Translational Repression of MCL-1 Couples Stress-induced eIF2α Phosphorylation to Mitochondrial Apoptosis Initiation

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The integrated stress response (ISR) integrates a broad range of environmental and endogenous stress signals to the phosphorylation of the α-subunit of eukaryotic translation initiation factor 2 (eIF2α). Although intense or prolonged activation of this pathway is known to induce apoptosis, the molecular mechanisms coupling stress-induced eIF2α phosphorylation to the cell death machinery have remained incompletely understood. In this study, we characterized apoptosis induction in response to classical activators of the ISR (tunicamycin, UVC, elevated osmotic pressure, arsenite). We found that all applied stress stimuli activated a mitochondrial pathway of apoptosis initiation. Rapid and selective down-regulation of the anti-apoptotic BCL-2 family protein MCL-1 preceded the activation of BAX, BAK, and caspases. Stabilization of MCL-1 blocked apoptosis initiation, while cells with reduced MCL-1 protein content were strongly sensitized to stress-induced apoptosis. Stress-induced elimination of MCL-1 occurred with unchanged protein turnover and independently of MCL-1 mRNA levels. In contrast, stress-induced phosphorylation of eIF2α at Ser51 was both sufficient and necessary for the down-regulation of MCL-1 protein in stressed cells. These findings indicate that stress-induced phosphorylation of eIF2α is directly coupled to mitochondrial apoptosis regulation via translational repression of MCL-1. Down-regulation of MCL-1 enables but does not enforce apoptosis initiation in stressed cells.

The integrated stress response (ISR) is a general stress response program conserved from yeast to mammals, that is known to integrate various types of environmental and endogenous stress signals, including endoplasmic reticulum stress, amino acid deprivation, infection with double-stranded RNA viruses, osmotic stress, UV light exposure, heme deficiency, and oxidative stress (1–4). Those diverse signals activate specific stress kinases, each of which converges on the phosphorylation at Ser51 on the α-subunit of eukaryotic translation initiation factor 2 (eIF2α) (1). Phosphorylation of eIF2α at Ser51 abrogates the function of eIF2α, required for the transfer of the initiator Met-tRNA^Met to the small ribosomal subunit. This leads to a shutdown of global mRNA translation due to reduced AUG initiator codon recognition, along with increased translation of a few selected mRNAs including ATF4, a basic zipper transcription activator (5–7). As a consequence of global translational arrest, the steady-state levels of most cellular proteins decrease with time, dependent on their respective protein half-life. This has recently been found to be essential for NF-κB activation mediated by down-regulation of 1κB proteins in response to eIF2α phosphorylation (8–10). These findings provide a first striking example of a signaling pathway activated by translational repression of a labile protein during eIF2α phosphorylation. Activation of the ISR also mobilizes stress-induced gene expression induced by ATF4 and its target genes, involved in cell growth, differentiation and apoptosis (11, 12). The biological consequences of activation of the ISR largely depend on the context. While activation of the eIF2α phosphorylation pathway is primarily thought to protect cells from ongoing damage (11, 13, 14), prolonged or extensive activation of this pathway has been found to induce apoptosis (15–18). However, the molecular mechanisms coupling activation of the ISR to the initiation of apoptosis have remained incompletely understood. In the vast majority of cell types, apoptosis depends on the mitochondrial pathway. Therein, disruption of mitochondrial integrity leads to the release of pro-apoptotic proteins such as cytochrome c, AIF, Smac/Diablo, EndoG, and HtrA2/Omi from the mitochondrial inner-membrane space into the cytosol (19). Cytochrome c contacts Apaf-1, thereby triggering the recruitment and auto-activation of pro-caspase-9. Caspase-9 then activates a cascade of specialized proteases, termed executioner caspases, which in turn execute cell death. Mitochondrial integrity is regulated by the members of the BCL-2 family of proteins (20). BAX and BAK, two pro-apoptotic multidomain BCL-2 proteins, directly or indirectly induce mitochondrial outer membrane permeabilization (MOMP) by oligomerization occurring upon their activation (21). MOMP is prevented by multidomain anti-apoptotic BCL-2 proteins such as BCL-2, BCL-xL, and MCL-1. Members of the BH3-only group of BCL-2 proteins undergo induction and/or activation by apoptotic

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2 The abbreviations used are: ISR, integrated stress response; eIF2α, α-subunit of eukaryotic translation initiation factor 2; CHAPS, 3-(3-cholamidopropyl)-dimethylammonio)-1-propanesulfonic acid; Z, benzyloxycarbonyl; fmk, fluoromethylketone.
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stimuli and either directly activate BAX-BAK or specifically neutralize anti-apoptotic multidomain BCL-2 proteins (22).

In this study, we aimed to uncover the molecular cross-talk between cellular stress signaling and apoptosis. We characterized apoptosis initiation in response to classical activators of the ISR and found that stress-induced apoptosis essentially involves the rapid and selective down-regulation of MCL-1 protein, mediated by stress-induced phosphorylation of eIF2α at Ser51.

