Differential requirements for caspase-8 activity in the mechanism of phosphorylation of eIF2α, cleavage of eIF4GI and signaling events associated with the inhibition of protein synthesis in apoptotic Jurkat T cells

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Abstract Previously we have reported that induction of apoptosis in Jurkat cells results in an inhibition of overall protein synthesis with the selective and rapid cleavage of eukaryotic initiation factor (eIF) 4GI. For the cleavage of eIF4GI, caspase-3 activity is both necessary and sufficient in vivo, in a process which does not require signaling through the p38 MAP kinase pathway. We now show that activation of the Fas/CD95 receptor promotes an early, transient increase in the level of eIF2α phosphorylation, which is temporally correlated with the onset of the inhibition of translation. This is associated with a modest increase in the autophosphorylation of the protein kinase activated by double-stranded RNA. Using a Jurkat cell line that is deficient in caspase-8 and resistant to anti-Fas-induced apoptosis, we show that whilst the cleavage of eIF4GI is caspase-8-dependent, the enhancement of eIF2α phosphorylation does not require caspase-8 activity and occurs prior to the cleavage of eIF4GI. In addition, activation of the Fas/CD95 receptor results in the caspase-8-dependent dephosphorylation and degradation of $p70^{S6K}$, the enhanced binding of 4E-BP1 to eIF4E, and, at later times, the cleavage of eIF2α. These data suggest that apoptosis impinges upon the activity of several polypeptides which are central to the regulation of protein synthesis and that multiple signaling pathways are involved in vivo.

Key words: Protein synthesis; Eukaryotic initiation factor 2α; Eukaryotic initiation factor 4G; Apoptosis; Caspase-8

1. Introduction

Fas (Apo-1/CD95) encodes a transmembrane protein belonging to the tumour necrosis factor (TNF) receptor family. Interaction of the receptor with its cognate ligand or cross-linking with anti-Fas IgM results in rapid programmed cell death characterised by a series of distinct morphological and biochemical changes [1–5]. The essential signaling events coupling the Fas/CD95 receptor to apoptosis have been studied intensively. Numerous proteins have been identified which interact with the intracellular domain of Fas/CD95 to assemble the death-inducing signaling complex (DISC). Recruitment of procaspase-8 (FLICE/MACH/Mch5) to the Fas/CD95 DISC results in its autocatalytic cleavage and activation [2,3,6], and the inhibition of caspase 8 activity is sufficient to prevent anti-Fas-IgM-induced apoptosis [7]. Downstream of the DISC, the Fas/CD95 signaling pathway has been shown to involve additional caspases (e.g. caspases 1, 3, 6–10), loss of mitochondrial integrity (reviewed in [2,5,6,8]), activation of the p38 MAP kinase and JNK stress kinases [9–12], the inhibition of protein synthesis and the selective cleavage of eukaryotic initiation factor (eIF) 4G [12–15]. Activation of caspase 3, a main downstream executioner in apoptotic pathways [2–4], is both necessary and sufficient for the proteolysis of eIF4G in vivo [16], in a process which does not require signaling through the p38 MAP kinase pathway [12].

eIF4G, which exists in two isoforms, plays an essential role in the mechanism of translation by acting as a molecular bridge between other components of the ribosomal initiation complex (reviewed in [17–19]). In vivo eIF4G exists partly in the form of a complex with the mRNA cap-binding protein eIF4E and the ATP-dependent RNA helicase eIF4A, constituting the initiation factor eIF4F [18–23]. Within the sequence of eIF4G there are domains that interact with eIF4E, eIF4A, eIF3, the poly(A)-binding protein (PABP) and the eIF4E kinase, Mnk1 (reviewed in [18,19]). Interaction of PABP with eIF4G has been suggested to facilitate the functional association of the 3′ end of an mRNA with the 5′ end [24] and the association of eIF4G with eIF4E markedly enhances the binding of the latter to the mRNA cap [25]. The phosphorylation of eIF4E, which has been correlated with enhanced translational activity in cells treated with mitogens and growth factors (reviewed in [19–23]), is facilitated by the binding of the eIF4E kinase Mnk1 to the C-terminus of eIF4GI [26,27]. The binding of eIF4E to eIF4G is inhibited by a small family of proteins, the 4E-BPs, which compete with eIF4G for the cap-binding factor (reviewed in [19–21]).

Another key initiation factor which can regulate the global rate of translation in response to physiological insults is eIF2, which is composed of three subunits (α, β, γ) [22,23,28]. It forms a ternary complex with initiator Met-$tRNA_f$ and guanosine triphosphate (GTP), which then interacts with the 43S

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Abbreviations: eIF, eukaryotic initiation factor; m^7GTP, 7-methyl guanosine triphosphate; PVDF, polyvinylidine difluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PABP, poly(A)-binding protein

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ribosomal subunit [17]. eIF2 recycling between successive rounds of initiation can be inhibited by phosphorylation of the α subunit on Ser-51 by a small family of protein kinases, which in turn are activated in response to starvation, viral infection, the induction of apoptosis and cell stress (reviewed in [28–31]). A well studied eIF2α protein kinase activated by double-stranded RNA (PKR), which is activated by double-stranded RNA, is capable of stimulating caspase-dependent apoptosis in response to bacterial lipopolysaccharide, serum deprivation and TNF-α. This response is mediated via the phosphorylation of eIF2α and perhaps also by increases in the transcription of target genes (reviewed in [28–36]).

