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Review

Initiation factor modifications in the preapoptotic phase

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Abstract

Recent studies have identified several mechanistic links between the regulation of translation and the process of apoptosis. Rates of protein synthesis are controlled by a wide range of agents that induce cell death, and in many instances, the changes that occur to the translational machinery precede overt apoptosis and loss of cell viability. The two principal ways in which factors required for translational activity are modified prior to and during apoptosis involve (i) changes in protein phosphorylation and (ii) specific proteolytic cleavages. In this review, we summarise the principal targets for such regulation, with particular emphasis on polypeptide chain initiation factors eIF2 and eIF4G and the eIF4E-binding proteins. We indicate how the functions of these factors and of other proteins with which they interact may be altered as a result of activation of apoptosis and we discuss the potential significance of such changes for translational control and cell growth regulation.
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Abbreviations: HRI, haemin-regulated inhibitor; PABP, poly(A)-binding protein; cbp, cap-binding proteins; IFN, interferon; TRAIL, TNFα-related apoptosis-inducing ligand

Introduction

In recent years, a great deal of effort has been focused on questions concerning the highly regulated processes of cell growth, proliferation and programmed cell death (apoptosis). Hormones, growth factors and other ligands exert pleiotropic effects through activation of specific cell-surface receptors, and via transmembrane signalling and activation of common protein kinase/phosphatase cascades inside the cell. These in turn trigger an array of cellular responses, culminating in either cell growth and division, differentiation or cell death. One of the obligatory, early responses in all of these processes is a modulation of the rate of protein synthesis, mediated by changes in the phosphorylation of translation initiation factor polypeptides, regulation of the association of these factors into functional complexes or targeted cleavage of factors by cellular proteases (reviewed in Morley[1]). Until recently, relatively little attention has been focused on the changes in protein synthesis that accompany the commitment and execution phases of apoptosis. This is in spite of the fact that the induction of apoptosis is associated with a rapid and substantial (but incomplete) inhibition of protein synthesis in several cell types (reviewed in Clemens *et al.*[2]). Here, we will discuss the most thoroughly studied examples of such regulation and will summarise the current views on the significance of this control for both global and mRNA-specific protein synthesis.

Apoptotic Targets

eIF2

Initiation factor eIF2 is an important target for regulation by several physiological stresses, including those that induce apoptosis. During the initiation phase of translation, eIF2 mediates the binding of the initiator Met-tRNA$_i$ to the 40S ribosomal subunit, resulting in the formation of a functional 43S preinitiation complex (reviewed in Hershey and Merrick[3]). This process requires the formation of a ternary complex between eIF2, Met-tRNA$_i$ and GTP. The 43S complex associates with mRNA, in a process requiring several other initiation factors,[3,4] and locates the initiating AUG codon, usually as a result of ribosomal scanning from the 5′-end of the mRNA. During the subsequent joining of the 60S ribosomal subunit, the GTP that is associated with the eIF2 molecule is hydrolysed to GDP and phosphate, concomitant with the dissociation of the initiation factor from the ribosome. The GDP remains associated with the eIF2 and has to be exchanged for another molecule of GTP in a reaction catalysed by the guanine nucleotide exchange factor, eIF2B.[5] The regulation of eIF2 activity is most frequently a consequence of changes in the phosphorylation state of its α subunit, the smallest of three subunits, on Ser-51. This leads to an increased affinity of the initiation factor for eIF2B, thus increasing the proportion of the latter that is trapped as an inactive complex with phosphorylated eIF2 and GDP.[3–5]

A small family of protein kinases, all with specificity for eIF2α Ser-51, is responsible for the phosphorylation of this protein. Each of the members of the family can be activated

by a specific set of cellular stress conditions. The kinases are as follows: HRI (haemin-regulated inhibitor), an enzyme prominent in red blood cell precursors that can be activated by iron or haem deficiency, heat-shock, osmotic and oxidative stress, nitric oxide and heavy metals; PKR, an interferon (IFN)-inducible, double-stranded RNA-dependent protein kinase that is activated during virus infections, as well as in response to many proapoptotic stimuli; the endoplasmic reticulum protein kinase PERK, regulated by the accumulation of unfolded proteins in the ER and a major player in the unfolded protein response; and mGCN2, the mammalian homologue of the *Saccharomyces cerevisiae* protein kinase that responds to amino-acid starvation (reviewed in Sonenberg and Dever[4] and Kaufman[6]). It is possible that any of these enzymes may be activated by individual apoptotic stimuli, but the best evidence so far concerns the role of PKR in cell death regulation (see below).

The eIF4F complex

The mRNA binding stage of translation is also a major site of regulation, requiring the activity of eIF4F, a heterotrimeric complex comprising eIF4E, eIF4GI or eIF4GII and eIF4A (Figure 1a). In terms of regulatory significance, this second phase of initiation has the potential to modulate both the overall rate of protein synthesis and the selective recruitment of specific mRNAs for translation. This step can be modulated during apoptosis both by the phosphorylation of key initiation factors and by changes in the levels and integrity of these factors (reviewed in Morley[1]).

A crucial step in mRNA binding is recognition of the 5′ m^{7}GpppG cap structure present on all cellular mRNAs by the phosphoprotein eIF4E (reviewed in Morley[1] and Hershey and Merrick[3]). The structure of eIF4E resembles a cupped hand,[7] with the concave surface of the protein binding to the cap, while the convex surface interacts with eIF4G.[8–10] The latter is in turn bound to eIF4A, the third subunit of the heterotrimeric eIF4F complex. eIF4E can bind the m^{7}GTP cap structure alone, but when it is part of the eIF4F complex, cap binding is greatly enhanced (reviewed in Morley,[1] Pain,[11] Dever,[12] Preiss and Hentze,[13] Gingras *et al*.[14,15] and Raught *et al*.[16]). Phosphorylation of eIF4E by the MAP kinase integrating kinases (Mnk1/2) in response to activation of the ERK and p38 MAP kinase pathways can also modulate the affinity of eIF4E for the m^{7}GTP cap.[15] Biophysical studies suggest that the phosphorylation of eIF4E actually decreases its cap-binding affinity, increasing the rate of dissociation of eIF4E from an immobilised m^{7}GTP cap structure.[17] In addition, the availability of eIF4E for interaction with eIF4G is controlled by a family of small eIF4E-binding proteins (the 4E-BPs), which act as competitive inhibitors of the interaction between eIF4E and eIF4G.[14] The extent of sequestration of eIF4E by 4E-BP1 and 4E-BP2 is determined by the state of phosphorylation of the 4E-BPs, which is regulated by a number of signalling pathways.[14,18] Phosphorylation at multiple sites strongly reduces the affinities of the 4E-BPs for eIF4E[9,19] (Figure 2).

