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eIF4G and its proteolytic cleavage products: Effect on initiation of protein synthesis from capped, uncapped, and IRES-containing mRNAs

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ABSTRACT

Rhinovirus 2A and foot-and-mouth disease virus Lb proteinases stimulate the translation of uncapped messages and those carrying the rhinovirus and enterovirus Internal Ribosome Entry Segments (IRESes) by a mechanism involving the cleavage of host cell proteins. Here, we investigate this mechanism using an artificial dicistronic RNA containing the human rhinovirus IRES as intercistronic spacer. Because both proteinases cleave eukaryotic initiation factor 4G (eIF4G), we examined whether the cleavage products of eIF4G could stimulate uncapped or IRES-driven translation. Addition of intact eIF4F to translation extracts inhibited IRES-driven translation and reduced the translation stimulation observed in reactions pre-treated with Lb proteinase. Prolonged incubation of translation extracts with Lb proteinase removed all endogenous eIF4G and a substantial amount of the primary C- and N-terminal cleavage products. The translation of all mRNAs was reduced in such extracts. Capped mRNA translation was rescued by the addition of intact eIF4F. In contrast, addition of pre-cleaved eIF4F stimulated translation of uncapped or IRES-bearing messages to the levels seen upon proteinase addition. Furthermore, fractions containing the C-terminal, but not N-terminal, cleavage product of eIF4G stimulated translation moderately. These results demonstrate that the Lb and 2A proteinases stimulate translation of uncapped RNAs and those carrying IRESes by the production of cleavage products of eIF4G that enhance translation and by the removal of intact eIF4G that interferes with this stimulation.

Keywords: picornavirus; translational control; translation initiation factors; viral proteinases

INTRODUCTION

Most eukaryotic mRNAs comprise a methylated cap at the free 5′ end, followed by a relatively short, unstructured, untranslated region before the translation initiation codon. The scanning hypothesis has been proposed to explain translation initiation on such mRNAs (Kozak, 1989). Under the dictates of this model, the 40 S ribosomal subunit interacts with the 5′ end of the mRNA and moves down the mRNA in an ATP-dependent manner to the initiation codon, where it is joined by the 60 S ribosomal subunit to form the translationally competent ribosome. Several eukaryotic initiation factors (eIF) are involved in this process (for a review, see Merrick, 1992). It has been proposed that eIF4F, a complex comprised of eIF4E (a phosphoprotein that recognizes the methylated cap) and eIF4A (a bidirectional ATP-dependent helicase) bound to eIF4G (previously known as eIF-4γ or p220), is the first initiation factor to bind the mRNA. eIF4G also binds eIF3, which facilitates binding of the 40 S ribosomal subunit to the mRNA, via the ternary complex (Benne & Hershey, 1978). It was reported recently that, whereas eIF4E binds to the N-terminal portion of eIF4G, eIF4A and eIF3 bind to the C-terminal portion (Lamphear et al., 1995; Mader et al., 1995).

A number of mRNAs have now been identified for which translation occurs by a mechanism that is both cap-independent and apparently 5′ end-independent. These include several viral eukaryotic mRNAs, among them the picornavirus mRNAs. The genomes of all picornaviruses studied to date possess an Internal Ribosome Entry Segment (IRES), which involves a large proportion (approximately 450 nt) of the unusually long viral 5′ untranslated region (5′-UTR), and which

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allows ribosomes to bind internally to the viral RNA (or to heterologous RNAs carrying the IRES) rather than to initiate translation after scanning from the 5′ end of the RNA (Pelletier & Sonenberg, 1988; Jang et al., 1989; for a review, see Meerovitch & Sonenberg, 1993). Although the boundaries of picornaviral IRESes have been mapped in terms of the minimal sequences required for activity, little is still known of the exact mechanism by which ribosomes are able to internally initiate RNA translation. Several RNA-binding proteins not defined previously as initiation factors have been implicated in internal initiation of translation (Borman et al., 1993; Hellen et al., 1993; Meerovitch et al., 1993). It is supposed that canonical initiation factors also play a role in this mode of translation. Indeed, several reports have shown a role for eIF4F in IRES function (Anthony & Merrick, 1991; Scheper et al., 1992; Pause et al., 1994); in addition, Svitkin et al. (1994) found that eIF2 and GEF stimulate translation of poliovirus (PV) RNA. The role of viral proteins in internal initiation of translation has not been defined clearly. It was shown recently that the 2A proteinases of rhinoviruses and enteroviruses and the Lb proteinase of foot-and-mouth disease virus (FMDV, an aphthovirus) stimulated translation initiation on uncapped RNAs and on RNAs carrying the PV and human rhinovirus (HRV), but not the encephalomyocarditis virus (EMCV) or FMDV IRESes (Hambidge & Sarnow, 1992; Liebig et al., 1993; Borman et al., 1995; Ohlmann et al., 1995; Ziegler et al., 1995a; 1995b). For both proteinases, translation stimulation depended upon enzymatic activity of the respective proteinases, as demonstrated by experiments using proteolytically inactive mutants (Hambidge & Sarnow, 1992; Ziegler et al., 1995a, 1995b), or using wild-type proteinases in the presence of specific proteinase inhibitors (Liebig et al., 1993; Ziegler et al., 1995a, 1995b). However, translation stimulation did not require the presence of active proteinase during translation, suggesting that stimulation was the result of proteinase-mediated modification of a cellular protein(s) (Ziegler et al., 1995a, 1995b). Furthermore, for uncapped mRNA, translation stimulation could be correlated with the presence of cleaved eIF4G (Ohlmann et al., 1995).

Although the 2A and Lb proteinases are distinct enzymes mechanistically, they are quite similar functionally. During the viral life-cycle, both proteinases cleave themselves from the respective viral polyprotein precursors (albeit at opposite termini of the proteinases) and cleave eIF4G at sites that are separated by only seven amino acids (Lamphear et al., 1993; Kirchweger et al., 1994). The cleavage of eIF4G results in a severely reduced capacity of capped mRNAs to recruite 40 S ribosomal subunits (Etchison et al., 1982). Thus, cleavage of eIF4G by the 2A and L proteinases is a key feature of the inhibition of host cell macromolecular synthesis, known as host cell shut-off (Penman & Summers, 1965; Summers & Maizel, 1967; Lloyd et al., 1988). In this manner, rhinovirus, enterovirus, and aphthovirus RNAs are able to compete efficiently with the plethora of host cell mRNAs for the cellular translation machinery, thus ensuring a productive viral infection.

