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Foot-and-Mouth Disease Virus Lb Proteinase Can Stimulate Rhinovirus and Enterovirus IRES-Driven Translation and Cleave Several Proteins of Cellular and Viral Origin

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Rhinovirus and enterovirus 2A proteinases stimulate translation initiation driven from the cognate internal ribosome entry segment (IRES) (S. J. Hambidge and P. Sarnow, Proc. Natl. Acad. Sci. USA 89:10272–10276, 1992; H.-D. Liebig, E. Ziegler, R. Yan, K. Hartmuth, H. Klump, H. Kowalski, D. Blaas, W. Sommergruber, L. Frasel, B. Lamphear, R. Rhoads, E. Kuechler, and T. Skern, Biochemistry 32:7581–7588, 1993). Given the functional similarities between the foot-and-mouth disease virus (FMDV) L proteinase and these 2A proteinases (autocatalytic excision from the nascent viral polyprotein and cleavage of eIF-4γ), we investigated whether the FMDV L proteinase would also be able to stimulate translation initiation. We found that purified recombinant FMDV Lb proteinase could stimulate in vitro translation driven from a rhinovirus or enterovirus IRES by 5- to 10-fold. In contrast, stimulation of translation initiation on a cardiovirus IRES by this proteinase was minimal, and stimulation of translation driven from the cognate FMDV IRES could not be evidenced. Studies using an inhibitor or a mutant Lb proteinase indicated that stimulation of IRES-driven translation is mediated via proteolysis of some cellular component(s). Our studies also demonstrated that the Lb proteinase is capable of stimulating initiation of translation on an uncapped cellular message. Unexpectedly, and in contrast to the 2A proteinases, the Lb proteinase specifically cleaved the products of the two reporter genes used in this study: *Xenopus laevis* cyclin B2 and influenza virus NS. Therefore, we also set out to investigate the requirements for substrate recognition by the Lb proteinase. Purified recombinant Lb proteinase recognized at least one mengovirus polypeptide and specifically cleaved human cyclin A and poliovirus replicase-related polypeptides. In the latter case, the site(s) of cleavage was located within the N-terminal part of polypeptide 3D. Sequence comparisons revealed no significant primary sequence similarities between the target proteins and the two sites already known to be recognized by the FMDV L proteinase.

Infection by picornaviruses leads to a reduction in host-cell macromolecular synthesis (22, 38). In the case of enteroviruses, rhinoviruses, and aphthoviruses, the inhibition of host-cell translation is well documented (39). Current evidence indicates that this inhibition is due at least in part to proteolytic cleavage of eIF-4γ (a component of the cap-binding complex eIF-4), which leads to a reduced capacity of the host cell to translate capped mRNAs (14, 15, 29). Cleavage of eIF-4γ is mediated directly by the viral 2A proteinases of rhinoviruses and enteroviruses and by the L proteinase of aphthoviruses (24, 28). The 2A and L proteinases fulfill similar roles in the respective virus life cycles: in addition to cleaving eIF-4γ in the same region, both are required for their own autocatalytic excision from the viral polyprotein.

However, although in all cases cysteine is the active-site nucleophile, the rhinovirus and enterovirus 2A proteinases are mechanistically very different from the aphthovirus L proteinase. The 2A proteinases show little overall amino acid sequence similarity to any other cysteine proteinase. In fact, they are homologous to the small bacterial trypsin-like serine proteinases (1). In contrast, the L proteinase has been classified as a papain-like thiol-proteinase on the basis of amino acid sequence homology (16). Inhibition of the L proteinase by E-64, a characteristic inhibitor of the papain-like thiol-proteinases, supports this (26). In addition, these two types of proteinases exhibit functional differences. Firstly, autocatalytic cleavage on the growing polyprotein occurs at the N terminus of the 2A proteinase, separating the P1 precursor to the viral structural polypeptides from the P2-P3 precursor to the nonstructural polypeptides (48). In contrast, the L proteinase autocleaves between its C terminus and the start of VP4, liberating itself from the rest of the viral polyprotein (45). Secondly, the cleavage of eIF-4γ by the 2A and L proteinases occurs at different sites, demonstrating that these proteinases have different cleavage specificities. Cleavage by the 2A proteinases of a rhinovirus and an enterovirus (human rhinovirus type 2 [HRV2] and coxsackievirus B4 [CVB4]) is between amino acids Arg-486 and Gly-487 (27), whereas the scissile bond of the L proteinase is between amino acids Gly-479 and Arg-480 (24). Thirdly, two lines of evidence suggest that the aphthovirus L proteinase is less restricted in the substrates that it recognizes than are the rhinovirus and enterovirus 2A proteinases. Although in both cases a primary cleavage site on eIF-4γ has been defined, further degradation of the products into smaller fragments occurs upon prolonged incubation with the L, but not the 2A, proteinase (24). Furthermore, the 2A autocleavage sites on the viral polyproteins of HRV2 and CVB4 and the site

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cleaved on eIF-4γ conform to a consensus sequence of I/LXTX↓GP (27, 44). In contrast, for the L proteinase no such consensus sequence which accommodates both the autocleavage site (36) and the eIF-4γ cleavage site (24) can be defined.

Picornavirus RNA translation can continue unabated upon cleavage of the cap-recognition complex, as translation initiation on these RNAs is via cap-independent internal ribosome entry (21, 37). This translation initiation mechanism requires a structured region of about 450 nucleotides (nt) of the 5′-untranslated region designated the IRES (for internal ribosome entry segment) (35). On the basis of IRES sequence conservation and secondary structure, picornaviruses can be classed into three distinct groups: rhinoviruses and enteroviruses, cardioviruses and aphthoviruses, and hepatitis A virus (19). Picornaviruses can be similarly classified according to the in vitro translation characteristics of mRNAs carrying the various IRESs. Cardiovirus and aphthovirus IRES-driven translation is accurate and efficient in the reticulocyte lysate system, whereas rhinovirus and enterovirus IRES-driven translation is extremely inefficient in this system and, in the case of poliovirus (PV), gives a high proportion of incorrect products resulting from initiation at internal (incorrect) sites (18). Rhinovirus and enterovirus IRES-driven translation is, however, efficient and accurate in extracts from HeLa cells or L cells or in reticulocyte lysates supplemented with such extracts. Indeed, translation initiation on the HRV2 IRES in reticulocyte lysates has been shown to have an almost absolute dependence on supplementation with proteins from HeLa cells (5).

Recently, it has been shown that in addition to down-regulating the initiation of translation of capped cellular mRNAs, the rhinovirus and enterovirus 2A proteinases actually stimulate the translation of mRNAs containing the cognate viral 5′-untranslated regions (17, 28). This was found to depend upon the enzymatic activity of the 2A proteinase: preincubation of the HRV2 2A proteinase with the inhibitor elastatinal abrogated translation stimulation (28), and a cleavage-defective mutant form of the PV 2A proteinase did not stimulate translation (17).

Given the functional similarities between the rhinovirus and enterovirus 2A proteinases and the aphthovirus L proteinase, we investigated the ability of the foot-and-mouth disease virus (FMDV) L proteinase to stimulate translation of mRNAs bearing picornaviral IRESs. The results presented here demonstrate that the FMDV L proteinase can functionally substitute for the rhinoviral and enteroviral 2A proteinases to stimulate rhinovirus or enterovirus IRES-driven translation initiation. However, it does not stimulate translation initiation from its cognate IRES. Cleavage of the two reporter gene products by the L proteinase also led us to investigate possible protein substrates for this enzyme. The results obtained emphasize that the rhinovirus and enterovirus 2A proteinases and the FMDV L proteinase have considerably different cleavage specificities.

