The leader proteinase (Lpro) of foot-and-mouth disease virus frees itself from the nascent polypeptide, cleaving between its own C terminus and the N terminus of VP4 at the sequence Lys-Leu-Lys↓-Gly-Ala-Gly. Subsequently, the Lpro impairs protein synthesis from capped mRNAs in the infected cell by processing a host protein, eukaryotic initiation factor 4GI, at the sequence Asn-Leu-Gly↓-Arg-Thr-Thr. A rabbit reticulocyte lysate system was used to examine the substrate specificity of Lpro and the relationship of the two cleavage reactions. We show that Lpro requires a basic residue at one side of the scissile bond to carry out efficient self-processing. This reaction is abrogated when leucine and lysine prior to the cleavage site are substituted by serine and glutamine, respectively. However, the cleavage of eIF4GI is unaffected by the inhibition of self-processing. Removal of the 18-amino acid C-terminal extension of Lpro slowed eIF4GI cleavage; replacement of the C-terminal extension by unrelated amino acid sequences further delayed this cleavage. Surprisingly, wild-type Lpro and the C-terminal variants all processed the polypeptide cleavage site in an intermolecular reaction at the same rate. However, when the polypeptide cleavage site was part of the same polypeptide chain as the wild-type Lbpro, the rate of processing was much more rapid. These experiments strongly suggest that self-processing is an intramolecular reaction.

Specific proteolysis is an essential component of the life cycle of many viruses. Virally encoded proteinases are required not only to process viral protein precursors into the mature proteins but also to cleave host proteins to modulate the physiology of the infected cell (1, 2). A classic example is the inhibition of host protein synthesis from capped cellular mRNA that occurs during the replication of most picornaviruses (3). These viruses mediate this through the cleavage of the two homologues of eukaryotic initiation factor 4G (eIF4G1 and eIF4GII) by either the Lpro of FMDV or by the 2A proteinase of human rhinoviruses, Coxsackie virus, and polioviruses (4–8). As a consequence of the cleavage of the eIF4G homologues, the domain of eIF4G that binds the cap-binding protein eIF4E is severed from the domain of eIF4G which binds eIF3, so that the infected cell is unable to recruit its own capped mRNA to the 40 S ribosome (Fig. 1A (9, 10)). Translation of viral mRNA is unaffected as it initiates internally via an IRES (11, 12).

The exact mechanism of the cleavage of eIF4GI and eIF4GII by the picornavriral proteinases is still unresolved. As this cleavage occurs before measurable amounts of viral proteins can be detected in vivo (13), it has been proposed that the viral proteinases induce cleavage of the eIF4G homologues by activating an as yet unidentified cellular proteinase (5, 14). This idea was supported by the inability to imitate in vitro the efficiency of eIF4GI cleavage using purified recombinant proteinases (15–17). However, we have recently shown that eIF4GI can be efficiently cleaved in RRLs by nascently translated proteinases at concentrations close to those calculated in vivo (18).

The Lpro of FMDV, which carries out the cleavage of the eIF4G homologues, is a papain-like cysteine proteinase (19, 20); it is the first protein encoded on the FMDV polyprotein (Fig. 1, B and C). Lpro frees itself by cleavage between its own C terminus and the N terminus of VP4. As the initiation of protein synthesis on the FMDV RNA can occur at one of two AUG codons lying 84 nucleotides apart, two forms of the Lpro are synthesized (designated Labpro and Lbpro). The reason for this is not clear, as both forms appear to have the same enzymatic properties (21). All work described here was carried out with the Lbpro form.

Recognition and cleavage of the sites on the polypeptide and on the eIF4G homologues present a number of questions. Cleavage at the junction of Labpro and VP4 occurs at Lys-Leu-Lys↓-Gly-Ala-Gly, whereas that on eIF4GI takes place at the sequence Asn-Leu-Gly↓-Arg-Thr-Thr (13). The sequence of Lbpro cleavage on eIF4GII has not yet been determined, but it would appear by comparison to be between Asn-Phe-Gly↓-Arg-Gln-Thr (22) at the usual specificity, the Lbpro has evolved to be able to bind basic residues at P1 or P1′, while maintaining the requirement of papain-like enzymes for a hydrophobic residue at P2 (20, 23). Nevertheless, the exact determinants of specificity are still not clear.