Here we demonstrate that activation of the Fas/CD95 receptor in Jurkat cells results in an early and transient increase in the phosphorylation of eIF2α and activation of eIF2α kinase activity. Using a cell line that is deficient in caspase-8 and resistant to anti-Fas-induced apoptosis, we show that the transient increase in the level of eIF2α phosphorylation does not require caspase-8 activity and occurs prior to detectable cleavage of eIF4GI. In addition, activation of the Fas/CD95 receptor results in the caspase-8-dependent dephosphorylation and degradation of $p70^{S6K}$, the enhanced binding of 4E-BP1 to eIF4E and, at later times, the cleavage of eIF2α. These data suggest that several proteins which impinge upon the control of protein synthesis are targets for caspase-dependent and -independent regulation in vivo during the induction of apoptosis.

2. Materials and methods

2.1. Chemicals and biochemicals

Materials for tissue culture were from Gibco Life Technologies, anti-Fas antiserum (clone CH-11) was from Upstate Biotechnology, antiserum against the cleavage product of poly(ADP-ribose) polymerase (PARP) was from Promega (UK), and that specific for the whole protein was from Boehringer Mannheim (UK). zVAD.fmk was from Alexis Corporation, Immobilon polyvinylidine difluoride (PVDF) was from Millipore, and microcystin was from Calbiochem (UK). Unless otherwise stated, all other chemicals were from Sigma.

2.2. Tissue culture

Wild-type human Jurkat T cells and a caspase-8-deficient Jurkat subclone (kindly provided by Dr. J. Blenis, Harvard Medical School, USA) were grown in RPMI 1640 with Glutamax™, supplemented with 10% foetal calf serum, as described [10].

2.3. Induction of apoptosis by anti-Fas antiserum

Cells (5×10^6 cells in 5 ml) were isolated by centrifugation, washed in ice-cold phosphate-buffered saline (PBS) and resuspended in 1 ml of RPMI 1640, without serum. Where indicated, cells were incubated on ice for 30 min with or without 250 ng/ml anti-Fas antiserum [37], isolated by centrifugation, washed in ice-cold PBS and resuspended in 5 ml of original culture medium for the times indicated in the individual figure legends. When used, zVAD.fmk (50 μM) was added to cultures for 60 min prior to isolation of the cells, and was present in all subsequent incubations.

2.4. Preparation of cell extracts

Following treatment, cells were isolated in a cooled centrifuge, the medium removed and cells washed with 1 ml PBS containing 40 mM β-glycerophosphate and 2 mM benzamidine. Pellets (5×10^6 cells) were resuspended in 100 μl ice-cold Buffer A (50 mM (3-[*N*-morpholino] propanesulfonic acid–KOH, pH 7.2, 2.5 mM ethylene glycol-bis(β-aminoethyl ether), 1 mM EDTA, 40 mM β-glycerophosphate, 1 μM microcystin, 50 mM NaCl, 7 mM 2-mercaptoethanol, 2 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 2 mM Na_3VO_4) and lysed by the addition of 0.5% (by volume) igepal and vortexing. Cell debris was removed by centrifugation in a microfuge for 5 min at 4°C and the resultant supernatants frozen in liquid N_2.

2.5. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting

Samples containing equal amounts of protein were resolved by PAGE as described previously [38,39]. Proteins were transferred to PVDF membrane and visualised by immunoblotting with the antisera described in individual figure legends, using alkaline-phosphatase-coupled secondary antibodies. In all cases, detection was within the linear response of the antiserum to the protein and immunoblots were quantified by densitometric scanning.

2.6. Isolation and analysis of eIF4E by 7-methyl GTP (m^7GTP)–Sepharose chromatography

eIF4E and associated proteins were isolated by m^7GTP–Sepharose chromatography and the association of eIF4E with 4E-BP1 was analysed by Western blotting using specific antisera, as described previously [40–42].

2.7. Protein kinase assays

Jurkat cell extracts were prepared as described above. Using equal amounts of protein, p38 MAP kinase activity was assessed by immune complex assays; the activity of the downstream kinase MAP kinase-activated kinase-2 was monitored using recombinant hsp25 as substrate, as previously described [41]. Immune complex assays for $p70^{S6K}$ activity, using rabbit polyclonal antisera to $p70^{S6K}$ (Santa Cruz) with 40S ribosomes as substrate [43], JNK activity, using GST-c-jun (1–79) as substrate [42], and immunocomplex autophosphorylation of PKR [36,44,45] were performed as previously described. All data were quantified using a Molecular Dynamics phosphorimager.

3. Results

3.1. The Fas-induced inhibition of protein synthesis and the cleavage of eIF4GI are severely attenuated in caspase-8 mutant Jurkat cells

Previously, we have shown that treatment of Jurkat cells with anti-Fas IgM antiserum resulted in the induction of apoptosis, the cleavage of eIF4GI and the inhibition of protein synthesis within 2 h of treatment [12]. Further studies using MCF-7 cells have shown that, during apoptosis, caspase-3 is both necessary and sufficient for the cleavage of eIF4GI in vivo and in vitro [16]. We have now examined the role of upstream caspases and signaling pathways in mediating the onset of the inhibition of protein synthesis by comparing a wild-type Jurkat T cell line, which is sensitive to killing by Fas antiserum, with a subclone which is resistant to Fas-induced apoptosis by virtue of a frameshift mutation in the caspase-8 gene [10]. These caspase-8 mutant cells fail to cleave PARP, caspase-2 or protein kinase C-δ in response to activation of the Fas receptor [10].