MATERIALS AND METHODS

Enzymes and reagents. Restriction endonucleases and DNA-modification enzymes (Boehringer-Mannheim, Bethesda Research Laboratories, or New England Biolabs) were used according to the manufacturers' instructions. The translation initiation inhibitor edeine was a gift from Richard Jackson. The proteinase inhibitor E-64 was from Sigma, as were chymotrypsin A, endoproteinase Glu-C from *Staphylococcus aureus* V8, pronase E, proteinase K, proteinase X from *Bacillus thermoproteolyticus rokko*, and trypsin. The CVB4 2A proteinase and the Lb proteinase from the O1k strain of FMDV were expressed in *Escherichia coli* and purified to greater than 98% homogeneity with an identical purification protocol, as described previously (references 28 and 24, respectively). A mutant form of the Lb proteinase in which Cys-21 was replaced by an Ala residue was generated as follows. Nucleotide sequences coding for VP4 and VP2 were removed from the expression vector pET11d/FMDV Lb (24), and a stop codon was introduced after the final Lb proteinase codon. Subsequently, codon 21 of the Lb proteinase was changed from TGC (Cys) to GCC (Ala). Similarly to the wild-type proteinase, this mutant proteinase was expressed in *E. coli* and purified to greater than 98% homogeneity. The purity of both the wild-type and the mutant Lb proteinase was verified by subjecting them to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and automated Edman degradation. Purified proteinases were stored in buffer A (50 mM NaCl, 50 mM Tris-HCl [pH 8.0 at 20°C], 1 mM EDTA, 5 mM dithiothreitol, 5% glycerol). Globin mRNA was isolated from rabbit reticulocytes by standard techniques.

Plasmid constructions. *E. coli* TG1 was used for the propagation of plasmids. Recombinant DNA procedures used were essentially as described previously (40).

The construction of plasmid pXLJO has been described previously (pXLJ Con [5]), as has that of pXLJ-HRV2 (pXLJ 10-585 [5]). Plasmid pXLJ-PV1 was constructed by digestion of pKK-C2 (6) with *Asp*718 and *Msc*I and insertion of the filled-in fragment corresponding to nt 67 to 629 of the PV type 1 (PV1) (Mahoney strain) genome into pXLJO which had been linearized with *Sal*I and whose overhanging ends had been filled in. Plasmid pXLJ-EMCV was constructed by digestion of p-CITE (Novagen) with *Eco*RI and *Nco*I and insertion of the filled-in fragment corresponding to the encephalomyocarditis virus (EMCV) (R strain) genome from the poly(C) tract to nt 848 into pXLJO which had been linearized with *Bam*HI and whose overhanging ends had been filled in. Similarly, plasmid pXLJ-FMDV was constructed by insertion of the filled-in *Eco*RI-*Sma*I fragment from pSP449 (30), containing nt 362 to 831 of the FMDV O1k genome into the filled-in *Bam*HI site of pXLJO. Thus, the NS′ open reading frame is of identical size in pXLJO, pXLJ-HRV2, and pXLJ-PV1 and comprises an N-terminal extension of 4 amino acids in the case of pXLJ-EMCV and 11 amino acids in the case of pXLJ-FMDV.

In vitro transcription and translation. Purified plasmid DNAs were linearized with *Eco*RI, and mRNAs were synthesized in vitro by using T7 RNA polymerase (Pharmacia; 0.6 U/μl), in the presence or absence of $m^7G(5')ppp(5')G$ (Pharmacia) to yield capped or uncapped transcripts, respectively (9). In vitro translation reactions were carried out in nuclease-treated rabbit reticulocyte lysates (20) supplemented with HeLa cell lysates prepared and treated with micrococcal nuclease as described previously (6). The composition of translation mixtures was as described previously (5), except that reaction mixtures generally contained 5% by volume of HeLa cell S10 extract. The final concentrations of added KCl and $MgCl_2$ in translation reaction mixtures were 69 and 0.5 mM, respectively, except for reactions programmed with pXLJ-PV1 mRNA, which contained 85 mM added KCl and no added $MgCl_2$, or reactions programmed with globin mRNA, which contained 100 mM added KCl, 0.5 mM added $MgCl_2$, and no added HeLa cell S10 extract. Exceptions to these conditions are indicated in the figure legends.

Translation reactions were carried out at 30°C for 1 h and 30 min in the presence of purified proteinases or buffer A, as indicated in the figure legends. Where pretranslated products were treated with proteinase, translation reactions were stopped by the addition of edeine to 10 μM and further incubated at 30°C for 20 min before the addition of proteinase. For certain experiments, E-64 was added to translation reaction mixtures to give a final concentration of 400 μM.

Preparation and immunoprecipitation of cytoplasmic extracts from HeLa cells. HeLa cell monolayers (80 to 90% confluent) were infected by PV1 (vKK17 [23]) or mengovirus (vM16 [13]) at a multiplicity of infection of 50 to 100 PFU per cell and incubated at 37°C for 4 h in the presence of actinomycin D (2.5 μg/ml). Subsequently, cells were incubated in methionine-free medium for 30 min before labelling for 75 min with 30 μCi of [^{35}S]methionine (3,000 Ci/mmol [Amersham]) per ml. Cytoplasmic extracts were then prepared: cells were resuspended in hypotonic buffer [20 mM MOPS (morpholinepropanesulfonic acid)-KOH (pH 7.2 at 20°C), 10 mM KCl, 2 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1.5 mM $Mg(CH_3COO)_2$, 7 mM β-mercaptoethanol], incubated at −70°C for 20 min, thawed at 37°C, and allowed to rupture during 20 min at 0°C. The nuclei were removed by centrifugation. Extracts were prepared from labelled mock-infected cells in an identical manner. Infections were carried out in parallel to check virus production after 8 and 24 h.

For immunoprecipitations, cytoplasmic extracts from HeLa cells infected with PV were incubated with polyclonal antibodies raised against PV P1 proteins or polypeptide 2C, 3C, or 3D. Protein-antibody complexes were recovered by using activated *S. aureus* cells (42).

Computer analysis. Peptide sequences of 12 amino acids derived from the FMDV L proteinase autocleavage site (KVQRKLKGAGNS [36]) and the cleavage site on rabbit reticulocyte eIF-4γ (PSFANLGRPALS [24]) were aligned against the sequences of the influenza virus NS′ (50), *Xenopus laevis* cyclin B2 (33), human cyclin A (49), and PV 3CD (25) proteins with the Bestfit and Fasta programmes within the Genetics Computer Group package.

RESULTS

Rationale for the study undertaken and presentation of the assay system used. The available evidence suggests a correla-

tion between 2A-mediated stimulation of IRES-driven translation initiation and proteolytic activity of the 2A proteinase (17, 28). Given the functional similarities between the rhinovirus and enterovirus 2A proteinases and the FMDV L proteinase, it could be predicted that the latter would also be capable of stimulating IRES-driven translation initiation.

