The proteolytic cleavage of eukaryotic initiation factor (eIF) 4G is prevented by eIF4E binding protein (PHAS-I; 4E-BP1) in the reticulocyte lysate

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A common feature of viral infection is the subversion of the host cell machinery towards the preferential translation of viral products. In some instances, this is partly mediated by the expression of virally encoded proteases which lead to the cleavage of initiation factor eIF4G. The foot-and-mouth disease virus encodes two forms of a cysteine proteinase (L protease) which bisects the eIF4G polypeptide into an N-terminal fragment containing the eIF4E binding site, and a C-terminal fragment which contains binding sites for eIF4A and eIF3 and which associates with the 40S ribosomal subunit. Previously, we have demonstrated that the cleavage of eIF4G by L protease stimulates the translation of uncapped transcripts encoding cellular proteins and supports internal initiation driven by picornavirus internal ribosome entry segment (IRES) elements. Use of reticulocyte lysates manipulated to deplete them of eIF4E and the N-terminal fragment suggests that the C-terminal fragment of eIF4G is responsible for these effects, and we have now confirmed this by purifying the C-terminal fragment and analysing its effects directly in the absence of L protease. Interestingly, we find that pre-incubation of reticulocyte lysates or ribosomal salt wash fractions with the specific eIF4E binding protein, PHAS-I (eIF4E-BP1), blocks the proteolytic cleavage of eIF4G by L protease. This effect can be reversed by addition of recombinant eIF4E. These data are consistent with a model whereby the L protease cleavage site in eIF4G is inaccessible until a change in conformation is induced by the binding of eIF4E. This may have implications for a role for eIF4E binding in triggering changes that expose other domains in the eIF4G molecule during initiation of translation.

Keywords: eIF4E/eIF4E-BP1/eIF4G/initiation factor/IRES

Introduction

The control of polypeptide synthesis plays an important role in cell proliferation, with physiological regulation of protein synthesis almost always exerted at the level of polypeptide chain initiation. Cap-dependent initiation of translation involves the assembly of initiation factors at the 5' end of mRNA, including the cap-binding protein,

eIF4E, the ATP-dependent RNA helicase, eIF4A, and the eIF4G polypeptide to form the eIF4F complex (reviewed in Hershey, 1989; Merrick, 1992; Rhoads, 1993; Morley, 1994, 1996; Pain, 1996). There is a strong requirement for eIF4E and eIF4G in cap structure-dependent initiation, and the eIF4F complex is believed to promote the unwinding of mRNA secondary structure to facilitate the binding of the 40S ribosomal subunit (reviewed by Morley, 1994, 1996; Pain 1996). Consistent with its proposed regulatory role, eIF4E exists in both phosphorylated and non-phosphorylated forms, and is believed to be regulated both by phosphorylation and the availability of the factor to enter the eIF4F complex. It is now becoming clear that eIF4E can interact specifically with either eIF4G or a new family of regulatory binding proteins (such as PHAS-I, also known as 4E-BP1; Lin *et al.*, 1994; Pause *et al.*, 1994a; Haghighat *et al.*, 1995; Mader *et al.*, 1995; Morley and Pain, 1995; Rau *et al.*, 1996). The amino acid sequence in eIF4G which binds to eIF4E is similar to the sequence in PHAS-I which binds to eIF4E (Haghighat *et al.*, 1995; Mader *et al.*, 1995). Hence, PHAS-I acts to sequester eIF4E and prevent its interaction with eIF4G and the formation of the eIF4F complex (Lin *et al.*, 1994; Pause *et al.*, 1994a; Haghighat *et al.*, 1995; Mader *et al.*, 1995; Azpiazu *et al.*, 1996; Beretta *et al.*, 1996; Kimball *et al.*, 1996; Mendez *et al.*, 1996; Whalen *et al.*, 1996).

Recent work has shed light on the potential role of eIF4G in translation initiation (Lamphear *et al.*, 1995; Mader *et al.*, 1995). These studies have identified an eIF4E binding motif on eIF4G in the N-terminal domain, and likely sites of interaction with eIF4A and eIF3 in the C-terminal domain. Thus, eIF4G seems to mediate joining of the mRNA and ribosomes by interaction with both the cap-binding protein, eIF4E, and with eIF3 already associated with the ribosome. eIF4A is believed to catalyse the unwinding of upstream secondary structures and is recycled through the eIF4F complex during successive rounds of initiation (Pause *et al.*, 1994b). When eIF4G is cleaved by viral proteases such as 2A of poliovirus, coxsackie virus and human rhinovirus (Etchison *et al.*, 1982; Liebig *et al.*, 1993) or the leader (L) protease of foot-and-mouth disease virus (FMDV) (Devaney *et al.*, 1988; Belsham and Brangwyn, 1990; Ohlmann *et al.*, 1995), translation of capped mRNAs is disrupted, but that of picornavirus RNA is maintained (Etchison *et al.*, 1982; Devaney *et al.*, 1988). Picornavirus RNAs are naturally uncapped and possess highly structured sequences [called internal ribosome entry segments (IRESes)] within their 5' untranslated regions (5' UTRs) which direct 40S ribosomal subunits to bind to internal elements rather than at the extreme 5' end (reviewed by Jackson *et al.*, 1990, 1994, 1995). mRNAs possessing an IRES element in the 5' UTR can still be translated when the eIF4G subunit is proteolytically cleaved (Liebig *et al.*, 1993; Ohlmann

et al., 1995, 1996; Ziegler *et al.*, 1995) and several reports have suggested that proteolytic cleavage of eIF4G, rather than merely discouraging translation of competing cellular mRNAs, may exert a positive role in promoting internal initiation on picornavirus RNAs in infected cells (Buckley and Ehrenfeld, 1987; Hambidge and Sarnow, 1992; Scheper *et al.*, 1992; Macadam *et al.*, 1994; Pause *et al.*, 1994b; Lamphear *et al.*, 1995). More direct studies with *in vitro* systems have demonstrated that cleavage of eIF4G enhances translation driven by enterovirus or rhinovirus IRES elements (Liebig *et al.*, 1993; Ziegler *et al.*, 1995), while translation of coding sequences downstream of a cardiovirus IRES element was not affected (Thomas *et al.*, 1992; Ohlmann *et al.*, 1995; Ziegler *et al.*, 1995).

