Translation initiation factor modifications and the regulation of protein synthesis in apoptotic cells

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Abstract

The rate of protein synthesis is rapidly down-regulated in mammalian cells following the induction of apoptosis. Inhibition occurs at the level of polypeptide chain initiation and is accompanied by the phosphorylation of the α subunit of initiation factor eIF2 and the caspase-dependent cleavage of initiation factors eIF4G, eIF4B, eIF2 α and the p35 subunit of eIF3. Proteolytic cleavage of these proteins yields characteristic products which may exert regulatory effects on the translational machinery. Inhibition of caspase activity protects protein synthesis from long-term inhibition in cells treated with some, but not all, inducers of apoptosis. This review describes the initiation factor modifications and the possible signalling pathways by which translation may be regulated during apoptosis. We discuss the significance of the initiation factor cleavages and other changes for protein synthesis, and the implications of these events for our understanding of the cellular changes associated with apoptosis. *Cell Death and Differentiation* (2000) 7, 603–615.

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Abbreviations: 4E-BP, eukaryotic initiation factor 4E binding protein; Apaf-1, apoptotic protease-activating factor 1; BOC-D-FMK, t-butoxycarbonyl-Asp(OMe)-fluoromethylketone; DISC, death-inducing signalling complex; dsRNA, double-stranded RNA; eIFs, eukaryotic initiation factors; FADD, Fas-associated death domain; IAP, inhibitor of apoptosis protein; MAPK, mitogen-activated protein kinase; PABP, poly(A) binding protein; PARP, poly(ADP-ribose) polymerase; z-VAD-FMK, Z-Val-Ala-Asp-fluoromethylketone

Introduction

Apoptosis is now recognized to be an important physiological process by which cell and tissue growth, differentiation and programmes of development are regulated. The molecular mechanisms of apoptosis have been the subject of intense research in recent years (for reviews see^{1–5}). Cell death is induced following the stimulation of specific cell surface receptors such as the CD95 (Apo-1/Fas) antigen or the tumour necrosis factor- α (TNF α) receptor-1 (TNFR-1).⁶ It can also result from intracellular events such as DNA damage or from a lack of specific growth factors. The relative importance of these different influences varies between cell types. The apoptotic process can be divided into a commitment phase and an execution phase. As described later in this review, the former phase involves the formation of multi-protein complexes which are either associated with specific cell membrane receptors (the death-inducing signalling complex (DISC))^{7,8} or are assembled in the cytoplasm ('aposomes' and 'apoptosomes')⁹. These complexes comprise various adapter proteins together with one or more cysteine proteases (caspases).^{10,11} In each case a specific caspase (usually caspase-8 or -9) is activated and this enzyme then catalyses the activation of further caspase cascades by limited proteolysis.^{12–14} During the execution phase the downstream caspases then target a variety of other cellular proteins for cleavage. As a consequence of such cleavages several further events occur, including the activation of DNA endonuclease activity, changes in cell permeability, cytoplasmic vacuolation and ultimately loss of cell viability.

In some systems cell death induced by CD95 or TNFR-1 activation can be completely blocked by caspase inhibitors (reviewed in reference¹⁵). However in other situations caspase inhibitors block only part of the overall apoptotic programme (e.g. following activation of the *c-myc* oncogene, treatment of cells with the DNA damaging agent etoposide, or induction of activity of the pro-apoptotic gene, *Bak*¹⁶), and it is likely that there are additional pathways involved (upstream of or parallel with caspase function).^{17,18} Activation of such pathways can lead to loss of cellular growth potential and result in some of the morphological changes characteristic of apoptosis even in the absence of caspase activity.^{16,19–21}

Until recently relatively little attention has been paid to the changes in protein synthesis, in terms of either overall translational activity or alterations in mRNA selection, that accompany the commitment and execution phases of apoptosis. However, recent findings have begun to shed some light on this topic and provide insights into the possible mechanisms involved. This review describes these developments and indicates the likely physiological significance of the data for our understanding of translational control during apoptosis.

Mechanism and regulation of initiation of protein synthesis

The initiation of protein synthesis involves complex interactions between a large number of protein initiation factors and RNA molecules.^{22,23} The process requires specific protein-protein binding between individual factors as well as between these factors and the ribosome (summarized in Figure 1). An early event in the initiation pathway is the association of eukaryotic initiation factor (eIF) 2 with the initiating methionyl-tRNA (Met-tRNA_i) and a molecule of GTP.²⁴ This ternary complex can then bind to the smaller (40S) ribosomal subunit. Another factor, eIF4E, binds directly to mRNA via the 5' methylated guanosine cap structure. eIF4E also has the ability to bind to the factor eIF4G. The latter is a large polypeptide which has binding sites for yet more protein synthesis initiation factors.^{25,26} One of these, the multi-subunit eIF3 protein, is itself able to bind to both ribosomes and

mRNA, as well as enhancing the ability of the eIF2 ternary complex to bind to 40S subunits.

