

Cleavage of Eukaryotic Translation Initiation Factor 4GII within Foot-and-Mouth Disease Virus-Infected Cells: Identification of the L-Protease Cleavage Site In Vitro

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Foot-and-mouth disease virus (FMDV) induces a very rapid inhibition of host cell protein synthesis within infected cells. This is accompanied by the cleavage of the eukaryotic translation initiation factor 4GI (eIF4GI). The cleavage of the related protein eIF4GII has now been analyzed. Within FMDV-infected cells, cleavage of eIF4GI and eIF4GII occurs with similar kinetics. Cleavage of eIF4GII is induced in cells and in cell extracts by the FMDV leader protease (L^{pro}) alone, generating cleavage products similar to those induced by enterovirus and rhinovirus 2A protease (2A^{pro}). By the use of a fusion protein containing residues 445 to 744 of human eIF4GII, it was demonstrated that the FMDV L^{pro} specifically cleaves this protein between residues G700 and S701, immediately adjacent to the site (V699/G700) cleaved by rhinovirus 2A^{pro} in vitro. The G700/S701 cleavage site does not correspond, by amino acid sequence alignment, to that cleaved in eIF4GI by the FMDV L^{pro} in vitro. Knowledge of the cleavage sites and the three-dimensional structures of the FMDV L^{pro} and rhinovirus 2A^{pro} enabled mutant forms of the eIF4GII sequence to be generated that are differentially resistant to either one of these proteases. These results confirmed the specificity of each protease and showed that the mutant forms of the fusion protein substrate retained their correct sensitivity to other proteases.

Foot-and-mouth disease virus (FMDV) is a member of the family *Picornaviridae*. Like other picornaviruses, FMDV has a positive-sense RNA genome that is infectious. The genome, about 8.3 kb, encodes a large polyprotein that is processed, mainly by virus-encoded proteases, to generate each of the mature virus proteins. Two unrelated *trans*-acting proteases encoded within the FMDV genome have been identified, the leader protease (L^{pro}) and the 3C protease (3C^{pro}). The L^{pro} is related to the papain-like cysteine proteases (15, 32, 34), whereas the 3C^{pro} is related to the chymotrypsin-like serine proteases (1). The L^{pro}, located at the N terminus of the polyprotein, is produced in two forms (termed Lab and Lb) from the viral RNA. The two proteins differ in their N termini since the initiation of protein synthesis on the viral RNA occurs at two distinct sites, separated by 84 nucleotides (2, 35). Both forms of the L^{pro} are able to cleave the L/P1 junction in *trans* (24) and probably also in *cis* (see reference 10). The L^{pro} also induces the cleavage of the eukaryotic translation initiation factor 4G (eIF4G), a component of the cap-binding complex eIF4F (7, 24). The eIF4F complex consists of three different polypeptides: eIF4E (which binds to the cap structure at the 5' terminus of all cytoplasmic cellular mRNAs), eIF4A (an RNA helicase), and eIF4G. The last component acts as a

scaffold protein that also interacts with eIF3 (associated with the small ribosomal subunit), the poly(A) binding protein, and Mnk-1 (an eIF4E kinase) (see review in reference 9).

Usually, cleavage of eIF4G roughly correlates with the loss of cellular cap-dependent protein synthesis within cells infected with poliovirus (PV) or FMDV and many other picornaviruses (see review in reference 3). However, under certain circumstances, when virus replication is inhibited, the complete loss of intact eIF4G could be observed while cellular protein synthesis was maintained, albeit at a much-reduced level (5, 30). This apparent paradox was resolved when it was discovered that a second species of eIF4G, now termed eIF4GII, is also present within cells (12).

The major form of eIF4G is now referred to as eIF4GI; this protein is about 41% identical to eIF4GII, and the two proteins appear functionally equivalent (12). Within cells infected by PV or human rhinovirus 14 (HRV14) there is a differential rate of cleavage of eIF4GI and eIF4GII (13, 39). The cleavage of eIF4GI occurs rapidly, while proteolysis of eIF4GII is observed only later. Both forms of eIF4G must be cleaved to achieve complete loss of cap-dependent protein synthesis.

In contrast to the observations made within PV- or HRV14-infected cells, the cleavage of eIF4GI and eIF4GII occurs simultaneously within cells infected by HRV2 (36) or the closely related HRV16 (14). The protein responsible for inducing cleavage of both forms of eIF4G within cells infected with PV or HRV is the 2A protease (2A^{pro}) (14, 19, 22). The difference in kinetics of eIF4GI and eIF4GII cleavage within cells infected with particular enteroviruses or rhinoviruses presumably reflects specific differences in the substrate binding

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efficiencies of the distinct 2A^{pro}. The enterovirus and rhinovirus 2A^{pro} is also related to the chymotrypsin-like family of proteases and hence are unrelated to the FMDV L^{pro} (31, 34, 37).