Here, we describe experiments to examine the mechanism of 2A and Lb proteinase-mediated translation stimulation, both for uncapped mRNAs and for translation directed by the HRV IRES. The results demonstrate that both proteinases stimulate translation via cleavage of eIF4G. It is shown that the products of eIF4G cleavage possess the capacity to specifically stimulate translation initiation both on uncapped mRNAs and on RNAs carrying the HRV IRES, and also that intact eIF4F interferes with proteinase-mediated stimulation of translation initiation driven from the HRV IRES.

RESULTS

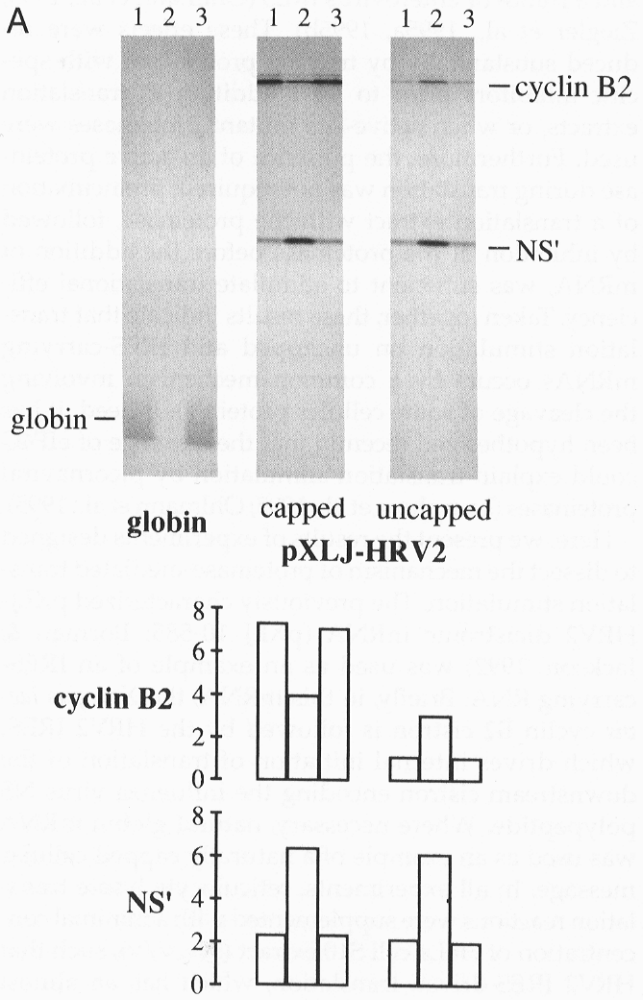
It has been shown previously that the presence of the FMDV Lb proteinase or a rhino- or enterovirus 2A proteinase in an in vitro translation reaction inhibits translation initiation from a capped mRNA and stimulates translation initiation from an uncapped mRNA and a rhino- or enterovirus IRES (Ohlmann et al., 1995; Ziegler et al., 1995a, 1995b). These effects were reduced substantially by treating proteinases with specific inhibitors prior to their addition to translation extracts, or when active-site mutant proteinases were used. Furthermore, the presence of an active proteinase during translation was not required; preincubation of a translation extract with the proteinase, followed by inhibition of the proteinase before the addition of mRNA, was sufficient to stimulate translational efficiency. Taken together, these results indicate that translation stimulation on uncapped and IRES-carrying mRNAs occurs by a common mechanism involving the cleavage of some cellular protein(s). Indeed, it has been hypothesized recently that the cleavage of eIF4G could explain translation stimulation by picornaviral proteinases (Lamphear et al., 1995; Ohlmann et al., 1995).

Here, we present the results of experiments designed to dissect the mechanism of proteinase-mediated translation stimulation. The previously characterized pXLJ-HRV2 dicistronic mRNA (pXLJ 10-585; Borman & Jackson, 1992) was used as an example of an IRES-carrying RNA. Briefly, in this mRNA, the *Xenopus laevis* cyclin B2 cistron is followed by the HRV2 IRES, which drives internal initiation of translation of the downstream cistron encoding the influenza virus NS polypeptide. Where necessary, natural globin mRNA was used as an example of a naturally capped cellular message. In all experiments, reticulocyte lysate translation reactions were supplemented with a minimal concentration of HeLa cell S10 extract (5%, v/v), such that HRV2 IRES-driven translation, which has an almost

absolute dependence on HeLa cell proteins (Borman & Jackson, 1992), was detectable but inefficient. Using this system, we have shown previously that HRV2 or PV1 IRES-driven synthesis of the NS protein could be stimulated by as much as sixfold in the presence of relatively high concentrations (up to 100 μg/mL final proteinase concentration) of active HRV2 2A or FMDV Lb proteinases (Ziegler et al., 1995a, 1995b).

Stimulation of IRES-driven translation initiation by the FMDV Lb proteinase

The nature of the experiments described here required that the concentrations of proteinase used were low enough to ensure complete inhibition of proteolytic activity for the duration of the translation reaction, and total abrogation of translation stimulation upon treatment with the proteinase inhibitors elastatinal (for the 2A proteinase) and E-64 (for the Lb proteinase). Figure 1A shows that the pretreatment of translation extracts with 10 μg/mL of Lb proteinase was largely sufficient to inhibit translation of naturally capped globin mRNA (compare lanes 1 and 2). This effect has been described previously for the 2A and Lb proteinases and is due to proteinase-mediated cleavage of the eIF4G component of the cap-binding protein complex (Liebig et al., 1993; Ziegler et al., 1995b). Similarly, the translation of the upstream cyclin B2 cistron in translations programmed with artificially capped pXLJ-HRV2 mRNA was reduced significantly, but not abrogated. We have observed previously that inhibition of artificially capped mRNA translation is less dramatic than that observed with naturally capped mRNAs, due to the intrinsic inefficiency of the in vitro capping reaction (Ziegler et al., 1995b). Such inhibition of translation could be prevented by pretreating the proteinase with E-64 (lanes 3). Conversely, 10 μg/mL of Lb proteinase was sufficient to stimulate HRV2 IRES-driven downstream cistron (NS') translation threefold using both capped and uncapped pXLJ-HRV2 mRNA, and also to stimulate upstream cyclin B2 cistron translation by a factor of 3 when uncapped pXLJ-HRV2 mRNA was translated. In both cases, translation stimulation was totally blocked by pretreatment of proteinase with E-64 (lanes 3).