From the above it will be apparent that eIF4G plays a crucial role by acting as a bridge between components such as the cap-binding protein eIF4E and the eIF3 complex, thus allowing the mRNA molecule to associate with the 40S ribosomal subunit (reviewed in^{23,25,27}). In mammalian cells eIF4G is a large polypeptide, originally thought to have a mass of 154 kDa,²⁸ but now known to be larger than this.^{29,30} This protein exists partly in the form of a trimeric complex with eIF4E and the ATP-dependent RNA helicase eIF4A, forming the initiation factor eIF4F.³¹ A second form of eIF4G (eIF4GII) has also been described.³² eIF4G has domains for the binding of eIF4E and eIF3 in its N-terminal and central regions respectively, as well as binding sites for eIF4A,^{33,34} the poly(A) binding protein (PABP)^{29,35–37} and the eIF4E kinase Mnk1³⁸ (see Figure 3 for the locations of these domains).

The association of eIF4G with eIF4E strongly enhances the binding of the latter to 5' mRNA cap structures.³⁹ However this association is inhibited by a family of 4E binding proteins (4E-BPs) which can compete with eIF4G for binding to eIF4E.^{40–43} The extent of complex formation between the best studied of these proteins, 4E-BP1,⁴⁴ and eIF4E is regulated by the phosphorylation of 4E-BP1.^{45–48}

To enable translation to begin at the correct site on an mRNA the [40S subunit-Met-tRNA_i] complex must locate the initiating AUG codon. This usually requires the ribosome to scan along the mRNA in a 3' direction from the cap site, a process which often requires the transient 'melting' of secondary structure in the mRNA.²² This function needs the RNA helicase activity of eIF4A, which is stimulated by another accessory factor, eIF4B. In some cases, the need for cap recognition and/or scanning can be by-passed by internal initiation, in which the [40S subunit-Met-tRNA_i] complex binds directly to an internal ribosome entry site (IRES). This is most commonly seen in the case of picornavirus mRNAs but is also the case for some cellular mRNAs.^{49–53} Certain mRNAs, such as *c-myc* or *c-sis*, may be capable of being translated by either mechanism, depending on the conditions in the cell^{54,55} (reviewed in reference⁵⁶). Interestingly some of these mRNAs also contain alternative start sites for protein synthesis and can encode functionally distinct products.^{57,58}

Following the location of the AUG initiation codon on the mRNA two additional events must occur. The GTP associated with the eIF2 is hydrolysed to GDP, in parallel with the dissociation of the previously bound initiation factors from the ribosome. GTP hydrolysis requires the involvement of the factor eIF5. The GDP that is generated remains associated with the eIF2 and must be exchanged for another molecule of GTP in a process catalysed by the guanine nucleotide exchange factor eIF2B.²⁴ The second event is the binding of the larger (60S) ribosomal subunit to the initiation complex to form the complete 80S initiation complex.

Dramatic changes occur in the integrity of eIF4G after infection of cells with picornaviruses such as polio or foot-and-mouth-disease virus (reviewed in²⁵). Within a few

Figure 1 Summary of the initiation of protein synthesis. A complex of the initiation factor eIF2 with GTP binds the initiator Met-tRNA_i to form a [Met-tRNA_i-eIF2-GTP] ternary complex. This then associates with a 40S ribosomal subunit carrying eIF3 (and other factors – not shown) to produce the 43S preinitiation complex. mRNA binding to this complex involves the participation of the trimeric eIF4F complex, comprising the cap-binding protein eIF4E, the scaffold protein eIF4G and the RNA helicase eIF4A, together with the helicase-stimulatory factor eIF4B. Joining of the 60S subunit and hydrolysis of the GTP, catalysed by eIF5, leads to the formation of the 80S initiation complex which can commence protein synthesis. The GDP formed as a result of GTP hydrolysis remains associated with the eIF2 and is later exchanged for another molecule of GTP by eIF2B. Modified from Figure 1 of reference²²

hours both eIF4GI and eIF4GII are cleaved by viral and/or cellular proteases at a region between the eIF4E and eIF3 binding sites,⁵⁹ with the effect on eIF4GI preceding that on eIF4GII.^{60,61} These cleavages destroy the bridging function of eIF4G in protein synthesis and lead to the inhibition of cap-dependent initiation.⁶² Recent work suggests that the kinetics of cleavage of eIF4GII correlate better than those of eIF4GI cleavage with the time of down-regulation of protein synthesis in picornavirus-infected cells.^{60,61} Several studies indicate that the remaining C-terminal fragment of eIF4G can still support initiation involving ribosome binding to internal sites on mRNAs (which is therefore cap-independent).^{63,64} As a result picornavirus RNA-directed protein synthesis, which occurs by internal initiation, is favoured. It is not yet clear whether a similar mechanism is used to inhibit cap-dependent protein synthesis in uninfected cells, in which some cellular mRNAs can also be translated by an internal initiation mechanism, although there have been reports of the cleavage of eIF4G under situations where protein synthesis is down-regulated.^{65,66}