To determine whether caspase-8 activity was required for the long-term, Fas-induced inhibition of protein synthesis [12], Jurkat cells were treated with anti-Fas antiserum and incubated in the presence of [^{35}S]methionine. Induction of apoptosis in the Fas-sensitive, wild-type cells resulted in a pronounced inhibition of protein synthesis, evident within 2 h of treatment, and a rapid loss of cell viability, as observed by others [10]. These responses were severely attenuated in the caspase-8 mutant cells (Fig. 1A) and were also prevented by preincubation of the wild-type cells with the cell-permeable caspase inhibitor, zVAD.fmk (data not shown but see Fig. 2). Correspondingly, within 1 h of Fas treatment, the cleavage of PARP, eIF4GI (Fig. 1B, lanes 1–3), and eIF4GII (data not shown) was evident in the wild-type cells, but not observed in the caspase-8 mutant cells (lanes 4–6). Cleavage of eIF4GI resulted in the appearance of a characteristic immunoreactive fragment, M-FAG, with an apparent molecular weight of

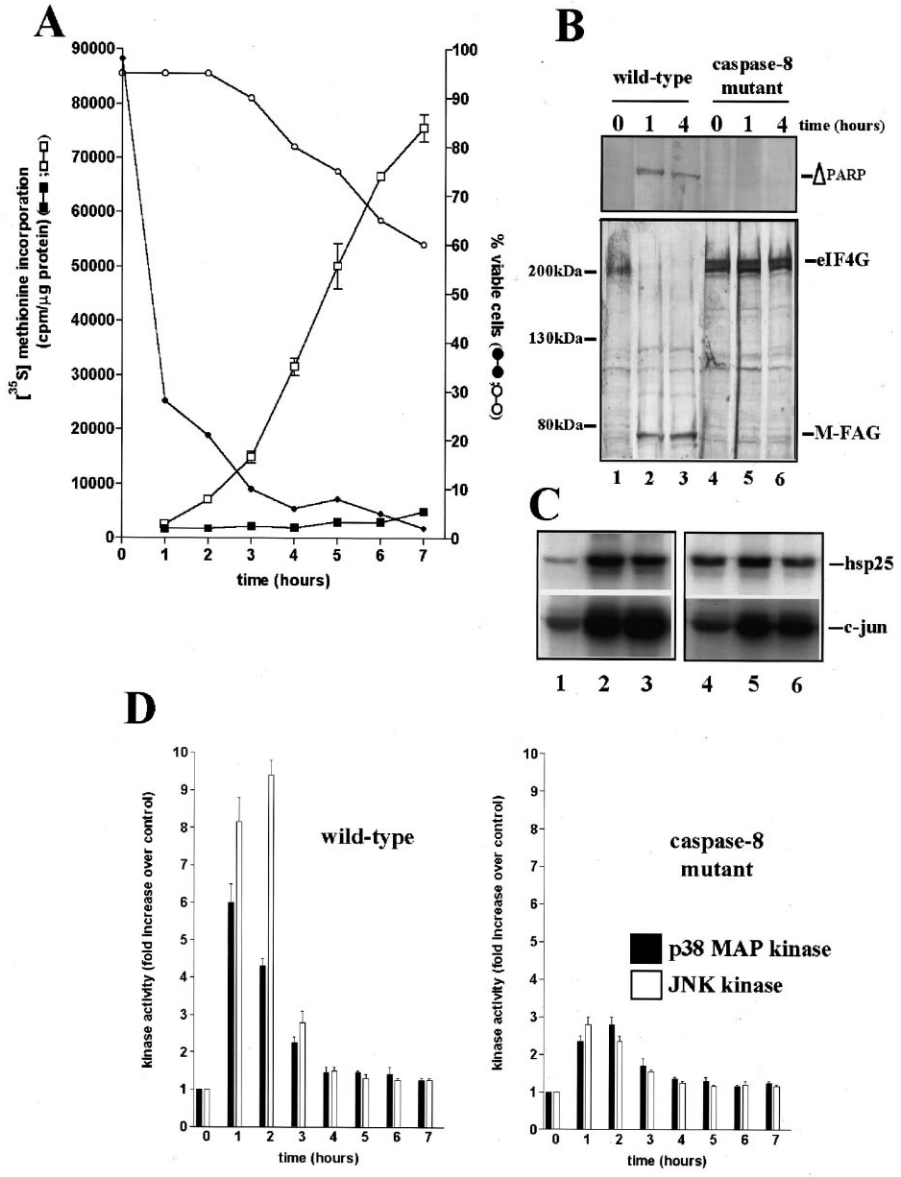

Fig. 1. The Fas-induced inhibition of protein synthesis and the cleavage of eIF4GI are severely attenuated in caspase-8 mutant Jurkat cells. A: Wild-type (closed squares) or caspase-8-deficient (open squares) Jurkat cells (5×10^6 cells) were isolated by centrifugation and incubated in the presence of 250 ng/ml anti-Fas antiserum for 30 min, as described in Section 2. Cells were isolated by centrifugation, washed in PBS and incubated in methionine-free RPMI 1640, in the presence of 10% (by volume) dialysed foetal calf serum and 50 μCi/ml [^{35}S]methionine for the times indicated. Incorporation of [^{35}S]methionine into protein was determined by TCA precipitation [12,41]. The data are the means ± S.D. (bars) of three separate experiments, each performed in triplicate. Parallel cultures of wild-type (closed circles) or mutant Jurkat cells (open circles) were used to determine cell viability, as estimated by trypan blue exclusion. B: Parallel cultures of anti-Fas-treated cells were used to prepare cell extracts at the times indicated, as described in Section 2. Equal amounts of protein (7.5 μg) were resolved by SDS–PAGE and the appearance of the 89 kDa cleavage fragment of PARP (upper panel) and the integrity of eIF4GI (lower panel) were visualised by immunoblotting. The position of the 89 kDa cleavage product of PARP, intact eIF4G and the p76 (M-FAG) cleavage product of eIF4G are indicated. The results are representative of those obtained in at least 10 experiments. C: Aliquots of extracts (containing 30 μg protein) prepared from wild-type (lanes 1–3) or caspase-8 mutant cells (lanes 4–6), incubated with anti-Fas antiserum for 0 h (lanes 1 and 4), 1 h (lanes 2 and 5) or 2 h (lanes 3 and 6) as described in A, were assayed for p38 MAP kinase (upper panel) or JNK activity (lower panel). The autoradiographs presented are from a single experiment. D: In vitro kinase assays from three independent experiments, conducted as in C, were quantified by use of a phosphorimager and are presented as the means ± S.D. (bars).