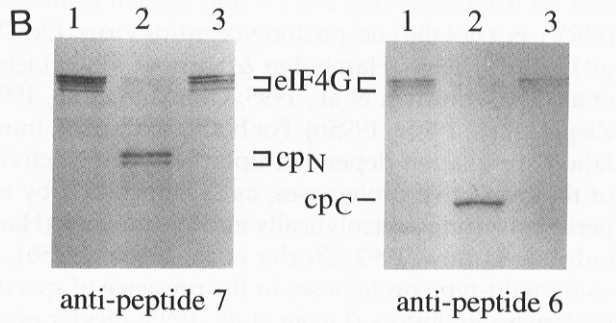


FIGURE 1. Effects of the Lb proteinase on initiation of translation and on eukaryotic initiation factor 4G (eIF4G). Reticulocyte lysates supplemented with 5% (v/v) HeLa cell S10 extract were incubated for 10 min at 30 °C with H100 buffer (lanes 1), 10 μg/mL final concentration of Lb proteinase in H100 buffer (lanes 2), or 10 μg/mL of Lb in H100 buffer that had been preincubated for 10 min at 4 °C with E-64 (final concentration in the translation extract of 400 μM) (lanes 3). Active Lb proteinase (lanes 2) was inhibited by adding E-64 to 400 μM and all samples were further incubated for 10 min at 4 °C before RNA was added and translations were conducted for 90 min at 30 °C. **A:** Translation reactions were programmed with natural globin mRNA (50 μg/mL final RNA concentration), or artificially capped or uncapped pXLJ-HRV2 mRNA (10 μg/mL) as indicated. Protein synthesis was analyzed by SDS-PAGE using a 20% polyacrylamide gel, and by subsequent exposure of the dried gel to Hyperfilm β-max for 16 h. Positions of the translation products are indicated. Densitometric quantification of the cyclin B2 and NS' translation products was conducted as described in Materials and Methods. Translation efficiency (in arbitrary units) is plotted below each lane. **B:** State of eIF4G in the translation extracts was examined after incubation for 90 min at 30 °C. Proteins were separated by SDS-PAGE through a 7.5% polyacrylamide gel. Proteins were then transferred to nitrocellulose by western blotting and blots were revealed using rabbit anti-eIF4G peptide 7 antiserum (left panel) or rabbit anti-eIF4G peptide 6 antiserum (right panel) as described by Liebig et al. (1993). Positions of intact eIF4G and the N- and C-terminal cleavage products (cp_N and cp_C, respectively) are indicated.

The state of eIF4G was examined by immunoblotting at the end of the translation reactions (Fig. 1B). Upon a 10-min treatment of the translation extract with active Lb proteinase, before the addition of inhibitor, intact eIF4G (lanes 1) is replaced by a series of polypeptides with an apparent molecular weight of 120 kDa (anti-peptide 7, lane 2) and by a single species with an apparent molecular weight of 100 kDa (anti-peptide 6, lane 2) corresponding to the N-terminal and C-terminal cleavage products of eIF4G, respectively (cp_N and cp_C). No detectable cleavage of eIF4G could be evidenced after a 90-min incubation with Lb proteinase that had been pretreated with the inhibitor E-64 (lanes 3). Similar results were obtained in a parallel experiment using 10 μg/mL of HRV2 2A proteinase and the inhibitor elastatinal (data not shown).

Influence of exogenous intact and proteinase-treated eIF4F on translation initiation

Translation stimulation by both the 2A and Lb proteinases depends on enzyme activity and involves cleavage of some cellular component in the translation extract. The C23A mutant Lb proteinase, which is inactive proteolytically (Ziegler et al., 1995b), could act as a competitor of both wild-type Lb and 2A proteinase-mediated stimulation of HRV2 IRES-driven internal initiation of translation (data not shown). This suggests that Lb and 2A proteinases act via the cleavage of a common cellular substrate, which can be bound and sequestered, but not cleaved, by an active-site mutant form of the Lb proteinase. The only such substrate known to date is eIF4G.

The cleavage of eIF4G by the 2A and Lb proteinases removes functional eIF4F from the translation reaction, generating the N-terminal and C-terminal cleavage products of eIF4G, cp_N, and cp_C. To investigate the effects on translation of removing intact eIF4G, we devised a series of experiments in which combinations of treatment with the Lb proteinase and addition of purified rabbit reticulocyte eIF4F were applied to provide a set of translation reactions with different amounts of intact and cleaved eIF4G.

The order of additions and the times and types of incubations are shown schematically in Figure 2A. The composition of the purified eIF4F used was such that, in addition to eIF4G, appreciable quantities of eIF4E are added into the system (Fig. 2B). The state of the eIF4G, eIF4A, and eIF4E in the translation extracts at the end of the reaction was examined by western blotting (Fig. 2C). As expected, this protocol led to translation reactions that contained: normal (lanes 1 and 5) or high (lanes 2 and 6) concentrations of intact eIF4G; low (lane 3) or high (lane 7) concentrations of cleaved eIF4G (although in the latter case, a significant amount of the secondary cleavage products cp_{C1} and cp_{C2} was evidenced in addition to cp_C); cleaved eIF4G, but also intact eIF4G (lane 4). It should be noted that an endogenous reticulocyte protein recognized by anti-eIF4G peptide 7 antibodies virtually co-migrates with cp_N (lane 1). This is not due to cleavage of eIF4G in the absence of added proteinase, because no corresponding C-terminal cleavage product was detected using anti-eIF4G peptide 6 antibodies. No cleavage of eIF4A or eIF4E was evidenced, and, although the concentration of eIF4E was approximately doubled by the addition of exogenous eIF4F (lanes 2, 4, 6, and 7), that of eIF4A was unchanged. The effects of these conditions on the translation of globin mRNA or capped and uncapped pXLJ-HRV2 mRNA were examined (Fig. 2D).

The addition of intact eIF4F to extracts preincubated without proteinase (lane 2, compare to lane 1) or with inactivated proteinase (lane 6, compare to lane 5) had no effect on translations programmed with globin mRNA (left panel). However, the addition of intact eIF4F rescued globin mRNA translation in proteinase-treated extracts (lane 4, compare to lane 3). These results support previous experiments showing that the endogenous eIF4F is sufficient to allow optimal translation of capped mRNAs in vitro. Generally, similar results were obtained for translation of the upstream cistron of artificially capped pXLJ-HRV2 mRNA (middle panel), although in this case some stimulation of translation was observed upon the addition of intact eIF4F to extracts (lane 2, compare to lane 1), and translation initiation was not completely inhibited in extracts treated with active proteinase (lane 3). The major difference between translation of naturally capped mRNA and that of artificially capped mRNA was when exogenous eIF4F was added to extracts together with active proteinase (lanes 7). In this case, whereas globin mRNA translation was abrogated (left panel), artificially capped mRNA translation was actually stimulated (middle panel). The explanation for this phenomenon probably again derives from the presence of both capped and uncapped molecules in the artificially capped mRNA preparation (Ziegler et al., 1995b).