The cleavage of eIF4GI that is induced by the enterovirus 2A^{pro} or by the FMDV L^{pro} occurs very early in infection and can be achieved in the absence of viral RNA replication (4, 5, 30). Recently, it has been shown that the 3C^{pro} of FMDV can also induce cleavage of eIF4GI within baby hamster kidney (BHK) cells (4). Subsequently it has been demonstrated that sequential cleavage of eIF4GI by the L^{pro} and then by 3C^{pro} occurs within BHK cells infected with wild-type (wt) FMDV (R. Strong and G. J. Belsham, unpublished results). The cleavage of eIF4GI by FMDV 3C occurs on the C-terminal side of the cleavage site induced by the L^{pro} (4). However, to date, there has been no identification of the precise cleavage sites in eIF4GI or eIF4GII within FMDV-infected cells.

The site within rabbit eIF4GI that is cleaved by incubation with recombinant FMDV L^{pro} in vitro has been identified as between G674 and R675 (18). (Note that throughout this paper the residues in eIF4GI have been numbered according to the system of Byrd et al. [6] for the largest form of eIF4GI, termed eIF4GIa, which is expressed from pSP4GI.) This site is just seven residues apart from the cleavage site (between R681 and G682) that is generated by 2A^{pro} from enteroviruses and HRV2 in vitro (20, 41). This latter site has been authenticated by analysis of the C-terminal cleavage product of eIF4GI generated within PV-infected cells (41; A. Gradi and N. Sonenberg, unpublished results). Recent studies have identified the site in eIF4GII that is cleaved by the 2A^{pro} in vitro (14). The scissile bond is between V699 and G700. It is noteworthy that this site does not correspond, by amino acid sequence alignment, to the position on eIF4GI that is cleaved by the 2A^{pro}.

It has recently been demonstrated that eIF4GII can be cleaved by the FMDV L^{pro} in vitro (23, 27). However, neither the L^{pro} cleavage site nor the in vivo kinetics of eIF4GII cleavage have been reported. Here, we investigate these issues and discuss the results in relation to the known specificity and three-dimensional structure of the Lb^{pro} (15).

MATERIALS AND METHODS

BHK cells were infected with FMDV (O1Kaufbeuren B64) as described previously (4). When appropriate, cells were labeled with [³⁵S]methionine and [³⁵S]cysteine in methionine- and cysteine-free Dulbecco modified Eagle medium for 15 min. Cell extracts were prepared and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography or for unlabeled extracts by immunoblotting with antibodies specific for the N- or C-terminal regions of eIF4GI or eIF4GII (see references 11 and 12) with appropriate peroxidase-labeled antispecies antibodies and chemiluminescence reagents.

Transient-expression assays within cells infected with the recombinant vaccinia virus vTF7-3, which expresses the T7 RNA polymerase (8), were performed as described previously (33). Briefly, human 293 cells were infected with vTF7-3 and transfected, with the use of Lipofectin (8 µg; Life Technologies), with plasmid DNA (2.5 µg) encoding the PV 2A^{pro} (pAΔ802) or the FMDV Lb^{pro} (pLb) as used previously (33). After 20 h cell extracts were prepared and analyzed by SDS-PAGE and immunoblotting as described above.

The expression and purification of recombinant eIF4E, FMDV Lb^{pro}, and the 2A^{pro} from Coxsackie virus B4 (CVB4) and HRV2 have been described previously (18, 21, 29).

Cytoplasmic extracts from uninfected 293 cells were prepared in the absence of protease inhibitors as described previously (12, 13). Cell extracts (100 µg) were incubated for 60 min at 30°C in buffer A (20 mM HEPES-KOH [pH 7.6],

150 mM KCl, 1 mM dithiothreitol, 1 mM EDTA) in a total volume of 35 µl in the presence or absence of purified recombinant FMDV Lb^{pro}. Before addition of the protease, the samples were preincubated for 5 min at 30°C with buffer alone or with recombinant eIF4E (1 µg). Following incubation, the reaction mixtures were divided into two samples and analyzed by SDS-PAGE and immunoblotting with polyclonal antibodies to the C-terminal cleavage products (CP_c) of eIF4GI and eIF4GII as described above.