EXPERIMENTAL PROCEDURES

Materials—The following drugs were purchased as indicated: Coumermycin (Fluka); ZVAD-fmk (Biomol); dithiothreitol and actinomycin D (Sigma); arsenite (Merck); MG-132, tunicamycin, thapsigargin, and cycloheximide (Calbiochem). Drugs were dissolved in appropriate solvent and further diluted in cell culture medium. Appropriate solvent controls and mock treatment were performed throughout the experiments.

Cell Culture and Treatment—HeLa, HEK293, MiaPaCa-2, and EGI-1 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum, 2 mmol/l L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. DANG and TFK-1 cells were cultured in RPMI 1640 supplemented identically. All cells were grown to a sub-confluent monolayer in a humidified atmosphere containing 5% CO2 at 37 °C. For UV irradiation, culture medium was removed and cells were exposed to UVC (254 nm) warmed phosphate-buffered saline. The lid of the cell culture dish was removed, and cells were covered with a small volume of pre-warmed phosphate-buffered saline. The lid of the cell culture dish was removed, and cells were exposed to UVC (254 nm) using a cross-linker (Stratagene). To generate osmotic stress, dishes were removed, and cells were exposed to cell culture medium. Appropriate solvent controls and mock treatment were performed after transfection.

Transient and Stable Transfection—Plasmids were delivered into cells using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s recommendations. Cells were transfected at a density of 70–80% and used for experiments 24 h after transfection. For stable expression of FLAG-MCL-1 in HeLa cells, the pcDNA3.2-FLAG-MCL-1 expression plasmid was linearized prior to transfection and the FLAG tag sequence was introduced at the 5′-side of MCL-1 cDNA. pCI-neo-EGFP was generated by PCR subcloning of EGFP cDNA obtained from pEGFP-C1 (BD Biosciences Clontech) into pCI-neo (Stratagene). pCI-neo-FLAG-eIF2α plasmids (wt, S51A, S51D) were generated by PCR subcloning of the respective eIF2α cDNAs obtained from expression plasmids kindly provided by David Ron. A FLAG tag was introduced at the 5′-side of the respective eIF2α cDNAs. pCI-neo-GyrB.PKR and the respective inactive mutant (pCI-neo-GyrB.PKR-K296H) were generated by PCR subcloning of GyrB.PKR cDNAs obtained from expression plasmids kindly provided by Tom Dever and described elsewhere (23). Integrity of all subcloned sequences was verified by automated DNA sequencing. Detailed cloning procedures and primer sequences are available on request.

Western Blot and Immunoprecipitation—For Western blot analysis, cells were harvested in Nonidet P-40 lysis buffer (50 mm HEPES, 150 mm NaCl, 1 mm EDTA, 0.5% Nonidet P-40, 10% glycerol), supplemented with protease inhibitors (Protease Inhibitor Mixture III; Calbiochem) and phosphatase inhibitors (17.5 mm β-glycerophosphate and Phosphatase Inhibitor Mixture I, Sigma). Western blot analysis was performed as described recently (24). Quantification of bands was performed from original scans using the Odyssey scanning software (Licor). Antibodies used for Western blot were: Rabbit anti-MCL-1 (Stressgen), rabbit anti-BAX (Santa Cruz Biotechnology), rabbit anti-BAX (Upstate), mouse anti-BCL-2 (Santa Cruz Biotechnology), rabbit anti-BCL-XL (Cell Signaling), rabbit anti-cleaved caspase-3 (Cell Signaling), rabbit anti-cleaved caspase-9 (Cell Signaling), mouse anti-β-actin (Sigma), mouse anti-PARP (Pharmingen), mouse anti-FLAG M2 (Sigma), rabbit anti-P-eIF2α (Ser51) (Epitomics), rabbit anti-eIF2α (Cell Signaling), rabbit anti-PKR (Cell Signaling).

Immunoprecipitations for active BAK and BAX were conducted after lysis of cells in CHAPS buffer (5 mm MgCl2, 137 mm KCl, 1 mm EDTA, 1 mm EGTA, 1% CHAPS, 20 mm Tris-HCl, pH 7.5), 1 mg/ml protein lysate was preclarified with 20 μl of A-/G-Sepharose (Santa Cruz Biotechnology) for 1 h. 2 μg of mouse anti-BAX 6A7 (Alexis) or mouse anti-BAX Ab-1 (Calbiochem), were added to 1 mg of cell lysate and incubated at 4 °C overnight. Antibodies were precipitated with 20 μl A-/G-Sepharose for 2 h and pellets were washed three times with PBS-PAGE and transferred to polyvinylidene difluoride membranes (Immobilon, Millipore). Precipitated BAX or BAK were detected by immunoblot as described above.

Quantitative RT-PCR—Total RNA was isolated from cells using the RNeasy kit (Qiagen). A DNase digestion step was included in all RNA isolation procedures to exclude DNA contamination. Specific mRNAs were quantified by real-time RT-PCR as described previously (24). Primer sequences are available on request. For detection of FLAG-tagged mRNAs, primer pairs specifically amplifying FLAG-tagged target sequences were chosen that did not amplify the corresponding untagged mRNAs or other FLAG-tagged sequences. Number of copies mRNA were determined by using standard curves generated by serial dilutions of plasmids containing the respective target sequence. Data are shown as relative number of copies mRNA. Data were obtained from at least three independent experiments.
Chromatin Condensation—Apoptotic nuclei were quantified by fluorescence microscopy after staining of chromatin with Hoechst 33342 according to the manufacturer’s recommendations. For each experiment, a minimum of 200 nuclei from three different microscopic fields were counted under blinded conditions.