Changes in translational activity during apoptosis

Induction of apoptosis has been shown to be associated with a rapid and substantial inhibition of protein synthesis in several cell types.^{67–71} In our own work we have observed decreases of about 60–70% within a few hours of induction of apoptosis by treatment of Jurkat T cells with anti-CD95 antibody or of MCF-7 breast cancer cells with TNF α . The mechanisms involved probably depend on the nature of the apoptotic inducer, since in Jurkat cells the down-regulation of translation following CD95 stimulation, but not that caused by the DNA damaging agent etoposide, can be completely abrogated by the broad spectrum caspase inhibitor z-VAD-FMK.⁶⁹ Similarly, in MCF-7 cells the effects of TNF α are prevented by z-VAD-FMK or another caspase inhibitor, BOC-D-FMK (IW Jeffrey, VJ Tilleray and MJ Clemens, unpublished data). Caspase-3 also partially inhibits protein synthesis in a cell-free system and this effect too is blocked by z-VAD-FMK (M Bushell, W Wood and SJ Morley, unpublished data). Thus the caspases play an important role in the down-regulation of protein synthesis during apoptosis, although there may also be caspase-independent components in the pathway(s) by which agents such as etoposide exert their effects on translation.

The inhibition of protein synthesis in apoptosing cells is associated with a substantial decrease in the proportion of ribosomes in polysomes,^{69,72} strongly suggesting that there is a block at the stage of polypeptide chain initiation. However we cannot rule out the possibility that other changes, such as altered rates of polypeptide elongation or decreased stability of ribosomes or mRNA (eg due to caspase-mediated activation of nucleases – see accompanying review by Degen *et al.*⁷³), also contribute to the effect.

Polypeptide chain initiation factors as substrates for caspase-mediated cleavage

Recent studies from our laboratories and others have demonstrated that a limited number of initiation factors are

targets for specific degradation by caspase-mediated mechanisms in a variety of cell types and in response to several inducers of apoptosis. In an investigation of the effects of deprivation of serum growth factors on protein synthesis in the human BJAB B cell lymphoma line we observed a very marked and progressive disappearance of eIF4G.⁶⁸ In contrast, under the same conditions there were no major decreases in the levels of several other initiation factors, including eIF4E and eIF4A. BJAB cells undergo apoptosis when placed in low serum concentrations, as indicated by the appearance of a specific cleavage product of the classical caspase substrate poly(ADP-ribose) polymerase (PARP). Further work showed that another physiological means of inducing apoptosis, *viz.* stimulation of the CD95 antigen, also led to pronounced cleavage of eIF4G in both BJAB and Jurkat cells.^{68,69} This cleavage was prevented by treating the cells with z-VAD-FMK. Similar results were seen using these and other cell types following induction of apoptosis with cycloheximide, etoposide, TNF α or the protein kinase inhibitor staurosporine.^{68–70,74} In each case, eIF4E remained quite stable under conditions where eIF4G disappeared with rapid kinetics. The eIF4G normally found in association with eIF4E in the eIF4F complex was vulnerable to cleavage. In all cases preincubation of cells with caspase inhibitors protected eIF4G from degradation.

The disappearance of intact eIF4GI is accompanied by the appearance of discrete breakdown products including a major fragment, originally designated p76⁶⁸ but which has been more recently termed M-FAG (middle fragment of apoptotic cleavage of eIF4G),⁷⁵ with a mass of ca. 72 kDa (Figure 2). The breakdown products of eIF4GI are reasonably stable in our experience, although others have suggested that only a small fraction of the protein generates discrete fragments.⁷⁶ The appearance of M-FAG and the other products is prevented by caspase inhibitors.^{68,75} Strikingly, in MCF-7 cells, which are deficient in caspase-3, eIF4GI is not cleaved to give rise to M-FAG during apoptosis.⁷⁴ The M-FAG fragment that appears in cells undergoing apoptosis is not the same as either of the fragments that appear as a result of specific proteolytic cleavage of eIF4GI in picornavirus-infected cells. Figure 3 shows a map of the locations of the caspase-3 cleavage sites on the eIF4GI sequence, determined by N-terminal microsequencing, and contrasts the characteristics of the cleavage products with those of the fragments generated by picornavirus-encoded proteases. An important difference between the two patterns of cleavage is that the viral enzymes cut eIF4GI at only one site but caspase-3 makes two cleavages. As a consequence, after caspase-3 cleavage the eIF4E binding domain remains attached to the central domain of eIF4GI in the M-FAG fragment, whereas the fragment C₁ generated by the viral proteases has lost the eIF4E binding site (Figure 3). M-FAG can also be produced by direct caspase-3 cleavage of eIF4GI *in vitro*, both in the reticulocyte lysate system and using recombinant proteins. This finding, together with the data from the MCF-7 cell studies, demonstrates that caspase-3 is both necessary and sufficient for eIF4GI cleavage. In this respect eIF4GI resembles the cytoskeletal protein α -fodrin in being one of

the few proteins which are apparently not substrates for other caspases in the cell.