The construction and expression of the wt, G700E, and Δ674-702 mutant glutathione *S*-transferase (GST)-eIF4GII-FLAG fusion proteins have been described previously (14). Additional mutant forms of the expression plasmid were prepared using overlap PCR mutagenesis. The S701P mutant was made in several stages. One reaction mixture was prepared using the wt GST-eIF4GII-FLAG plasmid as template with the 4GIIfor primer (dGTTTGGTGGTGGCCG ACCATC) and the 4GIIrevSP primer (dGATCTTCGTGGCCCAACATTC AAC) with *Pfu* DNA polymerase. A second reaction mixture contained the same template with the primers 4GIIforSP (dGTTGAATGTTGGGCCACGAAG ATC) and 4GIIrev (dCTCCGGGAGCTGCATGTGTC). The two products were gel purified and mixed, and a third PCR was performed using just the 4GIIfor and 4GIIrev primers. Similarly the G700K mutant was made using the same strategy with the 4GIIfor and 4GIIrev primers in conjunction with the mutant oligonucleotides 4GIIforGK (dGTTGAATGTTAAGTCACGAAG ATC) and 4GIIrevGK (dGATCTTCGTGACTTAACATTC AAC). In each case the final products were digested with EcoRI and NotI, and the fragments of ca. 900 nucleotides were used to replace the wt sequences within the similarly digested plasmid encoding the GST-eIF4GII-FLAG protein (14). The presence of the required mutations was confirmed by DNA sequencing, and the mutant fusion proteins were isolated as described above. For analytical assays, purified recombinant GST-eIF4GII-FLAG protein (2.5 µg) or the mutant derivatives were incubated in buffer A with purified FMDV Lb^{pro} or HRV2 2A^{pro} (the amount of proteases used is indicated in the figure legends) at 30°C for 60 min in the presence or absence of recombinant eIF4E (1 µg) as indicated. Samples were analyzed by SDS-PAGE (15% acrylamide) and immunoblotting with anti-FLAG M2 antibodies (Sigma or Upstate Biotechnology Inc.).

For the identification of the cleavage site, recombinant GST-eIF4GII-FLAG protein (35 µg) was incubated with purified FMDV Lb^{pro} (4 µg) at 30°C for 60 min in the presence of recombinant eIF4E (3 µg). The C-terminal FLAG-tagged cleavage fragment was captured using anti-FLAG M2 antibody resin (Sigma) as described previously (25) and purified using SDS-PAGE (15.5% acrylamide). After electrophoresis, the proteins were transferred in cyclohexylaminopropane-sulfonic acid buffer onto a membrane (ProBlott; 0.2-µm pore size) and stained with 0.1% Coomassie blue R-250 in 50% methanol. The band of interest was isolated and subjected to automated Edman degradation. The N-terminal sequence analysis was performed by the Sheldon Biotechnology Center, McGill University.

RESULTS

Cleavage of eIF4GII within FMDV-infected cells. The inhibition of host cell protein synthesis occurs very rapidly within FMDV-infected BHK cells (see reference 4). This is accompanied by the cleavage of eIF4GI. To examine the status of eIF4GII within FMDV-infected cells, a time course experiment was performed (Fig. 1). As observed previously (4), within 2 to 3 h postinfection, a dramatic decrease in host cell protein synthesis was detected by pulse-labeling of infected cells with [³⁵S]methionine-cysteine (Fig. 1A). Consistent with our previous results, cleavage of eIF4GI occurred very rapidly (Fig. 1B). In the same extracts we also monitored the status of eIF4GII. The loss of the intact eIF4GII protein was also readily detected early in infection (Fig. 1C). The rabbit anti-C-terminal antibody detected two distinct cleavage products (with apparent sizes of about 85 and 33 kDa) that were derived from eIF4GII in extracts of cells harvested at 2 h postinfection. An additional product of 45 kDa accumulated by 3 h postinfection and probably represents a breakdown product of the 85-kDa species. The kinetics of primary cleavage for eIF4GI and eIF4GII were very similar. In both cases, cleavage had started at 1 h postinfection and no intact protein was detect-

