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ORIGINAL PAPER

Translation termination factor eRF3 is targeted for caspase-mediated proteolytic cleavage and degradation during DNA damage-induced apoptosis

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Abstract Polypeptide chain release factor eRF3 plays pivotal roles in translation termination and post-termination events including ribosome recycling and mRNA decay. It is not clear, however, if eRF3 is targeted for the regulation of gene expression. Here we show that DNA-damaging agents (UV and etoposide) induce the immediate cleavage and degradation of eRF3 in a caspase-dependent manner. The effect is selective since the binding partners of eRF3, eRF1 and PABP, and an unrelated control, GAPDH, were not affected. Point mutations of aspartate residues within overlapping DXXD motifs near the amino terminus of eRF3 prevented the appearance of the UV-induced cleavage product, identifying D32 as the major cleavage site. The cleavage and degradation occurred in a similar time-dependent manner to those of eIF4G, a previously established caspase-3 target involved in the inhibition of translation during apoptosis. siRNA-mediated knockdown of eRF3 led to inhibition of cellular protein synthesis, supporting the idea that the decrease in the amount of eRF3 caused by the caspase-mediated degradation contributes to the inhibition of translation during apoptosis. This is the first report showing that eRF3 could serve as a target in the regulation of gene expression.

Keywords Apoptosis · Caspase · DNA damage · eRF3 · Translation termination

Abbreviations

eIF	Eukaryotic translation initiation factor
eRF	Eukaryotic translation releasing factor
PABP	Polyadenylate-binding protein
siRNA	Small interfering RNA
PMSF	Phenylmethylsulfonyl fluoride
N-terminal	Amino-terminal
C-terminal	Carboxyl-terminal

Introduction

In eukaryotes, mRNA translation consists of four steps; initiation, elongation, termination and ribosome recycling. Previous studies have focused on the regulation of translation mainly at the initiation step by reversible and irreversible modifications [1]. One important example of a reversible modification is the phosphorylation of initiation factors (eIF2a, eIF4E, eIF4G eIF4B, and eIF3) and associated proteins including 4EBP1. In addition, eIF4G, eIF3j, eIF4B, eIF2a and 4EBP are also known to be irreversibly cleaved by some proteases during apoptosis and viral infections [2–9]. However, it has not been elucidated if a translation termination factor is targeted for the regulation of translation.

In eukaryotes, the 5′ cap and 3′ poly(A) tail of mRNA physically and functionally communicate to synergistically enhance translation initiation [10–13]. The cap structure is recognized by the eukaryotic initiation factor eIF4E whereas the poly(A) tail is bound by the poly(A)-binding protein PABP. Both eIF4E and PABP bind to a scaffold protein eIF4G and bring about a circular organization of

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the mRNA. The formation of the closed loop structure is thought to contribute to the efficient recycling of the terminating ribosome to the next round of translation initiation. We have shown that the translation termination factor eRF3 binds with PABP [14] to form a ternary complex eRF3-PABP-eIF4G and is involved in the cap- and poly(A)-dependent synergic stimulation of translation [13, 15]. A previous study demonstrated that PABP as in the case of eIF4G is specifically degraded to contribute to translation inhibition during apoptosis [16]. Thus we reasoned that the termination factor might also be targeted for the regulation of translation.

In mammals we have identified two eRF3 genes GSPT1 and GSPT2 [17, 18], whose products were named later as eRF3a and eRF3b [19]. We have previously reported that an N-terminally processed form of eRF3a is present in cells, which harbors a conserved inhibitor of apoptosis (IAP)-binding motif (AKPF) in its N-terminus [20]. The processed form of eRF3a interacts with IAPs to promote caspase activation, IAP ubiquitination and apoptosis. These results suggest that processing of eRF3a into the IAP-binding isoform could promote apoptosis by liberating caspases from IAP inhibition. Although processing of eRF3a is suggested to be triggered by some cellular stress signals, such signals have not been reported.

Here, we demonstrate that eRF3 is targeted for DNA-damaging stress during apoptosis. eRF3 is selectively cleaved and degraded in a manner dependent on a group II caspase, with similar time-dependency to the cleavage of eIF4G, a previously established target of caspase-3 involved in the inhibition of translation during apoptosis. The caspase-mediated downregulation but not the cleavage of eRF3 likely contributes to the inhibition of translation during apoptosis, since the cleaved form of eRF3 still retained the ability to support translational activity. This is the first report showing that eRF3 could serve as a target in the regulation of gene expression.

Materials and methods

Cell culture and DNA/siRNA transfection

THP-1, Jurkat and U937 cells were cultured in RPMI-1640 medium (Nissui) supplemented with 5 % fetal bovine serum (Gibco) and maintained at 37 °C in 5 % CO_2. HeLa, NIH3T3 and MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (Nissui) supplemented with 5 % fetal bovine serum. THP-1 cells were transfected with plasmid using the Neon™ Transfection System (Invitrogen). HeLa cells were transfected with siRNA and plasmid using LipofectAMINE RNAiMAX and LipofectAMINE 2000 (Invitrogen), respectively.

siRNA

siRNAs against eRF3 (hGSPT1, hGSPT2) and luciferase consisted of 5′ r (GUC AAC CAU UGG AGG ACA A) d(TT) 3′, 5′ r (GAA CAC GUA AAU GUA GUA U) d(TT) 3′ and 5′ r (CGU ACG CGG AAU ACU UCG A) d(TT) 3′, respectively.

Plasmid

To construct pFLAG-CMV5-hGSPT1-His_6, the full-length ORF of hGSPT1 was PCR-amplified using primer pairs YH1S/YH2AS and pGH5 [18] as a template, and the cDNA fragment was digested with EcoRI and SalI, and inserted into pGPH6 [15]. The resulting plasmid pGPH6-hGSPT1 was then digested with EcoRI and NotI, and the 2.0 kb-fragment containing hGSPT1-His_6 cDNA was blunt-ended at the NotI site and inserted into the EcoRI and SmaI sites of pFLAG-CMV5 [21]. To construct pCMV-MycFLAG, oligonucleotides NH160/NH161 were inserted into the XhoI site of pCMV-Myc [21]. To construct pCMV-MycFLAG-Ub-hGSPT1, a human ubiquitin cDNA fragment fused with hGSPT1 cDNA (corresponding to aa 1-6 of eRF3a) at the 3′-end was amplified using the primer pair NH168/YH6 and as a template human ubiqutin cDNA obtained from HeLa cells, and hGSPT1 cDNA (corresponding to aa 1–637 of eRF3a) fused with ubiquitin cDNA (corresponding to aa 73–76 of ubiquitin) at the 5′-end was amplified using the primer pair YH5/CMV3 and pFLAG-CMV5-hGSPT1-His_6 as a template. These fragments were fused using NH168/CMV3 by overlapping PCR. The resulting hGSPT1 cDNA fragment (corresponding to aa 1–637 of eRF3a) fused with human ubiqitin cDNA at the 5′-end was digested with SalI, and inserted into the EcoRV and XhoI sites of pCMV-MycFLAG. To construct pCMV-MycFLAG-Ub-hGSPT1-33Q, -36 M, -54A and -73A, hGSPT1 cDNAs corresponding to aa 33–637, 36–637, 54–637 and 73–637 of eRF3a were amplified by inverse PCR using the primer pairs YH7/YH10, YH8/YH10, YH9/YH10 and YH3S/YH10, respectively, and pCMV-MycFLAG-Ub-hGSPT1 as a template. To construct pCMV-MycFLAG-Ub-hGSPT1 (D25A, D29A, D32A, D35A and D25A/D29A/D32A/D35A), the corresponding region of hGSPT1 cDNA was amplified by inverse PCR using YH14/YH18, YH15/YH17, YH11/YH8, YH12/YH8, and YH16/YH18, respectively, and pCMV-MycFLAG-Ub-hGSPT1 as a template. To construct pCMV-MycFLAG-Ub-hGSPT1-si-resist and -33Q-si-resist, the corresponding region of hGSPT1 cDNA was amplified by inverse PCR using YH19/YH20, and pCMV-MycFLAG-Ub-hGSPT1 and pCMV-MycFLAG-Ub-hGSPT1-33Q as a template, respectively. To construct pCMV-FLAG2-hluc2, the firefly luciferase gene was amplified using NH363/373 and pGL4.16 (Promega) and inserted into the HindIII and XhoI

sites of pCMV-FLAG [22]. All primers used are listed in Table 1.

