



Cleavage of eukaryotic translation initiation factor 4GII correlates with translation inhibition during apoptosis

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Abstract

Eukaryotic translation initiation factor 4G (eIF4G), which has two homologs known as eIF4GI and eIF4GII, functions in a complex (eIF4F) which binds to the 5' cap structure of cellular mRNAs and facilitates binding of capped mRNA to 40S ribosomal subunits. Disruption of this complex in enterovirus-infected cells through eIF4G cleavage is known to block this step of translation initiation, thus leading to a drastic inhibition of cap-dependent translation. Here, we show that like eIF4GI, the newly identified homolog eIF4GII is cleaved during apoptosis in HeLa cells and can serve as a substrate for caspase 3. Proteolysis of both eIF4GI and eIF4GII occurs with similar kinetics and coincides with the profound translation inhibition observed in cisplatin-treated HeLa cells. Both eIF4GI and eIF4GII can be cleaved by caspase 3 with similar efficiency *in vitro*, however, eIF4GII is processed into additional fragments which destroy its core central domain and likely contributes to the shutoff of translation observed in apoptosis. *Cell Death and Differentiation* (2000) 7, 1234–1243.

Keywords: translation initiation factor; eIF4G; caspase; apoptosis; protein synthesis inhibition

Abbreviations: CHAPS, % 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate; CHO, aldehyde; eIF, eukaryotic initiation factor; FITC, fluorescein isothiocyanate; PARP, poly-(ADP ribose) polymerase; pNA, para-nitroanilide; TNF α , tumor necrosis factor alpha; Z-VAD-FMK, benzyloxycarbonyl, VAD-fluoromethyl-ketone

Introduction

Apoptosis or programmed cell death is now recognized as a natural mechanism to remove damaged or even virus-infected cells from tissues. It has been known for some time that translation is inhibited in apoptotic cells but potential mechanisms of this inhibition have not been investigated

until recently. The components of the apoptotic machinery, i.e. the caspases, are already present in the cell, and therefore, *de novo* protein synthesis is not required for induction of apoptosis in most systems.^{1–4} However, there are several other systems in which protein synthesis is required for apoptosis to occur.^{5–8} In addition, drastic inhibition of cellular translation in virus-infected cells has long been considered a major mechanism of cell death. Therefore, regulation of translation could potentially play an important role in the induction or execution of apoptosis.

Eukaryotic translation initiation factor 4F (eIF4F) is required for binding the vast majority of capped mRNAs to ribosomes during the initial steps of translation. eIF4F consists of eIF4E, the cap-binding protein that specifically binds to the 5' cap structure (m⁷ GpppN) present on cellular mRNAs; eIF4A, an ATP-dependent RNA helicase; and eIF4G which functions as a molecular scaffold by simultaneously binding eIF4E, eIF4A, eIF3.^{9,10} Simultaneous binding of eIF3 with eIF4G and the 40S ribosomal subunit, is thought to enable ribosomes to bind capped mRNAs and initiate protein synthesis. Numerous studies with picornaviruses have shown that cleavage of eIF4G results in functional disruption of the eIF4F protein complex coincident with inhibition of translation.^{10–13} Cleavage of eIF4G results in production of two fragments, an N-terminal fragment containing the binding site for eIF4E and a C-terminal fragment bearing the binding site for eIF3 and eIF4A,⁹ thus separating domains responsible for binding capped mRNA (via eIF4E) and the ribosome (via eIF3). This is thought to result in drastic impairment of cap-dependent translation. Further, the rapid and drastic inhibition of cap-dependent translation during picornaviral infection has long been thought to be very important mechanism by which infected cells die. Recently, Goldstaub *et al*¹⁴ demonstrated that the expression of 2Apro in mammalian cells was sufficient to induce programmed cell death.

Recently, we discovered that eIF4GI was cleaved during apoptosis in a variety of cell types and that its cleavage correlated with translation inhibition in apoptotic cells.¹⁵ eIF4GI was the first mammalian translation factor found to be targeted during apoptosis and its degradation could account for much of the observed inhibition of protein synthesis in apoptotic cells. Recently, a new functional homolog of eIF4GI, named eIF4GII, was identified that is 46% identical to eIF4GI, which binds to eIF4E, eIF4A, eIF3, and is capable of restoring cap-dependent translation *in vitro* after degradation of eIF4G.¹⁶ Although the functional relevance of two eIF4G isoforms is currently not known, the presence of two forms of eIF4G has been conserved throughout evolution and can be observed in other species, including yeast,¹⁷ and plants.¹⁸ Intriguingly, in poliovirus-infected cells proteolysis of eIF4GII, but not eIF4GI, seems to correlate better with the observed translation inhibition.¹⁹

This may suggest that significant functional differences between the two forms of eIF4G do exist, yet more experiments need to be performed to elucidate those.

Here, we show that cleavage of eIF4GII coincides with eIF4GI cleavage in apoptotic cells and correlates with translation inhibition in cisplatin-treated HeLa cells. Interestingly, whereas eIF4GI is cleaved into only three fragments,¹⁵ eIF4GII appears to be cleaved by caspase 3 at multiple sites. Taken together, our results show cleavage of eIF4GII during apoptosis, thus providing an additional event which contributes to the mechanism of translational control during apoptosis.

Results

In vivo cleavage of eIF4GII

After our observation that eIF4GI was cleaved during apoptosis with kinetics which correlated with translation inhibition,¹⁵ we wanted to determine the fate of eIF4GII during apoptosis. Therefore, apoptosis was induced in HeLa cells by treatment with 100 μ M cisplatin, and monitored over a 16 h period. Cleavage of eIF4GII was analyzed by immunoblot using a N-terminal specific eIF4GII antibody (produced with a fragment of eIF4GII comprising aa 445–604), and indicated by eIF4GII_N. The data (Figure 1A) show that eIF4GII was rapidly cleaved in apoptotic cells, producing a dominant cleavage product of 97 kDa which could be detected by 3 h (Figure 1A) and less abundant products near 150, 120 and 82 kDa. Immunoblots using this antibody suggested that while cleavage of eIF4GII began by 3 h, uncleaved eIF4GII was still detectable until 8–9 h. Figure 1B is an immunoblot

showing eIF4GI cleavage kinetics from the same experiment shown in Figure 1A. Comparison of the kinetics of eIF4GI and eIF4GII cleavage shows an overall similarity in their cleavage rates, however, although eIF4GI cleavage was detected one hour later than eIF4GII cleavage, it seemed to reach completion more abruptly. It should be pointed out that comparison of immunoblots from numerous experiments revealed no consistent major differences in the kinetics of cleavage of eIF4GI and eIF4GII; they were essentially the same in most experiments, and minor variations are likely attributable to differential reactivity of antisera with antigens. The kinetics of cleavage of PARP, a well known cellular substrate of caspase 3, were also examined and suggest activation of caspases correlates well with the cleavage of eIF4GII. Therefore, cleavage of eIF4GII occurs simultaneously with caspase-3 dependent cleavage of eIF4GI and PARP. When apoptotic cells were measured by staining with annexin-FITC (Caltag) and immunofluorescence microscopy, we determined that the percentage of apoptotic cells were 2, 39, 77 and 98% at 0, 4, 8 and 16 h respectively. Taken together, this correlation with PARP cleavage and annexin staining suggests that under these conditions eIF4GII cleavage occurs relatively late during the execution phase of apoptosis.

