Caspase-mediated cleavage of eukaryotic translation initiation factor subunit 2α

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Eukaryotic translation initiation factor 2α (eIF-2α), a target molecule of the interferon-inducible double-stranded-RNA-dependent protein kinase (PKR), was cleaved in apoptotic Saos-2 cells on treatment with poly(I)·poly(C) or tumour necrosis factor α. This cleavage occurred with a time course similar to that of poly(ADP-ribose) polymerase, a well-known caspase substrate. In addition, eIF-2α was cleaved by recombinant active caspase-3 *in vitro*. By site-directed mutagenesis, the cleavage site was mapped to an Ala-Glu-Val-Asp^{300} ↓ Gly^{301} sequence located in the C-terminal portion of eIF-2α. PKR phosphorylates eIF-2α on Ser^{51}, resulting in the suppression of protein synthesis. PKR-mediated translational suppression was repressed when the C-terminally cleaved product of eIF-2α was overexpressed in Saos-2 cells, even though PKR can phosphorylate this cleaved product. These results suggest that caspase-3 or related protease(s) can modulate the efficiency of protein synthesis by cleaving the α subunit of eIF-2, a key component in the initiation of translation.

Key words: double-stranded-RNA-dependent protein kinase, phosphorylation, translational control.

INTRODUCTION

Eukaryotic translation initiation factor 2 (eIF-2), which is composed of three subunits (α, β and γ), is one of the key molecules in the initiation of translation. During the initiation phase, eIF-2 forms a ternary complex with methionyl-tRNA^{Met} and GTP; this ternary complex then binds the 40 S ribosomal subunit to generate a 43 S preinitiation complex. Before the joining of the 60 S ribosomal subunit, eIF-2-GTP is hydrolysed to eIF-2-GDP, then eIF-2-GDP is released from the ribosomal subunit for another round of initiation. For subsequent rounds of initiation, GDP bound to eIF-2 must be exchanged for GTP, a process catalysed by eIF-2B [1]. In mammalian cells, eIF-2α is phosphorylated at Ser^{51} by two kinases: the haem-controlled repressor ('HCR') [2] and the interferon (IFN)-inducible double-stranded-RNA-dependent protein kinase (PKR) [3]. Phosphorylated eIF-2α blocks the GDP–GTP exchange activity of eIF-2B, resulting in the suppression of protein synthesis [4]. Because eIF-2B exists in relatively small molar quantities with respect to eIF-2, the phosphorylation of small amounts (10–20 %) of eIF-2 can inhibit the exchange activity of eIF-2B [5]. Thus phosphorylation of eIF-2α is an important regulatory process in protein synthesis.

PKR has important roles in the cellular anti-viral defence mechanism, in cell growth control and in differentiation [6–9]. In anti-viral defence, PKR is activated by viral infection and activated PKR phosphorylates eIF-2α, resulting in the suppression of protein synthesis, which is believed to block viral replication in virus-infected cells [10,11]. In addition to these roles, it has been shown that PKR can mediate downstream events in tumour necrosis factor α (TNF-α)-induced apoptosis [12]; the phosphorylation of eIF-2α contributes to this process. These results suggested that the regulation of protein synthesis by PKR through eIF-2α phosphorylation is also important for apoptosis.

Apoptosis is the process of programmed cell death mediated by various stimuli, including DNA-damaging agents and Fas–Fas-ligand interaction [13]. This process is characterized by changes in the cytoplasmic membrane, the condensation of chromatin structure and the destruction of chromatin (laddering). During this process, specific proteases called caspases are activated and the cells undergo rapid death. Numerous studies have shown that the activation of caspases is central to apoptosis [14,15]. Among the caspases, caspase-3 has been studied most extensively and might be one of the downstream apoptotic effector molecules [16–19]. Caspase-3 cleaves various proteins including poly(ADP-ribose) polymerase (PARP), nuclear lamin, DNA-dependent protein kinase and the U1 RNA-associated 70 kDa protein [20–23]. Cleavage can lead to activation or inactivation of the substrate. However, it remains unclear whether these proteins are critical for the manifestations of apoptotic morphology and eventual cell death.

Here we demonstrate that transiently expressed eIF-2α is cleaved by caspase-3 or related proteases in apoptotic Saos-2 cells on treatment with poly(I)·poly(C) or TNF-α. The kinetics of eIF-2α cleavage were similar to those of a caspase substrate, PARP. When the C-terminally cleaved product of eIF-2α was overexpressed in Saos-2 cells, PKR could not suppress protein synthesis even though PKR phosphorylated this cleaved product. These observations suggest that the cleavage of eIF-2α modulates translational efficiency and the apoptotic process.

MATERIALS AND METHODS

Plasmid construction

The PKR cDNA encoding its open reading frame was obtained by reverse transcriptase-mediated PCR. Reverse transcription was performed with Superscript II (Gibco BRL), random primer (Takara) and total RNA isolated from IFN-α-treated PH5CH cells [24] by Isogen (Nippongene). Reverse transcriptase-reacted solution was used as the template for PCR with *Taq* polymerase (Takara) and primers (sense, 5′-CATTCTGCAGCCATGGCT-

Abbreviations used: DEVD-fmk, benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone; eIF-2, eukaryotic translation initiation factor 2; FLAG, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys; IEF, isoelectric focusing; IFN, interferon; PARP, poly(ADP-ribose) polymerase; PKR, double-stranded-RNA-dependent protein kinase; TNF-α, tumour necrosis factor α.

