Cleavage of translation initiation factor 4AI (eIF4AI) but not eIF4AII by foot-and-mouth disease virus 3C protease: identification of the eIF4AI cleavage site

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Abstract The translation initiation factor eIF4A is cleaved within mammalian cells infected by foot-and-mouth disease virus (FMDV). The FMDV 3C protease cleaves eIF4AI (between residues E143 and V144), but not the closely related eIF4AII. Modification of eIF4AI, to produce a sequence identical to eIF4AII around the cleavage site, blocked proteolysis. Alignment of mammalian eIF4AI onto the three-dimensional structure of yeast eIF4A located the scissile bond within an exposed, flexible portion of the molecule. The N- and C-terminal cleavage products of eIF4AI generated by FMDV 3C dissociate. Cleavage of eIF4AI by FMDV 3C is thus expected to inactivate it.

Key words: Picornavirus; Translation initiation factor eIF4A; Protein synthesis; 3C protease; Foot-and-mouth disease virus

1. Introduction

The translation initiation factor complex eIF4F has a central role in the initiation of protein synthesis in mammalian cells (reviewed in [1]) and comprises eIF4E, eIF4A and eIF4G. eIF4E binds to the 5′-cap-structure (m^7GpppN…) of mRNAs while eIF4A is the prototypic member of the DEAD box family of RNA helicases [2]. eIF4G is a scaffold protein that binds not only eIF4E and eIF4A but also eIF3 (associated with the ribosome), the poly(A) binding protein and Mnk-1 (an eIF4E kinase). Thus eIF4G bridges between the mRNA and the small ribosomal subunit [1].

The picornavirus family comprises many important human and animal pathogens including poliovirus (PV), foot-and-mouth disease virus (FMDV) and encephalomyocarditis virus (EMCV). Picornaviruses have a positive sense RNA genome of about 8 kb that acts like a mRNA. These RNAs are uncapped and have long (600–1300 nucleotides (nt)) 5′ untranslated regions that include internal ribosome entry site (IRES) elements (ca. 450 nt) which direct translation initiation on the picornavirus RNA (see [3–5]). Within cells infected by FMDV and PV, rapid inhibition of cellular cap-dependent protein synthesis occurs following virus-induced cleavage of eIF4G. Picornavirus IRES elements direct translation initiation using just the central and C-terminal domains of eIF4G, which has binding sites for eIF4A and eIF3 [6–8]. Cleavage of eIF4G is induced by the PV 2A protease [9] and by the unrelated FMDV leader (L) and 3C proteases [10–12] at distinct sites.

We have shown that eIF4A (ca. 48 kDa) is also cleaved in FMDV-infected cells to produce a stable C-terminal product (33 kDa) and that this cleavage requires the FMDV 3C protease [12]. The progressive cleavage of eIF4A, from about 3 h post-infection, is co-incident with the decline in the level of viral RNA translation [12]. Two cytoplasmic forms of mammalian eIF4A have been identified, termed eIF4AI and eIF4AII, that are 91% identical and appear to be functionally interchangeable [13]. eIF4A is the most abundant of the translation initiation factors [14], it exists within the eIF4F complex and also free within the cytoplasm [13]. Capped mRNAs and picornavirus IRES-containing RNAs require eIF4A to achieve efficient 48S initiation complex formation in vitro [6,15] and dominant negative mutants of eIF4A block both modes of translation initiation [16,17]. eIF4A has ATP-dependent RNA helicase activity which is stimulated by eIF4B [18]. The three-dimensional (3D) structure of yeast eIF4A has been determined [19–21].

No cleavage of eIF4A occurs in PV- or EMCV-infected cells [12,22] and currently it appears unique to FMDV-infected cells. We now show that eIF4AI (but not eIF4AII) is cleaved directly by FMDV 3C in vitro and identify the scissile bond. The cleavage separates critical regions within eIF4AI required for helicase activity.

2. Materials and methods

2.1. Expression plasmids for eIF4AI, eIF4AII and derivatives

The plasmid pcDNA3.1/GS4AI (Invitrogen) contains the human eIF4AI coding sequence with C-terminal hexa-His and V5-epitope tags under the control of CMV and T7 promoters. Derivatives of the eIF4AI cDNA lacking sequences encoding the N-terminus of the protein (Fig. 1) were prepared by polymerase chain reaction (PCR). The fragments generated were inserted using *Eco*RI and *Bam*HI into similarly digested pGEM3Z (Promega).