Many studies suggest that translation initiation is deregulated during tumorigenesis,[20,21] and it is of interest that the level of expression of eIF4E is often abnormally high in malignant cell lines and naturally occurring tumours.[22,23] Moreover, the experimental overexpression of eIF4E can cause malignant transformation (reviewed in Zimmer *et al*.[23]). The effects of eIF4E overexpression can result in the inhibition of apoptosis.[24,25] Conversely, enhanced expression or activity of the 4E-BPs inhibits cell growth, counteracts the transforming potential of eIF4E and sensitises cells to apoptosis. This depends on the ability of the 4E-BPs to sequester eIF4E.[26] A likely explanation for these phenomena is that high levels of eIF4E increase the translational efficiencies of mRNAs with roles in the promotion of cell proliferation or inhibition of apoptosis, whereas the 4E-BPs have the opposite effect. Examples of mRNAs regulated in this way are the src family member lck,[27] c-Myc,[28] cyclin D1[29] and VEGF.[30]

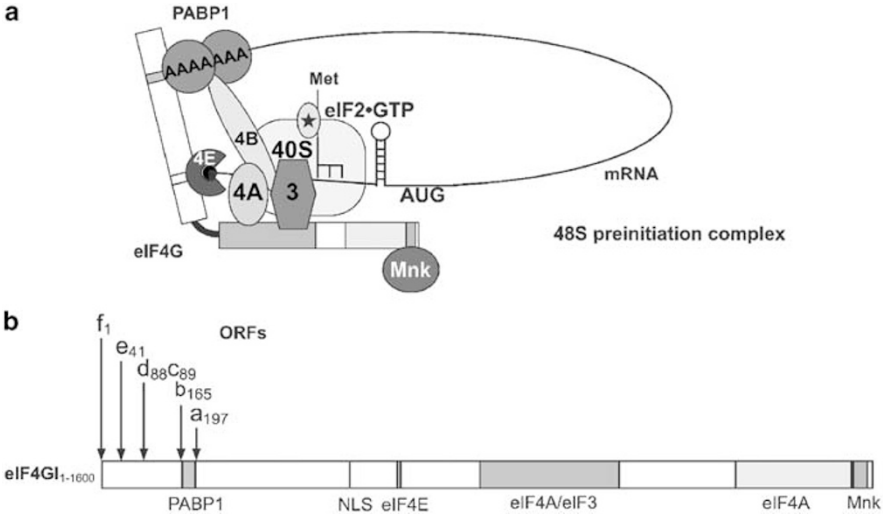

Figure 1 Role of eIF4G in translation initiation. (**a**) eIF4G plays a central role in assembly of the 48S preinitiation complex, where the black disc represents the mRNA cap structure. (**b**) Isoforms of eIF4GI arise through alternative translation initiation and differ in sequence at the N-terminus. All numbering of eIF4GI protein sequences is based on the N terminally extended sequence described in Bradley *et al*.[53] and Byrd *et al*.[54]

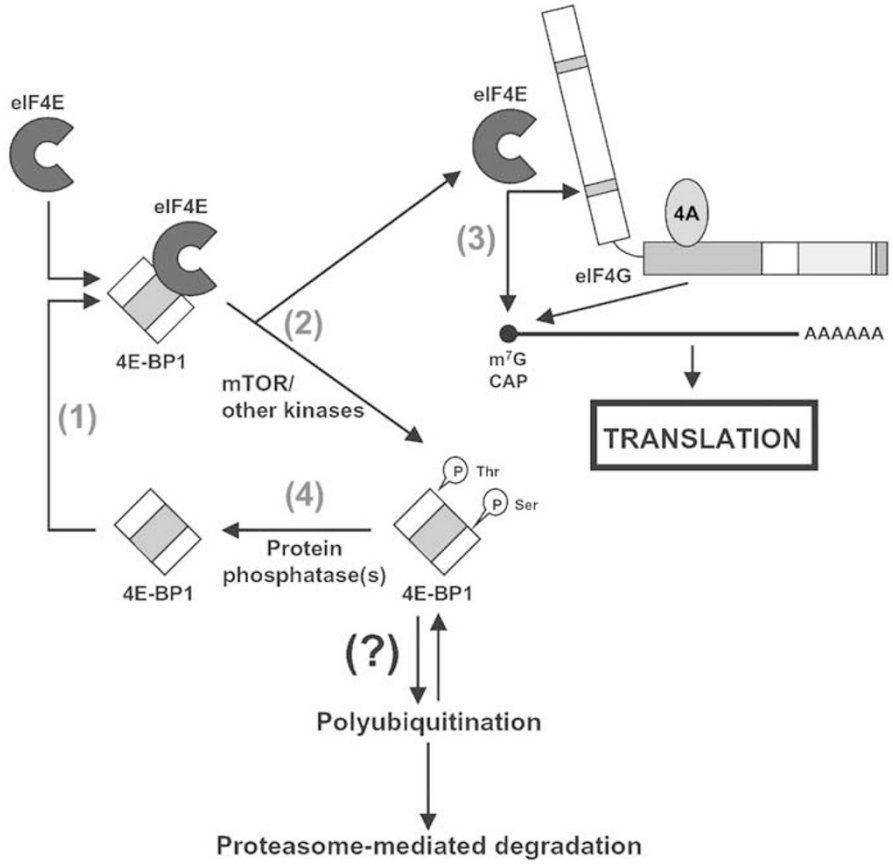

Figure 2 Model for regulation of eIF4E availability by 4E-BP1 phosphorylation and degradation. Hypophosphorylated 4E-BP1 binds to eIF4E and prevents the latter from interacting with eIF4G and participating in cap-dependent initiation. (1) 4E-BP1 is phosphorylated by an mTOR-dependent mechanism (2), resulting in its dissociation from eIF4E. The eIF4E can then interact with eIF4GI or II (3). Phosphorylated 4E-BP1 can be dephosphorylated by protein phosphatase(s) (4) and can then rebind eIF4E. Conditions that promote apoptosis lead to early changes in the state of phosphorylation of 4E-BP1 (preceding any loss of cell viability or overt cell death); this may be due to both inhibition of mTOR (and other protein kinases) and to activation of protein phosphatases. As well as causing a net dephosphorylation of 4E-BP1, some treatments (e.g. exposure of cells to TRAIL) lead to the accumulation of the protein. It remains to be determined whether the state of phosphorylation of 4E-BP1 regulates other post-translational modifications such as polyubiquitination that may signal the degradation of this protein

