Proteolytic cleavage of initiation factor eIF-4 γ in the reticulocyte lysate inhibits translation of capped mRNAs but enhances that of uncapped mRNAs

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ABSTRACT

Infection of cells with the foot-and-mouth-disease virus, a member of the picornavirus family, results in the shut-off of host protein synthesis. A major contributory mechanism is the proteolytic destruction of the γ subunit of the complex eIF-4, which functions in translation to promote the binding of the 43S ribosomal preinitiation complex to the 5' end of cellular mRNA molecules bearing a 5' terminal cap structure. Picornavirus RNA molecules, which are uncapped, use a distinct mechanism for translational initiation, which can operate in the absence, or at low levels, of eIF-4. The proteolysis of eIF-4 γ in cells infected by foot-and-mouth-disease virus results from expression of a virus-encoded cysteine proteinase known as Leader (or L) protease. We have used a transcription plasmid encoding this protease as a tool to deplete *in vitro* translation systems of eIF-4 γ in order to elucidate in more detail the role of this polypeptide in the control of translation. Using *in vitro* transcribed mRNAs we have observed a marked contrast between capped and uncapped transcripts in the response of their translation to the proteolysis of eIF-4 γ . Translation of capped mRNAs is, as expected, severely impaired, and is restored by addition of eIF-4 complex containing the intact γ -subunit. On the other hand, translation of uncapped transcripts, normally inefficient, is substantially enhanced. The data suggest that the translation of uncapped mRNAs may be stimulated in this system by one or more of the proteolytic degradation products of eIF-4 γ .

INTRODUCTION

A common feature of viral infection of mammalian cells is the diversion of the cell's translational capacity from the synthesis of normal cellular proteins towards the preferential translation of viral products. Viruses have evolved a wide variety of mechanisms to achieve this effect [see review by Sonenberg (1)]. A key mechanism utilized by some, but not all, picornaviruses involves the proteolytic destruction of one of the polypeptides (termed

eIF-4 γ or p220) which associate to form the translational initiation factor complex eIF-4 (2-4). This proteolysis results in a profound inhibition of translation of cellular mRNAs, most or all of which bear a 5'-terminal 7-methylguanosine cap, which is recognized by eIF-4 during the initiation process (5). Picornavirus RNAs, on the other hand, are naturally uncapped and bear long 5'-untranslated regions which include highly structured elements (internal ribosome entry segments, or IRES) that direct initiating 40S ribosomal subunits to bind internally rather than at the extreme 5' end. Cells infected with the picornaviruses polio, foot and mouth disease (FMDV) or human rhinovirus show severe inhibition of translation of host cell mRNAs, whereas that of picornavirus RNAs occurs actively. This suggests that translation of the picornavirus messages does not require intact eIF-4 complex.

The precise role of eIF-4 γ is still not clear, and the studies described above provide the strongest evidence that this polypeptide has an essential function in the translation of capped mRNAs. A number of recent reports have indicated a correlation between cellular rates of translation and the degree of association of eIF-4 γ with the cap-binding protein, eIF-4 α , to form the eIF-4 complex (6-8); this association in turn may be modulated by the phosphorylation of one or both components in response to physiological signals (9). Moreover, treatment of the isolated eIF-4 complex with protein kinases that preferentially phosphorylate eIF-4 γ (rather than eIF-4 α) was found to enhance its ability to bind mRNA and to promote translation in an *in vitro* system (10).