A further open question is the mechanism of self-processing (20, 21, 24). X-ray structural data provided evidence for an intramolecular mechanism, as the CTE of one Lbpro molecule was bound by the active site of the neighboring molecule (20). However, modeling studies using this structure suggested that a CTE would be able to reach into the active site of the same molecule, hinting that an intramolecular reaction was possible. Finally, it is not known whether self-processing is a prerequisite for cleavage of the eIF4G homologues.

In this paper, we examine these questions by expressing...
Fig. 1. The known biological activities of L\textsuperscript{prw}. The role of eIF4 proteins in the initiation of protein synthesis and the cleavage of eIF4GI by L\textsuperscript{prw} are shown in A. eIF4 proteins involved in protein synthesis and the outline of the 40S ribosomal subunit are named. The m'GDP 5' cap structure of cellular mRNAs (open circle) and the L\textsuperscript{prw} cleavage site (arrow) are indicated. The eIF4GI C-terminal domain still forms an initiation complex with IRES-containing mRNAs. The RNA genome of FMDV is shown in B. The single open reading frame is shown as an open box (with the mature viral proteins indicated), non-coding regions by a line, and the IRES as a closed box. L\textsuperscript{prw} forms are checkered. Protein synthesis on this mRNA is shown in C; L\textsuperscript{prw} self-processing is indicated as an intra- or intermolecular event. The L\textsuperscript{prw} CTE is indicated by an open box.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The plasmid pet\textit{sc}FMDV Lb\textsuperscript{prw} (encoding the mature Lb\textsuperscript{prw} that consists of amino acids 29–201 of the FMDV polyprotein) contains the FMDV nucleotides 892–1413 of the FMDV O1c cDNA (25) followed by two stop codons, cloned into the NcoI and BamHI restriction sites of the T7 polymerase expression vector pET8c (Novagen). The plasmid pet\textit{sc}FMDV Lb\textsuperscript{prw}VP4VP2 (encoding the mature Lb\textsuperscript{prw}, all 85 amino acids of VP4 and 78 amino acids of VP2) contains the FMDV nucleotides 892–1896 followed by two stop codons similarly cloned into pet\textit{sc}8c (13). In pet\textit{sc}FMDV Lb\textsuperscript{prw}VP4VP2, the active site cysteine 51 is replaced by an alanine.

The plasmid pet\textit{sc}\Delta Lb\textsuperscript{prw}VP was constructed by using PCR amplification to delete the amino acids 30–75 of Lb\textsuperscript{prw} in pet\textit{sc}FMDV Lb\textsuperscript{prw}VP4VP2 to give pet\textit{sc}FMDV Lb\textsuperscript{prw}VP4VP2. To introduce a fragment extending the number of VP2 amino acids encoded to 140, the required fragment was amplified from the original FMDV cDNA subclone p735 (25). Two stop codons followed by a BamHI site were also encoded by the 3' PCR primer. This sequence was then introduced into pet\textit{sc}FMDV Lb\textsuperscript{prw}VP4VP2 as an XbaI and BamHI fragment to give pet\textit{sc}\Delta Lb\textsuperscript{prw}VP. Following linearization with BamHI, transcription of this DNA gives an RNA encoding an inactive Lb\textsuperscript{prw} lacking 46 amino acids at the N terminus followed by all 85 amino acids of VP4 and 140 amino acids of VP2.

