

Proteasome inhibitors and immunosuppressive drugs promote the cleavage of eIF4GI and eIF4GII by caspase-8-independent mechanisms in Jurkat T cell lines

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Abstract Previously, we have shown that translation eukaryotic initiation factor (eIF) 4G is cleaved during anti-Fas-mediated apoptosis. Here, we have investigated the effects of the proteasome inhibitors, MG132 and lactacystin, and the immunosuppressants, 2-amino-2[2-(4-octylphenyl)ethyl]-1,3,propane diol (FTY720) and cyclosporin A, on the integrity of eIF4GI and eIF4GII in T cells. Using wild-type Jurkat T cells, we show that the proteasome inhibitors MG132 and lactacystin promote the cleavage of eIF4G, activate caspase-8 and caspase-3-like activities and decrease cell viability. Furthermore, MG132 also promotes the cleavage of eIF4G and the activation of caspase-3-like activity in a caspase-8-deficient Jurkat cell line which is resistant to anti-Fas-mediated apoptosis. Using specific anti-peptide antisera, we show that both eIF4GI and eIF4GII are cleaved in either cell line in response to MG132 and lactacystin. In response to such treatments, we demonstrate that the fragments of eIF4GI generated include those previously observed with anti-Fas antiserum together with a novel product which lacks the ability to interact with eIF4E. In contrast, cells treated with the immunosuppressants FTY720 and cyclosporin A appear to contain only the novel cleavage fragment of eIF4GI and to lack those characteristic of cells treated with anti-Fas antiserum. These data suggest that caspase-8 activation is not required for apoptosis and eIF4G cleavage mediated by proteasome inhibitors and immunosuppressants in human T cells. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: eIF4G; Apoptosis; Caspase-8; FTY720; MG132; Cyclosporin A

1. Introduction

Translation eukaryotic initiation factor (eIF) 4G, which exists in two forms (eIF4GI and eIF4GII), plays an pivotal role in the mechanism of translation by acting as a molecular bridge between other components of the ribosomal initiation complex (reviewed in [1–3]). 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 eIF4F (reviewed in [2–5]). Within the sequence of eIF4G, there are domains that interact with eIF4E [5–7], eIF4A [8–12], eIF3 [9,10,12], the poly(A)-binding protein (PABP; [13–16]) and the eIF4E kinase, Mnk1 [2,3,10,17]. Interaction of PABP with eIF4G has been suggested to facilitate the functional association of the 3' end of an mRNA with the 5' end [18], with the association of eIF4G with eIF4E markedly enhancing the binding of the latter to the mRNA cap [2,3,19].

Previously, it has been reported that induction of apoptosis in lymphocytes [20–23] or HeLa cells [24] results in an inhibition of overall protein synthesis and the selective cleavage of translation eIF4GI [20–22,25] and eIF4GII [26,27]. While caspase-8 activity is required for proteolysis of eIF4G in response to treatment of cells with anti-Fas antiserum [21], caspase-3 activity is both necessary and sufficient for the cleavage of eIF4G in vivo and in vitro [27,28]. Although anti-Fas treatment promoted a caspase-8-dependent increase in the activity of the p38 MAP kinase and JNK stress kinases [21,29], inhibitor studies showed that cleavage of eIF4G did not require signalling through the p38 MAP kinase pathway [20].

In addition to Fas/CD95 receptor activation, apoptosis can also be promoted in T cells by inhibition of proteasome activity or treatment of cells with immunosuppressive drugs. The proteasome is a multicatalytic proteinase complex responsible for intracellular protein degradation in mammalian cells [30]. Inhibition of the 26S proteasome with MG132 (*N*-carboxybenzoxyl-Leu-Leu-Leucinal) or lactacystin has been reported to induce activation of stress kinases and programmed cell death in a number of cell types [31–36]. These responses are also activated following treatment of cells with immunosuppressants such as 2-amino-2[2-(4-octylphenyl)ethyl]-1,3,propane diol (FTY720) and cyclosporin A (CsA; [37–41]). In vivo, consistent with its role as an immunosuppressant [42], FTY720 induces a drastic decrease in the level of T lymphocytes in the blood [40], possibly mediated by its direct effect on the inhibition of mitochondrial function resulting from cytochrome *c* release [41].

Here, we have investigated the effects of MG132, lactacystin, FTY720 and CsA on the integrity of eIF4GI and eIF4GII in T lymphocytes. We show that these agents induce the activation of caspase-like activities and the cleavage of eIF4G into discrete, novel fragments. This also occurs in caspase-8-deficient Jurkat T cells, which are resistant to anti-Fas-induced apoptosis. The proteolysis of both eIF4GI and eIF4GII is associated with a decrease in cell viability and the inhibition

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Abbreviations: eIF, eukaryotic initiation factor; m⁷GTP, 7-methyl guanosine triphosphate; PVDF, polyvinylidene difluoride; MOPS, 3-[*N*-morpholino] propanesulphonic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether); CsA, cyclosporin A; FTY720, 2-amino-2-[2-(4-octylphenyl)ethyl]-1,3,propane diol; MG132, *N*-carboxybenzoxyl-Leu-Leu-Leucinal; ALLN (MG101), *N*-acetyl-Leu-Leu-Nle-CHO

of protein synthesis in both cell types. These data suggest that eIF4G cleavage mediated by proteasome inhibitors and immunosuppressants in human T cells can be promoted by the activation of caspase-8-dependent and -independent pathways.