A considerable amount of effort has been directed towards identifying the enzymes responsible for the proteolysis of eIF-4 γ in picornavirus-infected cells. Important roles have been identified for the virally-encoded 2A proteinase in cells infected with poliovirus and human rhinovirus and for the Leader or L protease in FMDV-infected cells (3,11-14). However, in the former case there is conflicting evidence between recent work indicating direct proteolysis of eIF-4 γ by the 2A proteinase (13,15,16) and earlier studies implicating the activation of a cascade of cellular proteases (11,17). This uncertainty, however, has not prevented the recent exploitation of this mechanism to examine the requirement for intact eIF-4 complex for the translation of specific mRNAs in *in vitro* translation systems (13,18). The primary aim of the present work was to utilize the FMDV-encoded L protease to develop a reticulocyte lysate translation

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system depleted of eIF-4 γ , which could be used to investigate the function of this factor and to assay eIF-4 preparations derived from cells in different growth states. During the characterization of this system we found, unexpectedly, that L protease treatment of reticulocyte lysates significantly enhanced the translation of uncapped transcripts which did not bear the structural features of picornavirus RNAs that direct internal initiation. Our results suggest a role for the eIF-4 γ -derived degradation products in cap-independent translation.

MATERIALS AND METHODS

Preparation of L protease by *in vitro* transcription and translation

The plasmid pMM-1, encoding both the Lab and Lb forms of FMDV L protease behind the T7 promoter (19) was kindly provided by Dr G. Belsham, AFRC Institute for Animal Health, Pirbright, UK. Uncapped mRNA was prepared by *in vitro* transcription. Reactions, based on the method of Krieg and Melton (20), contained 40 mM Tris-HCl, pH 7.5, 50 mM NaCl, 8 mM MgCl₂, 2 mM spermidine, 30 mM dithiothreitol, 1.6 mM each of ATP, GTP, UTP and CTP, 300 U/ml placental ribonuclease inhibitor (RNAGuard, Pharmacia), 20 U/ml yeast inorganic pyrophosphatase, 40 μ g/ml linear template DNA and 400 U/ml T7 RNA polymerase (Ambion). Translation was carried out in messenger dependent reticulocyte lysates prepared in the laboratory as described (21). Translation reactions contained 50% messenger-dependent lysate plus the following (final concentrations): 12.5 μ M haemin, 75 mM KCl, 0.8 mM magnesium acetate, 50 μ M each amino acid (except methionine and leucine), 200 μ M leucine, 3 mM D-glucose, 5 mg/ml creatine phosphate, 25 μ g/ml creatine phosphokinase, 15 mM 2-aminopurine, 2 mM dithiothreitol, 50 μ g/ml calf liver tRNA, 300 U/ml human placental RNAase inhibitor (RNAGuard, Pharmacia) and 20 μ g/ml mRNA. After 50 min incubation at 30°C, the translation mixture was treated to remove pMM-1 mRNA by incubation for 10 min at room temperature in the presence of 2 mM additional CaCl₂ and 3 U micrococcal nuclease (Boehringer). This digestion was terminated by the addition of 4 mM EGTA. Postribosomal supernatant was then prepared by centrifugation of this mixture at 435 000 g for 30 min in the Beckman TL-100 ultracentrifuge and rapidly frozen in liquid nitrogen. This preparation is referred to as 'L protease' for the remainder of this paper. A control postribosomal supernatant was prepared in parallel from a translation mixture incubated in the absence of pMM-1 mRNA.

Preparation of messenger RNAs for translation experiments

Plasmids used to generate the *in vitro* transcripts used in this work are shown in Figure 1. Plasmid pXlcycaA, encoding *Xenopus laevis* cyclin A (22), and the bicistronic construct pXLJODA [referred to as pXLJODA 1099 in (23)], in which the first and second cistrons encoded *Xenopus* cyclin B2 and influenza NS' protein respectively, were kindly provided by Drs N. Standart and R. Jackson, University of Cambridge, UK. Plasmids pXlcdk2-21 and pXlcdk2-EPEX, both encoding *Xenopus* cdk2 but with different 5' untranslated regions, were kindly provided by Dr M. Philippe (Laboratory of Development and Genetics, CNRS 256, University of Rennes). *In vitro* transcription reactions (see above) were carried out to produce capped and uncapped mRNA in each