Cell treatment

For UV treatment, THP-1 cells growing exponentially in culture medium were resuspended in 1 ml of PBS at a density of 10^7 cells/ml, exposed to UV-C (254 nm) with a UV cross-linker (ATTO) and then resuspended in fresh cell culture medium at a density of 10^6 cells/ml. For etoposide treatment, THP-1 cells growing exponentially in culture medium were resuspended in fresh culture medium at 10^6 cells/ml, and etoposide was then added. AEBSF (Sigma), E-64 (Sigma), z-VAD-fmk (R&D Systems) and MG132 (Calbiochem) were added to the medium immediately after UV irradiation. Tumicamycin, thapsigargin and hydrogen peroxide were purchased from Wako.

Table 1 Primers used in this study

PCR oligos	Sequence 5′-3′
NH160	TCG AGG ATT ACA AGG ATG ACG ACG ATA AGT AG
NH161	TCG ACT ACT TAT CGT CGT CAT CCT TGT AAT CC
NH168	ATC ATG CAG ATT TTC GTG AAA AC
NH363	CAA TCC AAG CTT GTT GGT AAA GCC ACC ATG
NH373	TGC CTC GAG TTA CAC GGC GAT CTT GCC GCC
CMV3	CAC TGG AGT GGC AAC TTC CA
YH1S	ATC GAA TTC GAT CCG GGC AGT GGC
YH2AS	GTT GTC GAC GTC TTT CTC TGG AAC CAG
YH3S	GCC AAG CCC TTC GTG CCC
YH5	CTT CGT GGT GGT ATG GAT CCG GGC AGT GGC
YH6	GCC CGG ATC CAT ACC ACC ACG AAG TCT CAA
YH7	CAG GCG GAC ATG GAA GCC
YH8	ATG GAA GCC CCC GGG CCG
YH9	GCC GAG GCC CAG CGG GAG AAC
YH10	ACC ACC ACG AAG TCT CAA CAC AAG
YH11	GTC CGC CTG AGC CCA GCA GTC AGG CGC CGA GTC GCT
YH12	AGC CGC CTG GTC CCA GCA GTC AGG
YH14	GAC TGC TGG GAC CAG GCG GAC ATG GAA
YH15	GCT TGC TGG GAC CAG GCG GAC ATG GAA
YH16	GCT TGC TGG GCT CAG GCG GCT ATG GAA
YH17	AGG CGC CGA GTC GCT GCT GCT GCT GCC GCT GCT
YH18	AGG CGC CGA AGC GCT GCT GCT GCT GCC GCT GCT
YH19	TGC TGG CAA AAG CAC AAT AGG TGG TCA GAT AAT GTA TTT GAC TGG AAT G
YH20	TCT ACG TGC CCA ATG AAG ACA ACG TTC ACA TGC TCT TTC TTA GGA GCA C

Western blot analysis

Total cellular protein was prepared using buffer A (50 mM Tris–HCl (pH 6.8), 8 % glycerol, 2 % SDS, 2 % 2-mercaptoethanol) or RIPA buffer (20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 % NP-40, 0.1 % SDS, 1 % sodium deoxycholate, 2.5 mM EDTA, 1 mM dithiothreitol, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin and 2 μg/ml pepstatin A) and analyzed by western blotting using the following antibodies; anti-eRF3 (raised against His-tagged eRF3a(22-205)), anti-PABP (raised against His-tagged PABPC1), anti-eRF3a(179-190) (17), anti-eRF1 (17), anti-GAPDH (Millipore), anti-FLAG (Sigma), anti-His_6 (Qiagen), anti-Caspase-3 (Cell Signaling), anti-eIF4G (Cell Signaling), anti-eIF2a (Santa Cruz Biotechnology) and anti-eIF2a(phospho-Ser52) (Stressgen).

Measurement of translational activity

HeLa cells were labeled with 22 μCi/ml of the [^{35}S]-methionine/cysteine mixture in Dulbecco's modified Eagle's medium supplemented with 5 % fetal bovine serum for 3 h. After [^{35}S] pulse labeling, cells were washed with PBS and lysed in RIPA buffer (20 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1 % NP-40, 0.1 % SDS, 1 % sodium deoxycholate and 1 mM PMSF) and then analyzed by 12 % SDS-PAGE and autoradiography. Protein concentrations of the extracts were determined by DC protein assay (BIO RAD).

Results

DNA damage induces caspase-dependent cleavage and degradation of eRF3

We initially investigated the status of eRF3 in cells treated with various inducers of apoptosis. Among the conditions tested, UV-C irradiation appeared most prominently to induce some modifications of eRF3. So, we mainly tested the effect of UV-C irradiation on the eRF3 modifications. SDS-PAGE-western blot analysis revealed that UV-C irradiation of THP-1 cells at 100 J/m^2 induced a downward shift in the mobility of eRF3 with a concomitant decrease in the amount of the full-length protein (Fig. 1a). In this situation, caspase-3 resulted in cleavage and degradation with a similar time-dependency. As eRF3 was suggested to be posttranslationally modified during apoptosis, we further examined the effect of another DNA-damaging agent, etoposide. Treatment of THP-1 cells with 10 μM etoposide induced similar modifications of eRF3 within 4 h (Fig. 1b). As in the case of UV irradiation, the kinetics was analogous to that for the modifications of caspase-3. The sensitivity to apoptosis-inducing agents depends on cell lines, but similar

