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# Foot-and-mouth disease virus leader proteinase: Structural insights into the mechanism of intermolecular cleavage

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ABSTRACT

Translation of foot-and-mouth disease virus RNA initiates at one of two start codons leading to the synthesis of two forms of leader proteinase $L^{pro}$ ($Lab^{pro}$ and $Lb^{pro}$). These forms free themselves from the viral polyprotein by intra- and intermolecular self-processing and subsequently cleave the cellular eukaryotic initiation factor (eIF) 4G. During infection, $Lb^{pro}$ removes six residues from its own C-terminus, generating $sLb^{pro}$. We present the structure of $sLb^{pro}$ bound to the inhibitor E64-R-P-$NH_2$, illustrating how $sLb^{pro}$ can cleave between Lys/Gly and Gly/Arg pairs. In intermolecular cleavage on polyprotein substrates, $Lb^{pro}$ was unaffected by P1 or P1′ substitutions and processed a substrate containing nine eIF4GI cleavage site residues whereas $sLb^{pro}$ failed to cleave the eIF4GI containing substrate and cleaved appreciably more slowly on mutated substrates. Introduction of 70 eIF4GI residues bearing the $Lb^{pro}$ binding site restored cleavage. These data imply that $Lb^{pro}$ and $sLb^{pro}$ may have different functions in infected cells.
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## Introduction

Virally encoded proteinases play essential roles not only in the processing of the viral proteins but also in cleavage of host cell proteins in order to manipulate cellular processes to the advantage of the virus. One of the first such reactions to be documented was the modification of cellular translation factors during picornaviral replication leading to the shut-off of protein synthesis from capped cellular mRNA (Etchison et al., 1982; Leibowitz and Penman, 1971). This reaction was subsequently shown to be performed by the 2A proteinase ($2A^{pro}$) in enteroviruses (Kräusslich et al., 1987), a chymotrypsin-like cysteine proteinase (Petersen et al., 1999), whereas in aphthoviruses, the proteolysis is performed by the leader proteinase ($L^{pro}$, illustrated in Fig. 1) (Devaney et al., 1988), a papain-like cysteine proteinase (Guarné et al., 1998). The targets of both proteinases are the two homologues of the host protein eukaryotic initiation factor (eIF) 4G (Gingras et al., 1999). Cleavage of the eIF4G homologues prevents recruitment of capped mRNAs to the ribosome (Lamphear et al., 1995) whereas viral RNA can still be translated under these conditions as it initiates via an internal ribosome entry segment (IRES) (Martinez-Salas and Ryan, 2010). In addition, $L^{pro}$ has been shown to be involved in impairing the host innate immune defence by influencing NF-κB activation and to have deubiquitinase activity (de Los Santos et al., 2007, 2009; Skern and Steinberger, 2014).

Given these involvements in such different reactions as intramolecular and intermolecular self-processing, eIF4G cleavage and deubiquitination, it is not surprising that $L^{pro}$ has unusual specificity determinants. These are well illustrated by the sequences of the three $L^{pro}$ cleavage sites that have been determined directly by protein sequencing: KVQRKLK*GAGQSS for both intra- and intermolecular cleavage on the viral polyprotein between the C-terminus of $L^{pro}$ and VP4 (Strebel and Beck, 1986), PSFANLG*RTTLST on eIF4GI (Kirchweger et al., 1994) and VPLLNVG*SRRSQP on eIF4GII (Gradi et al., 2004). Studies on $L^{pro}$ intramolecular self-processing and cleavage of peptide substrates have revealed that $L^{pro}$ can cleave before or after basic residues provided that the other amino acid before or after the scissile bond is glycine (Glaser et al., 2001; Nogueira Santos et al., 2012; Santos et al., 2009). However, a peptide that contained basic residues before and after the scissile bond was refractory to

*Abbreviations:* FMDV, foot-and-mouth disease virus; $Lb^{pro}$, leader proteinase; $sLb^{pro}$, shortened leader proteinase (lacking 6 C-terminal amino acids); wt, wildtype; CTE, C-terminal extension; eIF, eukaryotic initiation factor