Initiation factor eIF4A is also an important component of the eIF4F complex. This factor is an RNA-dependent ATPase, and is the prototypic member of the DEAD box helicase family (reviewed in Morley,[1] Hershey and Merrick[3] and Raught *et al*.[16]). Mammalian eIF4A exists in three highly similar isoforms (eIF4AI, eIF4AII, eIF4AIII) that appear to differ in tissue distribution and developmental regulation.[31–35] It has been suggested that eIF4AI or II functions primarily as a subunit of the eIF4F complex, with eIF4A being required to recycle through the complex during translation.[3,15,36] In contrast, eIF4AIII binds spliced mRNA in the exon junction complex and is required for nonsense-mediated mRNA decay, but plays no role in mRNA translation.[37–39] In conjunction with eIF4B, eIF4A is believed to function to promote unwinding of mRNA secondary structure,[36,40] being absolutely required for mRNA-ribosome binding both in its free form and as part of the eIF4F complex.[36] Furthermore, eIF4AI/II is essential for translation of all mRNAs and for growth in yeast.[41] eIF4B is also required for mRNA binding to ribosomes and it stimulates the RNA helicase activity of eIF4A *in vitro*.[3,42] Three potential regulatory domains of eIF4B are revealed in the protein sequence: an RNA-binding domain (RRM), a hydrophilic region (DRYG), which mediates binding of a truncated form of eIF4B to the eIF3a/p170 subunit of the ribosome-associated eIF3 (Methot *et al*.;[43] see below) and a serine-rich region at the C-terminus.[44] *In vitro* studies with mutant proteins have indicated that the RNA-binding domain alone is insufficient to support interaction with mRNA, but may localise eIF4B to the ribosome.[45] A region in the DRYG domain, however, is important for both RNA binding and the ability of eIF4B to stimulate the helicase activity of eIF4A.[46] Thus, eIF4B has been postulated to act directly by binding to the 5′UTR of the mRNA and to the ribosome, and/or indirectly via its interaction with eIF3 to promote the mRNA/rRNA/initiator tRNA interaction at the AUG codon.[3] An eIF4B-related protein, termed eIF4H, has been identified that possesses an RRM domain but lacks the corresponding DRYG region.[47] eIF4H can substitute for eIF4B in a reconstituted translation system, increases the affinity of eIF4A for RNA, stimulates eIF4A helicase activity[48] and may function to

stabilise directly conformational changes in eIF4A that occur during initiation.[3]

Initiation factors eIF4GI and II, which are the largest components of eIF4F complexes, share 46% identity at the amino-acid level. Both proteins can act as adapter molecules on which many other initiation factors can bind (Figure 1a).[1,14,49] In addition, two families of proteins with sequence homology to eIF4G have been identified, referred to as p97/NAT1/DAP5 and Paip-1 (reviewed in Gingras *et al*.,[14,15] Raught *et al*.[16] and Morley *et al*.[50]). The original cDNA clone of eIF4GI obtained from a human brain cDNA library[51] was extended in a later study[52] to identify an N-terminal PABP binding site. More recently, two groups independently discovered a further N-terminal extension of 40 amino acids, finally identifying the longest possible open-reading frame of eIF4GI.[53,54] These studies suggest that five isoforms of eIF4GI exist in cells, generated by alternative translation initiation (Figure 1b). However, little is known as to the function of these different isoforms in the cell. Along with the interactions with eIF4E and eIF4A, the eIF4Gs also bind RNA, the multisubunit eIF3, the eIF4E kinase Mnk1 and the cytoplasmic form of PABP in the steady-state translation initiation complex (Figure 1a). In addition, eIF4GI has been implicated in a pioneer round of translation for newly transcribed mRNA, via interactions with the nuclear cap-binding proteins (cbp), cbp80 and cbp20.[55,56] PABP binds directly to a domain in the N-terminus of mammalian eIF4G via a conserved sequence of basic amino acids. This association is believed to mediate the circularisation of mRNA and promote the poly(A)- and PABP-dependent stimulation of mRNA translation.[57–65] The binding of PABP to eIF4G has also been suggested to increase the helicase activity of eIF4F,[66] while the association of eIF4GI with eIF4E markedly enhances the binding of the latter to the mRNA cap.[67] Phosphorylation of eIF4GI, possibly promoting conformational changes in the protein,[68] has been associated with the upregulation of cell growth.[50,69–73] In contrast, eIF4GII phosphorylation is increased primarily at the G_2/M phase of the cell cycle,[74,75] but roles for these phosphorylation events in modulating translation rates remain unclear.

Initiation factor eIF3

Initiation factor eIF3 was first isolated and purified as a high molecular weight complex from rabbit reticulocytes.[3] The mammalian factor (approx. 600 kDa) contains at least 12 nonidentical protein subunits, designated a-l in the order of decreasing molecular weight.[76] Specific functions for mammalian eIF3 have been identified by a variety of *in vitro* experiments,[3] showing that it binds directly to 40S ribosomal subunits and affects the association/dissociation of 80S ribosomes. In addition, eIF3 promotes the binding of Met-tRNA$_i$ and mRNA to the 40S ribosome, and binds directly to eIF1, eIF4B, eIF4G and eIF5, clearly playing a critical role in translation initiation.

It has been proposed that it structurally organises other translational components on the surface of the 40S ribosome.[77]

Modification of Translation Initiation Factors during Apoptosis

The downregulation of translation rates in apoptosing cells was initially observed in several independent studies (e.g. Deckwerth and Johnson,[78] Zhou *et al*.[79] and Scott and Adebodun[80]). However, it is only more recently that the underlying changes to the translation machinery that take place during apoptosis have been elucidated (Figures 3 and 4). For example, treatment of Jurkat T cells with anti-Fas antiserum results in a 60–70% decrease in the rate of protein synthesis within 2–4 h[81] and a loss of cell viability. The inhibition of protein synthesis is associated with a substantial decrease in the proportion of ribosomes in polysomes,[79,81] strongly suggesting that there is a block at the stage of polypeptide chain initiation at these early times. These events are prevented by the cell-permeable caspase inhibitor, z.VAD.FMK, indicating that caspase activity is required. However, the mechanisms involved in translational regulation during apoptosis depend on the nature of the apoptotic inducer (e.g. the downregulation of translation caused by the DNA-damaging agent etoposide is zVAD.FMK insensitive[81]).

