Distance effects during polyprotein processing in the complementation between defective FMDV RNAs

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Passage of foot-and-mouth disease virus (FMDV) in BHK-21 cells resulted in the segmentation of the viral genome into two defective RNAs lacking part of either the L- or the capsid-coding region. The two RNAs are infectious by complementation. Electroporation of L-defective RNA in BHK-21 cells resulted in the accumulation of the precursor P3 located away from the deleted sequence. Expression of L in trans led to the processing of P3, indicating that there is a connection between L protease activity and the secondary cleavages carried out by 3C protease within P3. These results suggest that the complementation mechanism between defective RNAs is not restricted to supplying the L and capsid proteins but that distance effects on polyprotein processing events are also implicated.

INTRODUCTION

Foot-and-mouth disease virus (FMDV) has a positive, single-stranded RNA genome that encodes a single polyprotein which is cleaved to generate the mature viral proteins. Polyprotein processing events start very rapidly, probably during the translation process, and the complete polyprotein is never observed within infected cells (Belsham, 1993). Virus-encoded proteins L (leader), 2A and 3C mediate three processing events: L protease cleavages to generate L, the capsid protein precursor P1-2A, and the replicative protein precursors P2 and P3, respectively. Subsequently, the precursors are subjected to ‘secondary’ cleavages by 3C and mature proteins are liberated.

The presence of a protease located at the N-terminal region of the ORF is only found in some members of the genus Aphthovirus within the Picornaviridae. FMDV RNA contains two functional initiation codons, 84 nt apart, which generate two different forms of L termed Lab and Lb that are found in all seven serotypes of FMDV (Carrillo et al., 2005; Clarke et al., 1985; Sangar et al., 1987). It has been proposed that when the ribosomes do not initiate protein synthesis at the first AUG, they continue scanning the RNA to start the polyprotein synthesis at the second AUG (Belsham, 1992). The removal of Lab and Lb from the viral polyprotein occurs via auto-cleavage at its C-terminus (Medina et al., 1993). This cleavage can also take place in trans. Construction of FMDVs with truncated L proteins showed that infectivity required that the residues that encode the terminal VP4 be positioned directly following the second functional Lb AUG codon (Belsham, 2013; Piccone et al., 1995a), and the virus displayed reduced fitness. The deletion of both AUGs results in a non-viable virus (Piccone et al., 1995a). When only the Lb protein is synthesized (due to the mutation of the Lab AUG), viable viruses can still be produced. However, mutation of the Lb AUG abolishes virus viability (Cao et al., 1995). The region between the two AUGs named ‘spacer’ region seems to be essential for some activity unrelated to its coding function, although deletion of the Lab ‘spacer’ region generates a functional Lb protease (Belsham, 2013).

More than 200 serial passages of FMDV in BHK-21 cells resulted in a variant version of the virus that included two defective RNAs, each with an in-frame deletion that maintained the long ORF (García-Arriaza et al., 2004). One of the segments included a deletion of 417 nt at the L-coding region (Δ417), and the other a 999 nt deletion at the VP3, VP1-coding region (Δ999). Each of the ΔRNAs is encapsidated into a separate particle, and the two viral forms infect by complementation (García-Arriaza et al., 2004; Moreno et al., 2014). The basis of this evolutionary transition was...
the extensive exploration of sequence space by the standard virus that reached a point at which the segmented versions displayed higher replicative fitness than the unsegmented parental genome (Moreno et al., 2014). Once the critical genetic event occurred, the selective advantage of the bipartite virus was reinforced by enhanced stability of the virions that encapsidated shorter genomes (Ojoñegros et al., 2011). Evidence that infectivity was dependent on complementation includes: (i) presence of genomes with the expected deletions with undetectable levels of standard RNA in individual viral plaques (García-Arriaza et al., 2004); (ii) two-hit kinetics for plaque formation (García-Arriaza et al., 2004; Manrubia et al., 2006); (iii) reconstruction of the complementation system by co-transfection with ΔRNAs transcribed from plasmids encoding FMDV RNA with the corresponding deletions (García-Arriaza et al., 2004); (iv) evidence that L protein plays a major role in proteolytic events that are associated with the complementation (Moreno et al., 2014).