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GGTGATCTTTCAGC-3′; anti-sense, 5′-ATCCCAAGCTTCT-AACATGTGTGTCGTTCAT-3′). To obtain pCMV/PKR, the PCR product was digested with *Pst*I and *Hin*dIII, then inserted into the *Pst*I–*Hin*dIII site of pKS(+)/CMV (in which CMV stands for cytomegalovirus) [25]. pCMV/eIF-2α, which produces eIF-2α protein, was constructed by PCR with the plasmid pSP72-2α encoding the eIF-2α gene (a gift from Dr. J. W. Hershey) and primers (sense, 5′-TTTTCTGCAGCCATGCC-GGGTCTAAGTTGTAG-3′; anti-sense, 5′-GGGAAGCTTTT-AATCTTCAGCTTTGGC-3′). This PCR product was digested with *Pst*I and *Hin*dIII, then inserted into the *Pst*I–*Hin*dIII site of pKS(+)/CMV, yielding pCMV/eIF-2α. To obtain mutant forms of eIF-2α replacing Asp^{300} with Glu, or Ser^{51} with Ala, designated eIF-2α D300E or eIF-2α S51A respectively, two-step PCR was conducted with pCMV/eIF-2α as a template. In the first-step PCR, products designated D300E-N, D300E-C, S51A-N and S51A-C were obtained with the following combinations of sense and anti-sense primers: D300E-N, 5′-TTTTCTGCAGCCATG-CCGGGTCTAAGTTGTAG-3′ and 5′-CATCATCATCTCCT-TCCACTTCGGCATTTTC-3′; D300E-C, 5′-AATGCCGAA-GTGGAAGGAGATGATGATGCAG-3′ and 5′-GGGAAGC-TTTTAATCTTCAGCTTTGGC-3′; S51A-N, 5′-TTTTCTGC-AGCCATGCCGGGTCTAAGTTGTAG-3′ and 5′-CGCCTT-CTGGCTAGCTCACTAAGAAGAATCATG-3′; and S51A-C, 5′-GTGAGCTAGCCAGAAGGCGTATCCGTTC-3′ and 5′-GGGAAGCTTTTAATCTTCAGCTTTGGC-3′. eIF-2α D300E and eIF-2α S51A were obtained by the second-step PCR with the use of combinations of D300E-N and D300E-C, and S51A-N and S51A-C respectively. The products, eIF-2α D300E and eIF-2α S51A, were digested with *Pst*I and *Hin*dIII, then inserted into the *Pst*I–*Hin*dIII site of pKS(+)/CMV to obtain pCMV/eIF-2α D300E and pCMV/eIF-2α S51A respectively. Plasmid pCMV/eIF-2α ΔC, expressing the C-terminal 14-residue-deleted form of eIF-2α, was constructed by inserting the *Pst*I–*Hin*dIII fragment of a PCR product amplified with pCMV/eIF-2α as a template with primers (sense, 5′-TTTTCTGCAGCCATGCCG-GGTCTAAGTTGTAG-3′; anti-sense, 5′-CCCCCCAAGCTTT-TAATCCACTTCGGCATTTTC-3′) into the *Pst*I–*Hin*dIII site of pKS(+)/CMV. Plasmids, pCMV/FLAG-eIF-2α series expressing the series of eIF-2α tagged with the Asp-Tyr-Lys-Asp-Asp-Asp-Lys (FLAG) epitope at their N-termini, were constructed by inserting an annealed oligodeoxynucleotide fragment (sense, 5′-AGAATTCCACCATGGACTACTACAAGGACG-ACGATGACAAGCCTGCA-3′; anti-sense, 5′-GGCTTGTCA-TCGTCGTCCTTGTAGTCCATGGTGGAATTCTTGCA-3′) into the *Pst*I site of the pCMV/eIF-2α series. The pCMV/eIF-2α-FLAG series plasmids, expressing a series of eIF-2α tagged with the FLAG epitope at the C-terminus, were constructed by inserting the *Pst*I–*Hin*dIII fragment of PCR products amplified with each of the pCMV/eIF-2α series as templates with primers (sense, 5′-TTTTCTGCAGCCATGCCGGGTCTAAGTTGTA-G-3′; anti-sense, 5′-GGGAAGCTTATCTTCAGCTTTGGC-3′) into the *Pst*I–*Hin*dIII site of pCMV/FLAG [26]. To express the eIF-2α-FLAG series *in vitro*, we constructed the pTZ/eIF-2α-FLAG series by inserting the *Pst*I–*Cla*I fragment of each member of the pCMV/eIF-2α-FLAG series into the *Pst*I–*Cla*I site of pTZ18U [27]. pCMV/Luc has been described previously [28].

Cell culture

Saos-2 cells were grown in Dulbecco's modified Eagle's medium supplemented with glutamine and 10% (v/v) fetal bovine serum. For treatment with double-stranded RNA, transfected or non-transfected Saos-2 cells were primed by treatment with 500 i.u./ml human IFN-α (Mochida) for 15 h; 100 μg/ml poly(I)·poly(C) (Pharmacia) was then added, followed by incubation for the durations shown in the figures. Where indicated, cycloheximide was added at 50 μg/ml. The caspase inhibitor benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone (DEVD-fmk; MBL) (20 μM) was added to cells 1 h before treatment with double-stranded RNA. TNF-α (Sigma) (10 ng/ml) was added with 50 μg/ml cycloheximide for 8 h.