2. Materials and methods

2.1. Chemicals and biochemicals

Materials for tissue culture were from Gibco Life Technologies and anti-Fas antiserum (clone CH-11) was from Upstate Biotechnology. FTY720 and CsA were kind gifts from Dr. J. Kay (Sussex, UK) and Dr. S. Matsuda (Yoshitama Pharmaceutical Industries, Tokyo), zVAD.fmk was from Alexis Corporation, Immobilon polyvinylidene difluoride (PVDF) was from Millipore, MG132, *N*-acetyl-Leu-Leu-Nle-CHO (ALLN; MG101) and synthetic lactacystin were from Calbiochem. Antiserum specific to the C-terminus of eIF4GII was a gift from Dr. N. Sonenberg (Montreal, QC, Canada) and 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 (provided by Dr. J. Blenis, Harvard Medical School, Cambridge, CA, USA) were cultured in RPMI 1640 supplemented with 10% foetal calf serum, as described [21,29].

2.3. Treatment of cells

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. Where indicated, cells were incubated on ice for 30 min with or without 250 ng/ml anti-Fas antiserum [43], FTY720, MG132 or CsA at the doses and for the times indicated in the individual figure legends. Cells were isolated by centrifugation, washed in ice-cold PBS and resuspended in 5 ml of original culture medium in the absence of these agents for the times indicated in the individual figure legends. When used, zVAD.fmk (50 μ M) or ALLN (10 μ g/ml) were added to cultures for 60 min prior to isolation of the cells, and were present in all subsequent incubations.

2.4. Preparation of cell extracts

Following treatment, cells were isolated in a cooled centrifuge and washed with 1 ml PBS containing 40 mM β -glycerophosphate and 2 mM benzamidine. Pellets (5×10^6 cells) were resuspended in 100 μ l ice-cold buffer A (50 mM 3-[*N*-morpholino] propanesulphonic acid (MOPS)-KOH, pH 7.2, 2.5 mM ethylene glycol-bis(β -aminoethyl ether) (EGTA), 1 mM EDTA, 40 mM sodium fluoride, 1 μ M microcystin, 80 mM NaCl, 7 mM 2-mercaptoethanol, 2 mM benzamidine, 1 mM phenylmethylsulphonyl fluoride (PMSF), 2 mM Na_3VO_4) and lysed by the addition of 0.5% (by vol.) 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. Measurement of AC-DEVD-AMC and AC-IETD-AMC cleaving caspase activity

Following treatment, cell extracts were prepared in buffer B (10 mM Tris-HCl, pH 8, 130 mM NaCl, 2 mM EDTA, 10 mM sodium pyrophosphate, 1% (v/v) Triton X-100, 1 mM PMSF, 0.25 μ g/ml pepstatin A) and adjusted to equal protein concentration. The fluorogenic caspase substrates AC-DEVD-AMC or AC-IETD-AMC (20 μ M; Biosource International, UK) were added and fluorescence was determined at 60 min intervals following excitation at 380 nm and reading at 440 nm using a SpectraMax (Molecular Devices, USA).

2.6. SDS-PAGE and immunoblotting

Samples containing equal amounts of protein were resolved by SDS-PAGE and processed, as described previously [21,44,45]. Antisera specific for the middle or C-terminal domains of eIF4GI were as described previously [26], and antiserum to the C-terminus of eIF4GII was as described previously [7,27].

2.7. Isolation of eIF4E and associated factors by 7-methyl guanosine triphosphate (m^7 GTP)-Sepharose chromatography

eIF4E and associated proteins were isolated by m^7 GTP-Sepharose

chromatography, as described previously [21,45–47], and the associated eIF4GI was visualised by immunoblotting.

3. Results

3.1. The proteasome inhibitors, MG132 and lactacystin promote cleavage of eIF4GI and eIF4GII by a caspase-8-independent mechanism