Here we re-examine the molecular basis of the complementation between the two forms of FMDV ΔRNA, and demonstrate that the complementation involves at least two sequential proteolytic activities. The first is carried out in trans by L to cleave the truncated L protein, and the second by 3C to liberate products directed by standard pMT28 and Δ417ev RNAs or by the full-length standard RNA (termed pMT28 RNA) was compared. The ΔRNAs used are those that acquired five point mutations within the 2C- and 3A-coding region that conferred the segmented version with a fitness advantage over the unsegmented counterpart (Moreno et al., 2014; the evolved RNAs are termed Δ417ev and Δ999ev. The expression products predicted from pMT28, Δ417ev and Δ999ev RNAs are depicted in Fig. 1. The actual precursor and processed expression products directed by standard pMT28 and Δ417ev or Δ999ev RNAs in electroporated cells were identified by electrophoretic analysis of labelled cellular extracts (Fig. 2). Expression from Δ417ev RNA revealed two new proteins absent with pMT28 that displayed a slightly lower mobility than VP0. Based on the molar mass of the proteins anticipated from the expression of Δ417ev RNA, the upper band is compatible with the predicted precursor Δ417-Lab-VP0 (Moreno et al., 2014; Fig. 2). The lower band is compatible with the initiation of L either from Lb or the newly generated AUG at position 39 (the size difference does not allow distinguishing between these two possibilities). The expression of capsid proteins from Δ999ev RNA revealed the absence of VP0, despite proteins VP4 and VP2 not being affected by the Δ999 deletion. Instead, a band compatible with Δ999-P1-2A was observed. Unexpectedly, precursor P3 accumulated when it was expressed from Δ417ev but not from Δ999ev or pMT28 (P<0.001, ANOVA test), and 3C was present in larger amounts when expressed from Δ999ev than from Δ417ev or pMT28 (Fig. 2).

To confirm the identity of the proteins expressed from ΔRNAs and from pMT28, Western blot analysis using specific antibodies against VP2, VP3, VP1, 2C, 3C and 3D was performed (Fig. 3). The presence of VP2 was identified in the band that displayed the mobility expected of VP0 as well as in other bands that displayed a slightly lower mobility than VP0 (Fig. 3a). No products including VP2 were identified among the expression products from Δ999ev, despite the VP2-coding region being present. The pattern of viral protein expression analysed by Western blot with specific antibodies against VP1 and VP3 was as expected. P3 accumulation was detected by densitometry of the relevant bands stained either by 3C- or 3D-specific antibodies (Fig. 3b) (P<0.01, ANOVA test). Thus, despite the deletion in Δ417ev affecting only the L-coding region, its presence limited the processing of P3 located away from the deleted sequence. This result agrees with the lack of precursor P3 accumulation following co-electroporation of cells with both Δ417ev and Δ999ev RNAs (Moreno et al., 2014), presumably because L is supplied by Δ999ev.

**RESULTS**

**Pattern of protein expression from ΔRNAs and from standard pMT28 RNA**

To investigate the molecular basis of the complementation activity, the intracellular expression of viral proteins directed either by ΔRNAs or by the full-length standard RNA (termed pMT28 RNA) was compared. The ΔRNAs used are those that acquired five point mutations within the 2C- and 3A-coding region that conferred the segmented version with a fitness advantage over the unsegmented counterpart (Moreno et al., 2014); the evolved RNAs are termed Δ417ev and Δ999ev. The expression products predicted from pMT28, Δ417ev and Δ999ev RNAs are depicted in Fig. 1. The actual precursor and processed expression products directed by standard pMT28 and Δ417ev or Δ999ev RNAs in electroporated cells were identified by electrophoretic analysis of labelled cellular extracts (Fig. 2). Expression from Δ417ev RNA revealed two new proteins absent with pMT28 that displayed a slightly lower mobility than VP0. Based on the molar mass of the proteins anticipated from the expression of Δ417ev RNA, the upper band is compatible with the predicted precursor Δ417-Lab-VP0 (Moreno et al., 2014; Fig. 2). The lower band is compatible with the initiation of L either from Lb or the newly generated AUG at position 39 (the size difference does not allow distinguishing between these two possibilities). The expression of capsid proteins from Δ999ev RNA revealed the absence of VP0, despite proteins VP4 and VP2 not being affected by the Δ999 deletion. Instead, a band compatible with Δ999-P1-2A was observed. Unexpectedly, precursor P3 accumulated when it was expressed from Δ417ev but not from Δ999ev or pMT28 (P<0.001, ANOVA test), and 3C was present in larger amounts when expressed from Δ999ev than from Δ417ev or pMT28 (Fig. 2).

