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The leader proteinase of foot-and-mouth disease virus: structure-function relationships in a proteolytic virulence factor

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Abstract

The leader proteinase (L^{pro}) of foot-and-mouth disease virus, inhibits the host innate immune response by at least three different mechanisms. The most well characterised is the prevention of the synthesis of cytokines such as interferons immediately after infection, brought about by specific proteolytic cleavage of the eukaryotic initiation factor 4G. This prevents the recruitment of capped cellular mRNA; the viral RNA can however be translated under these conditions. The two other mechanisms are the induction of NF- κ B cleavage and the deubiquitination of immune signalling molecules. This review focuses on the structure-function relationships in L^{pro} responsible for these widely divergent activities.

Keywords

Papain-like cysteine proteinase; substrate specificity; initiation of protein synthesis; NF- κ B inactivation; deubiquitination

Introduction

Viruses are obligate, intracellular parasites that must modulate host processes to ensure their successful replication. A major challenge for all viruses with an RNA genome is the manipulation of the protein synthesis of the host cell to inhibit translation of cellular proteins whilst permitting or even enhancing protein synthesis of viral RNA. Many viruses of the picornavirus family achieve this goal by encoding a proteinase that specifically cleaves the host cell protein, eukaryotic initiation factor (eIF) 4G (Morley et al., 1997). As detailed below, this leads to the inability of the host cell to translate capped cellular mRNA. In contrast, the uncapped picornaviral RNA can be used as a template for protein synthesis following cleavage of the eIF4G, as it initiates through an IRES (internal ribosomal entry site) in the 5' untranslated region (UTR). The viral proteinases that cleave the two homologues of eIF4G, termed eIF4GI and eIF4GII, are cysteine proteinases, but with completely different folds (Skern et al., 2002). The enteroviral (e.g. poliovirus and human rhinovirus) 2A proteinase ($2A^{\text{pro}}$) has a chymotrypsin-like fold with a truncated N-terminal domain (Petersen et al., 1999) whereas the aphthoviral (e.g. foot-and-mouth disease virus (FMDV) and equine rhinitis A virus) and erboviral (equine rhinitis B virus) leader

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proteinases (L^{pro}) have a papain-like cysteine fold (Guarné et al., 1998). L^{pro} has also been reported to have two further activities, namely the inhibition of NF- κ B activation (de Los Santos et al., 2007) and the deubiquitination for certain molecules involved in innate immune signalling (Wang et al., 2011). This review focuses on the structure-function relationships in FMDV L^{pro} necessary for these reactions.

Functions of L^{pro}

i Self-processing

The FMDV genome, like that of all members of the picornavirus family, possesses a single open reading frame (ORF) surrounded by a 5' UTR of around 1000 nucleotides containing the IRES and a 3' UTR of about 90 nucleotides (Fig. 1; (Forss et al., 1984)). The primary product of translation of the ORF is a polyprotein that is subsequently processed to the mature viral proteins by (a) proteinase(s) present in the polyprotein. L^{pro} is the first protein encoded in the ORF; it was first shown to be a proteinase by Strebel and Beck (Strebel and Beck, 1986). The initiation of translation on the FMDV genome is unusual in that there are two AUG start codons 84 nucleotides apart, so that two forms of the L^{pro} are produced in infected cells, differing by the presence of 28 extra amino acids at the N-terminus (Fig. 1A; (Sangar et al., 1987)). The longer form is termed Lab^{pro} whereas the shorter form is termed Lb^{pro} . The C-terminus of both forms is the same and is generated by cleavage of either Lab^{pro} or Lb^{pro} at their own C-terminus and the N-terminus of the subsequent protein on the polyprotein, VP4 (Fig. 1B; (Strebel and Beck, 1986)). Using a genetic approach, it has been shown by Cao et al (1995) that the physiologically relevant form is Lb^{pro} and not Lab^{pro} . In addition, during an FMDV infection, it has also been observed that Lb^{pro} is shortened by six or seven amino acids at the C-terminus (Sangar et al., 1988). The role, if any, of this form (termed sLb^{pro}) has not been elucidated, although it has been reported that the sLb^{pro} has an altered substrate specificity compared to Lb^{pro} (Cencic et al., 2007).