Inhibition of the 26S proteasome induces activation of stress kinases, promotes apoptosis [31–36] and potentiates the cleavage of eIF4GI in Jurkat cells treated with anti-Fas antiserum [20,22]. To determine whether proteasome inhibitors alone influence the integrity of eIF4GI, wild-type Jurkat T cells and caspase-8 mutant cells, which are resistant to anti-Fas-mediated apoptosis, were incubated for various times with MG132. Fig. 1A (upper panel) shows that a decrease in cell viability was evident in both cell lines within 8 h following treatment of cells with MG132. To examine whether caspase processing is involved in cell death mediated by proteasome inhibitors, we have monitored caspase-8 and caspase-3-like protease activities at 8 h of treatment with MG132 using fluorogenic substrates. Fig. 1B shows that as with anti-Fas activation, MG132 promotes the activation of caspase-3 and caspase-8-like activities in wild-type Jurkat cells, involving a process which is sensitive to the general caspase inhibitor, zVAD.fmk. These results are consistent with the finding that MG132 can activate a number of caspases in glioma cell lines [36]. In contrast, treatment of caspase-8 mutant cells with MG132 only leads to the activation of caspase-3-like activity. As predicted from earlier studies [21,29], caspase-8 and caspase-3-like activities are not increased in response to anti-Fas treatment in the mutant cell line (Fig. 1B). The integrity of eIF4GI in these extracts was visualised by immunoblotting. Fig. 1C (lanes 1–4) shows that treatment of cells with MG132 for 8 h also promotes the cleavage of PARP and the partial cleavage of eIF4GI with similar kinetics in both the wild-type and caspase-8 mutant cells. In addition to the partial cleavage of eIF4GI, eIF4GII phosphorylation levels increase by two-fold at 8 h, and protein synthesis rates decrease to 30% of control levels (data not shown). In addition, the loss of integrity of eIF4GI in response to MG132 in caspase-8 mutant cells is prevented by pre-treatment of cells with zVAD.fmk (Fig. 1C, lane 5 vs. 6), indicating a role for caspase-like activity downstream of caspase-8 in their generation. However, to effect total cleavage of eIF4GI, these cell lines require treatment with 2.5–10 μ g/ml MG132 for 20 h, resulting in a decrease in cell viability to less than 50% (Fig. 1D). Similar cytotoxic effects of 0.5–2.5 μ g/ml MG132 over 24 h have been reported for glioma cell lines [36]. At concentrations of MG132 greater than 2.5 μ g/ml, eIF4GI is efficiently cleaved into stable, discrete immunoreactive fragments in both wild-type (Fig. 1B; Fig. 2A, lanes 2 and 8 vs. lanes 1 and 7) and caspase-8 mutant cell lines (Fig. 1B; Fig. 2A, lanes 5 and 11 vs. lanes 4 and 10). Therefore, our studies show that MG132 promotes cell death and the cleavage of eIF4GI in Jurkat cells via caspase-8-dependent and -independent pathways.

Inhibition of 20S proteasome activity with 20 μ M synthetic lactacystin in either wild-type (Fig. 2A, lanes 3 and 9) or caspase-8 mutant cells (lanes 6 and 12) also results in the cleavage of eIF4GI, the appearance of M-FAG and C-FAG [25]; Fig. 2B), and a moderate increase in the novel 50 kDa