The translation of uncapped mRNA or HRV2 IRES-driven mRNA was affected rather differently by eIF4F addition (Fig. 2D, right panel). The addition of intact eIF4F to extracts preincubated without proteinase (lane 2, compare to lane 1) or with inactivated proteinase (lane 6, compare to lane 5) inhibited the translation of uncapped mRNA (cyclin B2). HRV2 IRES-driven translation (NS′) similarly was inhibited slightly under these conditions. Furthermore, when eIF4F was added to extracts that had been treated with active proteinase, stimulation of HRV2 IRES-driven translation was reduced (lane 4, compare to lane 3). Interestingly, such inhibition of IRES-driven translation was not evidenced using capped dicistronic mRNAs (Fig. 2D, compare middle and right panels), presumably because, in this case, the added eIF4F is sequestered by the 5′ cap. In

contrast, when exogenous eIF4F was added to extracts together with active proteinase (lane 7), IRES-driven translation was stimulated at least to the levels seen when extracts received only active proteinase (lane 3), and uncapped mRNA translation was increased significantly compared to when extracts received buffer (lane 1) or proteinase alone (lane 3).

Depletion of eIF4G in translation reactions using Lb proteinase

The results presented thus far demonstrate that stimulation of translation on IRES-driven and uncapped mRNAs correlates with eIF4G cleavage, and that such translation stimulation is reduced by the presence of intact eIF4G in the translation extracts. To provide fur-

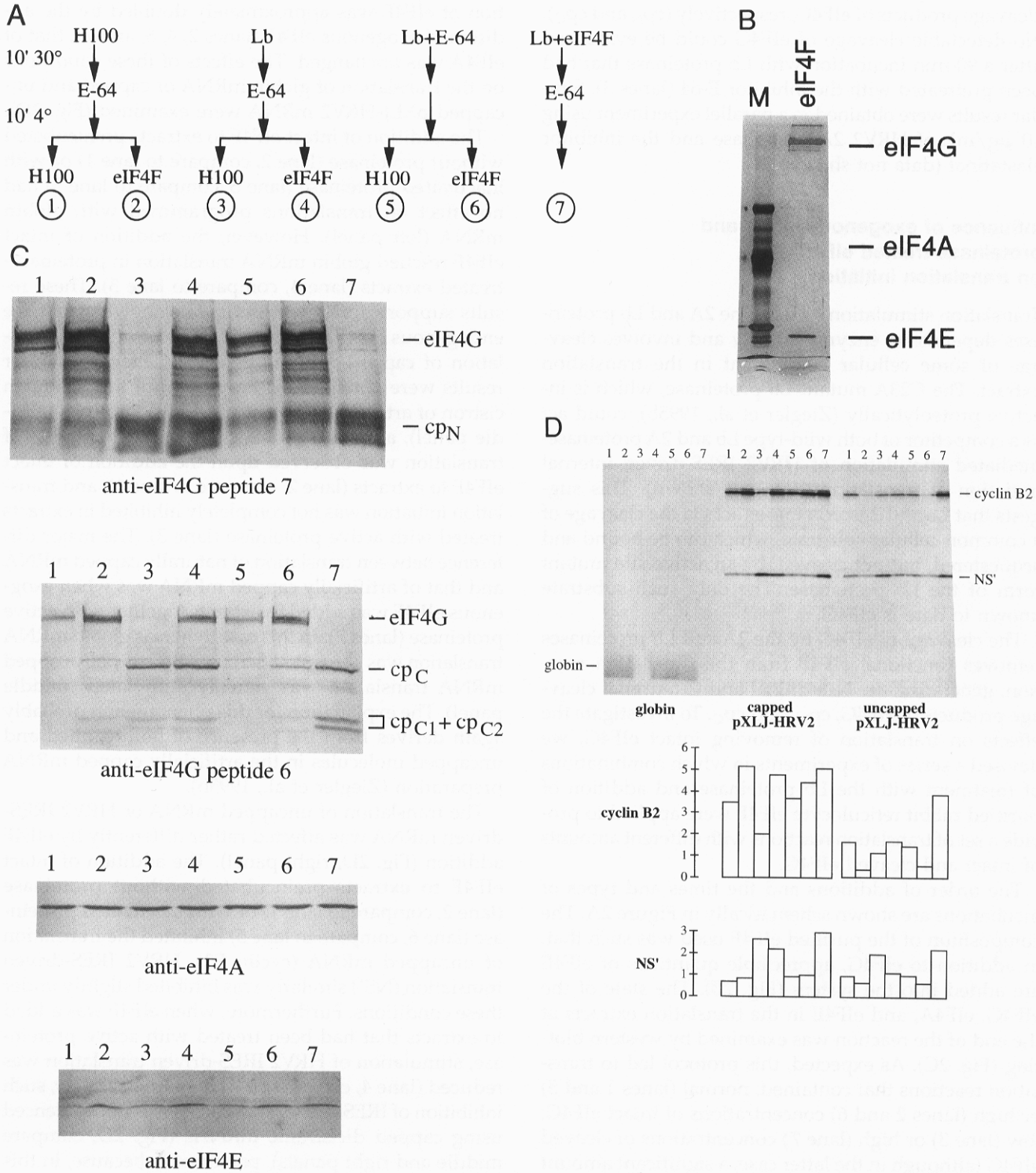


FIGURE 2. (*Legend on facing page.*)

ther support for this hypothesis, we wished to deplete translation reactions of eIF4G without generating the cleavage products cp_C and cp_N. Toward this end, we exploited a previous observation concerning the effects of treatment of eIF4G with Lb proteinase. During longer incubation of eIF4G with the Lb proteinase, the primary cp_C and cp_N cleavage products become degraded progressively by the proteinase, as a result of cleavage or trimming events at secondary sites (Kirchweger et al., 1994; Lamphear et al., 1995). Thus, by prolonged incubation of translation extracts with the Lb proteinase, it should be possible to remove eIF4G and then also cp_C and cp_N.

As shown in Figure 3A, HRV2 IRES-driven translation was stimulated after 5 or 10 min of pre-treatment of translation extracts with Lb proteinase (lanes 1 and 2). However, upon protracted incubation of the translation extract with proteinase, the efficiency of IRES-driven NS synthesis was reduced steadily. Translation initiation on the artificially capped upstream cyclin B2 cistron also decreased as the time of incubation with proteinase was prolonged. It should be noted that the degree of inhibition of capped cyclin B2 translation upon an incubation of the translation extract for 10 min with 10 μg/mL of Lb proteinase can vary from one experiment to another (compare Fig. 1A, middle panel, and Fig. 3A). This difference correlates with a difference in the efficiency of eIF4G cleavage (compare lanes 2 of Figs. 1B and 3B). Western blotting (Fig. 3B) showed that, in this experiment, 5 or 10 min of pretreatment of translation extracts with Lb proteinase resulted in cleavage of about 80–90% of eIF4G (compare lanes 1 and 2 to lane 0). All detectable eIF4G was cleaved by 20 min of preincubation of translation extracts with the proteinase (lanes 3), but, at this point, the level of cp_N was also beginning to fall, and the secondary cleavage products of cp_C (cp_{C1} and cp_{C2}) could be detected. Upon 60 min of preincubation, more than 50% of cp_N and cp_C had undergone further degradation (lane 5). When a similar control experiment was performed with inactivated proteinase, prolonged incubation of the translation extract had no effect on the translation efficiency of either cistron or on the state of eIF4G (data not shown).