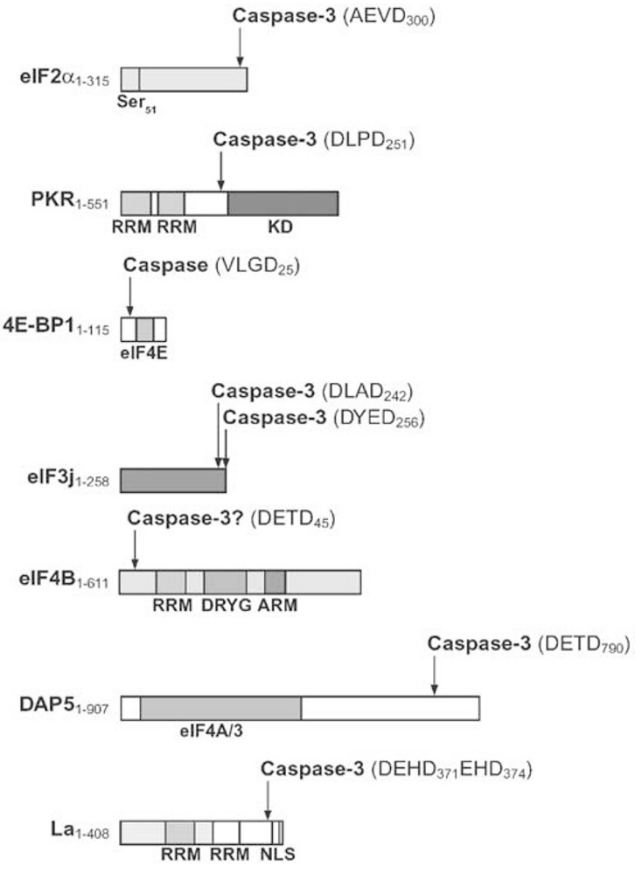

Figure 3 Modifications of other factors involved in translation initiation during apoptosis. See the text and references therein for details. Abbreviations: RRM, RNA recognition motif; KD, kinase domain; DRYG, region rich in aspartate, arginine, tyrosine and glycine; ARM, arginine-rich motif; NLS, nuclear localisation signal

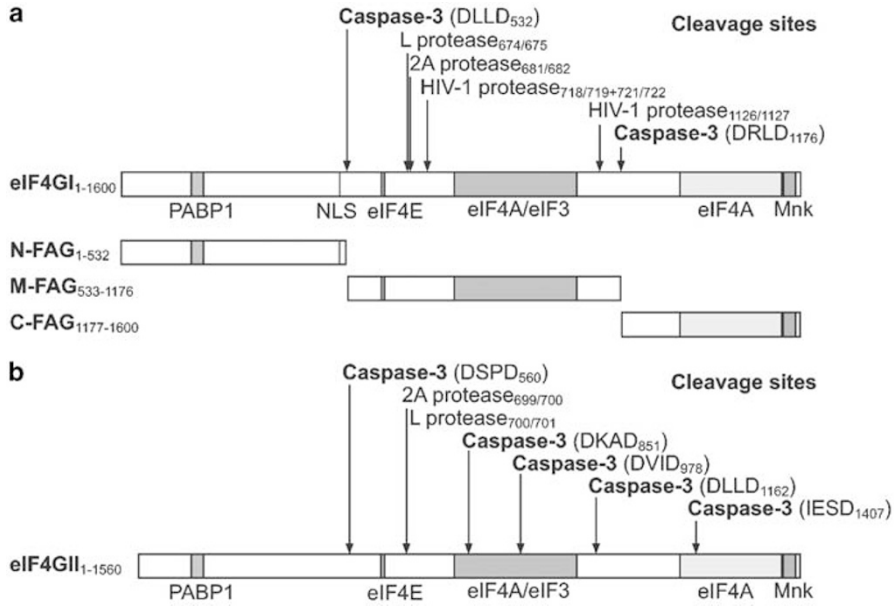

Figure 4 Caspase-mediated cleavage of eIF4G. (**a**) Cleavage of eIF4GI by caspase-3 generates three discrete fragments, designated 'Fragments of Apoptotic cleavage of eIF4G' (FAGs). These are different from the fragments produced after cleavage by picornaviral (L or 2A) proteases and HIV-1 protease. (**b**) Additional caspase cleavage sites are found in the eIF4GII molecule

eIF2

Initiation factor eIF2 is regulated during apoptosis in two ways. The extent of phosphorylation of the α subunit of this factor is often found to be increased in cells following exposure to a variety of proapoptotic stimuli.[82,83] In principle, any of the four protein kinases that phosphorylate Ser-51 on eIF2α could be responsible, but PKR is the strongest candidate because this enzyme has an important role in promoting cell death.[84,85] PKR is one of several gene products induced by IFN treatment. It is involved in both the antiviral and antitumour effects of these cytokines and appears capable of regulating both cell proliferation and apoptosis (reviewed in Clemens[21]). Studies with embryonic fibroblasts deficient in PKR have shown that this enzyme is essential for changes in the protein synthetic machinery associated with the response to TNFα.[83] In fact, the effects of the TNFα family on translation appear to require the participation of both PKR-mediated and caspase-dependent events. Consistent with these observations, we have found that the α subunit of eIF2 becomes highly phosphorylated in cells exposed to TNFα or TRAIL (TNFα-related apoptosis-inducing ligand), at times preceding any loss of cell viability or the onset of cell death.

TRAIL inhibits cell growth and induces programmed cell death in a wide variety of tumour types,[86–90] and acts preferentially on malignant cells.[91,92] A major effect of the association of this cytokine with its cell-surface receptors is the activation of caspase-8.[93–95] It is interesting to note, therefore, that in TRAIL-treated MCF-7 cells, the phosphorylation of eIF2α is itself a caspase-dependent process, which can be prevented by treatment of the cells with either the broad specificity caspase inhibitor z.VAD-FMK or the caspase-8-specific inhibitor z.IETD-FMK (IW Jeffrey and MJ Clemens, unpublished data). In contrast, there is no requirement for caspase-8 for the transient phosphorylation of eIF2α in Jurkat cells following activation of the Fas (CD95) receptor.[82]

In view of the PKR requirement for the effects of TNFα on protein synthesis in fibroblasts[83] and the marked ability of TRAIL to enhance the phosphorylation of eIF2α in MCF-7 cells, it is of interest to know whether PKR is activated following treatment of cells with TNFα or TRAIL. Immunoblotting of cell extracts with an antibody that only recognizes PKR that is phosphorylated at Thr-451, a site associated with the activation of the protein kinase, has suggested that TRAIL does not induce the activation of full-length PKR. However, it does result in the appearance of a PKR cleavage product of approximately 43 kDa that is phosphorylated at this site (IW Jeffrey and MJ Clemens, unpublished data). Although not formally proven to date, this may correspond to a catalytically active fragment of PKR; such a fragment, generated by caspase cleavage and highly active as an eIF2α kinase, can arise as a result of the removal of the inhibitory dsRNA-binding domain from the enzyme.[96]