Figure 1. Schematic diagram of constructs used (not to scale). (A) pXlcycaA1, encoding *Xenopus laevis* cyclin A1 [see (22)]; (B) pXlcdk-2 EPEX, encoding *Xenopus laevis* cdk2. The authentic 5' UTR is replaced by that encoding β -globin. The construct was cloned in pBluescript SK⁻ (Stratagene); (C) pXlcdk2-21, also encoding *Xenopus laevis* cdk2. In this case the authentic 5' UTR was truncated to the 21 nucleotides preceding the coding sequence. The construct was cloned in pBluescript KS⁺ (Stratagene); (D) pMM1, encoding the FMDV L protease, exactly as described in ref. 19; (E) pXLJODA, the bicistronic construct encoding *Xenopus laevis* cyclin B2 (first cistron) and Influenza virus NS' (second cistron), with the IRES from TMEV in the intercistronic space, as described in ref. 23.

case. For capping reactions GTP concentrations were reduced to 0.48 mM and m⁷GpppG cap analogue (New England BioLabs) added to 1.92 mM. Total reticulocyte poly(A)-rich RNA was extracted from reticulocyte polysomes as described (24). Translation reactions were as described above, with the addition of [³⁵S] methionine (ICN) at 200 μ Ci per ml of complete mixture.

Preparation of eIF-4 complex from rabbit reticulocyte lysate was by m⁷GTP affinity chromatography, as described in ref. 10.

Polyacrylamide electrophoresis and immunoblotting were as described previously (8). For analysis of the proteolysis of eIF-4 γ 7.5% polyacrylamide gels were used. The antibody used for immunoblotting was a polyclonal antiserum directed against Peptide 7 of eIF-4 γ , as described (25).

RESULTS

Figure 2 shows the effect of adding different amounts of L protease to a reticulocyte lysate translating endogenous mRNA. L protease was added in the form of a postribosomal supernatant derived from an *in vitro* translation system programmed with pMM1 mRNA, as described in Materials and Methods. Panel A shows a time course, which indicates that addition of 0.5 μ l or more of the L protease preparation per 10 μ l translation system resulted in very rapid inhibition of protein synthesis, with no further translation after 20 min. Panel B shows the inhibition of translation by increasing amounts of L protease, relative to control incubations where L protease was replaced by equivalent amounts of control postribosomal supernatant (see Methods). To examine the effectiveness of the L protease in cleaving eIF-4 γ in the reticulocyte lysate, samples were taken after 20 min of

Figure 2. L protease inhibits translation in a native reticulocyte lysate. (A) Rabbit reticulocyte lysate was incubated under translation conditions (10 μ l reactions) for up to 60 min at 30°C in the absence or presence of L protease: (A) 0.1 μ l, (B) 0.2 μ l, (C) 0.5 μ l, (D) 0.7 μ l, (E) 0.9 μ l, (F) 1.0 μ l. Aliquots (2 μ l) were removed at the indicated times and processed to measure incorporation of [³⁵S] methionine into protein. (B) Effect of increasing amounts of L protease (same experiment as panel A). For each concentration of L protease, the incorporation over a 40 min period is expressed relative to that in a parallel incubation in which L protease was replaced by an equivalent amount of control postribosomal supernatant.

Figure 3. Cleavage of eIF-4 γ in the reticulocyte lysate by different amounts of L protease. Reticulocyte lysate translation systems (10 μ l) were incubated at 30°C for 20 min in the presence of (A) 1.0 μ l, (B) 0.9 μ l, (C) 0.2 μ l, (D) 0.1 μ l L protease or (E) in the absence of L protease. Four μ l samples of each incubation mixture were analysed by SDS-polyacrylamide gel electrophoresis. The proteins were transferred to PVDF paper and visualized by immunoblotting, as described in Materials and Methods. The position of marker proteins (kDa) is shown. The migration positions of uncleaved eIF-4 γ ('p220') and of the N-terminal cleavage products (Cp) are indicated.

incubation and the state of the eIF-4 γ examined by Western blotting (Fig. 3). The antibody used was raised against Peptide 7 of eIF-4 γ , as defined by Yan *et al.* (25); this antibody recognizes the intact polypeptide and the proteolytic products N-terminal to the initial cleavage sites of both L protease and the poliovirus 2A proteinase (14,15). It can be seen (Fig. 3) that in the presence of relatively high concentrations of L protease virtually all the intact eIF-4 γ had disappeared by 20 min, but that some survived incubation with lower amounts (0.1 or 0.2 μ l) of the preparation. However, a characteristic pattern of cleavage products was clearly visible in all cases. The tendency for the degradation products to give a more pronounced signal in immunoblotting experiments than the intact protein has sometimes been observed by others (13).

