Endoplasmic Reticulum Stress-induced Apoptosis

MUTLIPLE PATHWAYS AND ACTIVATION OF p53-UP-REGULATED MODULATOR OF APOPTOSIS (PUMA) AND NOXA BY p53

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Endoplasmic reticulum (ER) stress-induced apoptosis has been implicated in the development of multiple diseases. However, the in vivo signaling pathways are still not fully understood. In this report, through the use of genetically deficient mouse embryo fibroblasts (MEFs) and their matched wild-type controls, we have demonstrated that the mitochondrial apoptotic pathway mediated by Apaf-1 is an integral part of ER stress-induced apoptosis and that ER stress activates different caspases through Apaf-1-dependent and -independent mechanisms. In search of the molecular link between ER stress and the mitochondrial apoptotic pathway, we have discovered that in MEFs, ER stress selectively activates BH3-only proteins PUMA and NOXA at the transcript level through the tumor suppressor gene p53. In p53<sup>-/-</sup> MEFs, ER stress-induced apoptosis is partially suppressed. The p53-independent apoptotic pathway may be mediated by C/EBP homologous protein (CHOP) and caspase-12, as their activation is intact in p53<sup>-/-</sup> MEFs. In multiple MEF lines, p53 is primarily nuclear and its level is elevated upon ER stress. To establish the role of NOXA and PUMA in ER stress-induced apoptosis, we have shown that, in MEFs deficient in NOXA or PUMA, ER stress-induced apoptosis is reduced. Reversely, overexpression of NOXA or PUMA induces apoptosis as evidenced by the activation of BAK and caspase-7. Our results provide new evidence that, in MEFs, in addition to PUMA, p53 and NOXA are novel components of the ER stress-induced apoptotic pathway, and both contribute to ER stress-induced apoptosis.

The endoplasmic reticulum (ER)<sup>1</sup> is the site for synthesis, folding, modification, and trafficking of secretory and cell-surface proteins. As a major intracellular calcium storage compartment, the ER also plays a critical role toward maintenance of cellular calcium homeostasis. Disruption of these physiological functions by ER stress has been implicated in a wide variety of human diseases, including Alzheimer disease, Parkinson disease, neuronal damage by ischemia, prion disease, cystic fibrosis, and diabetes mellitus.<sup>1</sup> ER stress could also be elicited in the cell culture system by pharmacological agents including tunicamycin (Tun), a protein N-glycosylation inhibitor; brefeldin A (BFA), which blocks protein transport from ER to Golgi; and thapsigargin (TG), which blocks ER uptake of calcium by inhibiting the sarcomplasmic/endoplasmic reticulum Ca<sup>2+</sup>-ATPase (SERCA) (2). A variety of ER stresses result in unfolded protein accumulation, which triggers the unfolded protein response (1, 3). For survival, the cells induce ER chaperone proteins to alleviate protein aggregation, transiently attenuate translation, and activate the proteasome machinery to degrade misfolded proteins. Nonetheless, under severe and prolonged ER stress, unfolded protein response activates unique pathways that lead to cell death through apoptosis (4).

Currently, several pathways have been directly implicated in ER stress-induced apoptosis. For example, the transcription factor CHOP/GADD153 is induced by ER stress at the transcript level, which sensitizes cells to ER stress through down-regulating BCL-2 and activating GADD34 and ERO1α, an ER oxidase (5, 6). ER stress also activates the ER transmembrane protein kinases type I ER membrane protein kinase (IRE1) and PKR-like ER kinase (PERK), which have been implicated in the activation of the pro-apoptotic c-Jun NH<sub>2</sub>-terminal kinase (JNK) (7, 8). Furthermore, ER stress leads to proteolytic cleavage of caspase-12 (C-12) in mouse and caspase-4 (C-4) in human, both of which localize to the cytoplasmic side of the ER membrane (9, 10). Evidence is also emerging that there is cross-talk between the ER and the mitochondria.

The mitochondria-initiated apoptotic pathway is a major one in mammalian cells and is tightly regulated by BCL-2 family proteins. BAX and BAK are pro-apoptotic members activated by a variety of apoptotic stimuli, leading to oligomerization and insertion into the mitochondrial outer membrane to release cytochrome c (4, 11, 12). Cytochrome c binds to Apaf-1 and caspase-9 (C-9), resulting in the activation of C-9 and the subsequent activation of caspase-3 (C-3) and caspase-7 (C-7) and ultimately cell death. Recent evidence reveals that BAX/BAK can also localize to the ER and are activated in response to ER stress, leading to calcium depletion and murine caspase 12 (C-12) activation (13, 14). In MEFs deficient in BAX or BAK, ER stress-mediated C-12 cleavage is abolished but can be restored by the introduction of ER-targeted BAK. However, because BAX/BAK can activate apoptosis from both the mitochondria and the ER, it remains to be determined how dependent the mitochondria-initiated apoptotic pathways are on ER stress-induced apoptosis <i>in vivo</i>. Also, this raises the important issue regarding the molecular links between ER stress and initiation of the mitochondrial apoptotic pathways.