Recently, we (Ohlmann *et al.*, 1996) and others (Ziegler *et al.*, 1995) have demonstrated that in *in vitro* translation systems the cleavage of eIF4G by FMDV L protease stimulates the translation of uncapped transcripts encoding cellular proteins, as well as supporting internal initiation driven by picornavirus IRES elements. Use of reticulocyte lysates manipulated to deplete them of eIF4E and the N-terminal domain of eIF4G suggests that the C-terminal domain of eIF4G is responsible for these effects (Ohlmann *et al.*, 1996). Curiously, we also found that translation of uncapped cellular mRNAs was inhibited by addition of PHAS-I, an effect prevented by prior treatment of the translation system with L protease (Ohlmann *et al.*, 1996). In order to confirm that these effects of L protease were due to generation of the C-terminal fragment, we have now purified this fragment of eIF4G and demonstrated that it stimulates the translation of uncapped mRNA when added directly to the reticulocyte lysate. In addition, we show that the action of L protease in cleaving eIF4G requires the interaction of eIF4E with eIF4G. Cleavage of eIF4G by L protease is prevented by pre-incubation of reticulocyte lysate or ribosomal salt wash with exogenously added PHAS-I, an effect reversed upon addition of excess recombinant eIF4E protein.

Results

The C-terminal domain of eIF4G stimulates the translation of uncapped cyclin mRNA

We showed previously that cleavage of eIF4G in reticulocyte lysates by the FMDV Lb protease exerted little effect on initiation driven by a TMEV IRES and was actually stimulatory for translation of an uncapped transcript encoding a cellular mRNA (Ohlmann *et al.*, 1995, 1996). Moreover, data from experiments employing modified lysate translation systems strongly suggested that the C-terminal cleavage product of eIF4G could support translation of uncapped or IRES-driven mRNAs under conditions where eIF4E and the N-terminal domain of eIF4G were severely depleted (Ohlmann *et al.*, 1996). The first aim of the present work, therefore, was to demonstrate a direct effect of the C-terminal fragment on translation and, accordingly, we attempted to isolate this product.

In the native reticulocyte lysate (not treated with micrococcal nuclease), most of the eIF4G is ribosome associated (Rau *et al.*, 1996). We therefore incubated a 0.5 M KCl wash from reticulocyte ribosomes (see Materials and methods) with L protease for 15 min, then added 300 μ M

elastatinal to inhibit further protease action (Ohlmann *et al.*, 1995, 1996; Ziegler *et al.*, 1995) and subjected the material to m⁷GTP-Sepharose affinity chromatography. The affinity resin absorbs virtually all the eIF4E, together with the N-terminal cleavage product of eIF4G, which includes the eIF4E binding site (Lamphear *et al.*, 1995; Mader *et al.*, 1995), whereas the C-terminal cleavage product is recovered in the run-through (Ohlmann *et al.*, 1996). The run-through fraction was then applied to an FPLC Mono-Q column, which was developed with a 50–600 mM KCl gradient in Buffer B. Figure 1A shows the protein elution profile from Mono-Q. Fractions were examined directly by SDS-PAGE and Western immunoblotting (Figure 1B), using antibodies recognizing eIF4E, eIF4A and the C-terminal half of eIF4G, as described previously (Ohlmann *et al.*, 1996). The elution profile of the C-terminal fragment was quite distinct from that of the residual intact eIF4G which eluted at higher salt. Although there was considerable overlap in the elution profiles of the C-terminal fragment and eIF4A, the bulk of eIF4A eluted in fractions 9 and 10. There is an eIF4A binding site in the C-terminal domain of eIF4G (Lamphear *et al.*, 1995), but it seems unlikely that the elution similarity was simply due to association between these two proteins since the peaks do not superimpose completely (Figure 1B). Each fraction, following concentration, was tested for its ability to influence the translation of capped and uncapped transcripts encoding *Xenopus* cyclin A. As shown in Figure 1C, the peak of stimulatory activity coincided exactly with that of the C-terminal fragment and eluted later than the peak of eIF4A. However, it should be noted that the peak fractions concentrated for use in the experiments to be described, and denoted 'Ct', contained significant amounts of eIF4A as well as the C-terminal domain of eIF4G. They were, however, free of eIF4E and the N-terminal domain of eIF4G. This preparation of the C-terminal fragment is referred to as 'Ct' in the remainder of this paper.

Figure 2 shows the effect of adding different amounts of Ct prepared in this way to translation systems (in the presence of 300 μ M elastatinal) based on native (RRL) and messenger-dependent (MDL) reticulocyte lysates. The addition of Ct did not affect the translation of endogenous globin mRNA in the RRL (Figure 2A) and only slightly stimulated translation of the influenza virus NS' coding sequence driven by the TMEV IRES (JODA 1099; Hunt *et al.*, 1993) in the MDL (Figure 2B). However, translation of uncapped cyclin A mRNA was enhanced in the presence of additional Ct in a dose-dependent manner (Figure 2C). This provides evidence for a direct stimulatory effect of Ct on the translation of uncapped mRNA, observable in the presence of intact eIF4G and in the absence of L protease. The result shown in Figure 2C was obtained with three separate preparations of Ct; in each case the stimulatory activity, though evident following one freeze-thaw cycle, was most pronounced when the fresh preparation was used (see the legend to Figure 1). In the remaining experiments shown in this paper, Ct was added to translation systems at 1 μ l/10 μ l assay. The immunoblots shown in Figure 2D (and Figure 4B) illustrate that this amount of added Ct is similar to the levels of endogenous eIF4G in the system. The samples analysed in Figure 2D were removed from the incubation mixtures at the end of

Fig. 1. Partial purification of the C-terminal domain of eIF4G. (A) Reticulocyte lysate ribosomal HSW was prepared as described in Materials and methods. An aliquot (3.5 ml) was treated with L protease and subjected to $m^7\text{GTP}$ -Sephacrose as described and the unbound material applied to a FPLC Mono Q column equilibrated in Buffer B. Bound proteins were resolved with a 50–600 mM NaCl gradient in the same buffer and 1 ml fractions collected. The resulting A_{254} profile is presented. (B) Samples (10 μl of 1 ml fractions) were resolved by SDS-PAGE (7.5% acrylamide), proteins transferred to PVDF and visualized by immunoblotting with antisera recognizing: upper panel, the C-terminal part of eIF4G; middle panel, eIF4A; lower panel, eIF4E. HSW refers to the starting material prior to cleavage with L protease and HSW+L that following cleavage. RT shows the material which was not retained by the $m^7\text{GTP}$ -Sephacrose column and was the fraction applied to the Mono Q column. (C) Following concentration and re-equilibration in Buffer A, samples (1 μl) were assayed for their ability to influence the translation of capped (hatched bars) or uncapped cyclin mRNA (filled bars) in the MDL translation system (10 μl), as described. Incubations were carried out in the presence of 300 μM elastatinal and were for 45 min at 30°C. Samples (2 μl) were then processed as described to determine the level of $[^{35}\text{S}]$ methionine incorporation into protein (Ohlmann *et al.*, 1996). With the uncapped transcript, incorporation by the same lysate in the absence of L protease treatment was 27 698 c.p.m./2 μl , and following L protease treatment was 97 322 c.p.m./2 μl . These data are representative of those obtained in at least three separate experiments.

the experiment, and the results (lanes 3 and 6) indicate that addition of the Ct preparation did not result in proteolysis of the endogenous eIF4G.