To construct pCITE Lb\textsuperscript{prw}VP4VP2, the Lb\textsuperscript{prw}VP4VP2 expression block was excised from pet\textit{sc}FMDV Lb\textsuperscript{prw}VP4VP2 using the XhoI site of pet\textit{sc}8c and the BamHI site at the 3' end of the expression block and subcloned into pBluescript. The Lb\textsuperscript{prw}VP4VP2 was then introduced as an NcoI PstI fragment into pCITE1 which had been cleaved by NcoI and PstI. In this construction, translation initiates at an AUG codon three codons upstream from the first Lb\textsuperscript{prw} codon. On linearization with SalI and transcription with T7 polymerase, a RNA is produced containing the encephalomyocarditis virus IRES which subsequently encodes the Lb\textsuperscript{prw} with three extra amino acids at the N terminus, all 85 amino acids of VP4 and 23 amino acids of VP2.

**In Vitro Mutagenesis**—Restriction sites for Bpu10I and SacI were introduced into pCITE Lb\textsuperscript{prw}VP4VP2 at the positions indicated in Fig. 2A using standard methods of PCR mutagenesis; the mutations do not change the amino acid sequence. Replacement of the 38-base pair Bpu10I SacI fragment with synthetic oligonucleotides enables the amino acid substitutions indicated in Table I to be introduced at the L\textsuperscript{prw} VP4 junction. Lb\textsuperscript{prw} C-terminal variants were generated in pet\textit{sc}Lb\textsuperscript{prw} by replacing the 47-base pair C-terminal BsiWI BamHI fragment with the required synthetic oligonucleotides as described previously (23). All mutations were confirmed by sequencing.

**In Vivo Transcription and Translation**—Plasmids were linearized with the indicated restriction enzyme and transcribed as described (18). In vitro translation reactions (typically 50 \(\mu\)l) contained 70% RRL (Promega), 20 \(\mu\)Ci of [\(\text{\textsuperscript{35S}}\)]methionine (1000 Ci/mmol, American Research Company), and amino acids (except methionine) at 20 \(\mu\)M. After preincubation for 2 min at 30°C, translation was started by addition of RNA. RNA concentrations (typically about 10 ng/ \(\mu\)l) were adjusted so that the Lb\textsuperscript{prw} concentration reached between 15 and 20 pg/\(\mu\)l after 8 min of incubation, unless otherwise stated. Aliquots (10 \(\mu\)l) were removed at the designated time points, and the reaction was stopped by immediate transfer to ice and the addition of unlabeled methionine and cysteine to a final concentration of 2 mM and Laemml sample buffer.

**Electrophoresis and Immunoblotting**—The PAGE system of Dasso and Jackson (26) was used for separation of translation products (gels contained 15% acrylamide) and for monitoring the state of eIF4GI (gels contained 6% acrylamide). Translation products were detected by fluorography; the state of eIF4GI was determined by immunoblotting using the anti-eIF4GI peptide 7 antiserum (27) as described (18).

**Quantification of Protein Synthesis in RRLs**—Quantification of protein synthesis using an Instant Imager (Canberra Packard) was as described previously, except that calculations were adjusted for the use of [\(\text{\textsuperscript{35S}}\)]methionine alone (18). Briefly, radioactivity in a particular band in the dried polyacrylamide gel was counted. By taking into account the counting efficiency (0.8%), the specific activity of the methionine in the assay (163 dpm/fmol), the number of methionines in Lb\textsuperscript{prw}, and the molecular weight of Lb\textsuperscript{prw} (19.8 kDa), the amount of protein present in a particular band can be estimated.

**RESULTS**

What Is the Relationship between Self-processing of Lb\textsuperscript{prw} and eIF4GI Cleavage?—To investigate whether self-processing of the Lb\textsuperscript{prw} from the growing polypeptide chain was a prerequisite for the cleavage of eIF4GI, it was necessary to produce mutant enzymes that could not process themselves from the growing polypeptide chain but that still possessed an intact active site and a correctly folded structure. Therefore, we introduced specific mutations into the cleavage site between Lb\textsuperscript{prw} and VP4VP2. To simplify this, we noted that restriction sites for Bpu10I and SacI could be introduced before and after the site of Lb\textsuperscript{prw} cleavage without affecting the amino acid sequence (Fig. 2A). However, the previously employed transcription vector pet\textit{sc}8c also contained a Bpu10I site, making it necessary to move the Lb\textsuperscript{prw}VP4VP2 expression cassette into pCITE1 which has itself no restriction sites for Bpu10I and SacI. The ensuing plasmid was designated pCITE Lb\textsuperscript{prw}VP4VP2 (Fig. 2A).