**Effect of expression of L in trans on the processing of P3**

Since the only region absent in Δ417ev RNA is that encoding L, we hypothesized that supply of L in trans might compensate for the absence of Δ999ev RNA. FMDV L expressed in trans cleaved the predicted chimeric precursor Δ417-VP0 yielding VP0 (Fig. 4). A Trans-expression of L resulted in a 14-fold increase of infectious Δ417ev RNA, indicating a key role of this protein in the complementation between ΔRNAs (Moreno et al., 2014). Here we demonstrate by quantification of the relevant FMDV protein bands that the presence of L expressed in trans largely compensated for the lack of processing of precursor P3 expressed from Δ417ev RNA (Fig. 4) (P=0.003, ANOVA test). This observation explains the absence of P3 when both Δ417ev and Δ999ev were co-expressed (Moreno et al., 2014).

In conclusion, the complementation activity that sustained the replication of the segmented FMDV genome was multifactorial and involved the leader L protease. Its presence was essential not only to produce the known L-dependent cleavages but also to effect processing of precursor P3 which lies far from the deletion site.

**DISCUSSION**

The truncated L protein expressed by Δ417ev lacks amino acids 39–178 of Lb, which results in loss of L function...
(Moreno et al., 2014). A similar construction containing an in-frame deletion of 192 nt lacking amino acids 35–98 in the middle of the L gene (named pRM-ΔL) was described previously (Piccone et al., 1995a, b). Transcripts from pRM-ΔL were translated efficiently in reticulocyte lysates but no viral plaques were observed upon transfection of BHK cells (Piccone et al., 1995a). Electroporation of BHK-21 cells with Δ417ev did not produce viral infectivity. In Δ417ev and in pRM-ΔL, the L amino acids which are linked to VP0 need to be liberated from the N-terminus of the polyprotein to permit the myristoylation of VP4 and the correct assembly of the capsid. Co-expression of L protein and Δ417ev resulted in the cleavage of Δ417-VP0 to yield the normal precursor VP0 (Fig. 4), suggesting that the deletion of 417 nt did not affect the cleavage site between L and VP0, and that protein L expressed

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by Δ999ev seems to be an essential protein for the complementation between Δ417ev and Δ999ev RNAs (Moreno et al., 2014).

P2 and P3 genomic regions remain intact in Δ417ev and Δ999ev, which would mean that the expression of intermediate and mature proteins from P2 and P3 regions could be attained from both ΔRNAs. Our results indicate that: (i) the precursor P3 is not equally processed upon the electroporation of Δ417ev or Δ999ev RNAs in BHK-21 cells; and (ii) there is an association between the expression of the L and the processing of the precursor P3 by 3C upon the electroporation of Δ417ev RNA. The accumulation of P3 upon electroporation of Δ417ev indicates that the primary cleavage mediated by 3C in cis in the 2C/3A site is not impaired, but the secondary cleavages in trans to yield the mature proteins 3A, 3B1–3, 3C and 3D are affected. FMDV 3C needs to be expressed at optimal levels in infected cells for two reasons: (i) expression of 3C is poorly tolerated by mammalian cells, presumably because several cellular proteins are cleaved (Armer et al., 2008; Belsham et al., 2000; Falk et al., 1990; Li et al., 2001; Strong & Belsham, 2004), and (ii) the optimal production of capsid proteins from P1-2A requires reduced levels of 3C expression relative to P1-2A (Polacek et al., 2013).