Previously, we found that the ability of MDL systems to translate either capped or uncapped cellular mRNAs and, to a lesser extent, IRES-driven cistrons, was impaired if the systems were pre-treated with $m^7\text{GTP}$ -Sephacrose

to deplete them of most of the endogenous eIF4E (Ohlmann *et al.*, 1996). A possible explanation for this effect is that translation of these mRNAs is sensitive to partial depletion of eIF4G, some of which is removed concomitantly with eIF4E on the affinity resin. In support of this possibility, we found that the inhibitory effect on translation of uncapped or IRES-bearing mRNAs was prevented by

Fig. 2. Fractions enriched for the C-terminal domain of eIF4G stimulate the translation of uncapped mRNA in the reticulocyte lysate in the absence of L protease. Translation assays (10 μ l) based on the reticulocyte lysate (A) or MDL (B and C) were set up as described in Materials and methods, in the presence of 300 μ M elastatinal and in the absence of (A) or presence of 25 μ g/ml uncapped influenza virus NS' driven by the TMEV IRES sequence [JODA 1099; (B)] or uncapped cyclin A (pXLCycA1) mRNA (C) and supplemented with Buffer A or Ct as indicated. Aliquots (2 μ l) were removed after incubation for 45 min at 30°C and processed to measure incorporation of [35 S]methionine into protein. These data are representative of those obtained on at least three separate occasions using different reticulocyte lysates and preparations of Ct. (D) Translation mixes either in the absence or presence of 1 μ l Ct were resolved by SDS-PAGE (7.5% acrylamide), proteins transferred to PVDF and visualized by immunoblotting with antiserum recognizing the C-terminal domain of eIF4G. Lanes 1–3 and 4–6 represent analysis of two separate preparations of Ct in two different lysates; the migration of the intact eIF4G and the C-terminal product is indicated. These data are representative of those obtained in at least three separate experiments.

inducing cleavage of eIF4G in the lysate with L protease prior to the treatment with m⁷GTP–Sephacrose. In the doubly treated lysate, eIF4E and the N-terminal fragment of eIF4G were both reduced to very low levels, but virtually all the C-terminal fragment generated by eIF4G cleavage was retained. The maintenance of full translational activity for uncapped and IRES-bearing mRNAs in such a lysate thus suggested that the requirement for eIF4G could be met by an equivalent molar concentration of the free C-terminal domain (Ohlmann *et al.*, 1996). We have now tested this directly by addition of partially purified Ct to m⁷GTP–Sephacrose-treated lysates. Figure 3A shows an immunoblot analysis of the lysates used in such an experiment. Treatment of an MDL with m⁷GTP–Sephacrose affinity resin resulted in the loss of nearly all the eIF4E, and concomitantly removed ~40% of the eIF4G (compare lanes 1 and 2), which was recovered following elution of the resin with m⁷GTP (lanes 3 and 4). The ability of the depleted and control lysates to translate uncapped cyclin mRNA and NS' driven by the TMEV IRES in the presence and absence of 1 μ l additional free Ct is shown in Figure 3B and C. In each case, prior treatment of the lysate with m⁷GTP–Sephacrose decreased translation, as observed previously (compare 1 and 3, and

5 and 7). Addition of Ct stimulated that of uncapped cyclin mRNA (Figure 3B) and restored the translation of the IRES-driven mRNA to the level seen in lysates treated with uncoupled Sephacrose resin (Figure 3C). Similar data (not shown) have been obtained with at least two different lysates and three preparations of Ct, although there were minor differences between them as to whether or not the addition of Ct completely equalized the translation in m⁷GTP–Sephacrose and control Sephacrose-treated samples (2 versus 4, and 6 versus 8).

Addition of PHAS-I prevents the action of L protease in the reticulocyte lysate

An additional way of depleting translation systems of functional eIF4E, without the complication of concomitant removal of variable amounts of eIF4G, is to add the specific eIF4E binding protein, PHAS-I or 4E-BP1 (Lin *et al.*, 1994; Pause *et al.*, 1994a). Previously, we used this approach to demonstrate that translation of uncapped transcripts encoding cyclin A or cyclin B2 was very sensitive to eIF4E depletion, whereas internal initiation directed by an IRES element was unaffected (Ohlmann *et al.*, 1996). Interestingly, however, the inhibitory effect of PHAS-I was prevented by prior treatment of the lysate

Fig. 3. Fractions enriched for the C-terminal domain of eIF4G are sufficient to promote translation of uncapped and IRES-driven translation in an eIF4E-depleted MDL. **(A)** Reticulocyte MDL (100 μ l) was subjected to batch absorption with m^7 GTP-Sepharose (1 and 3) or Sepharose 4B (2 and 4) as described and the unbound lysate fraction retained. The resins were washed with 4 vols of Buffer A and bound proteins eluted with 150 μ l SDS-PAGE sample buffer. Aliquots (1.5 μ l) of the unbound fraction or eluted fractions (10 μ l) were resolved by SDS-PAGE, proteins transferred to PVDF and visualized by immunoblotting with antisera recognizing eIF4G (upper panel) or eIF4E (lower panel). **(B)** and **(C)** Uncapped versions of cyclin A **(B)** or TMEV IRES-containing mRNA **(C)** were translated in assays based on the reticulocyte lysate (10 μ l) containing 300 μ M elastatinal and subjected to the pre-treatments described above, in the presence of 1 μ l Buffer A (1 and 3) or 1 μ l Ct (2 and 4) as indicated. Following incubation for 45 min at 30°C, aliquots (2 μ l) were removed to measure the incorporation of [³⁵S]methionine into protein. These data are representative of those obtained in at least three separate experiments.

