C) from the T7 promoter were transfected into BHK cells infected with the vaccinia virus vTF7-3, which expresses the T7 RNA polymerase (Fuerst et al., 1986). After 20 h, cytosolic extracts were prepared and analysed for the presence of FMDV capsid proteins, 3C\textsuperscript{pro} and β-actin by immunoblotting. As expected, no FMDV proteins were detected by the antisera in the absence of any transfected plasmid (Fig. 2a, b, lanes 1). In the absence of 3C\textsuperscript{pro}, the unprocessed P1-2A precursor was produced from both the serotype O and the serotype A cDNA cassettes (~85 kDa, Fig. 2a, b, lanes 2) as observed previously (Polacek et al., 2013). When the 3C\textsuperscript{pro} sequence was included in the cassettes then processing of the P1-2A occurred, but the extent of processing observed and the accumulation of the processed products varied with the level of 3C\textsuperscript{pro} activity that was expressed (see Fig. 2). The use of wt 3C\textsuperscript{pro} resulted in near complete processing of P1-2A (see Fig. 2a, b, lanes 3, 5 and 6) into the individual capsid proteins VP0 (~36 kDa) and VP1 (~28 kDa) in accordance with the expected sizes (33 and 23 kDa, respectively; Fig. 2, lanes 3–10). Note the VP3 capsid protein (~24 kDa) was not efficiently recognized by this polyclonal anti-FMDV antibody (M. Gullberg, C. Polacek & G. J. Belsham, unpublished results). For both serotypes O and A, the P1-2A-3CC142S constructs (with a mutant 3C\textsuperscript{pro}) yielded increased levels of the processed capsid proteins compared with the P1-2A-3Cwt constructs (Fig. 2a, b, compare lanes 3 and 4). Using serotype O cassettes, a VP1/VP1-2A doublet was observed (Fig. 2a; these products were identified using P1 and P1-2A FMDV cDNA cassettes; M. Gullberg, C. Polacek & G. J. Belsham, unpublished results). A higher level accumulation of the 3C\textsuperscript{pro} (C142S) mutant compared with the wt 3C\textsuperscript{pro} was detected by immunoblotting (Fig. 2), as observed previously in co-transfection experiments (Polacek et al., 2013). This is probably due to the fact that the 3C\textsuperscript{pro} C142S mutant does not inhibit its own synthesis (see Discussion). Among all of the different constructs and independent of serotype, the P1-2A-mIRES-3C cassettes containing the GTTA mIRES element with reduced IRES activity together with the wt 3C\textsuperscript{pro} produced the highest amounts of processed capsid proteins (Fig. 2a, b, lanes 7). In contrast, no increased capsid protein accumulation was observed for the construct containing the GAGA mutant compared with the wt IRES (Fig. 2a, b, compare lanes 5 and 6). As expected, the mIRES element with the tetraloop sequence GTTA, in combination with the 3C\textsuperscript{pro} C142S, reduced the 3C\textsuperscript{pro} enzyme activity to lower levels compared with the combination with 3Cwt. Proteolytic activity was still observed, but higher levels of unprocessed P1-2A precursor and intermediates (i.e. VP0–VP3 and VP3–VP1) were detected (Fig. 2a, b, compare lanes 7 and 10). The accumulation of 3C\textsuperscript{pro} itself was detected by immunoblotting from all the FMDV cDNA cassettes containing the coding sequence for 3C\textsuperscript{pro} (as expected) except for the P1-2A-IRESgtta3Cwt and P1-2A-IRESgtta3CC142S (see Fig. 2a, b, lanes 7 and 10). Taken together, the results showed that co-expression of the P1-2A precursor with the 3C C142S mutant, or use of the defective mIRES element (containing the GTTA motif) with the wt 3C\textsuperscript{pro}, produced the highest level of the processed FMDV capsid proteins.