In mitochondria-initiated apoptosis, the activation of BAX/BAK involves members of the BH3-only BCL-2 family proteins, which are essential initiators of apoptotic cell death. This large group of proteins share only the BH3 domain, which is used for binding to the anti-apoptotic members of the BCL-2 family and for inducing apoptosis (15, 16). Among this group of proteins, only a subset is under stringent transcrip-

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2. The abbreviations used are: ER, endoplasmic reticulum; BFA, brefeldin A; Etop, etoposide; FITC, fluorescein isothiocyanate; MEFs, mouse embryo fibroblasts; PBS, phosphate-buffered saline; PI, propidium iodide; TG, thapsigargin; Tun, tunicamycin; FACS, fluorescence-activated cell sorter; WT, wild-type; CMV, cytomegalovirus; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PUMA, p53-up-regulated modulator of apoptosis; CHOP, C/EBP homologous protein; BH3, BCL-2 homology domain 3.
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EXPERIMENTAL PROCEDURES

Cell Culture—Purified wild-type (WT) 
Apaf-1 
/+ and Apaf-1 
−/− MEFs (18) were provided by Dr. X. Wang (University of Texas Southwestern Medical Center, Dallas, TX). Paired early passage WT (Puma 
/+ and Puma 
−/− MEFs (19) were provided by Dr. G. P. Zambetti (St. Jude Children’s Hospital, Memphis, TN). Noxa 
/+ MEFs (20) were originally provided to Dr. G. Chinnadurai (St. Louis University, St. Louis, MO) by Dr. A. Strasser (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia). Paired WT (ps3 
−/− and pS3 
−/− MEFs (21) were provided by Dr. A. Green (La Jolla Institute for Allergy and Immunology, San Diego, CA). The fibroblasts and 293 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, l-glutamine and antibiotics. All cells were plated 1 day prior to drug treatment.

Plasmids—The full-length murine Noxa cDNA was made by reverse-transcribed PCR using total RNA from WT MEFs and the following primers: sense, 5′-TTC GAT GAT GCC CCG GAG AA-3′ and antisense, 5′-GGG AGG TCC CCT CCT GGA AA-3′. The PCR product was directly subcloned into PCR2.1 vector using the TA cloning kit (Invitrogen) and was verified by DNA sequencing. For Northern blots, pCMV-Puma (22) was provided by Dr. K. H. Vousden (Beatson Institute for Cancer Research).

Antibodies and Reagents—Detection of protein expression was performed by standard immunoblotting techniques using primary antibodies against C-3 (rabbit polyclonal antiserum, Cell Signaling), C-7 (mouse IgG1, Pharmingen), C-12 (rabbit polyclonal antiserum, Cell Signaling), CHOP (B3, mouse IgG1, Santa Cruz Biotechnology), GFP (mouse IgG, BD Biosciences), GRP78 (rabbit polyclonal antiserum, Stressgene), NOXA (M16, goat anti-mouse polyclonal antiserum, Santa Cruz Biotechnology), p53 (Pab241, mouse IgG2a for MEF immunostaining, Calbiochem; Pab240, mouse IgG1, for Western blot, Santa Cruz Biotechnology), PUMAα (N-terminal, rabbit polyclonal antiserum for mouse cells, Sigma; N-20, goat polyclonal antiserum for human cells, Santa Cruz Biotechnology), and β-actin (AC15, mouse IgG, Sigma). ER stress inducers TG, Tun, and BFA were purchased from Sigma. Etoposide (Etop) was from Calbiochem.

Detection of BAK Activation—Analysis of the conformational changes of BAK was performed as described previously (14). 293 cells were transfected with pEGFP or pEGFP-Noxa. Forty-eight hours later, a portion of the cells was used for GFP detection. The rest of the cells were fixed in 0.25% paraformaldehyde in phosphate-buffered saline (PBS) for 5 min. The cells were washed three times with PBS and incubated with 1:50 anti-mouse IgG1 (BD Biosciences) or anti-BAK (AM03; Oncogene Research Products) in 100 μg/ml digitonin (Sigma) in PBS for 30 min. After being washed with PBS three times, the cells were incubated with 1:100 fluorescein isothiocyanate (FITC)-conjugated anti-mouse Ig for 30 min. The cells were subjected to analysis by flow cytometry (FACStar; BD Biosciences).