The addition of intact eIF4F to extracts that had been treated with active Lb proteinase for 60 min (Fig. 3A, lane 7) was sufficient to totally rescue capped upstream cistron translation. This result demonstrates effectively that eIF4G was the only limiting translation initiation factor required for cap-dependent translation that had been destroyed during the 60 min of treatment with proteinase. In contrast, the addition of intact eIF4F did not stimulate HRV2 IRES-driven translation (Fig. 3A, lane 7). However, the addition of cleaved eIF4F (i.e., eIF4F that had been treated with 2A proteinase and then the proteinase inactivated; see Fig. 4A) to the overtreated translation extract stimulated the translation of both cistrons to the level attained upon relatively short preincubation of translation extracts with the Lb proteinase (Fig. 3A, compare lane 8 to lane 1). This was not due to some effect of the 2A proteinase per se on the translation extract, because proteinase-treated buffer had no effect on IRES activity (Fig. 3A, lane 9). Thus, in extracts that had been treated with Lb proteinase so as to remove intact eIF4G and a significant proportion of the primary cleavage products, the addition of exogenous cleavage products of eIF4G stimulated translation as efficiently as did the addition of active Lb or 2A proteinase to untreated extracts.

Effects of purified products of eIF4G cleavage on translation initiation

The results above show clearly that the cleavage products of eIF4G stimulate IRES-driven translation. We next wished to examine whether both cleavage products of eIF4G are required for translation stimulation or whether, as proposed by Lamphear et al. (1995), cp_C

FIGURE 2. Inhibition of HRV IRES-driven translation by intact eIF4F. Mixed reticulocyte-HeLa cell translation extracts (5%, v/v, HeLa cell S10 extract) were preincubated for 10 min at 30 °C with H100 buffer (lanes 1 and 2), 1 μg/mL final concentration of wild-type Lb proteinase in H100 buffer (lanes 3 and 4), 1 μg/mL of Lb in H100 buffer that had been preincubated for 10 min at 4 °C with E-64 (final concentration in the translation extract of 400 μM) (lanes 5 and 6), or 1 μg/mL of Lb and 200 ng of purified eIF4F in H100 buffer (lanes 7). E-64 was next added to translation extracts that had not already received this inhibitor, to a final concentration of 400 μM, and all samples were further incubated for 10 min at 4 °C. Translation extracts were then supplemented with H100 buffer (lanes 1, 3, and 5) or 200 ng of purified eIF4F in H100 buffer (lanes 2, 4, and 6), before RNA was added and translations were performed for 90 min at 30 °C. **A:** Flow-chart summarizing the treatment of translation extracts. **B:** Composition of the purified eIF4F used was examined by migration of 2 μL of the preparation through a 7.5% SDS-polyacrylamide gel, which was then subjected to silver staining. M, marker proteins. **C:** State of eIF4G, eIFA, and eIF4E in the translation extracts was examined after incubation for 90 min at 30 °C. Proteins were separated by SDS-PAGE through a 6% polyacrylamide gel. Proteins were then transferred to nitrocellulose by western blotting and blots were revealed (using the antiserum indicated under each panel) using the commercial DAB kit, as described in Materials and Methods. Positions of intact eIF4G and the primary N- and C-terminal cleavage products (cp_N and cp_C, respectively) are indicated, as well as those of the secondary cleavage products of cp_C (cp_{C1} and cp_{C2}). **D:** Translation reactions were programmed with natural globin mRNA (50 μg/mL final RNA concentration), or artificially capped or uncapped pXLJ-HRV2 mRNA (10 μg/mL) as indicated. Protein synthesis was analyzed by SDS-PAGE using a 20% polyacrylamide gel and by subsequent exposure of the dried gel to Hyperfilm β-max for 16 h. Densitometric quantification of the cyclin B2 and NS′ translation products was performed as described in Materials and Methods.

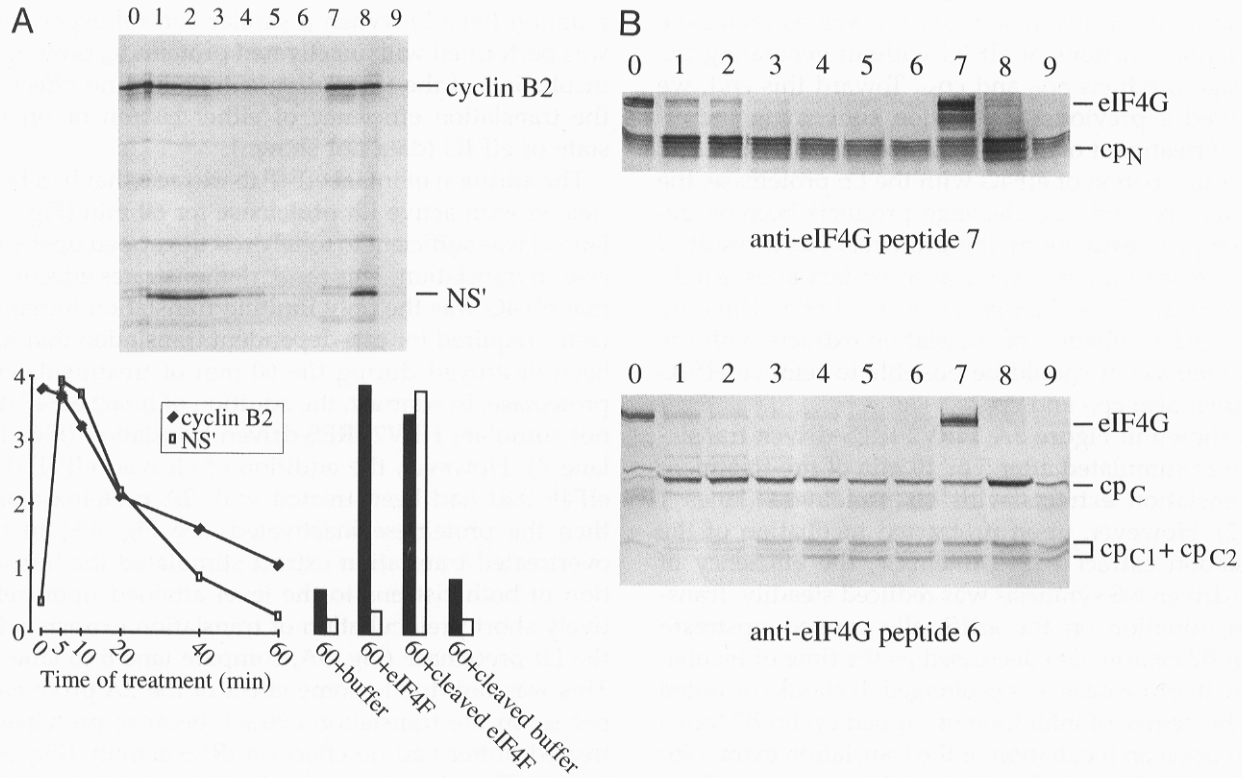
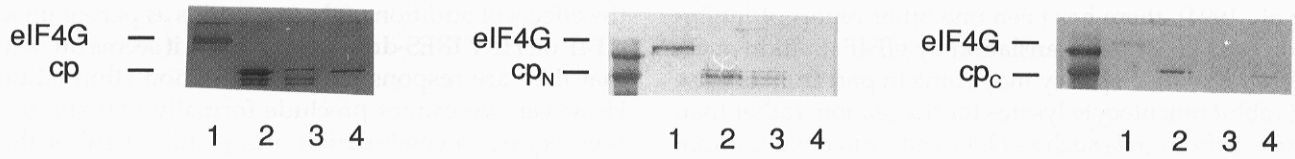