Transfection and protein expression

Saos-2 cells were transfected with pCMV-derived plasmids by using FUGENE 6 Transfection Reagent (Boehringer). Expression of protein in cells was examined by SDS/PAGE [10% (w/v) gel] followed by transfer to PVDF membranes. eIF-2α, FLAG-eIF-2α series and PARP were detected with anti-(eIF-2α) antibody (Santa Cruz), anti-FLAG antibody (Sigma) and anti-PARP antibody (Biomol) respectively. The signals were revealed with an enhanced chemiluminescence kit (NEN). For reporter gene assay, cells were harvested 24 h after transfection; cell lysates were prepared and assayed for luciferase in accordance with the manufacturer's protocol (Promega). Luciferase activity was normalized to the amount of total protein measured with a bicinchoninic acid protein assay kit (Pierce) to correct for transfection efficiency. Transfection experiments were repeated at least three times; average values are shown.

Apoptosis assay

The apoptosis-induced DNA ladder was prepared with an ApopLadder Ex™ kit (Takara) (only fragmented DNA could be recovered) as directed by the manufacturer. Isolated DNA was fractionated by electrophoresis in a 2% (w/v) agarose gel and detected by staining with ethidium bromide.

Cleavage *in vitro* by recombinant active caspase-3

[^{35}S]Methionine-labelled proteins were prepared by using expression plasmids, pTZ/eIF-2α-FLAG or pTZ/eIF-2α D300E-FLAG, and a TNT T7 quick-coupled transcription/translation system (Promega) in accordance with the manufacturer's instructions; they were then subjected to cleavage by recombinant active caspase-3 (MBL) in the amounts indicated in the figures in reactions containing 20 mM Pipes (pH 7.5), 100 mM NaCl, 0.1% (v/v) CHAPS, 10 mM dithiothreitol, 10% (w/v) sucrose and 1 mM EDTA at 37 °C for 90 min. The reactions were stopped with the addition of sample buffer [29], then analysed by SDS/PAGE [10% (w/v) gel]. The gel was dried and exposed to an imaging plate (Fuji Photo Film Co.).

Isoelectric focusing (IEF)–PAGE

Cells were collected with 100 μl of cold PBS, then sonicated and stored at −80 °C before use. The following reagents were added to aliquots of cell lysate: 8.5 M urea, 5% CHAPS, 5% 2-mercaptoethanol, 2% (v/v) Pharmalyte 4–6.5 (Pharmacia) and 50 mM NaF. The mixture was incubated at 37 °C for 30 min and then at 80 °C for 2 min. IEF was performed with Immobiline DryStrips (pH 4–7, 18 cm; Pharmacia) for 45501 V·h. Before use, the strips were rehydrated for 6 h in hydration buffer containing 8 M urea, 2% (v/v) CHAPS, 10 mM dithiothreitol, 0.2% (v/v) Pharmalyte 4–6.5, and 1% Orange G. After IEF, the gels were equilibrated with equilibration buffer [26] and then analysed by SDS/PAGE [10% (w/v) gel].

RESULTS

Cleavage of eIF-2α during apoptosis in Saos-2 cells

To establish the conditions for the induction of apoptosis by double-stranded RNA, we primed Saos-2 cells with IFN-α followed by treatment with poly(I)·poly(C) for 12 h, then analysed DNA ladder formation in these cells. However, under these conditions we detected no DNA ladder formation characteristic of apoptosis (results not shown). When cycloheximide was added to a final concentration of 50 μg/ml with poly(I)·poly(C), DNA ladder formation was observed even though cycloheximide alone did not cause laddering (results not shown). This is consistent with some apoptosis-inducing systems in which the inhibition of *de novo* protein synthesis enhances the progress of apoptosis [30–32]. To examine whether eIF-2α is targeted by caspases during apoptosis, we monitored the endogenous eIF-2α by immunoblotting. However, we could not detect eIF-2α with a commercially available anti-(eIF-2α) antibody; we therefore monitored the ectopically expressed eIF-2α. As shown in Figure 1(A), the antibody against eIF-2α detected the full-length eIF-2α in the control Saos-2 cells (indicated as 0 h), whereas an additional form of eIF-2α, migrating with slightly greater mobility than the full-length eIF-2α, was observed after the induction of apoptosis by the addition of poly(I)·poly(C) with cycloheximide. Conversion of eIF-2α to the faster-migrating form during apoptosis occurred with a time course similar to the cleavage of PARP, a known substrate of caspase-3. When FLAG-eIF-2α, eIF-2α with the FLAG-epitope tag fused to its N-terminus, was transiently produced in Saos-2 cells, the additional form of FLAG-eIF-2α, which is approx. 2 kDa smaller than the full-length molecule, was detected by anti-FLAG antibody during apoptosis; the full-length FLAG-eIF-2α disappeared completely 8 h after induction (Figure 1B). The detection of the faster-migrating form derived from FLAG-eIF-2α by anti-FLAG antibody suggested that cleavage of FLAG-eIF-2α and authentic eIF-2α occurred near the C-terminus of each molecule. To determine whether eIF-2α is cleaved by other inducers of apoptosis, we treated ectopically FLAG-eIF-2α-expressing Saos-2 cells with TNF-α plus cycloheximide. As shown in Figure 1(C), this treatment also resulted in the appearance of the cleaved form of FLAG-eIF-2α. These results indicate that full-length eIF-2α was converted to the shorter form of eIF-2α during apoptosis, probably by proteolytic cleavage.