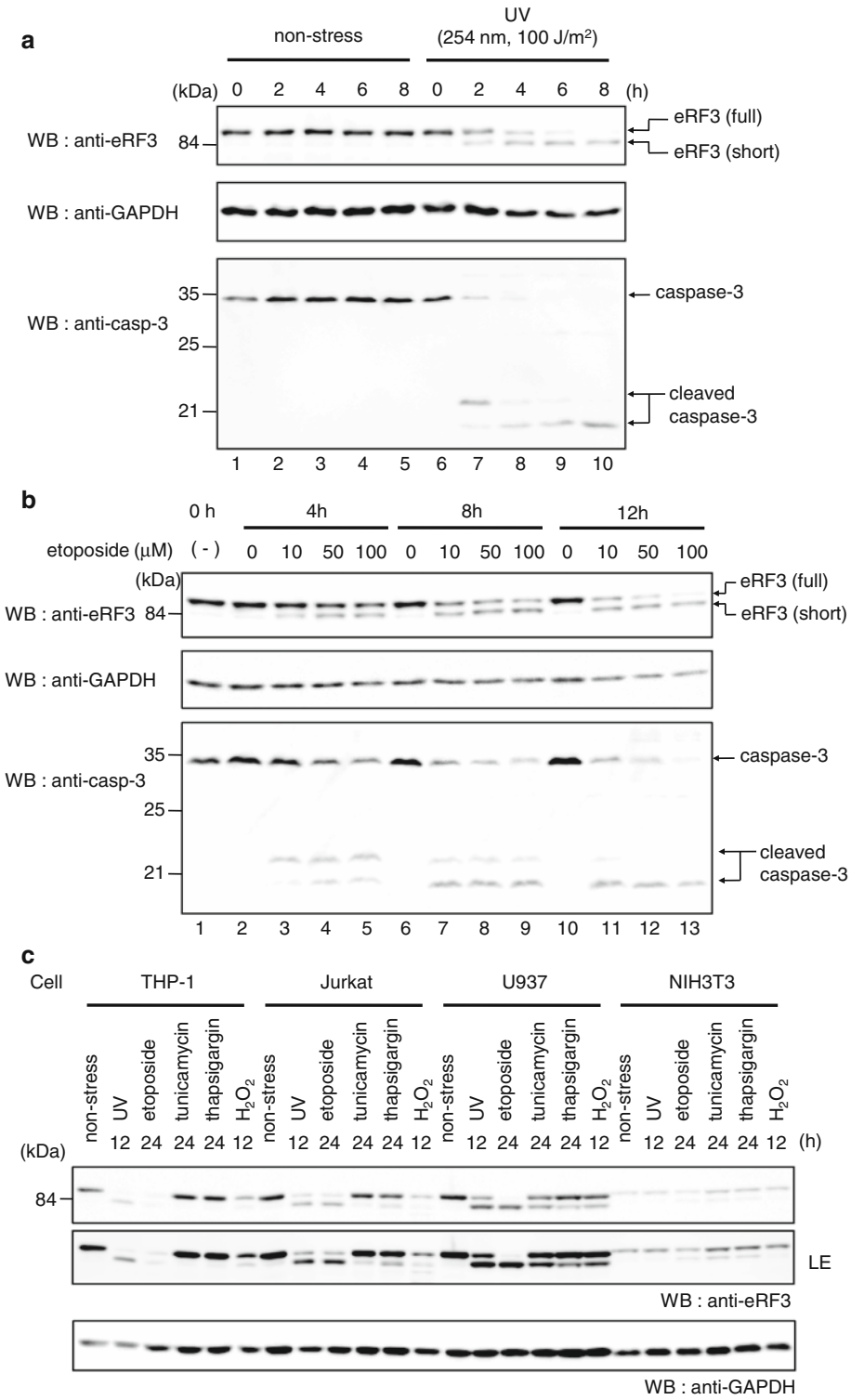

Fig. 1 Posttranslational modifications of eRF3 during DNA damage-induced apoptosis. **a** THP-1 cells were irradiated with UV-C (254 nm, 100 J/m^2) and then cultured for the indicated periods (*lanes* 6–10). As a control, cells cultured without UV-irradiation were prepared (*lanes* 1–5). Cells were lysed with RIPA buffer, and proteins were analyzed by western blotting using anti-eRF3 (*upper*), anti-GAPDH (*middle*) and anti-caspase-3 (*lower*). **b** THP-1 cells were treated with various concentrations of etoposide (0 μM (*lanes* 2, 6, 10), 10 μM (lanes 3, 7, 11), 50 μM (*lanes* 4, 8, 12) and 100 μM (*lanes* 5, 9, 13)) for 4 h (*lanes* 2–5), 8 h (*lanes* 6–9) and 12 h (*lanes* 10–13). Cells without incubation were prepared as a control (*lane* 1). Western blotting was performed as described in Fig. 1a. **c** THP-1, Jurkat, U937 and NIH3T3 cells were treated with various stress inducers and cultured for the indicated periods (UV-C (254 nm, 100 J/m^2, 12 h), etoposide (100 μM, 24 h), tunicamycin (5 μg/ml, 24 h), thapsigargin (5 μM, 24 h), hydrogen peroxide (H_2O_2) (3 mM, 12 h)). Cells without incubation were prepared as a control (non-stress). Proteins were analyzed by western blotting using anti-eRF3 and anti-GAPDH. *LE* indicates long exposure

modifications are observed with U937, Jurkat and NIH3T3 cells (Fig. 1c and Supplementary Fig. 1). In Jurkat and U937 cells, which are sensitive to DNA-damaging agents, eRF3 was also modified, though to a lesser extent than in THP-1 cells. Treatment with other types of apoptosis-inducing agents including tumicamycin, thapsigargin and hydrogen peroxide, also induced similar modifications. The ER-stressors tunicamycin and thapsigargin induced the modifications to a greater extent in Jurkat, U937 and NIH3T3 cells than in THP-1 cells. Thus, eRF3 modifications appear to occur generally in apoptotic cells. Since the eRF3 modifications are remarkable in DNA-damaging

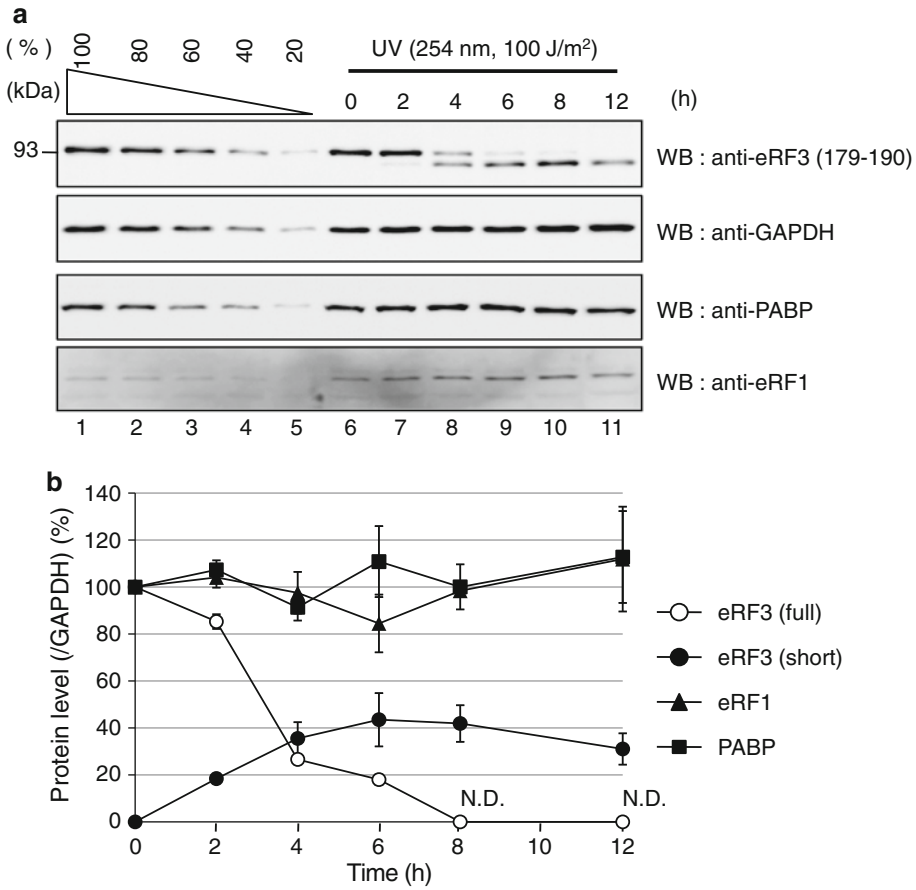

Fig. 2 Quantitative analysis of eRF3 protein. **a** After UV-C (254 nm, 100 J/m^2)-irradiation, THP-1 cells were collected at the indicated time points (lanes 6-11). The amount of protein was detected by western blotting using anti-eRF3 (GSPT1(179-190)), anti-GAPDH, anti-eRF1 and anti-PABP. The leftmost five lanes indicate the intensity of 100 % (*lane* 1), 80 % (*lane* 2), 60 % (*lane* 3), 40 % (*lane* 4) and 20 % (*lane* 5) protein levels. **b** The protein level of eRF3(*full*) (*open circle*) and eRF3(*short*) (*closed circle*) was measured by MultiGauge and normalized to that of GAPDH, where the normalized level of eRF3(*full*) at time 0 was defined as 100 %. The protein level of eRF1 (*closed triangle*) and PABP (*closed square*) was measured and normalized to GAPDH, where the normalized level of PABP and eRF1 at time 0 was defined as 100 %, respectively. *Error bars* represent the SD for three independent experiments. *N.D.* Not determined

agent-treated THP-1 cells, here we analyzed the effect of DNA-damaging agents in THP-1 cells.