FIGURE 3. Protein synthesis in translation extracts depleted of eIF4G by protracted treatment with Lb proteinase. Mixed reticulocyte-HeLa cell translation extracts (5%, v/v, HeLa cell S10 extract) were preincubated at 30 °C with 10 μg/mL final concentration of wild-type Lb proteinase for 5 min (lane 1), 10 min (lane 2), 20 min (lane 3), 40 min (lane 4) or 60 min (lanes 5 to 9). Then E-64 was added to a final concentration of 400 μM, and samples were further incubated for 10 min at 4 °C. Alternatively, translation extracts received wild-type Lb proteinase that had been inactivated by pre-incubation with E-64 for 10 min at 4 °C (lane 0). After inactivation of the Lb proteinase, translation extracts were supplemented with buffer B (lane 6) or 130 ng of purified eIF4F (lane 7), or 130 ng of purified eIF4F (lane 8) or buffer B (lane 9), both of which had been treated with 20 ng of 2A proteinase for 30 min at 30 °C, the 2A proteinase being inactivated with elastatinal (500 μM final concentration in the translation extract) for 10 min at 4 °C. **A:** Translation reactions were programmed with artificially capped pXLJ-HRV2 mRNA (10 μg/mL) and incubated for 90 min at 30 °C. Protein synthesis was analyzed by SDS-PAGE using a 20% polyacrylamide gel, and by subsequent exposure of the dried gel to Hyperfilm β-max for 16 h. Results of densitometric quantification of the cyclin B2 and NS translation products (as described in Materials and Methods) are shown below the autoradiograph, expressed in arbitrary units. **B:** Aliquots of the translation reactions shown in A were analyzed for the state of eIF4G by western blotting, as described in the legend to Figure 2C, using rabbit anti-eIF4G peptide 6 or peptide 7 antisera, as indicated.

alone would suffice. To this end, purified eIF4F was cleaved using the HRV2 2A proteinase, and the eIF4G cleavage products cp_C and cp_N were separated by m^7GTP-Sepharose column chromatography (see Materials and Methods; Fig. 4A). Although the column eluate and flow-through fractions were pure preparations in terms of their cp_N and cp_C content, respectively (Fig. 4A compare lanes 3 and 4), eIF4E co-purifies with cp_N and eIF4A and the 2A proteinase co-purify with cp_C (data not shown; Lamphear et al., 1995).

When translation reactions programmed with uncapped pXLJ-HRV2 mRNA were supplemented with the flow-through fractions that contained cp_C to a final concentration of 6 μg/mL, stimulation by a factor of approximately 2 of both uncapped mRNA translation and IRES-driven translation could be observed (Fig. 4B). In contrast, no translation stimulation was evidenced when the column eluate that contains cp_N was added to translation reactions (final cp_N concentration 20 μg/mL). It could be argued that translation stimulation was due to residual 2A proteinase that had co-purified with cp_C. However, when the cp_C and cp_N preparations were pre-treated with elastatinal before their addition to the translation reaction, translation was still stimulated in the presence of cp_C, excluding this possibility (Fig. 4B). Furthermore, western blotting at the end of the translation reaction showed no decrease in the amount of intact eIF4G in samples that had received the cp_C fractions, again indicating the absence of active 2A proteinase in the cp_C fraction (Fig. 4C). Thus, cp_C (together with eIF4A with which it co-purifies) are sufficient to moderately stimulate HRV IRES-driven translation and translation of uncapped mRNA. However, the translation stimulation observed is relatively weak compared to that achieved upon the addition of proteinase to translation extracts or upon the addition of cleaved eIF4G in the eIF4F complex (twofold, compared to three- to fourfold; see Fig. 1A

A



B

	buffer	cp_N/eIF4E	cp_C/eIF4A/2A	cp_N/eIF4E + elastatinal	cp_C/eIF4A/2A + elastatinal
cyclin B2	100	91	197	105	184
NS'	100	101	181	103	161

C

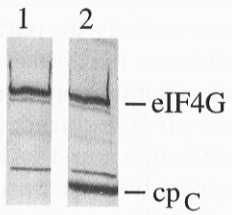


FIGURE 4. Stimulation of translation initiation by purified products of cleaved eIF4G. **A:** Purified eIF4F (lanes 1) was cleaved to completion with the HRV 2A proteinase (lanes 2) and fractionated into the eluate (lanes 3) and flow-through material (lanes 4) after passage over a m^7GTP-Sepharose column (see Materials and Methods). Composition of the different fractions was assessed following SDS-PAGE through a 7.5% polyacrylamide gel. Proteins were silver stained (left panel) or transferred to nitrocellulose by western blotting and blots were revealed using rabbit anti-eIF4G peptide 7 antiserum (middle panel) or rabbit anti-eIF4G peptide 6 antiserum (right panel) as described by Liebig et al. (1993). Positions of intact eIF4G and the N- and C-terminal cleavage products (cp_N and cp_C, respectively) are indicated. Marker proteins were migrated in parallel. **B:** Mixed reticulocyte-HeLa cell translation extracts (5%, v/v, HeLa cell S10 extract) were supplemented with H100 buffer, m^7GTP-Sepharose column eluate (cp_N/eIF4E) or m^7GTP-Sepharose column flow-through fractions (cp_C/eIF4A/2A) to final concentrations of 20 μg/mL cp_N or 6 μg/mL cp_C. Translation reactions were programmed with uncapped pXLJ-HRV2 mRNA (10 μg/mL) and incubated for 90 min at 30 °C. Protein synthesis was analyzed by SDS-PAGE using a 20% polyacrylamide gel, exposure of the dried gel to Hyperfilm β-max, and subsequent densitometric quantification of the cyclin B2 and NS' translation products (as described in Materials and Methods). Arbitrary figures of 100 were assigned to the reactions supplemented with H100 buffer for each product. **C:** State of the endogenous eIF4G at the end of the translation reactions supplemented with H100 buffer (lane 1) or m^7GTP-Sepharose column flow-through fractions (cp_C/eIF4A/2A; lane 2) was analyzed by western blotting, as described in the legend to Figure 2C, using rabbit anti-eIF4G peptide 6 antiserum.