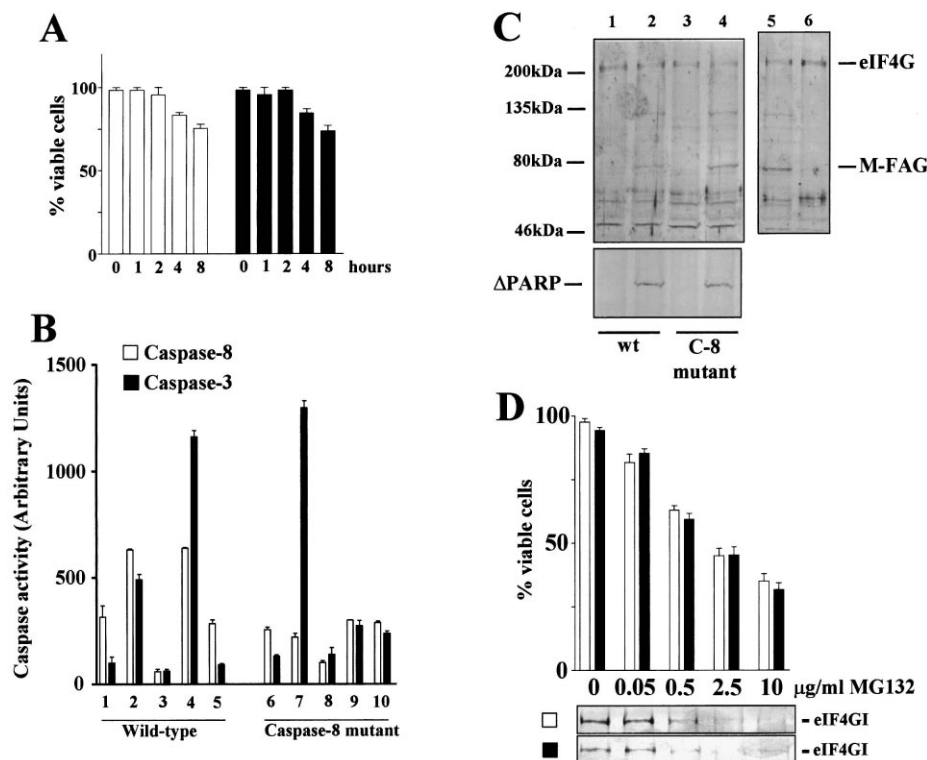


Fig. 1. The proteasome inhibitor MG132 decreases cell viability, activates caspase-like proteases and invokes the cleavage of eIF4GI. Panel A: Jurkat cells (5×10^6 cells in 5 ml) were incubated in the absence or presence of 2.5 μ M MG132 for the times indicated. Cell viability was estimated by trypan blue exclusion and the presented data are the means and S.D. (bars) of three separate experiments. Open symbols, wild-type cells; closed symbols, caspase-8 mutant cells. Panel B: Wild-type Jurkat cells (lanes 1–5) or caspase-8 mutant cells (lanes 6–10) were pre-incubated for 60 min in the absence (lanes 1, 2, 4, 6, 7, 9) or presence of 50 μ M zVAD.fmk (lanes 3, 5, 8, 10), prior to incubation with 2.5 μ M MG132 (lanes 2, 3, 7, 8) or 250 ng/ml anti-Fas antiserum (lanes 4, 5, 9, 10) for 8 h. Caspase-3-like activity was determined with the fluorogenic substrate AC-DEVD-AMC and caspase-8-like activity with AC-IETD-AMC, as described in Section 2. The presented data are the means and S.D. (bars) of two separate experiments, each performed in triplicate. Panel C: Wild-type Jurkat cells (lanes 1 and 2) or caspase-8 mutant Jurkat cells (lanes 3–6) were pre-incubated for 60 min in the absence (lanes 1–5) or presence of 50 μ M zVAD.fmk (lane 6), prior to incubation without (lanes 1, 3) or with 2.5 μ M MG132 (lanes 2, 4, 5, 6) for 8 h. Cell extracts were prepared and equal amounts of protein (7.5 μ g) were resolved by SDS-PAGE. The integrity of eIF4GI was visualised by immunoblotting using antiserum W specific to the middle domain of eIF4G (Fig. 2B) and the p85 cleavage fragment of PARP was visualised using commercial antiserum (Promega, UK). Results are representative of those obtained in three separate experiments. Panel D: Jurkat cells (5×10^6 cells in 5 ml) were incubated in the absence or presence of the indicated concentrations of MG132 for 20 h. Cell viability was estimated by trypan blue exclusion and the presented data are the means and S.D. (bars) of three separate experiments. Open symbols, wild-type cells; closed symbols, caspase-8 mutant cells. Lower: Extracts were prepared from the cultures and the integrity of eIF4GI was visualised by immunoblotting using antiserum W. Results are representative of those obtained in three separate experiments.

fragment, M1. Treatment of cells with a combination of MG132 and lactacystin did not appreciably accelerate these events, suggesting that both reagents are working via similar pathways in Jurkat cells (data not shown). Throughout this study, a low, variable level of the M1 cleavage fragment was present in control extracts from both Jurkat cell lines (see also Figs. 1C and 4A), possibly reflecting the presence of low levels of apoptotic cells in each population [29,48]. This 50 kDa fragment (M1) is recognised by antiserum specific to the central region of eIF4G (antiserum W; Fig. 2, lanes 1–6), but not by antiserum specific to the N-terminus (antiserum B; data not shown) or by antiserum to the C-terminus (antiserum E; Fig. 2, lanes 7–12), indicating that it is derived from the central region of eIF4GI (see also Fig. 3C). Use of anti-peptide antisera indicated that the other fragments generated following treatment of cells with lactacystin are similar to those observed with MG132 (Fig. 2A, lanes 2, 5, 8, 11). M-FAG and C-FAG resemble the fragments of eIF4GI generated exclusively in wild-type cells during anti-Fas-mediated apoptosis [21], only being visualised by antiserum W or antiserum E,

respectively (Fig. 2A). These data suggest that inhibition of proteasome activity generates M-FAG, C-FAG and the novel fragment of eIF4GI, M1, through activation of caspase-like activity downstream of caspase-8.

Another explanation is that the M1 fragment is derived from eIF4GII, which is also cleaved following the induction of apoptosis [26,27]. In contrast to eIF4GI, which has two caspase-3 cleavage sites [23,25,26], eIF4GII is postulated to be cleaved by caspase-3 at five distinct sites [27]. To address this possibility, extracts from wild-type cells subjected to different treatments were resolved on the same polyacrylamide gel, which was then cut into strips to facilitate analysis of the integrity of eIF4GI and eIF4GII (Fig. 2C). Using anti-peptide antiserum specific to the C-terminus of eIF4GII, these data show that eIF4GII is cleaved following treatment of these cells with MG132 (lane 5 vs. lane 4). Due to the low titre of this antiserum, it was not possible to further identify the cleavage fragments of eIF4GII. However, although these fragments generated with MG132 showed some similarity to those observed with anti-Fas treatment of cells (e.g. p140, p28; lane