Apoptosis Assays—Cell apoptosis of MEFs was assessed by flow cytometry after staining with annexin-V-FITC (Pharmingen) and propidium iodide (PI, Roche Applied Science) as described previously (23). In transient transfection assays, 293 cells were transfected with pEGFP, pEGFP-Noxa, pCMV-Puma alone, or in combination using Polyfect (Qiagen). The final concentration of the plasmid was adjusted to the same amount using pcDNA3 (Invitrogen). Forty-eight hours later, the cells were stained with phycocerythrin-conjugated annexin-V and 7-amino-actinomycin (7-AAD). The apoptosis of GFP-positive cells was analyzed by flow cytometry. For kinetics, cell apoptosis was assessed by flow cytometry and cell cycle analysis following permeabilization and staining with PI. Essentially, ER-stressed cells were trypsinized, washed in ice-cold PBS, and fixed in −20 °C 70% ethanol for 1 h at 4 °C. The cells were stored in −20 °C before preparation for analysis. The fixed cells were washed with cold PBS and treated with DNase-free RNase (200 units/ml, Roche Applied Science) for 30 min at 37 °C. Cellular DNA was stained with 50 μg/ml PI for at least 15 min at room temperature. The cells were analyzed by flow cytometry. Results represent the mean of triplicate determinations in which a minimum of 10,000 cells were assayed for each determination. Any sub-G1 population was counted as apoptotic cells.

Northern Blot Analysis—Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen) following the manufacturer’s procedure. Ten micrograms of total RNA per sample was subjected to Northern blot analysis, which was carried out as previously described (24). Probes were made by reverse-transcribed PCR using total RNA from WT MEFs and the following primers: BimL, sense, 5′-GCA CCC ATG AGT GTG GAC AA-3′ and antisense, 5′-TTCA AGG CCT CAT ACT ACC AG-3′; Bnip3 sense, 5′-GCT CCC AGA CAC CAC AAG AT-3′ and antisense, 5′-CAA GCC AAT GGC CAG CAG AT-3′; Dp-5 sense, 5′-GGG GAA AGC TGG TTC CTG TT-3′ and antisense, 5′-CCC ACC GGT CCA TGG AAG TT-3′; Nix sense, 5′-GAG ATG CAT ACC AGC AGG GA-3′ and antisense, 5′-CAC TTC ACA GGC CAC AGG AA-3′. The PCR products were subcloned into PCR2.1 and were verified by DNA sequencing. The cDNA probes were purified after digesting with EcoRI. Probes for Puma and Spike were prepared from CDNA clones (IMAGE identification numbers 6310857 and 2811290, respectively, Open Biosystems) after digesting with EcoRI/BamHI and EcoRI/HindIII, respectively. The probe for human Bik (25) was a gift from Dr. G. C. Shore (McGill University, Montreal, Quebec, Canada), and the probe for human Chop (26) was a gift from Dr. N. Holbrook (Yale University, New Haven, CT). Probes for Grp78 and GAPDH were prepared from expression plasmids as described previously (24). The mRNA levels were...
quantitated by a phosphorimaging device (Molecular Dynamics) using GAPDH as the loading control. The fold of induction was calculated by dividing the relative mRNA level by that of the untreated sample set as 1.

Western Blot Analysis—The cell lysates from treated or untreated cells were resuspended in lysis buffer (0.5% Nonidet P-40, 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, and proteinase inhibitor mixture (1 tablet/10 ml of lysis buffer, Roche Applied Science). After incubation on ice for 20 min, the homogenate was centrifuged at 14,000 revolutions/min for 15 min at 4°C. Thirty micrograms of total protein of the clarified supernatants was separated by 12 or 14% SDS-PAGE and transferred to nitrocellulose membrane. Western blot analysis was performed as described previously (23). All immunoblots were visualized by ECL (Amersham Biosciences).

Immunofluorescence Staining—Cells were plated in 4-well Lab-Tek II glass slides (Nalge Nunc International) a day before treatment and then exposed to TG (2 μM), Tun (3 μM), BFA (5 μg/ml), or Etop (50 μM) for 6, 24, and 20 h, harvested, and analyzed for apoptosis by PI staining and FACS analysis. Quantitation was performed by counting apoptotic cell population-exhibited sub-G₁ DNA content. Results are representative from three independent experiments. The standard deviations are shown.
mounting medium (Vector Laboratories) and visualized with a Zeiss LSM 510 dual photon confocal microscope.