Previously, we demonstrated that eIF4GI cleavage correlated with a decline in mRNA translation rates *in vivo*. Here, comparison of pulse label experiments (Figure 1C) with immunoblot results revealed that the progressive cleavage of intact eIF4GII also correlates with the observed drop in translation. In fact, the combined decline in both forms of eIF4G may account for much of the inhibition in translation observed here, although modification of other

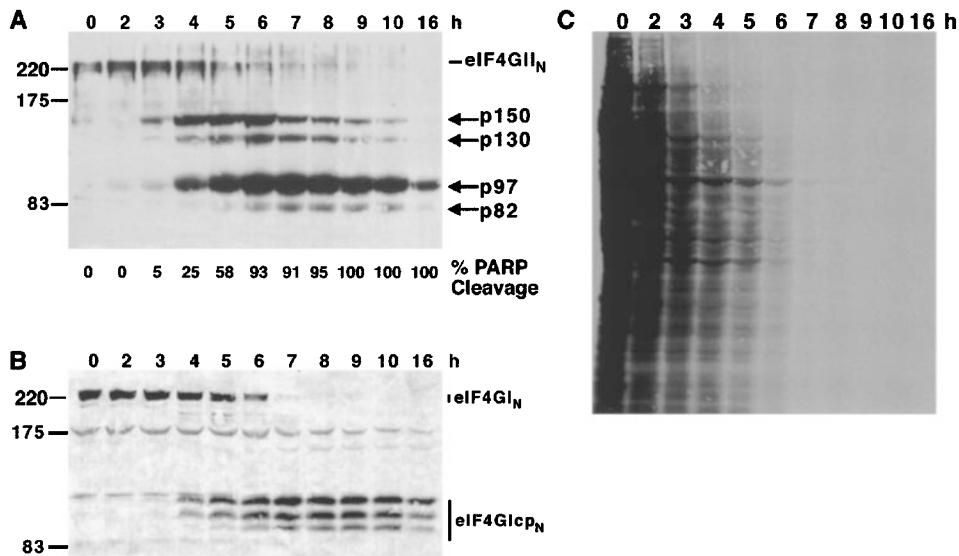


Figure 1 Kinetics of eIF4GI and eIF4GII cleavage during apoptosis in HeLa cells. **(A)** HeLa cells were treated with 100 μ M cisplatin and incubated for the indicated time periods. Cell lysates (50 μ g of protein) were loaded on a 7% acrylamide gel and immunoblotted with polyclonal antiserum specific for N-terminal eIF4GII. eIF4GII and cleavage products of eIF4GII are indicated by arrows on the right. Molecular weight markers are indicated on the left. The per cent cleavage of PARP detected at each timepoint is shown below the panel. **(B)** Cell lysates (50 μ g of protein) as described in **(A)** were loaded on a 7% acrylamide gel and immunoblotted with polyclonal antisera specific for N-terminal eIF4GI. eIF4GI and cleavage products of eIF4GI are indicated by arrows on the right. **(C)** After treatment with cisplatin for the time points (in hours) indicated, HeLa cells were pulse-labeled with [³⁵S]-methionine for 1 h, lysed, subjected to 12% SDS-PAGE, and analyzed by autoradiography

translation factors (e.g. eIF2 α , [Marissen, 2000 #3725]) is also occurring and likely contributes to translation regulation. Thus, we have now shown that both eIF4GI and eIF4GII are cleaved during apoptosis, and degradation of each could potentially contribute to inhibition of cap-dependent protein synthesis during apoptosis.

To further analyze the proteolytic processing of eIF4GII, we also used a different antibody directed against a C-terminal portion of eIF4GII (aa 1168–1192), indicated by eIF4GII_C (Figure 2). Cell lysates derived from the same experiment described in Figure 1 were analyzed by immunoblot, which again revealed complete cleavage of eIF4GII by 7 h after induction of apoptosis. The cleavage pattern of immunoreactive polypeptides included several potential transient cleavage products (p140, p120 and p58), which seem to accumulate into possible end products of 48, 35 and 28 kDa by 8 h. Putative cross-reactive polypeptides which migrate near 65, 60 and 34 kDa are also sometimes detected with this antisera, the latter is often degraded in apoptosis as well. Apparently, the eIF4GII cleavage fragments are not very stable as they often disappeared between 10–16 h after induction of apoptosis. In general, degradation of eIF4GII seemed to be more drastic and complete than eIF4GI, whose three major cleavage products appear to be more stable,¹⁵ (data not shown). Overall, analysis of eIF4GII cleavage using two different antibodies directed against either N- or C-terminal region of eIF4GII reveals that eIF4GII is cleaved into multiple fragments during apoptosis, and indicates several cleavage sites are utilized to degrade eIF4GII.

In vivo inhibition of eIF4GII cleavage

To test whether eIF4GII cleavage is caspase-dependent, HeLa cells were pretreated for 1 h with the broad spectrum caspase inhibitor benzyloxycarbonyl-VAD-fluoromethylketone (Z-VAD-fmk) before administration of various apoptosis-inducing agents (Figure 3A). Treatment of HeLa cells with cisplatin resulted in cleavage of eIF4GII resulting in similar