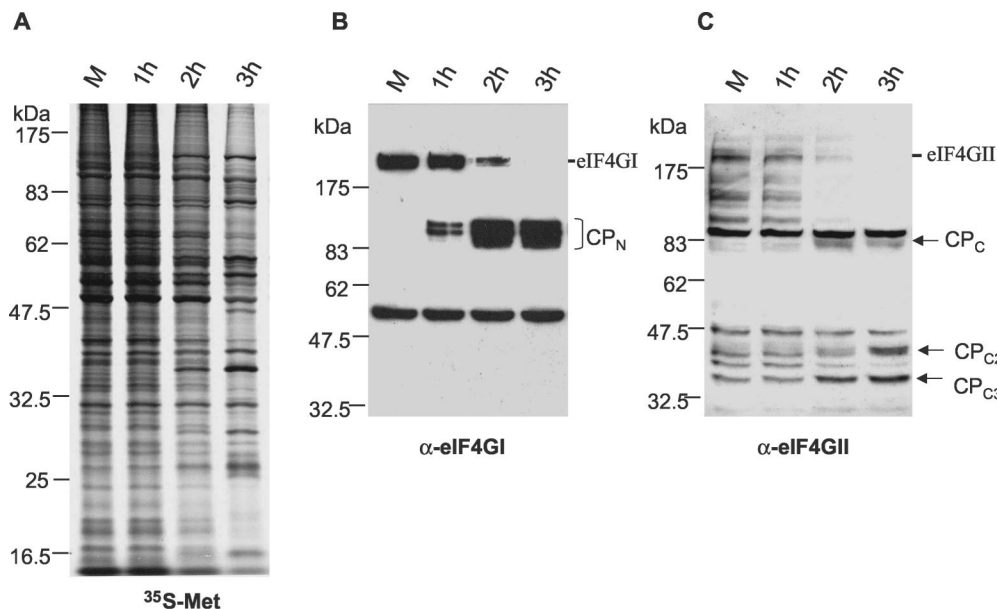


FIG. 1. Cleavage of eIF4GI and eIF4GII occurs simultaneously within FMDV-infected cells. BHK cells were infected with FMDV. (A) At the indicated times (hours postinfection) cells were incubated with ³⁵S-labeled amino acids for a further 15 min. Cell extracts were prepared and analyzed by SDS-PAGE (10% polyacrylamide) and autoradiography. (B and C) Cells infected in parallel were harvested at the indicated times, and cell extracts were analyzed by SDS-PAGE (6% polyacrylamide) and immunoblotting with rabbit polyclonal antibodies specific for eIF4GI (B) and eIF4GII (C) as described in Materials and Methods. Detection was achieved using peroxidase-labeled anti-rabbit antibodies and chemiluminescence reagents. The mobilities of molecular mass standards (kilodaltons) are indicated.

able by 3 h postinfection. The recognition of eIF4GII from BHK cells by the antibody was not as efficient as is observed with human cell extracts; presumably this reflects a difference in the sequences of the proteins from the two species.

Cleavage of eIF4GII by the FMDV L^{pro} within cells. We wished to determine whether the cleavage of eIF4GII was induced within cells by the expression of FMDV L^{pro} alone. A transient-expression assay was performed within human 293 cells (infected with the recombinant vaccinia virus vTF7-3 [8], which expresses the T7 RNA polymerase) by using plasmids that expressed either the PV 2A^{pro} or the FMDV L^{pro} (Fig. 2). The status of both eIF4GI and eIF4GII was examined in cell extracts by immunoblotting with antisera to the N-terminal cleavage products. As observed previously (4), eIF4GI cleavage was very efficiently induced by the PV 2A^{pro} and by the FMDV L^{pro} (Fig. 2A). Furthermore, it was apparent that both proteases also induced efficient cleavage of eIF4GII (Fig. 2B). The major cleavage products of eIF4GII generated by the expression of the two different proteases and detected by the anti-N-terminal antibody were indistinguishable, suggesting that the cleavage sites generated by these two proteases are near each other. However, it should be noted that an additional smaller product (marked by an asterisk in Fig. 2B) was also induced by the FMDV L^{pro}, presumably reflecting a second cleavage event within the N-terminal portion of the protein.

In vitro cleavage of eIF4GI and eIF4GII by the FMDV L^{pro}. The addition of eIF4E greatly stimulates the cleavage of eIF4GI and eIF4GII by HRV 2A^{pro} in vitro (12, 16). Similarly, the cleavage of eIF4GI by FMDV L^{pro} is stimulated by the presence of eIF4E (26). We wished to determine whether the cleavage of eIF4GII by the FMDV L^{pro} in vitro was also

modified by the addition of eIF4E. Cell extracts were prepared from uninfected human 293 cells and incubated with purified recombinant FMDV L^{pro} in the presence or absence of recombinant cap-binding protein eIF4E. A single C-terminal cleavage product (CP_C) of about 95 kDa was observed from both eIF4GI and eIF4GII in the presence of the FMDV L^{pro}. The products observed were indistinguishable from those generated by incubation with recombinant 2A^{pro} from CVB4 (Fig.

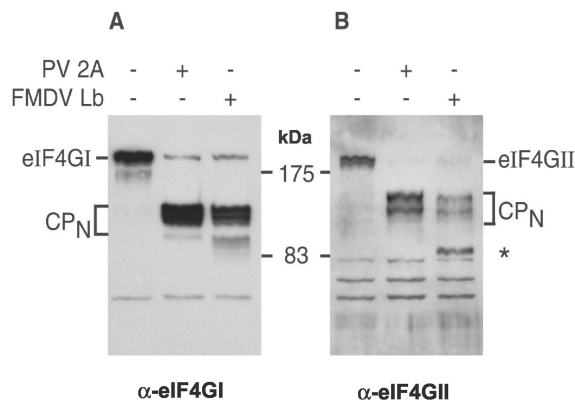


FIG. 2. The FMDV L^{pro} induces cleavage of eIF4GI and eIF4GII within cells. Human 293 cells were infected with the recombinant vaccinia virus vTF7-3 and transfected with the indicated plasmids that express FMDV L^{pro} (pLb) or the PV 2A^{pro} (pAΔ802) with Lipofectin. After 20 h, cell extracts were prepared and analyzed by SDS-PAGE and immunoblotting with antisera specific for the N termini of eIF4GI (A) and eIF4GII (B). The cleavage products and the full-length proteins are indicated. An additional cleavage product detected in extracts containing the FMDV L^{pro} is highlighted by an asterisk.

