Cell

ER Stress Triggers Apoptosis by Activating BH3-Only Protein Bim

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DOI 10.1016/j.cell.2007.04.027

SUMMARY

Endoplasmic reticulum (ER) stress caused by misfolded proteins or cytotoxic drugs can kill cells and although activation of this pathway has been implicated in the etiology of certain degenerative disorders its mechanism remains unresolved. Bim, a proapoptotic BH3-only member of the Bcl-2 family is required for initiation of apoptosis induced by cytokine deprivation or certain stress stimuli. Its proapoptotic activity can be regulated by several transcriptional or posttranslational mechanisms, such as ERK-mediated phosphorylation, promoting its ubiquitination and proteasomal degradation. We found that Bim is essential for ER stress-induced apoptosis in a diverse range of cell types both in culture and within the whole animal. ER stress activates Bim through two novel pathways, involving protein phosphatase 2A-mediated dephosphorylation, which prevents its ubiquitination and proteasomal degradation and CHOP-C/EBPα-mediated direct transcriptional induction. These results define the molecular mechanisms of ER stress-induced apoptosis and identify targets for therapeutic intervention in ER stress-related diseases.

INTRODUCTION

Eukaryotic cells have specific signaling pathways and effecter mechanisms to deal with the temporal and developmental variations in endoplasmic reticulum (ER) load (Ron, 2002). The upstream signal that activates these pathways is referred to as ER stress and is defined as an imbalance between the load of resident and transit proteins in the ER and the organelle's ability to process that load. ER stress can be provoked by a variety of patho-physiological conditions, such as ischemia, viral infection, or mutations that impair resident/secretory protein folding (Ron, 2002) and has been implicated as an initiator or contributing factor in a range of diseases, including liver cirrhosis or type 2 diabetes (Rutishauser and Spiess, 2002). The ER stress response can promote cellular repair and sustained survival by reducing the load of unfolded proteins through global attenuation of protein synthesis and/or upregulation of chaperones, enzymes, and structural components of the ER (Kaufman, 2002). When ER stress is overwhelming, cells undergo apoptosis, and although this was initially reported to be mediated by caspase-12 (Nakagawa et al., 2000), this has been challenged (Saleh et al., 2006), and the mechanism is therefore still unclear.

BH3-only proteins constitute a subgroup of proapoptotic members of the Bcl-2 protein family that are essential for initiation of programmed cell death and stress-induced apoptosis in species as distantly related as *C. elegans* and mice (Huang and Strasser, 2000). BH3-only proteins share with each other and the Bcl-2 family at large only the short (9-16 aa) BH3 (Bcl-2 Homology) region, which is essential for their ability to kill cells and bind to prosurvival Bcl-2-like proteins (Huang and Strasser, 2000). Apoptosis induced by BH3-only proteins requires a second proapoptotic subgroup of the Bcl-2 protein family, the Bax/Bak-like proteins, which in healthy cells are kept in check by the prosurvival Bcl-2 family members (Wei et al., 2001).

The proapoptotic activity of BH3-only proteins can be regulated by a variety of transcriptional as well as post-translational control mechanisms (Puthalakath and Strasser, 2002). For example, *noxa* and *puma/bbc3* were discovered as p53-inducible genes (Vousden and Lu, 2002) and are now known to be essential for DNA damage-induced apoptosis (Jeffers et al., 2003; Villunger

et al., 2003). Moreover, upon cytokine withdrawal *bim* is transcriptionally upregulated in hemopoietic cells by FOXO3A, which in healthy cells is kept in check by PI3K/Akt-mediated phosphorylation (Dijkers et al., 2000). Bim and Bmf can be regulated by their interaction with DLC1 or DLC2 dynein light chains, which causes their sequestration to the microtubular dynein motor complex or the actin-bound myosin V motor complex, respectively (Puthalakath et al., 1999, 2001). In addition, in growth factor-stimulated cells, Bim levels are kept low by ERK-mediated phosphorylation, which targets it for ubiquitination and proteasomal degradation (Ley et al., 2005).

Gene targeting studies in mice have shown that Bim is a critical regulator of lymphocyte homeostasis, serves as a barrier against autoimmune disease (Bouillet et al., 1999), and is required for deletion of autoreactive T (Bouillet et al., 2002) and B cells (Enders et al., 2003) and shutdown of T cell immune responses (Hildeman et al., 2002). Experiments with cultured cells have shown that Bim is essential for apoptosis induced by certain Bcl-2-inhibitable stress stimuli, including cytokine deprivation or treatment with ionomycin or taxol, but not others, such as stimulation with phorbol ester (Bouillet et al., 1999). Here, we report that ER stress, induced by a broad range of cytotoxic stimuli, requires Bim for initiating apoptosis in diverse cell types, both in vitro and even in a pathological setting within the whole animal. ER stress increases Bim levels through CHOP-C/EBPα-dependent transcriptional activation and posttranslationally by protein phosphatase 2A (PP2A)-mediated dephosphorylation, which prevents its ubiquitin-dependent proteasomal degradation. This definition of the ER stress-induced apoptosis signaling pathways identifies potential targets for therapeutic intervention in diseases associated with ER stress.

