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The eIF4G-eIF4E Complex Is the Target for Direct Cleavage by the Rhinovirus 2A Proteinase

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The 2A proteinases ($2A^{pro}$) of certain picornaviruses induce the cleavage of the eIF4G subunit of the cap-binding protein complex, eIF4F. Several reports have demonstrated that $2A^{pro}$ of rhinovirus and coxsackievirus B4 cleave eIF4G directly. However, it was suggested that in poliovirus infection, the $2A^{pro}$ induces the activation of a cellular proteinase which in turn cleaves eIF4G. Furthermore, it is not clear whether eIF4G is cleaved as part of the eIF4F complex or as an individual polypeptide. To address these issues, recombinant eIF4G was purified from Sf9 insect cells and tested for cleavage by purified rhinovirus $2A^{pro}$. Here we report that eIF4G alone is a relatively poor substrate for cleavage by the rhinovirus $2A^{pro}$. However, an eIF4G-eIF4E complex is cleaved efficiently by the $2A^{pro}$, suggesting that eIF4F is a preferred substrate for cleavage by rhinovirus $2A^{pro}$. Furthermore, $2A^{pro}$ drastically reduced the translation of a capped mRNA. An eIF4G-eIF4E complex, but not eIF4G alone, was required to restore translation.

Infection of cells by picornaviruses belonging to several genera results in a precipitous and dramatic inhibition of host cellular mRNA translation (8). In the case of poliovirus, this inhibition precedes any substantial synthesis of viral proteins (2). In contrast to cellular mRNA translation, viral RNA translation proceeds with high efficiency during the infection. The differential translation of viral mRNAs can be explained by the unique translational features of picornaviruses. Picornavirus RNAs, in contrast to cellular mRNAs, do not contain a 5′ cap structure (11, 23), and their translation is mediated by ribosome binding to an internal ribosome entry site (IRES) that is present in the 5′ untranslated region (27).

Picornaviruses induce both structural and functional modifications of the translational machinery. The ability of eukaryotic initiation factor 4F (eIF4F) to restore the translation of capped mRNAs in extracts prepared from poliovirus-infected cells initially implicated eIF4F as one target for such modifications (37). eIF4F is a cap-binding multisubunit complex which facilitates mRNA unwinding, and subsequent ribosome binding to mRNA (33). It is composed of three polypeptides: eIF4E, eIF4A, and eIF4G. eIF4E, a 24-kDa polypeptide, mediates the cap-binding function of the complex (34) and plays a critical role in the control of translation rates (33). eIF4A, a 50-kDa polypeptide, exhibits RNA-dependent ATPase activity and, in association with eIF4B, bidirectional RNA helicase activity (29, 30). Recent evidence suggests that eIF4G may serve as a scaffold: it interacts with both eIF4E and eIF4A, and its association with eIF3 is suggested to promote ribosome binding at the 5′ end of mRNAs (15, 22).

The *Enterovirus*, *Rhinovirus*, and *Aphthovirus* genera of picornaviruses cause cleavage of eIF4G into an N-terminal fragment of about 50 kDa (which migrates as a set of two or three polypeptides of 110 to 130 kDa) and a carboxy-terminal fragment of about 100 kDa. The 2A proteinase ($2A^{pro}$) of poliovirus, coxsackievirus, and rhinovirus is required for the cleavage of eIF4G, and mutants of poliovirus $2A^{pro}$ are defective in eIF4G cleavage (3). Similarly, the L proteinase of foot-and-mouth disease virus cleaves eIF4G (5, 12, 20). However, there is conflicting evidence whether these proteinases exert their effects directly or indirectly through the activation of cellular proteinases. Wyckoff et al. (38) had reported that the activity which cleaves eIF4G does not copurify with poliovirus $2A^{pro}$, suggesting an indirect mechanism whereby poliovirus-activated cellular proteases mediate proteolytic cleavage. In addition, anti-$2A^{pro}$ serum capable of inhibiting poliovirus polyprotein processing does not inhibit eIF4G cleavage (14, 21, 38). More recently, a role for eIF3 in the cleavage of eIF4G was reported. In these experiments eIF4G was not cleaved by an *Escherichia coli* extract expressing poliovirus $2A^{pro}$, but cleavage occurred when purified eIF3 was added (39). In contrast, experiments with recombinant $2A^{pro}$ of human rhinovirus 2 (HRV2) or of coxsackievirus B4 and the L proteinase of foot-and-mouth disease virus demonstrated direct cleavage of the eIF4G subunit in the eIF4F complex (12, 16, 18). Such an activity was not examined with purified poliovirus $2A^{pro}$. It is important to note that the eIF4G substrates used in the various studies were different. Wyckoff et al. (39) used eIF4G that was partially purified in a form dissociated from the other eIF4F polypeptides. On the other hand, the eIF4G substrate used in experiments with $2A^{pro}$ of rhinovirus and coxsackievirus (12, 16, 18) was purified as part of the intact eIF4F complex. While it is highly unlikely that the mechanism of action of poliovirus $2A^{pro}$ is different from those of coxsackieviruses and rhinoviruses, it is possible that eIF4G, in a complex with the other eIF4F subunits, assumes a conformation which renders it a substrate for $2A^{pro}$.