To examine Lb\textsuperscript{prw} self-processing and eIF4GI cleavage using
pcITE LbproVP4VP2, a standard translation reaction was performed (Fig. 2, B and C, left panels). The cleavage products Lbpro and VP4VP2 are visible on this fluorogram after 8 min. Lbpro encoded by pCITE LbproVP4VP2 has four methionines, whereas VP4VP2 has only two; thus, the intensity of the Lbpro band is about twice that of VP4VP2. The high efficiency of self-processing is indicated by the low amount of the uncleaved precursor (LbproVP4VP2). Table I shows the extent of self-processing at 12 min.

The cleavage of the endogenous eIF4GI in the RRL under these conditions occurs rapidly (Fig. 2C, left panel); 50% cleavage was observed between 4 and 8 min. The concentration of Lbpro at 8 min was determined to be 15 pg/μl by counting the band in the Instant Imager. These results are almost identical to those previously reported, using pet8c LbproVP4VP2 driven from an uncapped RNA that did not contain an IRES (18).

The mutations indicated in Table I were then introduced at the Lbpro VP4 cleavage site using synthetic oligonucleotides. First, the P1 lysine was replaced by glutamine (LbproVP4VP2-(K201Q)), as the Lbpro three-dimensional structure (20) had revealed that the P1 lysine residue is bound within a deep groove consisting of three glutamate residues. Their aliphatic side chains bind the lysine side chain, whereas the amino group of the lysine interacts with the carboxyl groups of the glutamates. We reasoned that the uncharged glutamine residue would lack the electrostatic interaction and that the shorter side chain would further cause the amide group to clash with the glutamate side chains. Indeed, the pattern of proteins synthesized from this RNA is different from that of the wild-type RNA (Fig. 2B, middle panel). After 12 min, self-processing drops from over 85% in the wild-type to 37.5% in the mutant protein (Table I). Thus, although the self-processing reaction of Lbpro is impaired by this mutation, it is not completely inhibited.

The activity of this mutant protein on the cleavage of endogenous eIF4GI in the RRL was then examined by immunoblotting (Fig. 2C, middle panel). In contrast to the effect on self-processing, no effect on the processing of eIF4GI was observed. Reduction of the amount of RNA in the RRL also failed to reveal any differences in the rate of eIF4GI processing between

| Table I Effect of amino acid substitutions at the Lbpro VP4 junction on self-processing |
|-----------------------------------------------|
| Substituted amino acids are shown in bold. % self-processing was determined as the amount of Lbpro as a percentage of the total amount of Lbpro and LbproVP4VP2 synthesized. |
|-----------------------------------------------|
| LbproVP4VP2 | VQRKLK | 85.5 |
| Lbpro-K201Q | VQRKLK | 37.5 |
| Lbpro-L200S,K201Q | VQRKLQ | 0 |
| Lbpro-K201G | VQRKLQ | 38 |
| Lbpro-K201G,G202R | VQRKLQ | 82.5 |
A basic residue before or after the cleavage site is essential for efficient self-processing. The indicated mRNAs (10 ng/µl; transcribed in vitro following linearization with StuI) were used to program RRLs. Analysis of protein synthesis (A) and the state of eIF4GI (B) was as in Fig. 2. Fluorographs were exposed for 15 h for Lb<sup>pro</sup>VP4VP2 and Lb<sup>pro</sup>VP4VP2 (K201G, G202R) and 20 h for Lb<sup>pro</sup>VP4VP2 (K201G) to enable self-processing at 8 min to be seen.