Binding of the FMDV capsid proteins to serotype-specific anti-FMDV antibodies and to the integrin α\textsubscript{vβ}6

We have shown previously (Polacek et al., 2013), that the P1-2A capsid precursor proteins, and the 3C\textsuperscript{pro}-processed
products, are recognized in serotype-specific ELISAs which were used to determine their antigenicity and also bind to the major cellular receptor for FMDV, \( \alpha_v\beta_6 \) integrin (Ferris et al., 2005). The expressed FMDV capsid proteins as analysed in Fig. 2, were also tested using these ELISAs (Fig. 3). As expected, the FMDV proteins bound to both serotype-specific anti-FMDV antibodies and to the \( \alpha_v\beta_6 \) integrin. Furthermore, considering these results together with the immunoblot analysis of protein expression, it was concluded that the optimal production of processed FMDV capsid proteins was achieved when the P1-2A precursor (of both serotypes) was expressed with low levels of 3Cpro activity within the P1-2A-IRESgtta3Cwt cassettes (Figs 2 and 3).

**Self-assembly of processed capsid proteins into empty FMDV-like particles**

To verify that the processed capsid proteins self-assembled into FMDV empty capsid particles (75S), cell lysates were analysed by sucrose gradient ultracentrifugation and the fractions were examined using the serotype-specific antigen ELISAs. Protomers (5S) were detected near the top of the gradient (Fig. 4a, b, fractions 2–4) and empty capsid particles were detected near the bottom of the gradient (Fig. 4a, b, fractions 13–15). Consistent with the results described above, expression of high amounts of 3Cpro were not beneficial for FMDV capsid assembly. The yield of empty capsid particles from the P1-2A-3Cwt plasmid was low but was increased with a mutant 3Cpro (P1-2A-3CC142S) and the highest level of assembled capsids was generated from the P1-2A-IRESgtta3Cwt plasmid. Western blot analysis of the empty capsid fractions from the sucrose gradients showed that they contained the expected structural proteins VP0 and VP1, while no intact P1-2A precursor protein was present (Fig. 4c, d). However, significant breakdown of the VP0 within the empty capsid fractions was detected using a VP2-specific antibody and the product matched the expected size for authentic processing of VP0 (~33 kDa) into VP2 (~24 kDa) and VP4 (~9 kDa, not detectable with this gel system).

**Empty FMDV-like capsid particles bind to the \( \alpha_v\beta_6 \) integrin receptor**

To characterize the properties of the protomers and assembled empty capsids (from both serotype O and A), these products were examined, directly after sucrose gradient purification, for their ability to bind to \( \alpha_v\beta_6 \) integrin in the presence or absence of EDTA. These assays showed that both the protomers and the assembled empty capsids can bind, in a divalent cation-dependent manner, to the purified RGD-dependent integrin \( \alpha_v\beta_6 \) (Fig. 5a, b) as observed with the whole virus (Jackson et al., 2000).

To extend and support the results described above, we further analysed the purified serotype O and A empty capsids in the serotype-specific ELISAs and in the \( \alpha_v\beta_6 \) integrin binding assay (Fig. 5c, d). As expected, no cross-reactions...
were observed between the empty capsid particles from the two different serotypes using the serotype-specific antigen ELISAs (Fig. 5c). Furthermore, the detection of serotype A empty capsids bound to \( \alpha_v\beta_6 \) integrin was also serotype specific (Fig. 5d). In contrast, in the ELISA using a guinea pig anti-type O FMDV antiserum, both the serotype O and the serotype A antigens, when bound to \( \alpha_v\beta_6 \) integrin, were detected with equal efficiency (Fig. 5d). Thus, one-way, cross-reactivity was observed with the assembled empty capsids in this assay.

Morphological similarities between purified FMDV serotype A empty capsids and the virus

To determine the size and the morphology of the assembled empty capsids, sucrose gradient-purified FMDV serotype A empty particles were examined by transmission electron microscopy and the images were used for three-dimensional reconstruction. Negatively stained purified empty FMDV particles appeared as uniform spherical empty virion-like particles with a densely stained core and a median size of approx. 30 nm diameter (Fig. 6a). In total, 138 empty capsid particles were used for the three-dimensional reconstruction with a final resolution of 36 Å (Fig. 6b). The reconstruction had a less defined surface compared with the low-resolution (8 Å) model calculated from the X-ray crystal structure of FMDV serotype A1061 (Fig. 6c) (Fry et al., 2005). However, the overall surface appearance of the reconstructed empty capsid particle was ordered and repetitive, and resembled FMDV morphologically with distinct fivefold, threefold and twofold axes of symmetry. The elevation of certain features on the particle surface appeared different from the structure determined for the virus by crystallography.