with L protease. To examine more directly the role of the C-terminal domain of eIF4G in this response, we tested the effect of adding PHAS-I, Ct and L protease to lysates in different combinations on the translation of uncapped cyclin A mRNA (Figure 4A). Low levels of L protease were employed in these studies to limit proteolysis of eIF4G to one primary cleavage event, separating the N- and C-terminal domains. The first set of data [condition (i)] shows control levels of translation of this mRNA and the stimulation resulting from the addition of either L protease or Ct at zero time. The apparently greater effectiveness of Ct relative to L protease treatment in stimulating translation probably reflects the fact that these components were added at the beginning of, rather than prior to, the period of translation measurement. There would, therefore, have been a lag period before C-terminal fragments generated by the low levels of L protease employed in these experiments would have accumulated and exerted their full effect. In condition (ii), PHAS-I was added at zero time in the absence or presence of these components. Under these conditions, PHAS-I alone inhibited the translation of uncapped cyclin A mRNA by 70%; these data confirmed the inhibitory effect of the

binding protein, and demonstrated that Ct added directly was able to prevent it. Essentially similar results were obtained [condition (iii)] if PHAS-I was added after a 10 min incubation with L protease or Ct. However, the most interesting result was obtained under condition (iv), where PHAS-I was added at zero time, followed by either L protease or Ct at 10 min. In this case, complete rescue was still achieved by addition of Ct, whereas the stimulatory effect of L protease was completely abrogated. To investigate the basis of this result, we compared the effectiveness of L protease in cleaving eIF4G when added to the lysate either at the same time as [condition (ii)] or 10 min after [condition (iv)] the addition of PHAS-I. The immunoblot in Figure 4B clearly demonstrates that cleavage of eIF4G occurred when L protease was added at the same time as PHAS-I (lane 5), but was severely inhibited when the lysate was pre-incubated for 10 min with the binding protein (lane 11). Since PHAS-I is generally regarded as acting specifically to sequester eIF4E and withhold it from interacting with eIF4G, this raised the interesting possibility that the susceptibility of eIF4G to cleavage by L protease was in some way dependent on the integrity of the eIF4F complex.

Fig. 4. Pre-incubation of MDL with PHAS-I prevents the stimulation of translation uncapped cyclin A mRNA by L protease. **(A)** Translation assays (10 μ l) based on the reticulocyte lysate (MDL) were carried out for 45 min at 30°C in the presence of Buffer A (1, 4, 7 and 10), 1 μ l L protease (2, 5, 8, 11) or 1 μ l Ct (3, 6, 9 and 12) with addition of 300 μ M elastatinal. Incubations were conducted as follows: condition (i), in the absence of PHAS-I (1–3); condition (ii), with PHAS-I added at the same time as the other components (4–6); condition (iii), PHAS-I added 10 min after the start of the translation assay (7–9); condition (iv), PHAS-I added during a 10 min pre-incubation at 30°C prior to the addition of L protease or Ct (10–12). Aliquots (2 μ l) were removed after 45 min further incubation at 30°C and processed to measure incorporation of [35 S]methionine into protein. These data are representative of those obtained on at least three separate occasions using different preparations of Ct. **(B)** At the end of the translation reaction, samples (2 μ l) from assays 4–6 and 10–12 were resolved by SDS-PAGE (7.5% acrylamide), proteins transferred to PVDF and visualized by immunoblotting with antisera recognizing the C-terminal domain of eIF4G; the migration of the intact eIF4G and C-terminal product is indicated. These data are representative of those obtained in at least three separate experiments.

Inhibition of L protease-induced cleavage of eIF4G by PHAS-I is reversed by eIF4E

The simplest explanation of our findings is that eIF4G is only vulnerable to cleavage by L protease when eIF4E is bound to it. A possible mechanism for this would invoke a model where the binding of eIF4E induced a change in conformation of eIF4G that led to the exposure of the cleavage site (see Discussion). Sequestration of eIF4E by the binding protein would therefore shift most of the eIF4G into the protected conformation. To test this model, we examined the ability of exogenously added eIF4E to facilitate cleavage of eIF4G in MDL pre-incubated with PHAS-I. In the experiment shown in Figure 5, samples of lysate were incubated with different amounts of PHAS-I (0.03–1 μ g) for 10 min, followed by the addition of L protease. Cleavage of eIF4G was monitored by immuno-

Fig. 6. The addition of recombinant wild-type, Ala-53, but not Ala-209 eIF4E, induces the cleavage of eIF4G in the presence of PHAS-I. (A) Recombinant (His₆-tagged) PHAS-I (1 µg, lane 1), Ala-53 eIF4E (1 µg, lane 2), wild-type eIF4E (1 µg, lane 3) or Ala-209 (1.5 µg, lane 4) were resolved by SDS-PAGE; the resulting Coomassie-stained gel is presented. The numbers on the left refer to the position of the molecular weight markers, in kDa. (B) Samples of unsupplemented MDL (7 µl) were pre-incubated with 1 µl of Buffer A or PHAS-I (1 µl containing 0.2 µg) for 10 min at 30°C, prior to the addition of 1 µl L protease as indicated. Following a further 10 min incubation, either 1 µl of Buffer A (lanes 1–3), 1 µl recombinant wild-type eIF4E (WT; containing 0.05, 0.1 and 0.5 µg; lanes 4–6), Ala-53 eIF4E (0.025, 0.05 and 0.25 µg; lanes 7–9) or Ala-209 eIF4E (0.1, 0.2 and 1 µg; lanes 10–12) was added for 10 min at 30°C. Aliquots (2 µl) were then resolved by SDS-PAGE, proteins transferred to PVDF and visualized by immunoblotting with antisera recognizing the C-terminal domain of eIF4G. The position of the intact eIF4G and C-terminal product is indicated. These data are representative of those obtained in at least three separate experiments.

Inhibition of L protease-induced cleavage of eIF4G by PHAS-I occurs in a ribosomal high-salt wash

To investigate this further, we have carried out similar experiments using a high-salt fraction from reticulocyte ribosomes to test whether the effect could be seen with eIF4G present in eIF4F complexes released from ribosomal association. Samples of high-salt wash (HSW) prepared as described were incubated with PHAS-I for 10 min prior to addition of L protease. The degree of eIF4G degradation after a further 20 min of incubation was assessed by immunoblotting (Figure 7). Virtually complete cleavage of eIF4G in response to L protease was observed in the control (lane 1), and this was strongly inhibited by prior incubation of the HSW with PHAS-I (lane 2). We then compared the effect on eIF4G cleavage of adding increasing quantities of either the wild-type or the Ala-209 mutant eIF4E protein. As with the experiment with MDL shown in Figure 6, amounts of wild-type eIF4E in excess of added PHAS-I were effective in restoring the ability of eIF4G to be cleaved by L protease. However, in the presence of exogenous PHAS-I, the inclusion of the Ala-209 mutant eIF4E up to levels 4-fold higher than that employed for the wild-type protein was ineffective in restoring the cleavage of eIF4G. A GST-4E fusion protein purified via m⁷GTP-Sepharose was also ineffective in

