Internal Ribosome Entry Site-mediated Translation of Apaf-1, but Not XIAP, Is Regulated during UV-induced Cell Death*

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Components of the cellular translation machinery are targets of caspase-mediated cleavage during apoptosis that correlates with the inhibition of protein synthesis, which accompanies apoptosis. Paradoxically, protein synthesis is required for apoptosis to occur in many experimental settings. Previous studies showed that two proteins that regulate apoptosis by controlling caspase activity, XIAP and Apaf-1, are translated by a unique, cap-independent mechanism mediated by an internal ribosome entry site (IRES) that is used preferentially under conditions in which normal cap-dependent translation is repressed. We investigated the regulation of XIAP and Apaf-1 following UVC irradiation. We show that UVC irradiation leads to the inhibition of translation and cell death. Furthermore, IRES-mediated translation of Apaf-1, but not XIAP, is enhanced by UVC irradiation, and this increase in Apaf-1 translation correlates with cell death. The enhanced Apaf-1 IRES-mediated translation is caspase-independent but is negatively modulated by the eIF2α kinase protein kinase RNA-like endoplasmic reticulum kinase. These data suggest that progression of UV-induced apoptosis requires IRES-mediated translation of Apaf-1 to ensure continuous levels of Apaf-1 despite an overall suppression of protein synthesis.

Normal cellular functions require complex control of cellular homeostasis, and apoptosis plays an essential role in this process. In this context, apoptosis is a cellular stress response that serves to eliminate cells that are too damaged to maintain their proper function. UV light is a common type of environmental stress that causes DNA damage and induces apoptosis in mammalian cells by engaging the mitochondrial branch of apoptotic signaling pathway (1). This pathway of apoptosis requires the release of cytochrome c from mitochondria into the cytoplasm, where it binds to the adaptor protein Apaf-1, triggering the formation of the apoptosome (2). Formation of the apoptosome results in the stepwise activation of caspases-9 and -3 that, in turn, cleave intracellular substrates leading to the final demise of the cell (3). The assembly of the apoptosome and autoactivation of caspase-9 represent critical events in the mitochondrial apoptotic pathway, and Apaf-1 plays a central role in this process. Conversely, XIAP,2 the most potent member of the IAP (inhibitor of apoptosis protein) family, also associates with the apoptosome to prevent both the activation and activity of caspase-3 (4). Thus, both Apaf-1 and XIAP play critical but opposing roles in the regulation of the activity of the apoptosome.

Previous studies have underlined the importance of Apaf-1 and XIAP in regulating UV-induced apoptosis. It was shown that increased Apaf-1 protein levels correlate with increased sensitivity to UV-induced apoptosis (5–7). For example, T cells from Apaf-1 knock-out mice (5), as well as Apaf-1-deficient K562 and CEM leukemic cells (6), all exhibited increased resistance to UV-induced apoptosis. In contrast, restoration of Apaf-1 protein levels by transient transfection restored the sensitivity of leukemic cells to UV-induced apoptosis (6). On the other hand, increased XIAP protein levels conferred resistance of MCF-7 cells to UV-induced apoptosis (8).

The importance of Apaf-1 and XIAP proteins in the control of the cellular response to UV irradiation, it is important to understand the mechanisms involved in regulating the expression of both of these proteins. Cells generally respond to environmental stress stimuli by inhibiting global translation. In contrast, a small proportion of mRNAs containing IRES (internal ribosome entry sequence) elements within their 5′-untranslated region (UTR) are refractory to this inhibition and continue to be efficiently translated (9). UV irradiation was shown to inhibit protein synthesis by inducing phosphorylation of the translation initiation factor eIF2α by either the PERK (10) or GCN2 (11, 12) eIF2α kinases. Interestingly, both Apaf-1 and XIAP contain IRES elements within their respective 5′-UTRs and exhibit distinct modes of regulation (13, 14). However, the regulation of Apaf-1 or XIAP translation during UV irradiation-induced apoptosis has not been explored.

In the present study we investigated the regulation of Apaf-1 and XIAP expression during UV stress. We found that UV irradiation inhibits protein synthesis and triggers dose-dependent cell death in 293T cells. However, Apaf-1 protein levels are significantly enhanced following UV irradiation. This paradox is explained by the translational induction of Apaf-1 expression mediated by the Apaf-1 IRES element in response to UV irradiation, which is refractory to UV-mediated inhibition of global protein synthesis. In contrast, translation of XIAP was inhibited to the same extent as global protein synthesis. We further found that the translation of Apaf-1 is independent of the phosphorylation status of eIF2α but is modulated by the eIF2α kinase PERK both in vitro and in vivo.
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EXPERIMENTAL PROCEDURES