To investigate this possibility, we studied the effects of purified recombinant FMDV Lb proteinase on in vitro translation of artificial capped dicistronic mRNAs generated from the previously described pXLJ-HRV2 (5). In this system, initiation of translation of the upstream cistron (*X. laevis* cyclin B2) is dependent on a standard cellular 5′-untranslated region. In contrast, translation initiation of the downstream cistron (influenza virus NS) is after direct ribosome entry into the HRV2 IRES which serves as the intercistronic spacer. Thus, the efficiency of internal initiation of translation is reflected by the levels of the NS′ translation product. Furthermore, a built-in internal control for RNA quality and quantity and nonspecific effects of protein and/or extract addition is provided by the relative efficiency of translation of the upstream cistron. Similar plasmids were designed to generate dicistronic mRNAs in which NS′ translation is driven from the IRES of PV (pXLJ-PV1), EMCV (pXLJ-EMCV), or FMDV (pXLJ-FMDV) (see Materials and Methods).

It should be noted that two forms of the L proteinase exist (Lab and Lb), which originate from initiation of translation at alternative in-frame AUG codons in the FMDV RNA (2, 41). Our studies have examined only the effects of the Lb form on translation, since the Lab form has proved refractory to expression in bacteria (24). Furthermore, although it was previously reported that the two forms of the L proteinase have similar enzymatic capacities (32), the Lb (but not the Lab) form is necessary for virus replication in vivo (8).

Effects of the Lb proteinase on translation. We have recently used the system outlined above to demonstrate specific stimulation of HRV2 IRES-driven translation by the cognate 2A proteinase (51). However, given the aims of the study described here, it was necessary to show that translation initiation on mRNAs carrying the HRV2 IRES could be stimulated by a similar but noncognate 2A proteinase. Therefore, we examined the effects of the enterovirus CVB4 2A proteinase on HRV2 IRES-driven translation (Fig. 1, left-hand panel). The initiation of translation driven by the HRV2 IRES has an almost absolute dependence on the addition of HeLa cell proteins to reticulocyte lysates (5). For the studies described here, translations were performed in rabbit reticulocyte lysates supplemented with limiting amounts of HeLa cell extract, such that HRV2 IRES-driven synthesis of NS′ is detectable but is very inefficient (Fig. 1, lanes N). Hence, even a minimal level of translation stimulation should be seen clearly. The addition of the CVB4 2A proteinase to translation reactions significantly enhanced translation initiated from the HRV2 IRES (Fig. 1, left-hand panel; compare yield of NS′ product between lanes a through c and lane N), with a maximal stimulation of about fivefold being observed at less than 10 μg of added proteinase per ml (compare lanes a and b). No shutoff of translation of the upstream cyclin B2 gene was observed, even at the highest concentration of added proteinase (Fig. 1, left-hand panel; compare lanes N and a). Thus, it can be excluded that the observed stimulation of NS′ translation is a result of reduced competition from the upstream cistron for canonical initiation factors.

Given that stimulation of HRV IRES-driven translation was possible with a heterologous 2A proteinase, we examined the effects of the FMDV Lb proteinase on translation initiation (Fig. 1, right-hand panel). Strikingly, in the presence of the

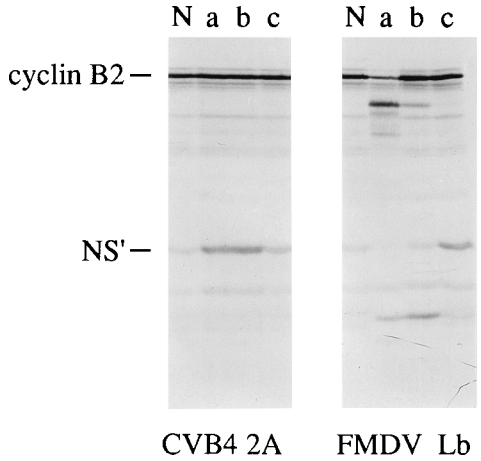

FIG. 1. Comparison of the effects of CVB4 2A and FMDV Lb proteinases on HRV2 IRES-driven translation initiation. Capped mRNA transcribed from pXLJ-HRV2 was translated in a mixed rabbit reticulocyte lysate-HeLa cell extract (5% [by volume] HeLa cell extract) at a final RNA concentration of 25 μg/ml. Reaction mixtures (10 μl) were supplemented with 2 μl of buffer A (lanes N) or 2 μl of various dilutions of CVB4 2A proteinase (left-hand panel) or FMDV Lb proteinase (right-hand panel), both diluted in buffer A. Final concentrations of added proteinase were 100 μg/ml (lanes a), 10 μg/ml (lanes b), and 1 μg/ml (lanes c). [^{35}S]methionine-labelled translation products were analyzed by migration through SDS–20% polyacrylamide gels and exposure of the dried gel to Hyperfilm β-Max.

FMDV Lb proteinase the size distribution of the observed translation products was greatly altered. Essentially, as the concentration of Lb proteinase was increased, the expected translation products were progressively replaced by one or more smaller proteins (compare lanes a through c with lane N). No such effect was seen in the presence of high concentrations of the CVB4 2A proteinase (Fig. 1, left-hand panel, lane a), even after overexposure of the gel (not shown). Despite this change in product profile, it appeared that NS′ synthesis was significantly increased in the presence of relatively low concentrations of Lb proteinase (Fig. 1, right-hand panel; compare lanes N and c). However, before we stated categorically that the FMDV Lb proteinase was capable of stimulating HRV2 IRES-driven translation, a more detailed investigation of the reasons for the altered product profile observed in the presence of the Lb proteinase was undertaken.

It could be postulated that the Lb proteinase either cleaved the authentic translation products or promoted translation initiation at alternative downstream AUG codons. To test the former hypothesis, we incubated presynthesized translation products with the Lb proteinase (Fig. 2, left-hand panel). For this experiment, pXLJ-PV1 mRNA was used (see Materials and Methods), rather than pXLJ-HRV2 mRNA. NS′ synthesis driven from the PV1 IRES is significantly more efficient than that driven from the HRV2 IRES under the same translation conditions (compare lane N of Fig. 2, left-hand panel, with lanes N of Fig. 1). Thus, the effects of Lb proteinase addition to presynthesized NS′ protein could be examined in the standard conditions (rabbit reticulocyte lysates supplemented with 5% HeLa cell extract) used elsewhere in these studies. The addition of the Lb proteinase to presynthesized cyclin B2 and NS′ (Fig. 2, left-hand panel) caused the same dose-dependent alteration of product profile seen when the proteinase was present during translation. This experiment suggests strongly that both the cyclin B2 and the NS′ proteins are substrates for the FMDV Lb proteinase. However, certain differences were observed in the product profiles depending on whether the Lb

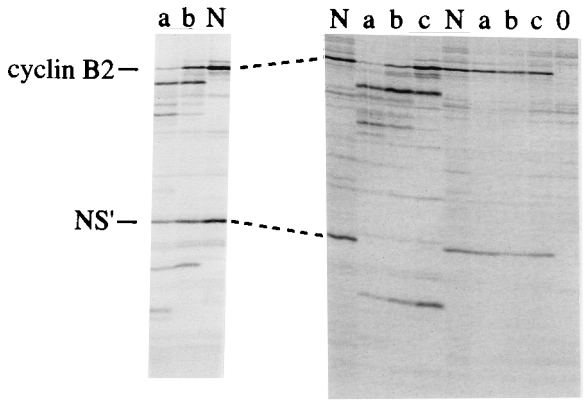

FIG. 2. Cleavage of cyclin B2 and NS′ by the Lb proteinase. Left-hand panel, a translation reaction (as described in the legend to Fig. 1 but without proteinase supplementation) was programmed with capped mRNA (25 μg/ml) transcribed from pXLJ-PV1. The reaction was stopped after 1 h 30 min by the addition of edeine, as described in Materials and Methods, and aliquots were then incubated for 1 h at 30°C with the following final concentrations of Lb proteinase (micrograms per milliliter): 500, lane a; 50, lane b; and 0, lane N. Aliquots were then analyzed by SDS-PAGE. Right-hand panel, translation reactions (as described in the legend to Fig. 1) were programmed with uncapped mRNA (25 μg/ml) transcribed from pXLJ-HRV2. Reaction mixtures (10 μl) were supplemented with 2 μl of buffer A (lanes N) or 2 μl of either wild-type FMDV Lb proteinase (first set of lanes) or the Ala-21 mutant Lb proteinase (second set of lanes) diluted in buffer A to give final concentrations of 100 μg/ml (lanes a), 10 μg/ml (lanes b), and 1 μg/ml (lanes c). A no-RNA control reaction mixture was included (lane 0). Samples were analyzed by SDS-PAGE.