RESULTS

Apaf-1 Is an Integral Component of ER Stress-induced Apoptosis—Apaf-1 binds to cytochrome c released from the mitochondria and recruits pro-C-9 to form the apoptosome and subsequently activates C-9 and downstream effector caspases (C-3, C-7). Thus, the activation of Apaf-1 serves as a major indicator for mitochondria-mediated apoptosis. To investigate the requirement of the mitochondrial apoptotic pathways, especially Apaf-1, in ER stress-induced apoptosis, Apaf-1<sup>-/-</sup> MEFs were either untreated or treated with TG (2 μM), Tun (3 μM), BFA (5 μg/ml), or Etop (50 μM) for 24 h. Cell lysates were analyzed for caspase activation following ER stress, cell lysates were prepared from Apaf-1<sup>-/-</sup> and matched WT (Apaf-1<sup>+/+</sup>) MEFs as described for Fig. 2. As shown in Fig. 1A, there was substantial reduction in the percentage of apoptotic cells in Apaf-1<sup>-/-</sup> MEFs under all treatment conditions, as compared with WT control. Although these results suggest that Apaf-1-mediated apoptotic pathways contribute significantly to cell death during the early phase (within 24 h) of ER stress treatment, it remains possible that, with more severe ER stress treatment and for a longer period of treatment, Apaf-1 could be dispensable. To test this, the same set of cells was treated with higher doses of TG, Tun, and BFA. The kinetics of apoptosis was determined by flow cytometry and cell cycle analysis following PI staining during the treatment period of up to 40 h. Our results reveal that, even under more severe ER stress conditions for prolonged periods of time, MEFs deficient in Apaf-1 were more resistant to apoptosis (20–40% apoptotic cells) as compared with WT cells (>80%) for all three ER stress inducers (Fig. 1B). The same was observed for Etop-treated cells. These observations provide direct evidence that Apaf-1 is an integral part of ER stress-induced cell death in vivo. On the other hand, it is also evident that the inhibition of apoptosis in Apaf-1<sup>-/-</sup> MEFs is not complete, indicating that pathways independent of Apaf-1 are in operation to execute ER stress-induced apoptosis.

ER Stress Activates Caspases through Apaf-1-dependent and -independent Pathways—To examine the effect of Apaf-1 deficiency on caspase activation following ER stress, cell lysates were prepared from Apaf-1<sup>-/-</sup> and their matched WT MEFs treated with ER stress inducers (TG, Tun, and BFA) and Etop, under conditions as described for Fig. 1A and matched WT (Apaf-1<sup>+/+</sup>) MEFs were either untreated or treated with ER stress inducers TG, Tun, and BFA. Following 24 h of ER stress treatment, the percentage of apoptotic cells was determined by flow cytometry after staining with annexin-V and propidium iodide. For comparison, the cells were also treated with Etop, a DNA-damaging agent known to initiate apoptosis through the mitochondrial pathway (28). As shown in Fig. 1A, there was substantial reduction in the percentage of apoptotic cells in Apaf-1<sup>-/-</sup> MEFs under all treatment conditions, as compared with WT control. Although these results suggest that Apaf-1-mediated apoptotic pathways contribute significantly to cell death during the early phase (within 24 h) of ER stress treatment, it remains possible that, with more severe ER stress treatment and for a longer period of treatment, Apaf-1 could be dispensable. To test this, the same set of cells was treated with higher doses of TG, Tun, and BFA. The kinetics of apoptosis was determined by flow cytometry and cell cycle analysis following PI staining during the treatment period of up to 40 h. Our results reveal that, even under more severe ER stress conditions for prolonged periods of time, MEFs deficient in Apaf-1 were more resistant to apoptosis (20–40% apoptotic cells) as compared with WT cells (>80%) for all three ER stress inducers (Fig. 1B). The same was observed for Etop-treated cells. These observations provide direct evidence that Apaf-1 is an integral part of ER stress-induced cell death in vivo. On the other hand, it is also evident that the inhibition of apoptosis in Apaf-1<sup>-/-</sup> MEFs is not complete, indicating that pathways independent of Apaf-1 are in operation to execute ER stress-induced apoptosis.

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FIGURE 2. Caspase activation by ER stress and inhibition in Apaf-1<sup>-/-</sup> cells. WT (Apaf-1<sup>+/+</sup>) and Apaf-1<sup>-/-</sup> MEFs were either untreated or treated with TG (2 μM), Tun (3 μM), BFA (5 μg/ml), or Etop (50 μM) for 24 h. Cell lysates were analyzed for caspase-7 pro-form (Pro-C7, 35 kDa) and cleaved form (Cleaved-C7, caspase-3 pro-form (Pro-C3, 35 kDa) and cleaved form (Cleaved C3, 17 kDa), caspase-12 pro-form (Pro-C12, 55 kDa) and cleaved form (Cleaved-C12, 42 kDa), GRP78 (as a positive ER stress response control), and β-actin (as the loading control) expression by Western blotting using antibodies sequentially.