and Fig. 2). This might suggest a role for cp_N in IRES-driven translation stimulation, either to stabilize cp_C or to act in conjunction with cp_C for maximal stimulatory capacity. However, in this respect, translation stimulation was not improved in experiments in which separated cp_N and cp_C were recombined and added to translation reactions (data not shown), suggesting that maximal stimulation does not require a combination of cp_N and cp_C. Alternatively, it could be postulated that cp_C becomes partially inactivated as a result of the purification procedures necessary for its production.

DISCUSSION

Previous investigations by ourselves and others demonstrated that the rhino- and enteroviral 2A proteinases and the FMDV Lb proteinase possess the ability to stimulate translation initiation on uncapped mRNAs and on mRNAs bearing rhino- or enteroviral IRESes (Hambidge & Sarnow, 1992; Liebig et al., 1993; Borman et al., 1995; Ohlmann et al., 1995; Ziegler et al., 1995a, 1995b). For both proteinases, and both types of mRNA, stimulation was dependent on the enzymatic activity of the proteinases. Here we have investigated the mechanism of 2A and Lb proteinase-mediated stimulation of translation. Initial experiments involving the use of inhibitors of proteinase activity demonstrated that translation stimulation was mediated via the cleavage of some cellular protein(s). Both Lb and 2A proteinase-mediated translation stimulation were reduced when an active site mutant form of the Lb proteinase was used as a competitor to preadsorb extracts, indicating that a common cellular target was involved in the stimulation of IRES-driven translation by the two proteinases (R. Kirchweger, A.M. Borman, and K.M. Kean, unpubl. data).

Subsequent experiments demonstrated that the common substrate protein was eIF4G, and that the effects of this protein on translation initiation were twofold. Cleaved eIF4G stimulated both the HRV2 IRES activity and the translation of uncapped mRNAs, whereas the addition of exogenous intact eIF4F inhibited such translation. This is in agreement with a recent report that described stimulation of uncapped mRNA translation in the presence of eIF4F in which eIF4G had been cleaved (Ohlmann et al., 1995). Furthermore, previous experiments indicated that the cleaved form of eIF4F is capable of stimulating PV mRNA translation (Buckley & Ehrenfeld, 1987). In contrast, the effects of intact eIF4F on the initiation of IRES-driven translation vary in the current literature. Although several groups find that eIF4F is required for such translation

(Anthony & Merrick, 1991; Scheper et al., 1992; Pause et al., 1994), there has been one other report of inhibition of IRES-driven translation by eIF4F (Svitkin et al., 1994). This discrepancy may come in part from the use of rabbit reticulocyte lysates for translation, rather than extracts from cells such as HeLa cells. Effectively, rhino- and enterovirus IRESes do not confer efficient or accurate translation initiation in rabbit reticulocyte lysates. Alternatively, the nature of the 5′ end of the mRNA may influence the initiation factor requirements even for internal initiation of translation. Interestingly, we found that eIF4F-induced inhibition of HRV2 IRES-driven translation could be evidenced essentially only on uncapped but not on capped mRNA (Fig. 2D, lanes 2 and 6). The explanation for this is not clear, although it is conceivable that, when eIF4F is sequestered by the 5′ cap structure, it is not free to interfere with IRES function. The physiological relevence of direct inhibition of IRES-driven translation as mediated by intact eIF4F is, for the moment, obscure. One could imagine that the intact eIF4F complex competes with the eIF4G cleavage products for binding of some component of the translation machinery, or for binding of the IRES itself. However, it remains to be determined whether the endogenous concentrations of eIF4G in untreated extracts and in cells are sufficient to downregulate translation initiation from the HRV IRES; if this is the case, then proteinase-mediated cleavage of this protein serves to remove an inhibitor of IRES activity, as well as to produce stimulatory cleavage products. These consequences of eIF4G cleavage are, of course, in addition to the inhibition of capped mRNA translation. Thus, a single cleavage event would have multiple effects that would contrive to ensure an efficient competition of viral mRNA with the numerous cellular mRNAs present during infection.

In eIF4G-depleted extracts, proteinase-cleaved eIF4F stimulated IRES-carrying RNA translation to the extent seen with proteinase itself in nondepleted translation extracts. An attempt to define the active cleavage product suggested that the C-terminal cleavage product of eIF4G (but not the N-terminal product) is the essential component required for translation stimulation. Effectively, when the C- and N-terminal cleavage products of eIF4G, which had been separated by column chromatography, were added to the translation extracts, HRV IRES activity and translation of uncapped mRNA was enhanced by the cp_C-containing fractions, but not the cp_N-containing fractions. These results are in accord with the recent report that cp_C, in the absence of cp_N and eIF4E, can support cap-independent translation of cellular mRNAs and translation from the IRES of either Theiler's murine encephalomyelitis virus or hepatitis C virus (Ohlmann et al., 1996). By the separation methods we used, cp_C and cp_N remain bound to eIF4A and eIF4E, respectively (Lamphear et al., 1995). Because neither eIF4E nor eIF4A are modified directly by the 2A or Lb proteinases, and, given the effects of addition of these proteins as part of intact eIF4F on HRV IRES-driven translation, it seems unlikely that they are responsible for translation stimulation. However, we cannot preclude formally that stimulation requires a combination of cp_C and eIF4A, or that cleavage of eIF4G liberates a more active form of eIF4A. Indeed, translation stimulation mediated by the cp_C-containing fraction was never as impressive as that observed upon the addition of wild-type proteinase. This may indicate that cp_C alone is not sufficient to ensure optimal stimulation of IRES activity.