Cell Culture and Transfection Reagents—Mouse embryonic fibroblasts from PERK-null (PERK−/−) or PERK wild-type (PERK+/+) mice were maintained in Dulbecco’s modified Eagle’s medium (Wisent Inc.) supplemented with heat-inactivated 10% fetal calf serum, 2 mM l-glutamine, 1% antibiotics (100 units/ml penicillin-streptomycin), 1% essential amino acids, and 0.01% β-mercaptoethanol. Human embryonic kidney (293T) cells were maintained in standard conditions in Dulbecco’s modified Eagle’s medium supplemented with heat-inactivated 10% fetal calf serum, 2 mM l-glutamine, and 1% antibiotics (100 units/ml penicillin-streptomycin). Transient transfection and co-transfection were done using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer’s protocol. Briefly, cells were seeded at a density of 3 × 10^5 cells/ml in 6-well plates coated with poly-d-lysine (5 μg/ml) and were transfected 24 h later in serum-free Opti-MEM medium (Invitrogen) with 2 μg of DNA and 4 μl of lipid per well. The transfection mixture was replaced 4 h later with fresh Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 1% antibiotics. Co-transfections of 293T cells were done as described above except that 1 μg of bicistronic plasmid was combined with 1 μg of expression vector.

Plasmids—The bicistronic vectors pβgal/hU78CAT (containing the human XIAP IRES) and pβgal/Apafl/CAT (containing the Apaf-1 IRES) were described previously (15, 16). The expression vectors for the FLAG-tagged GCN2 wild-type and the dominant-negative mutant GCN2 K → M (containing a point mutation that destroys the kinase activity of GCN2), the myc-tagged PERK wild-type and the T7-tagged PERKΔC expression vector (containing the coding sequence of the luminal domain of PERK), the myc-tagged IRE1b wild-type and the dominant-negative IRE1bΔC expression vector (containing the coding sequence of the luminal domain of IRE1b), and the elF2α wild-type and elF2α S51A mutant (containing a point mutation that prevents the phosphorylation of elF2α) were kindly provided by Drs. R. Wek, S. Wu, and D. Ron and were previously described (10, 17).

UV Irradiation—Cells were irradiated at room temperature in fresh medium with a 30-watt UVC light source (254 nm). The intensity of UVC light was measured prior to each experiment with a UVX radiometer (UVP Inc., Upland, CA). Transfected cells were irradiated 24 h post-transfection as described above. For the z-VAD-fmk experiments the cells were pre-treated with z-VAD-fmk (100 μM) or Me₂SO for 2 h before UVC treatment and for 24 h post UVC treatment. Cell viability was determined 24 h following UVC irradiation using the Vi-Cell cell viability analyzer (Beckman Coulter).

Analysis of Global Protein Synthesis—Both control and UV-irradiated cells were labeled with [35S]methionine (200 μCi/ml, Amersham Biosciences) for 1 h in methionine/cysteine-free minimal essential medium (Invitrogen) in which l-cysteine was added to a final concentration of 63 mg/liter. Cells were harvested in ice-cold phosphate-buffered saline, and cell extracts were prepared in radioimmune precipitation assay buffer (15 Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2, 2 mM EDTA, 0.1 M phenylmethylsulfonyl fluoride) containing 10 μg/ml each of aprotinin, pepstatin A, and leupeptin (all from Sigma). The lysates were then centrifuged at 14,000 × g for 10 min, and supernatants were collected. Protein concentration in the supernatant was determined by using a protein assay kit (Bradford Assay, Bio-Rad), and equal amounts of protein samples were separated by 10% SDS-PAGE. The total proteins were visualized by Coomassie Brilliant Blue (R-250) staining, and the [35S] incorporation was visualized by autoradiography.

Western Blot Analysis—Cells were harvested in ice-cold phosphate-buffered saline, and cell extracts were prepared in radioimmune precipitation assay buffer as described above. Equal amounts of protein samples were separated by 10% SDS-PAGE and analyzed by Western blotting. The antibodies used were as follows: mouse monoclonal anti-FLAG M2 (Stratagene), mouse monoclonal anti-c-Myc (Sigma), mouse monoclonal anti-T7 (Novagen), rabbit monoclonal anti-elF2α (Cell Signaling Technology), rabbit monoclonal anti-phospho elF2α (Ser-51, Cell Signaling Technology), rabbit polyclonal anti-XIAP (Agera), mouse monoclonal anti-Actin (Sigma), rat monoclonal anti-Apaf-1 (Chemicon), rabbit polyclonal anti-CREB2/ATF4 (Santa Cruz Biotechnology), and mouse monoclonal anti-processed caspase 3 (Cell Signaling Technology). The antibodies were used at the manufacturer’s suggested dilutions, and conditions were followed by secondary antibody (horseradish peroxidase-conjugated sheep anti-mouse or anti-rabbit IgG, Amersham Biosciences). Antibody complexes were detected using the ECL plus Western blotting detection system (Amersham Biosciences). If quantification of protein data was required the Western blots were performed as described above, but the secondary antibody used was Alexa Fluor 680 goat anti-mouse, anti-rat, or anti-rabbit IgG (LI-Cor Inc.). Antibody complexes were then detected and quantified using the Odyssey Infrared Imaging system (LI-Cor Inc.).