The plasmids pET4A [16] and p4AIIms [23] encoding the murine eIF4AI and eIF4AII respectively were used as templates in PCRs to permit insertion of *Bam*HI–*Sal*I fragments into similarly digested pGEX-4T (Amersham Pharmacia Biotech) to generate plasmids that expressed GST–eIF4AI and GST–eIF4AII fusion proteins.

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Abbreviations: FMDV, foot-and-mouth disease virus; IRES, internal ribosome entry site; PV, poliovirus; EMCV, encephalomyocarditis virus; IPTG, isopropyl β-D-thiogalactoside; PCR, polymerase chain reaction

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Modified forms of the GST–eIF4AI coding sequences containing single and double mutations were produced using PCR. The changes made were V144 (GTG) to M144 (ATG) alone or A142 (GCT) to N142 (AAT) alone or the two changes together within the eIF4AI coding sequence. The presence of the required mutations in the plasmids was verified by DNA sequencing.

2.2. Transient expression assays

Transient expression assays were performed as described previously [24]. Briefly, plasmids (2.5 μg) were transfected using Lipofectin (Life Technologies) into BHK cells (35 mm dishes) infected with the recombinant vaccinia virus vTF7-3 [25] which expresses T7 RNA polymerase. Cell extracts (400:1) were prepared at 20 h post-infection. Samples were analysed by SDS–PAGE and immunoblotting. Detection was achieved using horseradish peroxidase-conjugated anti-V5 antibodies (Invitrogen) and chemiluminescence reagents (Pierce).

2.3. Production of BHK cells expressing tagged eIF4AI

BHK-4AI cells that constitutively express the C-terminal V5-tagged human eIF4AI were prepared by transfection of BHK cells with pcDNA3.1/GS4AI using Lipofectin and selection of cells using Zeocin (125 μg/ml, Invitrogen). BHK-4AI cells were infected with FMDV O1K as described [12] and harvested at 1–5 h post-infection. The expression and cleavage of the V5-tagged eIF4AI in BHK-4AI cells were assessed by immunoblotting as above.

2.4. Expression and purification of GST–eIF4A fusion proteins

The production of GST fusion proteins in *Escherichia coli* JM109 was induced with 1 mM isopropyl β-D-thiogalactoside (IPTG). After cell lysis (by sonication), fusion proteins in phosphate-buffered saline (PBS) containing 1% Triton X-100 were bound to glutathione-Sepharose (Amersham Pharmacia Biotech), eluted (with 50 mM glutathione in 50 mM Tris, pH 8.0) and dialysed against PBS containing 20% glycerol, 1 mM EDTA (pH 8.0) and 1 mM dithithreitol (DTT) (buffer S). In each case, the purified protein (about 5 mg/l of culture) appeared homogeneous by SDS–PAGE.

2.5. Expression and purification of recombinant FMDV 3C protease

A *Xho*I–*Sal*I fragment from pSK3C (derived by *Kpn*I digestion and religation of pSKRH3C [12] to remove most of the FMDV IRES sequence) was inserted into the *Xho*I site of pET28b (Novagen) to generate pET-FMDV3CXS. This plasmid was transformed into *E. coli* BL21(DE3)LysS and recombinant protein expression was induced with IPTG (1 mM). The His-tagged 3C protease (the hexa-His tag replaces the normal C-terminal six residues PEPHHE) was bound to TALON resin (Clontech), eluted with 500 mM imidazole and dialysed against buffer S. The product (about 5 mg/l culture) appeared homogeneous by SDS–PAGE and by immunoblotting using anti-His tag and anti-FMDV 3C antibodies.

2.6. In vitro cleavage of recombinant eIF4A proteins

Purified GST–eIF4AI (5 μg) or GST–eIF4AII (5 μg) was incubated for 2 h at 37°C alone or with purified 3C protease (0.5 μg) in 100 mM sodium phosphate buffer (pH 7.4), 1 mM EDTA, 1 mM DTT and 5% glycerol (20 μl). Reactions were stopped directly by addition of SDS sample buffer or incubated with glutathione-Sepharose beads in PBS/0.5% Triton X-100 (0.5 ml). The bound proteins were washed and eluted with SDS sample buffer prior to analysis by SDS–PAGE and immunoblotting using antisera specific for the N- or C-terminus of eIF4A.

2.7. Antisera against eIF4A

Sheep antisera raised against synthetic peptides derived from the N-termini of eIF4AI and eIF4AII and a peptide derived from the C-terminal region common to both proteins were described previously [26].