Figure 2 Initiation factor cleavages during apoptosis. Apoptosis was induced in human BJAB cells (in this case by treatment with cycloheximide) and cell extracts were prepared at the times indicated. Proteins were separated by SDS gel electrophoresis and subjected to immunoblotting using antisera directed against the C-terminal third of eIF4GI (amino acids 1079–1560 in the revised numbering system of Imataka et al.²⁹), eIF4B, eIF3 and the apoptotic marker protein PARP. The full-sized protein and a specific cleavage product generated by caspase-dependent proteolysis are indicated for each panel. M-FAG is the middle fragment of eIF4GI (see Figure 3). Note that $\Delta 4B$ (the cleavage product of eIF4B) is only weakly recognised by the antiserum to this factor

Further investigations into the integrity of other initiation factors in cells undergoing apoptosis have now shown that, in addition to eIF4GI, there is selective cleavage of eIF4B and the smallest subunit of eIF3 (p35).⁷⁷ These cleavages occur in response to serum deprivation or treatment of cells with anti-CD95, etoposide or cycloheximide. In contrast, none of the other 10 subunits of eIF3 are degraded under the same conditions. As in the case of eIF4GI caspase-3 is both necessary and sufficient for eIF3 (p35) cleavage; however eIF4B is also cleaved during apoptosis in MCF-7 cells (IW Jeffrey and M Bushell, unpublished data), suggesting that other caspases may also target this protein for degradation. We have also found that small proportions of the α subunit of eIF2 and the eIF4E-binding protein 4E-BP1 are cleaved to give rise to discrete fragments.⁷⁷ Such changes have also been described by other laboratories⁷⁸ (A Tee and CG Proud, personal communication).

In BJAB cells the cleavages of eIF4B and eIF3(p35) occur with delayed kinetics relative to that seen for eIF4GI.⁷⁷ Purified recombinant caspase-3 is able to degrade eIF4B and eIF3(p35) *in vitro*, producing fragments of the same sizes as those seen in intact cells, and the cleavage sites in these proteins have now been identified. Cleavage of eIF4B occurs between amino acids 45 and 46, after the sequence DETD, and cleavage of eIF3(p35) occurs on the C-terminal side of amino acids 242 (after the sequence DLAD) and/or 256 (after the sequence DYED).⁷⁷

Figure 3 Caspase cleavage sites in eIF4GI and characteristics of the fragments generated. The domain structure of eIF4GI is shown in the centre of the Figure, with the cleavage products generated by picornavirus protease-mediated cleavage and caspase-mediated cleavage indicated above and below respectively. The amino acid numbering system of Imataka et al.²⁹ is used. Note that the L protease of foot-and-mouth-disease virus and the 2A protease of poliovirus cleave after residues 634 and 641 respectively, each producing two fragments of eIF4GI, N_t and C_t . This separates the eIF4E binding domain from the eIF3 and eIF4A binding domains. In contrast caspase-3 cleaves at two sites in eIF4GI (after residues 492 and 1136) to produce three fragments, N-FAG, M-FAG and C-FAG. The M-FAG fragment retains the ability to bind eIF4E, eIF3 and eIF4A but loses the binding sites for PABP and the eIF4E protein kinase Mnk1

We have also found that the second form of eIF4G recently described, eIF4GII, is cleaved during apoptosis. This happens with the same kinetics as eIF4GI cleavage and in this respect the behaviour of the two forms of eIF4G is different from the situation in poliovirus infection where eIF4GII is fragmented more slowly than eIF4GI.^{60,61}

Further aspects of translational regulation in apoptotic cells

Although the induction of apoptosis by stimulation of the CD95 or TNF α receptors inhibits overall protein synthesis in a way which can be completely prevented by treating cells with z-VAD-FMK, these data do not necessarily prove that eIF4G, eIF4B or eIF3(p35) are the critical targets responsible for down-regulation of translation. At early times during CD95-induced apoptosis in Jurkat cells or TNF α -induced apoptosis in MCF-7 cells we have also observed increased eIF2 α kinase activity and a transient increase in the phosphorylation of eIF2 α . However the increase in eIF2 α phosphorylation is not prevented by zVAD-FMK and it is not yet clear whether it is part of the apoptotic response or is a separate 'stress' response of cells to anti-CD95 or TNF α stimulation. Phosphorylation of eIF2 α is followed by the caspase-dependent cleavage of the factor at later times (S Morley, M Bushell and IW Jeffrey, unpublished data). Comparison of the behaviour of wild-type Jurkat cells and cells deficient in caspase-8 activity (which are therefore resistant to anti-CD95-induced apoptosis) has demonstrated that in both cases the timing of the transient increase in eIF2 α phosphorylation coincides with the onset of the inhibition of translation and occurs prior to detectable cleavage of eIF4GI. At slightly later times, when activation of the CD95 receptor results in the caspase-8-dependent cleavage of eIF4GI in wild-type Jurkat cells, dephosphorylation and degradation of the ribosomal protein S6 kinase p70^{S6K} have also been observed (S Morley, unpublished data).

Changes in the state of phosphorylation of eIF4E also occur during apoptosis. Although the level of total eIF4E in the cytoplasm does not change as apoptosis proceeds, a decline in the extent of phosphorylation of the factor is evident in both BJAB cells⁷⁵ and Jurkat cells (S Morley and L McKendrick, unpublished data). A similar phenomenon has been reported upon stimulation of an apoptotic response in immature thymocytes with anti-CD3 plus anti-CD4 antibodies.⁷⁹ We have also observed a substantial increase in the association of the binding protein 4E-BP1 with eIF4E at later times after induction of apoptosis in BJAB cells⁷⁷ and Jurkat cells (S Morley, unpublished data), whereas the level of total 4E-BP1 in the cytoplasm hardly changes over several hours after activation of the apoptotic response.