76 kDa [12,13,16,46,47]. Under these assay conditions, the M-FAG species of eIF4GI was stable for approximately 3 h before it was further processed (data not shown). In the wild-type cells, and to a lesser extent in the caspase-8 mutant cells, activation of the Fas receptor results in an early, transient increase in p38 MAP kinase and JNK kinase activity (Fig. 1C; [10]). More detailed analysis shows that these kinase activities peak at 1–2 h after treatment and decline with time (Fig. 1D). Quantification of these data indicates that caspase-8 activity is required for full Fas-mediated activation of the stress kinases.

To analyse the onset of inhibition of translation more directly, Jurkat cells were exposed to anti-Fas antiserum for 30 min on ice and then incubated at 37°C. Rates of protein synthesis at different times were estimated by measuring [^{35}S]methionine incorporation over the final 15 min before

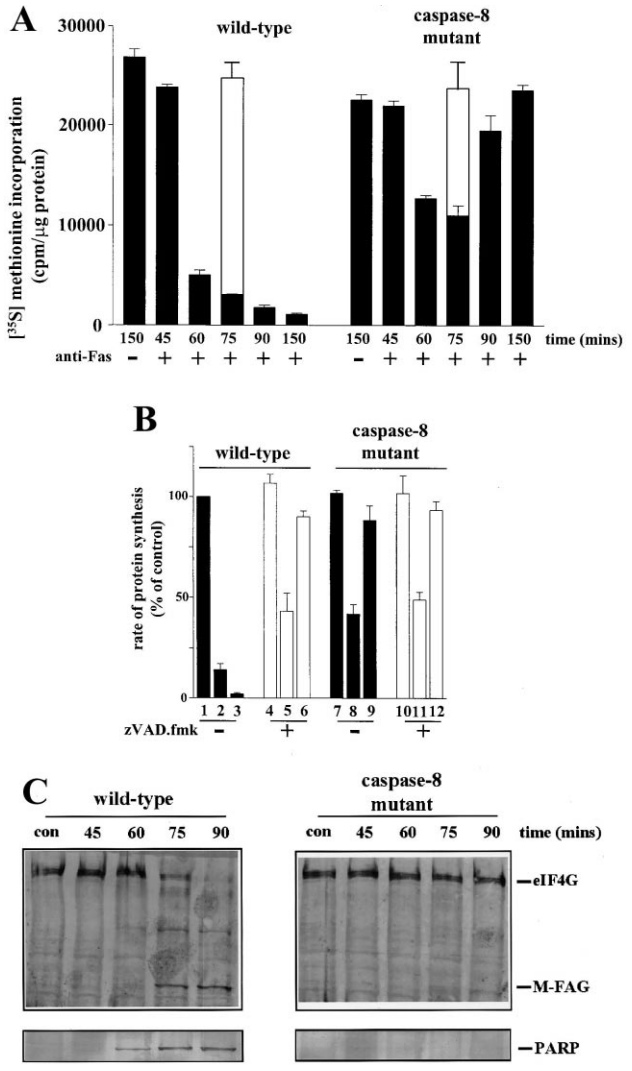

Fig. 2. A transient, caspase-independent inhibition of protein synthesis is observed at early times following anti-Fas treatment of Jurkat cells which is not associated with the cleavage of eIF4GI. A: Wild-type cells (left panel) or caspase-8-mutant cells (right panel) were incubated as in Fig. 1A, except that exposure to [^{35}S]methionine was for only 15 min prior to harvesting at the times indicated (these times include the 30 min incubation with anti-Fas antiserum on ice). Incorporation of [^{35}S]methionine into protein was measured by TCA precipitation [12,41]. The presented data are the means ± S.D. (bars) of five separate experiments; the incorporation of [^{35}S] methionine into protein for each cell line in the absence of anti-Fas antiserum (at 75 min incubation) is shown by the open bars. Similar results were obtained when cells were incubated with anti-Fas antiserum on ice in the presence of PBS rather than with RPMI 1640 (data not shown). B: Wild-type cells (columns 1–6) or caspase-8-mutant cells (columns 7–12) were preincubated for 1 h in the absence (columns 1–3, 7–9) or presence of 50 μM zVAD.fmk (columns 4–6, 10–12). Cells were exposed to 250 ng/ml anti-Fas antiserum as described above, and harvested after 15 min (lanes 1, 4, 7, 10) or incubated for 75 min (lanes 2, 5, 8, 11) or 5 h (lanes 3, 6, 9, 12). [^{35}S]Methionine was added for 15 min prior to harvesting at the time indicated and incorporation of [^{35}S]methionine into protein was monitored by TCA precipitation. The presented data are the means ± S.D. (bars) of three separate experiments, each performed in triplicate. C: Wild-type and caspase-8 mutant cells were incubated as in A and extracts were prepared at the times indicated. Equal amounts of protein (7.5 μg) were resolved by SDS–PAGE and the integrity of eIF4GI and PARP were visualised by immunoblotting. The position of the 89 kDa cleavage product of PARP, intact eIF4GI and the p76 (M-FAG) cleavage product of eIF4GI are indicated.
←