Fig. 7. eIF4E can reverse the inhibitory effect of PHAS-I on cleavage of eIF4G by L protease in the absence of ribosomes. Samples (2.5 µl) of HSW were diluted to 7 µl with Buffer A and pre-incubated (where indicated) with 2 µg PHAS-I for 10 min at 30°C. Where indicated, L protease (1 µl) was then added and the incubation continued for a further 10 min prior to the addition of 1 µl Buffer A (lanes 1 and 2), 1 µl wild-type eIF4E (containing 0.25, 0.5, 1, 2 µg; lanes 3–6) or Ala-209 eIF4E (0.25, 1, 2, 2.5 µg) for 10 min at 30°C. Samples (5 µl) were then resolved by SDS-PAGE, proteins transferred to PVDF and visualized by immunoblotting with antisera recognizing the C-terminal domain of eIF4G. The position of the intact eIF4G and C-terminal product is indicated. These data are representative of those obtained in at least three separate experiments.

these assays (data not shown). Possible reasons for this are discussed later.

Ribosomes isolated from reticulocyte lysate following prior treatment with PHAS-I are depleted in eIF4E and resistant to the action of L protease

In an attempt to understand how PHAS-I protects the eIF4G from cleavage by L protease, we have analysed the effect of PHAS-I pre-treatment on the subcellular distribution of eIF4E. To this end, MDL was incubated in the absence or presence of PHAS-I for 10 min and the ribosomes isolated as described previously (Ohlmann *et al.*, 1996). The initiation factors associated with the ribosomes were analysed by SDS-PAGE and immunoblotting. As shown in Figure 8A, addition of PHAS-I resulted in >90% depletion of eIF4E from the ribosomes, with loss of ~50% of the eIF4G. The resuspended ribosomes, depleted of eIF4E and PHAS-I, were then assessed for the ability of L protease to cleave the associated eIF4G. In the case of control ribosomes (isolated from MDL that had not been pre-treated with PHAS-I) with associated initiation factors (Figure 8A, lane 1), the addition of L protease resulted in the efficient cleavage of eIF4G (compare Figure 8B, lanes 1 and 2). When PHAS-I was added to these ribosomes for 10 min prior to the L protease, the eIF4G was protected even in the presence of low levels of added wild-type recombinant eIF4E (lane 3). However, the addition of a 2-fold excess of eIF4E relative to PHAS-I reversed the PHAS-I effect and allowed the cleavage of eIF4G. In the case of the ribosomes from the PHAS-I-pre-treated MDL, which were severely depleted in eIF4E (Figure 8A), the associated eIF4G was protected from cleavage by L protease (Figure 8C, compare lanes 1 and 2). Addition of wild-type eIF4E resulted in the efficient cleavage of this eIF4G (compare lanes 1, 3 and 4). We have also tested the ability of the Ala-53 and Ala-209 mutant eIF4E proteins to promote efficient cleavage of eIF4G in the PHAS-I-pre-treated ribosomes. As shown in Figure 8D, in the absence of PHAS-I and eIF4E, both the Ala-53 and Ala-209 variants

Fig. 8. Treatment of MDL with PHAS-I removes eIF4E from the ribosomes and prevents the cleavage of eIF4G by L protease on the ribosome. (A) Reticulocyte MDL (100 μ l) was incubated with Buffer A or PHAS-I (5 μ g) for 10 min prior to the sedimentation of ribosomes in a Beckman TL100 centrifuge, as described (Ohlmann *et al.*, 1996). Ribosomes were resuspended in 100 μ l Buffer A and samples (5 μ l) analysed by SDS-PAGE, and the proteins visualized by immunoblotting with antiserum recognizing eIF4E and eIF4G. Lane 1, control ribosomes; lane 2, ribosomes isolated from MDL pre-incubated with PHAS-I. The position of eIF4E and intact eIF4G is indicated. (B) Samples of the resuspended control ribosomes [5 μ l; (A), lane 1] were incubated for 10 min at 30°C in the absence (lanes 1, 2) or presence of PHAS-I (1 μ g; lanes 3, 4), prior to the addition of L protease (lanes 2–4) for 10 min at 30°C. Wild-type recombinant eIF4E (1 μ l containing 0.2 or 1 μ g; lanes 3, 4, respectively) was then added and the incubation continued for a further 10 min. Samples (10 μ l) were then resolved by SDS-PAGE, proteins transferred to PVDF and visualized by immunoblotting with antiserum recognizing the C-terminal domain of eIF4G. (C) Ribosomes (5 μ l) isolated from MDL pre-incubated with PHAS-I, and hence severely depleted in eIF4E [(A), lane 2], were incubated with 1 μ l of Buffer A (lane 1) or L protease (lanes 2–4) for 10 min at 30°C, prior to the addition of 1 μ l Buffer A (lanes 1 and 2) or wild-type recombinant eIF4E (containing 0.2 or 1 μ g; lanes 3, 4, respectively) for a further 10 min. Samples (10 μ l) were then processed as in (B). (D) Ribosomes (5 μ l) were incubated as in (C), in the absence (lane 1) or presence of L protease (lanes 2–5), prior to the addition of Buffer A (lanes 1 and 5) or Buffer A containing 1 μ g wild-type (wt, lane 2), Ala-53 (lane 3) or Ala-209 recombinant eIF4E (lane 4). Samples were then processed as in (B). These data are representative of those obtained in at least three separate experiments.

of eIF4E were able to promote cleavage of eIF4G to a level similar to that of the wild-type recombinant protein. These data, therefore, are consistent with the requirement for eIF4E in the efficient cleavage of ribosome-associated eIF4G.