In addition to changes in eIF2α phosphorylation, in cells treated with TRAIL and some other proapoptotic agents, a small fraction of eIF2α is often cleaved to give rise to a C terminally truncated product[97,98] (Figure 3). The physiological significance of this cleavage is unclear, however, since only a small proportion of the eIF2α population ever seems to be modified in this way (but see later in this review for a discussion of this point).

eIF4GI and eIF4GII

Many studies[81,82,99–105] have demonstrated that both eIF4GI and eIF4GII are targets for specific degradation during apoptosis (reviewed in Morley[1] and Clemens *et al*.[2]). Deprivation of serum growth factors, activation of the Fas receptor, or

treatment of a variety of cells with cycloheximide, MG132, cisplatin, etoposide, TNFα or TRAIL leads to the inhibition of translation and the progressive degradation of eIF4GI and eIF4GII. In contrast to the cleavage of eIF4G during picornavirus infection,[106] the loss of eIF4GI and eIF4GII during apoptosis occurs with similar kinetics.[82,99,101,104] Under the same conditions, there are no major decreases in the levels of several other initiation factors including eIF4E, eIF4A and PABP.[82,101] However, it has been shown recently that PABP can be cleaved by a partially caspase-dependent mechanism, although it is not a substrate for caspase-3 itself.[107] In most (but not all) cases, both the inhibition of translation and the cleavage of eIF4G can be prevented by cell-permeable caspase inhibitors, with the activity of the effector caspase, caspase-3, being both necessary and sufficient for the proteolysis of eIF4GI and eIF4GII *in vitro* and *in vivo*.[81,82,99,100,104]

The cleavage of eIF4GI and eIF4GII by picornaviral proteases bifurcates the molecule, separating the cap-binding component from the ribosome-binding portion (Figure 4) and providing an obvious mechanism for the inhibition of cap-dependent translation. In contrast, HIV-1 protease only cleaves eIF4GI, cutting the protein into novel, distinct fragments that serve to inhibit both cap-dependent and IRES-driven translation.[108,109] However, the cleavage of the eIF4Gs by intracellular caspases produces considerably different fragments. Caspase-3 cleaves eIF4GI at two sites, the first being between amino acids 532 and 533 downstream of a DLLD sequence and the second between amino acids 1176 and 1177, downstream of a DRLD sequence (Figure 1b). Initial cleavage events at either of these two sites generate intermediate fragments migrating at 120 and 150 kDa, respectively. The three distinct breakdown products of eIF4GI resulting from the two cleavages have been termed *F*ragments of *A*poptotic cleavage of eIF4*G* (FAGs)[99,102] and designated N-FAG, M-FAG and C-FAG (Figure 4a). During apoptosis, dephosphorylated eIF4E is maintained in a modified eIF4F complex with M-FAG, which is in turn still able to bind eIF4A, eIF3 and 40S ribosomes.[82,101] This complex was proposed to maintain some form of cap-dependent translation and an independent study defined a fragment closely corresponding to M-FAG as the minimal sequence of eIF4GI required to bind mRNA to ribosomes in an *in vitro* assay.[110]

4E-BP1

In proliferating cells, the 4E-BPs are phosphorylated and remain dissociated from eIF4E. In contrast, under conditions that block cell proliferation or induce apoptosis, 4E-BP phosphorylation decreases and the 4E-BPs associate with eIF4E. Many physiological stresses and growth-regulatory conditions affect the state of phosphorylation of the 4E-BPs.[14,111–113] Prominent among the agents that both cause dephosphorylation of 4E-BP1 and induce apoptosis are the broad specificity protein kinase inhibitor staurosporine, TRAIL, Fas ligand, DNA damage inducers and activated p53.[21,82,83] Previous studies have shown that TRAIL inhibits overall translation by a mechanism that involves increased association of eIF4E with 4E-BP1, and that these effects precede the development of overt cell death and loss of viability.[83] Consistent with its effects on the binding of 4E-BP1 to eIF4E, TRAIL also causes decreased binding of eIF4GI to eIF4E (I Jeffrey and MJ Clemens, unpublished data).

A key factor involved in promoting 4E-BP phosphorylation is the rapamycin-sensitive enzyme, mammalian target of rapamycin (mTOR;[113]) and the ability of rapamycin to inhibit 4E-BP phosphorylation is important for the proapoptotic effect of this drug.[114] The levels of 4E-BPs that are expressed can influence cellular sensitivity to rapamycin,[115] and recent developments in assessing rapamycin and its derivatives as potential anticancer agents[116,117] have included strategies targeted specifically at mTOR-mediated regulation of the 4E-BPs.[118] However, there is also evidence that some changes in the phosphorylation of 4E-BP1 (e.g. those seen after activation of p53) are independent of rapamycin-sensitive mTOR (C Constantinou and MJ Clemens, unpublished data).

There is also evidence that the *levels* of the 4E-BPs may change with differing physiological circumstances. For example, following fertilization of sea urchin eggs, 4E-BP1 is rapidly degraded, and this coincides with an increase in the rate of protein synthesis during early embryogenesis in this system.[119] Conversely, the level of 4E-BP1 increases rapidly and substantially when either human lymphoma or breast cancer cell lines are treated with TRAIL (I Jeffrey, A Elia and MJ Clemens, unpublished data). We do not yet know the mechanism of the TRAIL-mediated accumulation of 4E-BP1. Nevertheless, there are several precedents for a relationship between changes in protein phosphorylation and protein stability. Examples are the destabilization of the inhibitor of NFκB (IκB), as a result of phosphorylation-dependent polyubiquitination and subsequent degradation via the proteasome pathway,[120,121] and regulation of the turnover of β-catenin and p53.[122,123] However, it remains to be established whether 4E-BP1 is ubiquitinated prior to its degradation (Figure 2) and whether the phosphorylation status of 4E-BP1 influences its turnover in a TRAIL-sensitive manner.