To determine whether the modifications are specific to eRF3, we next examined the status of proteins related to eRF3; eRF1 and PABP, by quantifying their amounts. The amount of full-length eRF3 was decreased immediately after UV irradiation and reached ~20 % of the total eRF3 level within 6 h (Figs. 2a, b). In accordance with this, the modified form of eRF3 appeared within 2 h after UV irradiation and reached a maximum at 6 h. However, there was no detectable change in the amount or mobility of eRF1 and PABP, or of an unrelated control, GAPDH. These results indicate that the modifications are specific to eRF3.

We surmised the observed modifications of eRF3 to be proteolysis. Thus we examined the effect of a variety of protease inhibitors including the serine protease inhibitor AEBSF, cysteine protease inhibitor E-64, caspase inhibitor z-VAD-fmk and proteasome inhibitor MG132 on the modifications of eRF3. After UV irradiation, THP-1 cells were incubated in the presence or absence of the inhibitors and the cell lysates were analyzed by western blotting. The UV-induced modifications were completely inhibited by z-VAD-fmk (Fig. 3a). In contrast, no apparent effect was observed with AEBSF, E-64 and MG132. These results strongly suggest that the UV-induced modifications of eRF3 are proteolysis and that the proteolytic cleavage and degradation of eRF3 are a caspase-dependent event during apoptosis [23]. Since MCF-7 cells are deficient in caspase-3, we utilized MCF-7 cells to determine whether caspase-3 is involved in the cleavage and degradation of eRF3. In UV- or etoposide-treated MCF-7 cells, we could hardly detect any cleavage and degradation of eRF3 (Fig. 3b). These results strongly suggest that caspase-3 is the protease responsible for the cleavage and degradation of eRF3.

To determine if eRF3 is cleaved at the N terminus and/or the C terminus, we engineered a construct encoding an N-terminal FLAG- and C-terminal His_6-tagged eRF3a (Fig. 4b). The engineered eRF3a protein was transiently expressed in THP-1 cells by electroporation and the status of the eRF3a after UV irradiation of the cells was examined by western blotting using anti-FLAG and anti-His_6 antibodies. Anti-His_6 antibody but not anti-FLAG antibody detected the cleaved eRF3a (Fig. 4a). These results are consistent with the idea that UV-induced cleavage of eRF3a occurs at the N-terminus.

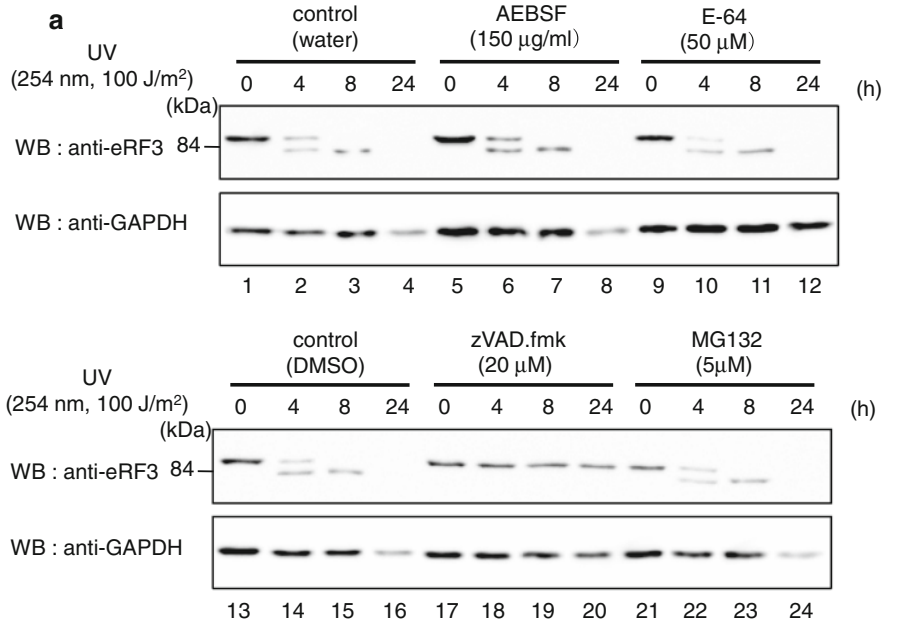

Fig. 3 Both cleavage and degradation of eRF3 are blocked by the general caspase inhibitor z-VAD-fmk. **a** THP-1 cells were treated with the indicated protease inhibitors (AEBSF (150 μg/ml) (*lanes* 5–8), E-64 (50 μM) (*lanes* 9–12), z-VAD-fmk (20 μM) (*lanes* 17–20) and MG132 (5 μM) (*lanes* 21–24)) after UV-C (254 nm, 100 J/m^2) irradiation. In the control experiments, cells were incubated in the culture medium containing vehicle alone (*lanes* 1–4 and 13–16). After UV-irradiation, cells were harvested at 4 h (*lanes* 2, 6, 10, 14, 18, 22), 8 h (*lanes* 3, 7, 11, 15, 19, 23) and 24 h (*lanes* 4, 8, 12, 16, 20, 24) and the effects of each protease inhibitor on the cleavage and degradation of eRF3 were analyzed by western blotting. **b** MCF-7 cells were treated with etoposide (50 μM) (*lane* 5–7) or irradiated with UV-C (254 nm, 200 J/m^2) (*lane* 8–10) and then cultured for the indicated periods (4, 8, 24 h). As a control, cells cultured without stress treatment were prepared (*lanes* 1–4). Proteins were analyzed by western blotting using anti-eRF3 and anti-GAPDH

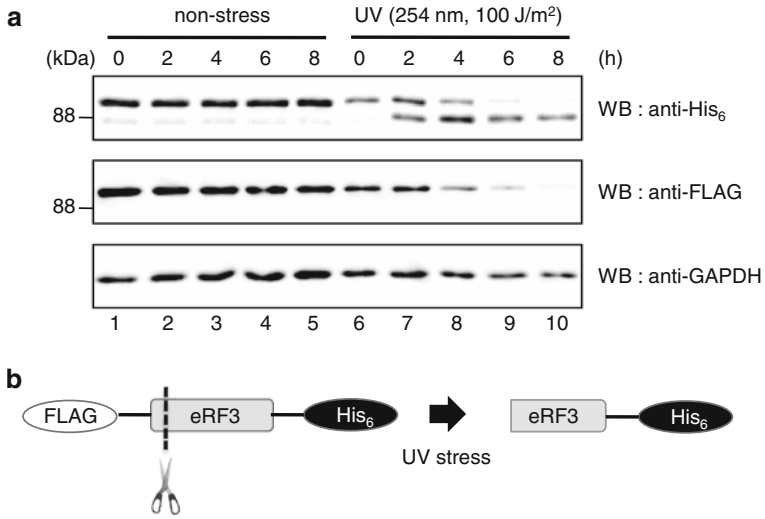

Fig. 4 eRF3 is cleaved in its N-terminal region. **a** THP-1 cells were electroporated with pFLAG-CMV5-hGSPT1-His$_6$ expressing FLAG-eRF3a- His$_6$. At 36 h after electroporation, THP-1 cells were irradiated with UV-C (254 nm, 100 J/m^2) and then cultured for the indicated periods (*lanes* 6–10). Cells cultured without UV-irradiation were used as a control (*lanes* 1–5). Proteins were analyzed by western blotting using anti-His$_6$ (*upper*), anti-FLAG (*middle*) and anti-GAPDH (*lower*). **b** A schematic model of eRF3 cleavage