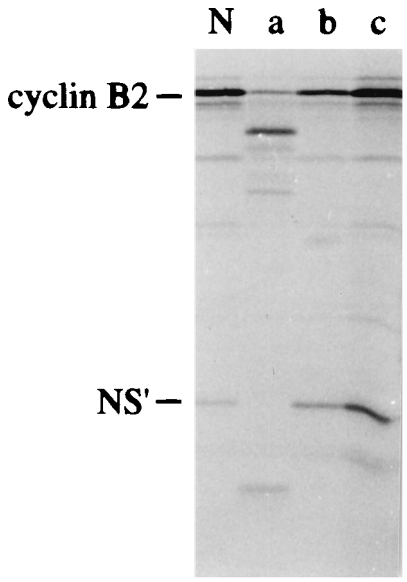

FIG. 3. Stimulation of HRV IRES-driven translation depends on enzymatic activity of the Lb proteinase. Translation reaction mixtures (as described in the legend to Fig. 1) were preincubated for 10 min at 30°C with buffer A (lane N); FMDV Lb proteinase (100-μg/ml final concentration) (lane a); FMDV Lb proteinase which had been preincubated with E-64 for 10 min at 4°C (lane b); and FMDV Lb proteinase, E-64 being added after preincubation (lane c). Reaction mixtures were then further preincubated for 10 min at 4°C, before being programmed with capped mRNA (25 μg/ml) transcribed from pXLJ-HRV2.

proteinase was present during translations or was added to pretranslated products (compare lanes a of Fig. 1, right-hand panel, and Fig. 2, left-hand panel). Notably, two or three extra protein species were observed upon cleavage of presynthesized cyclin B2 and NS′, and the authentic gene products appeared to be progressively processed into multiple smaller fragments as the proteinase concentration was increased. In addition, the efficiency of cleavage was reduced when proteins were presynthesized: for instance, in the case of the NS gene product, complete cleavage was not achieved even with proteinase added to 500 μg/ml (Fig. 2, left-hand panel, lane a). It could be argued that the recombinant Lb proteinase is contaminated by an *E. coli* proteinase. To ensure that the observed proteolytic activity is indeed a property of the Lb proteinase, we compared the product profiles when translation reactions were carried out in the presence of either wild-type Lb proteinase or a mutant in which Cys-21 is replaced by an Ala residue (Fig. 2, right-hand panel). This mutant was chosen since it had previously been suggested that Cys-21 is the active-site nucleophile (16). The mutant Lb proteinase had no effect on translation, even at a concentration of 100 μg/ml (compare the second set of lanes a, b, and c with lane N). In contrast, even at a concentration as low as 1 μg/ml, the particular batch of wild-type proteinase used for this experiment cleaved both translation products (compare the first set of lanes a, b, and c with lane N).

Taken together, the experiments described above demonstrate that the FMDV Lb proteinase stimulates HRV2 IRES-driven translation and also specifically cleaves the translation products.

Characterization of translation stimulatory capacities of the FMDV Lb proteinase. The Ala-21 mutant Lb proteinase did not cleave cyclin B2 or NS′ (Fig. 2, right-hand panel), nor did it cleave eIF-4γ (data not shown). Furthermore, it did not stimulate HRV2 IRES-driven translation (Fig. 2, right-hand panel), suggesting that stimulation of translation initiation depended on the proteolytic activity of Lb. To test this hypothesis, we used the chemical inhibitor E-64, which has been shown to inhibit the FMDV L proteinase (26). pXLJ-HRV2 mRNA was translated in the presence of Lb proteinase which had been treated with E-64 before its addition to the reaction (Fig. 3, lane b). The enzymatic activity of the Lb proteinase was blocked by the addition of E-64, as evidenced by the lack of cleavage of cyclin B2 and NS′ translation products (compare lanes a and b). Under these conditions, translation stimulation was barely detectable (compare lanes N and b). In contrast, when the translation reaction was preincubated with the Lb proteinase but E-64 was added before the RNA (Fig. 3, lane c), HRV2 IRES-driven translation was stimulated approximately 10-fold (compare lanes N and c). Again, the inhibition of proteolytic activity of the Lb proteinase during the actual translation reaction was witnessed by the lack of cleavage of the translation products. These results strongly suggest that a major factor contributing to translation stimulation by Lb is dependent on its proteolytic activity and that the effect is mediated indirectly via cleavage of some component of either the HeLa cell extract or the reticulocyte lysate.

We next set out to determine whether Lb proteinase-mediated translation stimulation is restricted to internal initiation on mRNAs carrying the HRV IRES. The effects of the Lb proteinase on translation driven by the poliovirus IRES were examined (Fig. 4, left-hand panel), since this IRES has been shown to be responsive to the 2A proteinase (17). This study was carried out with uncapped mRNAs in addition to artificially capped transcripts, since no inhibition of translation initiation of the upstream cistron in the presence of the Lb proteinase was observed in the experiments with artificially capped mRNAs described so far (for example, Fig. 1, lane b, or Fig. 3, lane c). This observation contrasts with results obtained in vivo, which suggested that the L proteinase inhibits cap-dependent translation (4). When the NS′ cistron was placed downstream of the PV1 IRES, translation enhancement could be

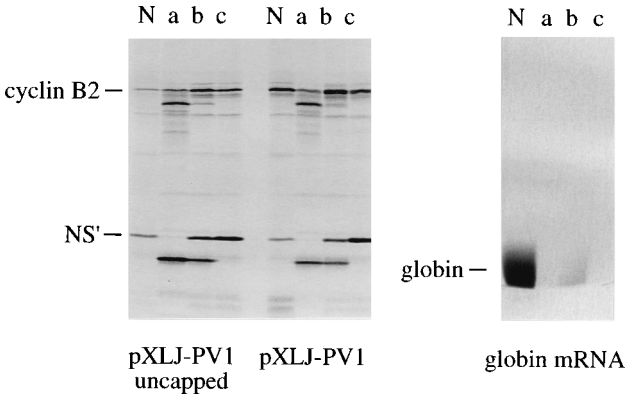

FIG. 4. Lb proteinase stimulates initiation of translation on an uncapped mRNA and internal initiation of translation on an enterovirus IRES. Left-hand panel, translation reactions (as described in the legend to Fig. 1) were programmed with capped or uncapped (as indicated) mRNA (25 μg/ml) transcribed from pXLJ-PV1. Reaction mixtures (10 μl) were supplemented with 2 μl of buffer A (lanes N) or 2 μl of FMDV Lb proteinase diluted in buffer A. Final concentrations of added proteinase were 100 μg/ml (lanes a), 10 μg/ml (lanes b), and 1 μg/ml (lanes c). Right-hand panel, natural capped globin mRNA (25 μg/ml) was translated in a rabbit reticulocyte lysate. Reaction mixtures (10 μl) were preincubated for 10 min at 30°C with 2 μl of buffer A (lane N), FMDV Lb proteinase diluted in buffer A to give a final concentration of 100 μg/ml (lane a) or 10 μg/ml (lane b), or FMDV Lb proteinase (final concentration, 100 μg/ml), E-64 being added after preincubation (lane c). Reaction mixtures were further preincubated for 10 min at 4°C before mRNA was added.