The self-processing of L^{pro} from the capsid protein VP4 makes this proteinase an interesting target for anti-viral compounds. Inhibition of the self-processing of the L^{pro} from VP4 will produce molecules of VP4 with an extremely long extension (Glaser et al., 2001). The presence of only a few such molecules of the 60 VP4 molecules required for the capsid will almost certainly be sufficient to disrupt the formation of an infectious capsid (Fig. 1 A). An analogous effect has been observed through mutation of the poliovirus $2A^{pro}$ that leads to an extension of VP1 (Crowder and Kirkegaard, 2005). This extra effect of the inhibition of L^{pro} or $2A^{pro}$ will amplify the anti-proteolytic effect of an anti-viral such that a complete inhibition of the proteinase in question will not be required to achieve a major reduction in viral replication. Indeed, the use of a soluble form of the general, non-specific papain-like cysteine proteinase inhibitor E-64 was shown over 20 years ago to inhibit FMDV replication in tissue culture (Kleina and Grubman, 1992).

ii Inhibition of host-cell protein synthesis

Inhibition of host cell protein synthesis through cleavage of the eIF4G homologues (Fig. 1C) was first documented during infection in cell culture by Devaney *et al* (1988) and characterised biochemically in vitro by Kirchweber *et al* (1994). The cleavage sites on the

two homologues (Table 1) have been determined only for Lb^{pro} by protein sequencing of cleavage fragments from full-length eIF4GI (Kirchweiger et al., 1994) and a fragment of eIF4GII (Gradi et al., 2004). Cleavage of the homologues occurs simultaneously within FMDV infected cells and prevents translation of host mRNAs (Gradi et al., 2004).

iii Interference with cell signalling

A mutant FMD virus from which the L^{pro} coding sequence (termed leaderless virus) (Piccone et al., 1995) had been synthetically removed showed that L^{pro} is a viral virulence factor. The leaderless virus, following aerosol infection of cattle, failed to spread beyond the site of infection and was thus attenuated (Brown et al., 1996). This was also the case for infection with swine. In cell culture, infection of IFN-competent cells with leaderless virus led to reduced virus yield and the absence of plaques that are normally observed with wild-type virus (Chinsangaram et al., 1999). These observations correlated with higher levels of IFN α / β in the cell culture supernatants and suggested that the insensitivity of the wild-type virus to IFN was due to the ability of Lb^{pro} to shutdown the host cell protein synthesis (Fig. 1C; (de Los Santos et al., 2006)). Additionally, work with the leaderless virus showed that L^{pro} can also inhibit the synthesis of IFN- β at the transcriptional level (de Los Santos et al., 2007).

In addition, Lb^{pro} has been reported to act as a deubiquitinase removing both K48- and K63-linked chains and thus prevent induction of interferon stimulated genes (ISGs) by the ubiquitin pathway (Wang et al., 2011) as well as at the transcriptional and translational levels as reported by Grubman and co-workers (de los Santos et al., 2009). This deubiquitinase activity was unexpected as comparisons with certain coronavirus proteinases indicated that Lb^{pro} would not possess such an activity owing to differences in the P2 specificity. The most C-terminal residues of ubiquitin are LeuXGlyGly, with deubiquitination occurring at the isopeptide bond of the final glycine. Thus, enzymes that can cleave at this isopeptide bond such as certain proteinases of coronaviruses generally prefer small residues at the P2 site whereas FMDV Lb^{pro} has a specificity for leucine and valine at this position (Sulea et al., 2006).

iv Other reactions

Recent work (Pineiro et al., 2012) has identified the proteins Gemin5 and the Fas-ligand Daxx as substrates of Lb^{pro} that are cleaved in FMDV infected cells. However, the exact role of these cleavages in the replication cycle of FMDV is at present unclear. It has been proposed that Gemin5 may downregulate FMDV proteins synthesis, so that Gemin5 cleavage may release this inhibition. Daxx has been proposed to play a role in the innate immune antiviral response, so that cleavage of this protein could hinder the host response (Pineiro et al., 2012). However, these ideas are at present only suppositions.