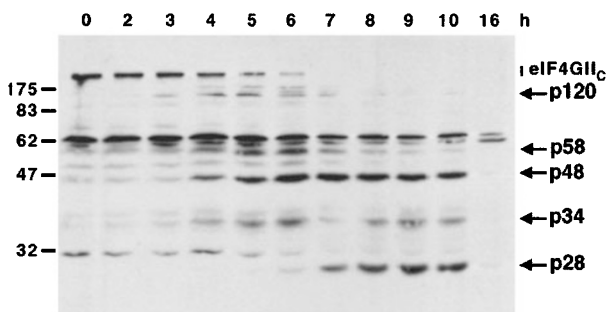


Figure 2 Analysis of eIF4GII cleavage during apoptosis using C-terminus specific eIF4GII antibody. Cell lysates described in Figure 1 were subjected to SDS-PAGE (12% acrylamide gel), and analyzed by immunoblotting with an antibody specific for the C-terminal portion of eIF4GII. eIF4GII and cleavage products of eIF4GII are indicated by arrows on the right. Molecular weight markers are indicated on the left. Immunoreactive bands at near 62 kDa are likely unrelated proteins which cross-react with this antisera. The identity of p34, which could be a minor alternate processing intermediate or an unrelated cross-reactive protein, has not been determined

fragments as shown in Figure 1A (using the N-terminal specific antibody). In addition, treatment with etoposide or TNF α also led to cleavage and processing of eIF4GII into the p97 cleavage fragment, indicating that cleavage of eIF4GII may be a common event during apoptosis generated by several types of inducers, producing similar fragments. Regardless of which apoptotic inducer was used, cleavage of eIF4GII could be blocked by pretreatment of cells with 75 μ M Z-VAD-fmk. This suggests that activation of caspases is a necessary event for the induction or catalysis of eIF4GII cleavage in apoptotic cells. Analysis of control cells (Figure 3A, lane C) revealed an apparent sporadic partial degradation of eIF4GII (evidenced by a p150 cleavage product) that was not accompanied by detectable eIF4GI degradation or PARP cleavage (data not shown). This was observed many times but did not correlate with increased presence of apoptotic cells in the culture and is possibly due to another unknown form of degradation.

Figure 3B shows that caspase 3-related activity can be detected in cell lysates by cleavage of DEVD-pNA substrate and pretreatment of cells with Z-VAD-fmk caspase-inhibitor significantly inhibited the degree of

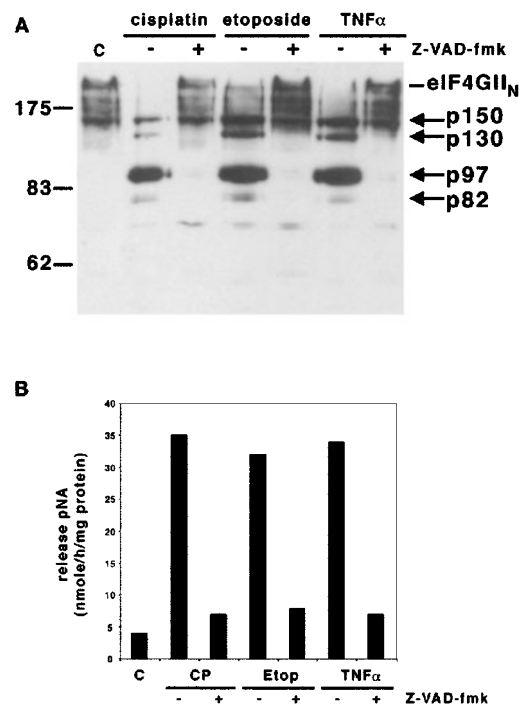


Figure 3 In vivo inhibition of eIF4GII cleavage in HeLa cells by Z-VAD-fmk. (A) Inhibition of eIF4GII cleavage *in vivo*. HeLa cells were pretreated with 75 μ M Z-VAD-fmk for 1 h as indicated by + before treatment with 100 μ M cisplatin, 50 μ M etoposide, 40 ng/ml TNF α plus 10 μ g/ml cycloheximide, for 16 h as indicated on top of the figure. Cell lysates (50 μ g of protein) were analyzed on 12% acrylamide gel and immunoblotted with specific antiserum against N-terminal eIF4GII. Lane C, untreated HeLa cells. (B) In vivo inhibition of caspase activity. Cell lysates (20 μ g of protein) as described in (A) were assayed for caspase activity by incubation with Ac-DEVD-pNA (0.2 mM). Release of pNA was analyzed by optical density at 405 nm, and caspase activity is displayed as the number of nanomoles of pNA released per hour per total milligram of protein as calculated from a standard curve by using free pNA. Lane C, untreated HeLa cells

caspase activation (by greater than 70%) observed in cell lysates. Thus, activation of eIF4GII cleavage activity correlates with activation of caspases in apoptotic cells, suggesting that caspases are involved in the induction or catalysis of eIF4GII cleavage.

Cleavage of eIF4GII in apoptotic K562 and Jurkat cells

To further address the degree to which eIF4GII cleavage is a general response to apoptosis, we induced apoptosis in two other cell types using a variety of well-characterized inducers. Immunoblot analysis of cell lysates from K562 pre-erythroid leukemia cells (Figure 4A) and Jurkat T cells (Figure 4B) reveal the generation of the same p97 eIF4GII cleavage product observed in HeLa cells, although the degree of cleavage was variable depending on the inducer tested. Treatment of K562 cells with cisplatin, MG132, and to a lesser

extent etoposide, or Jurkat cells with cisplatin, MG132, TNF α , all effectively induced apoptosis as determined by DNA fragmentation and PARP cleavage (data not shown). Treatment of cells with etoposide resulted in partial activation of apoptosis by these criteria, and also resulted in only partial cleavage of eIF4GII. Overall, we observed a good correlation between the per cent apoptotic cells in a population and the degree of eIF4GII cleavage. The p97 putative cleavage fragment was also present in untreated Jurkat cells, suggesting a proportion of apoptotic cells exist in Jurkat cultures as has been reported previously by others (e.g.²⁴) or another mechanism of eIF4GII degradation exists in untreated cells. Overall, these results show that induction of eIF4GII cleavage during apoptosis appears to be common to several different cell types and can be induced through several pathways.

In vitro cleavage of eIF4GI and eIF4GII by caspases

To address whether eIF4GII is a direct substrate for caspases, we tested a panel of purified caspases for their ability to cleave eIF4GII in purified preparations (Figure 5). Preparations of eIF4F complex purified from HeLa cells by m⁷GTP-sepharose affinity chromatography contain both eIF4GI and eIF4GII, as well as eIF4A and eIF4E,¹⁶ and analysis of these preparations by SDS-PAGE and silver stain reveals no observable contamination with other proteins.¹⁵ Therefore, purified eIF4F was incubated with purified caspases and analyzed by immunoblot using the C-terminal-specific antibodies for either eIF4GI (Figure 5A) or eIF4GII (Figure 5B). The results show eIF4GI can be cleaved by caspases 3, 8 and 10, however, only caspase 3 produced cleavage fragments of 120 and 46 kDa (arrows) that are also observed *in vivo* (see¹⁵). This suggests caspase 3 is primarily responsible for eIF4GI cleavage and agrees with our earlier conclusions.¹⁵ On the other hand, eIF4GII seems to be more