TRAIL is by no means the only inducer of apoptosis that affects 4E-BP1 phosphorylation and/or stability. Both dephosphorylation and caspase-mediated cleavage of 4E-BP1 have been shown to occur in cells exposed to staurosporine and the DNA-damaging agent etoposide.[124–126] Cleavage occurs at Asp-24, close to the N-terminus of the protein (Figure 3). The activation of p53 also results in the cleavage of 4E-BP1, but in this case, the cleavage is a z-VAD.FMK-insensitive event, suggesting that it is mediated by another type of protease (C Constantinou and MJ Clemens, unpublished data). However, the characteristics of the caspase-mediated and caspase-independent cleavage products of 4E-BP1 so far appear identical in all respects, and it is possible that the region of the protein close to Asp-24 is a particularly vulnerable site for proteolysis. Whether the site-specific cleavage(s) within this region of 4E-BP1 have a functional relationship to the turnover of the protein as a whole remains to be established.

eIF4B and eIF3j (p35)

Cleavages of eIF4B and the p35 (j) subunit of eIF3 have also been observed during apoptosis, with none of the other subunits of eIF3 being affected.[101] However, these

caspase-3-mediated events occur with delayed kinetics relative to that seen for eIF4GI. As with eIF4GI, caspase-3 is able to cleave both eIF3j and eIF4B *in vitro*; however, eIF4B is also cleaved during apoptosis in MCF-7 cells lacking caspase-3, suggesting that other caspases may be involved *in vivo*.[83] eIF4B is cleaved between amino acids 45 and 46 after a DETD sequence to produce a 60 kDa cleavage product (Figure 3). This cleavage site is in the N-terminal domain of the protein in a region that is required for its interaction with PABP. The N terminally truncated fragment of eIF4B was still able to coisolate with the mRNA cap-binding eIF4F complex[101] and still contains the DRYG motif that is essential for self-association of eIF4B molecules and for its interaction with the largest 170 kDa subunit of eIF3 (eIF3a).[127]

The appearance of the ΔeIF3j fragment (Figure 3), which is only slightly smaller than the intact protein, is evident within 4 h of cycloheximide-induced apoptosis of BJAB cells, with complete conversion to the truncated form evident at later times.[101] The main caspase cleavage site was mapped between amino acids 242 and 243, after a DLAD sequence, leading to the loss of the final 16 amino acids of the molecule (Figure 3). *In vitro* experiments also indicated that caspase-3 has the ability to cleave just two amino acids from the C-terminus of the molecule.[101] Although the exact function of the eIF3j subunit in the eIF3 multisubunit complex remained unresolved for a number of years,[3,128,129] recent studies have determined that it has a central role in mediating the stable association of eIF3 with the 40S ribosome.[77] eIF3j that has been processed to the shorter form by caspase-3 shows a dramatically reduced affinity for the 40S ribosome and consequently does not efficiently recruit eIF3 to the 40S ribosome.[77]

Other factors that regulate initiation

A gene variously called p97, DAP-5 or NAT1[130–132] encodes a protein that is homologous to the central and C-terminal parts of eIF4GI. This protein acts to protect HeLa cells against IFN-α-induced apoptosis.[131] In addition, recent studies have indicated that while DAP5 is not important for general translation, DAP5 function is required for controlling gene expression during cellular differentiation.[133] As with eIF4GI, DAP5 is also cleaved during apoptosis, downstream of $DETD_{790}$ (Figure 3), yielding an N-terminal fragment competent to bind eIF3 and eIF4A, which is distinct from M-FAG as it lacks the eIF4E binding site.[134]

The La (SS-B) autoantigen binds precursors to 5S rRNA and tRNAs and also promotes their maturation in the nucleus. Separate from this function, human La has been shown to positively modulate the translation of IRES-containing mRNAs. During apoptosis, La is also a target for caspase-mediated cleavage and dephosphorylation.[135,136] Cleavage at the C-terminus of the protein, downstream of $DEHD_{371}$ or $DEHD_{374}$ (Figure 3) results in the loss of the nuclear localisation signal, relocalising the truncated protein to the cytoplasm[136]). This relocalisation of La may promote the ability of the protein to influence translation initiation, including effects on internal initiation[137] and the inhibition of PKR activity.[138,139]

Effects of Modification of Initiation Factors on mRNA Translation during Apoptosis

The progress of apoptosis is characterised by a general but incomplete inhibition of cap-dependent translation and a complex programme of changes involving several initiation factors that function in recruiting mRNA to the ribosome. Apoptosis-associated modifications include the specific fragmentation of proteins (eIF4G, eIF4B, eIF3j), alterations in the state of phosphorylation of initiation factors (eIF2α, eIF4E, 4E-BP1) (reviewed in Morley[1] and Clemens *et al*.[2]) and interference with protein/protein interactions. An example of the latter is the caspase-mediated activation of the signalling molecule, Pak2, which impinges on eIF4F complex assembly. Once cleaved, this kinase has the ability to phosphorylate Mnk1 and, without influencing its kinase activity, reduce the binding of Mnk1 to eIF4GI[140] prior to cleavage of eIF4GI. Any or all of the above events could potentially contribute to the observed inhibition of protein synthesis, and it is likely that the relative importance of the various changes may be different at distinct stages of the ongoing apoptotic response. For example, in anti-Fas-treated Jurkat cells, it has been shown that the caspase-8-independent increase in eIF2α phosphorylation is associated with a reversible, general inhibition of protein synthesis and polysome disaggregation at early times.[141] At slightly later times, this is followed by the irreversible cleavage of eIF4GI, the loss of p70S6K activity and an increase in the binding of 4E-BP1 to eIF4E.[1]

As several factors with different functions in translation are modified before and during the early stages of apoptosis, it is difficult to dissect the individual contributions of the changes observed to the overall regulation of protein synthesis. As with many other aspects of apoptosis, the mechanisms of translational downregulation are almost certainly multifactorial. In addition, other events occur that can have a major impact on global protein synthesis and/or the translation of specific mRNAs. For example, recent studies have identified significant degradation of some mRNA species at early times after treatment of cells with various inducers of apoptosis.[142,143] There is also cleavage of 28S rRNA in ribosomes in apoptotic cells, although this may be a somewhat later event.[144] In spite of these uncertainties, with our knowledge of the roles of individual initiation factors, it is possible to identify several specific effects that can be predicted from the changes induced by the phosphorylation and/or cleavage of these proteins during the early stages of the induction of cell death.

Effects of eIF2α phosphorylation and cleavage

Increases in the extent of phosphorylation of eIF2α would be expected to have a strong impact on global protein synthesis because of the inhibitory effect of this modification on the guanine nucleotide exchange activity of eIF2B.[145] As far as we know, all cellular mRNAs require eIF2 and eIF2B activity for their translation. However, it is becoming clear that some mRNAs are less dependent than others on the availability of eIF2-containing ternary complexes, and, in certain notable

cases, the rate of translation of specific coding sequences in mRNAs with short upstream open-reading frames can even be *enhanced* by increased phosphorylation of eIF2α and decreased eIF2B activity.[146,147] Moreover, it has been reported that the truncated form of eIF2α that arises in apoptotic cells as a result of caspase activity shows very rapid exchange of GDP that is no longer dependent upon eIF2B.[97] Consistent with this, expression of the cleaved protein overcomes PKR-mediated translational suppression.[98] These results suggest that the caspase-mediated cleavage of eIF2α may in fact render the protein constitutively active in a manner independent of its state of phosphorylation. This may have the effect of protecting protein synthesis from complete downregulation since a basal level of activity could presumably be maintained by the small fraction of the factor that is cleaved. This could be especially relevant for the continued translation of IRES-containing mRNAs (see below) since although the utilisation of these mRNAs is independent of changes in eIF4G or 4E-BP function, it does require eIF2 activity. However, such a possibility has not yet been demonstrated experimentally. It is also not known whether there is any functional relationship between eIF2α phosphorylation and the susceptibility of the protein to cleavage by caspases.