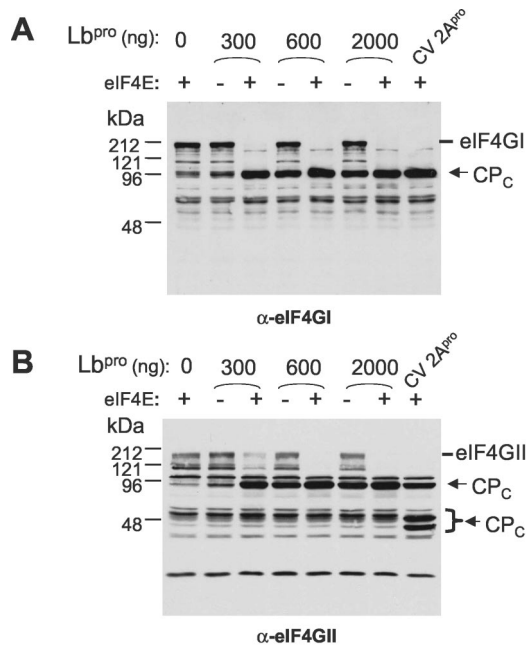


FIG. 3. Cleavage of eIF4GI and eIF4GII by the FMDV Lb^{PRO} in cell extracts is enhanced by the presence of added eIF4E. Extracts of uninfected human 293 cells (100 μg of protein) were incubated with the indicated amounts of recombinant FMDV Lb^{PRO} or CVB4 2A^{PRO} (7 μg) in the presence (+) or absence (-) of recombinant eIF4E (1 μg) as indicated. Samples were analyzed by SDS-PAGE (10% polyacrylamide) and immunoblotting with antibodies specific for the C terminus of eIF4GI (A) or eIF4GII (B). Proteins were detected using chemiluminescence reagents as in Fig. 1.

3) as used previously (14). The presence of added eIF4E greatly enhanced the cleavage of eIF4GI by the Lb^{PRO} in vitro, consistent with previous results (26). Furthermore, addition of eIF4E also strongly stimulated the cleavage of eIF4GII by the Lb^{PRO} in these assays (Fig. 3B).

In vitro cleavage of recombinant eIF4GII fusion proteins by the FMDV Lb^{PRO}. A recombinant fusion protein, GST-eIF4GII-FLAG (Fig. 4A), which contains an internal region (residues 445 to 744) of human eIF4GII (including the eIF4E binding site) linked to both GST and FLAG tags, was expressed in *Escherichia coli* and purified to homogeneity on glutathione-Sepharose as described previously (14). The protein was incubated in vitro with recombinant FMDV Lb^{PRO} in the presence or absence of added recombinant eIF4E. The GST-eIF4GII-FLAG fusion protein was completely cleaved by Lb^{PRO} under both conditions (Fig. 4B). We also analyzed the sensitivities to FMDV Lb^{PRO} of two similar fusion proteins (Fig. 4A) that contain a modified region of eIF4GII (Fig. 4B). These two mutant fusion proteins were initially prepared as part of an analysis of the in vitro cleavage of eIF4GII by the HRV2 2A^{PRO} (14). One mutant protein has a single point mutation, G700E, which confers resistance to cleavage by 2A^{PRO}, since the cleavage site is between residues 699 and 700. The second mutant protein has a deletion of 27 residues (between 674 and 702); this protein was also fully resistant to the 2A^{PRO} since the deletion removes the cleavage site (14). Surprisingly, both mutant proteins were also highly resistant to the action of the FMDV Lb^{PRO} in both the presence and the absence of eIF4E (Fig. 4B). A small yield of the C-terminal FLAG-tagged cleavage product was observed from the G700E mutant following incubation with a high level of the Lb^{PRO} (data not shown);