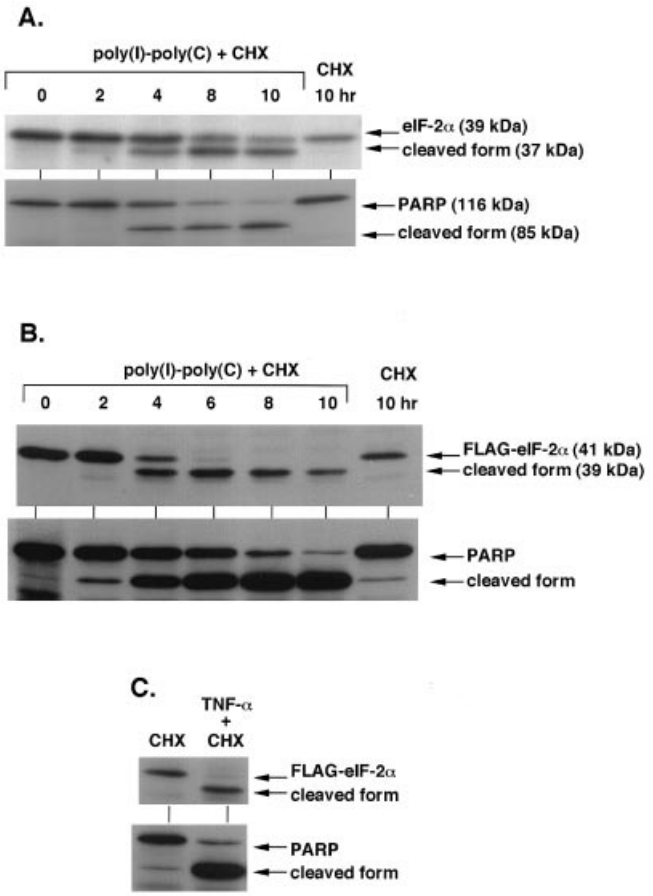

Figure 1 Cleavage of eIF-2α in apoptosis-induced Saos-2 cells

(**A**) Saos-2 cells transfected with pCMV/eIF-2α were treated with 100 μg/ml poly(I)·poly(C) and cycloheximide (CHX) in combination for the indicated times or with only 50 μg/ml CHX for 10 h; cell extracts were analysed for eIF-2α or PARP by Western blotting with anti-(eIF-2α) or anti-PARP antibody respectively. (**B**) Saos-2 cells transfected with pCMV/FLAG-eIF-2α were treated as in (**A**); cell extracts were analysed for FLAG-eIF-2α or PARP by Western blotting with anti-FLAG or anti-PARP antibody respectively. (**C**) Saos-2 cells transfected with pCMV/FLAG-eIF-2α were treated with 10 ng/ml TNF-α and 50 μg/ml CHX in combination or only 50 μg/ml CHX for 8 h; cell extracts were analysed as in (**B**).

Mapping of the cleavage site in eIF-2α

To examine whether caspase-3 contributes to the cleavage of eIF-2α, Saos-2 cells transiently producing FLAG-eIF-2α were pre-incubated with the tetrapeptide inhibitor DEVD-fmk for caspase-3/Cpp32, then treated with poly(I)·poly(C) plus cycloheximide for an additional 8 h. As shown in Figure 2(A), DEVD-fmk inhibited the appearance of the apoptotic fragment of FLAG-eIF-2α, suggesting that eIF-2α is cleaved by caspase-3 or a related protease during apoptosis. Because these observations suggested that caspase-mediated cleavage occurred close to the C-terminal portion of eIF-2α, we surveyed the amino acid sequence of this portion of eIF-2α for an appropriate caspase cleavage motif. We found an Asp residue at position 300 as a potential cleavage site for caspases (Figure 2B). To verify that this position was indeed the cleavage site in eIF-2α, we constructed mutant forms of FLAG-eIF-2α replacing Asp^{300} with Glu or truncating the C-terminal 14 residues (residues 301–314) of eIF-2α, designating them FLAG-eIF-2α D300E and FLAG-eIF-2α ΔC respectively. When Saos-2 cells transiently producing each FLAG-eIF-2α mutant were treated with poly(I)·poly(C) plus cycloheximide, the cleaved product, as seen in Saos-2 cells producing wild-type FLAG-eIF-2α, was not detected with anti-FLAG antibody (Figure 2C, lanes 2 and 4). FLAG-eIF-2α ΔC migrated with the same mobility as the apoptosis-induced cleaved product of FLAG-eIF-2α. These results indicated that the Asp^{300} in eIF-2α is the cleavage site or is an important amino acid residue targeted by proteolysis during apoptosis.

Cleavage of eIF-2α by recombinant caspase-3 *in vitro*

To confirm that eIF-2α can be cleaved by a caspase, we examined whether eIF-2α produced by using the TNT system *in vitro* with [^{35}S]methionine was cleaved by recombinant active caspase-3. When wild-type or mutant eIF-2α was produced with this TNT system, a slower-migrating band with the same mobility as eIF-2α and a faster-migrating band that was not further characterized were detected by autoradiography (results not shown). Because the faster-migrating band produced with the TNT system showed the same mobility as the apoptosis-induced cleaved product of eIF-2α, making it difficult to characterize the product by mobility-