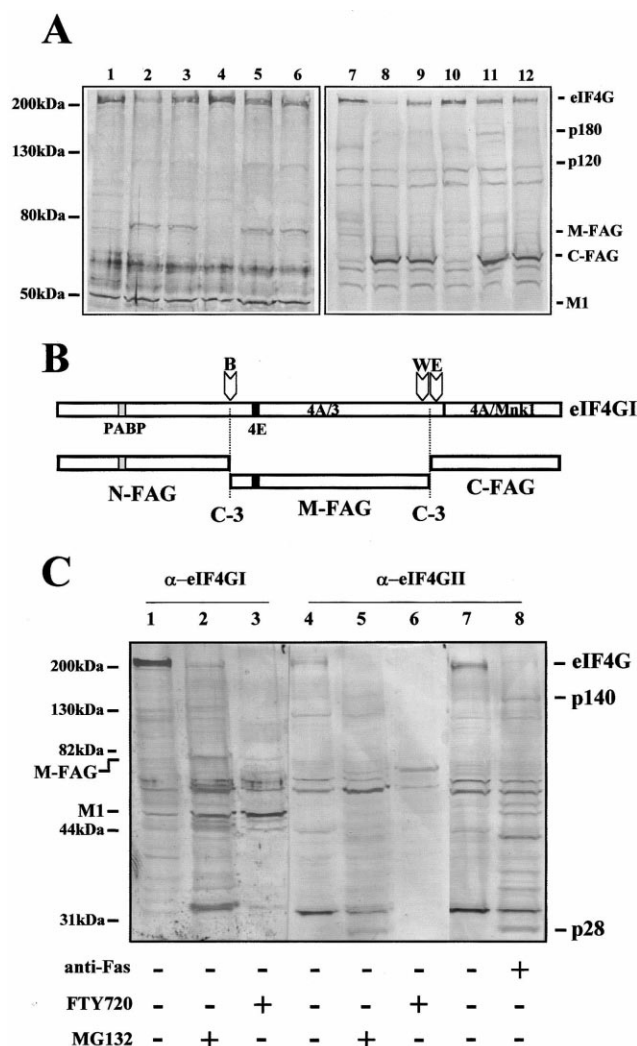


Fig. 2. MG132 and lactacystin promote the cleavage of eIF4GI and eIF4GII into discrete fragments. Panel A: Wild-type (lanes 1–3 and 7–9) or caspase-8 mutant Jurkat cells (lanes 4–6 and 10–12; 5×10^6 cells in 5 ml) were incubated in the absence (lanes 1, 4, 7, 10) or presence of 10 μ g/ml MG132 (lanes 2, 5, 8, 11) or 20 μ M lactacystin (lanes 3, 6, 9, 12) for 20 h. Equal amounts of protein (7.5 μ g) were resolved by SDS-PAGE and the integrity of eIF4GI was visualised by immunoblotting using antiserum W (to the middle domain (left panel)) or antiserum E (to the C-terminus of eIF4GI (right panel)). The migration of intact eIF4G, the M-FAG and C-FAG cleavage products and the novel cleavage fragment of eIF4G (M1) are indicated, and molecular weight markers are shown on the left. Results are representative of those obtained in five separate experiments. Panel B: Diagrammatic representation of eIF4GI, showing sites of cleavage by caspase-3 (C-3; [25,26]), location of peptide sequences used to generate antiserum used in these studies (B, W, E) and sites of interaction with PABP, eIF4E, eIF4A, eIF3 and Mnk1 (see Section 1 for details). Panel C: Jurkat cells (5×10^6 cells in 5 ml) were incubated in the absence (lanes 1, 4, 7) or presence of 250 ng/ml anti-Fas antiserum (lane 8), 10 μ g/ml MG132 (lanes 2 and 5), or 25 μ M FTY720 (lanes 3 and 6) for 6 h. Equal amounts of protein (7.5 μ g) were resolved by SDS-PAGE and the integrity of eIF4G was visualised by immunoblotting (lanes 1–3), eIF4GI (antiserum W; lanes 4–8), antiserum specific to the C-terminus of eIF4GII [7]. The migration of intact eIF4G, the M-FAG and M1 cleavage fragments of eIF4GI are indicated on the left and those specific to eIF4GII (p140, p28; [27]) on the right.

8 vs. lane 5), they were distinct from those observed with anti-eIF4GI antiserum (lanes 1 and 2). Similar results were obtained with the caspase-8 mutant cells and with both cell lines following their incubation with lactacystin (data not shown). This makes it unlikely that the M1 fragment is derived from the C-terminal region of eIF4GII. This region of eIF4GII was presumably processed into smaller fragments not resolved by this gel system. This supposition is confirmed by the fact that the only possible C-terminal immunoreactive fragments of eIF4GII of the correct molecular weight to correspond to the M1 fragment (48C, 58C; [27]), are not observed following these treatments (lanes 4 and 6). In contrast, the p28 cleavage fragment of eIF4GII [27] is clearly observed with MG132 (lane 5), anti-Fas antiserum (lane 8) and lactacystin (data not shown).

3.2. Immunosuppressive drugs promote the cleavage of eIF4G

Previously, we have shown that although the immunosuppressive macrolide, rapamycin, promotes the destruction of eIF4G in yeast [49], it does not induce the degradation of eIF4GI in B or T cells [20,22]. As FTY720 and CsA have been shown to induce apoptosis and activate stress kinases in numerous cell types [37–41,50], we have investigated whether they can promote the proteolysis of eIF4G in T cells. Incubation of wild-type Jurkat T cells with CsA for 2–4 h results in the cleavage of eIF4GI (Fig. 3A, lanes 1–4) and eIF4GII (data not shown), promoting a loss in cell viability (Fig. 3B). Similarly, FTY720 also promotes the cleavage of eIF4GI (Fig. 2C, lane 3 vs. lane 1; Fig. 3A, lanes 5–8), eIF4GII (Fig. 2C, lane 6 vs. lane 4) and decreased viability in both cell lines (Fig. 3B). In addition, these treatments result in the cleavage of PARP, activation of caspase-like proteases, the activation of p38 and JNK stress kinases, a 1.8-fold increase in eIF2 α phosphorylation and a drastic reduction in the rate of protein synthesis (data not shown). In contrast to MG132 and lactacystin (Fig. 2), proteolysis of eIF4GI in response to FTY720 or CsA does not result in the production of M-FAG or C-FAG. As with MG132, the predominant fragment of eIF4GI generated (M1) is recognised by antiserum W (Fig. 2A) but not with antiserum E (data not shown). As FTY720 and CsA also promote the proteolysis of eIF4GII in both cell lines (Fig. 2C and data not shown), these data suggest that caspase-8 activity is not required for cleavage of eIF4GI or eIF4GII in response to immunosuppressants.