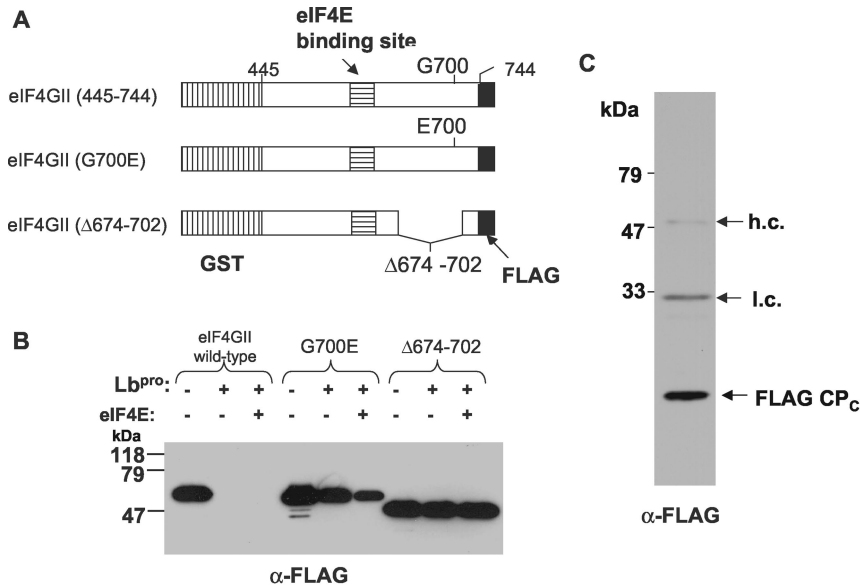


FIG. 4. In vitro cleavage of a fragment of eIF4GII by FMDV Lb^{PRO} permits identification of the cleavage site. (A) Structure of the GST-eIF4GII-FLAG (abbreviated GST-4GII-FLAG) fusion proteins as described previously (14). (B) Recombinant GST-4GII-FLAG protein (2.5 μg) or mutant forms with a G700E substitution or deletion of residues 674 to 702 were incubated in the presence (+) or absence (-) of recombinant FMDV Lb^{PRO} (1 μg) alone or with recombinant eIF4E (1 μg) as indicated. Samples were analyzed by SDS-PAGE (15% polyacrylamide) and immunoblotting with anti-FLAG M2 antibodies. Detection was achieved using chemiluminescence reagents. (C) The C-terminal cleavage product of the wt GST-4GII-FLAG protein, generated by the FMDV Lb^{PRO}, was isolated using an anti-FLAG M2 affinity resin. An aliquot of the material was analyzed by SDS-PAGE (15.5% polyacrylamide) and immunoblotting with anti-FLAG M2 antibodies as for panel B. The presence of antibody heavy chains (h.c.) and light chains (l.c.) as well as the FLAG-tagged C-terminal cleavage product (CP_c) is indicated.

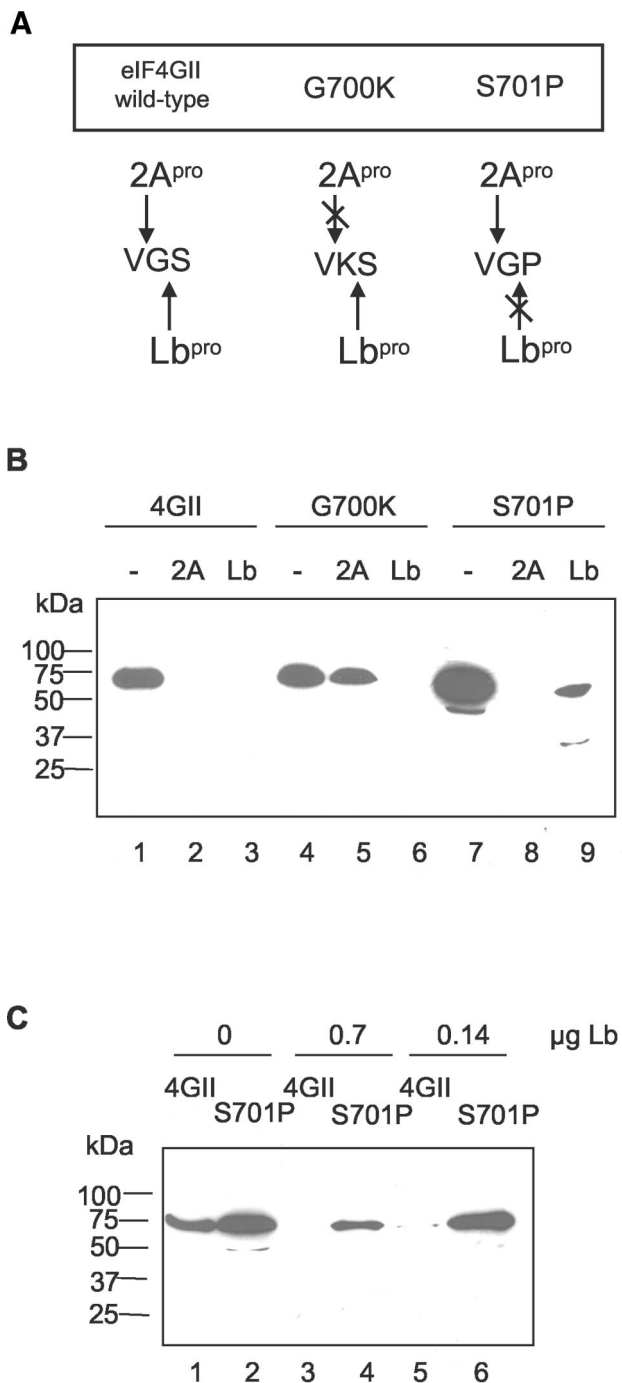


FIG. 6. Specific mutations in eIF4GII have differential effects on 2A^{pro} and Lb^{pro} cleavage. (A) Predicted cleavage activity of 2A^{pro} and Lb^{pro} on eIF4GII mutant proteins. (B) The indicated fusion proteins were incubated for 1 h at 30°C in buffer A alone (lanes 1, 4, and 7), with purified HRV2 2A^{pro} (1.2 μg; lanes 2, 5, and 8), or with purified FMDV Lb^{pro} (1.43 μg; lanes 3, 6, and 9). Protein samples were analyzed by SDS-PAGE and immunoblotting with a monoclonal antibody against the C-terminal FLAG tag (Upstate Biotechnology Inc.). (C) The wt and S701P mutant GST-eIF4GII-FLAG fusion proteins were incubated with the indicated concentrations of purified FMDV Lb^{pro} for 1 h at 30°C. Protein samples were analyzed by SDS-PAGE and immunoblotting as described for panel B.

strate to a proline. At the cleavage site on the viral polyprotein, a proline residue is observed in this position. This led us to believe that the S701P mutant protein would still be a substrate for HRV2 2A^{pro} (Fig. 6A).