RESULTS

A Mitochondrial Pathway of Apoptosis Initiation Is Activated in Response to Classical Activators of the ISR—To study apoptosis initiation in response to activation of the ISR, HeLa cells were exposed to a number of classical ISR activators: the ER-stress inductor tunicamycin (Fig. 1A), UVC (Fig. 1B), elevated osmotic pressure (Fig. 1C) and arsenite (Fig. 1D). All stress stimuli were applied at doses found to elicit a strong apoptotic response in HeLa cells. Activation of BAX and BAK was assayed by immunoprecipitation using conformation-specific antibodies (25, 26). Cleavage of caspase-9 and caspase-3 was detected by Western blot. All applied stress stimuli synchronously induced the activation of BAX and BAK as well as cleavage of caspase-9 and caspase-3 (Fig. 1, A–D, lanes 1–4). Biochemical signs of apoptosis were accompanied by characteristic morphological changes such as cytoplasmic shrinkage and condensation of chromatin (data not shown). Parallel experiments were conducted in the presence of ZVAD-fmk, a broad-spectrum caspase inhibitor. Addition of ZVAD-fmk strongly inhibited cleavage of caspase-9 and caspase-3 (Fig. 1, A–D, lanes 5–8) as well as condensation of chromatin (data not shown). However, application of ZVAD-fmk did not affect the activation of BAX and BAK, indicating that BAX and BAK were activated upstream of caspases and that apoptosis was initiated at the mitochondria.

FIGURE 1. Early and selective down-regulation of MCL-1 during apoptosis initiation in response to classical activators of the ISR. A–D, HeLa cells were exposed to (A) the ER-stress inductor tunicamycin (2 μg/ml), (B) UVC (100 J/m²), (C) elevated osmotic pressure (400 mosm/l NaCl in excess for 30 min), or (D) arsenite (200 μM) in the presence of 0.1% Me₂SO (DMSO) or ZVAD-fmk (50 μM). Initiation of apoptosis was assayed by immunoprecipitation for active BAX and BAK and by immunoblotting for cleavage of caspase-9 and -3 at indicated time points. Protein levels of pro- and anti-apoptotic (BCL-2, BCL-xL, and MCL-1) multidomain BCL-2 proteins were determined by immunoblotting. Membranes were stripped and re-probed with β-actin as a loading control.
Down-regulation of MCL-1 Precedes Apoptosis Initiation in Response to Cellular Stress—Mitochondrial integrity is preserved by the balance of pro- and anti-apoptotic members of the BCL-2 protein family. To uncover alterations within the levels of pro- and anti-apoptotic multidomain BCL-2 proteins during apoptosis initiation in response to the applied ISR activators, we performed Western blot analysis for BAX, BAK, BCL-2, BCL-xL, and MCL-1 (Fig. 1, A–D, lanes 1–4). Strikingly, all applied stress stimuli induced the rapid and selective down-regulation of the anti-apoptotic BCL-2 protein MCL-1. Down-regulation of MCL-1 preceded all further events of apoptosis initiation and was not affected by addition of ZVAD-fmk (Fig. 1, A–D, lanes 5–8). However, although the initial decrease in MCL-1 levels remained unaffected by ZVAD-fmk, reappearance of MCL-1 occurred in response to tunicamycin, osmotic stress, and arsenite when caspase activity was blocked (Fig. 1, A, C, D, lanes 5–8).

Down-regulation of MCL-1 in Response to Cellular Stress Is Not Restricted to a Single Cell Type—To ensure that the rapid down-regulation of MCL-1 in response to the applied stress stimuli is not restricted to a single cell type, we exposed several cancer cell lines to tunicamycin, UVC, elevated osmotic pressure, and arsenite, and studied MCL-1 protein levels. Cell lines examined were MiaPaCa-2 and DANG pancreatic cancer cells as well as TFK-1 and EGL-1 cholangiocarcinoma cells. As shown in Fig. S1, MCL-1 was rapidly lost in response to all of the applied stresses in all cell lines tested.