The results suggest that the cleavages performed by L and 3C are not independent but linked events that contribute to the complementation between ΔRNAs. We hypothesize that L expressed from Δ999ev cleaves in trans the truncated L from Δ417ev as an essential event in the complementation between ΔRNAs. Once the truncated L is liberated, 3C expressed from Δ417ev can act in trans to liberate mature proteins from precursor P3. 3C expressed from Δ999ev RNA could mediate the cleavage in trans of precursors expressed from both ΔRNAs. However, it is not clear why 3C expressed from Δ999ev did not process P3 expressed from Δ417ev. A connection between L and 3C proteolytic activities has been described previously in the sequential cleavage of the translation initiation factor eIF4GI in FMDV-infected cells (Strong & Belsham, 2004). A possibility is that L might play a role in the switching of RNA from translation to replication, a switch that in poliovirus is known to involve cleavage of RNA-binding protein poly(rC)-binding protein 2 (PCBP2) by 3CD (Chase et al., 2014). A distance effect on the processing of the FMDV polyprotein was also observed.

Fig. 2. Analysis of FMDV proteins expressed from pMT28 and ΔRNAs. Electropherogram of proteins expressed upon electroporation of BHK-21 cells with 25 μg of the RNAs indicated at the top of each lane (No RNA, mock-electroporated cells). Cells were labelled with [35S]Met-Cys for 1 h, at 4 h post-electroporation. Then, the extracts were analysed electrophoretically. New proteins expected from the deletions in ΔRNAs or proteins whose levels differ among the RNAs are highlighted in red. The amount of extract analysed was normalized using actin (bottom bands). The numbers on the left indicate molecular mass markers (Mr, KDa) for proteins. The asterisk next to Δ417-Lb-VP0 means that this product could be originated at L amino acid M29 or M39 (Moreno et al., 2014). Procedures are detailed in Methods.
Fig. 3. Western blot (WB) analysis of FMDV proteins expressed from pMT28 and ΔRNAs. The filled boxes indicate the viral protein (VP2, VP3, VP1, 2C, 3C or 3D) detected by the specific monoclonal antibodies for VP2, VP3, VP1, 2C and 3C, and a polyclonal antibody against 3D in the Western blot assay. The relevant proteins expected from the expression of each RNA are indicated by coloured boxes, with the deleted regions distinguished by white boxes delimited with discontinuous lines. The inverted Y (symbol for antibody molecule) indicates the protein expected to be visualized by the corresponding Western blot. Below the protein boxes, the actual Western blots are shown. The RNA (25 µg) used in the electroporation is shown at the top of each lane. The electrophoresis was performed as described in Fig. 2, and the proteins transferred to a nitrocellulose
membrane for protein visualization with the relevant antibodies. No RNA indicates mock-electroporated cells, and pMT28 indicates expression from pMT28 RNA used as a control. The amount of extract analysed was normalized using actin (Fig. 2). The position of FMDV proteins and precursors is shown at the right of each blot. The numbers on the left indicate molecular mass markers (M, kDa) for proteins. The asterisk next to Δ417-Lb-VP0 means that this product could be originated at L amino acid M29 or M39 (Moreno et al., 2014). Below each blot the percentage of each protein in each lane is given. Quantification was performed by densitometric analysis of all the bands visualized in the corresponding lane, taking as 100 % the sum of densitometric values for each lane. Colour codes for the different proteins are depicted in the key at the top of each plot. Procedures are detailed in Methods.

in a viral clone subjected to multiple plaque-to-plaque transfers: the VP3–VP1 cleavage was affected by substitution M54I in VP1, despite the VP1 substitution and the VP3–VP1 cleavage site being 54 aa away in the primary structure (Escarmis et al., 2009). Also, a linkage between the substitution E83K within the VP1 and the blockage of VP1/2A cleavage has been suggested (Gullberg et al., 2014).