Three-dimensional structure of Lb^{pro} and sLb^{pro}

The three-dimensional structures of both Lb^{pro} and sLb^{pro} have been determined to atomic resolution using X-ray crystallography (Guarné et al., 2000; Skern et al., 1998) and the behaviour in solution of both these proteins has been investigated by NMR (Cencic et al.,

2007; Steinberger et al., 2013). The structure of the Lb^{pro} and that of the cellular prototype papain is shown in Fig. 2A. Clearly, the enzymes possess the same overall fold. Two main differences are clearly visible. First, the Lb^{pro} has a much simpler structure, lacking many of the loops found in papain, including the pro-peptide binding loop (PBL). Secondly, Lb^{pro} possesses a C-terminal extension (CTE) of 18 amino acids that is absent from papain and almost all other papain-like enzymes (Guarné et al., 1998). Given its uniqueness, it is not surprising that the CTE plays important roles in the function of Lb^{pro}. These are discussed in detail below.

The Lb^{pro} active site has an imidazole-thiolate (C51-H148) ion pair and thus resembles that of papain (C25-H159) (Guarné et al., 2000) (Fig 2B). However, the Lb^{pro} residue that stabilises the ion pair is an aspartate residue (D163) instead of the asparagine residue (N175) found in almost all papain-like cysteine proteinases (Berti and Storer, 1995). Furthermore, the loop containing the stabilising aspartate contains three further acidic residues (D164, E165 and E166). However, in Lb^{pro}, there is no residue corresponding to the tryptophan residue W177 found in most papain-like enzymes (Fig 2B; (Berti and Storer, 1995)). Consequently, the active site of the Lb^{pro} is open to solvent and Lb^{pro} activity is very sensitive to the mono- and divalent cations (Guarné et al., 2000). The implications of these unique features for Lb^{pro} activity in the infected cell are not known.

Mechanisms ensuring specific L^{pro} cleavage on a protein

Cleavage sites for Lb^{pro} on the polyprotein and the two eIF4G homologues have been characterised by protein sequencing; those from the IRES binding protein Gemin5 and the Fas-ligand Daxx have been characterised by mutagenesis (Table 1). A cursory glance at the cleavage sequences reveals little conservation between the sequences. Indeed, it is surprising given the variation in the cleavage sites that more substrates have not been discovered. However, a number of mechanisms ensure that L^{pro} remains a specific proteinase despite being able to recognise a variety of cleavage sites. The first is that the Lb^{pro} can cleave before or after a basic residue, provided that the other residue next to the scissile bond is glycine or serine, as seen in three of the five known cleavage sites (Table 1). Replacement of the P1' glycine residue with a basic residue virtually eliminated cleavage by Lb^{pro} both in a self-processing system and a peptide assay. The proposed cleavage site on the Daxx protein does not fit into this scheme; however, as exact site of cleavage has not been determined for this protein, it remains to be seen whether this site genuinely represents an exception (Pineiro et al., 2012).

A second source of specificity is at P2, with the L^{pro} accepting only leucine, valine or alanine; phenylalanine and proline are not accepted at this position (Kuehnelt et al., 2004). The third source of specificity is the high number of subsites on the enzyme for binding substrates. Thus, analysis of the crystal structure of the Lb^{pro} showed the CTE of one molecule lying in the substrate binding site of the neighbouring one. The final seven residues of the CTE interacted with the substrate binding site, suggesting specific subsites from P1 to P7. This observation was confirmed and extended by cleavage of peptide substrates that indicated the presence of subsites from P7 to P5'. Thus, although being smaller than cellular

papain-like cysteine proteinases, Lb^{pro} has appreciably more subsites for substrate binding than papain or the cathepsins (Nogueira Santos et al., 2012; Santos et al., 2009).