Stabilization of MCL-1 by Proteasome Inhibition Prevents Stress-induced Apoptosis—We next aimed to determine whether stabilizing MCL-1 under stressful conditions would alter stress-induced apoptosis. MCL-1 is a high turnover protein that is rapidly degraded by the proteasome (27). Consequently, addition of a proteasome inhibitor (MG-132), but not adequate solvent control, completely prevented the decrease in MCL-1 levels in response to UVC, osmotic stress and arsenite (Fig. 2, A–C, compare lanes 1–4 and 5–8). Moreover, MG-132 completely blocked mitochondrial apoptosis initiation as demonstrated by absent precipitation of active BAX and non-detectable cleavage of caspase-3. Because inhibition of the proteasome affects degradation and thereby increases concentration of a myriad of proteins, we next asked whether inhibition of apoptosis by MG-132 was dependent on the observed stabiliza-
tion of MCL-1. To this end, we transfected cells either with control siRNA or siRNA specifically targeting MCL-1 (siMCL-1-1, see also Fig. 3) and exposed them to the respective stresses in the presence of MG-132. As shown in Fig. 2 (A–C, compare lanes 9–12 and lanes 13–16), MG-132 blocked apoptosis initiation in controls (lanes 9–12), but not in cells with strongly reduced MCL-1 protein content (lanes 13–16). These findings indicate that stabilization of MCL-1 at pre-existing levels accounted for the blockage of apoptosis initiation in the presence of MG-132. Of note, addition of MG-132 alone did not induce apoptosis during the time course of these experiments in both controls and cells with reduced MCL-1 abundance. However, significant toxicity occurred when cells were exposed to proteasome inhibitors for longer incubation times (~12 h). Therefore, we could not perform parallel experiments with ER-stress-inducing agents that required longer incubation times to induce apoptosis.

**FIGURE 3.** MCL-1 is a strong inhibitor of apoptosis in response to activators of the ISR. A, HeLa cells were either left untreated or were transfected with control siRNA or with each one of two different siRNAs specifically targeting MCL-1 (siMCL-1-1, siMCL-1-2). Protein lysates were assayed for MCL-1 protein content by immunoblotting 24 h after transfection. B, untreated HeLa cells (wt), and cells transfected with control siRNA or siRNA specifically targeting MCL-1 (siMCL-1-1, siMCL-1-2) were exposed to increasing doses of tunicamycin, UVC, osmotic stress, and arsenite as indicated. Cells were stained with H33342 after 4 h (UVC, osmotic stress, arsenite) or 24 h (tunicamycin). Chromatin condensation was assayed by fluorescence microscopy. C, HeLa cells transfected with nontargeting siRNA (control) or siMCL-1-1 were exposed to increasing doses of tunicamycin, UVC, osmotic stress, and arsenite as indicated. PARP cleavage was assayed by immunoblotting, β-actin served as loading control.
MCL-1 Is a Survival Factor in Response to Cellular Stress—In contrast to other anti-apoptotic BCL-2 proteins like BCL-xL and BCL-2, MCL-1 is a short-lived protein that undergoes continuous regulation at the level of transcription, translation, and degradation (28). We therefore aimed to determine whether alterations in MCL-1 protein levels would affect the cellular response to the applied ISR activators. To this end, we modified MCL-1 protein expression through RNA interference and conducted dose-response experiments. In these experiments, apoptosis was assayed by quantification of chromatin condensation (Fig. 3B) and immunoblotting for PARP cleavage (Fig. 3C). Fig. 3A demonstrates efficient knockdown of MCL-1 protein after transfection of cells with two different siRNAs (siMCL-1-1, siMCL-1-2) specifically targeting MCL-1. In line with findings reported by others (29), down-regulation of MCL-1 protein alone was not sufficient to induce cell death (Fig. 3, B and C). Furthermore, cells with reduced MCL-1 protein levels proliferated normally and exhibited no gross morphological changes (data not shown). However, cells with reduced MCL-1 protein expression levels were strongly sensitized to the applied stress stimuli and underwent apoptosis in response to very mild stress conditions (Fig. 3, B and C). These findings indicate that MCL-1 protein levels determine apoptosis sensitivity under stressful conditions.

Cellular Stress Does Not Affect MCL-1 Protein Turnover—We next asked for the molecular mechanisms mediating down-regulation of MCL-1 in response to the applied activators of the ISR. Throughout these experiments, the more rapidly acting ER-stress inducing drug thapsigargin was used instead of tunicamycin. As shown in Fig. 4A, elimination of MCL-1 in response to the applied stresses occurred very rapidly. To determine whether this was due to an increase in MCL-1 protein turnover, the kinetics of MCL-1 protein degradation were uncovered by treating cells with the translation inhibitor cycloheximide. At the same time, cells were exposed either to mock treatment (Fig. 4B, lanes 1–4) or to stress stimuli as indicated (Fig. 4B, lanes 5–8). Addition of cycloheximide uncovered a short constitutive MCL-1 protein half-life of ~30 min in HeLa cells (Fig. 4B, lanes 1–4). Notably, none of the applied stresses...
further accelerated the disappearance of MCL-1 protein when compared with mock treatment (Fig. 4B, compare lanes 5–8 and 1–4). These findings suggest that the rapid decrease in MCL-1 levels in response to cellular stress is not mediated by enhanced MCL-1 protein degradation.