the times indicated in Fig. 2A. Anti-Fas-treatment of wild-type cells resulted in an 80% decrease in the rate of protein synthesis between 45 and 60 min of incubation and thereafter, whereas protein synthesis rates were maintained in mock-treated cells (open bar). In contrast, incubation of the caspase-8 mutant cells with anti-Fas antiserum resulted in a transient inhibition of protein synthesis at 45–60 min, followed by recovery to control rates, an effect not observed in mock-treated cells (open bar). This biphasic response seen in caspase-8 mutant cells was also exhibited by wild-type cells treated with the caspase inhibitor, z.VAD.fmk (Fig. 2B, columns 4–6). This agent blocked the severe depression of protein synthesis rates seen in the wild-type cells after 5 h incubation (Fig. 2B, columns 3 and 6 vs column 1), but only partially relieved the earlier effect seen at 75 min (columns 2 and 5 vs column 1). These data suggest that the inhibition of translation rates at early times is, in part, mediated via a caspase-independent mechanism. In wild-type cells, inhibition of translation occurred co-incident with the cleavage of PARP but prior to the detectable cleavage of either eIF4GI (Fig. 2C) or eIF4GII (data not shown). The transient inhibition of protein synthesis rates seen in the caspase-8 mutant cells also occurred in the absence of cleavage of PARP or eIF4GI (Fig. 2C).

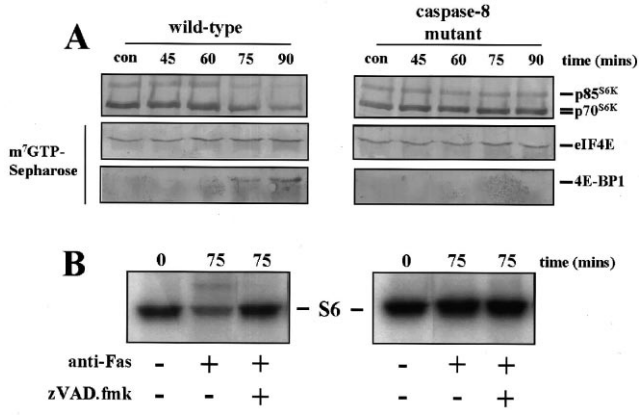

Fig. 3. The early inhibition of protein synthesis in wild-type Jurkat cells does not directly correlate with the inactivation of p70^{S6K} or the increased binding of 4E-BP1 to eIF4E. A: Wild-type and caspase-8 mutant cells were incubated as in Fig. 2A and extracts prepared at the times indicated. Equal amounts of protein (7.5 μg) were resolved by SDS–PAGE and the level and phosphorylation status of p70^{S6K} kinase were visualised by immunoblotting. The positions of the p85 and p70 forms of S6 kinase are indicated. In the case of p70^{S6K} kinase, the lower band of the doublet represents the unphosphorylated form of the enzyme. In addition, eIF4E and associated proteins were isolated by m^{7}GTP–Sepharose chromatography; the recovery of eIF4E and bound 4E-BP1 was visualised by immunoblotting, as indicated. Results are from a single experiment but are representative of those obtained in three separate experiments. B: Cells were incubated in the absence or presence of 50 μM zVAD.fmk, prior to incubation in the absence or presence of 250 ng/ml anti-Fas antiserum for 75 min. Extracts were prepared and aliquots (containing 30 μg protein) prepared from wild-type cells (left panel) or caspase-8 mutant cells (right panel) were assayed for p70^{S6K} kinase activity as described in Section 2. The autoradiograph presented is from a single experiment but is representative of those obtained on three separate occasions.

3.2. Fas induces the inactivation of $p70^{S6K}$ and enhances the binding of 4E-BP1 to eIF4E

The phosphatidylinositol 3-kinase-regulated pathway has been shown to have significant anti-apoptotic signaling properties [48], and modulates the activity of downstream targets including the target of rapamycin (mTOR), eIF4E-binding proteins (4E-BP1/4E-BP2), and $p70^{S6K}$. Activity of $p70^{S6K}$ is required for the up-regulation of translation of some specific mRNAs and for cell cycle progression (reviewed in [19,21,49,50]). Using wild-type and caspase-8 mutant Jurkat cells, we have analysed whether such intracellular signaling events play any role in modulating the Fas-induced inhibition of translation. Immunoblot analysis of extracts prepared at different times after the induction of apoptosis (Fig. 3A) shows that in the wild-type, but not the caspase-8 mutant cells, anti-Fas antiserum promoted the dephosphorylation and destruction of S6 kinase ($p70^{S6K}$). Immunocomplex kinase assays (Fig. 3B) confirmed a decrease in S6 kinase activity in wild-type cells co-incident with the dephosphorylation of $p70^{S6K}$. However, $p70^{S6K}$ activity was retained in the caspase-8 mutant cells and in the wild-type cells preincubated with zVAD.fmk. To see if increased association of 4E-BP1 with eIF4E coincided with the inhibition of translation at early times, eIF4E and associated proteins were isolated by m^7GTP–Sepharose chromatography. Immunoblot analysis showed that whilst the level of eIF4E did not change in either cell line, there was an increase in the association of 4E-BP1 with eIF4E in wild-type but not caspase-8 mutant cells. This was only evident from 75 min of incubation (Fig. 3A) and was prevented by preincubation of wild-type cells with zVAD.fmk (S. Morley, unpublished data).