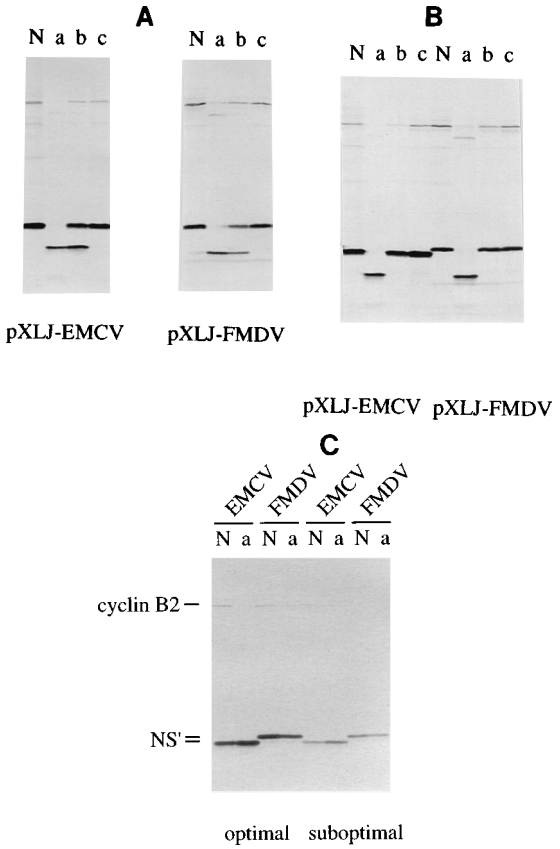

FIG. 5. Effects of the FMDV Lb proteinase on cardiovirus and aphthovirus IRES-driven translation. Translation reactions were programmed with capped mRNA transcribed from either pXLJ-EMCV or pXLJ-FMDV (as indicated) at a final RNA concentration of 25 μg/ml (A and B) or 10 μg/ml (C). (A) Reaction mixtures (10 μl) (as described in the legend to Fig. 1) were supplemented with 2 μl of buffer A (lanes N) or 2 μl of FMDV Lb proteinase diluted in buffer A. Final concentrations of added proteinase were 100 μg/ml (lanes a), 10 μg/ml (lanes b), and 1 μg/ml (lanes c). (B) Translation reaction mixtures (as described in the legend to Fig. 1) were preincubated for 10 min at 30°C with buffer A (lanes N), FMDV Lb proteinase (100-μg/ml final concentration) (lanes a), FMDV Lb proteinase which had been preincubated with E-64 for 10 min at 4°C (lanes b), and FMDV Lb proteinase, E-64 being added after preincubation (lanes c). Reactions were then further preincubated for 10 min at 4°C before being programmed with mRNA. (C) Translation reactions were performed in a reticulocyte lysate which contained 100 mM added KCl, 0.5 mM added $MgCl_2$, and no added HeLa cell S10 extract (optimal conditions) or 220 mM added KCl, 0.5 mM added $MgCl_2$, and 20% (by volume) HeLa cell S10 extract (suboptimal conditions). Reaction mixtures (10 μl) were preincubated for 10 min at 30°C with buffer A (lanes N) or FMDV Lb proteinase (100-μg/ml final concentration), E-64 being added after preincubation (lanes a). Reaction mixtures were then further preincubated for 10 min at 4°C before being programmed with mRNA.

seen in the presence of low concentrations of the Lb proteinase (Fig. 4, left-hand panel; compare lanes N and c). Thus, the Lb proteinase stimulates translation driven by both the PV1 IRES and the HRV2 IRES. Furthermore, upstream cistron translation from uncapped pXLJ-PV1 mRNA was enhanced by the presence of the Lb proteinase during translation reactions, whereas once again the Lb proteinase did not inhibit upstream cistron translation initiation on artificially capped mRNAs (compare the yield of cyclin B2 between lane N and lanes b and c in each case). It is known that capping of mRNAs in vitro is not very efficient (9), and only 40 to 70% of full-length transcripts would be expected to be capped with the protocols described in this report. Thus, the apparent absence of an effect of the Lb proteinase on the translation of the upstream cistron of a "capped" dicistronic mRNA could reflect an equilibrium between the stimulation of translation of uncapped mRNAs and inhibition of initiation on the mRNAs actually carrying the cap structure. To further verify this, the effects of the Lb proteinase on translation of a naturally capped mRNA were examined (Fig. 4, right-hand panel). The addition of Lb proteinase to translation reactions programmed with globin mRNA resulted in a dramatic reduction in the amount of protein detected (compare lanes a and b with lane N). This was due to inhibition of translation initiation, rather than to cleavage of globin by the Lb proteinase, since inactivation of the Lb proteinase with E-64 before the addition of mRNA did not increase the amount of globin detected (lane c).

To investigate whether Lb proteinase-mediated stimulation of internal initiation of translation was restricted to rhinovirus and enterovirus IRESs or extended to include cardiovirus and aphthovirus IRESs, the effects of the Lb proteinase on translation driven by the cognate FMDV IRES and the heterologous EMCV IRES were examined (Fig. 5). There was no obvious effect of low concentrations of the Lb proteinase on translation driven by either of these IRESs (Fig. 5A; compare lanes N and c). The demonstration of stimulation of translation initiation when E-64 was present during the reaction (Fig. 3) provided a tool to circumvent the complication of cleavage of translation products which precluded a direct analysis of the effects of the Lb proteinase at high concentrations (lanes a and b). However, no significant difference in the level of translation of pXLJ-EMCV or pXLJ-FMDV mRNAs was detected whether reaction mixtures were preincubated with buffer (Fig. 5B, lanes N), inactivated Lb proteinase (Fig. 5B, lanes b), or active Lb proteinase, E-64 being added before the RNA (Fig. 5B, lanes c). It remained possible that translation stimulation could not be evidenced merely because the base level of cardiovirus and aphthovirus IRES-driven translation initiation was too high (compare lanes N of Fig. 1 and Fig. 5A or 5B). Therefore, translation efficiency was reduced at least fourfold, by altering the composition of the translation mixtures (Fig.