RESULTS

ER Stress Results in Bim Upregulation

ER stress-induced apoptosis can be inhibited by overexpression of Bcl-2 or one of its homologs (Hitomi et al., 2004), indicating that this process is initiated by proapoptotic BH3-only proteins. We therefore treated MCF-7 breast carcinoma-derived cells with thapsigargin (TG), a selective inhibitor of SERCA (seroplasmic endoplasmic reticulum calcium ATPase) (Lewis, 2001) or, as a control, with taxol, a microtubule polymerizing agent that induces apoptosis in MCF-7 cells, and analyzed the levels of BH3-only proteins by Western blotting. Interestingly, treatment with thapsigargin but not exposure to taxol caused an increase in Bim protein levels (Figure 1A). Increased levels of *bim* mRNA were also seen in thapsigargin-treated MCF-7 cells (Figure 1B), but the magnitude of this increase ($\sim$2-fold) was less than the increase seen at the protein level ($\sim$5-fold) (compare Figures 1A and 1B). Thapsigargin-induced upregulation of Bim was also observed in mouse FDC-P1 myeloid cells (Figure 1C), embryonic fibroblasts (MEF) (Figure 1D) and thymocytes (Figure 1E). Moreover, treatment with other drugs that induce ER stress, such as tunicamycin, DTT or ionomycin, also caused a substantial increase in Bim in MCF-7 cells (Figure 1F). Induction of Bim appeared to be specific, because ER stress caused only a minor or no increase in the expression of the related BH3-only proteins Bid or Puma in FDC-P1 (Figure 1C) or MCF-7 cells (Figure 1G). Viral infection constitutes a patho-physiological stimulus that causes ER stress (Rutishauser and Spiess, 2002) and, remarkably, Bim levels rose in measles infected Vero monkey kidney epithelial cells (Figure 1H), coinciding with induction of the ER stress-induced transcription factor CHOP (see Figure S1 in the Supplemental Data available with this article online). Collectively, these results demonstrate that ER stress induced by diverse cytotoxic drugs or a patho-physiological stimulus causes an increase in *bim* mRNA and Bim protein levels in diverse cell types.

Bim Is Essential for ER Stress-Induced Apoptosis in Diverse Cell Types

We next used cells from Bim-deficient mice to examine whether this BH3-only protein is essential for ER stress-induced apoptosis. Upon treatment with tunicamycin or thapsigargin, $bim^{-/-}$ thymocytes survived significantly better than their WT counterparts (Figure 2A). Puma has been reported to be critical for ER stress-induced apoptosis of neuronal cells (Reimertz et al., 2003). We therefore also examined $puma^{-/-}$ thymocytes but found only minor resistance to tunicamycin and normal sensitivity to thapsigargin (Figure 2A) or ionomycin (E.M. and A.S., unpublished data).

Because we performed many of our biochemical analyses with MCF-7 cells, we wanted to verify that ER stress-induced apoptosis was also Bim-dependent in these cells. Stable transfection with an RNA interference (RNAi) construct (Bouillet et al., 2005) allowed us to generate MCF-7 cells with reduced Bim expression. These Bim-deficient MCF-7 cells were significantly resistant to thapsigargin-induced killing compared to parental cells or cells expressing a control RNAi construct (Figure 2B). However, after 72 hr, a substantial fraction of the Bim-deficient cells had died, although still significantly fewer compared to control cells (Figure 2B). This is likely a consequence of the fact that although the RNAi technique efficiently reduced *bim* mRNA and Bim protein levels in untreated MCF-7 cells, thapsigargin treatment was still able to increase Bim levels over time (Figures 2C and 2D). Knockdown of Bim expression using stable expression of an RNAi vector also protected Vero cells from apoptosis caused by measles infection (Figures 2E and 2F). These results demonstrate that diverse cell types require Bim for ER stress-induced apoptosis elicited by a broad range of cytotoxic or patho-physiological stimuli.

ER Stress Causes Bim Dephosphorylation

Because Bim is critical for ER stress-induced apoptosis, we examined the mechanisms that stimulate its proapoptotic activity. First, by using an intracellular calcium antagonist and cell surface calcium release activated channel