To determine whether the novel 50 kDa (M1) fragment of eIF4GI contains the eIF4E-binding site, eIF4E and associated proteins were recovered from extracts using m^7 GTP-Sepharose. As shown in Fig. 3C, M-FAG retains the ability to associate with eIF4E (lane 3 vs. lane 1). This is in agreement with our previous studies [25]. However, the M1 fragment derived from FTY720-treated cells, which contains the central domain of eIF4G (and thereby recognised by antiserum W) is not recovered in the eluate (lane 4 vs. lane 2). Similar results were obtained with extracts from MG132 and CsA-treated cells (data not shown). Together with the lack of immunoreactivity with antiserum B, these data indicate that the 50 kDa fragment of eIF4GI lacks the eIF4E-binding site.

3.3. Caspase and calpain inhibitors do not protect eIF4GI from cleavage induced by CsA and FTY720

To determine whether the cleavage of eIF4GI following immunosuppressant treatment requires caspase or calpain ac-

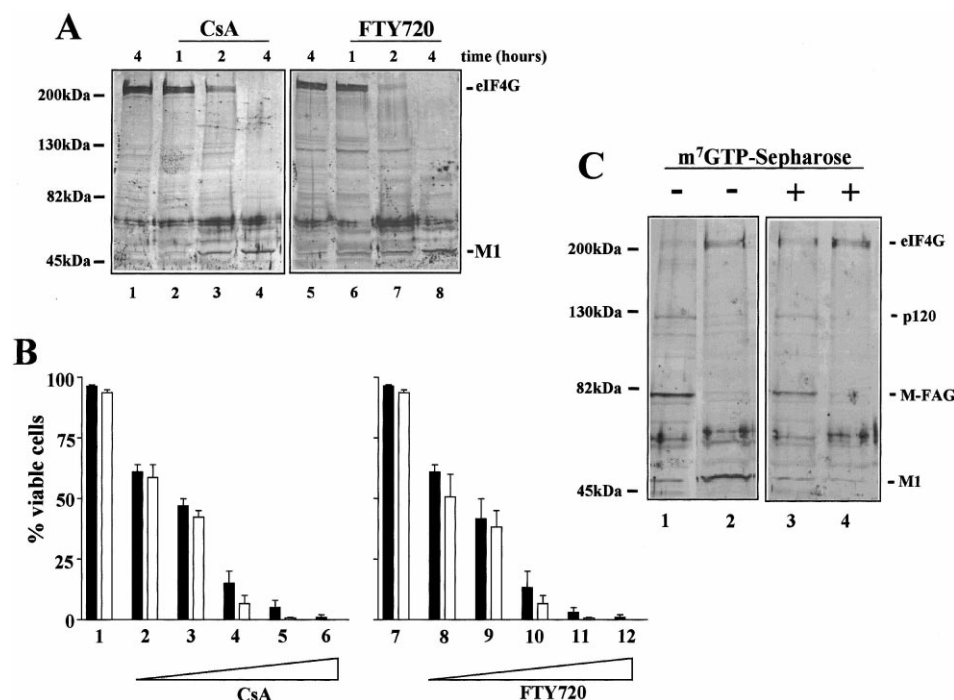


Fig. 3. CsA and FTY720 promote the caspase-8-independent cleavage of eIF4GI and eIF4GII. Panel A: Jurkat cells (5×10^6 cells in 5 ml) were incubated in the presence of CsA (12.5 μ M) or 25 μ M FTY720 for the times indicated. Extracts were prepared and the integrity of eIF4GI was visualised by immunoblotting with antiserum W. Results are from a single experiment, but are representative of those obtained on three separate occasions. Panel B: Wild-type (open symbols) or caspase-8 mutant cells (closed symbols; 5×10^6 cells in 5 ml) were incubated in the absence (lanes 1, 7) or presence of the following concentrations of immunosuppressants for the times indicated; lane 2, 3.12 μ M CsA; lane 3, 6.25 μ M CsA; lane 4, 12.5 μ M CsA; lane 5, 25 μ M CsA; lane 6, 50 μ M CsA; lane 8, 1 μ M FTY720; lane 9, 5 μ M FTY720; lane 10, 10 μ M FTY720; lane 11, 25 μ M FTY720; lane 12, 50 μ M FTY720. Cell viability was estimated by trypan blue exclusion and the presented data are the means and S.D. (bars) of three separate experiments. Panel C: Extracts were prepared from cells incubated with anti-Fas antiserum (lanes 1, 3) or FTY720 (lanes 2, 4) for 4 h were either not fractionated (lanes 1, 2), or eIF4E and associated factors were isolated by m⁷GTP-Sepharose chromatography. Recovered proteins were resolved by SDS-PAGE and eIF4GI, and its cleavage products were visualised by immunoblotting. The migration of eIF4G, p120, M-FAG and the novel cleavage fragment (M1) are indicated on the right with the molecular weight markers on the left. Results are representative of those obtained in three separate experiments.