To address these questions and to determine the substrate for $2A^{pro}$ (eIF4G or eIF4F), we examined directly whether recombinant eIF4G is a substrate for HRV2 $2A^{pro}$. Here, we

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demonstrate that 2A^{pro} from HRV2 cleaves purified recombinant eIF4G directly in vitro, although relatively poorly. In contrast, a complex of eIF4G with eIF4E is a preferable substrate for HRV2 2A^{pro}. We therefore propose that eIF4F, and not the eIF4G subunit alone, is the primary target for cleavage by HRV2 2A^{pro}. Consistent with these results, we show that restoration of cap-dependent translation in 2A^{pro}-treated extracts requires both the eIF4E and eIF4G subunits of the eIF4F complex.

MATERIALS AND METHODS

Cell culture, protein factors, and enzymes. *Spodoptera frugiperda* (Sf9) insect cells were cultured in Grace medium (GIBCO-BRL) supplemented with 10% fetal calf serum, TC Yeastolate, lactalbumin hydrolysate, 50 μg of gentamicin sulfate per ml, and 2.5 μg of amphotericin B (Fungizone) per ml in either T flasks or spinner flasks at 27°C as described previously (35). Glutathione-*S*-transferase (GST) fusion proteins of HMK-4E-BP1 and HMK-4E-BP1Δ were expressed in *E. coli* BL21 and purified as described previously (25). Murine eIF4E protein was expressed in *E. coli* K38 and purified as described previously (6). HRV2 2A^{pro} was expressed in *E. coli* BL21(DE3)pLysE and purified as described previously (18). m^{7}GDP column chromatography was performed as described previously (6). Polyclonal antibody to eIF4G was as described previously (1).

Generation of recombinant baculovirus. To generate a flag-HMK fusion of eIF4G in the baculovirus expression system, we first constructed a new baculovirus transfer vector, pVL1392flagHMK, derived from pVL1392 (Pharmingen). This vector contains the flag-HMK epitope (4) at an *Eco*RI site. The *Eco*RI fragment of eIF4G was excised from plasmid pSK(−)HFC1 (a kind gift from R. E. Rhoads [40]) and inserted blunt into the *Eco*RI site of pVL1392flagHMK, creating pVL1392flagHMK-eIF4G. Recombinant baculovirus was subsequently generated with the BaculoGold expression system (Pharmingen). At 5 days posttransfection, the virus released into the media was collected and amplified. The resulting high-titer virus was used for preparation of recombinant protein. The flag epitope-tagged protein was subsequently immunopurified on a commercial anti-flag affinity column (Kodak). Flag-eIF4G was eluted with flag peptide (100 μg/ml) in TEN buffer (20 mM Tris-HCl [pH 7.5], 0.1 mM EDTA, 150 mM NaCl). The eluate was then dialyzed against buffer A (20 mM Tris-HCl [pH 7.5], 100 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 5% glycerol).

HRV2 2A proteinase cleavage assays. Incubation of either HeLa S10 cell extracts or purified flag-eIF4G with the HRV2 2A^{pro} took place in buffer B (50 mM NaCl, 50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 5 mM dithiothreitol, 5% glycerol) in a final volume of 12 μl at 30°C for 30 min. Reactions were terminated by the addition of Laemmli sample buffer. Cleavage products of eIF4G were resolved on sodium dodecyl sulfate (SDS) 8% polyacrylamide gels and analyzed by immunoblotting with a rabbit anti-eIF4G polyclonal antibody (1).

Western blotting (immunoblotting). Nitrocellulose membranes were incubated for 90 min at room temperature in Tris-buffered saline containing 0.2% Tween 20 (TBST) and 5% dry milk. Next, membranes were incubated with rabbit anti-eIF4G polyclonal antibody overnight at 4°C. After extensive washing with TBST, the membranes were incubated with ^{125}I-protein A for 2 h, washed with TBST, and exposed to Dupont reflection film.

In vitro transcription and translation. The plasmids pSP64-CAT and pEMC-CAT were linearized with *Bam*HI. Transcription was performed with SP6 RNA polymerase as previously described (26). Capped transcripts were obtained in a reaction mixture containing 50 μM GTP and 500 μM m^{7}GpppG. The integrity of RNAs was analyzed on a formaldehyde-agarose gel, and the amounts were quantitated by spectrophotometry. Translations were performed in Krebs-2 ascites cell extracts as described previously (36) in a final volume of 14 μl. Where indicated, extracts were treated with HRV2 2A^{pro} or buffer B for 4 min at 30°C and then incubated for 10 min on ice in the presence of 0.7 mM elastatinal (Sigma). Initiation factors were then added, followed by the mRNA (200 ng) and other translation ingredients. Translation reaction mixtures were incubated at 30°C for 90 min and subsequently analyzed by SDS-polyacrylamide gel electrophoresis. Gels were fixed, treated with En^{3}Hance and processed for autoradiography.

RESULTS

Expression and purification of recombinant eIF4G from Sf9 insect cells. Human eIF4G was expressed as a fusion protein with flag-HMK epitope tag in insect cells by using a baculovirus expression system. High-titer virus (~2 × 10^8 PFU/ml) was generated and used to infect Sf9 insect cells. Cytoplasmic cell lysates were prepared at 72 h postinfection, and eIF4G was immunopurified on an anti-flag column (Fig. 1A). The eluate contained a major polypeptide of about 200 kDa (lanes 4 to 6). The identity of the eluted band was determined by immunoblotting with an anti-eIF4G polyclonal antibody (the antibody detects both the amino- and carboxy-terminal cleavage products of eIF4G [1]). Uninfected cells showed no immunoreactive material (Fig. 1B, lane 1). Flag-eIF4G was detected in the load (lane 2), flowthrough (lane 3), and eluate fractions (lanes 4 to 6). In HeLa S10 extracts, the antibody recognized eIF4G, which migrates at about 220 kDa (lane 7). Previously, we reported that HA-eIF4G expressed in Sf9 insect cells migrated at about 190 kDa (9). The slower migration reported here is most probably due to the flag epitope.