UV induces cleavage at amino terminal aspartate residues of eRF3a

To identify the cleavage sites in eRF3a during DNA damage-induced apoptosis, we performed N-terminal Edman sequencing of the cleavage product of eRF3a (eRF3a-cp). However, no sequence was detected probably due to N-terminal blockage by some artificial modifications (e.g. pyroglutamylation) occurring during electrophoresis or electroblotting. So, we decided to compare the cleavage

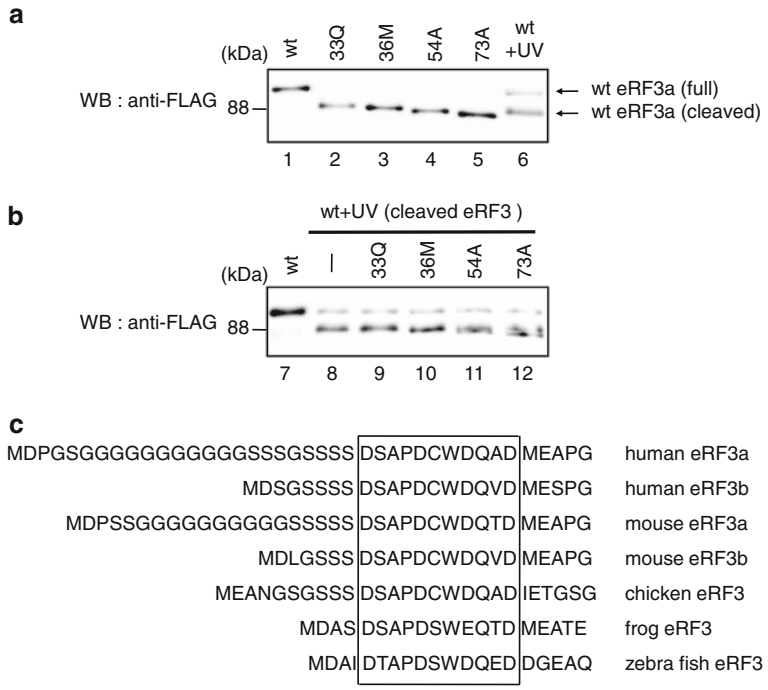

Fig. 5 eRF3a-cp is different from the IAP-binding isoform of eRF3a. **a** THP-1 cells were electroporated with pCMV-MycFLAG-Ub-hGSPT1 expressing Ub-eRF3a-FLAG. Cell lysates were prepared 4 h after UV-C irradiation (254 nm, 100 J/m^2) (*lane* 6). eRF3a standard samples wt, 33Q, 36 M, 54A and 73A (*lanes* 1–5) were prepared from HEK 293T cells transfected with pCMV-MycFLAG-Ub-hGSPT1, -33Q, -36M, -54A and 73A expressing Ub-(wt)/(33Q)/(36M)/(54A)/(73A)-hGSPT1-FLAG proteins. The cleaved-eRF3a isoforms were analyzed by western blotting with anti-FLAG antibody. **b** Cleaved eRF3 sample (wt + UV) was mixed with each of the eRF3a standard samples; 33Q (*lane* 9), 36M (*lane* 10), 54A (*lane* 11) and 73A (*lane* 12), and analyzed by western blotting using anti-FLAG. **c** Alignment of amino acid sequences around cleavage sites of eRF3. Amino acid sequences of human eRF3a, human eRF3b, mouse eRF3a, mouse eRF3b, chicken eRF3, frog eRF3 and zebra fish eRF3 are displayed. The UV/etoposide-induced cleavage site residues are boxed

product with a variety of amino-terminally deleted eRF3a fragments to estimate the cleavage site. We first constructed plasmids expressing ubiquitin (Ub)-tagged eRF3a-fusion proteins, in which ubiquitin is fused with eRF3a deleted of 32, 35, 53 and 72 amino-terminal amino acids (33Q, 36M, 54A and 73A in Fig. 5a). The fusion proteins were also engineered to contain a FLAG tag at their carboxyl terminus. As described previously, when expressed in cells, the Ub moiety is cleaved by multiple ATP-dependent proteases, and eRF3a fragments without Ub can be produced [20]. Ectopic expression of the Ub-eRF3a fusion proteins in HEK293T cells resulted in the generation of the amino-terminally deleted eRF3a proteins with the expected mobility on SDS-PAGE (Fig. 5a, lanes 2–5). Thus, we next prepared eRF3a-cp by UV irradiation of THP-1 cells expressing carboxyl-terminally FLAG-tagged eRF3a and compared its mobility to that of the eRF3a deletion mutants. The mobility of eRF3a-cp was clearly lower than that of eRF3a (73A): the IAP-binding isoform of eRF3a (Fig. 5a, lane 6). When eRF3a-cp and eRF3a fragments were mixed and separated by SDS-PAGE, the bands of 54A and 73A but not those of 33Q and 36M exhibited as doublets (Fig. 5b). These results clearly indicate that eRF3a is cleaved around 33Q and 36M, and eRF3a-cp is different from the IAP-binding isoform of eRF3a.

To more precisely define the caspase-dependent cleavage sites in eRF3a, we searched for aspartate residues, which could be cleaved by caspases. Although aspartate residues are present throughout the eRF3a sequence, we have found two overlapping DXXD motifs preferentially cleaved by group II caspases [24] only within the region around 33Q and 36M, which we identified as potential cleavage sites (Figs. 5b and 6a). The overlapping DXXD motifs are conserved in vertebrates (Fig. 5c). Therefore, we substituted one of the aspartate residues (D25, D29, D32 or D35) within and around the overlapping DXXD motifs in eRF3a with alanine by site-directed mutagenesis (Fig. 6a), and examined if the eRF3a mutant is susceptible to UV-induced cleavage in THP-1 cells. The D32A substitution led to a partial reduction in the UV-induced cleavage of eRF3a (Fig. 6b–d). However, since the cleavage was still observed in this mutant, we further substituted D25, D29 and D35 with alanine and examined its susceptibility to UV-induced cleavage. The eRF3a mutant (D25A/D29A/D32A/D35A) showed no cleavage products (Fig. 6d,