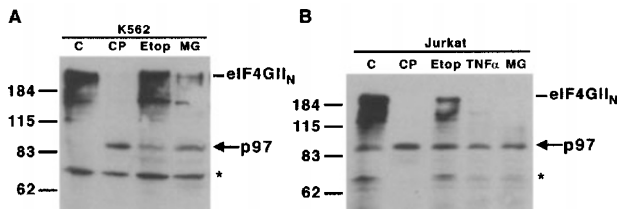


Figure 4 Cleavage of eIF4GII in apoptotic K562 and Jurkat cells. K562 cells (A) or Jurkat cells (B) were treated with 100 μ M cisplatin (CP), 50 μ M etoposide (Etop), 10 μ M MG132 (MG), or 40 ng/ml TNF α plus 10 μ g/ml cycloheximide (TNF α) as indicated above each lane for 20h at 37°C. Cell lysates were analyzed on 12% acrylamide gels, transferred to nitrocellulose, and immunoblotted with N-terminal specific eIF4GII antibody. eIF4GII and cleavage products of eIF4GII are indicated by arrows on the right. Asterisks denote an immunoreactive protein of unknown origin often observed in cell lysates from these cells. Molecular weight markers are indicated on the left

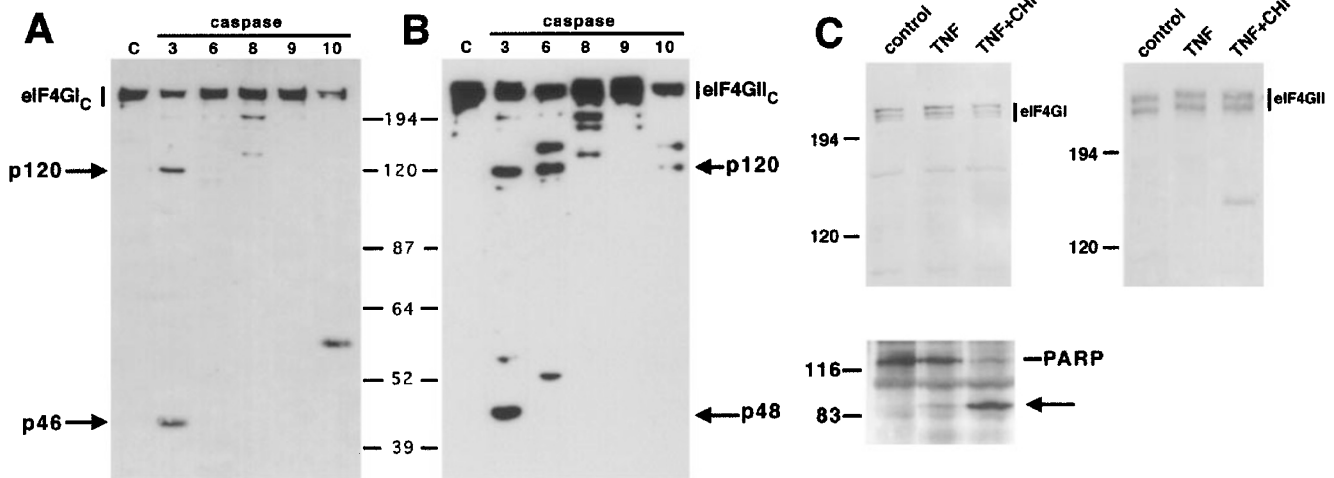


Figure 5 Identification of caspases which can cleave eIF4GII. Purified eIF4F was incubated with purified recombinant human caspases 3, 6, 8, 9, or 10 (10 U each) for 3h at 37°C. Samples were then analyzed on 8% acrylamide gels and immunoblotted with either antibody specific for C-terminal region of eIF4GI (A) or eIF4GII (B). Caspase 3 generated cleavage products of eIF4GI and eIF4GII are indicated by the arrows. The 34 and 28 kDa cleavage products observed *in vivo* (Figure 2) were not resolved from the solvent front on this gel. (C) MCF7 cells were treated with 40 ng/ml TNF α alone or TNF plus 10 μ g/ml cycloheximide for 20h at 37°C. Immunoblots of cell lysates were probed with antibody for eIF4GI, eIF4GII or PARP as indicated. The PARP cleavage product is designated with an arrow

susceptible to caspase cleavage and cleavage fragments can be produced by caspases 3, 6, 8 and 10 but not caspase 9. The eIF4GII cleavage patterns generated by each caspase are different, suggesting that each caspase utilizes different cleavage sites on eIF4GII, though some may be shared. Although caspase 3 treatment induced cleavage fragments of 120, and 48 kDa that resemble eIF4GII fragments observed *in vivo* (Figure 2), it is possible that other caspases are involved in cleavage of eIF4GII *in vivo* since many cleavage products are observed.

To further test if caspase 3 was the predominant eIF4GII cleavage activity *in vivo*, cell extracts from cisplatin-treated HeLa cells were incubated with eIF4GII present in initiation factor preparations purified from control cells. A partial eIF4GII cleavage activity was detected upon overnight incubation which was inhibited by addition of DEVD-CHO to lysates (data not shown), supporting a role for caspase 3 in eIF4GII cleavage. To further examine the role of caspase 3, we induced apoptosis in MCF7 breast carcinoma cells which lack caspase 3 via a functional gene deletion.²⁵ The data show (Figure 5C) that at later stages of apoptosis induced with TNF and cycloheximide, when PARP cleavage is nearly complete, there is no significant cleavage of eIF4GI, as has been previously reported.²⁶ Likewise, the majority of eIF4GII also remained intact in apoptotic MCF7 cells, although one potential cleavage product did appear at low levels. This could represent inefficient cleavage by other caspases (which are also able to cleave PARP²⁵). Similar efficient PARP cleavage combined with a lack of eIF4GII cleavage was observed when cisplatin was used to induce apoptosis in MCF7 cells (data not shown). Taken together, these results suggest that caspase 3 is the predominant caspase activity which degrades eIF4GII *in vivo*, but do not exclude ancillary roles of other proteases in eIF4GII processing.