Figure 4. Partially purified eIF-4 complex rescues translation in an L protease treated lysate. Translation systems containing messenger-dependent reticulocyte lysate but lacking exogenous mRNA and radioactive methionine were preincubated for 25 min at 30°C with 1 μ l of either control postribosomal supernatant (Tube 1) or L protease (Tubes 2 and 3). The protease inhibitor elastatinal was then added to a final concentration of 300 μ M and the tubes transferred to ice for 10 min. One μ l of a purified preparation of eIF-4 complex (1 μ g protein) was then added to Tube 2; Tubes 1 and 3 received an equal volume of control buffer. [³⁵S] methionine and reticulocyte poly(A) rich RNA were then added and the samples (final volume 10 μ l) were incubated at 30°C to measure translational activity. Results are expressed as c.p.m. incorporated into the protein of 2 μ l samples of translation mixture removed at the indicated times.

Translation of capped and uncapped mRNAs encoding cellular proteins in the messenger-dependent reticulocyte lysate

Figure 4 shows the effect of pre-treatment with L protease on the ability of a messenger-dependent lysate (MDL) to translate exogenous poly(A)-rich mRNA isolated from rabbit reticulo-

Figure 5. L protease treatment stimulates translation of uncapped transcripts. Indicated amounts of L protease were added to 10 μ l translation systems based on the messenger-dependent reticulocyte lysate. Transcripts, either capped (A–C) or uncapped (D–F), were prepared by *in vitro* transcription from plasmids following linearization: pXICYA1 (A, D), pXlck2-21 (B, E) and pXlck2 EPEX (C, F) (see Fig. 1). Results represent translation over 40 min and are expressed relative to protein synthesis in parallel incubations in which L protease was replaced by equivalent amounts of control postribosomal supernatant.

cytes. Samples of MDL were first incubated under translation conditions (see Methods) for 25 min in the presence of either L protease or an equivalent amount of control postribosomal supernatant. At the end of this period the protease inhibitor elastatinal was added to a concentration found in preliminary experiments (not shown) to block the proteolysis of eIF-4 γ by L protease under these conditions. This inhibitor was previously demonstrated to be effective against destruction of eIF-4 γ by the

human rhinovirus 2A proteinase (13). Reticulocyte poly(A)-rich RNA and [³⁵S] methionine were then added and protein synthesis estimated. Figure 4 shows that protein synthesis was almost completely blocked by prior incubation of the lysate with L protease. To confirm that this was due to the proteolysis of eIF-4 γ , and not to some non-specific effect of L protease, we examined the ability of exogenous eIF-4 complex to rescue protein synthesis. Figure 4 shows that this initiation factor complex was

extremely effective in restoring translational activity to the L protease-treated lysate. Essentially similar results were obtained using human rhinovirus 2A proteinase in HeLa cell-free systems translating rabbit globin RNA (13).