Recombinant eIF4E is able to form a stable complex with native eIF4G, recombinant GST-4G_{409–526} or PHAS-I

The most likely mechanism by which eIF4E potentiates the cleavage of eIF4G by L protease would involve interaction between the two factors to form an eIF4F complex. To test this directly, reticulocyte HSW was incubated with a low level of recombinant PHAS-I for 15 min and absorbed onto m⁷GTP-Sepharose to remove both eIF4E and PHAS-I. Aliquots of the unbound material were then incubated with either wild-type, Ala-53 or Ala-209 recombinant eIF4E for 10 min, and added eIF4E and the associated eIF4G were recovered by m⁷GTP-Sepharose affinity chromatography. As shown in Figure

9A (lane 1), as expected, no eIF4G was recovered in the absence of added eIF4E. However, following addition of any of the three forms of recombinant eIF4E (lanes 2–4), both intact eIF4G and Nt present in the preparation at low levels were recovered, directly indicating an interaction between eIF4G and exogenous eIF4E. This interaction was further analysed using a recombinant glutathione S-transferase (GST)–4G fusion protein (GST–4G_{409–526}) encompassing the eIF4E binding site (amino acids 409–526), as described previously by Mader *et al.* (1995). Glutathione-Sepharose-immobilized GST or GST–4G_{409–526} proteins were incubated with wild-type, Ala-53 or Ala-209 variants of eIF4E, the resin recovered, washed, and the amount of associated eIF4E visualized by SDS-PAGE and immunoblotting (Figure 9B). Although there was a low level of non-specific interaction of eIF4E with the control resin (lanes marked GST), recovery of recombinant eIF4E protein was greater with GST–4G_{409–526}, with all variants of eIF4E able to interact with the latter to a similar level in this type of assay. As shown

Fig. 9. (A) Added recombinant eIF4E forms a complex with native eIF4G, with recombinant GST-4G₄₀₉₋₅₂₆ or with PHAS-I. Reticulocyte HSW (200 μ l) was incubated with PHAS-I (5 μ g) for 15 min prior to m⁷GTP-Sepharose affinity chromatography. The unbound fraction (10 μ l) was incubated with Buffer A (lane 1), or 5 μ g of wild-type (wt, lane 2), Ala-53 (lane 3) or Ala-209 (lane 4) recombinant eIF4E for 10 min at 30°C and batch absorbed onto m⁷GTP-Sepharose [30 μ l of a 50% (v/v) solution in Buffer A] for 10 min on ice. The resin was isolated, washed three times with 0.25 ml Buffer A and the bound proteins eluted with 0.2 mM m⁷GTP in Buffer A. Samples were then resolved by SDS-PAGE, proteins transferred to PVDF and visualized by immunoblotting with antiserum recognizing the N-terminal domain of eIF4G. (B) Aliquots of GST or GST-4G₄₀₉₋₅₂₆ (8 μ g) were immobilized on glutathione-Sepharose (10 μ l), the resin washed and resuspended in Buffer A containing 100 mM NaCl and 50 μ g/ml bovine serum albumin (BSA), and incubated for 10 min at 30°C with 0.5 μ g/ml wild-type, Ala-53 or Ala-209 recombinant eIF4E as indicated. The resin was isolated by centrifugation, washed three times with 0.5 ml of the above buffer and retained proteins eluted with 25 mM reduced glutathione in Buffer A. Samples were then resolved by SDS-PAGE, proteins transferred to PVDF and visualized by immunoblotting with antiserum recognizing eIF4E. (C) His₆-PHAS-I (0.5 μ g) was incubated in 10 μ l Buffer A containing 100 mM NaCl and 50 μ g/ml BSA, in the presence of 0.05 μ g wild-type (lane 1), Ala-53 (lane 2) or Ala-209 eIF4E (lane 3) for 10 min at 30°C. NTA agarose [20 μ l of 50% (v/v) in Buffer A] or m⁷GTP-Sepharose [20 μ l of 50% (v/v) in Buffer A] was added for 10 min on ice, the resin isolated by centrifugation and washed three times with 0.5 ml of the above buffer. Protein retained by the NTA agarose (upper panel) was eluted with 100 mM EDTA in Buffer A and that retained by m⁷GTP-Sepharose (lower panel) by 0.2 mM m⁷GTP in Buffer A. Samples were then resolved by SDS-PAGE, proteins transferred to PVDF and visualized by immunoblotting with antiserum recognizing eIF4E (upper panel) or PHAS-I (lower panel). These data are representative of those obtained in at least three separate experiments.

in Figure 9C, following incubation of recombinant (His₆)-tagged PHAS-I with eIF4E, the wild-type (lane 1), Ala-53 (lane 2) and Ala-209 (lane 3) recombinant eIF4E proteins were equally able to interact with PHAS-I, whether the putative complex was isolated by affinity matrices that extracted the (His₆)-PHAS-I with nickel-agarose (NTA agarose, upper panel) or the eIF4E with m⁷GTP-Sepharose (lower panel).

Discussion

In this paper, we show first that an extensively purified preparation of the C-terminal fragment of eIF4G (Ct) can enhance translation of uncapped cyclin mRNA in unfractionated lysates (Figure 2), in lysates depleted of eIF4E and part of their eIF4G by treatment with m⁷GTP-Sepharose (Figure 3), and in lysates treated with PHAS-I to inactivate eIF4E (Figure 4A). As a precaution, we have included elastatinal (300 μ M) during the purification and use of Ct in order to negate any possible contribution from contaminating L protease in these assays (Ohlmann *et al.*, 1996). Therefore, these data confirm that the Ct preparation can reproduce the effects of eIF4G cleavage in the reticulocyte lysate, even in the presence of the intact factor.

In the course of our studies on the influence of PHAS-I treatment on the effects of Ct (Figure 4A), we revealed a particularly interesting effect, which led us to undertake the remaining work described in this paper. We observed that the effect of L protease, at a level normally very

potent in enhancing translation of uncapped cyclin A mRNA, was partially inhibited by the addition of PHAS-I at the start of the incubation [Ohlmann *et al.* (1996) and condition (ii)], but almost completely blocked by prior treatment of the lysate with PHAS-I [condition (iv)]. Examination of the endogenous eIF4G by immunoblotting (Figure 4B) revealed that the pre-incubation with PHAS-I had protected most of the factor from cleavage by L protease. This ability of PHAS-I to render the factor resistant to cleavage was dose dependent (Figure 5), and could also be demonstrated in a ribosomal salt wash (Figure 7) and, conversely, in a preparation of ribosomes isolated from a PHAS-I-treated lysate (Figures 8C and D). In the last case, virtually all the PHAS-I had been removed from the system prior to the treatment of the ribosomes with L protease, indicating that the inhibitory effect was not the result of direct interaction of the PHAS-I with the L protease.

A possible explanation for the observation that pre-treatment of the lysate or ribosomal salt wash with PHAS-I reduces the ability of eIF4G to be cleaved by L protease is that the accessibility of the primary cleavage site may depend on the association of eIF4G with eIF4E as part of an eIF4F complex. We therefore tested whether adding back a preparation of recombinant eIF4E would restore the sensitivity of eIF4G to L protease in a system that had been blocked by PHAS-I treatment. Figure 5 shows that addition of recombinant, wild-type eIF4E could indeed release the block on eIF4G proteolysis exerted by pre-incubation of lysates in the presence of PHAS-I; similar

effects were seen in experiments with ribosomal salt wash (Figure 7) and with ribosomes isolated from PHAS-I-treated lysates (Figure 8). The effectiveness of eIF4E in this assay depended, as would be expected, on the relative concentrations of the factor and whether PHAS-I was still present in the assay.