2.8. N-Terminal amino acid sequence analysis of eIF4AI cleavage product

The products generated in vitro by FMDV 3C (as described above) from 5 μg of GST–eIF4AI in a 20 μl reaction were separated by SDS–PAGE and transferred to polyvinylidene difluoride membrane. The blot was stained with GelCode Blue stain reagent (Pierce), the C-terminal fragment of eIF4AI was excised and subjected to nine cycles of automated Edman degradation by Dr Nick Morrice of the MRC Phosphorylation Unit (Dundee, UK). Residues were identified as their phenylthiohydantoin derivatives by high-performance liquid chromatography.

3. Results

3.1. eIF4AI is cleaved by FMDV 3C within cells

In our initial studies, it was demonstrated that eIF4A was cleaved within FMDV-infected cells or when the FMDV 3C protease was transiently expressed within BHK cells [12]. In these studies, which used antibodies that do not discriminate between eIF4AI and eIF4AII, it appeared that cleavage of eIF4A was incomplete. To analyse the modification of eIF4AI individually, an epitope-tagged version of human eIF4AI (Fig. 1) was introduced into BHK cells and a stable cell line (BHK-4AI) that expressed this protein was generated (see Fig. 2). Infection of these cells with FMDV resulted in progressive cleavage of the human eIF4AI and the production of a V5-tagged 33 kDa species (Fig. 2). This result confirmed that the large cleavage product of eIF4AI contains the C-terminus, indicating that cleavage occurs within the N-terminal region.

3.2. Characterisation of truncated eIF4AI proteins

A series of N-terminal deletion mutants of eIF4AI was constructed (see Fig. 1). These each contained a C-terminal V5-epitope tag and were efficiently expressed in a transient assay within BHK cells (Fig. 3). Extracts of the FMDV-infected BHK-4AI cells were analysed in parallel (Fig. 3). The C-terminal cleavage product generated during infection co-migrated closely with the truncated mutant of eIF4AI lacking the N-terminal 150 amino acids (Δ150) which provided a rough estimate for the location of the cleavage site.

3.3. Identification of site within eIF4AI cleaved by FMDV 3C protease

To identify the cleavage site precisely, the GST–eIF4AI and GST–eIF4AII proteins were incubated with FMDV 3C protease in vitro. The products were analysed by SDS–PAGE and immunoblotting (Fig. 4A). A 33 kDa C-terminal product was generated from GST–eIF4AI but not from the closely related GST–eIF4AII. The 3C-generated eIF4AI fragment was isolated and its N-terminal amino acid sequence was determined (VQKLQMEAP). This precisely matches the published sequence for eIF4AI residues 144–152 [23]. Thus the bond cleaved in eIF4AI by FMDV 3C is between E143 and

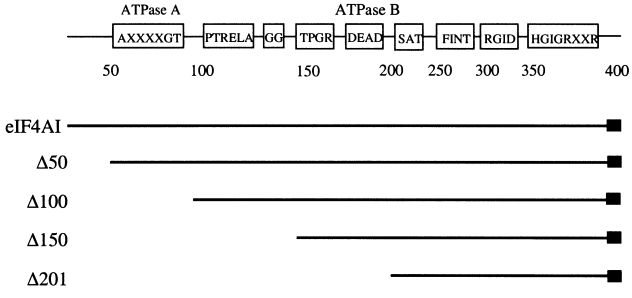

Fig. 1. Representation of the structure of eIF4AI cDNA and derivatives. The location of the nine conserved motifs (indicated by single letter amino acid code) present within the eIF4AI sequence (406 amino acids) is indicated. The structures of the truncated derivatives of eIF4AI are shown. The C-terminal His- and V5-epitope tags are depicted as filled rectangles.

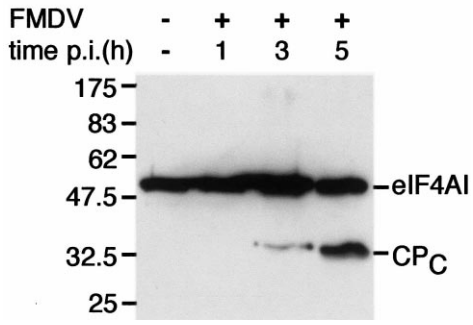

Fig. 2. Cleavage of V5-tagged eIF4AI is induced by FMDV 3C in cells. BHK-4AI cells, that express the C-terminal V5-tagged human eIF4AI, were infected with FMDV. At 1, 3 and 5 h post-infection the cells were lysed and analysed, in parallel with uninfected BHK-4AI cell extract, by SDS–PAGE and immunoblotting using anti-V5 antibodies. The C-terminal cleavage product (CP_C) is indicated.