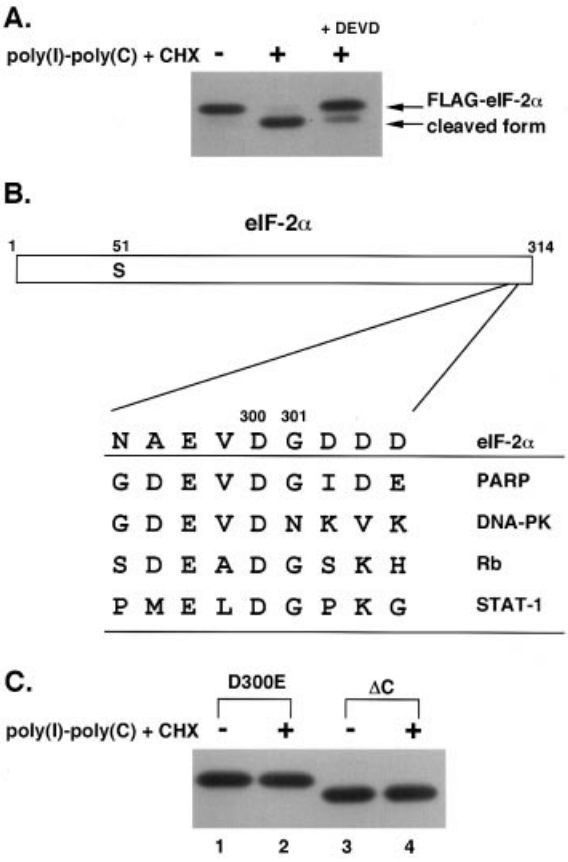

Figure 2 Mapping the cleavage site in eIF-2α

(**A**) Saos-2 cells transfected with pCMV/FLAG-eIF-2α were pretreated with 20 μM DEVD-fmk (DEVD), then treated with 100 μg/ml poly(I)·poly(C) and 50 μg/ml cycloheximide (CHX) in combination for 8 h; cell extracts were analysed for FLAG-eIF-2α by Western blotting with anti-FLAG antibody. (**B**) Alignment of the amino acid sequence in residues 296–304 of eIF-2α with the caspase-cleavage site in proteins including PARP, DNA-dependent protein kinase (DNA-PK), Rb (retinoblastoma gene product) and STAT-1 (signal transduction and activators of transcription-1). (**C**) Saos-2 cells transfected with pCMV/FLAG-eIF-2α D300E (D300E) or pCMV/FLAG-eIF-2α ΔC (ΔC) were treated with 100 μg/ml poly(I)·poly(C) and 50 μg/ml CHX in combination for 8 h; cell extracts were analysed for these FLAG-eIF-2α mutants by Western blotting with anti-FLAG antibody.

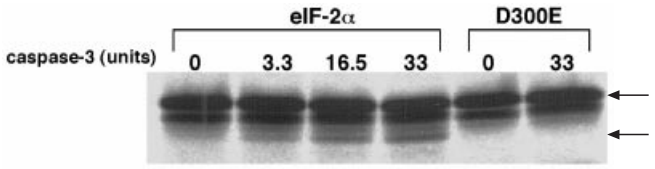

Figure 3 Cleavage of eIF-2α *in vitro* by recombinant caspase-3

[^{35}S]Methionine-labelled C-terminally FLAG-tagged eIF-2α (eIF-2α) or C-terminally FLAG-tagged eIF-2α D300E (D300E) was incubated with the indicated quantities of recombinant caspase-3. The upper and lower arrows at the right indicate the positions of the intact and cleaved forms of eIF-2α respectively.

shift assay, we generated eIF-2α-FLAG, in which the FLAG epitope tag was fused at the C-terminus of eIF-2α, to distinguish the cleaved product of eIF-2α by size from the uncharacterized band. As shown in Figure 3, wild-type eIF-2α-FLAG was cleaved by recombinant caspase-3 in a dose-dependent manner to give a

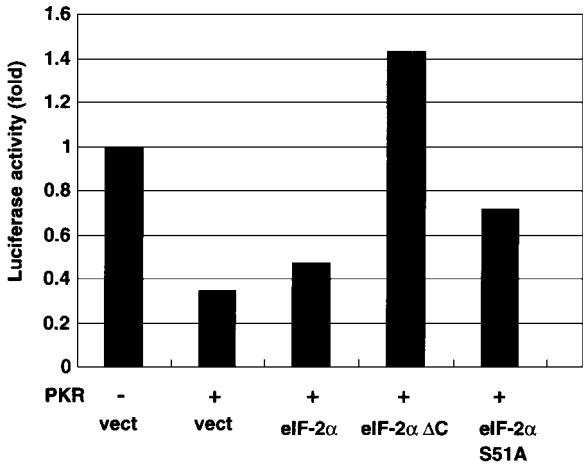

Figure 4 Repression by eIF-2α ΔC of the PKR-mediated suppression of protein synthesis *in vivo*

Saos-2 cells were transiently transfected with reporter construct pCMV/Luc and expression plasmids for FLAG-eIF-2α or FLAG-eIF-2α mutants with or without PKR expression plasmid. Lysates were prepared and assayed for luciferase activity in accordance with the manufacturer's protocol. Luciferase activity was normalized to the amount of total protein to correct for the transfection efficiency.

product approx. 4 kDa smaller than eIF-2α-FLAG. In contrast with wild-type eIF-2α-FLAG, recombinant caspase-3 could not cleave the eIF-2α-FLAG mutant in which Asp^{300} was changed to Glu (Figure 3, rightmost two lanes). These results confirmed that eIF-2α was cleaved by caspase-3. However, we could not exclude the possibility that a caspase-3-like protease was responsible for this cleavage.