The evolutionary success of drastic structural transitions in genomic RNA may depend on subtle reaccommodation of complex interactions among gene products, thus explaining their rare occurrence.

**METHODS**

**Cells and virus.** The origin of baby hamster kidney 21 (BHK-21) cells and procedures for cell growth in Dulbecco’s modification of Eagle’s medium (DMEM), and for FMDV plaque assays in semisolid agar medium have been described previously (Domingo et al., 1980; Sobrino et al., 1983). FMDV C-88c1 (expressed from plasmid pMT28; Toja et al., 1999) has the genomic sequence of a plaque-purified virus of the European serotype C, natural isolate C; Santa-Pau Spain 70 (Sobrino et al., 1983). FMDV C-S8p260 is a viral population obtained after 260 serial cytopathic passages of C-88c1 at high m.o.i. in BHK-21 cells (2–4 p. f.u. per cell) for each passage 2 × 10^6 BHK-21 cells were infected with the virus contained in 200 µl of supernatant from the previous infection, which included 2 × 10^5– 4 × 10^5 p.f.u.) (Garcia-Arriaza et al., 2004, 2005, 2006; Osses et al., 2011). The Genbank accession numbers for the viral genomes used in the present study are AJ133357 (C-88c1), DQ409183 (Δ417ev) and DQ409184 (A999ev).

**Transcription of viral RNA and electroporation of BHK-21 cells.** Plasmid DNA was linearized by cleavage with the appropriate restriction enzymes (NdeI for pMT28 derivatives), purified (Wizard PCR Prep DNA purification resin, Promega) and transcribed (Riboprobe in vitro transcription system, Promega). The mixture contained transcription buffer (Promega), 10 mM DTT, 0.48 units RNasin µl^-1, 1 mM each of the standard ribonucleoside–triphosphates, 4 ng linearized plasmid DNA µl^-1 and 0.3 units SP6 polymerase µl^-1; it was incubated for 2 h at 37 °C. The RNA concentration was estimated by absorbance gel electrophoresis, with known amounts of rRNA as standard.

To electroporate BHK-21 cells with RNA transcribed in vitro, subconfluent cells were washed, heated with ice-cold PBS and resuspended in PBS at a density of about 2.5 × 10^5 cells ml^-1. Aliquots (50–80 µl) of transcription mixture, including the appropriate amount of RNA, were added to 0.4 ml of cell suspension, and the mixtures were transferred to 2 mm electroporation cuvettes (Bio-Rad). Electroporation was performed at room temperature by two consecutive 1.5 kV, 25 µF pulses using a Gene Pulser apparatus (Bio-Rad) as described by Liljestrom & Garoff (1991). As a control, BHK-21 cells were electroporated with 50–80 µl of transcription mixture (without RNA) in PBS to monitor absence of contamination. The cells were then resuspended in growth medium and seeded onto culture plates. At 4 h post-electroporation, samples of the culture medium were withdrawn and stored at −70 °C. Mock-co electroporated cultures were treated in parallel and served as a control for the experiments of protein labelling and titration of extracellular virus, as indicated in the control panels in the corresponding figures. No evidence of viral contamination was obtained in any of the experiments.

**Protein analysis and fluorography.** Proteins were labelled by the addition of 60 µCi of [35S]Met-Cys (Amersham) per ml contained in methionine-free DMEM, at the time post-electroporation indicated for each assay. After 1 h of incubation of the cell monolayers with the radioactive medium, the medium was removed and the cells were harvested in 0.1 ml of sample buffer (160 mM Tris-HCl, pH 6.8, 2 % SDS, 11 %, v/v, glycerol, 0.1 M DTT, 0.033 % bromophenol blue). The samples were boiled for 5 min, and aliquots were analysed by SDS-PAGE at 200 V, and subjected to fluorography and autoradiography. The amount of extract analysed was normalized using actin, identified with a specific mAb (anti-β-actin clone AC-15; Sigma). Actin was chosen due to its long intracellular half-life, and the amount of extract analysed corresponded to the linear region of the relationship between the amount of extract and the intensity of the actin band by Western blot (Perales et al., 2007).