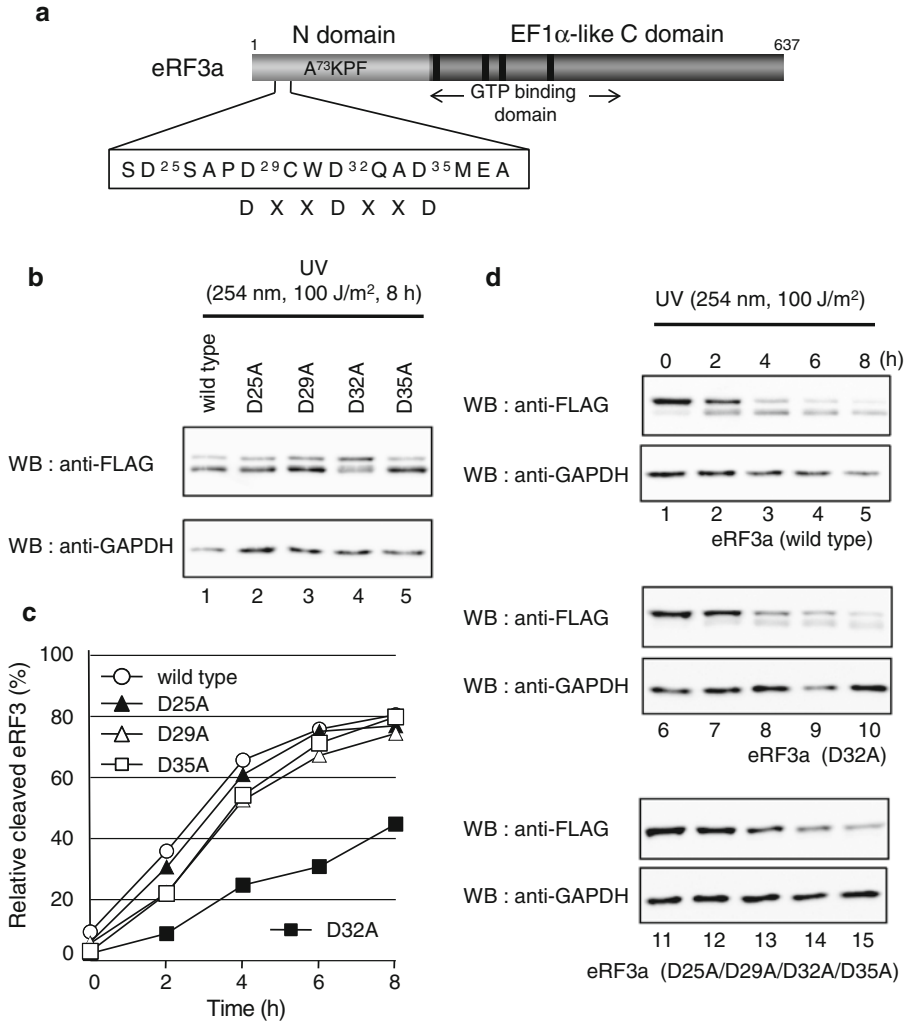

Fig. 6 Identification of the caspase-mediated cleavage sites in eRF3a. **a** Schematic representation of the N-terminal region of eRF3a. **b** An eRF3a/GSPT1 gene wild type or single (D25A, D29A, D32A, D35A) mutant was introduced into THP-1 cells by electroporation. At 36 h after transfection, THP-1 cells were irradiated with UV (254 nm, 100 J/m^2). The status of the eRF3 wild type and mutants at 8 h after UV irradiation was evaluated by western blotting using anti-FLAG (*upper*) and anti-GAPDH (*lower*). **c** The relative cleavage levels of eRF3a(wild type) (*open circle*), eRF3a(D25A) (*closed triangle*), eRF3a(D29A) (*open triangle*), eRF3a(D32A) (*closed square*) and eRF3a(D35A) (*open square*) after UV irradiation were measured by MultiGauge and calculated as the proportion of cleaved eRF3a to total (full + cleaved) eRF3a. **d** Wild-type, single (D32A) mutated or multiple (D25A/D29A/D32A/D35A) mutated eRF3a was introduced into THP-1 cells by electroporation. At 36 h after transfection, THP-1 cells were irradiated with UV (254 nm, 100 J/m^2) and then cultured for the indicated periods. The eRF3a proteins (wild type (*lanes* 1–5), D32A (*lanes* 6–10) and D25A/D29A/D32A/D35A (*lanes* 11–15)) were analyzed by western blotting using anti-FLAG (*upper*), and endogenous GAPDH was analyzed using anti-GAPDH as an internal control (*lower*)

compare lanes 1–5 with lanes 11–15). From these results we conclude that UV-induced cleavage occurs at aspartate residues in the N-terminal region of eRF3a, and D32 is the major cleavage site.

The data shown in Fig. 2b demonstrate that the cleavage occurs prior to the degradation of eRF3a. Two hours after UV irradiation, ~20 % of eRF3a was converted to the cleaved form with no apparent reduction in the total amount of eRF3a. While within the next 2 h, full-length eRF3a decreased rapidly to ~25 % and the amount of cleaved eRF3a reached ~35 %. These results led us to speculate that cleavage may be necessary for the degradation of eRF3a. Thus, we next examined the half-lives of eRF3a and the eRF3a (D25A/D29A/D32A/D35A) mutant resistant to the UV-induced cleavage. The eRF3a (D25A/D29A/D32A/D35A) mutant ectopically expressed in THP-1 cells showed a half-life of ~6 h after UV irradiation, which is similar to that of wild-type eRF3a (Fig. 7a, b). In addition, the major cleavage product of eRF3a starting at amino acid residue 33Q showed a similar half-life. These