As the K201Q mutant protein still possessed some self-processing activity, we wished to examine whether a complete inhibition of self-processing would eliminate the mature Lb<sup>pro</sup> from the reaction and thus affect the kinetics of eIF4GI processing. Therefore, we substituted the P2 leucine with serine in addition to the P1 mutation would inhibit any self-processing activity. Fig. 2B (right panel) shows that this is indeed the case. After 12 min, only the uncleaved product was visible. Remarkably, however, no effect on the cleavage of eIF4GI was observed (Fig. 2C, right panel); the kinetics of cleavage are almost identical to those of the wild-type enzyme.

In summary, these results support the predictions of the three-dimensional structure about the substrate specificity of Lb<sup>pro</sup> and demonstrate clearly that Lb<sup>pro</sup> which is still covalently connected to the capsid proteins can efficiently cleave eIF4GI. Thus, self-processing of the Lb<sup>pro</sup> is not a prerequisite for the efficient cleavage of eIF4GI.

Role of the C Terminus on the Activity of the Lb<sup>pro</sup>—The above experiments confirmed the high efficiency of eIF4GI cleavage in RRLs by Lb<sup>pro</sup>, regardless of whether self-processing had occurred or not. What are the features of the Lb<sup>pro</sup> that enable it to cleave eIF4GI so efficiently? One unique feature of Lb<sup>pro</sup>, which is not found in papain, is the presence of the 18-amino acid CTE in Lb<sup>pro</sup>. We wished to examine whether this feature was involved in some way in eIF4GI cleavage.

To investigate this, we used a second construction pet8cLb<sup>pro</sup> that contains a synthetic stop codon after the Lys<sup>201</sup> residue, so that a mature Lb<sup>pro</sup> is expressed without the need for self-processing (Fig. 4A). The kinetics of eIF4GI cleavage are the same as those with pet8c Lb<sup>pro</sup>VP4VP2 (18). C-terminal deletions were introduced into pet8cLb<sup>pro</sup> by replacing the wild-type BsiWI BamHI fragment with synthetic oligonucleotides. Initially, two deletions in Lb<sup>pro</sup> were made; the first (designated Lb<sup>pro</sup>-6; Table II) lacked the six most C-terminal amino acids, i.e., just those which had been found in the active site of the neighboring molecule in the crystal structure. In the second (Lb<sup>pro</sup>-18), the entire 18 amino acids of the CTE were removed. RNAs were prepared from both deletion mutants and were used to drive protein synthesis in RRLs; synthesis of Lb<sup>pro</sup> (Fig. 4B) and cleavage of eIF4GI (Fig. 4C) were analyzed as before. The mutant protein Lb<sup>pro</sup>-6 cleaved eIF4GI with kinetics sim-
ilar to that of the wild-type enzyme (Fig. 4, B and C, left and middle panels); however, the mutant protein Lbpro-18 had a reduced ability to cleave eIF4GI, cleavage was still incomplete after 12 min (Fig. 4, B and C, left and right panels). This was despite the fact that about twice as much Lb pro-18 had been synthesized than in the wild-type reaction.

Fig. 4 shows that the deletion Lbpro-6 migrates more slowly than the wild-type Lbpro. This is reminiscent of an observation of Sangar et al. (28) who demonstrated that prolonged incubation (i.e., longer than 1 h) of Lbpro in RRLs led to a modification of the protein which caused it to migrate more slowly on SDS-PAGE. This modified form appeared to be deleted at its C terminus because it could also be generated by carboxypeptidase A digestion (28). Thus, removal of a small number of amino acids decreases the mobility of Lbpro on SDS-PAGE. We believe that the missing 6 amino acids of the Lbpro-6 deletion are responsible for the reduced mobility of this protein.