**Different Types of Stress Exhibit Different Effects on MCL-1 mRNA**—We next studied MCL-1 mRNA behavior in response to the applied stress stimuli. As shown in Fig. 4C, no uniform effect on MCL-1 mRNA was observed. Exposure of cells to UV light induced a rapid and sustained loss of MCL-1 mRNA, while...
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UV-induced MCL-1 mRNA Degradation Is Dispensable for UV-induced Down-regulation of MCL-1 Protein—The rapid decrease of MCL-1 mRNA in response to UVC paralleled the kinetics of MCL-1 protein disappearance. To determine whether UV-induced down-regulation of MCL-1 mRNA was required for the down-regulation of MCL-1 protein, we further characterized UV-induced loss of MCL-1 mRNA. As shown in Fig. 5A, UVC induced a dose-dependent decline in MCL-1 mRNA levels. Treatment of cells with actinomycin D to block novel mRNA synthesis uncovered a MCL-1 mRNA half-life of ~2 h (Fig. 5B). Exposure of actinomycin D-treated cells to UVC strongly enhanced MCL-1 mRNA decay, followed by a stabilization of the remaining pool (Fig. 5B), indicating that UV light interfered with MCL-1 mRNA stability. Regulation of mRNA stability during cellular stress has been found to depend on regulatory elements located within the 5'- and 3'-untranslated regions of eukaryotic mRNAs (30). Therefore, we tested whether transgenic MCL-1 mRNA devoid of 5'- and 3'-untranslated regions would undergo UV-induced degradation. Two independent HeLa cell clones (37 and 9) raised after stable transfection with pcDNA-3.2-FLAG-MCL-1 were exposed to UV light and both mRNA (Fig. 5C) and protein (Fig. 5D) levels of FLAG-tagged MCL-1 and endogenous MCL-1 were studied. As shown in Fig. 5C, endogenous MCL-1 mRNA was rapidly down-regulated in response to UVC, while FLAG-MCL-1 mRNA remained largely unaffected. However, at protein level, both FLAG-tagged MCL-1 and endogenous MCL-1 protein were eliminated with identical kinetics (Fig. 5D), indicating that the down-regulation of MCL-1 protein occurred independently of MCL-1 mRNA behavior. In summary, all of the applied stresses affected MCL-1 protein abundance downstream of MCL-1 mRNA levels, and upstream of MCL-1 protein degradation, suggesting blockade at the level of mRNA translation.

Phosphorylation of eIF2α Parallels the Suppression of MCL-1 Protein—Manifold stresses have been shown to induce global translational arrest by inducing the phosphorylation of the α-subunit of eukaryotic translation initiation factor 2 (eIF2α) at Ser51, leading to decreased formation of the ternary complex required for the binding of Met-tRNA<sub>Met</sub> to the 40 S ribosomal subunit (5). To determine whether stress-induced down-regulation of MCL-1 was related to eIF2α phosphorylation, the kinetics of eIF2α phosphorylation and MCL-1 down-regulation were studied in parallel. As shown in Fig. 6, all of the applied stresses induced a strong phosphorylation of eIF2α that was temporally paralleled by the down-regulation of MCL-1. In detail, exposure of cells to UVC induced a rapid, strong and sustained eIF2α phosphorylation that inversely correlated with MCL-1 protein levels (Fig. 6A). Osmotic stress induced a strong but transient phosphorylation of eIF2α, which was paralleled by a sudden but transient decrease of MCL-1 protein (Fig. 6B). Similarly, the reducing agent and ISR activator DTT, arsenite (Fig. 6C), thapsigargin and tunicamycin (Fig. 6D) induced eIF2α phosphorylation and down-regulation of MCL-1 protein in parallel.

FIGURE 6. Stress-induced down-regulation of MCL-1 is paralleled by phosphorylation of eIF2α. A, HeLa cells were exposed to UVC (100 J/m²), and cells were harvested in whole cell lysis buffer supplemented with phosphatase inhibitors as described under “Experimental Procedures.” Phosphorylation of eIF2α at Ser51 was detected by immunoblotting using a phosphospecific antibody. Total eIF2α, MCL-1, and β-actin were visualized after stripping of the membrane. B, HeLa cells were exposed to elevated osmotic pressure (400 mosm/liter NaCl in excess for 30 min), and immunoblotting was performed as indicated. C, treatment of HeLa cells with the reducing agent and ISR activator dithiothreitol (DTT, 1 mM) or arsenite (200 μM) as indicated. D, HeLa cells were exposed to thapsigargin (1 μM) or tunicamycin (2 μg/ml) and immunoblotting was performed as indicated.
Phosphorylation of eIF2α Is Essential for the Down-regulation of MCL-1 Protein Levels in Stressed Cells—To determine whether phosphorylation of eIF2α at Ser37 is essential for the down-regulation of MCL-1 in response to activators of the ISR, we overexpressed a non-phosphorylatable eIF2α mutant (pCI-neo-FLAG-eIF2α-S51A) in HeLa cells. Overexpression of eIF2α-S51A has been shown to abrogate the eIF2α phosphorylation pathway and enable ongoing mRNA translation during cellular stress (31). Controls were transfected with pCI-neo-FLAG-eIF2α-wt. To study MCL-1 protein alterations specifically in transfected cells, we co-transfected cells with an expression plasmid encoding for FLAG-MCL-1 (pcDNA3.2-FLAG-MCL-1) and either wild-type eIF2α (pcDNA3.2-FLAG-eIF2α-wt) or a non-phosphorylatable eIF2α mutant (pcDNA3.2-FLAG-eIF2α-S51A). 24 h later, cells were exposed to (A) UVC (100 J/m²), (B) elevated osmotic pressure (400 mosm/liter NaCl in excess for 30 min), (C) arsenite (200 μM), or (D) tunicamycin (2 μg/ml), and harvested after indicated time periods. Protein levels of FLAG-MCL-1 and FLAG-eIF2α were determined by Western blot for FLAG and were distinguished from each other by their different molecular weight. Total eIF2α and β-actin protein levels were determined by Western blot after stripping and re-probing the membrane.