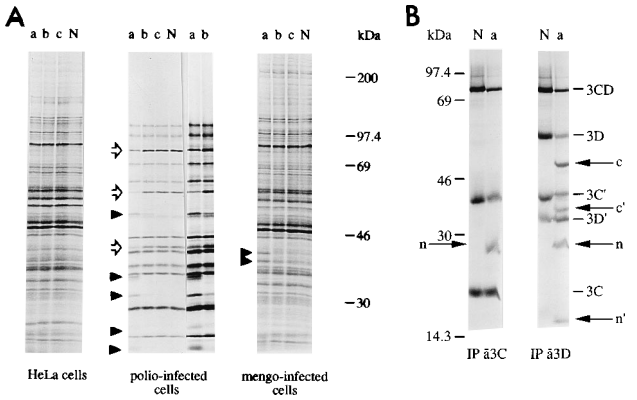

FIG. 6. Lb proteinase specifically cleaves the PV replicase. (A) Aliquots (corresponding to 10^4 cells) of [^{35}S]methionine-labelled cytoplasmic extracts from HeLa cells (left-hand panel) or from HeLa cells infected with PV (middle panel) or mengovirus (right-hand panel) were incubated for 1 h with varying concentrations of Lb proteinase (micrograms per milliliter): lanes a, 100; lanes b, 10; lanes c, 1; and lanes N, 0 (no proteinase). Samples were then analyzed by migration through SDS–15% polyacrylamide gels. An overexposure of lanes a and b of the middle panel is shown to the right of this panel. Positions of molecular mass markers are shown to the right of the figure (sizes in kilodaltons). New protein species detected in the presence of Lb proteinase (closed arrowheads) and PV-specific polypeptides reduced in quantity (open arrows) are indicated. (B) After incubation for 1 h with Lb proteinase (100 μg/ml [lanes a]) or with buffer A (lanes N), aliquots of [^{35}S]methionine-labelled cytoplasmic extracts from PV-infected HeLa cells were immunoprecipitated with antibodies directed against PV polypeptide 3C (left-hand panel) or 3D (right-hand panel). Samples were then analyzed by SDS-PAGE. Positions of molecular mass markers are shown to the left of the figure (sizes in kilodaltons).

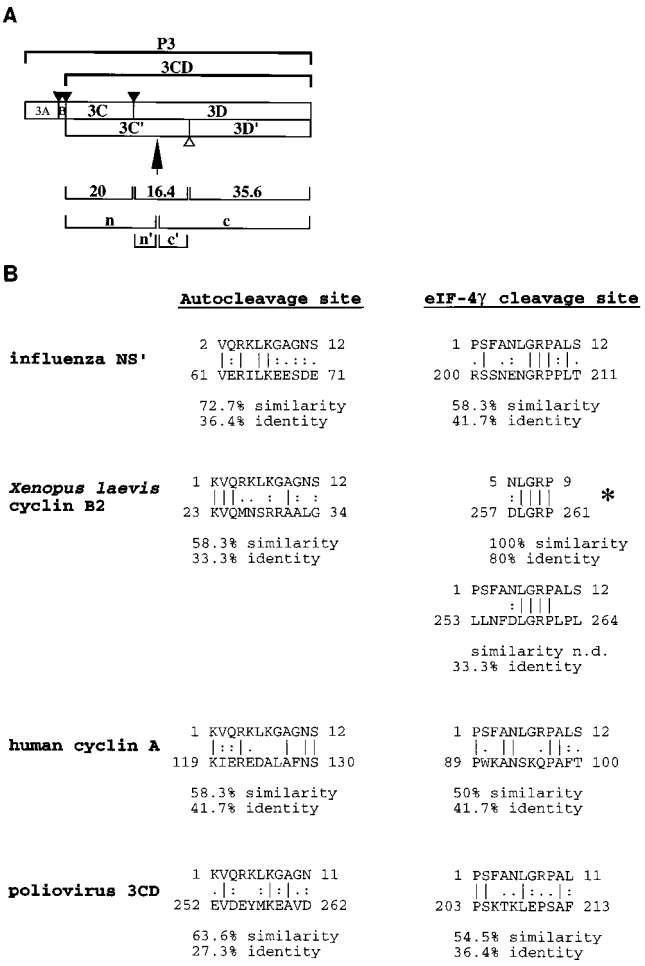

FIG. 7. Potential target sites for cleavage by the FMDV Lb proteinase. (A) A schematic representation of the P3 region of the PV polyprotein (to scale; compiled from data in reference 25). The positions of the PV 3C and 2A proteinase-specific cleavage sites are indicated (closed and open triangles, respectively), as is the putative position of the Lb proteinase cleavage site (arrow). Sizes (in kilodaltons) are indicated below the figure, as are the putative locations of the products of cleavage by the Lb proteinase. (B) Sequence comparisons between the known cleavage sites of the Lb proteinase and the target proteins. Shown are the optimal Bestfit alignments (without gaps) of peptides spanning the autocleavage site (cleavage is at the KG dipeptide) or the eIF-4γ cleavage site (cleavage is at the GR dipeptide) with the full amino acid sequences of the indicated target proteins. The same results were obtained with the Fasta programme. The percent amino acid identity and similarity over the aligned region are given in each case. Where the optimal alignment involved substantially fewer than the 12 amino acids corresponding to the known cleavage site, both the computer-predicted alignment (*) and a manual extension of this alignment are shown.

5C; compare lanes N). Then, when reaction mixtures were preincubated with active Lb proteinase, E-64 being added before the RNA (Fig. 5C, lanes a), minimal stimulation of translation initiation on the EMCV IRES could be evidenced. In contrast, stimulation of FMDV IRES-driven translation could still not be demonstrated, and in fact, the presence of the Lb proteinase seemed to slightly inhibit translation efficiency.

Cleavage potential of the FMDV Lb proteinase. It is noteworthy that the translation products of both the reporter genes used in this study were specifically cleaved by the Lb proteinase. Furthermore, similarly to *X. laevis* cyclin B2, we have found that the Lb proteinase processes human cyclin A (data not shown). We set out to examine the potential of other proteins to serve as substrates for the Lb proteinase. Initially, for ease of identification of any cleaved species, the effects of the Lb proteinase on PV-specific polypeptides were examined. To this end, infected HeLa cells were transiently labelled with [^{35}S]methionine, and cytoplasmic extracts were prepared and incubated with the Lb proteinase (Fig. 6A, middle panel). The shutoff of host cell protein synthesis was essentially complete at the time of labelling, such that all proteins detected were specific to PV (Fig. 6A; compare middle and left-hand panels). The quantities of three polypeptides were clearly reduced upon incubation with the Lb proteinase (Fig. 6A, middle panel, open arrows; compare lanes N and a), and at least five new protein species could be detected (closed arrowheads).

To determine the approximate locations of the cleavage sites in the viral polyprotein, immunoprecipitations were performed with antibodies directed against PV polypeptide 2C, 3C, or 3D, or P1 proteins (Fig. 6B and data not shown). Only anti-3C and anti-3D antibodies revealed different patterns of viral proteins in cytoplasmic extracts which had been treated with the Lb proteinase (Fig. 6B, lanes a) compared with untreated samples (lanes N). The viral polypeptides affected by incubation with the Lb proteinase could be identified as 3CD, 3D, and 3C′, since once again reduced quantities of these polypeptides were detected in samples treated with the Lb proteinase, whereas the amounts of polypeptides 3C and 3D′ were not reduced. In addition, one extra 3C-specific polypeptide was seen in proteinase-treated extracts (left-hand panel; see arrow). This polypeptide, along with three of the other new species generated by treatment with the Lb proteinase, was also reactive towards antibodies directed against polypeptide 3D (right-hand panel; see arrows).