We also examined the ability of two mutant forms of eIF4E to reverse the effect of PHAS-I. A variant in which Ser-53 was mutated to Ala, which is reported to be inactive in the formation of initiation complexes (Joshi-Barve *et al.*, 1990), was just as effective as the wild-type protein, whereas mutation of Ser-209 to Ala rendered the protein ineffective (Figures 6 and 7). In a similar manner, whilst wild type and Ala-53 eIF4E were both effective, Ala-209 eIF4E was unable to rescue translation in a PHAS-I-treated mRNA-dependent reticulocyte lysate. However, under conditions where the PHAS-I and endogenous eIF4E had been removed prior to the addition of L protease, the Ala-209 eIF4E was able to: form a complex with eIF4G which remained in a ribosomal HSW following treatment with PHAS-I (Figure 9A); in a purified system, form a complex with either a recombinant GST-4G₄₀₉₋₅₂₆ fusion protein containing the eIF4E binding site (Figure 9B; Mader *et al.*, 1995) or with recombinant PHAS-I (Figure 9C), to a similar level seen with the wild-type protein. It is important in interpreting these data to remember that whilst Ser-209 has been identified as the major physiological phosphorylation site on eIF4E in mammalian cells (Flynn and Proud, 1995; Joshi *et al.*, 1995), it is not clear at present whether inability to be phosphorylated at this site plays any role in the effect we observe here.

These observations would be consistent with a working model shown in Figure 10, whereby, in the absence of eIF4E, eIF4G assumes a conformation in which the L protease cleavage site is inaccessible to enzyme attack. In the present work, pre-incubation of the system with excess PHAS-I would serve to sequester the eIF4E away from the eIF4G and push it into this conformation. Binding of eIF4E to the specific region identified in the N-terminal domain of eIF4G (Lamphear *et al.*, 1995; Mader *et al.*, 1995) would result in a change of conformation of the protein, exposing the protease-sensitive hinge region. In addition, one might speculate, as suggested in Figure 10, that at the same time, the binding site for eIF4A in the C-terminal domain (Lamphear *et al.*, 1995) becomes accessible. Such a mechanism might explain our rather puzzling observations (Figure 4A; Ohlmann *et al.*, 1996) that (i) PHAS-I is severely inhibitory to the translation of uncapped transcripts and (ii) the inhibition is rescued by prior cleavage of eIF4G or by addition of free Ct, both of which could recruit eIF4A to the ribosome. The apparent requirement for eIF4E for translation of an uncapped transcript may thus have little to do with its cap-binding activity, but reflect a role for the factor in promoting a change in conformation of eIF4G which opens up the binding sites for other initiation factors on this scaffold protein. This step would, of course, be by-passed in cells infected by rhinoviruses, enteroviruses and FMDV, which encode proteases that bring about cleavage of eIF4G to liberate free C-terminal domains that are accessible to binding by eIF4A and/or other factors. Recently, in the yeast *Saccharomyces cerevisiae*, it has been shown that poly(A) binding protein can specifically interact with

Fig. 10. Potential mechanism for the ability of PHAS-I and eIF4E to influence the susceptibility of eIF4G to cleavage by L protease. The model is modified from that of Lamphear *et al.* (1995). When eIF4E is fully sequestered by PHAS-I, eIF4G assumes a conformation, possibly involving interaction between the N-terminal and C-terminal domains, that protects the intervening hinge region from attack by L protease. The factor is depicted in association with the 40S ribosomal subunit (Joshi *et al.*, 1994), most likely via eIF3, but this is not essential to the model proposed. When eIF4E is added, or released from association with PHAS-I, it binds to the N-terminal domain of eIF4G (Mader *et al.*, 1995) and triggers a conformational change that renders the primary cleavage site in the hinge region of the molecule more susceptible to L protease. More speculatively, we propose that the binding of eIF4E may disrupt the interaction between the N- and C-terminal domains of eIF4G, and thus facilitate the binding of eIF4A to a site near the C-terminus (Lamphear *et al.*, 1995). This would be consistent with our observation that translation of uncapped cyclin A mRNA is severely inhibited by PHAS-I when eIF4G is intact, but potentiated when eIF4G is pre-cleaved with L protease (this paper, Figure 4; Ohlmann *et al.*, 1996).

eIF4G. The site of interaction has been mapped to a site proximal to the eIF4E binding site of eIF4G in the Nt domain (A. Sachs, personal communication). It is plausible, although not directly tested, that poly(A) binding protein can also bind to eIF4G and alter its conformation in a manner similar to that of eIF4E.

In the experimental context of our present work, it appears that the addition of recombinant eIF4E facilitates eIF4G cleavage by binding directly, but we cannot discount the possibility that it interacts with the PHAS-I to release the endogenous eIF4E. The inability of the Ala-209 mutant form of eIF4E to rescue eIF4G proteolysis in the presence of added PHAS-I is of considerable potential interest, but there is no evidence at present to link this result to the function of Ser-209 as a phosphorylation site. Indeed, although phosphorylation of eIF4E has been reported to increase its affinity for the cap structure (Minich *et al.*, 1994), clearly phosphorylation of eIF4E is not a pre-requisite for complex formation with eIF4G under the conditions employed above (Figure 9). It is possible that the Ala-209 mutation results in a change in conformation that affects its ability to interact with endogenous PHAS-I and release associated eIF4E, or affects its interaction with other, as yet unidentified, potential proteins involved

in eIF4F complex formation. In this context, it is of interest to note that a GST-eIF4E fusion protein also failed to work in this assay (data not shown). Finally, we wish to point out that, while models of the initiation pathway are still at variance as to whether or not the interaction between eIF4G and eIF4E occurs on the 40S ribosomal subunit (reviewed in Pain, 1996), our results here appear to be similar for experiments conducted in the lysate (Figure 5 and 6), in the absence of ribosomes (Figure 7) or with ribosome-associated eIF4G (Figures 8 and 9).