Collectively these data therefore suggest that several proteins which impinge upon the control of protein synthesis are direct or indirect targets for caspase-mediated regulation *in vivo* (summarized in Table 1). However, it is not yet possible to rank the relative importance of these various events in mediating the inhibition of translation that occurs during apoptosis.

Signal transduction mechanisms in apoptosis and their relevance to control of translation

Over recent years, a large amount of effort has gone into understanding how death signals are propagated in cells to promote apoptosis. The cytoplasmic compartment contains the core apoptotic machinery in a latent or sequestered form. It is now apparent that activation of these proteins requires specific protein sequence domains which facilitate translocation to and from the plasma membrane, mitochondria and nucleus. However, relatively little is yet known about the signalling mechanisms employed by cells to bring about the inhibition of protein synthesis which is associated with the induction of apoptosis, or the effects that apoptotic signalling

Table 1 Summary of initiation factor changes associated with apoptosis

Initiation factor	Modification	Consequences
eIF4GI and eIF4GII	Caspase-mediated cleavages after amino acids 492 and 1136 in eIF4GI	Generation of discrete cleavage fragments; loss of 'bridging' function of eIF4G; possible inhibition of mRNA circularization; potential changes in mRNA recruitment
eIF4B	Caspase-mediated cleavage after amino acid 45	Generation of N-terminally truncated cleavage product
eIF3 (p35 subunit)	Caspase-mediated cleavages after amino acids 242 and/or 256	Generation of C-terminally truncated cleavage product(s)
eIF2 α	Caspase-mediated cleavage after amino acid 300	Generation of C-terminally truncated cleavage product; protection of protein synthesis from inhibition by phosphorylation of eIF2 α
eIF2 α	Phosphorylation at Ser ⁵¹	Inhibition of guanine nucleotide exchange on eIF2 (catalysed by eIF2B), with consequent impairment of eIF2 function
4E-BP1	Caspase-mediated cleavage (site unknown)	Generation of truncated cleavage product; functional consequences not known
4E-BP1	Possible dephosphorylation	Increased binding to (and potential sequestration of) eIF4E
eIF4E	Dephosphorylation	Possible inhibition of cap-binding activity

This table summarizes the various modifications of polypeptide chain initiation factors observed in apoptosing cells

pathways may have on the phosphorylation and activity of translation initiation factors. The current information concerning the pathways involved is summarized in Figure 4.

Transmembrane receptors of the tumour necrosis factor (TNF) receptor family, such as CD95, can promote rapid programmed cell death upon interaction with their cognate ligands or agonistic antibodies.^{80,81} The essential signalling events in this process have been the subject of numerous reviews.^{10,82–85} The activated receptors recruit key signalling proteins to form the DISC complex.^{8,84,85} An adapter protein, Fas-associated death domain protein (FADD or MORT-1),^{86,87} is translocated and recruited via its C-terminal 'death domain' and this in turn recruits pro-caspase-8 (FLICE/MACH α 1/Mch5) to the DISC in association with the Flice-associated huge protein (FLASH).^{85,88–91} Following the dimerisation and autocatalytic cleavage of pro-caspase-8 at internal aspartate residues

an active tetrameric caspase-8 complex (p18/p10)₂ is released into the cytosol.^{10,14,84,85,88,89} This enzyme then initiates the death cascade by proteolytic activation of downstream targets such as caspase-6 and caspase-3^{85,89,92–94} and other substrates.^{95,96} These events ultimately result in the loss of mitochondrial integrity, the release of cytochrome c and the activation of caspase-9 via interaction of the latter with Apaf-1.^{10,84,85} Numerous studies have demonstrated that the inhibition of caspase-8 activity is sufficient to prevent CD95-induced apoptosis.^{10,84,92,93,97} However caspase-3, which is also activated by a variety of other death stimuli, plays a major role as downstream executioner in the apoptotic pathway, both in the cytoplasm and the nucleus.^{10,13,84,85,96,98–100} Irreparable damage to the genome caused by mutagens, pharmaceuticals that inhibit DNA repair or ionising radiation also leads to apoptosis, primarily via activation of caspase-9.¹⁰¹

In the last few years it has also become increasingly clear that the eIF2 α kinase PKR¹⁰² is capable of contributing to caspase-dependent apoptosis in cells treated with interferons or double-stranded RNA (dsRNA), or following cell stress.^{103–107} It has been suggested that PKR may act as a 'receptor/inducer' for dsRNA signalling, having inputs into the apoptotic pathways at more than one level.^{108,109} One input is via the phosphorylation of eIF2 α ,^{105,106} with TNF α -induced apoptosis being partially prevented by the over-expression of a non-phosphorylatable form of eIF2 α . Consistent with this, the forced over-expression of a mutant eIF2 α (S51D), which mimics phosphorylation at position Ser51, was sufficient to induce apoptosis in COS-1 cells.¹⁰⁵ At another level, the PKR-dependent cellular response to dsRNA has been shown to require the activity of FADD, and it has been suggested that PKR may promote DISC formation.¹⁰⁹ In addition, signalling through NF- κ B and p53 may also have a role to play in PKR-induced apoptosis.^{105,110–113}