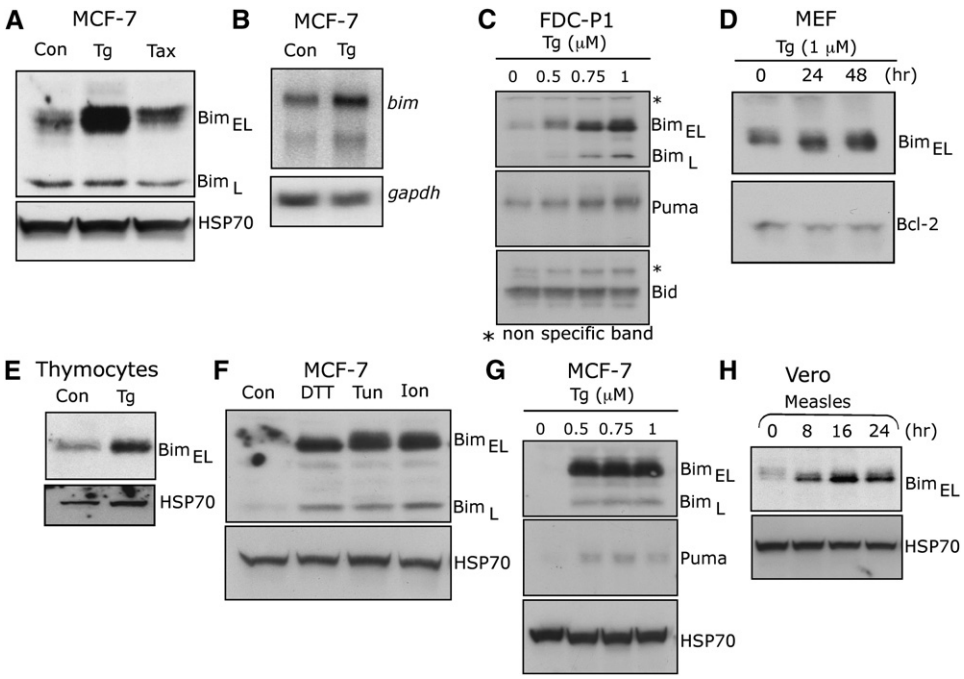

Figure 1. ER Stress Elicited by Diverse Stimuli Causes Increased Bim Expression in Various Cell Types

(A) MCF-7 cells were treated for 36 hr with either 1.5 μM thapsigargin (Tg) or 200 nM paclitaxel (Taxol, Tax) or were left untreated (Con) and protein samples (20 μg) were analyzed by Western blotting for Bim expression.

(B) PolyA+ RNA was isolated from MCF-7 cells that were left untreated (Con) or had been treated for 24 hr with thapsigargin (Tg, 1.5 μM) and subjected to Northern blot analysis for levels of *bim* mRNA (3 μg of polyA+ RNA/lane). Hybridization to a *gapdh* probe was used as a loading control.

(C) FDC-P1 myeloid cells were treated for 24 hr with the indicated concentrations of thapsigargin and Western blotting performed to determine the levels of Bim, Puma, and Bid.

(D) Mouse embryonic fibroblasts (MEF) were analyzed for Bim expression by Western blotting after treatment for 0, 24, or 48 hr with 1.0 μM thapsigargin.

(E) Mouse thymocytes were treated for 7 hr with 37.5 nM thapsigargin and analyzed for Bim expression by Western blotting.

(F) MCF-7 cells were left untreated (Con) or treated for 24 hr with different compounds that induce ER stress (1 mM Dithiothreitol [DTT], 10 μg/mL tunicamycin [Tun], or 10 μg/mL ionomycin [Ion]) and Bim protein levels analyzed as in (A).

(G) MCF-7 cells were treated for 36 hr with the indicated concentrations of thapsigargin and analyzed by Western blotting for expression of Bim or Puma.

(H) Vero African green monkey kidney epithelial cells were infected with measles virus and harvested at the time points indicated. Protein samples (20 μg) were analyzed by Western blotting for Bim expression. Probing with antibodies to HSP70 or Bcl-2 was used as a loading control in all Western blots.

(CRAC) blockers, we showed that calcium is not required for the ER stress-induced Bim upregulation (Figure S2). Second, in MCF-7 cells, ER stress augmented *bim* mRNA levels by only ~2-fold, whereas Bim protein levels increased by ~5-fold (compare Figures 1A and 1B), indicating that only a component of Bim protein induction is due to increased *bim* transcription. We therefore investigated whether ER stress can regulate Bim levels through a posttranslational mechanism. In contrast to treatment with the chemotherapeutic drug taxol (Puthalakath et al., 1999), ER stress did not result in substantial release of Bim from the dynein motor complex (Figure S3). Bim can also be regulated by ERK-mediated phosphorylation and this has been associated with protection from apoptosis in lymphocytes, fibroblasts, and osteoclasts (Ley et al., 2005). In five different cell types (MCF-7, thymocytes, FDC-P1, MEF, and Vero), Bim (particularly the BimEL isoform but also BimL) underwent a mobility shift (faster migration on SDS-PAGE) during ER stress (see Figures 1A, 1C–1F, and 3A), but this change did not occur after exposure to taxol (Figure 1A). This prompted us to examine whether ER stress causes a change in Bim phosphorylation. Treatment of protein extracts from healthy MCF-7 cells with λ phosphatase, a Ser/Thr and Tyr phosphatase, but not exposure to the tyrosine phosphatase LAR resulted in mobility shifts of BimEL and BimL that were similar to those observed in Bim from extracts of MCF-7 cells exposed to thapsigargin (Figure 3B). This indicated that ER stress causes a change in Ser/Thr phosphorylation of Bim. This was investigated in detail by separating proteins from extracts of healthy or thapsigargin-treated MCF-7 cells by 2D gel electrophoresis. BimEL and BimL from healthy MCF-7 cells each migrated on 2D gels as at least 5 spots (Figure 3C), indicating the presence of