β-Galactosidase and CAT Analysis—Cells were briefly washed in ice-cold phosphate-buffered saline and harvested in the CAT ELISA kit lysis buffer (Roche Molecular Biochemicals) according to the protocol provided by the manufacturer. β-Galactosidase enzymatic activity in cell extracts was spectrophotometrically determined using o-nitrophenyl-β-D-galactopyranoside (18). The CAT levels were determined using the CAT ELISA kit (Roche Molecular Biochemicals) and the protocol provided by the manufacturer. The relative IRES activity was determined as a ratio of CAT/β-galactosidase in three independent experiments performed in triplicates. All data are shown as an average ± S.D. of three independent experiments performed in triplicates.

RNA Interference—293T cells were transfected in a 6-well plate with control siRNA (CY3-labeled, U47296, Dharmacon) or with a PERK-targeting Smartpool (M-004883-01, Dharmacon) using RNAiFect (Qia-gen) as per the manufacturer’s instructions. The cells were replated on the next day, and the levels of PERK message were assessed by quantitative RT-PCR 72 h post-transfection. For detection of Apaf-1 IRES activity, the PERK or control siRNA transfected cells were transfected the day following siRNA transfection with the Apaf-1 bicistronic plasmid pβgal/Apafl/CAT. The UVC irradiation and β-galactosidase and CAT analyses were performed as described above.

Ribonuclease Protection Assay—Total RNA was isolated from UVC-treated or control cells with TRIzol reagent (Invitrogen) and treated with 1 unit of DNase I. The 52P-labeled antisense RNA probes spanning 162 nucleotides of the XIAP 5′-UTR (coordinates 0 to −162), the complete Apaf-1 5′-UTR, or human glyceraldehyde-3-phosphate dehydrogenase (BD Pharmingen) were synthesized using the MaxiScript T7 transcription kit (Ambion) and gel-purified. RPA analyses were performed using the RPA III kit (Ambion) and the protocol provided by the manufacturer. Briefly, 10 μg of total RNA was hybridized overnight at 42 °C with 5 × 10^5 cpnm of high specific activity probe and then digested with an RNase A/RNase T1 mix. The digested samples were ethanol-precipitated and separated on a denaturing polyacrylamide gel. The protected fragments were visualized by x-ray film and quantified on a Bio-Rad Model GS-670 imaging densitometer.

Quantitative RT-PCR—Total RNA was isolated from UVC-treated or control cells that were previously transfected with the pβgal/Apafl/CAT reporter plasmid as described above. For quantitative RT-PCR,
reverse transcription was carried out using the First-Strand cDNA Synthesis kit (Amersham Biosciences) with NotI-d(T)16 primers. The quantitative PCR was performed using the QuantiTect SYBR green PCR kit (Qiagen) and analyzed on an ABI Prism 7000 sequence detection system using the ABI Prism 7000 SDS Software. Quantitative PCRs were carried out to detect β-galactosidase (5′-ACTATCCGACGGCCTTACT-3′ and 5′-CTGTAAGCGGTGATGTGAAAACCT-3′) and CAT (5′-CCGTGTATACGGTGAAMACCT-3′ and 5′-GGCGGAGAAGTTGTGCCATA-3′) as described previously (19). The primers for detection of PERK were as follows: forward, 5′-CTCACAGGCAAAGGAAAGGAG-3′, and reverse, 5′-AACACTTCCAAGGACACCAC-3′.

RESULTS

UV Irradiation Induces Cell Death and Inhibits Protein Synthesis—
To investigate the regulatory mechanism of UVC-induced cell death, we first determined the sensitivity of 293T cells to UVC irradiation. Twenty-four hours after exposure to increasing doses of UVC the percentage of surviving 293T cells was determined by viable cell count using the Beckman Coulter Vi-Cell 1.01 cell viability analyzer. UVC irradiation was found to induce cell death in a dose-dependent manner, with 50% cell survival at a UVC dose of 300 J/m² and only 10% survival at 450 J/m² (Fig. 1A). The 300 J/m² dose was therefore chosen for subsequent experiments.

We next asked whether the exposure of 293T cells to UVC irradiation also inhibits protein synthesis in 293T cells as have been shown previously for other cell lines (10–12). UVC-irradiated 293T cells were metabolically labeled with [35S]Met for 1 h, and total cell extracts (30 μg/lane) were resolved by SDS-PAGE as described under “Experimental Procedures.” The protein gel was then stained with Coomassie Blue R-250 to detect total proteins, followed by autoradiography to visualize newly synthesized proteins.

samples isolated from treated and untreated cells by RPA analysis we noted that the levels of Apaf-1 mRNA did not change in response to UVC irradiation (Fig. 2B). This suggests that the observed increase in Apaf-1 protein levels following UVC irradiation could be due to translational up-regulation of Apaf-1.