Fig. 3. Binding of expressed FMDV capsid proteins to serotype-specific anti-FMDV antibodies and to \( \alpha_v\beta_6 \) integrin. Samples were prepared using serotype O cDNA cassettes as described in Fig. 2 and equal amounts of cell lysates were analysed using an FMDV serotype O-specific antigen ELISA (a) and an ELISA for FMDV antigen bound to \( \alpha_v\beta_6 \) integrin (b). Samples were prepared using serotype A cDNA cassettes and analysed using an FMDV serotype A-specific antigen ELISA (c) and an ELISA for FMDV antigen bound to \( \alpha_v\beta_6 \) integrin (d). Results are presented as the mean from two independent experiments. Note: The samples were analysed in the same order as the samples in Fig. 2.
Attempts to study purified FMDV serotype O empty capsids were not successful, due to stability issues.

**DISCUSSION**

In this study, we have analysed different strategies to determine the optimal system for the production, processing and assembly of FMDV capsid proteins within mammalian cells by the efficient expression of the structural protein precursor (P1-2A) together with a limited amount of the 3C<sup>pro</sup> that is sufficient to achieve P1-2A processing but without adverse effects on the expression system. Recently, Porta et al. (2013a) showed that a low level of 3C<sup>pro</sup> activity can be sufficient to achieve FMDV P1-2A processing in insect cell systems. Similarly, it has been demonstrated that co-transfection of a plasmid encoding P1-2A with low levels of a second plasmid encoding 3C<sup>pro</sup> was sufficient to achieve efficient processing of the P1-2A to the mature FMDV empty capsid proteins within mammalian cells (Polacek et al., 2013). The studies presented here have now extended...
these observations using an mIRES-containing dicistronic mRNA expression system to achieve a low level of 3Cpro expression relative to the P1-2A.

The cDNA sequences encoding the capsid protein precursor (P1-2A) and 3Cpro, with a mutant IRES located between them, were expressed from a dicistronic mRNA. Production of 3Cpro was dependent on a modified FMDV IRES, with a mutation in the GNRA motif (GCGA to GTTA) known to inhibit the EMCV IRES element (Robertson et al., 1999). Using this system it was possible to achieve the highest expression and processing of the mature empty capsid components (i.e. VP0, VP1 and VP3). Furthermore, these proteins self-assembled into empty capsid particles (or virus-like particles), as determined by sucrose gradient analyses and electron microscopy. The strategy was reproducible since very similar results were obtained for the two most common serotypes, namely O and A.

It is noteworthy that the GAGA tetraloop mIRES, previously shown to decrease the efficiency of the EMCV IRES (Robertson et al., 1999), was as active as the wt FMDV IRES element even though the FMDV IRES is predicted to have a secondary structure that is closely related to the EMCV IRES (Pilipenko et al., 1989). The precise role of the GNRA tetraloop in IRES function is unknown; no protein interactions have been localized to this site, but it is believed to be important for RNA–RNA interactions required to maintain the IRES tertiary structure (Fernández-Miragall et al., 2009).

As an alternative strategy for expressing a reduced level of 3Cpro activity, a monocistronic expression vector with the FMDV capsid protein precursor linked to the 3Cpro C142S mutant (Sweeney et al., 2007) was also evaluated. The results indicated that the 3Cpro mutant does not limit its own expression as effectively as the 3Cpro wt (as may be expected due to its lower catalytic activity) and hence the level of mutant 3Cpro protein expression was higher compared with the 3Cpro wt (Fig. 2a, b). These results are consistent with the fact that 3Cpro not only cleaves the viral precursor proteins, but also a variety of cellular substrates associated with translation (Belsham et al., 2000) and transcription (Falk et al., 1990).