tivity, wild-type and caspase-8 mutant cells were pre-incubated in the absence or presence of zVAD.fmk or ALLN (MG101; calpain inhibitor II) prior to exposure to anti-Fas antiserum, CsA or FTY720 (Fig. 4). Under these conditions, ALLN alone does not influence the integrity of eIF4GI (data not shown). In wild-type cells, anti-Fas antiserum promotes the appearance of M-FAG with little change in the levels of the M1 fragment (Fig. 4A, upper panel lane 2 vs. lane 1). The cleavage of eIF4GI is insensitive to ALLN (lane 3 vs. lane 2) but sensitive to zVAD.fmk (lane 4 vs. lane 2). Similarly, the cleavage of eIF4GI in wild-type cells in response to either CsA or FTY720 is insensitive to ALLN (lane 6 vs. lane 5 and lane 9 vs. lane 8, respectively). However, under these conditions, the generation of the novel M1 fragment of eIF4GI is insensitive to zVAD.fmk (lane 7 vs. lane 5 and lane 10 vs. lane 8). In agreement with previous data, anti-Fas treatment of the caspase-8 mutant cells does not induce cleavage of eIF4GI (lower panel, lanes 1–4). However, as with the wild-type cells, cleavage of eIF4GI in response to either CsA (lanes 5–7) or FTY720 (lanes 8–10) is refractory to pre-treatment of cells with ALLN or zVAD.fmk. These data suggest that activation of caspase-8 or calpains are not required for the cleavage of eIF4GI in response to immunosuppressants *in vivo*.

Throughout this study, it has been clear that a low level of the M1 cleavage fragment observed with proteasome inhibitors and immunosuppressants was present in untreated cells

(Fig. 2). To gain insight into the relationship of this fragment to M-FAG, cells were incubated with anti-Fas antiserum for longer periods of time. At extended times of exposure of wild-type cells to anti-Fas antiserum, M-FAG is further processed into a fragment which co-migrates with that induced by CsA (Fig. 4B, lane 3 vs. lane 4). These data suggest that the novel fragment of eIF4G observed with proteasome inhibitors and immunosuppressive drugs is also generated at later times during anti-Fas-mediated apoptosis.

4. Discussion

Stimulation of the Fas/CD95 receptor results in apoptosis and the activation of stress kinases [21], the proteolysis of eIF4GI and a severe, but incomplete inhibition of protein synthesis [20–23,25,26]. We now show that the induction of apoptosis following inhibition of the 26S proteasome with MG132 or the 20S proteasome with lactacystin promotes the cleavage of eIF4GI and eIF4GII in a caspase-8-independent manner (Figs. 1 and 2). The sensitivity of this response to zVAD.fmk (Fig. 1C) indicates the involvement of caspase-like activities, which function downstream of caspase-8. An ideal candidate would be caspase-3, which is strongly activated in both cell lines in response to MG132 (Fig. 1B), and which has been shown to be both necessary and sufficient for the cleavage of eIF4G *in vitro* and *in vivo* [23,26,28]. These findings