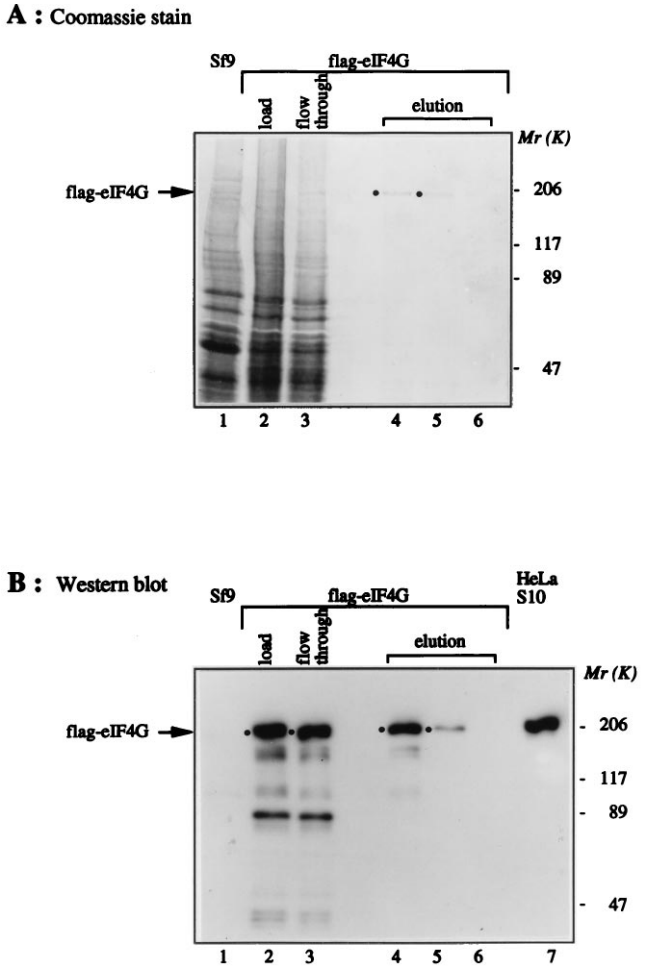

FIG. 1. Expression of recombinant flag-eIF4G in Sf9 insect cells and purification on an anti-flag column. Samples were resolved on an SDS–8% polyacrylamide gel and analyzed by Coomassie blue staining (A) and Western blotting (B). The following samples were loaded on the gel: lane 1, ~70 μg of uninfected Sf9 insect cell lysate; lane 2, ~70 μg of infected Sf9 insect cell lysate (load); lane 3, ~70 μg of flowthrough; lanes 4 to 6, 10 μl (from a total of 1 ml for each fraction) of eluate. The Western blots of duplicate samples contain one-fifth of the material used in the Coomassie blue stain. Lane 7, ~12 μg of a HeLa S10 extract. Molecular masses of protein standards (Bio-Rad) are indicated on the right. The position of flag-eIF4G is indicated by an arrow and black dots.

To examine whether insect eIF4E copurifies with the recombinant eIF4G, the ability of eIF4G to be retained on an m^{7}GDP-coupled agarose resin was determined. Recombinant flag-eIF4G alone did not bind to the resin, as determined by Western blotting (data not shown). Flag-eIF4G was retained on the m^{7}GDP-coupled agarose resin only in the presence of exogenous recombinant murine eIF4E (data not shown) (9). This finding also demonstrates that the recombinant eIF4G expressed in insect cells exhibits eIF4E-binding activity.