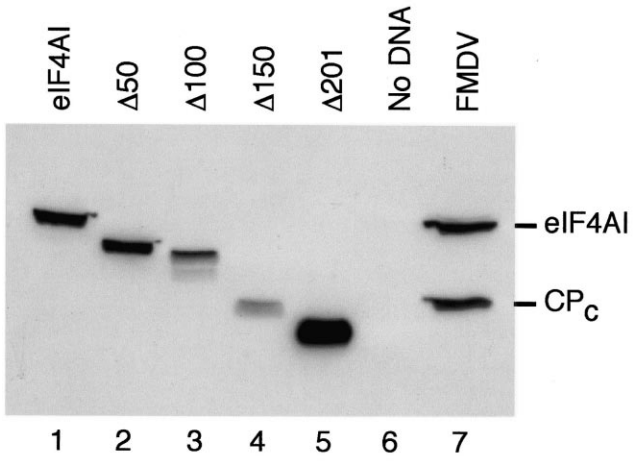

Fig. 3. Characterisation of the FMDV-induced eIF4AI C-terminal cleavage product. Extracts from vTF7-3 infected BHK cells that had been transfected with plasmids encoding the indicated C-terminal V5-tagged forms of eIF4AI were prepared at 20 h post-infection. Samples were analysed, using anti-V5 antibodies, in parallel with an extract (labelled FMDV) from FMDV-infected BHK-4AI cells as in Fig. 2. The positions of the full-length eIF4AI and its C-terminal cleavage product (CP_C) are indicated.

V144. This result is entirely consistent with the close co-migration of the C-terminal cleavage product generated in FMDV-infected cells with the Δ150 product shown in Fig. 3.

3.4. The N- and C-terminal cleavage products of eIF4AI dissociate

To be able to interpret the effect of eIF4AI cleavage, it was important to determine whether the N- and C-terminal cleavage products of eIF4AI remained associated. The GST–CP_N fusion product but not the C-terminal cleavage product (CP_C) was bound by glutathione-Sepharose (Fig. 4B,C) indicating that the two fragments dissociated from one another.

3.5. Analysis of the specificity of FMDV 3C for eIF4AI but not eIF4AII

Although there is a high degree (ca. 90%) of identity between eIF4AI and eIF4AII, there are differences between these proteins in the region of the cleavage site identified in eIF4AI (Fig. 5A). Substitution of residue V144 for M144 within eIF4AI did not alter its sensitivity to FMDV 3C (Fig. 5B). Similarly, the A142N mutant remained susceptible to cleavage by the 3C protease (Fig. 5B). However the double substitution, A142N and V144M, within the GST–eIF4AI sequence rendered it resistant to FMDV 3C cleavage in vitro (Fig. 5B), consistent with the resistance of GST–eIF4AII to this protease.

3.6. Mapping the 3C cleavage site within eIF4AI onto the 3D structure of yeast eIF4A

The 3D structure of yeast eIF4A has been determined [19–21]. Human/murine eIF4AI is 65% identical to yeast eIF4A and the presence of several conserved motifs facilitated alignment of the yeast and mammalian eIF4A sequences (Fig. 5A). The cleavage site within human/murine eIF4AI is located between the GG and TPGR conserved motifs (Fig. 5A) and the sequence similarity between the yeast and mammalian proteins between these motifs is rather low. We have selected residues E132/D133 in the yeast sequence to indicate the position of the scissile bond on the structure of the N-terminal domain of yeast eIF4A (Fig. 6) as determined [20]. The cleavage site is within an exposed, flexible corner of the molecule. In the first determination of the structure of the N-terminal