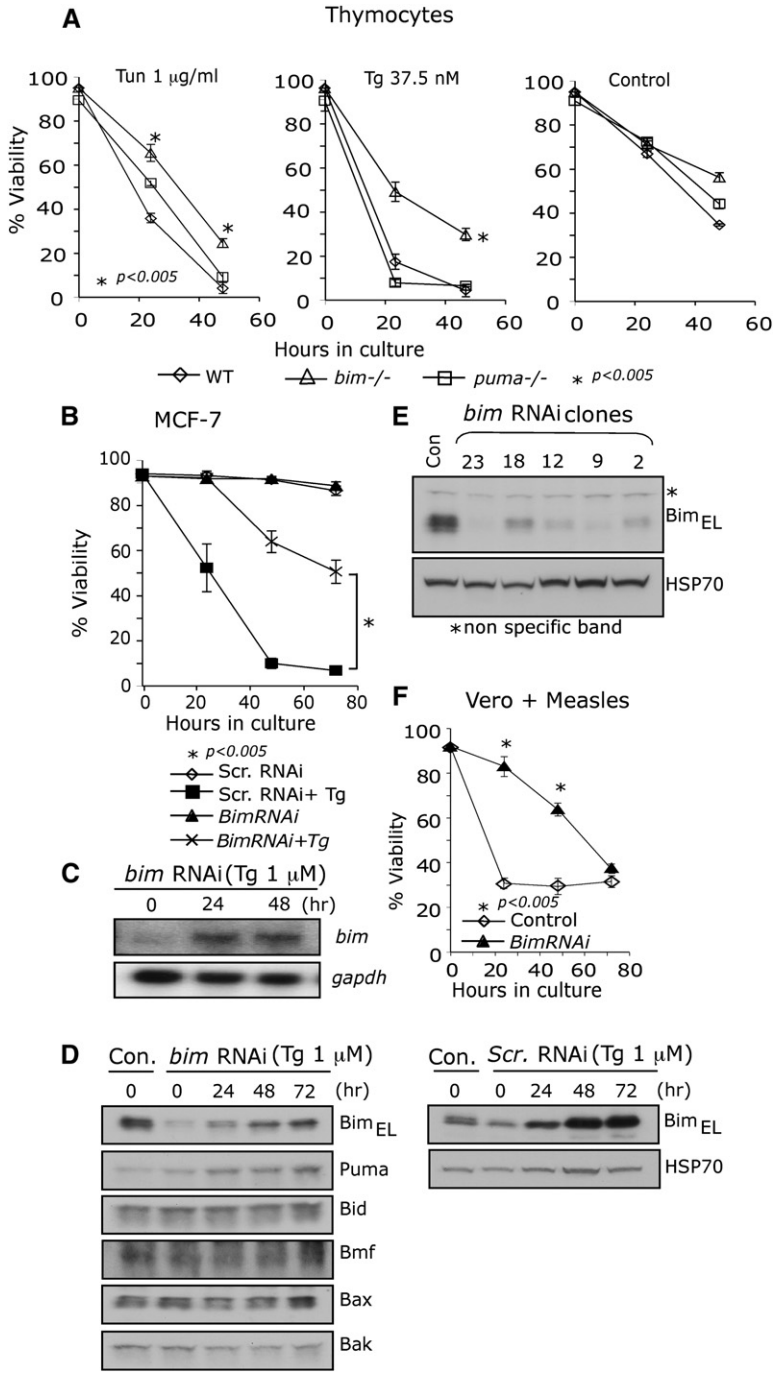

Figure 2. Bim Is Essential for ER Stress-Induced Apoptosis in Diverse Cell Types

(A) Thymocytes from WT, *puma*$^{-/-}$, or *bim*$^{-/-}$ mice were treated for 24 or 48 hr with 1 μg/mL tunicamycin (left) or 37.5 nM thapsigargin (middle) or were cultured in simple medium (right panel) and their survival analyzed in a FACScan after staining with FITC-conjugated Annexin V and propidium iodide. Data represent means ± SD from three mice of each genotype.

(B) MCF-7 cells stably transfected with a *bim*-RNAi or a control scrambled (scr.) RNAi construct were treated for 20, 48, or 72 hr with 1 μM thapsigargin or cultured in simple medium and cell survival determined as in (A). Data represent means ± SD from three independent pools of cells for each type of transfectant.

(C) *bim*-RNAi transfected MCF-7 cells were treated for 24 or 48 hr with 1 μM thapsigargin and analyzed for *bim* mRNA expression by Northern blotting. Probing for *gapdh* transcripts was used as a loading control.