The above experiment suggested that the presence of the CTE was required for full enzymatic activity. This notion was further strengthened by a serendipitous observation made during the synthesis of the above deletion mutants. A variant plasmid was obtained in which an incorrect, truncated oligonucleotide was inserted between the BsiWI site and the BamHI site at the 3’ end of the Lbpro (Fig. 4A and Table II). As a consequence, the reading frame is shifted from amino acid 183 onwards and there are only 8 amino acids before the BamHI site. Thus, translation of an RNA transcribed from a template linearized with BamHI produces a variant encoding a C-terminal extension of 8 amino acids, all differing from the wild-type (Table II). This variant was designated Lbpro-11*, as it lacks 11 amino acids compared with the wild-type Lbpro; the asterisk indicates the aberrant amino acid sequence of the CTE. In addition, RNA encoding a second variant was produced by linearizing the same plasmid with HindIII, which cleaves 481 nucleotides downstream of the BamHI site; due to the position of the first stop codon encountered, the CTE in this case has 28 amino acids, 9 longer than the wild-type. Once again, they are of different sequence (Table II), so that the variant was designated Lbpro-9*. Although the mutant sequences have a relatively high alanine content and are somewhat hydrophobic, they are not otherwise unusual.

Nevertheless, examination of these variants in the RRL system (Fig. 5) showed that both Lbpro-11* and Lbpro-9* had an impaired ability to cleave eIF4GI. With the Lbpro-11* variant, about 90% eIF4GI cleavage is observed after 30 min of incubation, even though after 4 and 8 min similar protein concentrations to those of the wild-type experiment shown in Fig. 4B are present. Processing with the variant Lbpro-9* (Fig. 5, right panels) was reduced even more dramatically, as 50% eIF4GI cleavage did not occur until 30 min after cleavage, even though the RNA concentration had been adjusted to ensure that twice the amount of protein was synthesized in this experiment compared with the wild-type Lbpro in Fig. 4.

The Lbpro C-terminal Variants Are Not Affected in Their Ability to Process a Polyprotein Substrate—In the above exper-

![Fig. 4. Effect of C-terminal deletions in the Lbpro on the processing of eIF4GI. The structure of the expression block Lbpro is shown in A. Restriction enzyme sites mentioned in the text are indicated. The indicated mRNAs (5 ng/μl for Lbpro and 10 ng/μl for the deletion mutants; transcribed in vitro following linearization with BamHI) were used to program RRLs. Analysis of protein synthesis (B) and the state of eIF4GI (C) are as in Fig. 2. All fluorograms were exposed for 15 h.](image)
ments, the activity of the Lbpro C-terminal variants was monitored only on the endogenous eIF4GI present in the RRL extract. We wished to investigate whether these variants were also impaired in their ability to recognize the polyprotein cleavage site. To generate a suitable substrate, the construction ∆LbproVP was prepared (Fig. 6A). Following linearization with BamHI, transcription generates an RNA encoding an inactive Lbpro with a 46-amino acid deletion, all 85 amino acids of VP4, and 140 amino acids of VP2. The deletion in the Lbpro part and the extension of the VP2 part make the cleavage products of LbproVP4VP2 and ∆LbproVP distinguishable from each other on PAGE.

The protein produced in RRL from the pet8c∆LbproVP construction is of apparent molecular mass of 46 kDa (Fig. 6B, far right lane) and is proteolytically inactive (Fig. 6B, C, far right lanes). The ability of ∆LbproVP to serve as substrate was examined by simultaneously translating its RNA with the pet8cLbpro RNA (Fig. 6B, left panel). Initially, after 8 min of incubation, two products of translation were observed, the mature Lbpro and the uncleaved substrate ∆LbproVP (Fig. 6B, left panel). Subsequently, a third product of apparent molecular mass of 34 kDa becomes visible, representing the VP part of the substrate following cleavage at the junction between ∆Lbpro and VP. 50% cleavage of ∆LbproVP is achieved between 15 and 30 min. Eventually, all ∆LbproVP is converted to product, as sufficient proteinase is presumably present to cleave rapidly any newly synthesized substrate so that it is no longer observed. The ΔLbpro part of the substrate has a molecular mass of 14.2 kDa; despite this, however, it is not resolved by this gel system.