FIGURE 3. Effect of ER stress on the transcript levels of BH3-only proteins. A, WT (Apaf-1<sup>+/+</sup>) MEFs were treated with TG (2 μM) for various periods (0, 2, 4, 8, 16 h). Total RNAs isolated were subjected to Northern blot analysis. The blot was sequentially hybridized with Bnip3, Nix, Spike, and GAPDH cDNA probes (lower panel), or BimL, Bik, Chop, and GAPDH cDNA probes (upper panel). B, WT (Apaf-1<sup>+/+</sup>) MEFs were treated with TG (2 μM), Tun (3 μM), BFA (5 μg/ml), or Etop (50 μM) for various periods (0, 2, 4, 8, 16 h). Total RNAs were subjected to Northern blot analysis. The blots were sequentially hybridized with Noxa, Puma, and GAPDH probes. C, the mRNA levels in cells treated with TG for 0, 2, and 4 h were quantitated, normalized against the GAPDH loading control, and the fold of induction plotted with the level in untreated cells (0 h) set as 1.
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and subjected to Western blot analysis. As shown in Fig. 2, for WT cells, C-7 was activated by TG, Tun, and BFA, as indicated by the detection of the cleaved form of C-7. For C-3, its cleaved form was observed in cells treated with TG but was below the detection limit in cells treated with Tun or BFA. As a control, Etop strongly activated both C-3 and C-7. In Apaf-1−/− MEFs, the activation of both C-3 and C-7 was largely inhibited (Fig. 2). In contrast, C-12 activation by TG and BFA, as evidenced by the appearance of its cleaved form, was independent of Apaf-1 (Fig. 2). Interestingly, in MEFs, Tun only weakly activated C-12, and its cleavage was detected after Etop treatment. The effectiveness of the ER stress inducers was confirmed by their ability to increase the level of the ER chaperone protein GRP78/Bip, and the loading of the protein samples were monitored by β-actin levels. These results indicate that ER stress strongly activates C-7 primarily by mitochondrial pathways downstream of Apaf-1, whereas C-12 activation by ER stress, such as with TG and BFA, is Apaf-1-independent and may contribute to Apaf-1-independent apoptosis in murine MEF cells.

The BH3-only Proteins PUMA and NOXA Are Selectively Activated at the Transcript Level upon ER Stress—BH3-only BCL-2 family proteins are regulators of BAK/BAX; as such, they are initiators for the mitochondrial apoptotic pathway. Among the mammalian BH3-only proteins, the transcript levels of BimL, Dp-5, Bnip-3, Nix, Puma, and Noxa could be induced by a variety of stimuli. Their mRNA levels, as well as those encoding Bik and Spike, were measured in primary MEFs following treatment with TG, Tun, and BFA by Northern blot. The induction of Chop served as a positive control, and mRNA loading was monitored by GAPDH levels. Examples of the Northern blots are shown in Fig. 3A and B, and the relative mRNA levels after TG treatment were quantitated, normalized against the loading control, and summarized in Fig. 3C. Our results show that Bnip-3, Spike, Nix, Bik, and BimL transcript levels were either not affected by ER stress or that the effect was transient and marginal. The level of Dp-5 mRNA was undetectable in MEFs (data not shown). In contrast, Puma and Noxa mRNA levels increased by ~3–4-fold within 2–4 h under all three ER stress treatment conditions and persisted for at least 16 h. The doublet appearance of the Puma transcripts is consistent with alternative splicing as previously reported (22, 29). As a positive control, their mRNA levels were also strongly activated in MEFs treated with Etop (Fig. 3B).

Puma and Noxa are target genes of p53 and are critical mediators of the apoptotic responses induced by p53. To test whether the ER stress induction of Puma and Noxa is p53-dependent, mRNA levels of Puma and Noxa were measured in p53−/− and matched WT (p53+/+) MEFs treated with either ER stress inducers (TG, Tun, BFA) or Etop as a positive control. ER stress induction was monitored by Chop mRNA activation and mRNA loading by GAPDH levels. Examples of the Northern blots are shown in Fig. 4A, and the results are summarized in Fig. 4B. We observed that, in primary MEFs, induction of Noxa by all three ER stress inducers was highly dependent on p53, as the induction level was suppressed drastically in p53−/− MEFs. For Puma, mRNA induction by TG, Tun, and BFA was also dependent on p53, although a low level of residual activation could be detected at 4 h. As a positive control, Etop induction of Noxa and Puma transcripts were blocked in p53−/− MEFs. These results indicate that, in MEFs, both Puma and Noxa mRNA induction by ER stress is largely p53-dependent.

Suppression of ER Stress-induced Apoptosis in p53−/− MEFs—As induction of Puma and Noxa by ER stress is largely dependent on p53 in MEFs and p53 itself has recently been shown to have a direct signaling role at the mitochondria in the induction of apoptosis (30), we investigated the requirement of p53 in ER-stress-induced apoptosis by utilizing the matched pair of WT (p53+/+) and p53−/− MEFs. Etop treatment was used as a positive control. As shown in Fig. 5A, apoptosis induced by TG treatment for 24 h was inhibited by ~50% in p53−/− MEFs compared with WT cells. This result was confirmed when the kinetics of apoptotic cell death were monitored during a 40-h period (Fig. 5B). These results reveal p53 as a novel component of the ER stress-induced apoptotic signaling pathway in MEFs. Nonetheless, the suppression of apoptosis in the p53 null cells was only partial, indicating that other independent pathways are involved in ER stress-induced apoptosis.