C-terminally truncated eIF-2α repressed the PKR-mediated suppression of protein synthesis in mammalian cells

To characterize the caspase-mediated cleaved product of eIF-2α, designated eIF-2α ΔC, we examined the effect of this product on the PKR-mediated suppression of protein synthesis. As expected, when the luciferase reporter plasmid pCMV/Luc was co-transfected with pCMV/PKR, which produces wild-type PKR, the translation of luciferase was decreased to approx. 35% of that without PKR. In the presence of FLAG-eIF-2α S51A, which could not be phosphorylated by PKR, the PKR-mediated suppression of luciferase translation was moderately repressed (Figure 4), as reported previously [33,34]. These observations confirmed that the phosphorylation of Ser^{51} of eIF-2α by PKR contributed to the suppression of protein synthesis. In the presence of FLAG-eIF-2α ΔC, the PKR-mediated suppression of protein synthesis was completely abolished. Moreover, luciferase activity was higher than that in the control (exogenous PKR-negative and vector; Figure 4), suggesting more efficient translation in cells expressing eIF-2α ΔC. We observed that the expression of different amounts of FLAG-eIF-2α (half or double that shown in Figure 4) did not affect the PKR activity. Moreover, FLAG-eIF-2α ΔC, but not FLAG-eIF-2α, suppressed the PKR activity when the same amount of each protein was expressed (results not shown). This suggested that eIF-2α ΔC suppresses PKR activity efficiently, or that eIF-2α ΔC contributes to protein synthesis more efficiently than wild-type eIF-2α. A similar result was also obtained when the same assay was conducted with NIH 3T3 cells (results not shown), suggesting that this characteristic feature of eIF-2α ΔC is not limited to certain cell lines.

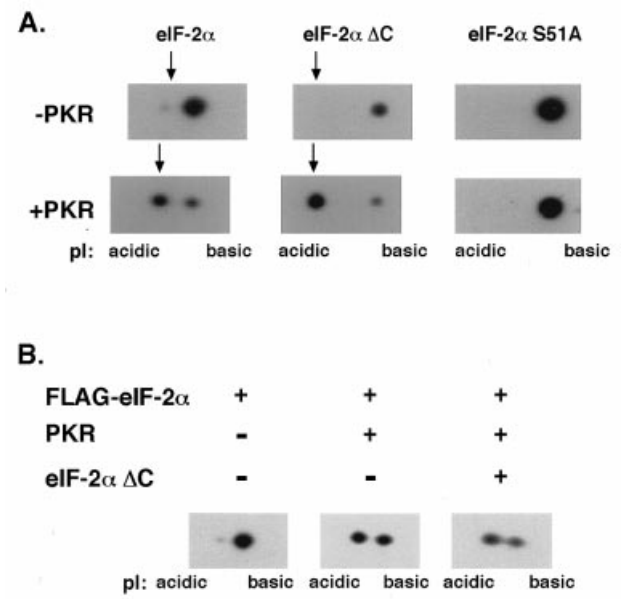

Figure 5 eIF-2α ΔC was phosphorylated by PKR

(**A**) eIF-2α ΔC and wild-type eIF-2α were phosphorylated by PKR. Saos-2 cells were transiently transfected with various FLAG-eIF-2α mutant expression plasmids with or without PKR expression plasmid; 24 h after transfection, cell extracts were prepared and separated by IEF–PAGE as described in the Materials and methods section. The presence of FLAG-eIF-2α mutants was detected by Western blotting with anti-FLAG antibody. The arrow indicates the position of the phosphorylated (acidic) form of each FLAG-eIF-2α mutant. (**B**) Simultaneous production of eIF-2α ΔC did not interfere with the PKR-mediated phosphorylation of wild-type eIF-2α. Saos-2 cells were transfected as indicated above the panels; 24 h after transfection, cell extracts were prepared and separated by IEF–PAGE as described in the Materials and methods section. The presence of wild-type FLAG-eIF-2α was detected by Western blotting with anti-FLAG antibody.

Phosphorylation of FLAG-eIF-2α ΔC by PKR, as in wild-type FLAG-eIF-2α

To examine whether Ser^{51} in eIF-2α ΔC was phosphorylated by PKR, we analysed the phosphorylation state of this molecule *in vivo*. IEF–PAGE was conducted to resolve eIF-2α isoforms differing in phosphorylation on Ser^{51}. When FLAG-eIF-2α was produced together with PKR in Saos-2 cells, the phosphorylated form was detected mainly by anti-FLAG antibody, whereas the unphosphorylated form was detected mainly in the absence of PKR (Figure 5A, left panels). FLAG-eIF-2α ΔC was also phosphorylated efficiently by PKR (Figure 5A, middle panels). When FLAG-eIF-2α ΔC S51A, in which Ser^{51} of FLAG-eIF-2α ΔC had been replaced by Ala, was produced with PKR, only one form corresponding to the unphosphorylated form of FLAG-eIF-2α ΔC was detected (results not shown). This indicated that Ser^{51} of eIF-2α ΔC, as well as wild-type eIF-2α, was phosphorylated by PKR. FLAG-eIF-2α ΔC had a higher pI than wild-type FLAG-eIF-2α because the former had lost a very acidic region in the C-terminal 14 residues of eIF-2α. Consistent with the result of a previous study [35] was the observation that only an unphosphorylated form of FLAG-eIF-2α S51A was detected in the presence or absence of PKR (Figure 5A, right panels). Next we examined whether eIF-2α ΔC interfered with the PKR-mediated phosphorylation of wild-type eIF-2α *in vivo*. As shown in Figure 5(B), the PKR-mediated phosphorylation level of FLAG-eIF-2α was not affected even in the presence of eIF-2α ΔC. These results indicate that eIF-2α ΔC overcame the PKR-mediated suppression of protein synthesis by decreasing the inhibitory effect of eIF-2α phosphorylation on translation initiation, rather than decreasing the proportion of phosphorylated eIF-2α.

eIF-2α ΔC did not enhance apoptosis induced by poly(I)·poly(C)

To determine whether eIF-2α cleavage can alter the apoptotic response in Saos-2 cells, we examined the effects of transiently produced FLAG-eIF-2α mutants on the sensitivity of apoptosis induced by poly(I)·poly(C). We transiently transfected Saos-2 cells with various pCMV/FLAG-eIF-2α mutants along with pMACS Kk and then selected transfected cells by using the MACSelect Kk system (Miltenyi Biotec Corp.). Isolated cells were pretreated with IFN-α for 15 h after the addition of poly(I)·poly(C); 24 h later, cell viability was analysed with an MTT assay kit (Promega). None of the wild-type, mutant or truncated FLAG-eIF-2α forms had any significant effect on cell death (results not shown).