Comparison of caspase 3-induced cleavage of eIF4GI and eIF4GII

Since it appeared that caspase 3 could generate eIF4GII cleavage products similar to those observed *in vivo*, we further compared *in vitro* cleavage of purified eIF4GI and eIF4GII present in eIF4F preparations by caspase 3 using the C-terminal specific antibodies (Figure 6). Incubation of eIF4F with caspase 3 results in rapid cleavage of both eIF4GI (Figure 6A) and eIF4GII (Figure 6B), and is complete by 3 h of incubation at 37°C. The difference in eIF4G cleavage efficiency by caspase 3 as detected here, compared to Figure 5 in which eIF4G was not completely cleaved by 3 h, is most likely due to variable activity of caspase 3 preparations. No apparent differences are observed between the efficiency of cleavage of eIF4GI and eIF4GII and both seem equally susceptible to caspase 3-induced cleavage. Interestingly, caspase 3 generates additional eIF4GII cleavage products containing the antibody epitope than eIF4GI cleavage products (arrows), which indicates recognition of additional cleavage sites on eIF4GII. It should be mentioned that both antisera used here are directed against the same region (which is not conserved) of their respective eIF4G homolog (aa 1159–1181 of eIF4GI, aa 1168–1192 of eIF4GII). In

conclusion, both eIF4GI and eIF4GII can serve as substrates for caspase 3, however, eIF4GII is processed into more fragments by caspase 3 than eIF4GI.

Analysis of eIF4GII cleavage *in vitro*

Characterization of eIF4GII cleavage fragments was further addressed by monitoring a time course of caspase 3 induced cleavage of eIF4GII present in HeLa crude initiation factor fragments (Figure 7). Caspase 3 generates p150, and p97 cleavage fragments (Figure 7A) which were recognized by N-terminus specific antibody, similar to the fragments observed above in apoptotic cell lysates. The p150 polypeptide is likely

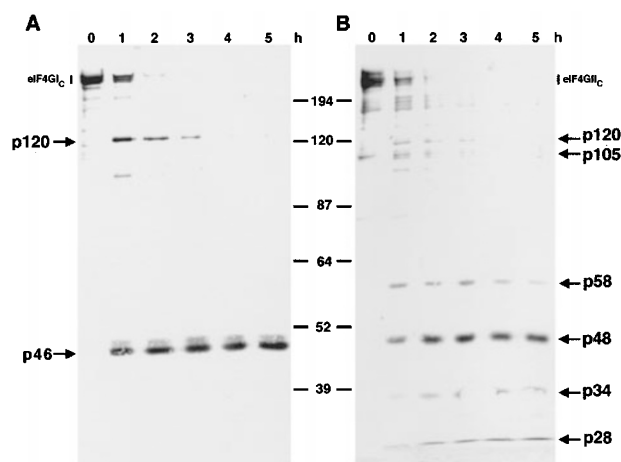


Figure 6 Cleavage of eIF4GI and eIF4GII by caspase 3 *in vitro*. Purified eIF4F was incubated with purified recombinant human caspase 3 (17 U) at 37°C and aliquots were taken at time points indicated on top of the figure. Samples were analyzed on 9% acrylamide gels and immunoblotted with either specific C-terminal eIF4GI (A) or eIF4GII (B) antibody. Caspase 3 generated cleavage products of eIF4GI and eIF4GII are indicated by arrows

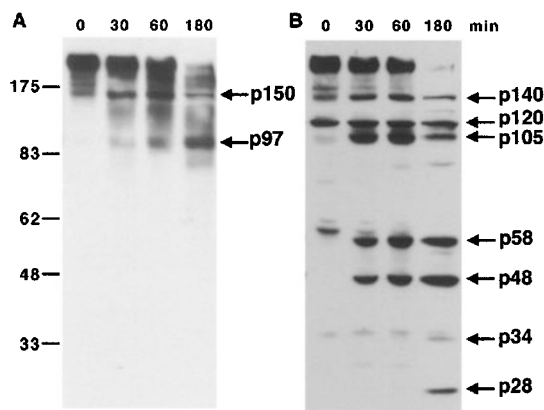


Figure 7 Immunoblot analysis of eIF4GII processing scheme *in vitro*. HeLa RSW (4 μ l) was incubated at 37°C with purified caspase 3 (17 U) in a total reaction volume of 50 μ l. Time points were taken as indicated above each lane and were analyzed by SDS-PAGE and immunoblotting with either N- (A) or C-terminal (B) specific eIF4GII antibody. Caspase 3 generated cleavage products of eIF4GI and eIF4GII are indicated by arrows. Molecular weight marker is indicated on the left

to be an intermediate eIF4GII cleavage product, which is further processed by caspase 3 resulting in p97. Analysis of the same samples with C-terminal specific antibody (Figure 7B) reveals generation of several eIF4GII cleavage fragments p140, p105, p58, p48, p34 and p28 which contain the C-terminal antibody epitope. Again, the slower migrating p140, and p105 polypeptides appear to be further processed into the faster migrating end products p58, p48, p34, and p28 as incubation time increased. These results are in good agreement with the cleavage pattern observed *in vivo* (Figure 2), suggesting that caspase 3 could be responsible for the majority of eIF4GII cleavage observed *in vivo*.

Analysis of the amino acid sequence of eIF4GII reveals four consensus caspase 3 sites (DxxD)²⁷ at positions 557–560, 848–851, 975–978 and 1159–1162. However, our data presented so far do suggest that other non-conventional caspase 3 sites may actually be utilized by caspase 3. In order to visualize all the caspase 3-generated cleavage products, not just those containing recognizable epitopes, we radiolabeled eIF4GII with [³⁵S]-methionine and cysteine by programming rabbit reticulocyte translation lysates with eIF4GII mRNA and full-length radiolabeled eIF4GII was purified by affinity chromatography on m⁷GTP-sepharose (lanes a–d, Figure 8) before cleavage reactions were performed. Cap-column purified eIF4GII was then incubated at 37°C with purified caspase 3 for the amount of time indicated. Figure 8 (lanes b–e) shows the cleavage products derived from eIF4GII in two independent experiments. Incubation with caspase 3 led to proteolytic processing of eIF4GII into multiple cleavage products, some of which appear to be similar to those observed on immunoblots in previous figures, including p97, p58, p48 and p28. Many new bands, previously not seen on immunoblots are also detected here, including p82, p72, p62, p52 and p35, and several proteins which migrate faster than p28. Many of these proteins likely represent cleavage fragments derived from the middle third of eIF4GII that are not recognized by the antibodies used in this study but could result from cleavage at classic caspase 3 recognition sites. After extended incubation to 18 h, many bands have disappeared, notably p97 and p58, indicating that both of these can be further processed. It is possible that besides the four consensus caspase 3 cleavage sites mentioned above, additional cleavage sites may be used by caspase 3 inefficiently that do not have the classic consensus motif DxxD (see below). Alternatively, slow developing cleavage events could also arise from trace amounts of another protease which could be present in either the caspase preparation or the eIF4GII, despite the high level purification of both. In addition, it seems that more polypeptide products are produced from this highly purified substrate than we usually observed *in vivo*. It is possible that certain cleavage sites on eIF4GII may be masked by the binding of other proteins to eIF4GII *in vivo* that are not present on eIF4GII translated *in vitro*. Alternatively, conformations of eIF4GII may exist when highly purified, which could expose additional sites.