It has been demonstrated previously that IRES-dependent translation is preferentially employed during conditions of cellular stress (13–15, 20). Because both the Apaf-1 and XIAP mRNAs have IRES elements within their 5′-UTRs, we examined the effect of UVC irradiation on the activity of Apaf1 and XIAP IRES elements. To study IRES-mediated translation exclusively, we used the previously described bicistronic pβgal/CAT reporter plasmid that has the β-galactosidase gene and chloramphenicol acetyltransferase gene as first and second cistrons, respectively, with the IRES to be tested inserted between the two cistrons (15). In this system, expression of the second cistron (CAT) is dependent on IRES activity. Bicistronic reporter plasmids carrying either the Apaf1 IRES or the XIAP IRES were transfected into 293T cells, and IRES-dependent translation of CAT was determined 24 h after treatment with increasing doses of UVC irradiation (Fig. 2C). The activity of the XIAP IRES (as measured by CAT expression) was inhibited to the same extend as global protein synthesis (measured by β-galactosidase expression). In sharp contrast, the activity of the Apaf-1 IRES paralleled the increase in UVC dose, with a 5-fold increase in activity at an UVC dose of 200 J/m², and an 8-fold increase in activity at 450 J/m² (Fig. 2C and Table 1).

It is formally possible that the observed induction of Apaf-1 IRES activity is due to transcriptional induction, cryptic promoter activity, or spurious splicing of the bicistronic RNA transcript (βgal/Apaf1/CAT) produced from the pβgal/Apaf1/CAT plasmid, thus altering the ratio of CAT protein to β-galactosidase protein. We therefore determined the effect of UV irradiation on the integrity of the βgal/Apaf1/CAT bicistronic RNA transcript using quantitative RT-PCR as described previously (19). Total RNA was isolated 24 h post-UVC irradiation and subjected to quantitative RT-PCR of the Apaf-1 bicistronic mRNA, and this confirmed that the increase in Apaf-1 IRES activity was not due to transcriptional induction, cryptic promoter activity, or spurious splicing of the bicistronic mRNA (Fig. 2D).

The results presented above demonstrate that increasing doses of UVC irradiation induce Apaf-1 IRES-dependent translation that correlates with a loss of viability in 293T cells. These data are in agreement
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A.

**WB:**
- Apaf-1
- XIAP
- Actin

**RPA:**
- XIAP
- GAPDH

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**TABLE 1**

| Construct  | β-Galactosidase | CAT | Relative IRES activity |
|------------|-----------------|-----|------------------------|
| Apaf-1 (0 J/m²) | 0.184 (±0.031) | 0.213 (±0.049) | 1.157 |
| Apaf-1 (450 J/m²) | 0.105 (±0.023) | 1.021 (±0.093) | 9.724 |
| XIAP (0 J/m²) | 0.210 (±0.038) | 2.173 (±0.098) | 10.347 |
| XIAP (450 J/m²) | 0.098 (±0.029) | 0.638 (±0.078) | 6.518 |

*Cells were transfected with the indicated plasmids and exposed to 0 or 450 J/m² UVC 24 h post-transfection as described under “Experimental Procedures.”

β-Galactosidase (cap-dependent) and CAT (IRES-dependent) levels were determined 24 h post-U/V irradiation as described under “Experimental Procedures” and are shown per 1 µg of total protein.

Relative IRES activity was determined as CAT/β-galactosidase.

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FIGURE 2. UVC induces the expression of Apaf-1 but not XIAP via IRES-mediated translation. A, 293T cells were treated with the indicated doses of UVC irradiation, total protein lysates were prepared 24 h later and resolved by 10% SDS-PAGE. Endogenous expression of Apaf-1 and XIAP proteins was determined by Western blot analyses using antibodies against Apaf-1 and XIAP, respectively. The expression of β-actin in both UVC-treated and untreated cells was used as a control. B, ribonuclease protein assay (RPA) of Apaf-1, XIAP, and glyceraldehyde-3-phosphate dehydrogenase mRNAs in treated and untreated cells (same as A). C, 293T cells were transiently transfected with the XIAP or Apaf-1 IRES bicistronic reporter constructs and the effect of UVC irradiation on XIAP (white bars) and Apaf-1 IRES (black bars) translation were determined 24 h later by measuring the β-galactosidase and CAT activities as described under “Experimental Procedures.” The relative IRES activity in non-irradiated cells was set as 1 for each construct. The bars represent the average ± S.D. determined from three independent experiments performed in triplicates. D, quantitative RT-PCR was performed on DNase I-treated total RNA isolated from 293T cells transfected with Apaf-1 IRES bicistronic plasmids. The RNA was isolated and reverse transcribed as described under “Experimental Procedures.” The quantitative real-time RT-PCR was performed using the Quantitect SYBR green RT-PCR kit (Qiagen) and analyzed on an ABI Prism 7000 sequence detection system using the ABI Prism 7000 SDS Software. Quantitative PCR reactions were carried out to detect the β-galactosidase and CAT cistrons of the bicistronic mRNA. The CAT/β-galactosidase ratio was calculated as 2^ΔCt(CAT)−ΔCt(gal). The bars represent the mean ± S.E. of three experiments.