3.3. Anti-Fas treatment of both wild-type and caspase-8 mutant cells results in increased activity of PKR and the phosphorylation of eIF2α

As the cleavage of eIF4GI and eIF4GII (data not shown),

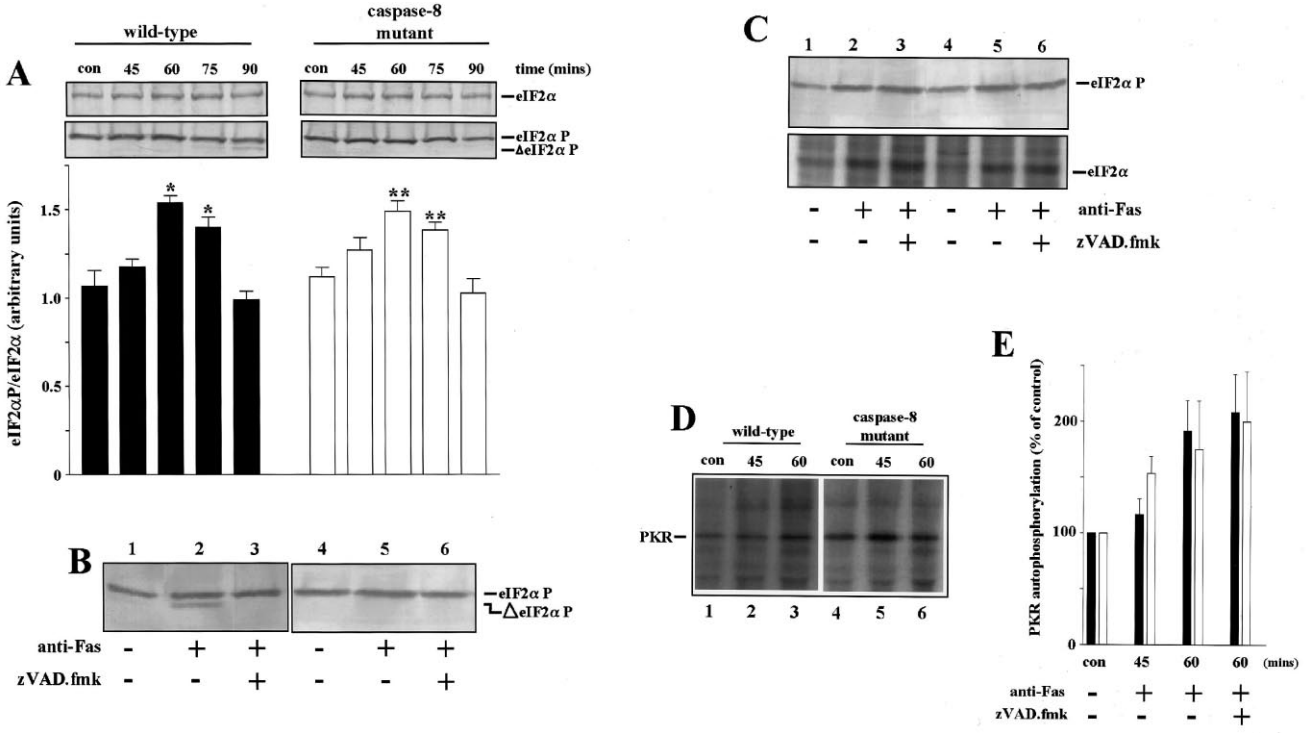

Fig. 4. The Fas-induced increase in the phosphorylation of eIF2α, but not the cleavage of eIF2α, is independent of caspase-8. A: Aliquots of extracts (7.5 μg) prepared from wild-type cells (left panel) or mutant cells (right panel), as described in Fig. 2A, were resolved by SDS–PAGE and total eIF2α protein or eIF2α in the phosphorylated form were visualised by immunoblotting, as indicated. An immunoreactive, phosphorylated cleavage fragment of eIF2α is also visible (wild-type cells, 90 min). Data presented are from a single experiment; results from seven separate experiments were quantified by densitometric scanning and are expressed as the level of eIF2α in the phosphorylated form divided by the total level of eIF2α (lower panel). Presented data are the means ± S.D. (bars); left panel, *, $P < 0.0001$; right panel, **, $P < 0.0021$ (Student's paired *t*-test). B: Wild-type (lanes 1–3) or caspase-8-mutant cells (lanes 4–6) were incubated for 90 min in the absence (lanes 1 and 4) or presence of anti-Fas antiserum (lanes 2, 3, 5, 6), without (lanes 1, 2, 4, 5) or following pretreatment with z.VAD.fmk (lanes 3 and 6). Extracts prepared as described in A were resolved by SDS–PAGE and the amount of eIF2α in the phosphorylated form was visualised by immunoblotting; the cleavage fragment of eIF2α (lane 2) is indicated. C: Wild-type (lanes 1–3) or caspase-8-mutant cells (lanes 4–6) were incubated for 60 min in the absence (lanes 1 and 4) or presence of anti-Fas antiserum (lanes 2, 3, 5, 6), without (lanes 1, 2, 4, 5) or following pretreatment with zVAD.fmk (lanes 3 and 6). Extracts were prepared and aliquots containing equal amounts of protein were resolved by SDS–PAGE; the amount of eIF2α in the phosphorylated form was visualised by immunoblotting (upper panel). In addition, eIF2α kinase activity was monitored by incubation of extracts with recombinant eIF2α and [γ^{32}P]ATP for 10 min; the resulting autoradiograph is presented (lower panel). The data are from a single experiment but are representative of those obtained on five separate occasions. D: Wild-type (lanes 1–3) or caspase-8-mutant cells (lanes 4–6) were incubated for the times indicated in the absence (lanes 1 and 4) or presence of anti-Fas antiserum (lanes 2, 3, 5, 6). Extracts were prepared and PKR activity was visualised by immunoprecipitation and an immunocomplex autophosphorylation assay. The data are from a single experiment but are representative of those obtained on six separate occasions. E: Wild-type (filled bars) or caspase-8-mutant cells (open bars) were incubated without or with anti-Fas antiserum for the times indicated in the absence or presence of zVAD.fmk, as indicated. Extracts were prepared and PKR autophosphorylation was measured as in D; presented data are the means ± S.D. (bars) of five separate experiments.

the inactivation of $p70^{S6K}$ and the increased binding of 4E-BP1 to eIF4E all appeared to be initiated later than the onset of the inhibition of translation, we have also analysed the phosphorylation of eIF2α during induction of apoptosis. In many cell systems, relatively small changes in the phosphorylation of this protein are associated with the inhibition of global protein synthesis (reviewed in [28,29,31,35,36,51]). Using antisera which recognise either total eIF2α or eIF2α in the phosphorylated form, we found that the level of phosphorylated eIF2α was significantly increased in both the wild-type (Fig. 4A, left panel) and