Effects of modifications of the eIF4F complex

The modification of the eIF4F complex observed in picornaviral infection, cutting the cap-binding components and the ribosome binding components, serves a clear function in allowing the translation of only the uncapped picornaviral mRNAs by internal ribosome entry. However, the very different modifications of the eIF4F complex during apoptosis require us to make different hypotheses as to how such changes may assert themselves in those circumstances.

In several apoptotic systems, such as cycloheximide-treated BJAB cells and anti-Fas-treated Jurkat cells, cleavage of eIF4GI results in the production of a modified form of eIF4F, containing eIF4E and eIF4A, but with the central M-FAG fragment in place of full-length eIF4GI (Figure 5). This complex, which is distinct from the steady-state eIF4F complex observed during cell growth, remains stable for several hours in apoptosing cells before M-FAG is further degraded, with the loss of the eIF4E binding site.[141] As such, the modified eIF4F complex present in apoptotic cells at early times may still be able to support either cap-dependent[110] or -independent initiation.[149] In addition, the decrease in the phosphorylation state of eIF4E during apoptosis may reflect the activation of Pak2,[140] but it could also be a consequence of eIF4G cleavage because the binding site for the eIF4E kinases, Mnk1/2, is not present in M-FAG.[148]

When HeLa cells are exposed to the cytotoxic ligand TRAIL, apoptosis is rapidly induced and translation rates are severely, but not completely, inhibited.[105] The presence of either the c-myc or HRV2 IRES between the cistrons of a dicistronic luciferase reporter mRNA[150] was able to maintain the translation of the downstream Firefly luciferase sequence under these conditions. In contrast, the translation of the upstream *Renilla* luciferase cistron, which is translated in a cap-dependent manner, was found to decrease over time.

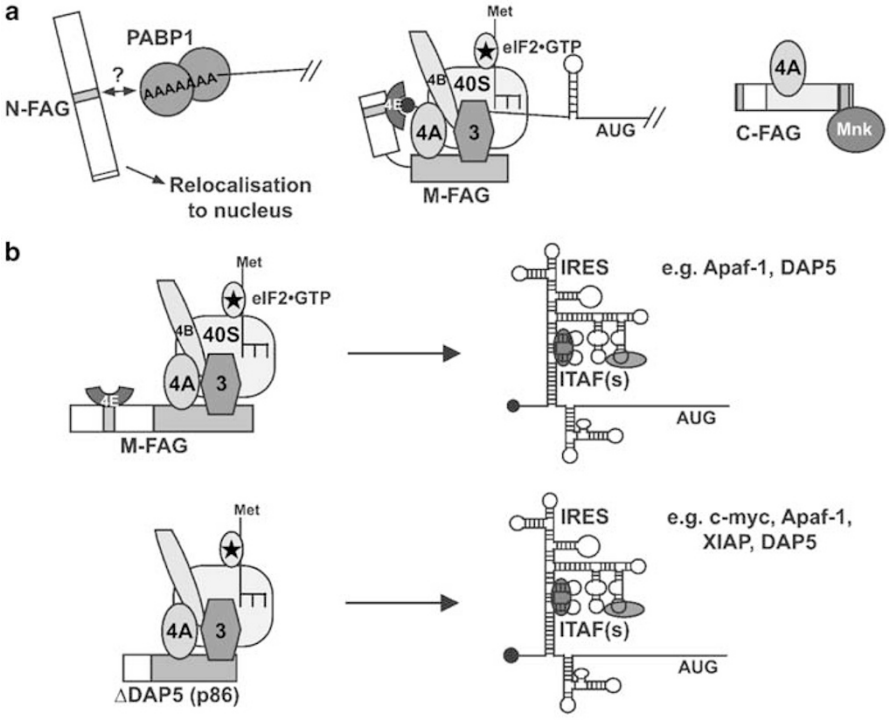

Figure 5 Translation initiation during apoptosis. (**a**) N-FAG has been postulated to be involved in sequestering PABP1. (Note, however, that N-FAG expressed from a cDNA is observed to relocalise to the nucleus without influencing the localisation of PABP1.) M-FAG is present in a modified eIF4F complex, which may be able to direct some cap-dependent translation. A function for C-FAG is yet to be determined, but it may sequester Mnk1 and/or eIF4A. (**b**) The modified eIF4F complex, containing either M-FAG or cleaved DAP-5, is still able to maintain the translation of some, but not all, cellular IRES-containing mRNAs

C-myc mRNA itself can initiate translation in either a cap-dependent manner or an IRES-dependent manner,[151] and the continued expression of the c-myc protein from the IRES may be important for the later stages of apoptosis. Although a definite function is still not known, the c-myc protein may be important in activating the transcription of genes that are involved in ensuring the appropriate engulfment of the apoptotic cell. As the c-myc protein has a very short half-life (around 20 min[152,153]), it would have to be continuously synthesised to maintain suitable levels of the protein.

It has also been shown that the IRES present in the mRNA encoding the XIAP protein is still active during apoptosis.[154,155] Moreover, the DAP5 IRES is used to maintain translation initiation during apoptosis in cells treated with anti-Fas antibodies.[134] Work has also identified IRES activity within the 5′UTR of the La mRNA,[156] and it is tempting to hypothesise that the La protein may be able to regulate expression of itself at the level of translation initiation. These results suggest that this may be a common mechanism in ensuring maintenance of the expression of IRES specific *trans*-acting factors to allow translation of other IRES-driven messages under conditions when cap-dependent translation is compromised. However, it is clear that not all mRNAs that can be translated by an IRES-dependent mechanism continue to be translated during apoptosis. When dicistronic luciferase reporter plasmids containing the Apaf-1 and BAG-1 IRESs were transfected into HeLa cells and an apoptotic programme was induced by TRAIL, the Apaf-1 and BAG-1 IRESs were unable to maintain translation of the downstream Firefly luciferase reporter gene beyond the first 2 h of TRAIL treatment (MJ Coldwell, unpublished results). This is in contrast to the results seen with the c-myc and HRV2 IRESs, where Firefly luciferase expression was observed throughout an 8 h time course, after which 95% of the cells were apoptotic.[105]