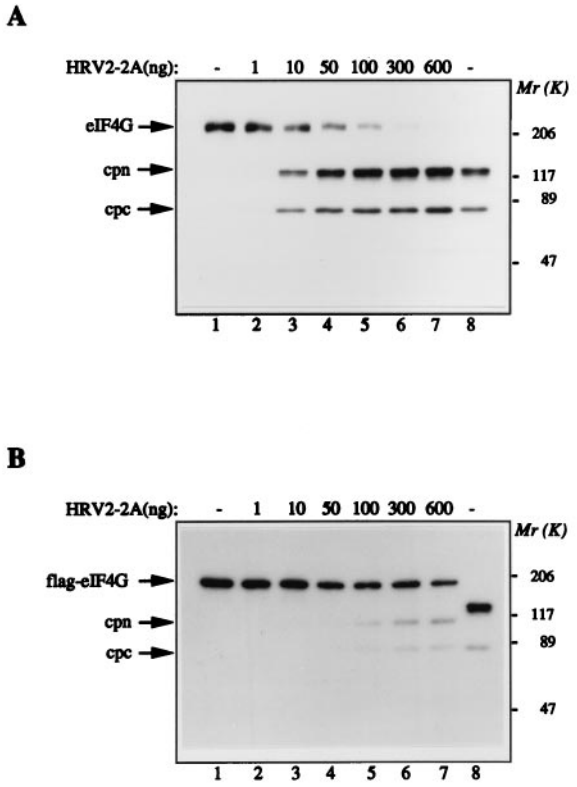

FIG. 2. Cleavage of eIF4G by the HRV2 $2A^{pro}$ in vitro. (A) HeLa S10 extract (~12 μg) was incubated at 30°C for 30 min with the indicated amounts of HRV2 $2A^{pro}$. Samples were analyzed as described in Materials and Methods. Lanes: 1, buffer B; 2 to 7, HRV2 $2A^{pro}$; 8, ~12 μg of poliovirus-infected HeLa S3 extract. (B) A ~80-ng portion of flag-eIF4G, equivalent to the eIF4G content of ~12 μg of HeLa S10 extract, as determined by Western blotting, was treated as in panel A. Lanes: 1, buffer B; 2 to 7, HRV2 2A proteinase; 8, ~12 μg of poliovirus-infected HeLa S3 extract. The positions of intact eIF4G and the N-terminal (cpn) and C-terminal (cpc) cleavage products are indicated by arrows.

Inefficient cleavage of pure eIF4G by HRV2 $2A^{pro}$ in vitro. To test whether recombinant eIF4G is cleaved by purified HRV2 $2A^{pro}$, HeLa S10 extract or purified flag-eIF4G was incubated with increasing amounts of the $2A^{pro}$. The cleavage of eIF4G was monitored by immunoblotting with an anti-eIF4G polyclonal antibody (1). eIF4G in the HeLa S10 extract was stable when incubated with buffer alone (Fig. 2A, lane 1) but was efficiently cleaved into its characteristic cleavage products when incubated with increasing amounts of HRV2 $2A^{pro}$ (lanes 2 to 7), consistent with the reports that $2A^{pro}$ is the only virally encoded protein required for the induction of eIF4G cleavage (14, 18). Under these conditions, 10 ng of HRV2 $2A^{pro}$ cleaved approximately 50% of the eIF4G, as reported by Klump et al. (13). The migration of the cleavage products resembled the pattern observed in extracts from poliovirus-infected HeLa S3 cells (lane 8). The cleavage products derived from the amino and carboxy termini were designated cpn (for cleavage product N terminus) and cpc (for cleavage product C terminus).

In contrast to the efficient cleavage of eIF4G in the HeLa S10 extract, recombinant flag-eIF4G was a relatively poor substrate for cleavage by the HRV2 $2A^{pro}$ (Fig. 2B). While buffer alone had no effect on the stability of flag-eIF4G (lane 1), 600 ng of HRV2 $2A^{pro}$ was required to cleave about 60% of the flag-eIF4G (lanes 2 to 7 [note that the antibody recognizes the flag-eIF4G cleavage products less efficiently than it recognizes the intact protein; the reason for this is not known—see also Fig. 3 and 4]). The cleavage product derived from the carboxy terminus of flag-eIF4G comigrated with the corresponding fragment in the control lane, whereas the amino-terminal product displayed a higher mobility than its counterpart in the control lane (compare lanes 7 and 8). In addition, the cpn derived from flag-eIF4G migrated as a single band at about 110 kDa whereas two or three bands are observed following cleavage of the authentic protein. Neither the heterogeneity observed with the authentic protein (8) nor the aberrant mobility exhibited by the amino-terminal third of flag-eIF4G is understood. A conformational change or the absence of a posttranslational modification in insect cells at the amino terminus may account for the altered mobility.

eIF4E enhances the cleavage of eIF4G by HRV2 $2A^{pro}$. To determine whether eIF4E enhances eIF4G cleavage, recombinant eIF4G was preincubated with eIF4E to allow for complex formation, and then increasing amounts of HRV2 $2A^{pro}$ were added. Incubation in the presence of buffer alone did not induce any cleavage (Fig. 3A, lane 1). While no significant cleavage of eIF4G in the presence of eIF4E occurred with 1 ng of HRV2 $2A^{pro}$ (lane 2), 10 ng of HRV2 $2A^{pro}$ cleaved more than 75% of the input flag-eIF4G (lane 3). Approximately 9% of input eIF4G was resistant to cleavage by $2A^{pro}$ (lanes 4 to 7). Increasing amounts of eIF4E did not enhance cleavage of the resistant material (data not shown), which might be misfolded and unable to interact with eIF4E. To address this possibility, flag-eIF4G was preincubated with excess eIF4E and subsequently purified as a complex with eIF4E by chromatography on an m^7GDP-coupled agarose resin. This procedure is expected to eliminate the misfolded eIF4G that cannot interact with eIF4E. Incubation with buffer alone did not result in cleavage of eIF4G (Fig. 3B, lane 1), ruling out the possibility that eIF4E induces the cleavage of eIF4G. However, about 20% and in excess of 80% of the eIF4G was cleaved with 1 and 10 ng of the HRV2 $2A^{pro}$, respectively (lanes 2 and 3), and cleavage was complete with increasing amounts of enzyme (lanes 4 to 7). Addition of eIF4A or eIF3 did not change the rate of appearance or the mobility of the cleavage products (data not shown). Taken together, these results indicate that eIF4G in a complex with eIF4E is more susceptible to cleavage by HRV2 $2A^{pro}$ than is eIF4G alone.