Mechanisms to ensure specific cleavage and/or increase the efficiency of the reaction have been identified for Lb^{pro} in both intramolecular and intermolecular self-processing as well as for the eIF4G homologues. For intramolecular self-processing, the 18 residue CTE (residues 183 to 201; Fig. 3) has to reach back into the substrate binding site of its own globular domain. Residues 183 to 188 are on the opposite face of the molecule to the substrate binding site, with L188 being the last residue to interact with the globular domain. Residues 189 to 194 connect this part of the CTE to the terminal seven residues (195 to 201) that interact with the substrate binding site, as mentioned above. Mutational analysis of the C-terminal residues N189, G190 and E191 has shown a profoundly detrimental effect on intramolecular self-processing without affecting eIF4G cleavage (Glatz, Schultes, O'Niell and Skern, unpublished; Fig. 3). This indicates that occupancy of these residues that appear to correctly orient the CTE is also critical for accurate self-cleavage and can therefore also be considered as a specificity determinant.

Given that L^{pro} has to carry out intermolecular cleavages in the infected cell, it is important that the CTE is released following cleavage. NMR studies of sLb^{pro} have shown that the truncated CTE is indeed flexible (Steinberger et al., 2013). However, NMR studies of the Lb^{pro} showed the presence of a dimer formed by the interaction of the CTE of one molecule with the substrate binding domain of the second one and vice versa (Cencic et al., 2007). The K_D for dimer formation is in the millimolar range indicating that the dimer is probably not present in the infected cell. To examine the behaviour of the full-length CTE on a monomeric Lb^{pro}, it was therefore necessary to introduce the mutation L200F (Kuehnelt et al., 2004). This prevented dimer formation whilst allowing the CTE to interact with the globular domain, as measured by NMR. Again, the CTE of Lb^{pro}L200F was flexible. In addition, the loss of signals in the substrate binding site compared to those observed with the corresponding residues in sLb^{pro} indicate that the modified CTE was moving in and out of the substrate binding site in the milli-second time range (Steinberger et al., 2013).

Intermolecular self-processing can be observed *in vitro* when a polypeptide fragment containing an inactive Lb^{pro} (i.e. one that is incapable of self-processing) linked to the following VP4 and VP2 proteins is incubated with an active Lb^{pro}. Cleavage is at the same position as in intramolecular self-processing. However, the kinetics between the two reactions are quite different (Glaser et al., 2001). Thus, the kinetics of the intramolecular reactions appear to be zero-order (ie concentration-independent) whereas those of the intermolecular reaction appear to be first-order (Glaser et al., 2001). In addition, evidence has been presented that the sLb^{pro}, lacking 6 amino acids at the C-terminus, behaves differently in intermolecular processing when compared to the wild-type. Thus, Lb^{pro} and sLb^{pro} cleaved a fragment bearing the wild-type junction between Lb^{pro} and VP4 at essentially the same rate. However, the sLb^{pro} was incapable of cleaving a fragment that contained nine residues of the eIF4GI cleavage sequence in place of the wild-type junction (Cencic et al., 2007). This was interpreted to mean that the presence of at least one intact Lb^{pro} CTE was required for optimal intermolecular cleavage. Whatever the mechanism

behind this, it is rare that sequences 40 Å from the active site can influence the efficiency of the cleavage reaction.

For the efficient cleavage of the eIF4G homologues, Lb^{Pro} has evolved an exosite that enables binding to the eIF4G homologues approximately 20 to 30 amino acids N-terminal from the cleavage site (Fig. 3). Evidence for this comes from several directions. First, both peptides corresponding to the cleavage sites on the eIF4G homologues are poor substrates for Lb^{Pro}. Second, deletion of the 18 amino acid CTE or replacement with another irrelevant sequence leads to a reduction in the rate of cleavage of the eIF4G homologues. Third, specific mutations in part of the CTE (e.g. residues 184 to 188) or residue C133, which is adjacent to the CTE, severely affect the binding to and cleavage of the eIF4G homologues by Lb^{Pro}. In contrast, these mutations did not affect intramolecular self-processing.