The indicated mutations were therefore introduced into the GST-eIF4GII-FLAG plasmid, and the fusion proteins were expressed and purified by affinity chromatography on glutathione-Sepharose. The wt and mutant fusion proteins were then incubated alone or with one of the viral proteinases. The products were analyzed by SDS-PAGE and immunoblotting with anti-FLAG antibodies (Fig. 6B). As expected, the wt protein was fully digested by both proteinases (lanes 2 and 3). The G700K mutant was indeed resistant to 2A^{pro} of HRV2 as predicted (lane 5), whereas, in contrast, the S701P mutant was still efficiently cleaved by this protease. As anticipated, the G700K protein was still cleaved, like the wt construct, by the Lb^{pro}. However, unexpectedly the S701P mutant protein was not completely resistant to cleavage by Lb^{pro}; a small amount of a novel cleavage product of about 30 kDa was detected (lane 9). The production of this 30-kDa protein appeared to represent an aberrant cleavage. Hence, we attempted to determine whether the S701P protein and the wt protein would exhibit markedly different sensitivities to the FMDV Lb^{pro} at lower concentrations of the enzyme. Thus, the concentration of Lb^{pro} was reduced twofold (Fig. 6C, lanes 3 and 4) or fourfold (Fig. 6C, lanes 5 and 6) compared to that used in Fig. 6B. Under these conditions, the S701P fusion protein remained intact whereas the wt fusion protein was almost completely processed (Fig. 6C, lanes 5 and 6). In addition, the 30-kDa aberrant cleavage product was not detected. We conclude that the G700/P701 site is resistant to Lb^{pro} as predicted. Thus, the two mutant forms of the GST-eIF4GII-FLAG fusion protein exhibit sensitivity to one protease while being resistant to the other. These results support the view that the structure of the fusion proteins is similar in each case and it is a reflection of the particular specificity of each protease that the mutated sites confer resistance.

DISCUSSION

The results presented here demonstrate that cleavage of both eIF4GI and eIF4GII occurs rapidly, simultaneously, and completely within FMDV-infected cells. These results are reminiscent of those obtained with HRV2 (36) and HRV16 (14) but contrast with the differential rates of cleavage of these proteins observed within cells infected by PV or HRV14 (13, 39). Transient-expression assays demonstrated that the FMDV Lb^{pro} alone is able to induce cleavage of eIF4GII by itself within cells (Fig. 2); these results mirror earlier studies with eIF4GI (4, 24).

The direct cleavage of eIF4GI by the FMDV Lb^{pro} in vitro was demonstrated by Kirchweiger et al. (18), who also identified the cleavage site as between residues G674 and R675. The cleavage of purified eIF4GI is rather inefficient and requires high levels of Lb^{pro}. In contrast, cleavage of eIF4GI within FMDV-infected cells occurs early in infection before detectable virus protein expression and can be achieved even in the absence of RNA replication (see reference 4). These data indicate that a low level of protease is required to initiate cleavage of eIF4GI within cells. It has been suggested that the

2A^{PRO} of enteroviruses and rhinoviruses and the L^{PRO} of FMDV may induce cleavage of eIF4G indirectly within cells by activating a cellular protease (see review in reference 3). It is certainly the case that cellular proteases can cleave eIF4G (41). However, it has also been demonstrated that the addition of eIF4E to eIF4G greatly enhances the efficiency with which eIF4G can be cleaved by 2A^{PRO} and FMDV Lb^{PRO} in vitro (12, 14, 16, 26). These results suggested that the conformation of eIF4G may be altered by this (and possibly other) interaction(s), resulting in a complex that may be more susceptible to cleavage than the isolated protein is. Indeed, an earlier observation had indicated that eIF3 (which also interacts with eIF4G) also enhanced the susceptibility of eIF4G to 2A^{PRO} (40). The results presented here (Fig. 3) demonstrated that eIF4E greatly enhanced the cleavage of eIF4GI and eIF4GII within cell lysates by recombinant FMDV Lb^{PRO}; these results confirm and extend those of Ohlmann et al. (26), who analyzed only eIF4GI cleavage. Interestingly, when a fragment of eIF4GII (residues 445 to 744) was used as part of a GST fusion protein, it was found that this fragment was susceptible to cleavage by FMDV Lb^{PRO} in the presence or absence of eIF4E (Fig. 4B).