Proteolytic processing of the VP0 precursor into VP4 and VP2 is required for virion maturation and infectivity but the mechanism of cleavage is unknown (Ansardi et al., 1992; Moscufo et al., 1991). Previously, it was believed that RNA encapsidation was essential to trigger the cleavage of...
The empty FMDV particles displayed the VP2 antibody, indicated that a large proportion of the VP0 had been processed (as indicated by the formation of VP2). The results presented in Fig. 4 (c, d), using an anti-FMDV RNA. Under the experimental conditions described here, cleavage of VP0 in FMDV does not require the presence of integrin then the guinea pig anti-FMDV antibodies antigen and when the type O antigen was captured using separate antisera for the capture and detection of the serotype-specific ELISAs also indicated that the serotype-specificity of the assay relies on the use of two FMDV virus particles and are therefore capable of serving as antigens to detect FMDV-specific antibodies. However, the serotype-specificity of the assay relies on the use of two separate antisera for the capture and detection of the antigen and when the type O antigen was captured using the integrin then the guinea pig anti-FMDV antibodies failed to distinguish between serotype O and serotype A.

The empty FMDV particles displayed the αβ6 integrin binding activity of the native virion, as demonstrated by ELISAs (Fig. 5). As expected (DiCara et al., 2008; Goodwin et al., 2009), the Ca2+-dependent binding of FMDV capsid proteins to αβ6 integrin was completely inhibited by 10 mM EDTA, a strong inhibitor of divalent cation-dependent integrin–ligand interaction. The results from the serotype-specific ELISAs also indicated that the assembled capsids possess antigenicity similar to that of FMDV virus particles and are therefore capable of serving as antigens to detect FMDV-specific antibodies. However, the serotype-specificity of the assay relies on the use of two separate antisera for the capture and detection of the antigen and when the type O antigen was captured using the integrin then the guinea pig anti-FMDV antibodies failed to distinguish between serotype O and serotype A.

The characteristics of the empty capsid particles suggested that these FMDV empty capsid particles may have a similar tertiary structure as the virus. Indeed, when the self-assembled empty capsid particles were examined by electron microscopy and three-dimensional reconstruction, the serotype A empty capsid particles were found to closely resemble FMDV viruses in size and overall particle morphology (but see below). We did not succeed in observing the serotype O empty capsid particles using electron microscopy due to stability issues. It has been noted previously that serotype A particles are more robust than serotype O particles (Abrams et al., 1995; Porta et al., 2013a). Lack of assembled empty capsid proteins might explain why a candidate vaccine based on a replication-deficient adenovirus vector containing the capsid coding region of serotype O and the 3CPro induced less effective protection than the equivalent serotype A system (Moraes et al., 2002, 2011; Pacheco et al., 2005).

Previous comparisons of the crystal structures of virions and natural empty capsids of FMDV A (Curry et al., 1996, 1997; Fry et al., 2005) revealed identical packaging of the capsid proteins in both particles. The virions have a more ordered structure than the empty capsid particles in the region of the threefold axes of symmetry and the observed differences, on the interior surface of the capsid, were correlated with the presence of the RNA. In the reconstructions of the expressed empty capsid particles obtained using electron microscopy which are presented here, the elevation of different features of the particle surface seems distinct from that observed in the crystal structures (see Fig. 6b, c). The low resolution three-dimensional reconstruction exaggerates some of the surface features visible on the map simulated from the crystal structure. Namely the pore-like depression at the fivefold vertex and the elevated threefold plateaus are more pronounced. However, this may be due to lack of detail from the limited resolution or an effect of particles being coated with heavy metal stain in the rather harsh process. Thus we cannot rigorously compare them as they are not of equal quality, but we can rely on the low resolution result to be largely accurate overall. This methodology, based on analytical scale production of the empty capsids using a