To further identify the origin of some of the cleavage fragments, we purified several C-terminal truncated forms of eIF4GII which were cleaved with caspase 3. The data

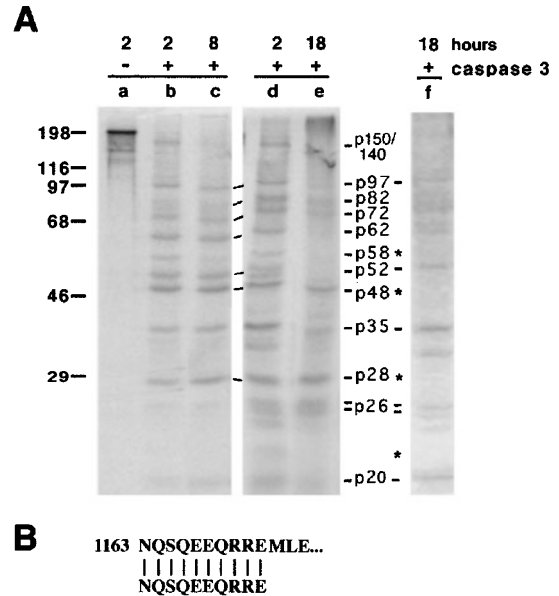


Figure 8 Analysis of processing of purified radiolabeled eIF4GII by caspase 3. (A) *In vitro* translated eIF4GII was purified on methyl-7-GTP-sepharose and eluted with 100 μM m⁷GTP. Purified eIF4GII was incubated with purified caspase 3 (17 U) and samples were analyzed by SDS-PAGE and autoradiography. Lane a, *in vitro* translated eIF4GII (start); lanes b–e, eIF4GII incubated with caspase 3 for indicated times. Similarly, truncated eIF4GII generated from *SpeI*-linearized cDNA was also purified and cleaved with caspase 3 (lane f). Migration of molecular weight markers is indicated on the left; dominant eIF4GII cleavage products are indicated on the right. Asterisks indicate cleavage products of eIF4GII that are not detected when truncated eIF4GII (lane f) was used as substrate. (B) Identification of caspase 3 cleavage site. Purified recombinant eIF4GII was incubated with purified recombinant caspase 3 for 5 h at 37°C, transferred to PVDF, and protein bands were subjected to amino acid sequencing. Resulting amino acid sequence (bottom) was matched with amino acid sequence in eIF4GII (top) starting at position 1163

(Figure 8, lane f) is presented from one such truncated eIF4GII (at aa 1126) which reveal p58, p48 and p28 were no longer produced whereas p35, p19 and a p26 doublet was still produced. The p97 fragment was also still visible, although only faintly due to continued processing. These data support the assignment of p58, p48 and p28 fragments to the C-terminal portion of eIF4GII, in agreement with earlier immunoblot data.

In addition, the p28 cleavage fragment was purified and its sequence analyzed by Edman degradation, which revealed a perfect match with the sequence immediately downstream from a consensus caspase cleavage site DLLD¹¹⁶²N in the C-terminal region of eIF4GII (Figure 8B). Although this site is not exactly conserved between eIF4GI and eIF4GII, the site is in a similar region of the protein as the downstream eIF4GI cleavage site recently identified (see Figure 9). Interestingly, cleavage at position 1162 would result in a C-terminal fragment of approximately 48 kDa. Thus, p28 must be generated by additional cleavage of this C-terminal fragment by caspase 3 at a site downstream of 1162. This site is most likely the sequence IESD at position 1407, which would generate fragments of approximately 28 and 20 kDa.

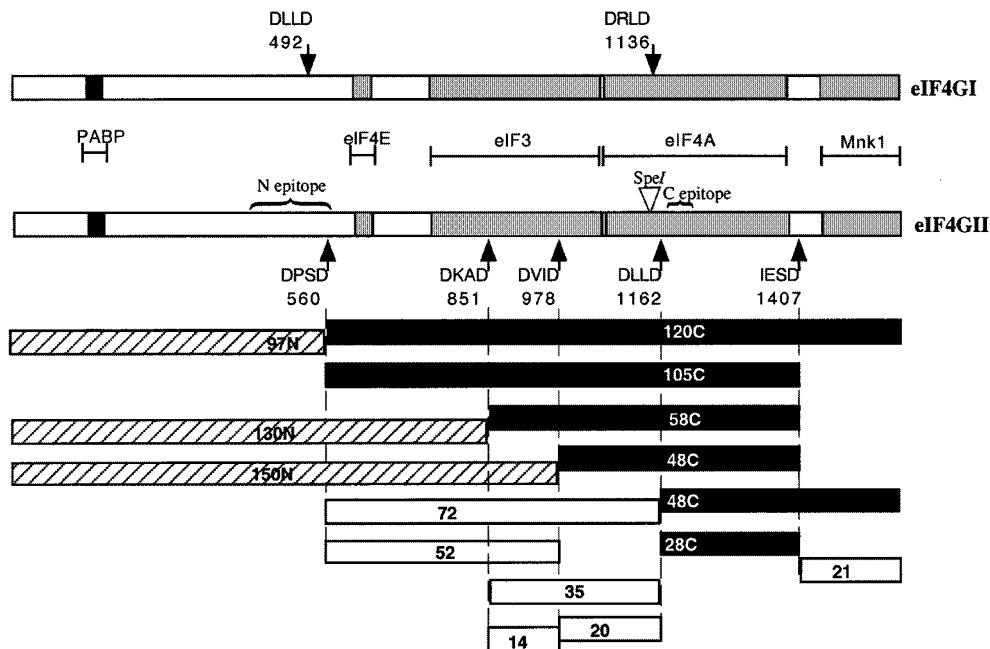


Figure 9 Proposed processing scheme for eIF4GII. Previously determined cleavage sites for eIF4GI are shown for comparison and bars indicate regions of eIF4GI and eIF4GII which interact with other factors. The region of eIF4GII containing a N-terminus or C-terminus-specific antibody epitope is also shown. Only fragments resulting from cleavage at the four canonical caspase 3 cleavage sites plus the additional IESD site are diagrammed. The putative apparent molecular weight of each fragment is indicated by the number and N or C indicate potential reactivity with the respective antisera