With previously published findings that repression of Apaf-1 expression using small interfering RNA (siRNA) to Apaf-1 (21), or by deletion of the Apaf-1 gene (5–7) resulted in the suppression of UV-induced apoptosis. Conversely, restoration of Apaf-1 protein levels reseeditized cells to UV-induced apoptosis (6). Taken together, these data suggest that continued translation of Apaf-1 afforded by the Apaf-1 IRES element is required for cell death induced by UVC irradiation.

**UVC-mediated Induction of Apaf-1 Is Caspase-independent**—We previously demonstrated that the activation of caspases is required for the stimulation of Apaf1 IRES activity in etoposide-treated cells (13). To determine if Apaf-1 IRES activity is regulated by the same mechanism during UVC-induced cellular stress, 293T cells were transfected with either the Apaf-1 IRES or XIAP IRES bicistronic plasmids, treated with the broad-range caspase inhibitor z-VAD-fmk, and then irradiated. As shown in Fig. 3A, pre-treatment of cells with z-VAD-fmk had no effect on the induction of Apaf1 IRES activity following UVC irradiation, although it was sufficient to inhibit caspase-3 cleavage (Fig. 3B). These data suggest that the induction of Apaf-1 IRES activity by UVC irradiation is caspase-independent.

**UVC-mediated Induction of Apaf-1 Is Independent of eIF2α Phosphorylation Status, but Is Modulated by PERK**—Previous studies have shown that diverse cellular stresses can stimulate the IRES-mediated translation of some mRNAs via a mechanism that requires transient phosphorylation of the α-subunit of initiation factor 2 (eIF2α) (22). Recently, two distinct eIF2α kinases, GCN2 and PERK, were implicated in the phosphorylation of eIF2α and the consequent inhibition of protein synthesis during UVC irradiation (10–12). We therefore investigated whether GCN2, PERK, or phosphorylation of eIF2α are involved in the modulation of Apaf-1 IRES activity by UVC irradiation. To test the requirement for GCN2, 293T cells were co-transfected with each of the bicistronic constructs containing the XIAP IRES and Apaf-1 IRES and one of the following expression vectors: pCI, FLAG-GCN2, or FLAG-GCN2K→M, a catalytically inactive dominant-negative GCN2 mutant. The expression of GCN2 or GCN2K→M had the expected effect on the phosphorylation status of eIF2α (Fig. 4A). The relative IRES activities of...
XIAP and Apaf-1 IRESs were then determined in both UVC-treated and untreated cells. XIAP IRES activity did not change in response to UVC irradiation regardless of the GCN2 expression vector used (Fig. 4B). Similarly, 293T cells co-transfected with the Apaf-1 bicistronic plasmid and either of the GCN2 expression plasmids did not exhibit any changes in Apaf-1 IRES activity following UVC irradiation (Fig. 4B). These results indicate that UVC-induced Apaf-1 IRES activity is independent of GCN2 activity.

To determine whether PERK plays a role in Apaf-1 IRES activity following UVC irradiation, 293T cells were co-transfected with each of the bicistronic constructs containing the XIAP IRES and Apaf-1 IRES and one of the following vectors: pCI, myc-PERK, or T7-PERKΔΔC, a catalytically inactive dominant-negative PERK mutant. Again, the expression of PERK or PERKΔΔC had the expected effect on the phosphorylation status of eIF2α (Fig. 4C). Twenty-four hours post-transfection, cells were UV-irradiated. We observed that XIAP IRES activity was not affected by the overexpression of either PERK or its inactive mutant, PERKΔΔC, in UVC-irradiated 293T cells (Fig. 4D). In contrast, overexpression of the dominant negative PERKΔΔC mutant resulted in a significant increase in the UVC-mediated induction of Apaf-1 IRES activity when compared with the control vector (Fig. 4D). We determined the effect of PERKΔΔC overexpression on the integrity of the βgal/Apaf-1/CAT bicistronic RNA transcript using quantitative RT-PCR. Total RNA was isolated 24 h post-UVC irradiation and subjected to quantitative RT-PCR. As shown in Fig. 4E, the ratio of CAT and β-galactosidase transcripts was unchanged in PERKΔΔC-transfected cells treated with UVC as compared with non-irradiated cells. These data confirm that the integrity of the bicistronic RNA transcript produced from the βgal/Apaf-1/CAT reporter plasmid is not affected by PERKΔΔC overexpression, and therefore spurious splicing did not contribute to the apparent Apaf-1 IRES activity.

It is possible that overexpression of the PERK dominant-negative mutant PERKΔΔC has an inadvertent effect on IRE1, another ER-resident- and ER-stress-activated protein, which results in the observed changes in Apaf-1 IRES activity. Although this notion is unlikely, we nevertheless wanted to test this possibility. Thus, 293T cells were co-transfected with the bicistronic constructs containing the Apaf-1 IRES and one of the following expression vectors: pCI, myc-IRE1bΔ, or myc-IRE1bCΔ, a dominant negative IRE1b mutant. The overexpression of IRE1b or IRE1bΔ had no effect on the Apaf-1 IRES activity following UVC irradiation (supplementary Fig. 1S). These results indicate that the effect of PERKΔΔC on UVC-induced Apaf-1 IRES activity is not mediated through inadvertent inhibition of IRE1b.