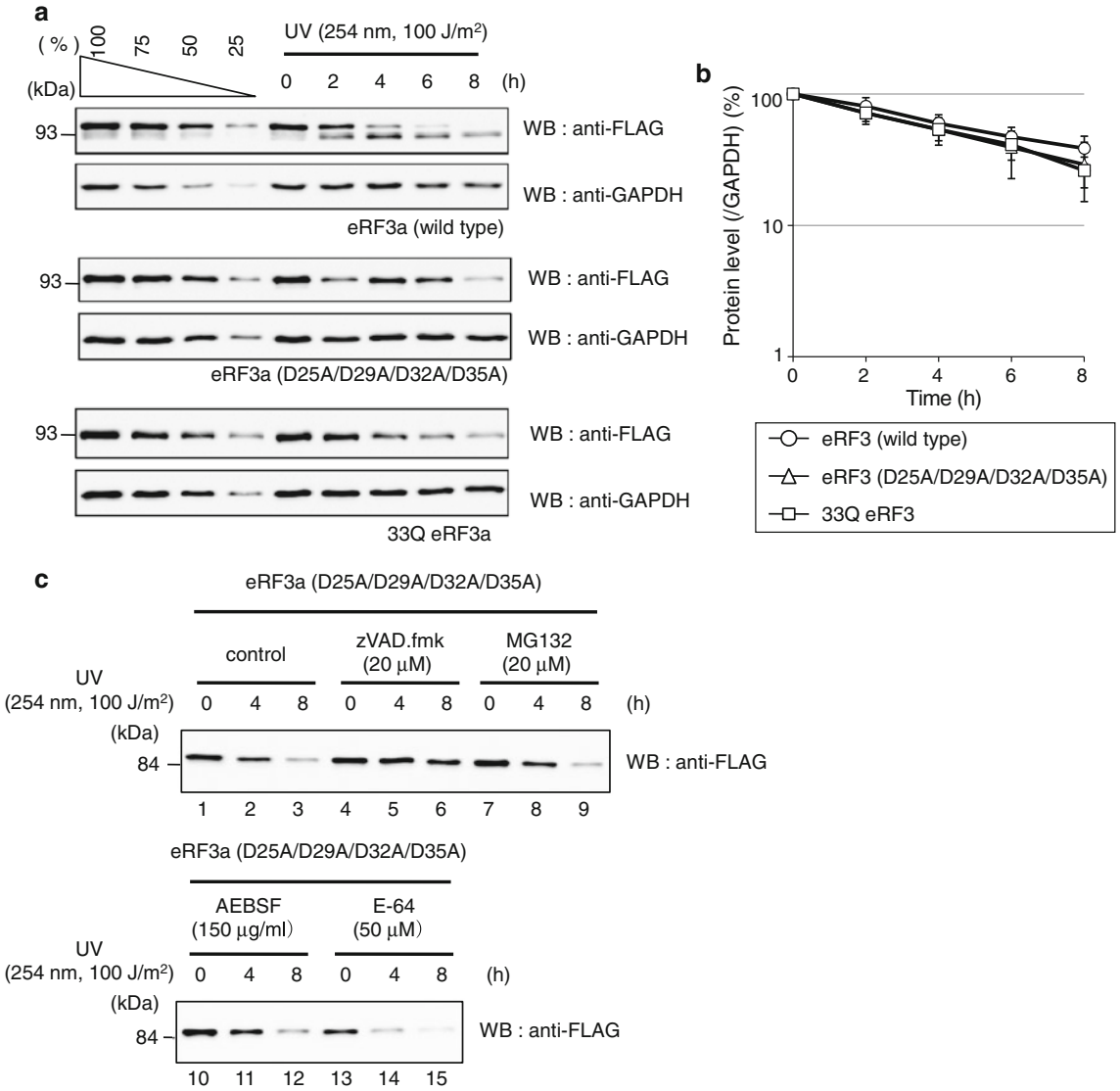

Fig. 7 Cleavage is not necessary for the degradation of eRF3a. **a** THP-1 cells were electroporated with plasmid expressing either wild-type eRF3a, the cleavage resistant eRF3a (D25A/D29A/D32A/D35A) or the major cleavage product 33Q eRF3a. At 36 h after transfection, THP-1 cells were irradiated with UV-C (254 nm, 100 J/m^2) and then cultured for the indicated time periods. Proteins were analyzed by western blotting using anti-FLAG and anti-GAPDH. The *leftmost four lanes* indicate the intensity of 100, 75, 50 and 25 % of protein levels. **b** The protein level of the wild-type eRF3a (*open circle*), eRF3a (D25A/D29A/D32A/D35A) mutant (*open triangle*) and 33Q eRF3a (*open square*) was measured by MultiGauge and normalized to that of GAPDH, where the normalized level of individual eRF3a at time 0 was defined as 100 %, respectively. *Error bars* represent the SD for three independent experiments. **c** THP-1 cells were electroporated with plasmid expressing the cleavage-resistant eRF3a (D25A/D29A/D32A/D35A). At 36 h after transfection, THP-1 cells were irradiated with UV-C (254 nm, 100 J/m^2) and treated with the indicated protease inhibitors (z-VAD-fmk (20 μM) (*lanes* 4–6), MG132 (20 μM) (*lanes* 7–9) AEBSF (150 μg/ml) (*lanes* 10–12) and E-64 (50 μM) (*lanes* 13–15)). Cells not treated with a protease inhibitor were prepared as a control of eRF3 modifications (*lanes* 1–3). After UV-irradiation, cells were harvested at 0 h (*lanes* 1, 4, 7, 10, 13), 4 h (*lanes* 2, 5, 8, 11, 14) and 8 h (*lanes* 3, 6, 9, 12, 15) and the effects of each protease inhibitor on the cleavage and degradation of eRF3 were analyzed by western blotting using anti-FLAG

results indicate that the preceding cleavage is not necessary for the degradation of eRF3a.

It should be noted that the cleavage-resistant mutant still undergoes UV-induced degradation similar to WT. To verify the mechanism for the UV-induced degradation of the cleavage-resistant mutant, we have examined the effect of a variety of protease inhibitors on the degradation of the mutant (Fig. 7c). The UV-induced degradation of the mutant was almost completely inhibited by z-VAD-fmk, while no apparent effect was observed with AEBSF, E-64

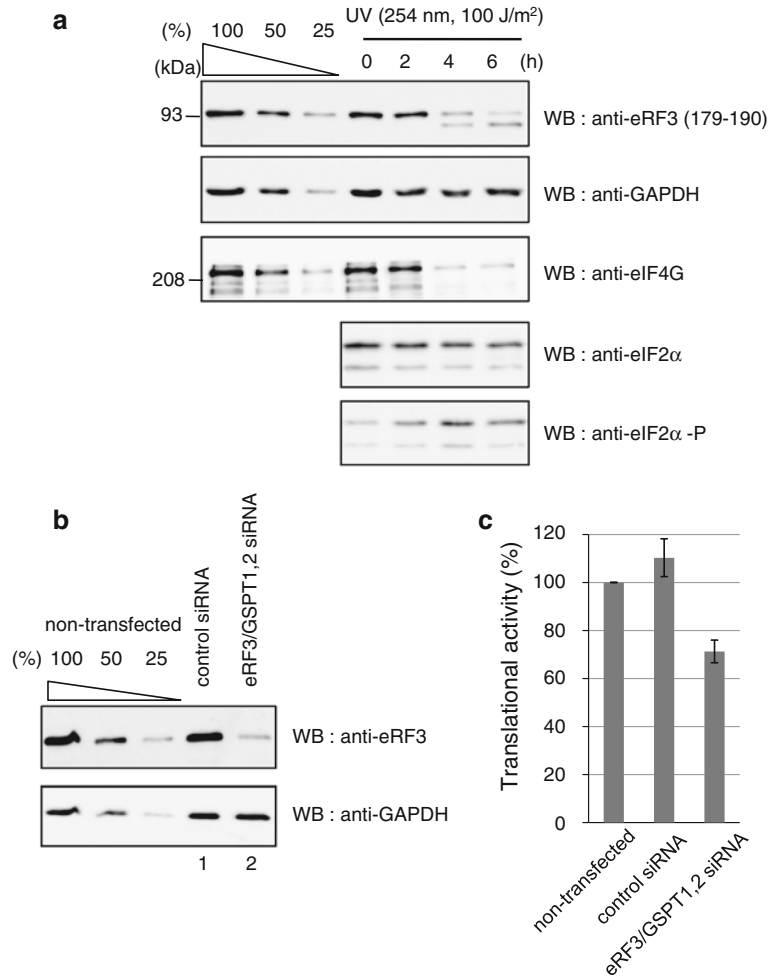

Fig. 8 Downregulation of eRF3 expression leads to translation inhibition. **a** THP-1 cells were irradiated with UV-C (254 nm, 100 J/m^2) at 6 h, 4 h and 2 h before harvest. An untreated sample was used as a standard (time 0). Cells prepared at the same time were lysed in SDS-PAGE sample buffer and analyzed by western blotting using anti-eRF3, anti-GAPDH, anti-eIF4G, anti-eIF2a and anti-eIF2a-P. Two-fold dilutions of standard sample in the leftmost three lanes indicate that this analysis is semiquantitative. **b** Total lysate of cells transfected with siRNA against luciferase (*lane* 1) or eRF3 (GSPT1/2) (*lane* 2) for 72 h was analyzed by western blotting using anti-eRF3 and anti-GAPDH. Two-fold dilutions of non-transfected sample in the *leftmost three lanes* indicate that this analysis is semiquantitative. **c** HeLa cells were transfected with either luciferase or eRF3 (GSPT1/GSPT2) siRNA. At 72 h after siRNA transfection, protein synthesis in cells was measured by metabolic labeling with [^{35}S]-methionine/cysteine. The translation efficiency of non-transfected HeLa cells was defined as 100 %. *Error bars* represent the SD for three experiments

and MG132 (even at 20 μM). These results strongly suggest that a caspase is responsible for the degradation of the cleavage-resistant mutant and strengthen our conclusion that DNA damaging agent-induced degradation is caspase-dependent. Although we could not detect any cleavage intermediates besides the eRF3a-cp, caspase or an unknown protease activated by caspase is thought to mediate the degradation of eRF3a.

Downregulation of eRF3 expression leads to translation inhibition

Previous studies have demonstrated that translation is specifically inhibited during apoptosis by a mechanism involving phosphorylation of eIF2a and caspase-mediated cleavage of eIF4G, respectively, at early and late phases of apoptosis [2–4, 25]. To gain insight into the biological significance of the cleavage and degradation of eRF3 during UV-induced apoptosis, we have compared the behavior of eRF3 with that of translation initiation factors (eIF4G and eIF2a). As shown in Fig. 8a, phosphorylation of eIF2a was observed at an early phase of apoptosis as reported previously (within 2 h) and peaked at 4 h after UV irradiation. In contrast, the caspase-mediated cleavage and degradation of both eRF3 and eIF4G occurred with a similar time-dependency (4 h after UV irradiation). These results are consistent with the idea that eRF3 is involved in the caspase-mediated inhibition of translation during

apoptosis. To clarify whether a reduction in the amount of eRF3 by itself could lead to the inhibition of translation, we next examined the effect of small interfering RNA (siRNA)-mediated knockdown of eRF3 on cellular protein synthesis. When eRF3 was knocked down by siRNAs against eRF3 (GSPT1 and GSPT2) to ~30 % of the level in non-transfected control cells, translational activity measured by the incorporation of [^{35}S]-methionine/cysteine into cellular proteins was reduced to ~70 % of that in non-transfected cells (Fig. 8b, c). These results indicate that downregulation of eRF3 expression could contribute to the inhibition of translation during apoptosis.