ER Stress Induces p53 Level in MEFs and Activates CHOP and C-12 Independent of p53—Because ER stress-induced apoptosis was inhibited in p53−/− MEFs, we examined whether p53 itself undergoes ER stress-induced changes. In WT (Puma+/+) MEFs, by 16 h of TG stress treatment, increase in p53 protein was evident (Fig. 6A). As a positive control, the level of CHOP was elevated by ER stress, and the p53 level was strongly induced by Etop (Fig. 6A). The induction of p53 by ER stress is not restricted to this particular WT MEF line, because in the
matched pair of WT (p53+/+) and the p53−/− MEFs, p53 induction by TG and Etop was readily detected in the WT cells (Fig. 6B). The PUMA protein exists in multiple isoforms due to alternative splicing (22, 29). The best-characterized form is PUMAα, which is readily detected by specific antibodies and is the focus of this study. In p53−/− MEFs, PUMA protein was strongly induced by TG and Etop. Interestingly, a weak but detectable induction of PUMA was observed in the p53−/− MEFs, where p53 expression was completely abolished, suggesting that, although PUMA induction by ER stress is mediated largely by p53 in MEFs, it may also occur via p53-independent pathways, albeit at a much lower level (Fig. 6B). We also attempted to detect endogenous NOXA protein expression by Western blot with commercially available antibodies. However, the signal in MEFs was below the detection limit (data not shown).

Utilizing the same matched pair of WT and the p53−/− MEFs, we investigated whether CHOP induction and C-12 activation by ER stress is p53-dependent. As shown in Fig. 6, B and C, TG treatment induced CHOP expression and C-12 activation, as demonstrated by the decrease of pro-C-12 and the increase of cleaved C-12, in both WT (p53+/+) and p53−/− MEFs. These results suggest that ER stress induction of CHOP and activation in C-12 may account in part for the p53-independent apoptosis in ER-stressed MEFs.
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Previously, it was reported that ER stress results in cytoplasmic relocation of p53 in some cell types within 3 h of treatment, leading to destabilization (31). In examining the distribution of endogenous p53 in the WT (p53+/−) MEFs by confocal microscopy, we observed that p53 is primarily nuclear throughout the TG treatment period (Fig. 6D). Further, consistent with the Western blot results, there was detectable increase in p53 staining at 24 h, even under exposure conditions to detect low levels of cytosolic staining. The p53+/− MEFs treated with Etop for 24 h showed substantial nuclear destruction with concomitant cytosolic distribution of p53 (Fig. 6D). As expected, the p53−/− MEFs did not show any p53 staining.

**ER-induced Apoptosis Is Suppressed in MEFs Deficient in Puma or Noxa**—Next we investigated the induction of PUMA by ER stress in independently derived MEFs. It is noted that, although ER stress results in a general transient translational arrest, CHOP translation is specifically elevated (32). As shown in Fig. 7A, the CHOP protein level was elevated in WT (Puma+/−) MEFs treated with all three ER stressors (TG, Tun, BFA) for 4 h, and by 16 h, the PUMA protein level was also elevated under all three stress conditions. Using MEFs derived from Apaf-1−/− or Apaf-1−/− mice, induction of PUMA was also observed in cells treated with TG (Fig. 7B). These results show that induction of PUMA by ER stress is independent of the origin of MEFs and is upstream of the Apaf-1 pathway. To test whether PUMA is required for ER stress-induced apoptosis in MEFs, Puma+/− and its matched WT (Puma+/+) control were subjected to ER stressors TG or Tun to assess apoptosis. The lack of PUMA expression in the Puma+/− MEFs was confirmed by Western blot (Fig. 7B). As shown in Fig. 7, C and D, apoptosis induced by TG or Tun was inhibited by ~50% in Puma+/− MEFs as compared with the WT cells. Direct comparison of the matched pair of Noxa+/− and Noxa−/− MEFs also revealed that apoptosis was reduced by ~30 and 60%, respectively, in TG- and Tun-treated cells (Fig. 7D). Therefore, both PUMA and NOXA contribute to ER stress-induced apoptosis.

**Overexpression of NOXA Activates BAK and C-7 and Induces Apoptosis**—Because NOXA is induced and required for ER stress-induced apoptosis in MEFs, it represents a novel component of the ER stress apoptotic signaling pathway. To establish further the pro-apoptotic role of murine NOXA, we constructed a fusion protein in which GFP was fused to the amino end of murine NOXA (33). Following transfection into 293 cells, its expression was detected by anti-GFP as well as murine-specific anti-NOXA antibodies (Fig. 8A). Overexpression of NOXA resulted in the conformational change of BAK that was recognized by a specific anti-Bak antibody indicative of BAK activation (Fig. 8B). Expression of NOXA also activated C-7 (Fig. 8C) and induced apoptosis, as measured by annexin-V labeling (Fig. 8D). Similarly, overexpression of human PUMA activated C-7 and induced apoptosis (Fig. 8, C and D). Further, co-expression of NOXA and PUMA induced more apoptosis than expression of NOXA or PUMA alone.