mutant cells (right panel), coincident with the onset of the inhibition of protein synthesis (Fig. 2A). However, by 90 min the extent of eIF2α phosphorylation had returned to the control value in each cell line. At later times, cleavage of a small proportion of phosphorylated eIF2α was evident only in the wild-type cells (Fig. 4B, lane 2 vs lane 5) and this was prevented by preincubation of the cells with zVAD.fmk prior to exposure to anti-Fas antiserum (lane 3 vs lane 2). It should be noted that whilst the cleavage of eIF2α was not observed in the caspase-8 mutant cells, phosphorylation of eIF2α did occur over a similar time course as described for the wild-type cells (Fig. 4A).

We have also investigated eIF2α kinase activity during apoptosis in Jurkat cells. As shown in Fig. 4C, incubation of either wild-type (lanes 1–3) or caspase-8 mutant cells (lanes 4–6) with anti-Fas antiserum resulted in a transient increase in overall eIF2α kinase activity (lower panel) which corresponded in timing with the increase in eIF2α phosphorylation (upper panel) and the inhibition of protein synthesis (Fig. 2A). These events were not prevented by zVAD.fmk in either cell line indicating that they occur via a caspase-independent mechanism. We have investigated the possible identity of the eIF2α kinase involved in these effects. Immunoblotting analysis of extracts revealed that total PKR levels did not alter significantly during the onset of apoptosis in either wild-type or caspase-8 mutant cell lines (data not shown). As activation of PKR results in both autophosphorylation and enhanced kinase activity [28,31,36], we have used immunocomplex autophosphorylation assays to monitor PKR activity during apoptosis. Fig. 4D shows that in both wild-type cells and caspase-8 mutant cells, anti-Fas antiserum promoted a moderate, but reproducible increase in the autophosphorylation of PKR (quantified in Fig. 4E). However, similar assays employed to examine the activity of another eIF2α kinase, PEK [45], indicated that PEK activity was not appreciably increased following anti-Fas treatment of either wild-type or caspase-8 mutant cells (data not shown). As described for the phosphorylation of eIF2α (Fig. 4B), the increase in autophosphorylation of PKR was unaffected by pretreatment of either cell line with z.VAD.fmk (Fig. 4E).

4. Discussion

Cells undergoing apoptosis in response to different stimuli display a rapid decrease in the rate of protein synthesis [12,14,15,52]. Previously, we have described that activation of the Fas/CD95 receptor in Jurkat and BJAB cells results in a severe, but incomplete, inhibition of protein synthesis [12,13], with the selective and rapid cleavage of eIF4GI. In the present study, we have further examined early events in apoptosis using two Jurkat T cell lines; wild-type cells which are sensitive to killing by Fas antiserum and caspase-8 mutant cells, which are resistant to Fas-induced apoptosis by virtue of a frameshift mutation in the caspase-8 gene [10]. Our data indicate that with wild-type cells, activation of the Fas receptor results in a severe decrease in the rate of protein synthesis between 45 and 60 min of incubation, with translation rates subsequently maintained at a low level (Fig. 2). In contrast, although incubation of the caspase-8 mutant cells with anti-Fas antiserum resulted in a transient inhibition of protein synthesis, this was followed by recovery to control rates (Fig. 2). The differential sensitivity of the early and late components of this biphasic response to z.VAD.fmk in wild-type cells suggests that the inhibition of translation rates is mediated via both caspase-independent and -dependent mechanisms, respectively.