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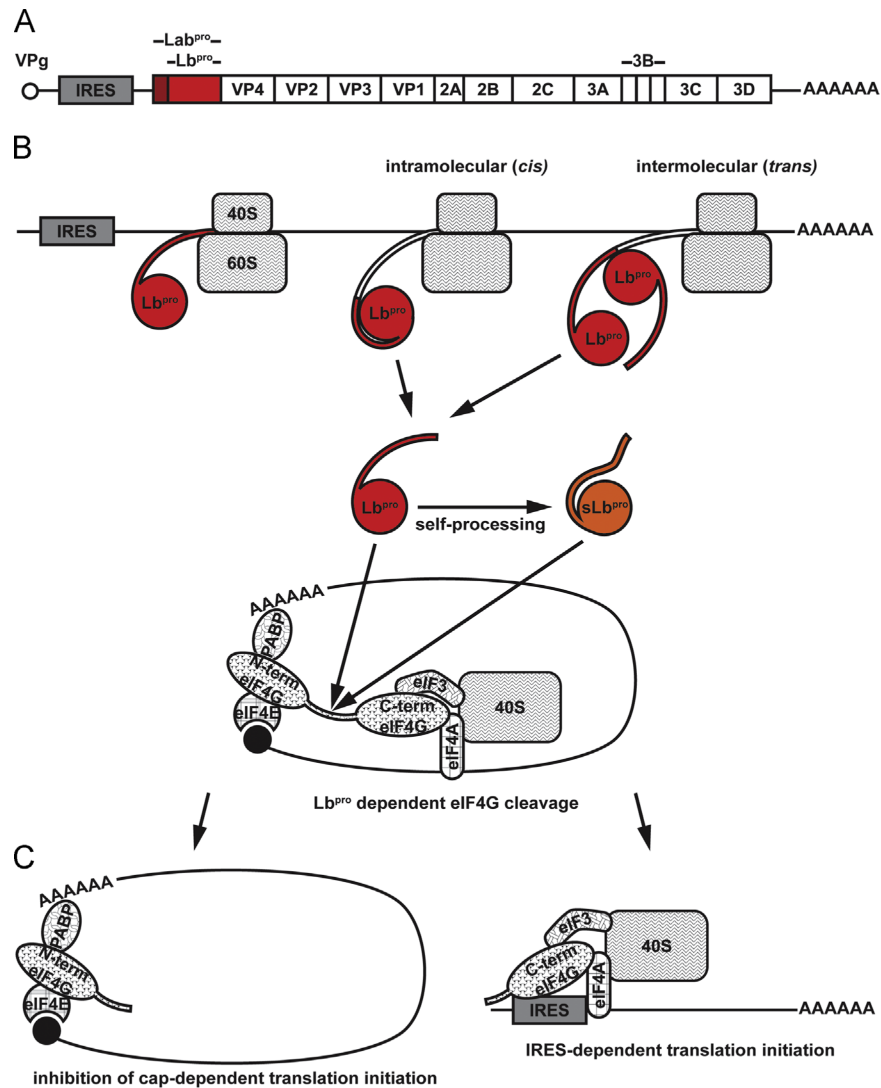


**Fig. 1.** Schematic drawing of FMDV $L^{pro}$ self-processing and eIF4G cleavage. (A) The FMDV RNA genome is shown as a black line, the single open reading frame as a box with the names of the mature proteins and the position of the IRES. $L^{pro}$, being expressed either as $Lab^{pro}$ or $Lb^{pro}$, is indicated in red. (B) Synthesis of the polyprotein from the FMDV genome showing that $Lb^{pro}$ can either be freed by an intramolecular or intermolecular reaction. $sLb^{pro}$ (shown in orange) is generated by self-processing at the C-terminus of $Lb^{pro}$. (C) The effect of eIF4G cleavage by $Lb^{pro}$ or $sLb^{pro}$. The cellular mRNA is shown as a black line with the cap structure as a filled circle. $Lb^{pro}$ and $sLb^{pro}$ are shown in red and orange, respectively. The 40S ribosomal subunit, the polyA-binding protein (PABP), eIF4G, eIF4E, eIF4A and eIF3 are shown in different shades of grey. Following cleavage of eIF4G by $Lb^{pro}$ or $sLb^{pro}$, 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.

cleavage and was subsequently shown to be an inhibitor in the micromolar range (Santos et al., 2009). This information was then used to develop a nanomolar epoxide inhibitor based on E64, termed E64-R-P-$NH_2$ (Santos et al., 2009); the structure and inhibitor parameters are shown in Fig. 2, together with those of the other inhibitors used or referred to in this work. The slow formation of the tight enzyme–inhibitor complex indicates that inhibition follows slow-binding kinetics (Santos et al., 2009; Zhou et al., 1998).

The structural basis for this unusual specificity has not been elucidated, as the present structures determined by X-ray crystallography and NMR (Cencic et al., 2007; Guarné et al., 1998, 2000; Steinberger et al., 2013) only provide information on the S binding region but not on the S′ binding region of $L^{pro}$. The nomenclature for sites (S) on the enzyme binding to residues of substrate (P) is that of Schechter and Berger (1967); prime site residues are those C-terminal to the scissile bond. Indeed, information on the nature of the S′ region from related papain-like proteinases is also sparse (Turk et al., 2012), with structural information only being available for cathepsin B (Stern et al., 2004; Turk et al., 1995; Yamamoto et al., 1997) determined with inhibitors similar to E64-R-P-$NH_2$ (Fig. 2). However, cathepsin B is also unusual in being an exopeptidase, with an occluding loop that prevents access beyond the S2′ site, that is the site on the enzyme interacting with the P2′ residue of the substrate (Stern et al., 2004). Thus, any information on the S′ binding region of FMDV $L^{pro}$ will shed light on the nature of this region in papain-like cysteine proteinases generally.

Understanding of the mechanism of $L^{pro}$ is complicated by the presence of different forms of the protein in the infected cell (Sangar et al., 1987, 1988). Two isoforms, $Lab^{pro}$ and $Lb^{pro}$ (Fig. 1), arise from the presence of two in-frame AUG codons for the initiation of protein synthesis on the viral RNA (Sangar et al., 1987). Consequently, the $Lab^{pro}$ possesses an additional 28 amino acids at the N-terminus than $Lb^{pro}$. Cao et al. (1995) demonstrated in cell culture that $Lb^{pro}$ was essential whereas $Lab^{pro}$ was not; nevertheless, there may still be as yet unknown roles for $Lab^{pro}$ during infection in the host organism. In addition, a shortened form of $Lb^{pro}$ ($sLb^{pro}$) lacking 6 or 7 amino acids at the C-terminus has long been known (Sangar et al., 1988). The truncation arises through $Lb^{pro}$ self-cleavage (Sangar et al., 1988) and

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