Expression of a Phosphomimetic eIF2α Mutant Is Sufficient to Suppress MCL-1 Protein Expression—We next asked whether phosphorylation of eIF2α at Ser37 is sufficient to down-regulate MCL-1 protein. Therefore, we transiently expressed eIF2α mutants mimicking different phosphorylation states of eIF2α in HEK293 cells, and studied endogenous MCL-1 protein levels. Of note, the respective eIF2α mutants act by mimicking different eIF2α phosphorylation states and not by altering endogenous eIF2α phosphorylation (32), which therefore was not assayed in these experiments. As shown in Fig. 8A, expression of the phosphomimetic mutant eIF2α-S51D (pCI-neo-FLAG-eIF2α-S51D) suppressed MCL-1 protein expression (lane 3), while expression of the non-phosphorylatable mutant eIF2α-S51A (pCI-neo-FLAG-eIF2α-S51A) induced a slight increase in MCL-1 protein levels (lane 4) when compared with cells transfected with pCI-neo-EGFP (lane 1) or pCI-neo-FLAG-eIF2α-wt (lane 2). In contrast, MCL-1 mRNA levels remained unaffected by these interventions (Fig. 8A, middle panel). Notably, FLAG-eIF2α mRNA abundance was virtually equal in all groups (Fig. 8A, lower panel), while FLAG-eIF2α protein expression was reduced in cells transfected with pCI-neo-FLAG-eIF2α-S51D (Fig. 8A, lane 3), suggesting that the inhibition of translation induced by FLAG-eIF2α-S51D also interfered with its own protein expression. Transfection efficiency in HEK293 cells did not exceed 70–80%, potentially leading to an underestimation of effects occurring within transfected cells. Therefore, parallel experiments were conducted with HeLa cells co-transfected with pcDNA3.2-FLAG-MCL-1 and the respective eIF2α mutants. In these experiments, expression of the phosphomimetic mutant FLAG-eIF2α-S51D almost entirely suppressed FLAG-MCL-1 protein abundance and when compared with cells expressing FLAG-eIF2α-wt and FLAG-eIF2α-S51A (Fig. 8B, compare lane 2 and lanes 1 and 3).

Activation of PKR Induces the Elimination of MCL-1—To test whether phosphorylation of eIF2α by one of its upstream kinases results in elimination of MCL-1, we used a chemically activable fusion construct containing the catalytic domain of the dsRNA-activated protein kinase (PKR) and Escherichia coli gyrase B (23). PKR is one of four kinases known to phosphorylate eIF2α at Ser37 (33). The GyrB.PKR fusion protein undergoes rapid dimerization and activation upon addition of coumermycin. As shown in Fig. 8C, HEK293 cells were transfected with pCI-neo expression plasmids encoding for EGFP (lanes 1 and 2), GyrB.PKR-wt (lanes 3 and 4) or the catalytically inactive mutant GyrB.PKR-K296H (lanes 5 and 6). Addition of coumermycin induced an increase in eIF2α phosphorylation as well as a partial loss of MCL-1 protein in cells expressing
GyrB.PKR-wt (lane 4), but not in controls (lane 2) or in cells expressing GyrB.PKR-K296H (lane 6). Furthermore, activation of GyrB.PKR-wt lead to a decrease of its own protein expression (lane 4), reminiscent of the findings obtained with expression of FLAG-eIF2α-S51D (pCI-neo-FLAG-eIF2α-S51D) or the non-phosphorylatable mutant eIF2α-S51A (pCI-neo-FLAG-eIF2α-S51A). Cells were harvested 16 h later, and immunoblot analysis of MCL-1, FLAG-eIF2α, and β-actin protein content were performed. In parallel experiments, MCL-1 mRNA (middle panel) and FLAG-eIF2α mRNA (lower panel) expression levels were determined by real time RT-PCR using primer pairs amplifying MCL-1 and specifically FLAG-tagged eIF2α. B, HeLa cells were co-transfected with expression plasmids encoding for FLAG-tagged MCL-1 (pDNA3.2-FLAG-MCL-1) and either wild-type eIF2α (pCI-neo-FLAG-eIF2α-wt), the phosphomimetic mutant eIF2α-S51D (pCI-neo-FLAG-eIF2α-S51D) or the non-phosphorylatable mutant eIF2α-S51A (pCI-neo-FLAG-eIF2α-S51A). Cells were harvested 16 h after transfection. Protein levels of MCL-1, FLAG-eIF2α, and β-actin were determined by Western blot. MCL-1 mRNA (middle panel) and FLAG-eIF2α mRNA levels (lower panel) were determined by real-time RT-PCR in parallel experiments. C, HEK293 cells were transiently transfected with expression plasmids for wild-type eIF2α (pCI-neo-FLAG-eIF2α-wt), the phosphomimetic mutant eIF2α-S51D (pCI-neo-FLAG-eIF2α-S51D) or the non-phosphorylatable mutant eIF2α-S51A (pCI-neo-FLAG-eIF2α-S51A). Cells were harvested 16 h after transfection. Immunoblotting for MCL-1, PKR, P-eIF2α, eIF2α, and β-actin proteins was performed. D, HeLa cells were co-transfected with expression plasmids encoding for FLAG-tagged MCL-1 (pDNA3.2-FLAG-MCL-1) and either EGFP (pCI-neo-EGFP; lanes 1), GyrB.PKR-wt (pCI-neo-GyrB.PKR; lanes 3), or GyrB.PKR-K296H (pCI-neo-GyrB.PKR-K296H; lanes 5). 24 h after transfection, coumermycin or solvent control was added, and cells were harvested for Western blot 4 h later.