To further substantiate this conclusion, a HeLa S10 extract was preincubated with eIF4E and increasing amounts of HRV2 $2A^{pro}$ were added. Incubation with buffer alone did not generate the characteristic cleavage products (Fig. 3C, lane 1). Strikingly, in the presence of excess eIF4E, cleavage of the authentic eIF4G was complete with 10 ng of HRV2 $2A^{pro}$ (lanes 2 to 7), compared with more than 300 ng in the absence of exogenous eIF4E (Fig. 2A, lanes 2 to 7). These results further confirm the stimulatory effect of eIF4E on the cleavage of eIF4G by HRV2 $2A^{pro}$. The quantitative analysis of the above data is depicted in Fig. 3D and E. (Since the antibody does not recognize the cleavage products of flag-eIF4G as efficiently as the intact form, cleavage was calculated as the percentage of intact eIF4G. To ensure that the reduction in intact eIF4G is not due to accidental loss, the experiment was performed three times, with similar results.)

4E-BP1 reverses the stimulatory effect of eIF4E on the cleavage of eIF4G. Additional experiments were designed to demonstrate that the stimulatory effect of eIF4E is a result of complex formation with eIF4G. The activity of eIF4E is modulated by two specific binding proteins (BPs), termed 4E-BP1 and 4E-BP2 (19, 25). 4E-BP1 competes with eIF4G for binding to eIF4E and represses cap-dependent translation (9). It is therefore predicted that 4E-BP1 would reverse the stimulatory

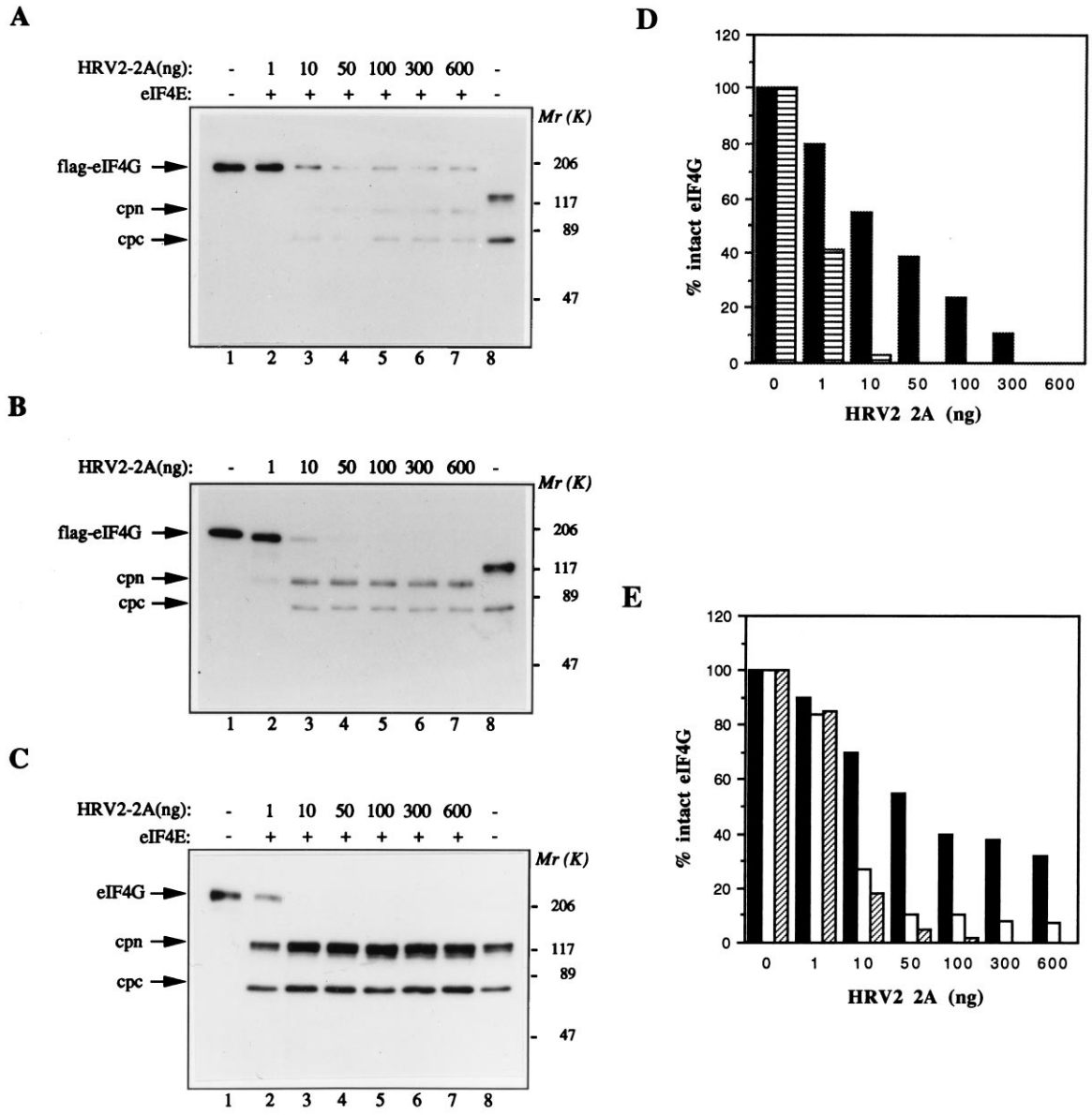

FIG. 3. Cleavage of eIF4G by the HRV2 2A^{pro} is enhanced by eIF4E. (A and C) Similar amounts of flag-eIF4G (A) or HeLa S10 extract (C) to those in Fig. 2 were preincubated with buffer A (lane 1) or 50 ng of murine eIF4E for 5 min at 30°C. The mixture was subsequently treated as in the experiment in Fig. 2. (B) Purified flag-eIF4G was incubated with purified murine eIF4E, and the mixture was applied to an m^7GDP-coupled agarose resin. The eluted eIF4G-eIF4E complex in buffer A, containing the same amount of eIF4G as in panel A, was treated with either buffer B (lane 1) or HRV2 2A^{pro}, as in the experiment Fig. 2). (D) Quantitative analysis of the results in panel C. Symbols: ■, HeLa S10; ▤, HeLa S10 plus eIF4E. (E) Quantitative analysis of the results in panels A and B. The amount of intact eIF4G present in each lane was quantitated with a Bas 2000 phosphorimager and is presented as a percentage of input eIF4G. Symbols: ■, eIF4G; □, eIF4G plus eIF4E; ▨, eIF4F.