Because activation of both GCN2 and PERK results in the phosphorylation of eIF2α, one possible mechanism explaining how PERKΔΔC could modulate Apaf-1 IRES activity is via the phosphorylation of eIF2α. To directly test this hypothesis, 293T cells were co-transfected with the Apaf-1 IRES or XIAP IRES bicistronic plasmids and a plasmid expressing either eIF2α or eIF2αS51A, a non-phosphorylatable mutant of eIF2α. We observed that overexpression of either eIF2α or eIF2αS51A did not alter the Apaf-1 IRES activity following UVC irradiation (Fig. 5A), although the levels of eIF2α phosphorylation were significantly higher in eIF2α- than in eIF2αS51A-transfected cells (Fig. 5B).

Taken together, these results suggest that the induction of Apaf-1 IRES activity by UVC irradiation is independent of the phosphorylation status of eIF2α. Furthermore, the expression of the dominant-negative catalytically inactive mutant of PERK increases the activity of the Apaf-1 IRES following UV stress, suggesting that PERK may function as a negative regulator of Apaf-1 IRES-mediated translation.

To further verify the notion that PERK plays a role in Apaf-1 IRES activity we utilized siRNA-mediated knock-down of PERK. 293T cells were transfected either with control or PERK-targeting siRNA and the efficiency of knock-down was determined in transfected cells by quantitative RT-PCR to be ~90% (Fig. 6A). To functionally confirm that PERK levels were lowered sufficiently, control or PERK siRNA-transfected cells were exposed to thapsigargin (1 μM), an inducer of ER stress, for 2 h, and the levels of ATF4 were determined by Western blot analysis (Fig. 6B). ATF4/CREB2 is a transcription factor that is translationally induced in a PERK-dependent manner in response to ER stress (23). Indeed, upon PERK depletion, we observed a significant reduction in ATF4 in thapsigargin-treated cells, thus confirming our ability to sufficiently reduce PERK levels. To determine the effect of PERK knockdown on Apaf-1 IRES activity we transfected the control or PERK siRNA-transfected cells with the Apaf-1 IRES bicistronic reporter plasmid and exposed the cells to UVC irradiation. We observed that in PERK siRNA-transfected cells the activity of Apaf-1 IRES was significantly enhanced in response to UVC (Fig. 6C). However, the extent of Apaf-1 IRES activation was not as great as that observed with PERKΔΔC co-expression. This is likely due to the fact that we were only able to

**FIGURE 3.** UVC-mediated induction of Apaf-1 IRES activity is independent of caspase activation. A, 293T cells were transiently transfected with either Apaf-1 IRES or XIAP IRES containing bicistronic plasmids. 24 h following transfection the cells were pre-treated with z-VAD-fmk (100 μM) for 2 h and then exposed to the indicated doses of UVC irradiation. Cell lysates were prepared 24 h post irradiation as described under “Experimental Procedures,” and the IRES activity was determined. The relative IRES activity in the non-irradiated cells was set as 1 for each IRES. The bars represent the average ± S.D. determined from three independent experiments performed in triplicates. B, the activity of z-VAD-fmk was verified by Western blot analysis using antibodies against cleaved caspase-3.
FIGURE 4. PERK kinase but not GCN2 kinase regulates UVC-mediated induction of Apaf-1 IRES activity. A. 293T cells were co-transfected with either Apaf-1 or XIAP IRES-containing bicistronic plasmids and FLAG-GCN2 or FLAG-GCN2K→M expressing vector, and the expression levels of FLAG-GCN2 and FLAG-GCN2K→M proteins were determined by Western blot analysis using the anti-FLAG antibodies. The levels of eIF2α phosphorylation were determined in UVC-irradiated cells (300 J/m²) using the anti-eIF2α and anti-phospho-eIF2α antibodies, respectively. 3 h post-UVC irradiation. The levels of eIF2α and phosphorylated eIF2α were determined using LI-COR Odyssey (version 1.1), and the ratio of eIF2α-P/eIF2α is shown below the blot. B. co-transfected cells (as in A) were irradiated with 300 J/m² UVC 24 h post-transfection, and the relative IRES activity was determined 24 h after UVC irradiation for each IRES as described under “Experimental Procedures.” The IRES activity in pCI-transfected, non-irradiated cells was set as 1. The bars represent the average ± S.D. of three independent experiments performed in triplicates. C. 293T cells were co-transfected with either Apaf-1 or XIAP IRES-containing bicistronic plasmids and myc-PERK or T7-PERK→AC expressing vector. Expression levels of myc-PERK and T7-PERK→AC proteins were determined by Western blot analysis using the anti-myc or anti-T7 antibodies. The levels of eIF2α phosphorylation were determined in UVC-irradiated cells (300 J/m², as in A). D. co-transfected cells (as in C) were irradiated with 300 J/m² UVC 24 h post-transfection, and the relative IRES activity was determined 24 h after UVC irradiation for each IRES as described under “Experimental Procedures.” The IRES activity in pCI-transfected, non-irradiated cells was set as 1. The bars represent the average ± S.D. of three independent experiments performed in triplicates. E. quantitative RT-PCR was performed on DNase I-treated total RNA isolated from 293T cells co-transfected with Apaf-1 IRES bicistronic plasmid and T7-PERK→AC-expressing vector. The RNA was isolated and reverse transcribed as described under “Experimental Procedures.” The quantitative real-time RT-PCR was performed using the Quantitect SYBR green RT-PCR kit (Qiagen) and analyzed on an ABI Prism 7000 sequence detection system using the ABI Prism 7000 SDS software. Quantitative PCR reactions were carried out to detect the β-galactosidase and CAT cistrons of the bicistronic mRNA. The CAT/β-galactosidase ratio was calculated as 2^[-ΔC(T)(Ct(CAT)-Ct(βGal))]. The bars represent the mean ± S.E. of three experiments.