Evidence also points to the importance of several other protein phosphorylation events in the induction and regulation of apoptosis. Although there appears to be no conclusive evidence that kinases are required for the execution of the cell death programme, these enzymes may impinge upon the death machinery by amplifying or integrating incoming signals to swing the balance between cell survival and cell death. During apoptosis several protein kinases are cleaved and enzymatically activated, including MEK kinase 1 (MEKK-1), p21-activated kinase 2 (PAK2) and protein kinase C- δ (PKC- δ).⁹⁶ Cleavage-activation of MEKK-1 and PKC- δ probably contributes to cell death by altering the substrate specificity and subcellular localisation of these kinases in dying cells, and PAK2 can activate stress signalling pathways.^{96,114–116} The caspase-dependent cleavage of signalling proteins essential for cell survival has also been examined,⁹⁶ with the cleavage of Raf-1 and Akt (PKB) kinases impairing their activity and leading to the inhibition of cell survival signalling pathways.^{85,117,118} Treatment of haemopoietic cells with survival factors (such as IGF-1 and IL-3) results in the activation of the phosphoinositide-3 kinase pathway, culminating in the Akt-mediated phosphorylation

Figure 4 Signalling pathways by which protein synthesis may be regulated during apoptosis. When cells are induced to undergo apoptosis executioner caspases (e.g. caspase-3) become activated by mechanisms involving death receptors such as the CD95 (Fas) receptor, and/or by a pathway involving mitochondrial-derived proteins such as cytochrome c and Apaf-1. The executioner caspases cleave many protein substrates including the protein synthesis initiation factors eIF4G1 and II, eIF4B, eIF3(p35) and eIF2 α , leading to inhibition of translation. In addition, the eIF2 α kinase PKR can be activated by a FADD-dependent pathway. PKR is involved in the phosphorylation of both eIF2 α and NF- κ B, either or both of which may contribute to the regulation of apoptosis. Finally, the activation of the JNK and p38 stress kinase pathways (by caspase cleavage-dependent activation of PAK2 and MEKK1 and/or by the Daxx kinase pathway) can control both protein synthesis (perhaps via phosphorylation of eIF4E) and cell death itself. For further details see the text

of the pro-apoptotic proteins BAD and caspase-9.^{85,117–119} Phosphorylation maintains caspase-9 in an inactive state and allows BAD to interact with a 14-3-3 protein, such that it is retained in the cytoplasm as an inactive heterodimer, promoting cell survival. Conversely, dephosphorylation of BAD mediated by calcineurin induces apoptosis.¹²⁰

In Jurkat cells, CD95-mediated inhibition of signalling via the Akt pathway has been associated with decreased phosphorylation and cleavage of both p70^{S6K} and the eIF4E-binding protein 1 (4E-BP1) (S Morley, unpublished data). Down-regulation of p70^{S6K} would be predicted to result in a decrease in the translation of mRNAs containing a 5' polypyrimidine tract,¹²¹ while the dephosphorylation of 4E-BP1 should result in the sequestration of eIF4E away from eIF4G and a general inhibition of translation (reviewed in ^{22,25,122–124}).

The roles of the mitogen-activated protein kinase (MAPK) signalling pathways in the control of apoptosis are more controversial. Activation of the ERK MAP kinase has been generally associated with cell survival,¹²⁵ with inhibition of this pathway found to sensitize HeLa cells to CD95-mediated apoptosis.¹²⁶ Surprisingly, ERK kinase is transiently activated by CD95 stimulation during apoptosis,^{96,126} but it is then down-regulated following the cleavage of Raf-1.⁹⁶ In contrast, the p38 MAP kinase and *c-jun* N-terminal kinases (JNK)^{94,97,127–132} are strongly activated in a caspase-dependent manner during apoptosis, and have been implicated as possible mediators of the apoptotic process.^{94,97,131,133} Their activation may occur via numerous possible routes which include: JNK activation following the recruitment of Daxx kinase rather than FADD to the activated CD95 receptor;¹³⁴ activation via the cleaved, activated forms of MEKK-1 or PAK2, which are downstream of caspases but upstream of JNK,^{96,133,135} resulting in amplification of apoptotic signals; or activation through typical signalling pathways utilising Ras and Rac.¹³⁶ However, the story does not seem to be a simple one and mechanisms may be cell-type specific. For instance, signalling through the p38 MAP kinase pathway has been implicated as a requirement for cell survival in response to TNF α ¹³⁵ and inhibition of the p38 pathway has been reported to induce apoptosis.¹³² Furthermore, p38 and/or JNK activities are not required for apoptosis in all cell types.^{137–139}