effect of eIF4E on 2A^{pro} cleavage. HeLa S10 extract was preincubated with increasing amounts of GST–4E-BP1 before the addition of HRV2 2A^{pro}. Buffer alone had no effect on the stability of eIF4G in the extract (Fig. 4A, lane 1). Addition of HRV2 2A^{pro} generated the expected cleavage products (lane 2). Significantly, preincubation of the HeLa S10 extract with increasing amounts of GST–4E-BP1 rendered eIF4G more resistant to cleavage by 2A^{pro} (lanes 3 to 5). In addition, while exogenous eIF4E enhanced the rate of appearance of the characteristic cleavage products (compare lanes 2 and 6), preincubation of the exogenous eIF4E with increasing amounts of GST–4E-BP1 repressed the stimulatory activity of eIF4E (lanes 7 to 9).

To demonstrate the specificity of the effect of GST–4E-BP1 on the cleavage of eIF4G, a mutant of 4E-BP1 containing a deletion of the 4E binding domain (GST–4E-BP1Δ) was used. This mutant does not prevent the interaction of eIF4G with eIF4E and does not repress translation (9, 22). Preincubation of GST–4E-BP1Δ with the HeLa S10 extract had no effect on the rate of eIF4G cleavage (Fig. 4B, compare lanes 2 and 6). Furthermore, the deletion mutant did not reverse the stimulatory effect of eIF4E (compare lanes 3 and 7), whereas wild-type GST–4E-BP1 prevented the stimulatory activity of eIF4E (lanes 3 and 4).

Similar experiments were extended to the flag-eIF4G preparation to examine the specificity of eIF4E stimulatory effect

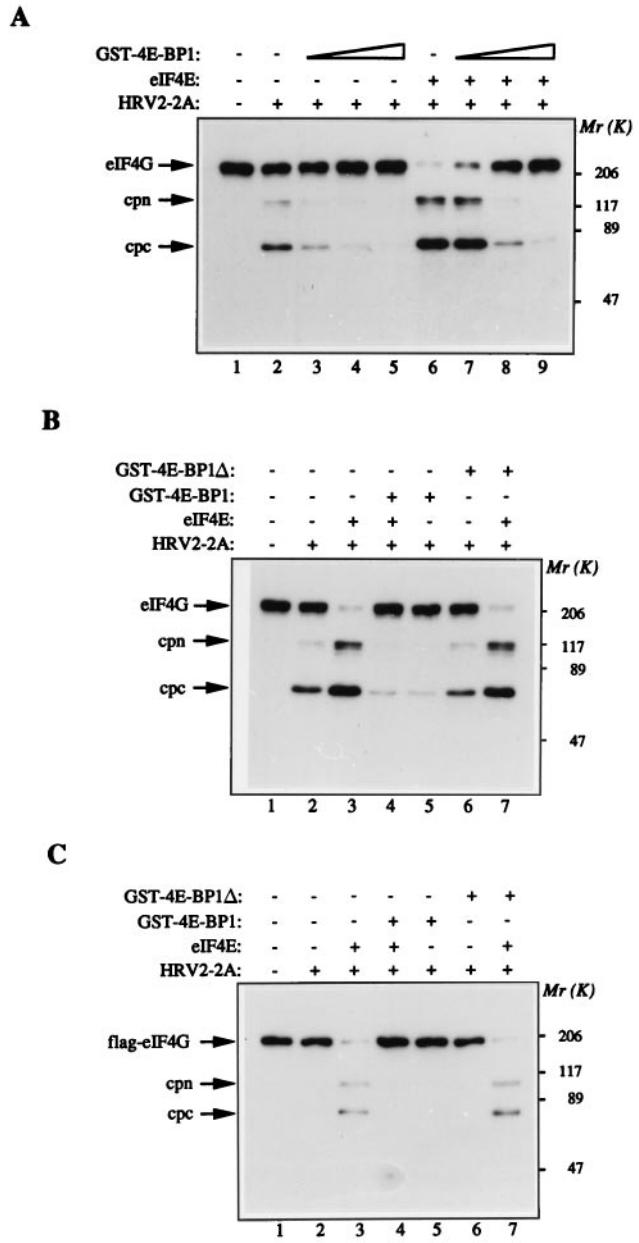

FIG. 4. Effect of 4E-BP1 on the cleavage of eIF4G in vitro. (A) HeLa S10 extract (12 μg) was preincubated with eIF4E, GST–4E-BP1, or both at 30°C for 5 min before the addition of 10 ng of HRV2 $2A^{pro}$. Lanes: 1, buffer A; 2 to 9, HRV2 $2A^{pro}$. The amounts of GST–4E-BP1 were as follows: lanes 3 to 5, 10, 50, and 100 ng, respectively; lanes 7 to 9, 10, 50, and 100 ng, respectively. Where indicated, 50 ng of eIF4E was added to the extracts. (B) As in panel A, except that the deletion mutant GST–4E-BP1Δ was used as a control (lanes 6 and 7). Where indicated, 50 ng of eIF4E, 50 ng of GST–4E-BP1, 50 ng of GST–4E-BP1Δ, or a combination of two, was preincubated with the extracts before the addition of HRV2 $2A^{pro}$. (C) As in panel B, except that ~80 ng of flag-eIF4G was used. Samples were processed for Western blotting as in Materials and Methods.