FIGURE 5. UVC-mediated induction of Apaf-1 IRES activity is independent of the phosphorylation of eIF2α. A. 293T cells were co-transfected with either Apaf-1 or XIAP IRES-containing bicistronic plasmids and eIF2α or eIF2αS51A-expressing vector. Cells were irradiated with 300 J/m² UVC 24 h post-transfection, and the relative IRES activity was determined 24 h after UVC irradiation for each IRES as described under “Experimental Procedures.” The IRES activity in pCI-transfected, non-irradiated cells was set as 1. The bars represent the average ± S.D. of three independent experiments performed in triplicates. B. expression levels of eIF2α and eIF2αS51A proteins and their level of phosphorylation were determined by Western blot analysis using the anti-eIF2α and anti-phospho-eIF2α antibodies.
PERK (PERK−/−) to the corresponding level of Apaf-1 expression in normal MEF cells (PERK+/+). Western blot analysis revealed that the basal level of Apaf-1 in PERK−/− cells is 3- to 4-fold higher than the level of Apaf-1 expression in PERK+/+ cells (Fig. 7A). Similarly, the induction of the Apaf-1 expression following exposure to UVC irradiation was greatly enhanced in the PERK−/− cells (Fig. 7A). Moreover, the increased level of Apaf-1 protein in the PERK−/− cells correlated with enhanced sensitivity of the PERK-null cells to UVC irradiation (Fig. 7B). These findings suggest that PERK might function as a negative regulator of Apaf-1 translation in vivo, and thus a negative regulator of Apaf-1-mediated cell death following UVC irradiation.

**DISCUSSION**

To gain insight into the control of UVC-induced cell death, the present study aimed to investigate the mechanisms that regulate expression of two proteins involved in the control of apoptosis, the anti-apoptotic protein XIAP, and the pro-apoptotic protein Apaf-1. Importantly, XIAP and Apaf-1 play opposing roles in the regulation of apoptosis. Whereas Apaf-1 is essential for the formation of the apoptosome and consequent activation of the caspase cascade, XIAP is a potent intrinsic inhibitor of caspases (19, 23). In addition, both Apaf-1 and XIAP are found associated with the apoptosome (4), suggesting that their coordinated function is required for the fine tuning of the apoptotic response. To date, many studies have been conducted to determine the involvement of the XIAP and Apaf-1 proteins in cell survival regulation during UV-induced cellular stress. It was demonstrated that an increase in XIAP protein levels parallels a decrease in sensitivity to UV-induced apoptosis (8). Moreover, overexpression of XIAP inhibited UV-induced cell death in MCF-7 cells (8). Similarly, the levels of XIAP protein decreased in keratinocytes exposed to increasing doses of UVB irradiation (24). Conversely, it was shown that an increase in Apaf-1 protein levels parallels an increase in sensitivity to UV-induced apoptosis (5–7, 21). Lassus et al. (21) showed that repression of Apaf-1 expression using siRNA to Apaf-1 resulted in the prevention of UV-induced apoptosis in IMR90E1A cells. Furthermore, T cells from Apaf-1 knock-out mice (5), as well as the Apaf-1-deficient K562 and CEM leukemic cell lines (6, 7), all exhibited increased resistance to UV-induced apoptosis. Restoration of Apaf-1 protein levels by transient transfection of the Apaf-1-deficient leukemic cells with an Apaf-1 plasmid re-sensitized these cells to UV-induced apoptosis (6). Taken together, these findings provide substantial evidence for the importance of the antagonistic roles of XIAP and Apaf-1 proteins in regulating UV-induced apoptosis.