Finally, we tested if the cleaved form of eRF3 still retains the ability to support translation. The results in Fig. 9a show that ectopic expression of the truncated form of eRF3a (33Q eRF3a) in cells that had been depleted of endogenous eRF3 by siRNA-mediated knockdown led to an increased expression of the luciferase reporter gene in a dose-dependent manner, which is comparable to the activity of wild-type eRF3a. The expression of the eRF3a proteins was confirmed by western blotting (Fig. 9b). Taken together these results suggest that downregulation of eRF3 expression by caspase-mediated degradation but not cleavage contributes to the inhibition of translation during DNA damage-induced apoptosis.

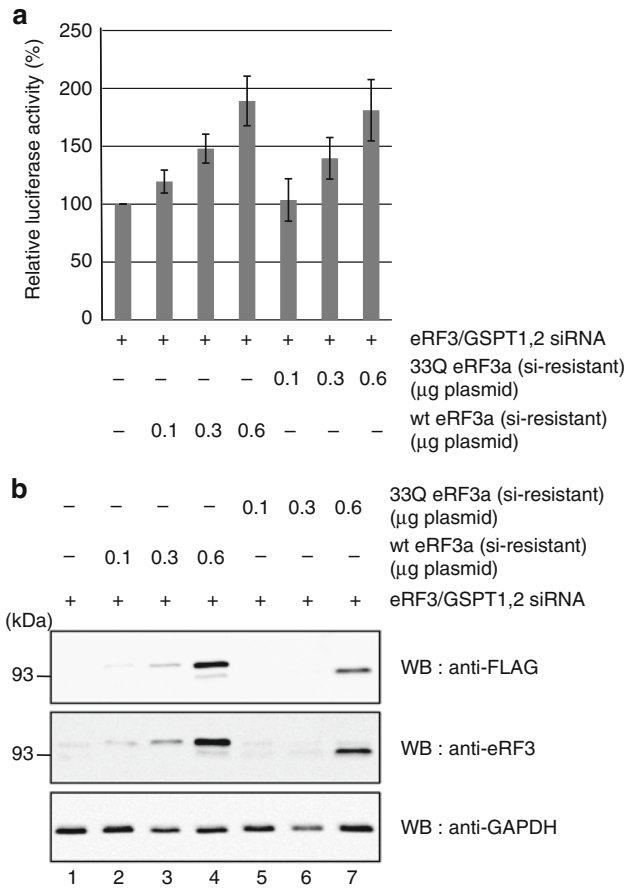

Fig. 9 Cleaved form of eRF3 retains the ability to support translation. **a** HeLa cells were transfected with eRF3 (GSPT1/GSPT2) siRNA. At 48 h after transfection, cells were co-transfected with eRF3 (GSPT1/GSPT2) siRNA, pCMV-FLAG2-hluc2 as a reporter and the indicated amount of either pCMV-MycFLAG-Ub-hGSPT1-si-resist, pCMV-MycFLAG-Ub-(33Q)-hGSPT1-si-resist, or pCMV-MycFLAG as a control. At 24 h after the second transfection, a luciferase assay was performed. Relative luciferase activity was quantitated, with the value for cells transfected with pCMV-MycFLAG defined as 100 %. *Error bars* represent the SD for three independent experiments. **b** Expression of 1M eRF3a (*lanes* 2–4) and 33Q eRF3a (*lanes* 5–7) was analyzed by western blotting using anti-FLAG (*upper*), anti-eRF3 (*middle*) and anti-GAPDH (*lower*)

Discussion

In this study, we have shown for the first time that eRF3 is modified (cleaved and degraded) and targeted for the regulation of translation during DNA damage-induced apoptosis. eRF3 functions as a general regulator of gene expression not only at translation termination but also at post-termination events including ribosome recycling [15] and initiation of mRNA decay [21, 26]. Specifically, eRF3 binds with PABP and eIF4G to form a ternary complex eRF3-PABP-eIF4G and accelerates cap- and poly(A)-dependent translation [15]. Thus, it is reasonable to assume that the caspase-mediated degradation of eRF3 contributes to the inhibition of cellular protein synthesis observed during DNA-damage-induced apoptosis. In fact, the siRNA-mediated knockdown of eRF3 significantly inhibited cellular protein synthesis. In this regard, a previous study demonstrated that eIF4G is also selectively targeted for cleavage and degradation during apoptosis, which contributes to the inhibition of cap-dependent translation [2–4]. Here, we have shown that the modifications of eRF3 and eIF4G occur with a similar time-dependency. Although it is difficult to assess the relative contribution of each translation factor to the inhibition of translation during apoptosis, the finding that the translation termination factor eRF3 as well as initiation factor eIF4G is targeted for the regulation of translation during apoptosis is suggestive of a "fail-safe" mechanism to ensure that the most energy-consuming process, protein synthesis, is certainly shut off during apoptotic cell death.

Thus, regulation of the amount of eRF3 might be a key to the control of translation. Previously, we and others have reported that eRF3 expression is up-regulated during G1-to-S phase transition [18, 27], where a general activation of translation occurs. Also, eRF3 is overexpressed in intestinal type gastric tumours [28]. These situations are reminiscent of the cap-binding translation initiation factor eIF4E; overexpression of eIF4E enhances translation, proliferation, suppression of apoptosis and cellular transformation, whereas reduced expression of eIF4E suppresses cellular translation and transformation [29]. It is therefore interesting to assume that the regulation of the amount of

eRF3 plays pivotal roles not only in translational control but also pathological processes including tumor development. In addition to the caspase-mediated regulation described in this study, the eRF3 protein level is also regulated by a ubiquitin–proteasome system [30]. Although the proteasome system is described as a mechanism that adjusts the level of eRF3 to that of another translation termination factor eRF1 during the formation of the translation termination complex (eRF1–eRF3), it might also be used for the regulation of translation and some cellular functions.

In this study, we initially aimed to identify the signals that lead to production of the IAP-binding isoform of eRF3. Unexpectedly, we identified another eRF3 isoform (eRF3-cp), which is generated by caspase-mediated cleavage during DNA damage-induced apoptosis. The IAP-binding isoform of eRF3 was demonstrated to promote caspase activation, IAP ubiquitination, and apoptosis through interaction with IAPs [20]. In contrast to the IAP-binding isoform of eRF3, the biological significance of the generation of eRF3-cp is as yet unclear. The caspase-mediated cleavage of eRF3 appeared neither to affect translation, nor to affect degradation of the eRF3 protein itself; the caspase-mediated cleavage is not necessary for the degradation of eRF3 (Fig. 7) and the new isoform could activate translation as in the case of wild-type eRF3 (Fig. 9). Recently, an important observation has been made by Brito's group: polyglycine expansion in eRF3a is associated with cancer susceptibility [31, 32]. Patients with a 12-Gly allele had a 20-fold increased risk of gastric cancer. However, it is not known why the polymorphic expansion of eRF3a leads to the increased risk for cancers. As the polymorphic region is adjacent to the caspase-mediated cleavage sites identified in this study, it is tempting to speculate that the polymorphism in polyglycine expansion affects the caspase-mediated cleavage of eRF3 during apoptosis and this might affect the risk for cancer development. These possibilities are now under investigation in our laboratory.

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Conflict of interest The authors declare that they have no conflict of interest.

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