**DISCUSSION**

It is well established that prolonged ER stress can lead to cell apoptosis. Several novel pathways have been identified that can offer explanations on how cells trigger programmed cell death when faced with irreparable damages that cannot be rescued by the unfolded protein response. Despite these important discoveries, the in vivo molecular mechanisms underlying ER stress-induced apoptosis are just emerging. Furthermore, it is unclear whether observations derived from specialized cell lines reflect tissue-specific or general mechanisms and whether results from in vitro reconstitution assay systems apply to endogenous cellular mechanisms. In the current study, as proof-of-principle, we utilized matched pairs of genetically deficient MEFs to dissect the contribution and functional relationship of molecular components implicated in the onset of ER stress-induced apoptosis. In the process, we have identified a novel moiety, a BH3-only protein called NOXA, which in concert with PUMA can act to induce the apoptotic cascade. Further, our studies reveal that the tumor suppressor gene p53 is a novel component of the ER stress apoptotic pathway. Based on our findings, a summary for the interrelationship between the ER mitochondrial and nuclear signaling pathways leading to ER stress-induced apoptosis is summarized in Fig. 9.

First, we investigated whether the mitochondrial apoptotic pathway plays an essential role in ER stress-induced cell death. A previous study performed with the Apaf-1−/− immortalized the mouse embryo fibroblast cell line Sak2, and cell-free assays gave the first indication that ER stress-mediated caspase activation and cell death could proceed to some extent without Apaf-1 (34). Through the use of a matched pair of Apaf-1+/− and Apaf-1−/− primary MEFs and following the kinetics of the induction of cell death subjected to three different ER stress inducers at different doses, we provide evidence that the mitochondrial branch, as represented by Apaf-1-regulated pathways, plays a major role in ER stress-initiated cell death throughout the time course of stress treatment, as Apaf-1−/− MEFs resist ER stress-induced activation of C-3 and
FIGURE 7. PUMA protein induction by ER stress is upstream of Apaf-1 and contributes to ER stress-induced apoptosis. WT (Puma+/+) MEFs (A) or WT (Apaf-1+/+) and Apaf-1-/- MEFs (B) were treated with TG (2 μM) or Tun (3 μM) or BFA (5 μg/ml) for various periods (0, 4, 16 h). Cell lysates were analyzed for PUMA, CHOP, and anti-β-actin (as the loading control) expression by Western blotting using antibodies sequentially. Lysate from Puma-/- MEFs was used as a negative control.

C, WT (Puma+/+) MEFs were untreated or treated with TG (2 μM) or Tun (3 μM) for 24 h, harvested, stained with annexin-V-FITC and PI, and analyzed by flow cytometry. D, quantitation of the percentage of apoptotic cells (annexin-V-positive) from C and Noxa-/- MEFs with the same treatment and analysis. Results are representative from three independent experiments. The standard deviations are shown.
C-7 and cell death. However, there clearly are compensating pathways independent of Apaf-1 that account for the residual cell death in Apaf-1-/-/MEFs.

Activation of murine C-12 may represent one such pathway, because we observed that C-12 is proteolytically cleaved upon ER stress in both wild-type and Apaf-1-/-/MEFs. Our results also showed that, under in vivo conditions, despite activation of C-12 in Apaf-1-/-/MEFs, activation of C-3 or C-7 by ER stress was still inhibited in these same cells. Thus, in MEFs, it appears unlikely that in vivo C-12, upon activation, activates C-9, which in turn activates C-3 and C-7, as shown in vitro (35). Vice versa, it was also reported that C-12 could be activated by C-7 in vitro (36). However, C-7 is unlikely to be the major activator for C-12 in vivo, because in Apaf-1-/-/MEFs, there is no activation of C-7 in ER-stressed cells, yet C-12 is cleaved by TG and BFA. Nonetheless, one likely explanation for the apparent discrepancies is cell type-specific differences, as a recent report shows that Apaf-1-deficient myoblasts and MEFs differ in the processing of procaspase-9 in response to cytotoxic drugs (37).

The role of murine C-12 in mediating ER stress-induced cell death has been collaborated on in multiple systems using both gene knockout and C-12 suppressor protein approaches (10, 14, 38); nonetheless, there is still the unresolved issue of C-12-specific substrates and tissue-specific differences in its functionality (39, 40). Although human C-12 is confined to a subpopulation and has no role in apoptosis (41), a possible functional counterpart of C-12 in humans is C-4, which is the caspase most closely related to C-12 and is activated by ER stress in human cell cultures. Further, reduction of C-4 expression in human neuroblastoma cells by small interfering RNA
inhibits ER stress-induced cell death (9). Nonetheless, the contribution of C-12 and C-4 to ER stress-induced cell death may differ among cell types and differentiation stages.