Discussion

Here, we have shown that induction of apoptosis results in rapid and complete cleavage of not only eIF4GI, but also eIF4GII, and this cleavage occurs concomitantly with the drastic inhibition of cellular translation. Cleavage of eIF4GII could be inhibited by Z-VAD-fmk indicating the direct involvement of caspases in the cleavage of eIF4GII as was shown for eIF4GI.¹⁵ In addition, both eIF4GI and eIF4GII were cleaved by caspase 3 *in vitro* indicating that caspase 3 could be responsible for the observed eIF4G cleavage *in vivo*. This was further supported by the fact that of the cleavage patterns generated by incubation of purified eIF4F with individual purified caspases, caspase 3-generated fragments most closely matched with eIF4GII cleavage products generated *in vivo*. Both eIF4GI and eIF4GII play a crucial role in the assembly of the initiation complex and recruitment of ribosomes to capped mRNAs. In poliovirus-infected HeLa cells, it was reported that proteolysis of eIF4GII, rather than eIF4GI, more closely correlates with translation inhibition in poliovirus- or rhinovirus-infected HeLa cells because its cleavage kinetics lagged significantly behind eIF4GI cleavage, especially under experimental conditions which limited viral replication.^{19,29} Further, replenishment of both isoforms of eIF4GII in reticulocyte lysates pretreated to cleave endogenous eIF4GI and eIF4GII, was capable of restoring cap-dependent mRNA translation,¹⁶ supporting the hypothesis that translation inhibition in picornavirus-infected cells is primarily mediated by eIF4G cleavage. Therefore, the biological importance of eIF4G cleavage during apoptosis may also be significant and it is possible that cleavage of both eIF4GI and eIF4GII may hasten the execution phase of

apoptosis. In addition, eIF4GI and eIF4GII are cleaved in HeLa cells as well as Jurkat T cells, and K562 cells when treated with a variety of apoptosis-inducing agents as well as in BJAB cells treated with cycloheximide.³⁰ Thus, it seems that cleavage of both eIF4GI and eIF4GII is a common event during apoptosis, supporting an important role for eIF4G cleavage in translation shutoff during the execution phase of apoptosis and suggesting that eIF4GII cleavage may be another marker for cell death. Continued investigation of the fate of translation initiation factors during apoptosis has revealed an interesting pattern of proteolysis. Translation factors which are not proteolyzed at all by caspases tested include two other members of the cap-binding protein complex, eIF4E, eIF4A, most of the subunits of eIF3.^{15,30} In contrast, we and others have recently discovered that eIF2 α is cleaved at its extreme C-terminus^{31,32} as well as processing of eIF4B.³⁰ Further experiments are required to determine if execution of apoptosis actually requires any of these cleavage events or drastic inhibition of cellular translation in order to reach completion.

Figure 9 shows a proposed processing scheme which was derived from compiling data from the experiments shown above as well as numerous others which were not shown. To account for all the dominant fragments observed, we hypothesize eIF4GII is cleaved at all four classical caspase 3 cleavage sites (DxxD) at positions 557–560, 848–851, 975–978 and 1159–1162 plus an additional IESD at position 1407. Recently, the caspase 3 cleavage sites on eIF4GI were identified²⁸ at positions DLLD⁴⁹²A, and DRLD¹¹³⁶R (see Figure 9). Although neither of these sites are conserved between eIF4GI and eIF4GII, the potential caspase 3 cleavage sites DPSD⁵⁶⁰L and

DLLD¹¹⁶²N are found in similar regions within eIF4GII and could generate a similar tripartite fragmentation of eIF4GII, including release of a 48 kDa C-terminal product (which was observed in several experiments). Indeed, we have verified by direct amino acid sequencing that caspase 3 does recognize the DLLD motif at position 1162 (Figure 8B), however, this sequence was found on a smaller 28 kDa polypeptide, providing strong evidence for processing by caspase 3 at a non-canonical IESD site at position 1407. Since caspase 3 is known to autocatalytically cleave itself at IETD¹⁷⁵S, and is known to cleave other proteins after IETD,³³ the IESD motif in eIF4GII seems a likely candidate. Interestingly, further comparison between eIF4GI and eIF4GII reveals that this IESD site is not conserved between the two proteins and no 28 kDa cleavage fragment is generated by caspase 3-induced cleavage of eIF4GI.¹⁵ The location of antibody epitopes on polypeptide fragments proposed in this processing scheme is consistent with the major fragments observed in our experiments and it also accounts for the dominant cleavage fragments observed using truncated eIF4GII substrates (data not shown). However, several minor fragments which appear late in incubation periods likely result from inefficient cleavage at additional sites not identified here. For instance, p97 which represents the N-terminal third of eIF4GII, was often further degraded during our experiments, particularly during long incubations with highly purified substrate, yet no consensus DxxD caspase sites exist within its sequence. Additional work will be required to confirm this proposed processing scheme and to identify additional sites that are less efficiently recognized by caspase 3. However, it is very clear that eIF4GII is cleaved into many more fragments by caspase 3 than eIF4GI,²⁸ which likely destroys any useful function of eIF4GII fragments in translation.

An interesting question is why eIF4GII is cleaved differently compared to eIF4GI. It is not known if eIF4GII contains any functions in translation which are slightly different than its homolog. For instance, since large regions of eIF4GI and eIF4GII are not homologous, it is possible that eIF4GII binds to proteins that do not interact with eIF4GI, possibly providing altered or additional functions. Thus, eIF4GII may require a higher degree of proteolytic processing than eIF4GI further to inactivate these functions during apoptosis. There is also a possibility that the ordered cleavage of eIF4GI and eIF4GII might result in a generation of eIF4G cleavage fragments that display new properties that might aid in the apoptotic process or the regulation of its induction. It has been shown in the case of poliovirus that upon cleavage, the C-terminal cleavage product of eIF4GI can stimulate cap-independent, IRES driven translation.^{34,35} Further, a core region of eIF4GI spanning the binding domains for eIF4E and eIF3 has been shown to be sufficient to support cap-dependent translation.^{36,37} Conceivably, generation of eIF4G cleavage products during apoptosis could potentially lead to specific upregulation of certain pro-apoptotic genes due to altered characteristics of eIF4GI and eIF4GII cleavage products. Since eIF4GI is fragmented into only three portions which releases an intact core domain, whereas eIF4GII is

fragmented into five or more segments which destroys the core domain, it is more likely that gained functions would reside with eIF4GI fragments than eIF4GII fragments. However, further studies are required to test these new hypotheses and more finely assess the exact impact of eIF4G cleavage on the apoptotic process.