(D) *bim*-RNAi or scramble (scr.) RNAi transfected MCF-7 cells were treated with thapsigargin (1 μM) for various time points and expression of the proteins indicated was determined by Western blotting. Control (Con.) sample on both panels refers to untreated parental MCF-7 cells.

(E) Vero cell clones stably expressing a *bim*-RNAi construct were compared with control vector transfected cells (Con) for Bim protein expression by Western blotting.

(F) *bim*-RNAi and control vector transfected Vero clones were infected with measles virus and analyzed after 24, 48, or 72 hr for survival as described in (A). Data represent means ± SD from three independent pools of cells for each type of transfectant.

multiple differentially phosphorylated forms of these proteins. Bim immunopurified from MCF-7 cells exposed to thapsigargin or Bim immunopurified from healthy cells and then treated in vitro with λ phosphatase migrated as five spots with different mobility, with the major spot being the least phosphorylated form (migrating toward the cathode; see arrowheads in Figure 3C). Loss of Bim phosphorylation did not appear to be due to a reduction in ERK activity, because Western blot analysis of extracts from MCF-7 cells undergoing ER stress-induced apoptosis found no decrease in ERK phosphorylation (Figure S4). Upon treatment of MCF-7 cells with thapsigargin, Bad, another BH3-only protein that can be regulated by phosphorylation (Puthalakath and Strasser, 2002), did not undergo a noticeable change in phosphorylation or migration on SDS-PAGE (Figure 3A). These results demonstrate that Bim is dephosphorylated specifically in response to ER stress.

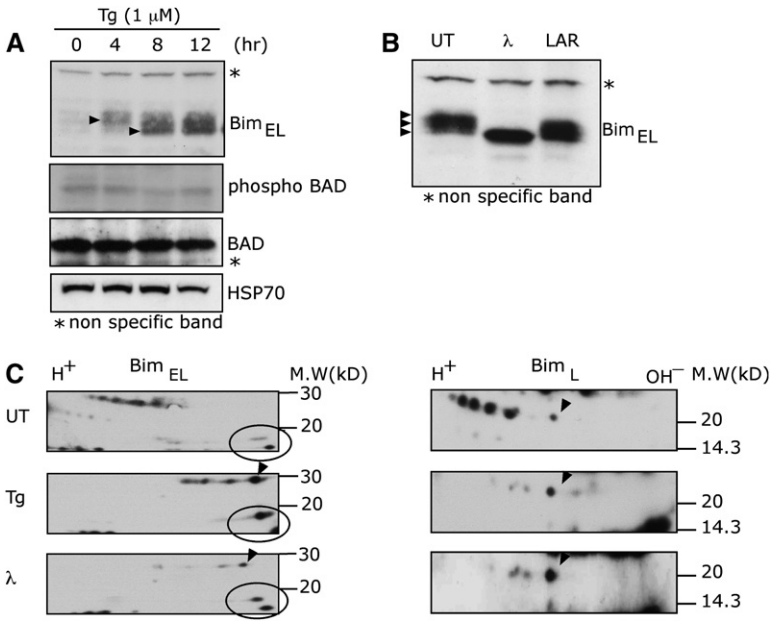

Figure 3. Bim Is Serine/Threonine Phosphorylated and Becomes Dephosphorylated during ER Stress

(A) FDC-P1 cells were treated for the times indicated with 1 μM thapsigargin and changes in phosphorylation and migration on SDS-PAGE of Bim and Bad analyzed by Western blotting using antibodies to Bim, phospho-Bad, or total Bad. Arrowheads indicate the mobility shift in BimEL. Probing with antibodies to HSP70 was used as a loading control.

(B) Lysates from healthy MCF-7 cells were treated either with λ phosphatase (serine, threonine, and tyrosine phosphatase) or human leukocyte antigen related (LAR) phosphatase (tyrosine phosphatase) and analyzed by Western blotting for changes in migration of Bim in SDS-PAGE.

(C) IEF 2D analysis of Bim immunoprecipitated from healthy MCF-7 cells (UT) or MCF-7 cells treated for 36 hr with 1.5 μM thapsigargin or Bim immunoprecipitated from healthy cells and then treated in vitro with λ phosphatase. Since the BimL isoform is expressed at substantially lower levels than BimEL, autoradiograms to show results for BimL and BimEL were scanned separately after different exposure times. Arrowheads indicate totally dephosphorylated forms of BimEL and BimL. The circles represent nonspecific spots (proteins cross-reacting with the anti-Bim antibodies) that can be used as the reference points to visualize the mobility shift of BimEL.