Materials and methods

Expression and purification of recombinant proteins

The recombinant Lb form of the L protease protein (0.03 µg/µl) and (His)₆-tagged PHAS-I protein (5 µg/µl) were expressed in BL21(DE₃) bacteria (plasmids provided by T.Skern, Institute of Biochemistry, University of Vienna, and J.Lawrence, Jr, Washington University School of Medicine, WA, respectively) and the proteins purified as described previously (Ohlmann *et al.*, 1996). The purified Lb preparation is referred to as L protease. Wild-type recombinant eIF4E (wt), eIF4E containing a Ser-53 to Ala-53 substitution (Ala-53; plasmids provided by R.Jagus, Maryland Biotechnology Institute, Center for Marine Biotechnology, MD) or a Ser-209 to Ala-209 substitution (Ala-209; plasmid provided by Dr Z.Damuni, Pennsylvania State University, Hershey, PA) were expressed in BL21(DE₃) bacteria, and the proteins purified by staged dialysis and m⁷GTP-Sepharose chromatography as described by Jagus *et al.* (1993). During the final step for the above proteins, an overnight dialysis was carried out against Buffer A [20 mM MOPS-KOH (pH 7.2), 10 mM NaCl, 25 mM KCl, 1.1 mM MgCl₂, 0.1 mM EDTA, 7 mM β-mercaptoethanol]; therefore, Buffer A was added to parallel control incubations throughout the experiments described. Plasmids encoding GST and a GST-4G fusion protein (GST-4G₄₀₉₋₅₂₆) containing the eIF4E binding site (amino acids 409–526) were provided by S.Mader and N.Sonenberg, McGill University, Montreal, Canada, and recombinant proteins expressed as described (Mader *et al.*, 1995).

m⁷GTP-Sepharose chromatography

For batch absorption, m⁷GTP-Sepharose 4B (Pharmacia) resin was washed extensively in Buffer A, pre-treated with cytochrome C [100 µg/ml in a 50% (v/v) suspension of resin in buffer] and Buffer A was removed by aspiration using a needle attached to a vacuum line. Depletion of eIF4E was carried out by adding 1 vol of m⁷GTP-Sepharose resin to 3 vols of MDL, the mix gently agitated for 10 min on ice, centrifuged for 30 s in a microfuge, and the unbound fraction removed and utilized for *in vitro* translation. In each case, a parallel incubation was carried out with the control matrix Sepharose-4B. For column chromatography of the HSW fraction, m⁷GTP-Sepharose 4B (1 ml) was equilibrated in Buffer B [40 mM MOPS-KOH (pH 7.2), 50 mM NaCl, 2 mM benzamidine, 10 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM EDTA, 7 mM β-mercaptoethanol, 0.5 mM EGTA].

Preparation of the ribosomal high-salt wash fraction

Rabbit reticulocyte lysate (200 ml) was supplemented with 2 mM benzamidine, 10 µg/ml leupeptin and 1 mM PMSF prior to centrifugation at 42 000 r.p.m. for 2 h in a Beckman Ti60 rotor at 4°C. The resulting ribosomal pellet was resuspended in 20 ml of Buffer C [40 mM MOPS-KOH (pH 7.2), 500 mM KCl, 6.1 mM MgCl₂, 0.1 mM EDTA, 7 mM β-mercaptoethanol, 2 mM benzamidine, 10 µg/ml leupeptin, 1 mM PMSF], re-centrifuged as above and the HSW fraction removed. The HSW was concentrated by the addition of solid ammonium sulfate to 60% saturation, the precipitate isolated by centrifugation, resuspended in Buffer B and dialysed overnight at 4°C against the same buffer. The HSW fraction (7 ml) was frozen in liquid nitrogen in aliquots prior to use.

Isolation of the C-terminal domain of eIF4G

HSW (3.5 ml) was incubated with L protease (10.5 µg) for 15 min at 30°C prior to the addition of 300 µM elastatinal (Sigma) and 1 mM PMSF. The HSW, containing cleaved eIF4G, was then absorbed onto m⁷GTP-Sepharose (in Buffer B supplemented with 300 µM elastatinal) to remove the eIF4E and N-terminal domain of eIF4G (Ohlmann

et al., 1996). The run-through fraction was then subjected to FPLC chromatography on Mono Q (Pharmacia) and the bound proteins resolved with a 50–600 mM NaCl gradient in Buffer B. Fractions enriched for the C-terminal domain were concentrated and desalted into Buffer A using Microcon-10 units (Amicon) prior to storage in aliquots at –70°C. This preparation is referred to as 'Ct' in the text.

mRNA preparation

Plasmid DNA encoding *Xenopus laevis* cyclin A (pXLCycA; kindly provided by N.Standart) was linearized with *Bam*HI (Ohlmann *et al.*, 1995, 1996). Plasmid DNA encoding the influenza virus NS' reporter driven by Theiler's murine encephalomyelitis virus (TMEV) IRES sequence (JODA 1099; Hunt *et al.*, 1993; kindly provided by A.Kaminski) was linearized with *Eco*RI; uncapped mRNA was transcribed *in vitro* with T7 RNA polymerase (Promega) as described previously (Ohlmann *et al.*, 1995, 1996).

Translation reactions

Rabbit reticulocyte lysates (RRL) and mRNA-dependent reticulocyte lysates (MDL) were prepared in the laboratory by the method of Jackson and Hunt (1983). In all cases, translation reactions contained 50% (v/v) reticulocyte lysate with the addition of the following (final concentrations): 12.5 µM haemin, 75 mM KCl, 0.8 mM magnesium acetate, 50 µM each amino acid (except methionine and leucine), 200 µM leucine, 3 mM D-glucose, 5 mg/ml creatine phosphate, 25 µg/ml creatine phosphokinase, 15 mM 2-aminopurine, 2 mM dithiothreitol, 50 µg/ml calf liver tRNA, 300 U/ml human placental RNase inhibitor and 25 µg/ml mRNA. Translation reactions were performed with 200 µCi [³⁵S]methionine/ml assay in the absence or presence of 300 µM elastatinal as indicated; at the times shown in the individual figure legends, aliquots (2 µl) were removed and processed to measure trichloroacetic acid-precipitable radioactivity.

SDS-PAGE analysis and immunoblotting

SDS-PAGE and immunoblotting were as described previously (Morley and Pain, 1995; Ohlmann *et al.*, 1995, 1996). Antibodies used for immunoblotting were: polyclonal antiserum directed against either peptide 7 (Morley and Pain, 1995) or peptide 6 (a kind gift from Dr R.E.Rhoads, Louisiana State University, LA) of the eIF4G polypeptide [following cleavage with L protease, peptide 7 is in the N-terminal part and peptide 6 in the C-terminal part of eIF4G, as described previously (Yan *et al.*, 1992)]; eIF4E polyclonal rabbit anti-peptide antiserum (Morley and Pain, 1995); eIF4A polyclonal rabbit anti-peptide antiserum raised against the peptide sequence DLPANRENYIHRTGRGGRFGRK. Immunoblots were visualized with an alkaline phosphatase-coupled second antibody (Sigma), as per the manufacturer's instructions.

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