At first sight, the observed immunoprecipitation profile can be accommodated within the hypothesis of a single cleavage event in the N-terminal part of the 3D coding region, upstream

of the 3C′-3D′ cleavage site (Fig. 7A). Thus, cleavage of polypeptide 3CD would give a 3D-specific C-terminal product (c in Fig. 6B and 7A) and a 3C- and 3D-reactive N-terminal product (n). Cleavage of polypeptide 3D would generate a smaller, 3D-specific, N-terminal product (n′ in Fig. 6B and 7A) and the same C-terminal product (c). Polypeptide 3C′ would be cleaved into the same N-terminal product generated by cleavage of polypeptide 3CD, and a 3D-specific C-terminal product smaller than that generated by cleavage of 3CD (c′ in Fig. 6B and 7A). However, an apparent size of over 30 kDa for the c′ cleavage product (Fig. 6B) does not accord with the theoretical calculation of between 0 and 16.4 kDa (Fig. 7A). We have previously reported the abnormal migration of PV 3D-related polypeptides on SDS-polyacrylamide gels (3). However, it seems unlikely that such a phenomenon could explain the protein profiles shown in Fig. 6. It is more probable that there is a second Lb-specific cleavage site, again in the N-terminal part of 3D, such that in fact the polypeptide labelled as c′ in Fig. 6B corresponds to polypeptide c truncated at its N terminus. This hypothesis would take into account the fact that at least one of the Lb cleavage products seen in total cytoplasmic extracts from PV-infected cells was not detected by immunoprecipitation. It is possible that some Lb-cleavage products cannot be recognized by the antisera tested, and some may be too small to be resolved by the gel system used.

Similarly, we examined the capacity of the Lb proteinase to cleave proteins in extracts from mengovirus-infected cells (Fig. 6A, right-hand panel). The shutoff of host-cell protein synthesis was very poor in the mengovirus-infected cells (compare lanes N of left-hand and right-hand panels). Therefore, it was verified that the mengovirus used was indeed alive, by determining the virus production in a single cycle (approximately 200 PFU per cell [data not shown]). Despite the poor shutoff, two new polypeptides could be evidenced after treatment of cytoplasmic extracts with the Lb proteinase (closed arrowheads; compare lane a with lane N). It seemed that these two new polypeptides were virus specific, since no evidence could be found for cleavage of labelled HeLa cell proteins in mock-infected cell extracts, even at high concentrations of Lb proteinase (Fig. 6A, left-hand panel; compare lanes N and a). However, to date we have been unable to identify by immunoprecipitation the protein(s) cleaved in extracts from mengovirus-infected cells (data not shown).

Amino acid sequence comparisons of Lb proteinase substrates. Most of the proteins shown in this report to be substrates for the FMDV Lb proteinase are of known sequence (see Materials and Methods). Therefore, we attempted to locate motifs which could constitute potential cleavage sites. The LysGly dipeptide found at the L proteinase autocleavage site within the FMDV polyprotein (36) is not present in any of the cyclin A, cyclin B2, or influenza virus NS′ proteins, nor in the region of the PV polyprotein implicated by immunoprecipitation experiments as the target for cleavage by the Lb proteinase. Similarly, while the GlyArg dipeptide cleaved by the Lb proteinase within eIF-4γ (24) is found in cyclin B2 and influenza virus NS′, it is not present either in cyclin A or in the region of the PV polyprotein implicated as the target. In an attempt to define a consensus cleavage site, the sequences of peptides corresponding to the 12 amino acids surrounding the two sites known to be cleaved by the L proteinase were compared with those of proteins shown to be substrates for proteolysis by Lb (Fig. 7B). In all cases, the peptide spanning the autocleavage site within the FMDV polyprotein was best aligned with a different sequence from that which best matched the peptide spanning the eIF-4γ cleavage site. Interestingly, no significant primary sequence homology could be found between peptides spanning the two known cleavage sites of the FMDV L proteinase (within eIF-4γ and the autocleavage site [24]). This could reflect a major difference in the determinants required for efficient *cis* or *trans* cleavage and may be indicative of a mechanistic difference between the two types of proteolysis.

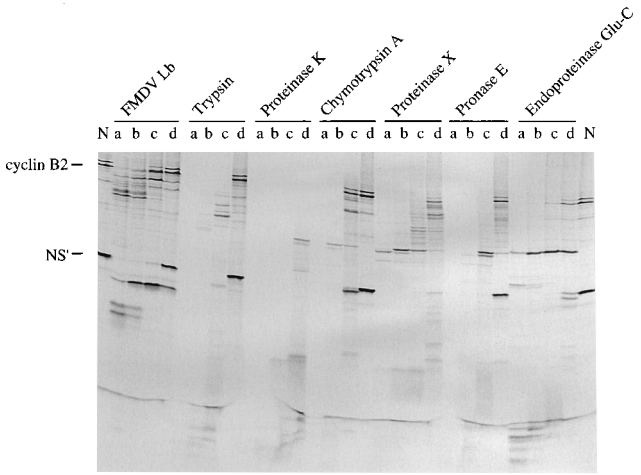

FIG. 8. Cleavage of presynthesized cyclin B2 and NS′ by different proteinases. A translation reaction (as described in the legend to Fig. 1 but without proteinase supplementation) was programmed with capped mRNA (25 μg/ml) transcribed from pXLJ-PV1. The reaction was stopped after 1 h and 30 min by the addition of edeine, as described in Materials and Methods, and aliquots were then incubated for 20 min at 30°C with the indicated proteinase at the given final concentrations (micrograms per milliliter): lanes a, 500; lanes b, 100; lanes c, 10; lanes d, 1; and lanes N, no proteinase. Aliquots were then analyzed by SDS-PAGE.

No significant pattern emerged when the different potential cleavage sites were manually compared with each other in a search for partial amino acid conservation either upstream or downstream of the putative cleavage site. Furthermore, although human cyclin A and *X. laevis* cyclin B2 are closely related proteins (52.7% amino acid similarity and 36.2% identity [data not shown]), each of the peptides corresponding to a known L proteinase cleavage site aligned with different regions of these two proteins (Fig. 7B). We could not find a single conserved sequence within the two cyclin proteins with which either peptide would reasonably align. Furthermore, no similarities could be seen between the cleavage profile observed upon treatment of presynthesized cyclin B2 and NS′ with the Lb proteinase and those obtained with commercially available proteinases of known cleavage specificities (Fig. 8).

DISCUSSION

The results described here demonstrate that the FMDV Lb proteinase is capable of stimulating, by 5- to 10-fold, rhinovirus and enterovirus IRES-driven initiation of translation in vitro. Interestingly, the FMDV Lb proteinase did not stimulate translation driven by its cognate IRES and only barely stimulated that driven by a heterologous cardiovirus IRES. Thus, the biological significance of Lb proteinase-mediated stimulation of translation seems at first sight to be obscure. On the basis of IRES sequence conservation and secondary structure, a distinct split exists between, on the one hand, the rhinoviruses and enteroviruses, and, on the other hand, the cardioviruses and aphthoviruses (19). Thus, it is probable that studies concerning translation transactivation by the FMDV Lb proteinase are of

relevance to an understanding of rhinovirus and enterovirus translation.

We generated a mutant Lb proteinase in which Cys-21 is replaced by an Ala residue. This mutant proteinase was devoid of any detectable proteolytic activity, providing the first direct experimental evidence to support the suggestion that Cys-21 is the active-site nucleophile (16). We used this mutant and E-64, a potent inhibitor of papain-like proteinases and of the FMDV L proteinase, to show that Lb proteinase-mediated stimulation of IRES-driven translation is largely dependent on the enzymatic activity of the proteinase. Pretreatment of translation extracts with active Lb proteinase, prior to inhibition of the proteinase and the subsequent addition of mRNA, was sufficient to allow translation initiation to be stimulated to the same extent as when active Lb proteinase was present throughout the translation reaction. This strongly suggests that translation stimulation is mediated indirectly via cleavage of some cellular component present in either the HeLa cell extract or the reticulocyte lysate.