Cleavage of eIF4GI also takes place under these conditions. Examination of the fate of the eIF4GI in the RRLs shows that over 50% is cleaved using Lbpro before 8 min (Fig. 6C, left panel; Table III); this is comparable with the experiments in which only one RNA was translated. The Lbpro concentration was estimated to be about 20 pg/μl by counting the band. This agrees with previous observations in which 50% cleavage of eIF4GI was achieved when the Lbpro concentration had reached 15 pg/μl (18).

This experiment was then repeated using mRNAs encoding Lbpro − 18 and Lbpro + 9*. The kinetics of cleavage of ∆LbproVP by these two variants are very similar to those of the wild type (Fig. 6B, compare the middle and right panel with the left one; Table III), indicating that the absence of the CTE or the presence of an aberrant one do not affect the ability of the variants to process the polyprotein substrate in an intermolecular reaction. In contrast, cleavage of eIF4GI by the C-terminal variants was once again delayed (Fig. 6C, middle and right panels; Table III). For the variant lacking the CTE, Lbpro − 18, 50% cleavage was achieved between 8 and 15 min at a proteinase concentration between 20 and 52 pg/μl; with the variant with the aberrant C terminus, Lbpro + 9*, 50% cleavage was obtained after about 45 min at a concentration of 75 pg/μl.

Comparison of Intra- and Intermolecular Lbpro Cleavage—In the experiment shown in Fig. 6, Lbpro can only be cleaving ∆LbproVP and eIF4GI intermolecularly. It has been a longstanding question, however, as to whether the initial cleavage of the Lbpro from the growing polyprotein (i.e. from LbproVP4VP2) is also an intra- or an intermolecular event (20, 21, 24). To investigate this, we translated the ∆LbproVP RNA together with that of LbproVP4VP2 to see whether the cleavage kinetics of ∆LbproVP or eIF4GI were changed (Fig. 7). This can be achieved because, as stated above, all the cleavage products from LbproVP4VP2 and ∆LbproVP can be separated from one another by SDS-PAGE.

The result is shown in Fig. 7A. Four products are visible during the time course. These are the uncleaved ΔLbproVP (46 kDa) and the VP cleavage products (34 kDa) (the 14.2-kDa ΔLbpro cleavage product is once again not resolved) as well as the mature Lbpro (19.8 kDa) and the VP4VP2 (15 kDa) cleavage product. However, the uncleaved protein LbproVP4VP2 is not a major product of the reaction; the uncleaved precursor is essentially only visible with the inactive leader proteinase mutant Lbpro/C51A VP4VP2 (Fig. 7A, far right lane). Therefore, the processing of the Lbpro from the growing LbproVP4VP2 polypeptide chain must be extremely efficient, strongly implying that this reaction is an intramolecular one.

The processing of the endogenous eIF4GI by Lbpro expressed as LbproVP4VP2 is comparable with that observed when the Lbpro is translated as a mature protein (compare Fig. 6C with Fig. 7B). The cleavage of ΔLbproVP with Lbpro expressed from LbproVP4VP2 is also comparable to that with Lbpro (compare Fig. 6B with Fig. 7A) and is therefore not delayed by self-processing. Furthermore, the self-processing of LbproVP4VP2...
the cleavage site at the Lb pro VP4 junction and to produce of the cellular substrate eIF4GI. By using mutagenesis to vary virus examined are those that are cleaved during replication of the could be ascertained. As the polyprotein and eIF4GI substrates C-terminal variants, factors affecting the rate of both reactions posited at the Lbpro VP4 junction. In summary, the reactions in order of efficiency catalyzed by the Lbpro are the intramolecular processing at the Lb pro VP4 junction and the cleavage of eIF4GI as part of the eIF4F complex followed by the much less efficient intermolecular cleavage at the Lbpro VP4 junction.

**DISCUSSION**

The experiments described here examine the relationship of the self-processing reaction of the FMDV Lbpro to the cleavage of the cellular substrate eIF4GI. By using mutagenesis to vary the cleavage site at the Lbpro VP4 junction and to produce C-terminal variants, factors affecting the rate of both reactions could be ascertained. As the polyprotein and eIF4GI substrates examined are those that are cleaved during replication of the virus in vivo, these findings are directly relevant to the biological activity of the Lbpro.