The question still remains as to what actually drives the selective translation of these IRES-containing mRNAs during the apoptotic process. At this time, there is still some confusion over any possible role for the cleavage fragments of eIF4GI in modulating translation rates during early apoptosis (Figure 5). N-FAG, which can be stable in apoptosing cells for at least 24 h,[103] contains the PABP binding site and was postulated to inhibit cap-dependent translation initiation by sequestering PABP in an inactive complex that was unable to recycle ribosomes between rounds of termination and initiation. However, *in vitro* experiments using the reticulocyte lysate system and purified, recombinant N-FAG have suggested that this is probably not the case (SJ Morley, unpublished data). In addition, a lack of any effect of N-FAG on the translation of a number of dicistronic reporter genes expressed in 293 cells has confirmed these findings.[157] Recent studies have shown that N-FAG actually accumulates in the nucleus and that this occurs due to the presence of a basic nuclear localisation signal in N-FAG (in the absence of the putative nuclear export signal that lies in the C-terminus of eIF4GI). These changes relocalise N-FAG into a cellular compartment where it cannot influence translation rates directly.[158] Moreover, they occur without a concomitant relocalisation of PABP. However, it is unclear whether N-FAG is able to exert an effect on translation after the collapse of the nucleus that occurs at later stages of apoptosis. C-FAG, which is predominantly cytoplasmic, contains one of the eIF4A binding sites and also the binding site for the eIF4E-kinase Mnk1. However, it also does not influence translation rates *in vitro* (SJ Morley, unpublished data). Effects on IRES-driven translation are also unlikely to be a result of the cleavage of eIF4GII during apoptosis (Figure 4b). The N-FAG equivalent that lacks the KRRRK nuclear localisation signal found in eIF4GI[158] remains intact and is predicted to be cytoplasmic; whether this can influence translation rates is unknown at this time. Further cleavages of eIF4GII at $DKAD_{851}$ and $DVID_{978}$ result in the production of fragments additional to those observed in the processing of eIF4GI.[104] The eIF4A/eIF3 binding sites are destroyed, suggesting that the fragments are unlikely to participate in any ongoing translation. An additional, noncanonical caspase-3 cleavage event at amino acid 1407 after an IESD sequence also bifurcates the eIF4GII counterpart of C-FAG (Figure 4b).

There has been one report that has suggested that M-FAG can influence the translation of reporter mRNAs containing a cellular IRES sequence. In this study, Apaf-I IRES-driven translation was responsive to the expression of M-FAG in cells that were not undergoing apoptosis.[157] However, both our own unpublished work and an independent study have concluded that overexpression of M-FAG alone has no effect on c-myc, XIAP or Apaf-1 IRES-driven translation.[159] The reasons for these differences are unclear, but the various studies have used different reporter systems, with various levels of expression of proteins in the cells. Alternatively, such assays may need to be carried out in cells during the early phases of apoptosis for consistent effects to be observed.

The most plausible candidate for modulating selective IRES-driven translation during apoptosis is DAP5. Studies have shown that DAP5 is a caspase-activated translation factor, able to maintain the translation of its own mRNA during apoptosis.[134] Removal of the C-terminus of the protein allows it to stimulate IRES-driven translation and abrogates its ability to inhibit cap-dependent translation (Figure 5b). As such, the cleaved form of DAP5 (p86) has been demonstrated to stimulate translation from a reporter mRNA containing Apaf-1, c-myc or XIAP IRES sequences.[157,159] Consequently, it has been proposed that DAP5 plays a central role in allowing the cell to rapidly and simultaneously upregulate expression of both pro- and antiapoptotic proteins for a short period of time, generating a fine balance between apoptosis and survival in the presence of the apoptotic trigger. Consistent with this is the report that the Apaf-1 and BAG-1 IRESs were unable to maintain reporter gene translation beyond the first 2 h of TRAIL treatment (MJ Coldwell, unpublished results). As Apaf-1 is proapoptotic, the cell may not require new synthesis of the protein once the caspase cascade is underway. Similarly, although BAG-1 is an antiapoptotic protein, it can only inhibit the early stages of the apoptotic programme by enhancing the function of Bcl-2. This protein is thought to prevent the release of cytochrome *c* into the cytosol, where it can bind to Apaf-1 and activate the apoptosome. This part of the apoptotic pathway is upstream of the site of action of XIAP, a protein that is itself translated by a cap-independent mechanism and binds to caspases to prevent their activation. The Apaf-1, BAG-1 and XIAP proteins may be required to regulate the complex interplay between the pro- and antiapoptotic pathways during the initial stages of apoptosis. However, once the

death of the cell is inevitable, only those IRESs required for maintenance of expression of short-lived proteins during the latter stages of apoptosis may remain functional. As a result of the cleavage of eIF4GI, the late cleavage of PABP[107] and the decreased ability of eIF4E to participate in the initiation process, *de novo* cap-dependent translation would be severely inhibited.[1]

Effects of modifications of 4E-BP1

Several previous studies have shown that during the early phase of the cellular response to inducers of apoptosis, the extent of phosphorylation of 4E-BP1 decreases,[83,101,124,125] and that the hypophosphorylated 4E-BP1 thus generated binds to eIF4E in competition with eIF4GI or II. This decreases the availability of the eIF4F complex for initiation of translation.[9,19] As described earlier, 4E-BP1 is also subject to cleavage (by both caspase and noncaspase enzymes), and it is possible that the cleavage product(s) may also serve as inhibitors of protein synthesis by sequestering eIF4E and preventing association of the latter with eIF4G[126] (C Constantinou and MJ Clemens, unpublished observations). Thus, the combined effects of the dephosphorylation of 4E-BP1 and the cleavages of both eIF4G and 4E-BP1 that occur prior to and during apoptosis appear to constitute a multifactorial mechanism leading to the sequestration of eIF4E.

The downregulation of cap-dependent translation is inevitably a consequence of these changes, but again there is the possibility of differential effects on different mRNAs, depending on the requirement for the level of eIF4F in the cell. Moreover, IRES-driven translation may well escape the inhibition altogether because it is an eIF4E-independent process. A further variable that could influence the outcome of 4E-BP dephosphorylation and cleavage may be the ratio of eIF4E to the 4E-BPs in the cell. High levels of eIF4E expression are related to cell transformation and resistance to apoptosis, whereas 4E-BP abundance can have growth inhibitory and proapoptotic effects. These cellular phenotypes may reflect the selective translation of different cell death-inhibiting or -promoting proteins, respectively, and such phenomena may in turn be influenced by the structures of the corresponding mRNAs for these critical proteins.

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