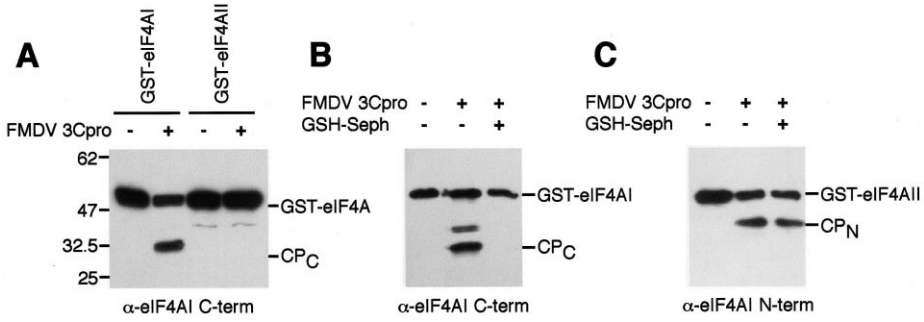

Fig. 4. Direct cleavage of GST–eIF4AI, but not GST–eIF4AII, by FMDV 3C protease results in dissociation of the two fragments. A: GST–eIF4AI (5 μg) and GST–eIF4AII (5 μg) fusion proteins were incubated alone or with FMDV 3C protease (0.5 μg). Reactions were terminated and analysed by SDS–PAGE and immunoblotting using an antibody specific for the C-terminus of eIF4A. The full-length fusion protein and C-terminal cleavage product of eIF4AI are indicated. B and C: Purified GST–eIF4AI was incubated alone or with FMDV 3C protease as indicated (as for A). Samples were analysed directly or after incubation with glutathione-Sepharose (GSH-Seph) beads. The bound proteins were analysed in parallel with the initial reaction products by SDS–PAGE and immunoblotting using antisera specific for the C-terminus (B) or the N-terminus (C) of eIF4AI. The eIF4AI C-terminal cleavage product (CP_C) and the GST–CP_N fusion protein are indicated.

yeast eIF4A [19], this portion of the molecule (residues 125–134) was not visible and was considered disordered.

4. Discussion

The FMDV 3C protease cleaves the viral polyprotein at several positions. The most common amino acid pair cleaved by FMDV 3C is E/G (glutamate/glycine) but E/S, Q/L, Q/I and Q/T pairs are also cut. This contrasts with the predominant use of Q/G (glutamine/glycine) cleavage sites by the enterovirus (e.g. PV) and cardiovirus (e.g. EMCV) 3C proteases. This difference in specificity is consistent with the ability of just the FMDV 3C protease to cleave the E143/V144 bond within eIF4AI. The cleavage of eIF4AI but not eIF4AII by FMDV 3C was surprising in view of the high degree of identity between the two proteins. However, co-expression of the FMDV 3C with tagged eIF4AI or tagged eIF4AII within mammalian cells also provided evidence for cleavage of eIF4AI but not of eI4FAII by this protease (data not shown). Furthermore, we have shown that two amino acid differences between eIF4AI and eIF4AII in the vicinity of the cleavage site are sufficient to account for this specificity. The modification of residue V144 to M alone did not render eIF4AI resistant to cleavage even though this residue is adjacent to the scissile bond. Similarly, the substitution of residue A142 by N alone did not block cleavage, but the presence of both modifications together, as in eIF4AII, conferred resistance to the protease.