It was suggested recently that poliovirus 2A proteinase affected cognate IRES function via a direct interaction between proteinase and RNA. This hypothesis was based upon the rescue of translationally defective IRES mutants in vivo by second-site suppressor mutations in the 2A gene (Macadam et al., 1994). Given the results of the experiments described here, in which translation stimulation could be achieved by adding 2A proteinase-treated eIF4F, this hypothesis now seems unlikely. It seems more feasible that the second-site mutations resulted in a 2A proteinase with increased enzymatic activity. Improved cleavage of eIF4G would allow a more efficient shut-off of host cell RNA translation in vivo (and hence reduced competition between cellular messages and a weak IRES), and also a more rapid production of the stimulatory cleavage products of eIF4G. In the future, it will be interesting to examine the infectivity of such revertants in cell lines that express a form of the eIF4G protein that is not cleavable by the 2A proteinase.

MATERIALS AND METHODS

Enzymes and reagents

Restriction endonucleases and DNA-modifying enzymes were from Boehringer Mannheim, Gibco-BRL, or New England Biolabs, and were used according to the suppliers' instructions. Rabbit reticulocyte lysate (Flexi™) was from Promega. $m^7G(5')ppp(5')G$ was from Pharmacia. Elastatinal and E-64 were from Sigma. All other chemicals were of reagent grade.

Proteinases, proteinase inhibitors, and antibodies

The purification of recombinant HRV2 2A and FMDV Lb proteinases has been described previously (Liebig et al., 1993; Kirchweger et al., 1994), as has that of rabbit eIF4F (Lamphear & Panniers, 1990). For the experiments described here, unless otherwise stated, the Lb and 2A proteinases were in buffer A (50 mM Tris, pH 8.0, 50 mM NaCl, 5 mM DTT, 1 mM EDTA, 5% glycerol), purified eIF4F was in buffer B (20 mM MOPS, pH 7.6, 100 mM KCl, 0.25 mM DTT, 0.1 mM EDTA, 0.02% Tween 20, 10% glycerol), and the purified N- and C-terminal cleavage products of eIF4G were in H100 buffer (10 mM HEPES-KOH, pH 7.5, 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 7 mM β-mercaptoethanol). Elastatinal and E-64 were used as inhibitors of the 2A and Lb proteinases,

respectively. Final inhibitor concentrations in translation reactions were 500 μM for elastatinal and 400 μM for E-64. Proteinases were treated with inhibitors at 4 °C for 10 min. Purification of the products of HRV2 2A proteinase-mediated cleavage of eIF4F by m^7GTP-Sepharose column fractionation has been described previously (Lamphear et al., 1995). Briefly, eIF4F (27 μg/mL) was incubated with recombinant HRV2 2A proteinase (8 μg/mL) at 30 °C for 60 min. After inhibition of the 2A proteinase by addition of elastatinal to 500 μM, the reaction mixture was applied to a 100 μL m^7GTP-Sepharose column equilibrated in buffer C (20 mM MOPS, pH 7.6, 100 mM KCl, 0.25 mM DTT, 0.1 mM EDTA, 50 mM NaF, 10% glycerol). The flow-through material was obtained by washing the column with 300 μL of buffer C; bound protein was eluted with buffer C containing 70 μM m^7GTP. The flow-through material and eluate were subjected to buffer exchange for H100 buffer and simultaneous concentration in centricon-10 cells (Amicon). Rabbit anti-eIF4G peptide 6 antiserum (raised against a synthetic peptide corresponding to residues 1230–1248 of human eIF4G) and rabbit anti-eIF4G peptide 7 antiserum (raised against residues 327–342) have been described elsewhere (Yan et al., 1992), as has rabbit anti-eIF4E antiserum (Kerekatte et al., 1995). Mouse monoclonal anti-eIF4A antibody was kindly provided by Dr. Hans Trachsel (Bern, Switzerland).

In vitro transcriptions and translations

The plasmid pXLJ-HRV2 (formerly pXLJ 10-585) has been described previously (Borman & Jackson, 1992). Briefly, it consists of the genes for *X. laevis* cyclin B2 and influenza virus NS protein under the control of a bacteriophage T7 promotor. The two genes are separated by a cDNA fragment corresponding to nt 10–585 of the HRV2 genome, which includes the IRES, such that the AUG codon, which serves for the initiation of NS protein synthesis, is the initiation site for ribosomes that have entered the IRES internally. The plasmid pXLJ-HRV2 was linearized by *Eco*R I for in vitro transcription reactions, which were performed as described previously for the synthesis of both uncapped and artificially capped mRNAs (Ziegler et al., 1995b). Natural globin mRNA was purified from rabbit red blood cells using standard protocols (Sambrook et al., 1989).

In vitro translation reactions were performed in nuclease-treated rabbit reticulocyte lysates (Flexi™-reticulocyte lysate, Promega) that were supplemented with 5% (by volume) HeLa cell S10 extract as described previously (Ziegler et al., 1995b). Translation reactions were programmed with pXLJ-HRV2 or globin mRNAs at 10 and 50 μg/mL, respectively (final RNA concentrations). The volume of reticulocyte lysate was maintained at 70% of the final reaction volume in all cases, and the final concentrations of added KCl and $MgCl_2$ were maintained at 80 mM and 0.5 mM, respectively. All reaction mixtures contained either purified proteins in their different buffers, or the equivalent volume of the appropriate buffer. Protein additions were made in minimal volumes such that the final reactions were always 11 μL.

Analysis of translation products and western blotting

The ^{35}S-methionine-labeled products of in vitro translation reactions were analyzed by SDS-PAGE, as described previously (Dasso & Jackson, 1989), using gels containing 20% acrylamide. Dried gels were exposed to Hyperfilm β-max (Amersham) for 4–24 h. Quantification of translation efficiencies was by densitometric scanning of autoradiographs, using a Sharp JX-330 analysis system linked to MacIntosh image analysis software (NIH image). Three different exposures were scanned to ensure that the linear response range of the film was respected. Where appropriate, translation reactions were also analyzed by western blotting. Electrophoretic separation of proteins was performed as described by Laemmli (1970), using gels containing 6% or 7.5% acrylamide. Transfer of proteins to Hybond C-super (Amersham, Inc.) was acheived using the milli-blotting (Millipore) system; transfer was performed at 4 °C for 2 h at 200 mA. Blots were revealed using the above-mentioned rabbit anti-eIF4E or anti-eIF4G peptide 6 and 7 antisera and with HRP-conjugated anti-rabbit secondary antibodies, or using the mouse monoclonal anti-eIF4A antibodies and with HRP-conjugated anti-mouse secondary antibodies as described previously (Liebig et al., 1993). Alternatively, to increase sensitivity of blotting, detection was performed using the commercial DAB peroxidase substrate kit (Vector Laboratories, Inc.). Silver staining of SDS-polyacrylamide gels was performed as described previously (Morrissey, 1981).

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