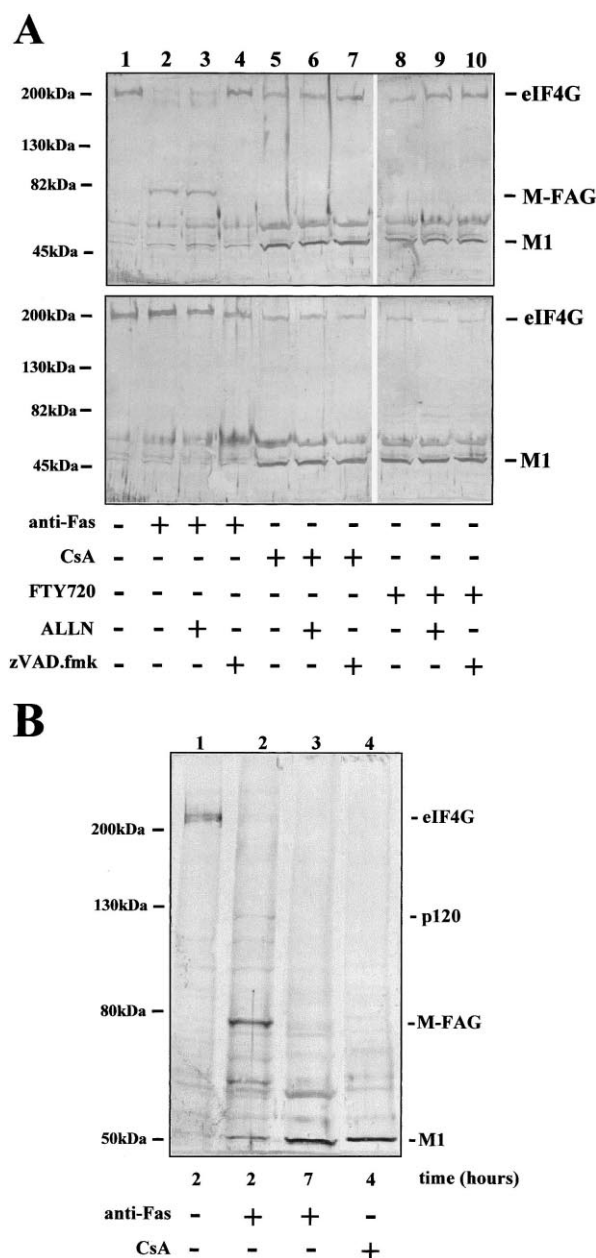


Fig. 4. eIF4GI is processed by a caspase- and calpain-independent process in response to CsA and FTY720. Panel A: Wild-type (upper panel) or caspase-8 mutant (lower panel) Jurkat cells (5×10^6 cells in 5 ml) were pre-incubated in the absence or presence of 50 μ M zVAD.fmk or 10 μ g/ml ALLN, prior to incubation for 2 h in the presence of 250 ng/ml anti-Fas antiserum (lanes 1–4), 1 h with 12.5 μ M CsA (lanes 5–7) or 2 h with 25 μ M FTY720, as indicated. Extracts were prepared, equal amounts of protein (7.5 μ g) were resolved by SDS-PAGE, and the integrity of eIF4G was visualised by immunoblotting using antiserum W specific for the central domain of eIF4GI. The migration of intact eIF4G, M-FAG and the novel cleavage fragment of eIF4G (M1) are indicated on the right and molecular weight markers are shown on the left. Results are representative of those obtained in four experiments. Panel B: Jurkat cells (5×10^6 cells in 5 ml) were incubated in the absence (lane 1) or presence of 250 ng/ml anti-Fas antiserum (lanes 2 and 3) or 12.5 μ M CsA (lane 4) for the times indicated. Extracts were prepared and the integrity of eIF4GI was visualised by immunoblotting as in panel A. Results are representative of those obtained in three separate experiments.

are also consistent with reports that MG132 will activate caspase-3 in HL-60 cells [51] and cortical neurones [35], and activates caspases-2,3,7,8 and 9 in glioma cells [36]. Furthermore, the latter study also showed that caspase-8 cleavage is dispensable for MG132-induced apoptosis [36].

Immunosuppressants such as FTY720 and CsA also activate stress kinases (data not shown) and induce the cleavage of both eIF4GI and eIF4GII (Figs. 2 and 3). Activation of caspase-8 was also not required for this effect, in agreement with the report that FTY720 is able to induce apoptosis in splenocytes isolated from Fas-knockout mice [52]. Consistent with its biological effects, FTY720 induces a drastic decrease in levels of T lymphocytes in the blood [40,42], possibly reflecting activation of caspase activity as a consequence of cytochrome *c* release from mitochondria [41,53]. In contrast to the results with MG132, cleavage of eIF4GI in both wild-type and caspase-8 mutant cells in response to FTY720 and CsA was insensitive to zVAD.fmk and ALLN (Fig. 4). These data suggest that cellular proteases other than caspases and calpains can also play a role in the degradation of eIF4GI. Further work will be needed to identify the proteases responsible for cleavage of eIF4GI in response to these immunosuppressants.

The data presented here indicate that MG132, lactacystin, FTY720 and CsA promote the caspase-8-independent cleavage of eIF4GI to yield a novel 50 kDa immunoreactive fragment (M1) which is not accumulated at early times during Fas-induced apoptosis. However, it is present at low levels in the Jurkat cell population (Fig. 2A), possibly reflecting the presence of apoptotic cells in the cultures [48]. Prolonged exposure of wild-type cells to anti-Fas antiserum promoted the conversion of M-FAG to this novel fragment of eIF4GI (Fig. 4B). Studies with anti-peptide antisera specific to distinct regions of eIF4GI (Fig. 2B), in conjunction with the lack of recovery of the FTY720-induced product on m^7 GTP-Sepharose (Fig. 3C), indicate that it does not contain the extreme N- or C-termini or the eIF4E-binding site. As such, it is presumed that the 50 kDa fragment would not function in recruiting capped mRNA to the ribosome, but may function in recruitment of IRES-containing mRNAs at later times during apoptosis [23]. The lack of detection of the M1 fragment with antiserum specific to the C-terminus of eIF4GII confirmed that it was most likely derived from eIF4GI (Fig. 2C). Furthermore, eIF4GII is proteolysed into five or more fragments at later times during cisplatin-induced apoptosis, destroying the core central domain, which may contribute to the inhibition of protein synthesis observed during apoptosis [27].

We have postulated that the accumulation of the caspase-generated eIF4GI cleavage fragments may directly influence mRNA translation rates *in vivo* (reviewed in [23]). eIF4GI-derived M-FAG 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. Indeed, M-FAG occurs in both ribosome-bound and soluble forms, retains the ability to associate with eIF4E and eIF4A [25,26], and thus is able to recruit all the necessary factors for basal translation and to interact with mRNA [3,10,54]. As such, M-FAG may still support cap-dependent and/or cap-independent translation, albeit at a low rate, during apoptosis [23,55]. It is interesting to note that MG132 has been reported to increase the expression of stress-related proteins such as hsp27 and hsp70 [31], as

well as the over-expression of transcriptional regulators ATF3, GADD153 and MAD1 [56]. Further work will be needed to address the potential role of the fragments of eIF4-GI in controlling the level of gene products involved in proliferation and the cellular stress response.

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