We then investigated the effect of L protease treatment on the translation of a range of capped and uncapped cellular mRNAs (transcribed from the plasmids shown in Fig. 1). In these experiments, unlike that shown in Figure 4, there was no preincubation period with L protease and translation was monitored from the time of its addition. As expected from the results of Figures 2 and 4, the translation of the capped transcripts was inhibited in the presence of L protease (Fig. 5A–C). However, in contrast, a pronounced stimulation of translation of the uncapped transcripts was seen in the L protease-treated system (Fig. 5D–F). While the nature of these effects was similar for all three mRNAs, its extent, particularly the degree of stimulation of translation of the uncapped transcripts, varied markedly. The most striking results were obtained with the mRNA transcribed from plasmid pXlcyca, which encodes *Xenopus laevis* cyclin A (Fig. 5D). Similar, though somewhat less pronounced, effects (Fig. 5E and F) were seen with two sets of transcripts which each encoded the *Xenopus* cdk2 protein, but differed in their 5' untranslated region (see Fig. 1). Figure 6 confirms that L protease does indeed stimulate the translation of the full-length Cyclin A product from the uncapped mRNA, although the labelling of the series of lower-molecular-weight products always observed with this mRNA was enhanced in proportion. These products probably represent incomplete nascent polypeptide chains, since they are mostly sedimented with ribosomes by centrifugation (not shown).

An interesting feature of the results with all three mRNAs is that the concentrations of L protease required to inhibit translation of capped transcripts were considerably higher than those required to stimulate translation of their uncapped counterparts. Analysis of the complete time courses of translation from which the data in Figure 4 were compiled (not shown) suggests that at low doses of L protease the stimulation of translation of the uncapped transcripts occurred prior to the disappearance of intact eIF-4 γ , and correlated more closely with the appearance of the cleavage products. We have therefore carried out a further experiment to investigate whether the cleavage products could stimulate translation of uncapped transcripts in the presence of intact eIF-4 γ . In this experiment a sample of purified eIF-4 complex was pre-treated with L protease, followed by addition of elastatinal.

Figure 6. L protease stimulates translation of uncapped Cyclin A1 mRNA. Uncapped Cyclin A1 mRNA was incubated at 20 μ g/ml in MDL translation systems (10 μ l) for 40 min at 30°C in the presence of either different amounts of L protease: (A) 0.2 μ l, (C) 1 μ l, or of control postribosomal supernatant: (B) 0.2 μ l, (D) 1 μ l or (E) water. Four μ l samples of each incubation mixture were analysed by SDS–polyacrylamide gel electrophoresis and autoradiography. The migration of marker proteins (kDa) is indicated and the arrow shows the position of full-length Cyclin A1.

Figure 7. Translation of uncapped Cyclin A1 mRNA can be stimulated by addition of a purified preparation of eIF-4 complex following partial cleavage with L protease. A preparation of eIF-4 complex ($\sim$ 2 μ g) was preincubated for 5 min at 30°C with 0.5 μ l of either L protease or control postribosomal supernatant in a volume of 10 μ l. The protease inhibitor elastatinal was then added to a concentration of 300 μ M. Different amounts of the preincubation mixtures were then added to 10 μ l MDL translation systems containing either capped or uncapped Cyclin A1 mRNA. Translation was measured for 35 min. (A) The translation in systems containing eIF-4 pre-treated with L-protease, expressed as a percentage of that in systems that received the corresponding amount of eIF-4 pre-treated with control postribosomal supernatant. (B) Immunoblot demonstrating the partial cleavage of eIF-4 γ during the preincubation of eIF-4 complex with L protease. Four μ l aliquots of each of the preincubation mixtures described in A were analysed by SDS–polyacrylamide gel electrophoresis and immunoblotting. Lane 1: sample preincubated with L protease; lane 2: sample preincubated with control postribosomal supernatant.

Addition of this cleaved preparation to a reticulocyte lysate again resulted in stimulation of translation of the uncapped Cyclin A transcript (Fig. 7). Figure 7B shows a Western blot of the combined (added + endogenous) eIF-4 γ present in the translation mixture at the end of the incubation and confirms the presence of a substantial amount of the intact protein. Taken together these results support a direct effect of the proteolytic cleavage products of eIF-4 γ on the translation of uncapped transcripts.