Materials and Methods

Cell culture

HeLa S3 cell monolayers were grown in S-MEM (Irvine Scientific), Jurkat and K562 cells were grown in RPMI (Irvine Scientific) supplemented with 10% bovine calf serum, 0.5% fetal calf serum (Summit Biotech.), 100 U penicillin and 100 μ g streptomycin per ml (Sigma) in a humidified chamber containing 5% CO₂. For the induction of apoptosis, various concentrations of cisplatin, etoposide, cycloheximide (Sigma), MG132 (Calbiochem), TNF α (Peprotech), stock solutions were diluted with S-MEM and then incubated with cells at 37°C for the time indicated in each figure. For *in vivo* inhibition experiments cells were first preincubated with the cell-permeable caspase inhibitor Z-VAD-fmk (Enzyme Systems Products) at indicated concentrations for 1 h at 37°C before the cisplatin was added to the cell cultures.

Antibodies

Antibodies for eIF4GI^{15,20} and eIF4GII^{14,16} have been previously described. Immunoblot analysis for PARP cleavage was performed as previously described¹⁵ using a monoclonal anti-PARP antibody (Zymed Labs). Levels of PARP cleavage were quantitated from scanned immunoblots using NIH Image software.

Preparation of HeLa cell extracts

Cell extracts were prepared as described.¹⁵ Briefly, HeLa cells were washed with phosphate-buffered saline (PBS), resuspended in CHAPS lysis buffer (20 mM Tris pH 7.2, 0.1 M NaCl, 1 mM EDTA, 10 mM DTT, 0.5% 3-[[3-cholamidopropyl]dimethyl-ammonio]-1-propanesulfonate (CHAPS) (Research Organics), 10% sucrose) and incubated on ice for 30 min. Cell lysates were then centrifuged for 10 min at 10 000 \times *g* at 4°C, supernatants were collected and stored at -80°C.

Metabolic labeling of proteins

After treatment of the cells with apoptosis inducers as indicated in the figures, the medium was replaced with methionine depleted medium (1 ml), and cells were pulse-labeled with 27 μ Ci Tran³⁵S (ICN) for 1 h at selected time points. Cytoplasmic extracts were prepared as described above and then analyzed (50 μ g of protein) by SDS-PAGE and autoradiography with Kodak Biomax MR film.

Caspase assays

Cell lysates were analyzed for caspase activity using the colorimetric peptide substrate Ac-DEVD-pNA (acetyl-DEVD-para-nitroanilide) (Quality Controlled Biochemicals). Assays (0.1 ml) contained 20 μ g of total protein from samples indicated in the figure legends and 0.2 mM pNA substrate (final concentration). Samples were incubated for 2 h at 37°C and release of pNA was monitored at 405 nm using a Beckman DU-70 UV Spectrophotometer.

Expression and purification of caspases

The full length cDNAs encoding human caspases 3, 6, 9, and the cDNAs encoding caspase 8 (Ser-217 to Asp-479) and caspase 10 (Ile-162 to Ile-479)²¹ cloned into pET15b (caspase 8), pET21b (caspases 6, 9, 10) and pET23b (caspase 3) (all Novagen) were expressed in *Escherichia coli* BL21(DE3)pLysS. Caspases 3 and 8 were a kind gift of Dr. Claudius Vincenz; caspases 6, 9 and 10 were a kind gift of Dr. Emad Alnemri. The expressed proteins were purified by affinity chromatography on TALON metal affinity resin (Clontech) according to the manufacturer's instructions. Working concentrations of active caspases were standardized based on *in vitro* colorimetric cleavage assays using appropriate substrate peptides coupled to para-nitroanilide (pNA). Caspase activity units are defined as the amount of caspase required to hydrolyze 1 nmol pNA substrate per minute. Various caspases were used in cleavage reactions at concentrations ranging from 10–60 $\mu\text{g/ml}$ as determined appropriate by these assays.

Purification of eIF4F and eIF4GII

eIF4F was purified from spinner HeLa cells as described.²² In short, a ribosomal salt wash preparation (RSW) (2 ml) was loaded on a 15–30% sucrose gradient in buffer F (20 mM Hepes pH 7.6, 0.5 M KCl, 0.5 mM EDTA, 2 mM β -mercaptoethanol), and centrifuged for 18 h at 3°C in a Beckman SW28 rotor. Fractions (1 ml) were collected and analyzed by immunoblot for the presence of eIF4F. Fractions containing eIF4F were pooled, diluted four-fold with buffer A (20 mM Mops pH 7.6, 0.25 mM DTT, 0.1 mM EDTA, 50 mM NaF, 10% glycerol), and loaded onto a 7-Methyl GTP-Sepharose 4B column (Pharmacia) equilibrated in buffer B110 (buffer A plus 110 mM KCl). eIF4F was eluted from the column with buffer B110 plus m^7 GTP (70 μM) and fractions were analyzed by Coomassie stain.

Purification of *in vitro* translated eIF4GII was carried out by mixing *in vitro* translated eIF4GII (50 μl) with 100 μl m^7 GTP sepharose beads equilibrated in buffer B110. After incubation for 1 h at room temperature, the beads were washed with 1.5 ml buffer B110, and bound proteins were eluted with 100 μl m^7 GTP in buffer B110.

Plasmid constructs

pcDNA3-HA-4GII was constructed as described.²³ The plasmid was linearized with *Xma*I to generate full-length eIF4GII via *in vitro* transcription, purified by phenol/chloroform extraction, and concentrated by alcohol precipitation for further use in transcription reactions.

Transcription and translation

Transcription reactions for eIF4GII using T7 RNA polymerase (Promega) were carried out according to manufacturer's recommendations followed by purification of the RNA using spin columns (Eppendorf-5 Prime). Translation reactions were performed in rabbit reticulocyte lysate (RRL) (Promega) according to the manufacturer's recommendations in a 50 μl reaction volume and incubated for 90 min at 30°C. Samples (2 μl) were subjected to SDS-PAGE and analyzed by autoradiography or otherwise, used to purify the *in vitro* translated eIF4GII as described above. C-terminal truncated forms of eIF4GII were synthesized by linearizing plasmid DNA at restriction sites within the eIF4GII open reading frame (*Spe*I or *Xho*I) followed by transcription and translation *in vitro*. Truncated radiolabeled eIF4GII was purified by cap affinity chromatography as described above before analysis.

Amino acid sequencing

To determine the caspase 3 cleavage site on eIF4GII, purified baculovirus-expressed eIF4GII (approximately 40 μg) was incubated with purified caspase 3 for 5 h at 37°C, loaded onto 15% acrylamide gel, and blotted to polyvinylidene fluoride (PVDF). Blot was stained briefly with Coomassie stain, destained briefly, and the protein band was cut out and subjected to Edman degradation at the Protein Chemistry Core Facility, Baylor College of Medicine.

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