**Antibodies and Western blot analysis.** FMDV proteins were detected using mouse MAb 6F2, which recognizes capsid protein VP2, MAB 6C2, which recognizes capsid protein VP3, MAB SD6 which recognizes capsid protein VP1 (Mateu et al., 1987), MAB 1C8 which recognizes protein 2C (kindly provided by E. Brocchi), MAB 2D2, which recognizes protein 3C (kindly provided by E. Brocchi), and rabbit polyclonal serum anti-3D (polymerase), raised against purified recombinant 3D protein, expressed in E. coli (Arias et al., 2005; Ferrer-Orta et al., 2004). Proteins were separated electrophoretically by SDS-PAGE, and transferred to a nitrocellulose membrane (Bio-Rad) in transfer buffer [25 mM Tris-HCl, pH 8.3, 190 mM glycine, 20 % methanol, 0.1 % SDS] at 200 mA for 15 h. The membrane was saturated with a solution of uncreamed powdered skimmed milk (5 %, w/v) in PBS for 1 h with gentle stirring. Then, an adequate dilution in PBS (with 0.1 % powdered milk) of the primary antibody was added, and the membrane incubated for 2 h. The membrane was then washed three times for 15 min in TBBS (Tween 20 at 0.05 %, v/v, in PBS) and incubated with the corresponding secondary antibody conjugated to peroxidase (1 : 10 000 dilution in TBPS). After 1 h of incubation, three washes with PBS were carried out, and the membrane was developed with chemiluminescence (ECL; Amersham) (Herrera et al., 2008; Moreno et al., 2014; Perales et al., 2007). The amount of cell extract used for electrophoretic analysis was normalized to a constant amount of cellular actin, measured by reactivity with monoclonal antibody (anti-β-actin clone AC-15, Sigma), and corresponded to a concentration of protein in the linear region of the relationship between the Western blot signal and the protein concentration.

**Expression of FMDV L.** Plasmid pTM1-L was obtained with primers 5‘Ncol-L and 3‘BamHI-L, which were designed for PCR amplification of the Lab-coding region from pMT28 (Toja et al., 1999). The resulting
Fig. 4. Role of proteinase L in the complementation between ΔRNAs. Western blot (WB) analysis of FMDV proteins expressed upon electroporation of BHK-21 cells with 25 µg of Δ417 RNA in the absence or presence of L expressed from plasmid pTM1-L (30 µg) at 4 h post-electroporation. Proteins separated by electrophoresis were transferred to a nitrocellulose membrane for protein visualization with the relevant antibodies as described in Fig. 3. No RNA indicates mock-electroporated cells, and pMT28 indicates expression from pMT28 RNA used as a control. The filled boxes indicate the viral protein (VP2, VP3, VP1, 2C, 3C or 3D) detected by the specific monoclonal antibodies for VP2, VP3, VP1, 2C and 3C, and a polyclonal antibody against 3D in the corresponding Western blot assay. The amount of extract analysed was normalized using actin. The position of FMDV proteins and precursors is shown at the right of each blot. The numbers on the left indicate molecular mass markers (Mr, kDa) for proteins. The asterisk next to Δ417-Lb-VP0 means that this product could be originated at L amino acid M29 or M39 (Moreno et al., 2014). Below each blot the percentage of each protein in each lane is given. Quantification was performed by densitometric analysis of all the bands visualized in the corresponding lane, taking as 100 % the sum of densitometric values for each lane. Colour codes for the different proteins are depicted in the key at the top of each plot. Procedures are detailed in Methods.
DNA fragment was digested with Ncol and BamHI and ligated to plasmid pTM1 containing the 5′ untranslated region of encephalomyocarditis virus (kindly provided by Dr L. Carrasco). The in vitro transcription and polyadenylation were performed with T7 polymerase (Promega) and poly(A) polymerase (Gibco), respectively, as specified by the manufacturers.

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