DISCUSSION

Several proteins are cleaved by caspases. However, it remains unclear whether these proteins are critical for eventual cell death or contribute to some extent to it. Here we have demonstrated that eIF-2α, a subunit of eIF-2, was proteolytically cleaved at its C-terminal portion to generate a fragment, which we designated eIF-2α ΔC, during the execution phase of apoptosis induced by double-stranded RNA or TNF-α. On the basis of the results of mutagenesis studies *in vitro* and *in vivo*, the possible cleavage site in eIF-2α was mapped between residues 300 and 301 with the amino acid sequence of Ala-Glu-Val-$Asp^{300}\downarrow Gly^{301}$ present near the C-terminus; this cleavage was mediated by caspase-3 *in vitro*. Because the cleavage site of eIF-2α was XaaXaaXaaAsp↓, which is slightly different from the caspase-3 consensus sequence, we cannot exclude the possibility that other caspases or apoptosis-induced proteases cleave this site.

The phosphorylation of eIF-2α by PKR sequesters eIF-2B in an inactive complex, resulting in a decrease in the level of the eIF-2-GTP complex and the inhibition of protein synthesis [4]. In the present study we demonstrated that eIF-2α ΔC repressed the PKR-mediated suppression of protein synthesis even though it was phosphorylated by PKR, suggesting that eIF-2α ΔC does not inhibit PKR activity but decreases the inhibitory effect of phosphorylated eIF-2 on translation initiation. It has been reported previously that yeast eIF-2α mutants with one point mutation in each molecule overcome the toxicity of constitutively activated forms of GCN2, a functional homologue of mammalian PKR, even though these mutants were phosphorylated by GCN2 [36]. These results suggest that not only the phosphorylation but also the mutation or truncation of eIF-2α affects eIF-2B activity. It has been reported that the phosphorylation of eIF-2α alters its conformation to form a stable inactive complex with eIF-2B, resulting in a decrease in the level of active eIF-2B [37]. This raises the possibility that eIF-2α ΔC might overcome the inhibitory effect of eIF-2α phosphorylation on translation initiation; removal of the C-terminal portion of eIF-2α can no longer alter the conformation of eIF-2 to form an inactive complex with eIF-2B even if this cleaved product is phosphorylated. Reconstitution studies *in vitro* are required to clarify this possibility.

Little is known about the role of translational regulation in cell death. In many apoptosis-inducing systems such as double-

stranded-RNA- and TNF-α-stimulated apoptosis, protein synthesis *de novo* is not required for the induction of apoptosis; moreover, the inhibition of protein synthesis efficiently induces apoptosis. It was demonstrated recently [38] that during cisplatin- and etoposide-induced apoptosis in HeLa cells, eIF-4G, which functions in translational initiation as a molecular scaffold by binding simultaneously with eIF-4E, eIF-4A and eIF-3 to enable mRNA to bind ribosomes, was cleaved by caspase-3, resulting in the generation of an inactive form of eIF-4G. The extent of this cleavage was correlated with the onset and extent of observed inhibition of cellular translation, suggesting that the inactivation of eIF-4G by caspase-3-mediated cleavage contributes to the inhibition of protein synthesis causing apoptosis [38]. In contrast, our results suggested that cleavage of eIF-2α during apoptosis does not contribute to the inhibition of protein synthesis. Although we did not observe any significant effect of transiently produced eIF-2α ΔC on poly(I)·poly(C)-induced apoptosis, we cannot exclude the possibility that the cleavage of eIF-2α contributes to progression of apoptosis by an as yet unknown mechanism.

It was demonstrated recently that human tumour cell lines often express high levels of a caspase-cleaved product of MDM2 in non-apoptotic cells; this cleaved MDM2 might function in the regulation of p53 [39]. It is possible that caspases are activated in non-apoptotic cells in response to certain signals. Because eIF-2α ΔC has a suppressive effect on the inhibition of protein synthesis by phosphorylating Ser^{51}, it might function as a dominant-negative regulator by competing with full-length eIF-2α for interaction with eIF-2B. It is therefore possible that caspases activated under certain conditions regulate protein synthesis by cleaving eIF-2α. Further studies are required to clarify the role of the cleavage of eIF-2α in apoptotic and non-apoptotic cells.