The exact mechanism leading to the inhibition of the activation of NF-κB has not been elucidated. The degradation of the p65/RelA subunit was demonstrated, although defined cleavage products were not observed (de Los Santos et al., 2007). Subsequent work showed that the destruction of this NF-κB subunit required catalytic activity of L^{Pro} and that this reaction could be prevented by the mutation of both residues I83 and L86 to alanine (de los Santos et al., 2009). These mutations were also shown to prevent localisation of the L^{Pro} to the nucleus. However, the ability of the enzyme to carry out self-processing or cleavage of eIF4G homologues was not affected (de los Santos et al., 2009). The most parsimonious explanation of these observations is that the region of L^{Pro} containing residues I83 and L86 (Fig. 3) is required for binding to either the p65/RelA subunit itself or to a proteinase that then degrades this subunit. Evidence to support this hypothesis has however not yet been obtained (de los Santos et al., 2009).

Concluding remarks

Viruses of the aphthovirus and erbovirus genera are unique amongst the picornaviruses in encoding a leader proteinase. This specific proteinase, comprising just 173 amino acids in the FMDV Lb^{Pro} form, has a structure related to papain. It can perform both intra- and intermolecular cleavage at its own C-terminus and efficiently cleave eIF4G to prevent host translation of proteins required for the innate immune reaction. In addition, in at present undefined ways, L^{Pro} appears to disrupt signalling pathways involved in host defences by initiating the degradation of the vital transcription factor NF-κB and by deubiquitinating messenger proteins using residues distinct from those involved in eIF4G binding.

Future work will undoubtedly provide us with more surprises on the gamut of reactions performed by this remarkable little proteinase.

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Figure 1. Schematic drawing of FMDV L^{pro} self-processing and eIF4G cleavage

A. The FMDV RNA genome is shown as a black line, the single open reading frame as a box with the names of the mature proteins. The IRES, required for initiation of protein synthesis on the RNA genome, is in grey and the VPg, a covalently linked relic from the synthesis of the RNA, is in yellow. B. Synthesis of the polyprotein from the FMDV genome showing that Lb^{pro} can either be freed by an intramolecular or intermolecular reaction. C. The effect of eIF4G cleavage by Lb^{pro}. The cellular mRNA is shown as a black line with the cap structure as a filled circle. Lb^{pro} is in red, the 40S ribosomal subunit in dark blue, the polyA-binding protein (PABP) in light blue, eIF4G in purple, eIF4E in grey green, eIF4A in

dark green and eIF3 in light green. Following cleavage of eIF4G by Lb^{pro}, the capped mRNA is no longer connected to the 40S subunit and cannot be translated. In contrast, the viral RNA can bind to the C-terminal fragment of eIF4G and thus to the 40S subunit via eIF3.

Figure 2. Comparison of the structures of Lb^{pro} and papain

A. The overall structures of the two proteinases are shown, with the α -helices in green and β -strands in cyan. The active site residues are shown as sticks. The CTE of Lb^{pro} and the PBL of papain are shown in purple and are labelled. B. The active sites of Lb^{pro} and papain are shown. Residues mentioned in the text are labelled. α -helices and β -strands are coloured as in A.

Figure 3. Structure/function relationships in Lb^{pro}

Residues defined as playing a role in the activities of Lb^{pro} are shown as sticks and labelled with the appropriate function. A. Standard view of papain-like enzymes. B. View in A rotated 90° around the y-axis.

Table 1Cleavage sites recognised by L^{pro}.

Polyprotein	KVQRKLK ²⁰¹	↓	GAGQSS
eIF4GI	PSFANLG ⁶⁷⁴	↓	RTTLST
eIF4GII	VPLLNVG ⁷⁰⁰	↓	SRRSQP
Gemin5	IKKRKAR ⁸⁴⁶	↓	SLLPLS
Daxx	VLARRLR ³⁶⁰	↓	ENRSLA

Cleavage sites for the polyprotein and the eIF4G homologues have been determined by N-terminal sequencing of the cleavage products (Gradi et al., 2004; Kirchweiger et al., 1994; Strebel and Beck, 1986). Those for Gemin5 and Daxx were determined by mutagenesis (Pineiro et al., 2012).