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**Fig. 6.** FMDV imaging and maps. (a) Electron micrograph of assembled FMDV serotype A empty capsids. BHK cells were infected with the vaccinia virus vTF7-3 and transfected with the serotype A FMDV P1-2A-IRESgtta3Cwt plasmid. At 20 h post-transfection, cells were harvested and empty capsids were purified by sucrose gradient centrifugation and concentrated. The capsids were stained with NanoVan and data collected by electron microscopy. Bar, 50 nm. (b) Surface-rendered three-dimensional reconstruction of FMDV serotype A empty capsids at a resolution of 36 Å. The icosahedral asymmetrical unit is indicated by the scale bar that depicts the most interior features in red gradating to the most exterior features in dark blue. (c) For comparison, a low-resolution map was calculated from the X-ray crystal structure of FMDV serotype A1061 (Fry et al., 2005) to a resolution of 8 Å. The empty capsid is shown at a contour level of 1 s coloured radially as indicated by the scale bar that depicts the most interior features in red gradating to the most exterior features in dark blue.
transient expression system, may be suitable for screening the effect of modifications to the virus proteins on capsid assembly.

The optimised co-expression of FMDV proteins, using the P1-2A-mIRES-3C cassettes, should serve as a platform for the improved production of FMDV empty capsids using a virus vector system and contribute to the development of improved and safer next generation FMD vaccines and the production of non-infectious diagnostic reagents. These systems should also facilitate future studies on capsid assembly, circumventing the handling of large amounts of infectious virus.

METHODS

Plasmid constructions. The FMDV cDNA cassettes used are shown in Fig. 1. The plasmids were prepared by standard methods (Sambrook et al., 1989) and primers used are listed in Table S1 (available in JGV Online). The cDNA sequences corresponding to the O1 Manisa FMDV (pGEM-3Z-O-P1-2A) and the A22 FMDV (pGEM-3Z-A-P1-2A) capsid precursors have been described (Polacek et al., 2013; Porta et al., 2013a). The IRES element from FMDV OIK cDNA (as in pU7T53; Ellard et al., 1999) was amplified with a T3 primer and a primer (IRES_SacI_BamHI_Re) flanked by SacI_BamHI sites using, as template, a monocistronic reporter plasmid, pFMDRluc (G. J. Belsham, unpublished, derived from pBBRluc; Belsham et al., 2008). This IRES wt segment was digested with EcoRI and BamHI and ligated into similarly digested pGEM-3Z (Promega) to produce pGEM-3Z-IRESwt. To construct the two IRES mutants, the wt GNRA tetraloop sequence GCCG was converted to GAGA or GTTA (Robertson et al., 1999) using primers containing a Sty1 site (IRES_gaga_SacI_Fw or IRES_gaga_Sty1_Fw) and the reverse primer (IRES_SacI_BamHI_Re) as above. The amplified fragments were digested with Sty1 and BamHI and ligated into the same sites of pGEM-3Z-IRESwt to create pGEM-3Z-IRESgaga and pGEM-3Z-IRESgtta, respectively. The plasmids encoding the wt FMDV 3Cpro and a modified 3Cpro with reduced protease activity (C95K and C142S) (Birtle et al., 2005; Sweeney et al., 2007), were kindly provided by S. Curry, Imperial College, London, UK. The 3Cwt or 3CC142S cDNA sequences were amplified with primers flanked by SacI and BamHI sites (3C_SacI_Fw and 3C_SacI_BamHI_Re) and digested into similar fragments using the same IRES wt vectors containing IRESwt, IRESgaga or IRESgtta. The 3Cwt, 3CC142S and IRES3C fragments were amplified using the 3Cwt or IRES3C plasmids as templates and primers containing an AscI site (3C_AscI_Fw or IRES_AscI_Fw and 3C_AscI_Re). The PCR products were digested with AscI and then ligated into the AscI-digested backbone of pGEM-3Z-P1-2A to produce the expression plasmids pGEM3-Z-P1-2A-3C (Polacek et al., 2013), pGEM3-Z-P1-2A-3C142S and the six different combinations of pGEM3-Z-P1-2A-IRES3C (IRESwt3Cwt, IRESgaga3Cwt, IRESgtta3Cwt, IRESwt3C142S, IRESgaga3C142S and IRESgtta3C142S) (see Fig. 1). All constructs were propagated in Escherichia coli Top10 cells (Invitrogen), purified (Midiprep kit; Fermentas) and verified by sequencing.