The proteolysis of a cellular component(s) is also a major factor contributing to rhinovirus and enterovirus 2A proteinase-mediated stimulation of translation initiation (17, 28, 51). Given the functional similarities between the two types of proteinase, it seems likely that a common mechanism can be invoked in both cases of stimulation of rhinovirus and enterovirus IRES-driven translation. A rhinovirus or enterovirus IRES per se can be considered to be a weak translation initiation element compared with a cardiovirus or aphthovirus IRES (compare lanes N of Fig. 1, 4, and 5). It is tempting to speculate that physiological rhinoviral and enteroviral translation relies on cleavage of some cellular protein by the 2A proteinase to enhance translation efficiency and thus compensate for an inefficient IRES. The ability of the FMDV Lb proteinase to functionally substitute for the 2A proteinase to stimulate translation in vitro would suggest that in fact rhinoviral and enteroviral translation profits from a proteolytic function destined primarily to play some other role in the viral replicative cycle.

An obvious candidate for the cellular component implicated in proteinase-mediated translation stimulation is eIF-4γ, since this cellular translation initiation factor is cleaved directly by both the Lb and the 2A proteinases, and the sites cleaved are only 7 amino acids apart on the polypeptide chain. It could be that one of the products of cleavage of eIF-4γ is responsible for the observed IRES-dependent translation stimulation. Indeed, it has been reported that the cleavage products of eIF-4γ stimulate, albeit moderately, the translation of PV RNA in vitro (7). Conversely, the addition of purified intact eIF-4 to in vitro translation reactions programmed with PV RNA led to a dramatic reduction in the synthesis of the P1 polypeptide (46). Thus, it remains to be proven whether the translation enhancement observed is actually due to stimulation of initiation by a cleavage product(s) or rather to the destruction of an inhibitor. Nevertheless, at the present time it cannot be ruled out that cleavage of some other, as yet unidentified, cellular protein is required for Lb and 2A proteinase-mediated stimulation of IRES-driven translation initiation.

In the experiments described in this report, initiation of translation of the upstream cistron was not inhibited by addition of the Lb proteinase to translation extracts when mRNAs were artificially capped and was even enhanced when mRNAs were uncapped. We have no concrete explanation for such effects of Lb proteinase addition on initiation of translation of a cellular mRNA. It could have been expected that the initiation of translation of capped mRNAs would be reduced because of the cleavage of eIF-4γ by the Lb proteinase (4, 12). Indeed, translation of natural capped globin mRNA was severely reduced in the presence of the Lb proteinase. Thus, our results concerning artificially capped mRNAs most probably reflect an equilibrium between inhibition of translation initiation on capped messages and stimulation of translation initiation on uncapped messages by the Lb proteinase, as outlined above. The observed stimulation of translation initiation on an uncapped cellular message raises several interesting questions. It remains to be proven whether stimulation by the Lb proteinase of IRES-driven translation and translation of uncapped mRNAs occurs by a common mechanism. Furthermore, it remains to be determined whether stimulation of translation on uncapped mRNAs is a general phenomenon or whether it is restricted to certain specific messages. It should be stressed that, during picornaviral infection, although cellular translation is generally shut off, translation initiation on certain cellular mRNAs continues (34). In one case, this was found to be by a cap-independent process of internal ribosome binding (31), but it is conceivable that in other cases it may be through cap-independent, but 5′-end-dependent, translation initiation.

The results presented here show clearly that the FMDV Lb proteinase has a much broader specificity than do the rhinovirus and enterovirus 2A proteinases. This difference first became apparent with the observation that eIF-4γ was completely degraded upon prolonged incubation with the Lb proteinase but not with the 2A proteinases (24). We have found that other proteins recognized by the FMDV Lb proteinase include *X. laevis* cyclin B2 and the related human cyclin A, influenza virus NS, poliovirus 3CD, and at least one mengovirus protein. No cleavage of these proteins by the CVB4 2A proteinase could be evidenced (Fig. 1 and data not shown). Furthermore, although the initial cleavage of these proteins by the Lb proteinase was specific and apparently occurred at single, or at most two, sites, prolonged incubation, or the addition of proteinase to high concentrations, resulted in degradation or trimming of the primary cleavage products. Our data raise questions as to the effects of the presence of such a proteinase on the viral life cycle. It is interesting to speculate that the cleavage of cellular targets by the L proteinase accounts, at least in part, for the dramatic cytopathic effects associated with FMDV infection. The cleavage of cyclin proteins may be relevant to the establishment, or maintenance, of persistent FMDV infections (10, 11). Furthermore, the Lb-mediated cleavage of the PV 3D replicase protein and at least one, as yet unidentified, mengovirus-specific protein suggests that FMDV genome evolution may have been constrained by the need to avoid possible sites for Lb cleavage within its own structural and nonstructural proteins.

A broad-range substrate specificity of the FMDV Lb proteinase was substantiated by manual and computer-assisted comparisons of the primary amino acid sequences of the proteins identified in this report as being cleaved. In most cases, we could find no significant sequence conservation between the substrate and either of the two previously determined Lb-specific cleavage sites. In fact, Bestfit alignments between peptide sequences spanning the known Lb-specific cleavage sites and the target protein sequences showed an overall lack of similarity between the different optimal alignments. This suggests that primary sequence determinants alone are insufficient to confer susceptibility on the proteinase. We would speculate that, in fact, recognition of sequence motifs per se plays a minimal role in proteolysis by the FMDV Lb proteinase and that the specificity determinants of the Lb proteinase are predominantly structural.

Indeed, when the predicted secondary structures of the target proteins were examined (with the Peptidestructure pro-

gram of the Genetics Computer Group package), it was found that the motifs which aligned best with the peptide spanning the site on eIF-4γ cleaved by the Lb proteinase were all flexible sequences which should in fact form turns (data not shown). Furthermore, they were hydrophilic motifs with a strong probability of being found at the surface of the protein, except for the motif within cyclin B2, a protein singularly devoid of regions predicted to form surface turns (data not shown). It is possible that an Lb-specific cleavage site is constituted by a combination of minimal primary sequence requirements and molecular accessibility. This hypothesis would be in keeping with the observation that Lb proteinase-mediated cleavage of the NS′ and cyclin B2 gene products was less efficient when the proteins had been presynthesized than when the proteinase was added during translation reactions (compare Fig. 1 and 2). Similarly, it could be postulated that secondary trimming or degradation of the primary cleavage products occurs at sites which were inaccessible in the intact native protein. Given the mechanistic similarities between the FMDV L proteinase and papain, we attempted to cleave the papain-sensitive monoclonal antibody 8F5 into its Fab and Fc fragments (43, 47). This could not be achieved with purified Lb proteinase (data not shown). Thus, structural determinants recognized by the Lb proteinase are unlikely to be analogous to the hinge region found in antibody proteins. The development of a model for a consensus Lb proteinase cleavage site (and the resolution of the roles that structural constraints play in processing) must await microsequencing of the products of Lb proteinase action on the newly identified target proteins. These questions are currently being addressed, since the FMDV proteinase may prove to be useful in studies involving specific, limited proteolysis as an additional tool in the rapidly growing battery of well-characterized restriction proteinases.

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