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REFERENCES

1 Proud, C. G. (1986) Trends Biochem. Sci. **11**, 73–77
2 Chen, J. J., Throop, M. S., Gehrke, L., Kuo, I., Pal, J. K., Brodsky, M. and London, I. M. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 7729–7733
3 Samuel, C. E. (1993) J. Biol. Chem. **268**, 7603–7606
4 Pain, V. M. (1996) Eur. J. Biochem. **236**, 747–771
5 Pain, V. M. (1986) Biochem. J. **235**, 625–637
6 Petryshyn, R., Chen, J. J. and London, I. M. (1984) J. Biol. Chem. **259**, 14736–14742
7 Petryshyn, R., Chen, J. J. and London, I. M. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 1427–1431
8 Chong, K. L., Feng, L., Schappert, K., Meurs, E., Donahue, T. F., Friesen, J. D., Hovanessian, A. G. and Williams, B. R. (1992) EMBO J. **11**, 1553–1562
9 Lee, S. B., Melkova, Z., Yan, W., Williams, B. R., Hovanessian, A. G. and Esteban, M. (1993) Virology **192**, 380–385
10 Meurs, E. F., Watanabe, Y., Kadereit, S., Barber, G. N., Katze, M. G., Chong, K., Williams, B. R. and Hovanessian, A. G. (1994) J. Virol. **66**, 5805–5814
11 Lee, S. B. and Esteban, M. (1994) Virology **199**, 491–496
12 Srivastava, S. P., Kumar, K. U. and Kaufman, R. J. (1998) J. Biol. Chem. **273**, 2416–2423
13 Thompson, C. B. (1995) Science **267**, 1456–1462
14 Hale, A. J., Smith, C. A., Sutherland, L. C., Stoneman, V. E., Longthorne, V. L., Culhane, A. C. and Williams, G. T. (1996) Eur. J. Biochem. **236**, 1–26
15 Alnemri, E. S., Livingston, D. J., Nicholson, D. W., Salvesen, G., Thornberry, N. A., Wong, W. W. and Yuan, J. (1996) Cell **87**, 171
16 Faleiro, L., Kobayashi, R., Fearnhead, H. and Lazebnik, Y. (1997) EMBO J. **16**, 2271–2281
17 MacFarlane, M., Cain, K., Sun, X.-M., Alnemri, E. S. and Cohen, G. M. (1997) J. Cell Biol. **137**, 469–479
18 Martins, L. M., Kottke, T., Mesner, P. W., Basi, G. S., Sinha, S., Frigon, Jr., N., Tater, E., Tung, J. S., Bryant, K., Takahashi, A. et al. (1997) J. Biol. Chem. **272**, 7421–7430
19 Takahashi, A., Hirata, H., Yonehara, S., Imai, Y., Lee, K.-K., Moyer, R. W., Turner, P. C., Mesner, P. W., Okazaki, T., Sawai, H. et al. (1997) Oncogene **14**, 2741–2752
20 Lazebnik, Y. A., Kaufmann, S. H., Desnoyers, S., Poirier, G. G. and Earshaw, W. C. (1994) Nature (London) **371**, 346–347
21 Lazebnik, Y. A., Takahashi, A., Moir, R. D., Goldman, R. D., Poirier, G. G., Kaufmann, S. H. and Earshaw, W. C. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 9042–9046
22 Casciola-Rosen, L. A., Miller, D. K., Anhalt, G. J. and Rosen, A. (1994) J. Biol. Chem. **269**, 30757–30760
23 Song, Q., Lees-Miller, S. P., Kumar, S., Zhang, Z., Chan, D. W., Smith, G. C., Jackson, S. P., Alnemri, E. S., Litwack, G., Khanna, K. K. and Lavin, M. F. (1996) EMBO J. **15**, 3238–3246
24 Kato, N., Ikeda, M., Mizutani, T., Sugiyama, K., Noguchi, M., Hirohashi, S. and Shimotohno, K. (1996) Jpn. J. Cancer Res. **87**, 787–792
25 Hijikata, M., Mizushima, H., Akagi, T., Mori, S., Kakiuchi, N., Kato, N., Kimura, K. and Shimotohno, K. (1993) J. Virol. **67**, 4665–4675
26 Hirota, M., Satoh, S., Asabe, S., Kohara, M., Tsukiyama-Kohara, K., Kato, N., Hijikata, M. and Shimotohno, K. (1999) Virology **257**, 130–137
27 Hijikata, M., Mizushima, H., Tanji, Y., Komoda, Y., Hirowatari, Y., Akagi, T., Kato, N., Tanaka, T., Kimura, K. and Shimotohno, K. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 10773–10777
28 Akagi, T., Ono, H., Nyunoya, H. and Shimotohno, K. (1997) Oncogene **14**, 2071–2078
29 Laemmli, U.K. (1970) Nature (London) **227**, 680–685
30 Piedrafita, F. J. and Pfahl, M. (1997) Mol. Cell. Biol. **17**, 6348–6358
31 Ray, R. B., Meyer, K., Steele, R., Shrivastava, A., Aggarwal, B. B. and Ray, R. (1998) J. Biol. Chem. **273**, 2256–2259
32 King, P. and Goodbourn, S. (1998) J. Biol. Chem. **273**, 8699–8704
33 Kaufmann, R. J., Davies, M. V., Pathak, V. K. and Hershey, J. W. D. (1989) Mol. Cell. Biol. **9**, 946–958
34 Choi, S. Y., Scherer, B. J., Schnier, J., Davies, M. V., Kaufmann, R. J. and Hershey, J. W. D. (1992) J. Biol. Chem. **267**, 286–293
35 Pathak, V. K., Schindler, D. and Hershey, J. W. B. (1988) Mol. Cell. Biol. **8**, 993–995
36 Carlos, R., Aldana, V., Dever, T. E. and Hinnebusch, A. G. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 7215–7219
37 Kimball, S. C., Heinzinger, N. K., Horetsky, R. L. and Jefferson, L. S. (1998) J. Biol. Chem. **273**, 3039–3044
38 Marissen, W. E. and Lloyd, R. E. (1998) Mol. Cell. Biol. **18**, 7565–7574
39 Pochampally, R., Fodera, B., Chen, L., Shao, W., Levine, E. A. and Chen, J. (1998) Oncogene **17**, 2629–2636

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