Phosphatase PP2A Mediated Dephosphorylation Causes an Increase in Bim Protein Levels during ER Stress

Having established that Bim undergoes dephosphorylation during ER stress induced apoptosis, we wanted to identify the phosphatase responsible. Thapsigargin is known to activate calcineurin (PP2B), a phosphatase that has been implicated in cell killing and dephosphorylation of Bad during calcium flux-induced apoptosis of rat hippocampal neurons (Wang et al., 1999). However, treatment with FK506 (600 nM; sufficient for blocking calcineurin) had no effect on thapsigargin-induced Bim dephosphorylation or apoptosis in MCF-7 cells (Figure S5 and data not shown). In contrast, in extracts from MCF-7 cells treated with thapsigargin plus okadaic acid (OA) or with OA alone, hyperphosphorylated forms of both BimEL and BimL could be seen (indicated by arrowheads in Figure 4A). Although OA can inhibit both protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), it is much less effective against PP1 (*Ki* of 147 nM versus 0.032 nM, respectively). In MCF-7 cells, specific inhibition of PP2A has previously been achieved with 1 μM OA (Favre et al., 1997), and we found that as little as 50 nM OA could inhibit thapsigargin-induced Bim dephosphorylation (Figure 4A). PP2A immunoprecipitated from MCF-7 cells undergoing ER stress, but not PP2A isolated from healthy cells, could dephosphorylate immunopurified Bim in vitro (Figure 4B), demonstrating that PP2A acts directly on Bim.

Interestingly, in MCF-7 cells treated with OA, we observed not only hyperphosphorylation of Bim but also a dramatic reduction in the levels of BimEL and BimL. This was particularly striking in cells treated with 100 nM OA and appeared to be specific to Bim, because the levels of Bcl-2 and HSP70 did not change (Figure 4A). Similar results were obtained with another PP2A inhibitor, calyculin A, even at concentrations as low as 1 nM (Figure S6). As a control, we performed Northern blot analysis to confirm that OA (100 nM) had no effect on thapsigargin-induced upregulation of *bim* mRNA in MCF-7 cells (Figure 4C). Collectively, these observations indicate that PP2A dephosphorylates Bim during ER stress-induced apoptosis and thereby increases its levels.

PP2A-Mediated Dephosphporylation Prevents Ubiquitination and Proteosomal Degradation of Bim

Since phosphorylation controls ubiquitination and proteasomal degradation of Bim (Ley et al., 2005), we investigated whether ER stress-induced dephosphorylation affects Bim turnover. Addition of the proteosome inhibitor PS 341 restored Bim protein levels in MCF-7 (Figure 4D) and FDC-P1 cells (data not shown) treated with thapsigargin plus OA. Moreover, protein half-life analysis demonstrated that Bim turnover was more rapid in healthy MCF-7 cells compared to cells undergoing ER stress (Figure 4E). This indicates that dephosphorylation may inhibit ubiquitin-dependent proteasomal degradation thereby causing Bim accumulation during ER stress. Indeed, immunoprecipitation with anti-Bim antibodies followed by Western blotting using antibodies to Bim or ubiquitin demonstrated that a significant fraction of Bim was ubiquitinated in healthy MCF-7 cells but no ubiquitinated Bim was seen in immunoprecipitates from