The cleavage of this GST-eIF4GII-FLAG fusion protein by Lb^{PRO} in vitro was used to identify the cleavage site within eIF4GII as between G700 and S701. This site is just 1 amino acid C terminal of the site cleaved by the HRV 2A^{PRO} in vitro (14). The identification of this site was entirely consistent with the resistance of the G700E mutant form of the GST-eIF4GII-FLAG protein to cleavage by Lb^{PRO}. Similarly, the deletion mutant lacking residues between 674 and 702 from eIF4GII was also completely resistant to cleavage by Lb^{PRO}. There have been no reports of successful expression of full-length recombinant eIF4GII in cells, but a His-tagged truncated form of this protein, lacking the first 143 residues, has been produced with the baculovirus expression system (see reference 17a). Treatment of this protein (kindly supplied by S. Morley, University of Sussex, United Kingdom) with the recombinant FMDV Lb^{PRO} generated cleavage products (N. Foeger and T. Skern, unpublished results), but attempts to determine N-terminal sequence information from these products were unsuccessful. Thus, we have been unable to confirm the location of the cleavage site within the full-length protein. However, there are additional strands of evidence that support the view that the cleavage site identified in our work is authentic. Firstly, we demonstrated that the cleavage products of eIF4GII generated in cells and in cell extracts by the PV (and CVB4) 2A^{PRO} and the FMDV Lb^{PRO} comigrate (Fig. 2 and 3); this clearly suggests that the cleavage sites are near each other. Secondly, in the GST-eIF4GII-FLAG fusion protein (Fig. 4 and 6), we are using about 300 amino acids from eIF4GII, and yet individual modification of a single amino acid on either side of the identified scissile bond (G700/S701) was enough to confer resistance to cleavage by Lb^{PRO}.

On the basis of the known properties of 2A^{PRO} and Lb^{PRO}, it was possible to design mutant forms of the eIF4GII sequence which were differentially resistant to either one of these proteases. Thus, although it is apparent that multiple sites within hamster eIF4GII may be cleaved by proteases within FMDV-infected cells (Fig. 1), there is high specificity in the cleavage of the GST-eIF4GII-FLAG fusion protein by the Lb^{PRO} in vitro. It

was interesting, however, that the S701P mutant protein (which was no longer cleaved by Lb^{PRO} at the usual position) was cleaved at a novel site with the use of a high concentration of the protease. An additional cleavage event in the N-terminal region of eIF4GII by the Lb^{PRO} was detected in human cells that expressed this protease (Fig. 2B). We have also observed that FMDV 3C^{PRO} can cleave eIF4GII in vitro and within cells (A. Gradi and G. J. Belsham, unpublished results), and hence, this protease could contribute to the additional fragments of eIF4GII observed within FMDV-infected cells.

Comparison of the Lb^{PRO} cleavage site in eIF4GII (PLLNV G⁷⁰⁰ SRRSQP) with those in eIF4GI (SFANLG⁶⁷⁴ RTTLST [18]) and the viral polyprotein (strain O1K; VQRKLLK²⁰¹ GA GQSS [28]) reveals a number of differences. Most strikingly, the eIF4GII cleavage site does not possess a basic residue at either the P1 or P1' position that flanks the scissile bond (although it can accept one, as in the G700K mutant). Until now, the presence of such a residue had been regarded as a major determinant of Lb^{PRO} specificity (10, 15). It seems possible, however, that the arginine (R) residue at P2' may provide the positive charge required for Lb^{PRO} substrate binding. In addition, the presence of a serine (S) residue has not previously been observed at P1' in an Lb^{PRO} cleavage site. On the P (N-terminal) side of the cleavage site, the presence of asparagine (N) at P3, valine (V) at P2, and glycine (G) at P1 fits well into the pattern of residues observed at these positions in the other cleavage sites.

It is noteworthy that the site cleaved in eIF4GII by the Lb^{PRO} does not correspond, by amino acid sequence alignment, to the position cleaved by this protease within eIF4GI (Fig. 5). The same is true for the sites in eIF4GI and eIF4GII that are cleaved by HRV 2A^{PRO} (14). The resistance of the eIF4GII mutant proteins G700E and S701P to Lb^{PRO} cleavage shows that the region of eIF4GII that corresponds to the cleavage site on eIF4GI (Fig. 5) is not recognized by Lb^{PRO}, even when the correct site is blocked. Why should this be so? Despite the availability of the crystal structure and other studies of Lb^{PRO} specificity, this question cannot be answered fully at present. Given that lysine (K) or asparagine (N) is found in the other cleavage sites at the P3 position, it seems possible that the presence of aspartate (D683) in the eIF4GII sequence might be detrimental to cleavage. In addition, although leucine (L) and phenylalanine (F) are often regarded as having similar properties, it is possible that the Lb^{PRO} can distinguish between the presence of these two residues at the P2 position. However, as papain accepts both residues well at P2 (17) and has a P2 binding pocket almost identical to that of Lb^{PRO} (15), it remains to be investigated whether Lb^{PRO} can discriminate so finely at this position.

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