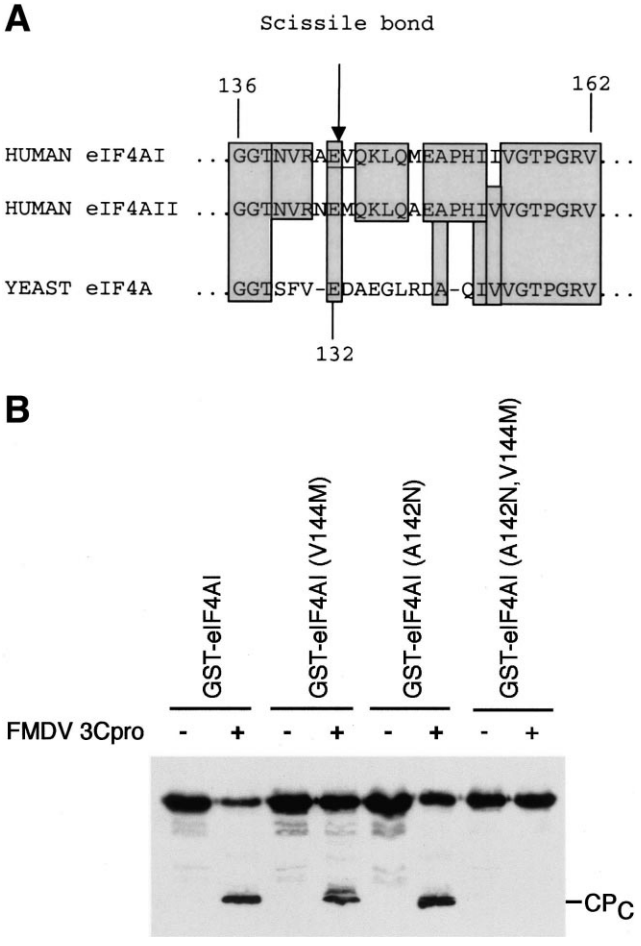

Fig. 5. Two amino acid substitutions in GST–eIF4AI confer resistance to cleavage by FMDV 3C protease in vitro. A: Sequence comparison of human/murine eIF4AI and eIF4AII with yeast eIF4A between the conserved GG and TPGR sequence motifs (n.b. for both eIF4AI and eIF4AII the human and murine amino acid sequences are the same). Residues that are identical in the different sequences are boxed and shaded. The location of the scissile bond in eIF4AI is indicated. Selected residues in eIF4AI and yeast eIF4A are numbered. B: The wild-type and mutant GST–eIF4AI fusion proteins (with single or double amino acid substitutions) were incubated alone or with FMDV 3C protease in vitro and analysed as in Fig. 4A. The C-terminal cleavage product (CP_C) is indicated.

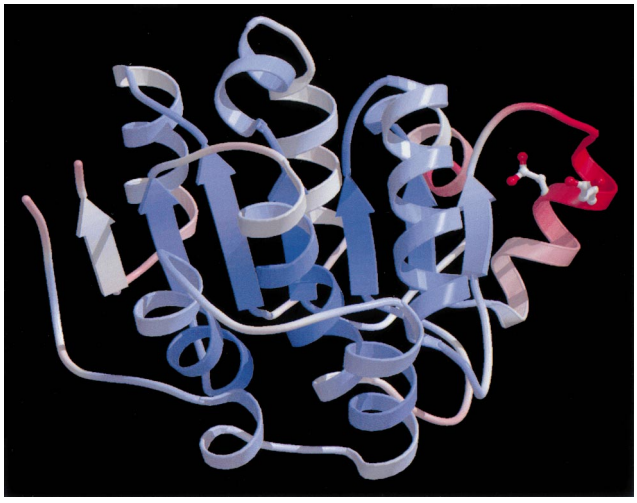

Fig. 6. Localisation of the cleavage site in eIF4AI recognised by FMDV 3C protease on the 3D structure. The 3D structure of the N-terminal region (residues 1–223) of yeast eIF4A as determined by X-ray crystallography [20] is shown. The structure is colour-coded according to the *b*-value. The red regions are most flexible while the blue regions are least mobile. Residues E132 and D133 corresponding to the cleavage site are indicated by the presence of the side chains.

Cleavage of eIF4AI by FMDV 3C resulted in dissociation of the N-terminal and C-terminal fragments (Fig. 4). Thus the cleavage separates three of the nine conserved regions that are found in all members of the DEAD box family of proteins (see [2] and Fig. 1). Modification of the ATPase A motif alone blocks ATP binding and the helicase activity of eIF4A. Similarly, modification of the conserved GG and TPGR motifs, adjacent to the cleavage site, also blocks ATPase activity [17]. Thus the truncated C-terminal fragment of eIF4AI lacks critical features of the eIF4A molecule and is accordingly expected to be inactive as a helicase.

The cleavage of eIF4AI within FMDV-infected cells does not go to completion but it should be noted that eIF4A is the most abundant of the translation initiation factors [14]. The progressive cleavage of eIF4AI co-incides with the decrease in the level of viral RNA translation that occurs in FMDV-infected cells [12]. However, it is unclear whether the decrease in viral protein synthesis is entirely attributable to the loss of eIF4AI. Indeed, since we can also observe decay in the primary cleavage products of eIF4GI (G.J.B. and N.R.-S., unpublished results and [12]), it is likely that several different processes contribute towards this effect.

The conservation of sequences between the mammalian and yeast eIF4A proteins allowed us to locate the FMDV 3C cleavage site on the known 3D structure of yeast eIF4A [20]. The flexible and exposed location of the cleavage site may explain the susceptibility of this region to the protease.

The peptide bond cleaved by FMDV 3C may be within a region that can associate with other molecules, e.g. eIF4G or eIF4B. If so, the partial cleavage of eIF4A observed in FMDV-infected cells may reflect modification of only the fraction of eIF4A that is not complexed with other proteins. Clearly the cleavage of one population of eIF4AI molecules may have significant effects if each population has distinct functional roles. Even if this is not the case, the partial cleavage of eIF4A by FMDV 3C may modify the amount of eIF4A associated with eIF4G or eIF4B and hence affect their activity.

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