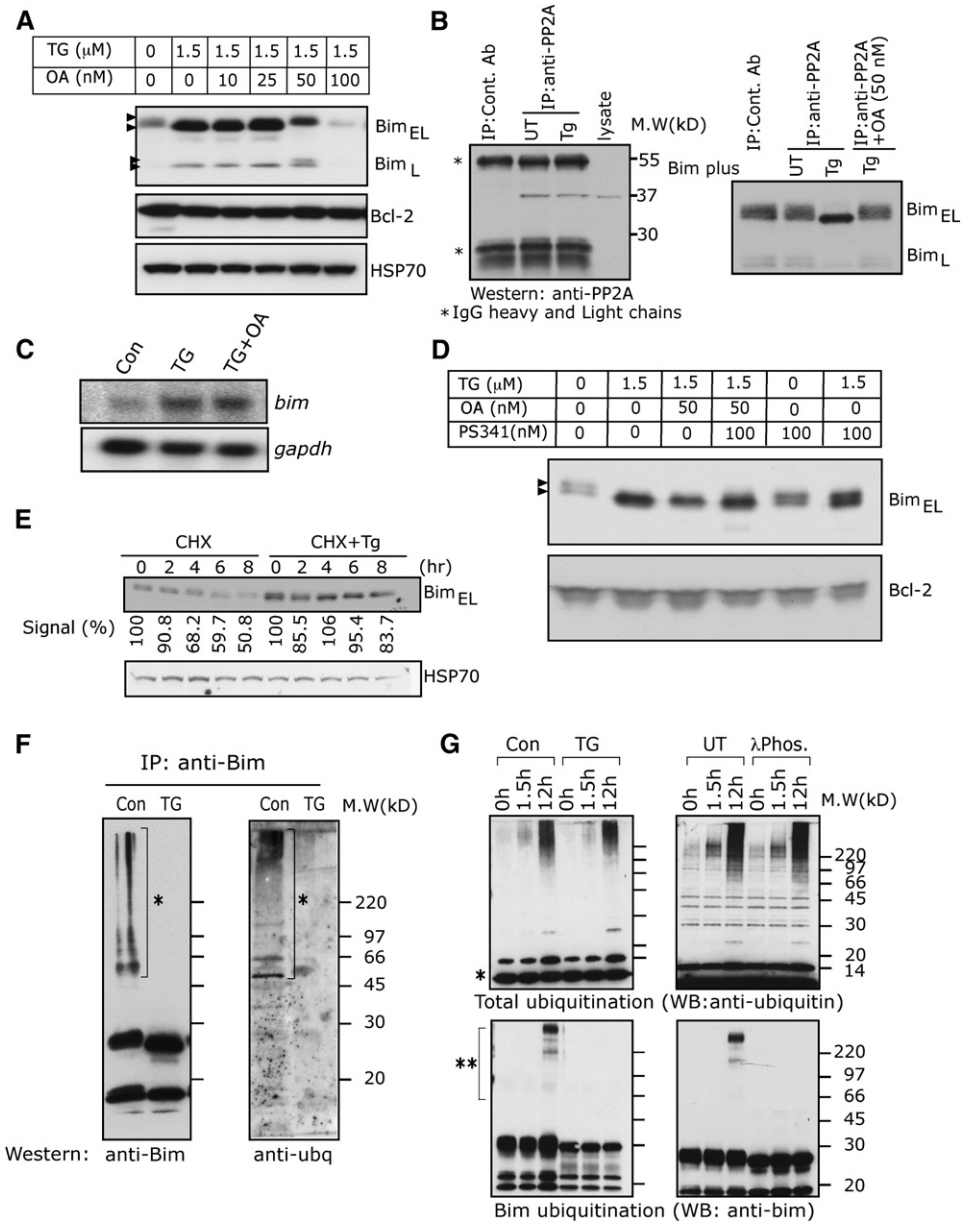

Figure 4. Bim Protein Is Dephosphorylated by Protein Phosphatase 2A during ER Stress and Is Thereby Rendered Resistant to Ubiquitin/Proteasome-Mediated Degradation

(A) MCF-7 cells were treated for 16 hr with 1.5 μM thapsigargin with or without addition of the indicated concentrations of okadaic acid and analyzed by Western blotting for Bim protein levels. The blot was then probed with antibodies to Bcl-2 and HSP70 (loading control).

(B) The left panel shows a Western blot probed with an antibody to Protein Phosphatase 2A (PP2A) to visualize PP2A immunoprecipitated from healthy or thapsigargin-treated (1.5 μM) MCF-7 cells. As controls, we show PP2A levels in total lysates from healthy MCF-7 cells and probing of the material immunoprecipitated by a control antibody. The right panel shows a Western blot probed with an antibody to demonstrate that Bim immunopurified from healthy MCF-7 cells can be dephosphorylated by PP2A immunoprecipitated from MCF-7 cells treated with thapsigargin but not by PP2A isolated from healthy cells. As controls, we show that okadaic acid (OA 50 nM) blocks the activity of PP2A immunopurified from thapsigargin-treated cells and that material immunoprecipitated with control antibodies has no effect on Bim.

(C) PolyA+ mRNA was isolated from MCF-7 cells that were left untreated (Con) or treated for 16 hr with thapsigargin (1.5 μM) with or without addition of OA (50 nM) and analyzed by Northern blotting (3 μg of polyA-RNA/lane) for *bim* mRNA levels. The blot was probed for *gapdh* to provide a loading control.

(D) MCF-7 cells were treated for 16 hr with thapsigargin (1.5 μM), with or without addition of okadaic acid (50 nM) and the proteosomal inhibitor PS341 (100 nM) and analyzed by Western blotting for Bim. Probing with antibodies to Bcl-2 was used as a loading control. Arrowheads in (A) and (D) indicate the position of phosphorylated and dephosphorylated BimEL.

thapsigargin-treated cells (Figure 4F). The relationship between phosphorylation and ubiquitination of Bim was further examined by exposing immunopurified Bim to a reaction system that supports ubiquitination in vitro. In this assay, only phosphorylated Bim immunopurified from healthy MCF-7 cells could be ubiquitinated, whereas no such modification was observed with Bim purified from cells exposed to thapsigargin (which is mostly dephosphorylated; Figure 4G, bottom left panel) or with λ phosphatase-treated Bim immunopurified from healthy MCF-7 cells (Figure 4G, bottom right panel). These results demonstrate that during ER stress, Bim protein levels rise because PP2A-mediated dephosphorylation renders it refractory to ubiquitination and proteasomal degradation.