FIGURE 8. Phosphorylation of eIF2α is sufficient to eliminate MCL-1. A, HEK293 cells were transiently transfected with control vector (pCI-neo-EGFP) or expression plasmids for wild-type eIF2α (pCI-neo-FLAG-eIF2α-wt), the phosphomimetic mutant eIF2α-S51D (pCI-neo-FLAG-eIF2α-S51D) or the non-phosphorylatable mutant eIF2α-S51A (pCI-neo-FLAG-eIF2α-S51A). Cells were harvested 16 h later, and immunoblot analysis of MCL-1, FLAG-eIF2α, and β-actin protein content were performed. In parallel experiments, MCL-1 mRNA (middle panel) and FLAG-eIF2α mRNA (lower panel) expression levels were determined by real time RT-PCR using primer pairs amplifying MCL-1 and specifically FLAG-tagged eIF2α. B, HeLa cells were co-transfected with expression plasmids encoding for FLAG-tagged MCL-1 (pDNA3.2-FLAG-MCL-1) and either wild-type eIF2α (pCI-neo-FLAG-eIF2α-wt), the phosphomimetic mutant eIF2α-S51D (pCI-neo-FLAG-eIF2α-S51D) or the non-phosphorylatable mutant eIF2α-S51A (pCI-neo-FLAG-eIF2α-S51A). Cells were harvested 16 h after transfection. Protein levels of MCL-1, FLAG-eIF2α, and β-actin were determined by Western blot. MCL-1 mRNA (middle panel) and FLAG-eIF2α mRNA levels (lower panel) were determined by real-time RT-PCR in parallel experiments. C, HEK293 cells were transiently transfected with expression plasmids encoding for FLAG-tagged MCL-1 (pDNA3.2-FLAG-MCL-1) and either wild-type eIF2α (pCI-neo-FLAG-eIF2α-wt), the phosphomimetic mutant eIF2α-S51D (pCI-neo-FLAG-eIF2α-S51D) or the non-phosphorylatable mutant eIF2α-S51A (pCI-neo-FLAG-eIF2α-S51A). Cells were harvested 16 h after transfection. Protein levels of MCL-1, FLAG-eIF2α, and β-actin were determined by Western blot. MCL-1 mRNA (middle panel) and FLAG-eIF2α mRNA levels (lower panel) were determined by real-time RT-PCR in parallel experiments. D, HeLa cells were co-transfected with expression plasmids encoding for wild-type eIF2α (pCI-neo-FLAG-eIF2α-wt), the phosphomimetic mutant eIF2α-S51D (pCI-neo-FLAG-eIF2α-S51D) or the non-phosphorylatable mutant eIF2α-S51A (pCI-neo-FLAG-eIF2α-S51A). Cells were harvested 16 h after transfection. Protein levels of MCL-1, FLAG-eIF2α, and β-actin were determined by Western blot. MCL-1 mRNA (middle panel) and FLAG-eIF2α mRNA levels (lower panel) were determined by real-time RT-PCR in parallel experiments. 

GyrB.PKR-wt (lane 4), but not in controls (lane 2) or in cells expressing GyrB.PKR-K296H (lane 6). Furthermore, activation of GyrB.PKR-wt lead to a decrease of its own protein expression (lane 4), reminiscent of the findings obtained with expression of FLAG-eIF2α-S51D (Fig. 8A, lane 3). Again, transfection efficiency in HEK293 cells was 70–80%. Parallel experiments were conducted with HeLa cells co-transfected with pcDNA3.2-FLAG-MCL-1 and the respective PKR constructs. As shown in Fig. 8D, addition of coumermycin induced a virtually complete elimination of FLAG-MCL-1 paralleled by increased eIF2α phosphorylation specifically in cells expressing GyrB.PKR-wt (Fig. 8D, compare lanes 3 and 4 to lanes 1 and 2 and 5 and 6). Taken together, these data show that phosphorylation of eIF2α is sufficient to rapidly and profoundly down-regulate MCL-1 protein.