Transient-expression assays. Monolayers (35 mm wells) of baby hamster kidney (BHK) cells (90% confluent) were infected with vTF7-3, a recombinant vaccinia virus that expresses T7 RNA polymerase (Fuerst et al., 1986) as described previously (Belsham et al., 2008). Cell lysates were prepared 20 h post-transfection with 20 mM Tris/HCl (pH 8.0), 125 mM NaCl and 0.5% NP-40 and clarified by centrifugation at 18 000 g for 10 min at 4 °C.

Western blot analysis. Samples were mixed with Laemmli sample buffer, resolved by SDS-PAGE (12.5 or 15% polyacrylamide) and electroblotted onto PVDF membranes (Millipore) as described previously (Polacek et al., 2013). The following primary antibodies were used: anti-FMDV O1 Manisa serum, anti-FMDV 3C 1G1 (kindly provided by E. Brocchi, Brescia, Italy, as used previously; Belsham et al., 2000), serotype-independent anti-FMDV VP2 4B2 (kindly provided by L. Yu, Harbin, China; Yu et al., 2011), and anto-actin (Abcam). Immunoreactive proteins were visualized using species-specific anti-lg secondary antibodies conjugated to HRP (Dako) with an ECL Prime detection system (Amersham) on a Bio-Rad Chemi-Doc XRS system.

ELISAs. Serotype-specific FMDV antigen ELISAs, for serotype O and A as appropriate, were performed as described previously (OIE, 2008; Polacek et al., 2013; Roeder & Le Blanc Smith, 1987) and the absorbance was read at 450 and 620 nm. The ELISA to detect FMDV antigen binding to the integrin receptor αvβ6, was performed essentially as described (Ferris et al., 2005, 2011), with some minor modifications (Polacek et al., 2013). Non-specific binding was determined in the presence of 10 mM EDTA. Detection of the bound FMDV antigen was achieved using guinea pig anti-FMDV polyclonal serum as for the serotype-specific FMDV antigen ELISAs.

Sucrose gradient centrifugation. Cell extracts (400 μl from one 35 mm well per gradient) were loaded onto gradients of 10–30% (w/v) sucrose in 40 mM sodium phosphate buffer (pH 7.6), 100 mM NaCl (buffer P) and centrifuged at 245 000 g in a SW55 Ti rotor (Beckman Coulter) for 2.5 h at 10 °C. Fractions were collected from the top of the gradient and viral proteins were detected by serotype-specific ELISAs (as above). To obtain assembled empty FMDV capsids, the particles were separated from the cell lysates (three six-well plates) by centrifugation through a 30% sucrose cushion (w/v) in buffer P at 245 000 g for 2 h at 10 °C. The pellet was dissolved in buffer P and treated with 0.1 mg RNase A ml−1 for 5 min before fractionation on a sucrose gradient as described above. Fractions containing empty capsids were concentrated using an Amicon Ultra 100 kDa centrifuge filter device (Millipore).

Electron microscopy and reconstruction. An aliquot of 3 μl of sucrose-gradient-purified FMDV serotype A empty capsids was placed on a freshly glow-discharged continuous carbon-coated copper grid. NanoVan stain (Nanoprobe) was applied by the standard drop method, and the sample was examined in a JEOL 1400 transmission electron microscope at 120 kV. For the three-dimensional reconstruction, 40 CCD (charge-coupled device) micrographs were recorded with a Gatan Orius SC 1000 camera with Digital Micrograph at a calibrated magnification of ×33 090. In total, 138 particles were selected using RobEM (Yan et al., 2007) and 127 were used for the reconstruction. The defocus distance ranged from 0.67 to 2.08 μm. The final pixel size was 2.72 Å. The reconstruction processes were performed using isocahedral averaging with the program AUTO3D, which generated a random model directly from the raw data as the initial starting structure (Yan et al., 2007). The final resolution of 36 Å was determined from where the Fourier shell correlation fell below 0.5. The final reconstruction was coloured radially using the program Chimera (Pettersen et al., 2004). The 3 Å calculated map was made using the X-ray crystallography structure of FMDV A1061 from the Protein Data Bank file, accession number 1ZBE (Fry et al., 2005), using the Situs program pdb2vol (Wrighers, 2010).

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