Several lines of evidence suggest that the phosphorylation of eIF4E can be associated with the stimulation of translation initiation and that eIF4E phosphorylation is decreased when protein synthesis rates are depressed (reviewed in ^{25,122–124}). The phosphorylation of eIF4E is mediated via the activation of the ERK and/or p38 MAP kinase signalling pathways, which recruit and activate the enzyme Mnk1. This kinase, which interacts with eIF4G, can phosphorylate eIF4E at the physiological site *in vitro* and *in vivo*. However, in spite of the fact that these kinase cascades are activated in CD95-stimulated Jurkat cells, they do not bring about the phosphorylation of eIF4E; on the other hand, etoposide-induced apoptosis does result in a robust phosphorylation of eIF4E.⁶⁹ The reasons for these discrepancies are unclear but may reflect differential regulation of eIF4E phosphatase activity. Indeed, Santoro

*et al.*¹⁰⁰ have shown that phosphatase 2A is cleaved and activated during apoptosis and this activity may have a role in preventing eIF4E phosphorylation, thus potentiating the inhibition of translation. Further work is required to address this possibility.

Mechanisms of regulation of protein synthesis during apoptosis

As indicated above, the progress of apoptosis is characterized by a complex programme of changes involving several initiation factors. This programme includes the specific fragmentation of certain factors and alterations in the state of phosphorylation or state of association with binding proteins of others (Table 1). Any or all of these events can potentially contribute to the inhibition of protein synthesis that is seen under these conditions and it is likely that the relative importance of the various changes may be different at distinct stages of development of the apoptotic response.

One of the earliest effects of apoptotic inducers is an increase in the phosphorylation of eIF2 α . This effect may in some cases be transient but could be responsible for the very early inhibition of protein synthesis. As well as becoming phosphorylated eIF2 α can also be cleaved to give rise to a C-terminally truncated form, eIF2 α Δ C.^{77,78} Although it can still be phosphorylated by PKR eIF2 α Δ C has been reported to block the PKR-mediated suppression of reporter gene expression.⁷⁸ Thus the caspase-mediated cleavage of eIF2 α may provide a mechanism for reversing the effects of phosphorylation on the function of initiation factor eIF2.

Cleavage of eIF4G is also a relatively early event and our data indicate that a modified form of eIF4F, containing eIF4E and eIF4A but with the central M-FAG fragment in place of full-length eIF4G1, is present for at least several hours in apoptosing cells (Figure 5). Unlike the situation in picornavirus-infected cells, where cleavage of eIF4G separates the site that interacts with eIF4E from other functional regions,^{34,59,63,140} the 'mini-eIF4F' present in apoptotic cells may still be able to support some cap-dependent initiation (Figure 5). Consistent with this, the M-FAG fragment of eIF4G1 remains associated with ribosomes in apoptotic cells.⁷⁵ Recent reports have shown that a central core domain of eIF4G can still recruit ribosomes to mRNA for cap-dependent translation.^{141,142} This can occur in the absence of both the N-terminal and C-terminal regions of the protein, which are required for the binding of other factors such as PABP and Mnk1 respectively. We have suggested that the M-FAG-containing form of eIF4F will be deficient in PABP-dependent mRNA circularization and re-initiation activity³⁷ since M-FAG does not contain the PABP binding site^{68,75} (see Figures 3 and 5). The decrease in the phosphorylation state of eIF4E during apoptosis may also be a consequence of eIF4G cleavage because the binding site for the eIF4E kinase, Mnk1, is not present in M-FAG.

It is also possible that one or more of the eIF4G cleavage fragments may show a gain of function. For example N-FAG, which can be stable in apoptosing cells for at least 24 h,⁷⁰ may impair poly(A)-dependent initiation

or its regulation by sequestering PABP,²⁹ and C-FAG may interfere with eIF4E phosphorylation by titrating out Mnk1.³⁸ In this connection it is of interest that a fragment of a gene variously called p97, DAP5 or NAT1,^{143–145} that encodes a mammalian homologue of the central part of eIF4GI, acted in a dominant negative fashion to protect HeLa cells against interferon- γ -induced apoptosis.¹⁴⁴ This suggested that full-length p97/DAP5 may itself be pro-apoptotic. The protein can bind both eIF4A and eIF3 and inhibits

translation when overexpressed in transfected cells.^{143,145} Although M-FAG still possesses the eIF4E binding site, which is missing from p97/DAP5, it otherwise has some resemblance to the latter (32% sequence identity). C-FAG also has regions of homology with p97/DAP5 (25% sequence identity). It is possible therefore that one or both fragments may function in a comparable pro-apoptotic manner to p97/DAP5.

We have reported elsewhere⁷⁷ that the fragment of eIF4B which is generated in apoptotic cells retains the domain required for self-association and for interaction with eIF3.¹⁴⁶ The cleaved eIF4B can also still interact with the cap binding complex. It is too early to say whether the cleavage of eIF4B has functional significance but this issue is currently being addressed in our laboratories. There is an essential RNA-binding domain close to the N-terminus of eIF4B^{146,147} and the caspase-3-mediated cleavage which occurs at a site adjacent to this motif may impair function. Similarly, although the role of the p35 subunit of eIF3 in the function of this multi-subunit factor remains unknown,¹⁴⁸ and it is not possible to predict the consequences of the caspase-mediated cleavage of eIF3(p35) during apoptosis, the possibility that p35 cleavage contributes to the down-regulation of translation cannot be ruled out.