The regulation of XIAP and Apaf-1 expression during UV-induced cellular stress, however, has not been investigated. Interestingly, both XIAP and Apaf-1 mRNAs are translated by a cap-independent mechanism facilitated by IRES elements located in their respective 5′-UTRs. It emerged recently that the IRES mechanism is utilized preferentially during conditions when normal cap-dependent translation initiation is compromised (9, 25, 26). These conditions include diverse cellular stress, differentiation, mitosis, and apoptosis. In fact, while only a small fraction of cellular mRNAs contain IRES elements in their respective 5′-UTRs, the majority of these elements are found in genes involved in the control of cell growth, proliferation, and survival (9, 25–27).

UV radiation inhibits nascent protein synthesis (10–12). Paradoxically, protein synthesis is required for UV-induced cell death in 293T cells, because treatment of cells with the protein synthesis inhibitor cycloheximide significantly protects cells from UV-induced death (data not shown). We and others have shown previously that IRES-mediated translation escapes the control mechanisms that regulate cap-dependent translation during cellular stress (13, 14, 20, 22, 28, 29). We
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We have also demonstrated that IRES-mediated translation of pro-survival proteins is activated during transient stress conditions, whereas IRES-mediated translation of pro-death proteins is activated during severe apoptotic conditions (13). Thus, we hypothesized that low dose (sublethal) UVC irradiation would result in an increase in XIAP IRES activity, whereas high dose (lethal) UVC irradiation would be accompanied by an increase in Apaf-1 IRES-mediated translation. Contrary to what we expected, exposure to various doses of UVC did not result in significant changes in XIAP IRES activity or XIAP protein levels. However, we observed that increasing doses of UVC irradiation resulted in an increase in Apaf-1 IRES activity, paralleled by an increase of the endogenous Apaf-1 protein. This increase in Apaf-1 protein synthesis was observed despite the general cessation of protein synthesis evoked by UVC irradiation. Thus, during UVC stress, Apaf-1 IRES activity and the corresponding increase in Apaf-1 protein levels is required for cell death. Our finding that increasing doses of UVC irradiation do not affect XIAP IRES activity, but significantly increase the activity of the Apaf-1 IRES, points to the distinct regulation of IRES-mediated translation during cellular stress, which we previously suggested (13, 30).

We have previously demonstrated the activation of Apaf-1 IRES activity during etoposide-induced cell death (13). We further showed that Apaf-1 IRES activation is caspase-dependent and is mediated by the caspase-cleaved fragments of the eIF4G translation initiation factor family members eIF4GI and p97/DAP5/NAT1 (13). Surprisingly, the UVC-mediated induction of Apaf-1 IRES activity does not appear to be caspase-dependent, because z-VAD-fmk treatment of cells prior to UVC irradiation did not affect Apaf-1 IRES-mediated translation. This suggests that cells may have evolved distinct regulatory pathways to regulate expression of apoptotic proteins in response to divergent physiological stresses.

It has been demonstrated that some, but not all, IRES elements are regulated by the phosphorylation of the eukaryotic initiation factor eIF2α (22, 31). Because eIF2α is phosphorylated in response to UV stress (10–12), we investigated the involvement of eIF2α in UVC-induced Apaf-1 IRES activity. The phosphorylation of eIF2α is important for the control of cap-dependent translation, because an increase in phosphorylation of eIF2α attenuates formation of the ternary complex, resulting in a reduction in global protein synthesis rates (32). The phosphorylation of eIF2α during UV stress is facilitated by two eIF2α kinases, GCN2 (11) and PERK (10). Our results indicate that eIF2α phosphorylation is not required for UVC-mediated induction of Apaf-1 IRES activity, because the overexpression of the wild-type or the non-phosphorylatable mutant of eIF2α did not affect Apaf-1 IRES activity following irradiation. Furthermore, activation of the GCN2 kinase is also not required, because the overexpression of a wild-type or a catalytically inactive dominant-negative mutant GCN2 did not affect Apaf-1 IRES activity following UV irradiation. Surprisingly, however, although Apaf-1 IRES activity following UVC irradiation was not affected by the overexpression of the PERK kinase, Apaf-1 IRES activity was considerably increased by the overexpression of the PERK dominant-negative catalytically inactive mutant PERKΔC or in cells with depleted PERK levels. The involvement of PERK in the regulation of Apaf-1 expression was further confirmed in PERK-null MEFs that exhibit increased levels of Apaf-1 and display enhanced susceptibility to UVC-mediated death.

Our findings support the hypothesis that there exists specific regulatory mechanisms that control translation of distinct IRES elements in response to cellular stress (9, 13, 22, 30). In addition, we provide the first evidence that enhanced Apaf-1 IRES-mediated translation assures the continued expression of the Apaf-1 protein during UVC stress. This increase in Apaf-1 IRES activity is required for cell death following UVC irradiation. Although the exact molecular mechanism that induces Apaf-1 IRES-mediated translation following UVC irradiation needs to be further investigated, we show that Apaf-1 IRES activity is negatively regulated by the PERK kinase. PERK is the primary kinase involved signal transduction during endoplasmic reticulum stress and unfolded protein response (33). In this context, the regulation of Apaf-1 during endoplasmic stress and endoplasmic stress-induced apoptosis warrants further examination.

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