on the cleavage of flag-eIF4G by $2A^{pro}$. No cleavage products were detected in the presence of either buffer alone (Fig. 4C, lane 1) or small amounts of HRV2 $2A^{pro}$ (lane 2). As observed above, eIF4E significantly enhanced the cleavage rate of flag-eIF4G (lane 3). The effect of eIF4E was diminished by GST–4E-BP1 (lane 4), whereas the deletion mutant did not prevent the accelerated cleavage of eIF4G in the presence of eIF4E (lane 7). Taken together, these results and those in Fig. 3 demonstrate that eIF4G in a complex with eIF4E is a better substrate for HRV2 $2A^{pro}$ than is free eIF4G.

Both eIF4E and eIF4G are required for restoration of cap-dependent translation following $2A^{pro}$ treatment. The amino terminus of eIF4G is stably associated with eIF4E in picornavirus-infected cells, because it can be purified as a complex by chromatography on an m^7GDP-coupled agarose resin (17). Consequently, eIF4E is sequestered by the amino-terminal half of eIF4G following cleavage with the picornavirus 2A or L proteinases. It is predicted, therefore, that restoration of cap-dependent translation would require the addition of both eIF4E and eIF4G. The availability of purified eIF4G allowed us to directly address this prediction. Krebs-2 ascites cell extracts were treated with an excess of HRV2 $2A^{pro}$ to ensure a rapid cleavage of eIF4G. Prior to the addition of initiation factors, extracts were treated with elastatinal to inhibit the HRV2 $2A^{pro}$ (18). Extracts were subsequently programmed with a capped transcript (m^7GpppG-CAT). The translation of CAT mRNA in control Krebs-2 ascites cell extracts was efficient (Fig. 5A, lane 1). Treatment of the extract with $2A^{pro}$ resulted in complete cleavage of eIF4G (data not shown) and abolished translation, as expected (lane 2). Addition of either eIF4E alone (lanes 3 and 4), or eIF4G alone (lanes 5 and 6) did not restore translation. However, addition of both eIF4E and eIF4G to a $2A^{pro}$-treated extract restored translation to almost control levels (lane 7). eIF4F, used as a positive control, also exhibited similar restoring activity, consistent with earlier results (lane 8) (7, 37).

To assess the cap specificity of the inhibition, duplicate samples were programmed with an mRNA which initiates translation by a cap-independent mechanism (EMC-CAT; the chloramphenicol acetyltransferase (CAT) open reading frame is preceded by the IRES of encephalomyocarditis virus). Similar to the results with the capped mRNA, CAT was efficiently

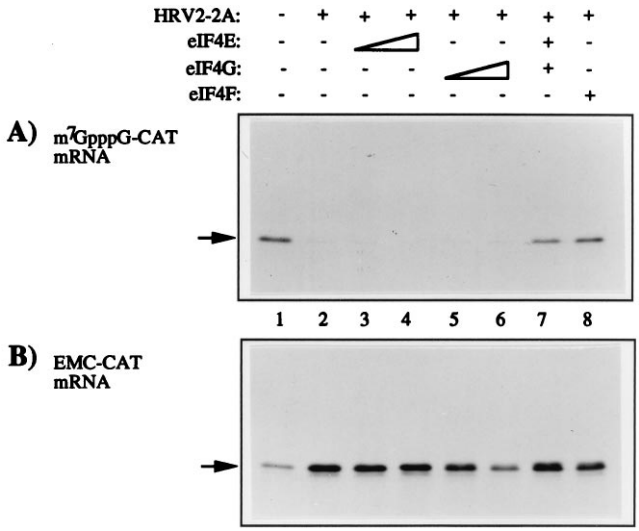

FIG. 5. Restoration of cap-dependent translation in HRV2 $2A^{pro}$-treated Krebs-2 ascites cell extracts. Extracts were treated with either buffer B (lane 1) or HRV2 $2A^{pro}$ (lanes 2 to 8) for 4 min at 30°C followed by a 10-min incubation on ice in the presence of 0.7 mM elastatinal. Extracts were then supplemented with the purified initiation factors and mRNA as indicated. (A) Translation of m^7GpppG-CAT mRNA. (B) Translation of EMC-CAT mRNA. Initiation factors were added as follows: lanes 1 and 2, buffer alone; lanes 3 and 4, 0.2 and 0.4 μg of eIF4E; lanes 5 and 6, ~0.2 and ~0.4 μg of flag-eIF4G; lane 7, 0.2 μg of eIF4E and ~0.4 μg of flag-eIF4; lane 8, 0.75 μg of eIF4F. The position of the CAT product is indicated by an arrow to the left of each panel.

translated in the control extract (Fig. 5B, lane 1). Treatment of the extracts with 2A^{pro} enhanced translation (lane 2), in agreement with earlier results (10, 18, 24). Addition of eIF4E and eIF4G, either alone (lanes 3 and 4 and lanes 5 and 6, respectively) or together (lane 7), had no effect on the translation of EMC-CAT mRNA in the treated extracts (the inhibition by the larger amount of eIF4G in lane 6 was not reproducible). eIF4F did not further stimulate translation either (lane 8). Taken together, these results and those in Fig. 4 directly support the hypothesis that eIF4E is sequestered by the amino terminus of eIF4G following cleavage by the HRV 2A^{pro}. Furthermore, an intact eIF4E-eIF4G complex is required for restoration of cap-dependent translation in picornavirus-infected cells.