In this cell system, the initial inhibition of protein synthesis cannot be directly attributed to the cleavage of either eIF4GI (Fig. 2C) or eIF4GII (data not shown), as the transient effect observed in the caspase-8 mutant cells occurred without eIF4G cleavage. Furthermore, although anti-Fas antiserum induced the cleavage of eIF4GI and increased the association of 4E-BP1 with eIF4E in wild-type cells, these events were not required for the initial inhibition of protein synthesis (Figs. 2C and 3A). Similarly, the activation of stress kinases (Fig. 1C,D), the inhibition of $p70^{S6K}$ activity (Fig. 3B), the cleavage of a proportion of the eIF2α pool (Fig. 4A), and the partial cleavage of initiation factor eIF4B and the 35 kDa subunit of eIF3 (data not shown; [46]) all occurred at relatively late times in Fas-stimulated wild-type cells, subsequent to the inhibition of protein synthesis (Fig. 2). Although these data suggest that none of these events is crucial for the initial inhibition of translation, a later role in the apoptotic response, when the synthesis of selected proteins is maintained [52–55], remains possible. Indeed, it is well established that both eIF4GI and eIF4GII are susceptible to rapid cleavage in picornavirus-infected cells, favouring conditions for the IRES-dependent translation of viral mRNAs [56–58]. In addition, following the cleavage of reticulocyte lysate eIF4G with L protease in vitro, changes in the translation capacity of the system can be correlated with the appearance of specific cleavage fragments of eIF4G [59,60]. Therefore, in an analogous manner, the accumulation of the caspase-generated eIF4G cleavage fragments may directly influence mRNA translation rates in vivo. For example, M-FAG (Fig. 1; [46,47]) may have a direct role in the translation of specific cellular mRNAs during apoptosis under conditions where the interaction between the 5′ and 3′ ends of the mRNA has been disrupted (reviewed in [14]). M-FAG, being able to recruit all the necessary factors for basal translation [27,47] and mRNA interaction [27,61,62], may still support cap-dependent and/or cap-independent translation, albeit at a low rate, during apoptosis. Target mRNAs for M-FAG-mediated recruitment may include those encoding *c-myc* [54,63], Apaf-1 [31,53], and XIAP [52], whose synthesis is maintained in vivo during apoptosis. Each of these mRNAs contains an IRES element and their translation behaviour is reminiscent of the translational control associated with cellular differentiation [31,64], stress responses [65], mitosis [66] and starvation responses in yeast [67]. Moreover, studies have shown that the death-associated protein 5 (DAP5/p97/NAT1), a member of the eIF4G family of initiation factors which interacts with eIF3 and eIF4A but not with eIF4E, is cleaved during apoptosis. This generates a stable fragment which can maintain the synthesis of DAP5

itself via a functional IRES element in vitro [55]. Further work will be required to determine whether such in vivo effects on specific mRNA translation are directly mediated by the caspase-generated cleavage fragments of the eIF4G family of proteins.

One of the earliest effects of apoptotic inducers on Jurkat cells is an increase in the phosphorylation of eIF2α; although this effect is transient, in contrast to the cleavage of eIF4G, eIF2α phosphorylation correlates with the onset of the inhibition of protein synthesis. In this study, increased phosphorylation of eIF2α could, in part, be ascribed to a modest increase in activation of PKR (Fig. 4D,E), with little or no effect on the activity of PEK over a similar time course (data not shown). It has been suggested that PKR may be acting as a 'receptor/inducer' for cell signaling by pro-apoptotic agents such as dsRNA, having inputs into the apoptotic pathways at more than one level [29–31,68,69]. These may include direct [33,34] or indirect [31,70] effects via both the phosphorylation of eIF2α and the activity of the DISC-associated protein, FADD. In addition to the promotion of DISC formation [71], signaling through NFκB and p53 may also play a role in PKR-induced apoptosis [29–31,69,71]. Our present data suggest that PKR activation or eIF2α phosphorylation are independent of caspase-8 and thus functions upstream of this enzyme.

During apoptosis, in addition to becoming more highly phosphorylated, a population of eIF2α is also cleaved to give rise to a C-terminally truncated form [46,72,73]. Although it can still be phosphorylated by PKR, the truncated form of eIF2α has been reported to block the PKR-mediated suppression of reporter gene expression [72], possibly by allowing eIF2B-independent GDP exchange on the truncated eIF2 complex [73]. Thus the caspase-mediated cleavage of eIF2α may provide a mechanism for reversing the effects of phosphorylation on the function of initiation factor eIF2 during apoptosis. Whether this is responsible for the recovery of protein synthesis at later times in the caspase-8 mutant cells is not known. However, this recovery was not inhibited by zVAD.fmk (Fig. 2B).

Alternative possibilities which need to be addressed are that transient eIF2α phosphorylation may initially promote the progression of cell death by causing a general inhibition of protein synthesis, facilitated by the maintained translation of pro-apoptotic proteins, such as Bax and Fas [2,74,75], or by impaired synthesis of anti-apoptotic proteins [73]. As Bax and Fas mRNAs possess upstream open reading frames (uORFs), and increased synthesis of Bax protein in some cells coincides with increased levels of eIF2α phosphorylation [71], it is possible that translational control of Bax mRNA occurs during apoptosis in a manner similar to that described for GCN4 in *Saccharomyces cerevisiae* (reviewed in [29,76]). Further work is required to resolve these possibilities.

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