Several authors have suggested that the products of proteolysis of eIF-4 γ by picornavirus proteases may specifically stimulate translation of uncapped, virally-encoded mRNAs bearing an internal ribosome entry site or IRES (26–30). We have therefore investigated the effect of *in vitro* treatment with L protease on

Figure 8. Effect of L protease on translation of a bicistronic transcript containing an internal ribosome entry segment. Bicistronic mRNAs were prepared by *in vitro* transcription from the plasmid pXLJODA in capped (A, B) and uncapped (C, D) form. They were translated in MDL systems for 30 min at 30°C in the presence of 1 µl of either L protease (A, C) or control (B, D) postribosomal supernatant. Four µl samples of each incubation mixture were analysed by SDS-polyacrylamide gel electrophoresis and autoradiography. The positions of the Cyclin B2 and NS' products are designated by the arrows.

translation of the second cistron of a bicistronic construct containing an IRES from Theiler's murine encephalomyelitis virus (TMEV) inserted in the intercistronic space (23) (see Fig. 1). The results, shown in Figure 8, indicate little or no stimulation of translation of the IRES-driven coding sequence by L protease, even under conditions where the 5' terminal cistron of the uncapped transcript showed enhancement (compare lanes C and D).

DISCUSSION

The initial aim of this work was to establish conditions for the selective depletion of eIF-4γ from an *in vitro* translation system to provide a tool for functional studies on this factor. Treatment with FMDV L protease had the expected effect of reducing the ability of the reticulocyte lysate system to translate capped mRNAs. Moreover, the ability of exogenous eIF-4 complex to rescue translation in the depleted system provides evidence that the principal effect of L protease treatment on translation under these conditions was indeed due to removal of eIF-4γ. However, when we investigated the effect of the protease treatment on translation of uncapped mRNA transcripts we observed an unexpected stimulation. This was particularly striking in the case of the cyclin A transcript (Figs 5 and 6).

The enhancement of translation of uncapped transcripts could be elicited by addition of a lower amount of L protease than was required either to inhibit translation of capped transcripts (Fig. 5) or to eliminate the bulk of the reticulocyte eIF-4γ during a 20 min incubation (Fig. 3). This suggests that the appearance of one or more of the proteolytic degradation products of eIF-4γ, rather than the depletion of the intact polypeptide, may be more responsible for the stimulatory effect on utilization of uncapped transcripts. Such a mechanism has been suggested previously to contribute to the enhancement of translation of picornavirus mRNAs possessing an IRES element in poliovirus-infected cells or in extracts derived from them (26–30).

While this work was in progress, Whetter *et al.* (31) reported an investigation utilizing cells engineered to express bacteriophage T7 polymerase constitutively in the cytosol. In these cells transfection of plasmid DNAs containing a T7 promoter resulted in production of uncapped transcripts and thus permitted the study of translation of uncapped mRNAs *in vivo*. Under the standard conditions of this experiment, significant translation was

only seen in the case of mRNAs possessing a strong IRES element. However, if the cells were induced to co-express the poliovirus 2A protease they were able to translate an uncapped mRNA lacking this element. The degree of enhancement of translation of this mRNA by the co-expression of the viral protease was similar to the best stimulation seen in our *in vitro* system. Whetter *et al.* (30) suggested that this enhancement was most likely due to elimination of competition for translation by capped cellular mRNAs, due to degradation of eIF-4γ. However, our results, using a nuclease-pretreated translation system directed by only a single mRNA, would argue in favour of a more specific mechanism. Further investigations are required to elucidate whether specific proteolytic degradation products of eIF-4γ are involved in this stimulation and to establish the mechanism.

In our system, L protease treatment did not stimulate translation of the second cistron of the mRNA derived from the bicistronic construct pXLJODA, in which the IRES element from TMEV was interposed between the two cistrons (Fig. 8). This was unexpected in the light of previous data from both *in vivo* and *in vitro* systems of enhanced translation of uncapped mRNAs possessing IRES elements from either polio or EMC virus by viral proteases that degrade eIF-4γ (17,18,28,31). However, the effect does not appear to be universal even *in vivo*, since co-expression of the polio 2A protease was recently found to inhibit translation directed by the Hepatitis A virus IRES in either a mono- or bi-cistronic transcript (31). Further work is needed to elucidate the influence of different IRES elements in this response.

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