In support of the diversity of the ER stress-induced apoptotic response, CHOP is another example of an Apaf-1-independent ER stress apoptotic pathway that may act in a tissue- or differentiation-specific manner (42). ER stress induces CHOP at the transcript level via multiple pathways, including ATF4, ATF6, and XBP-1 (43). Overexpression of CHOP results in cell growth arrest and apoptosis, effects that are antagonized by BCL-2 (6). CHOP-deficient mice show normal development and normal fertility but exhibit reduced apoptosis of the kidney proximal tubule epithelium in response to the ER stress agent Tn (42, 44). The pro-apoptotic property of CHOP is probably due to its promoting protein synthesis and oxidation in the stressed ER (5). Recently, two other molecules, valosin-containing protein and apoptosis-linked gene-2, are also implicated in mediating ER stress-induced cell death (45).

Previous studies using MEFs deficient in both Bak and Bax established that Bak/Bax play a major role in ER stress-induced apoptosis, both from ER and mitochondria sites (13, 14). Bak/Bax undergoes activation upon ER stress, resulting in ER calcium depletion and C-12 activation. This raises the question, what are the regulators of Bak/Bax that are triggered by ER stress? We show here that ER stress selectively induces BH3-only proteins PUMA and NOXA at the transcript level and that both Puma−/− and Noxa−/− MEFs resist TG stress-induced apoptosis. PUMA and NOXA are able to promote mitochondrial translocation and oligomerization of Bak and BAX, resulting in mitochondrial apoptosis (46), and ER stress has been reported to cause cytochrome c release from the mitochondria (47). It has been shown that overexpression of PUMA or NOXA induces apoptosis (22, 29, 33, 48). Murine PUMA shares 92% sequence identity with human PUMA. Human NOXA consists of 54 amino acids and contains one BH3 domain, whereas murine NOXA has 103 amino acids with two BH3 domains. We further establish here that murine NOXA can activate Bak and C-7, leading to apoptosis. Therefore, one consequence of the induction of PUMA and NOXA by ER stress may be the activation of the mitochondrial apoptotic pathway through Bak/BAX activation. A recent report further shows that PUMA is associated with a small chaperone protein p23 under normal culture conditions, and ER stress disrupts this interaction, which may activate PUMA (49).

Our finding that Noxa and Puma induction by ER stress is largely p53-dependent in MEFs and that ER stress-induced apoptosis is suppressed in p53−/− MEFs provide the first evidence that p53 is a novel regulatory component of ER stress-mediated cell death. PUMA and NOXA, both identified as p53 target genes, are essential mediators of p53-dependent apoptotic pathways (20, 33, 50). Nonetheless, ER stress still activates C-12 and induces CHOP in p53−/− MEFs, which explains that suppression of apoptosis p53−/− is partial and that multiple, compensatory pathways are operative for ER stress-induced apoptosis.

It has been reported that ER stress increases cytoplasmic localization and degradation of endogenous p53 in human primary WI-38 cells and to a lesser extent in HT1080 cells within 3 h of ER stress treatment (31). In contrast, our analysis of MEFs showed that p53 remained primarily nuclear up to 24 h of TG treatment. Although the protein level of p53 remained unchanged or showed a slight decrease within the first few hours of ER stress treatment in some cases, the p53 level was elevated by 16 h. Additional analysis of the p53 level in three independently derived MEFs and two cancer cell lines (MCF-7 and HCT116) following ER stress by Western blot showed that, although PUMA level is induced by ER stress in all of the cell lines, p53 level was most highly elevated in MEFs and to a lesser degree in MCF-7 and although the p53 level was not elevated in HCT116, there was no decrease following ER stress (data not shown). Plausible explanations for these apparent differences include the differential ability of different cell lines to regulate the relocation and degradation of p53 upon ER stress and that the outcome varies with the duration of stress treatment. The dependence of PUMA induction on p53 also differs among cell types. In particular, cancer cell lines may have evolved multiple pathways to induce PUMA following ER stress, consistent with a previous report that human osteosarcoma SAOS-2 cells lacking p53 can induce PUMA when treated with TG (17, 51) and our observation that HCT116 cells devoid of p53 show similar induction of PUMA by ER stress.

In summary, recent developments indicate that the ER is emerging as a new focal site for the initiation of endogenous cell death pathways. Evidence is accumulating that ER stress-induced apoptosis is an important factor in contributing to a variety of diseases, especially in neurodegenerative diseases, diabetes mellitus, and cancer. Investigations on the molecular mechanism underlying ER stress-induced apoptosis will provide important information for understanding disease progression and may provide specific targets for therapeutic intervention.

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