We began by investigating whether the inhibition of self-processing affected the rate of eIF4GI cleavage. For this, we mutated the Lbpro to prevent self-processing without affecting either the catalytic residues or the structure of the enzyme. Initially, mutations were introduced at the P1 position (Table 1). The substitution of the P1 lysine with either glycine or glutamine impaired self-processing but did not inhibit it completely. Replacement of the P1' glycine with arginine (thus mimicking the P1' residue in the eIF4GI cleavage site) in the mutant protein containing glycine at P1 restored self-processing to wild-type levels, thus confirming the unusual requirement of this enzyme for a basic residue at either the P1 or P1' positions.

Complete inhibition of the self-processing reaction was obtained by the introduction of a second mutation, namely leucine to serine at P2, in the P1 lysine to glutamine mutant protein. This stresses the importance of the presence of a hydrophobic residue at P2, in the P1 lysine to glutamine mutant protein. This implies that the enzyme is active as part of the growing polypeptide chain and that it may be active while the polypeptide chain is still bound to the ribosome.

Why is the cleavage of eIF4GI so efficient, despite the lack of self-processing? To begin, it can be assumed that the substrate eIF4GI is in the optimal conformation, as the binding of eIF4E to eIF4GI stimulates Lbpro cleavage of eIF4GI (31), and most of the eIF4GI in RRLs is complexed to eIF4E (29). For the enzyme, the C-terminal deletion experiments indicate a role for the C terminus. Thus, the mutant protein lacking the entire CTE cleaved eIF4GI at a significantly reduced rate; however, a much more drastic reduction in the reaction rate could be seen with the variants possessing an aberrant CTE. Nevertheless, all C-terminal variants recognized the Lbpro VP4 cleavage site in the intermolecular cleavage at the same rate. Thus, the CTE is required for efficient intermolecular cleavage of eIF4GI but not for that on the polyprotein substrate.

Three explanations for this observation appear plausible. First, the C terminus may be involved in a direct interaction with eIF4GI, perhaps fitting specifically into a pocket, or even eIF4E; removal of the CTE would prevent this interaction.
However, it is not clear from this theory why the presence of aberrant CTEs would have a further effect on eIF4GI processing than the variants lacking a CTE. Second, the CTE could stabilize the Lbpro in a conformation that favors eIF4GI cleavage but that is not required for intramolecular polyprotein processing. The lack of the CTE would obviate the stabilizing effect; however, the presence of the aberrant CTE might destabilize this conformation or even prevent it from being adopted.

The third possibility would require that binding of the Lbpro CTE to eIF4GI induces a conformational change in this protein that exposes the cleavage site. In this case, proteolysis would be the rate-limiting step; in the CTE deletion mutants, the rate-limiting step would be the exposure of the active site in the absence of the CTE.

It is interesting to note that, compared with papain, the Lbpro achieves its active conformation without a pro-domain and without activation at low pH (32). Indeed the experiments with the CTE variants appear to suggest a much more important role for the C terminus, a region of Lbpro that has no equivalent in papain (20).

Finally, cleavage reactions of the Lbpro on intra- and intermolecular polyprotein substrates were examined simultaneously. Thus, RNAs were translated so that the rate at which the substrate when part of the same chain was much more rapid, suggesting that this reaction is an intramolecular one, with the C terminus of Lbpro folding into the active site of the same molecule, as proposed by modeling studies based on the crystal structure (20). This also implies that the ΔLbproVP substrate adopts a conformation competent for the intramolecular reaction and must unfold so that it can be processed in trans.

In summary, we have shown that self-processing is not a prerequisite for cleavage of eIF4GI by Lbpro and have established the hierarchy of the Lbpro cleavage reactions. The first two are the extremely efficient intramolecular self-processing and the intermolecular cleavage of eIF4GI followed by the much less efficient intermolecular cleavage of the polyprotein sequence.

Acknowledgments—We thank Dieter Blaas and Joachim Seipel for critical reading of the manuscript.

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