Potential physiological consequences of changes in the protein synthetic machinery associated with apoptosis

Studies of the responses of a large variety of cell systems to many different physiological controls have shown that protein synthesis can be modulated by both changes in the state of phosphorylation of initiation factors and changes in the levels of these factors in the cell. These effects allow rapid modification of the overall rate of translation as well as post-transcriptional regulation of gene expression due to changes in the relative selection of different mRNA species. The key proteins that have most often been associated with the control of initiation are eIF2/eIF2B, eIF4E/4E-BPs and eIF4G. Overexpression of eIF4E, eIF4G or a non-phosphorylatable form of eIF2 α in NIH-3T3 cells results in transformation to a malignant phenotype^{149–151} (reviewed in ^{152,153}). The mechanisms responsible could include the enhanced translation of inefficient mRNAs¹⁵⁴ and/or the inability of the cell to down-regulate translation in response to negative regulators. Consistent with this, down-regulation of eIF4E strongly inhibits protein synthesis, decreases cell growth and impairs malignancy.^{155,156} (Note, however, that the level of eIF4G can also fall dramatically when eIF4E is depleted,¹⁵⁵ so it is not possible to say which factor is more important in bringing about these effects). High levels of expression of eIF4E and eIF4G have been found in various human tumours.^{157–160}

In view of these observations it is important to establish the molecular mechanisms by which changes in the levels or activities of the key polypeptide chain initiation factors affect the phenotype of the cell. In the case of eIF4G we know that truncated forms of the protein retain various biological activities, such as the ability to support cap-independent and/or internal initiation,^{63,141,161} and it is possible that the selective regulation of gene expression

Figure 5 Modifications to the 40S ribosomal initiation complex as a result of initiation factor cleavages in apoptotic cells. (A) In non-apoptotic cells a complex pattern of interactions between several initiation factors and between these factors and either the mRNA or the ribosome results in the formation of the complex shown. Note that the 5' end of the mRNA is bound to eIF4E (yellow) via the cap structure (small black circle) whilst the 3' end of the mRNA interacts with PABP (shaded star symbol) via the poly(A) tail. Since both eIF4E and PABP interact with eIF4G this effectively leads to 'circularisation' of the mRNA. The sizes of the various components are not drawn to scale. Modified from Figure 1 of reference ²⁵. (B) In apoptotic cells the initiation complex is likely to be modified in several ways. eIF4G is cleaved to generate the fragment M-FAG, which retains the ability to bind eIF4E and eIF3 (and hence the 5' end of mRNA and the 40S ribosomal subunit) but loses the interaction with PABP. eIF4B is truncated at its N-terminus, also leading to loss of PABP interaction (M Bushell and SJ Morley, unpublished data). As a result of these changes mRNA circularisation is likely to be impaired, with consequent loss of the efficiency of translational reinitiation. For other changes associated with apoptosis (not shown here) please see the text

at the translational level is perturbed by the accumulation of eIF4G cleavage products during apoptosis. Several cellular mRNAs are now known to be capable of translation by mechanisms other than the standard cap-dependent process under certain conditions.^{30,49,56,162} It is therefore of considerable interest that the mRNAs encoding a number of proteins associated with cell death such as the inhibitor of apoptosis XIAP, the *c-myc* oncogene and the pro-apoptotic proteins p97/DAP5 and Apaf-1 fall into this category^{52,76,163} (and AE Willis, personal communication). Furthermore the translation of these mRNAs appears to be resistant to the general inhibition of protein synthetic activity that occurs in apoptosing cells. In the case of p97/DAP5 recent evidence⁷⁶ additionally indicates that this protein (which itself is a substrate for cleavage during apoptosis) can enhance its own IRES-mediated translation. Enhanced expression of proteins of the IAP family^{164,165} would be expected to limit the apoptotic response, whereas synthesis of p97/DAP5 or Apaf-1 would have the opposite effect, providing a positive feedback and accelerating apoptosis. Which of these outcomes predominates might depend on the cell type, its state of differentiation and other conditions (see for example¹⁶⁶). Further work is required to address the possible roles of the eIF4G fragments N-FAG, M-FAG or C-FAG in mediating cap-dependent and/or -independent translation in apoptosing cells and to determine whether the continued translation of specific mRNAs such as those described above is influenced by these proteins.

Finally, consideration needs to be given to the possible relationship between the initiation factor cleavages that occur during apoptosis and the development of autoimmunity. Causal links between dysregulation of apoptosis and some autoimmune diseases have been proposed,¹⁶⁷ and it has been suggested that some caspase substrates may function as autoantigens.¹⁶⁸ It is certainly the case that some RNA binding proteins that may be involved in translational regulation, such as the La antigen,^{169,170} are frequently found to be targets for autoimmune antibodies. A recent report¹⁷¹ indicates that the La protein is indeed both dephosphorylated and cleaved during apoptosis. This protein is also a target for cleavage by the cytotoxic T lymphocyte-derived enzyme granzyme B.¹⁷² However as yet there are no reports of eIF4G or other caspase- or granzyme B-cleavable protein synthesis factors acting as autoantigens in human disease.

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