**DISCUSSION**

Down-regulation of MCL-1 Enables but Not Enforces Apoptosis Initiation in Stressed Cells—In this study, we show that apoptosis initiation in cells challenged with stimuli activating the integrated stress response involves the rapid down-regulation of the anti-apoptotic BCL-2 family protein MCL-1. Elimination of MCL-1 has been shown to be required for apoptosis induction in response to a number of stimuli including DNA-damaging agents (29), adenovirus infection (34), anoxia (35),
and IL-3 withdrawal (36). Our findings point to a crucial role of MCL-1 in regulating the onset of apoptosis in stressed cells. In line, we found that artificial down-regulation of MCL-1 was sufficient to strongly sensitize cells to very mild stress conditions, suggesting that MCL-1 protein levels play a crucial role in determining cell fate under stressful conditions. This is of special importance since MCL-1 is unique among the group of anti-apoptotic members of the Bcl-2 family in that it is a highly regulated short lived protein responsive to multiple intracellular and extracellular signaling events (28). In line with other reports (29, 37), we found that down-regulation of MCL-1 alone was not sufficient to induce apoptosis. These results implicate that additional apoptotic events must coincide with down-regulation of MCL-1 to disrupt mitochondrial integrity in stressed cells. Activation of BIM has been found to be involved in UV-induced apoptosis (38) and BIM-deficient lymphocytes showed resistance toward apoptosis induction in response to ionomycin (39), a calcium ionophore known to induce ER-stress. Others have found induction of NOXA and PUMA to be involved in ER-stress-mediated apoptosis (40, 41). Further studies will be required to identify additional apoptotic events involved and to determine whether these signals are elicited in a stimulus-specific manner or generally occur with activation of the integrated stress response.

**Down-regulation of MCL-1 is the Consequence of Stress-induced Phosphorylation of eIF2α**—Our experiments show that down-regulation of MCL-1 in stressed cells is the consequence of stress-induced phosphorylation of eIF2α at Ser51. This conclusion is supported by the following observations: 1) None of the applied stress stimuli significantly affected MCL-1 protein turnover. 2) A profound and persistent decrease of MCL-1 mRNA occurred exclusively in response to UVC and was dispensable for UV-induced down-regulation of MCL-1 protein. 3) Phosphorylation of eIF2α at Ser51 temporally paralleled the decrease in MCL-1 and abrogation of this pathway prevented decrease of MCL-1 in stressed cells. 4) Mimicking eIF2α phosphorylation and phosphorylation of eIF2α by PKR in the absence of external stress stimuli was sufficient to down-regulate MCL-1. Various mechanisms have been shown to lead to a down-regulation of MCL-1 during apoptosis. In response to DNA damaging agents, elimination of MCL-1 occurred with unchanged protein turnover due to a stop of novel MCL-1 protein synthesis (29). Others have found that interaction with NOXA enhanced MCL-1 degradation (37, 42). Upon IL-3 withdrawal, accelerated degradation of MCL-1 occurred in response to phosphorylation by glycogen synthase kinase-3 (36). Our study presents a rather conclusive pathway, explaining why MCL-1 protein is rapidly lost in stressed cells. Manifold stresses converge on the phosphorylation of eIF2α at Ser51, leading to a global shutdown of mRNA translation, affecting virtually all mRNAs with a few exceptions (5). Consequently, expression levels of most cellular proteins will decrease with time, according to their respective protein half-lives. The consequential rapid decrease in the steady-state level of high-turnover proteins has recently been found to lead to the activation of NF-κB in response to eIF2α phosphorylation, mediated by the down-regulation of IkB proteins (8–10). The down-regulation of MCL-1 protein in response to eIF2α phosphorylation follows the same basic model. The decrease of MCL-1 occurs so rapidly, because its constitutively high protein turnover (27) demands a high rate of protein synthesis to maintain a high steady state level of MCL-1 protein. Thus, the global inhibition of mRNA translation arising from eIF2α phosphorylation in stressed cells exhibits specific effects on mitochondrial apoptosis signaling by rapidly removing MCL-1, acting as essential inhibitor of apoptosis initiation in stressed cells.

**Down-regulation of MCL-1 Couples Cellular Stress Signaling to Apoptosis**—Activation of the ISR has been found to promote both, death or survival, depending on the physiological context (13, 15). While the transcriptional branch of the ISR has been found to recruit death pathways requiring induction of the ATF4 target CHOP (43, 44), the translational branch, centered by eIF2α phosphorylation, has mainly been considered a protective pathway (11). This credo has been challenged by recent studies showing that eIF2α phosphorylation may also act as an important cellular death stimulus (15–18), raising the question of how eIF2α phosphorylation contributes to cell death. Our findings show that phosphorylation of eIF2α is coupled to the rapid down-regulation of the highly unstable anti-apoptotic protein MCL-1. We further found that this decrease of MCL-1 is required for apoptosis initiation in stressed cells. However, because down-regulation of MCL-1 alone is not sufficient to disrupt mitochondrial integrity, phosphorylation of eIF2α does not automatically induce cell death, making this pathway compatible with both, a pro-death and a pro-survival role of eIF2α phosphorylation, depending on concomitantly occurring signaling events. Further studies will be required to better dissect the interwoven pathways of death and survival activated by eIF2α phosphorylation in stressed cells, improving our understanding of how the life or death decision is made under stressful conditions.

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