DISCUSSION

The cleavage of eIF4G during the replication of certain picornaviruses has been well documented (8). However, there has been much debate on the possible involvement of a cellular proteinase and other translation initiation factors such as eIF3 (8). In addition, the use of eIF4G alone or as a complex with eIF4E has led to different conclusions with regard to the substrate requirements.

Recently, the availability of pure recombinant 2A^{pro} from HRV2 and coxsackievirus B4 and the leader proteinase of FMDV allowed the demonstration that they cleave eIF4G as part of the eIF4F complex without a requirement for cellular proteins (12, 18). Moreover, cleavage of eIF4G by the two different proteinases takes place at sequences determined to be optimal for 2A cleavage (16, 31), further strengthening the idea of a direct mechanism of cleavage. In this regard, it is also of interest that the cleavage activity in infected cells exhibits an almost identical inhibitor profile to both poliovirus and rhinovirus 2A^{pro}. In particular, *N*-ethylmaleimide and iodoacetamide but not E64 inhibit the activity in infected cells (32, 39).

In this work, we have expressed human flag-eIF4G by using a baculovirus expression system, and immunopurified the recombinant protein on an anti-flag column. The ability of flag-eIF4G to act as a substrate for HRV2 2A^{pro} and to restore cap-dependent translation in HRV2 2A^{pro}-treated extracts was then examined. The cleavage of flag-eIF4G alone by HRV2 2A^{pro} was inefficient (Fig. 2). However, addition of exogenous eIF4E to a molar ratio of 4:1 increased the cleavage efficiency by at least 50-fold (Fig. 3E). Furthermore, complete cleavage of flag-eIF4G was obtained only after isolation of the eIF4G-eIF4E complex (Fig. 3B). These results indicate that eIF4E binding to eIF4G changes the conformation of eIF4G, rendering it more susceptible to cleavage by 2A^{pro}. Furthermore, the data suggest that the cleavage region functions as a hinge between the amino and carboxy portions of eIF4G. Stimulation of eIF4G cleavage in a HeLa S10 extract by eIF4E was also clearly evident (Fig. 3C), indicating that the stimulation is not restricted to the recombinant eIF4G that is produced in baculovirus.

The data presented here could explain the discrepancies in the literature concerning the mechanism of cleavage of eIF4G. Recombinant 2A^{pro} of HRV2 and CVB4 expressed in *E. coli* directly cleave rabbit reticulocyte eIF4F to produce the characteristic cleavage products that are observed in vivo (16, 31). However, poliovirus 2A^{pro} has not been tested on intact eIF4F in a similar fashion. Instead, the activity of poliovirus 2A^{pro} has been tested on eIF4G alone that has been separated from eIF4E during the purification (39). Addition of eIF3 was required for cleavage of eIF4G by poliovirus 2A^{pro} (39). eIF3 preparations have been shown to contain eIF4E (34), and it is possible that eIF4E in the eIF3 preparation formed a complex with eIF4G to provide a preferable substrate for poliovirus 2A^{pro}.

The data shown in this paper provide the strongest evidence yet that rhinovirus 2A^{pro} can cleave directly, and without intermediates, the cap-binding protein complex eIF4F. Because previous studies were performed with eIF4F purified from rabbit reticulocyte lysate, it could be argued that this complex contained some trace amounts of other initiation factors or other proteins that could promote or catalyze the proteolytic cleavage. In this study, all components tested in the reactions were recombinants except for eIF3. eIF4G can be cleaved by 2A^{pro}. However, complex formation between eIF4G and eIF4E enhanced the reaction rate and decreased the amount of uncleaved material. These results show that the eIF4G-eIF4E complex is the preferred substrate for 2A^{pro}. Cleavage was not influenced by the addition of either eIF4A or eIF3.

Cleavage of eIF4G by picornavirus 2A^{pro} yields an amino-terminal fragment of about 50 kDa (which migrates as a set of two or three polypeptides of 110 to 130 kDa) that is bound to eIF4E and a carboxy-terminal cleavage product of about 100 kDa that binds eIF4A and eIF3 and associates with ribosomes (15, 22, 28). Thus, cleavage of eIF4G leads to the uncoupling of the cap recognition function of eIF4E from the helicase and ribosome-binding activities of eIF4A and eIF3. Moreover, eIF4E remains sequestered by the amino terminus of eIF4G. The outcome of the cleavage of eIF4G is the specific inactivation of eIF4F function and inhibition of cap-dependent translation. In contrast, translation via internal ribosome binding to the IRES is stimulated (18, 24). It has been suggested that the stimulation is effected by the carboxy-terminal two-thirds of eIF4G, which has a higher affinity for the IRES than does intact eIF4G (24). This is consistent with the idea that cleavage of eIF4G by some picornaviruses is a strategy for stimulating their IRES-driven translation.

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