ER Stress-Induced Transcriptional Upregulation of *bim* Is Mediated by CHOP-C/EBPα Heterodimers

An ~2-fold increase in *bim* transcript levels was observed during ER stress induced apoptosis (see Figures 1B and 4C). This could be attributed to a transcriptional increase rather than a change in mRNA stability, because treatment of MCF-7 cells with actinomycin D blocked thapsigargin-induced accumulation of *bim* mRNA levels (Figure S7). It has previously been shown that *bim* is transcriptionally induced by FOXO3A in response to IL-3 withdrawal in a pro-B cell line (Dijkers et al., 2000). In thapsigargin-treated MCF-7 cells, FOXO3A and FOXO1 did, however, not undergo dephosphorylation prior to the time of Bim induction (in fact, FOXO3A appeared to be degraded; see Figure S8), indicating that these transcription factors are not critical for ER stress-induced apoptosis. Consistent with this notion, FOXO3A-deficient thymocytes were normally sensitive to thapsigargin and tunicamycin (Figure 5A). Involvement of the known ER stress-induced transcription factor ATF6 could also be ruled out because Pefabloc, a serine protease inhibitor, which blocks ATF6 activation (Okada et al., 2003), had no effect on thapsigargin-induced transcriptional induction of *bim* (data not shown).

The transcription factor CHOP is activated by ER stress and a range of other cytotoxic stimuli, including amino acid starvation, γ-irradiation or treatment with DNA damaging drugs (Marciniak et al., 2004 [and references therein]). Since many of these stimuli also upregulate Bim expression (HP, LO'R, and AS, manuscript in preparation) and since we observed concomitant induction of CHOP (plus its heterodimeric partner C/EBPα) and Bim in several cell types undergoing ER stress (see Figures 5B, S9, and S10), we investigated whether the ER stress-induced increase in Bim is mediated by CHOP. Remarkably, although thapsigargin treatment caused a readily detectable increase in *bim* mRNA levels in thymocytes from WT mice, this was not observed in *chop*$^{-/-}$ thymocytes (Figure 5C). We therefore investigated whether CHOP can directly upregulate *bim* transcription. Sequence analysis failed to detect a CHOP binding site within the conventional *bim* promoter (Bouillet et al., 2001), but a perfect CHOP-C/EBPα heterodimer binding site (TGCAAT) was found within the first intron of the mouse and human *bim* genes (at 447–452 nt from the translation initiation site within the second exon of mouse *bim*; Figure 5D). This indicated that there might be an alternate promoter within the first intron of the *bim* gene. Indeed, database searches revealed that many entries of *bim* transcripts start within the first intron (see legend to Figure S11). Semiquantitative RT-PCR and Q-PCR analysis using primers specific to noncoding exon 1, the internal transcription initiation site and a common 3′ primer binding to coding exon II revealed that both promoters are active in cells undergoing ER stress (Figure S11). To test whether CHOP-C/EBPα can activate this site within the *bim* gene, we generated a luciferase reporter construct containing 0.8 kb from the conventional promoter, the first noncoding exon and the first intron. Cotransfection of this reporter in combination with CHOP, C/EBPα, or both demonstrated that CHOP together with C/EBPα could activate transcription from this site (Figure 5D). Mutating this putative binding site abolished the induction by CHOP plus C/EBPα (Figure 5D). In addition, electrophoretic mobility shift assays (EMSA) showed that although CHOP or C/EBPα by themselves failed to interact with a radiolabeled probe containing the putative binding site, together these two proteins produced a marked mobility shift, which could be super-shifted with antibodies specific to CHOP or C/EBPα (Figure 5E). No binding was seen using a probe in which the critical residues had been mutated (Figure 5E) and the mobility shift of the radiolabeled WT probe could be prevented by adding a 5-fold molar excess of the cold WT probe, whereas addition of the mutant cold probe had no effect (Figure S12). These results demonstrate that during ER stress CHOP and C/EBPα cooperate to induce *bim* transcription by binding to a site within the first intron.

(E) MCF-7 cells were treated with cycloheximide (50 μg/mL) with or without the addition of thapsigargin (1.5 μM) and Bim levels were determined by Western blotting. Probing with an antibody to HSP70 was used as a loading control. The % values represent the Bim signal intensity compared to the 0 hr time point.

(F) Bim was immunopurified from healthy MCF-7 cells (Con) or from MCF-7 cells treated for 24 hr with thapsigargin (TG, 1.5 μM). Western blot analysis of immunoprecipitated proteins was performed on two filters, one probed with antibodies to Bim and the other with antibodies to ubiquitin. Asterisks indicate ubiquitinated Bim appearing as a smear of bands with a higher molecular weight.

(G) Bim proteins immunopurified from healthy MCF-7 cells (Con) or from cells treated for 20 hr with thapsigargin (TG, 1.5 μM) and Bim immunoprecipitated from healthy cells (UT) and then treated in vitro with λ phosphatase (λphos) were subjected to an in vitro ubiquitination reaction and samples were removed at the time points indicated for Western blot analysis using antibodies to ubiquitin (upper panels) or Bim (lower panels). The asterisk in the upper panel indicates monomeric ubiquitin used in the reaction. The smear which increases with time represents ubiquitination of all proteins from the lysate of the MCF-7-*bim RNAi* cells, which was used in the reaction as the source of ubiquitin ligase(s), and this serves as a positive control for our reaction system. The lower panel represents the Western blot analysis using anti-Bim monoclonal antibody. The double asterisks indicate ubiquitinated forms of Bim.

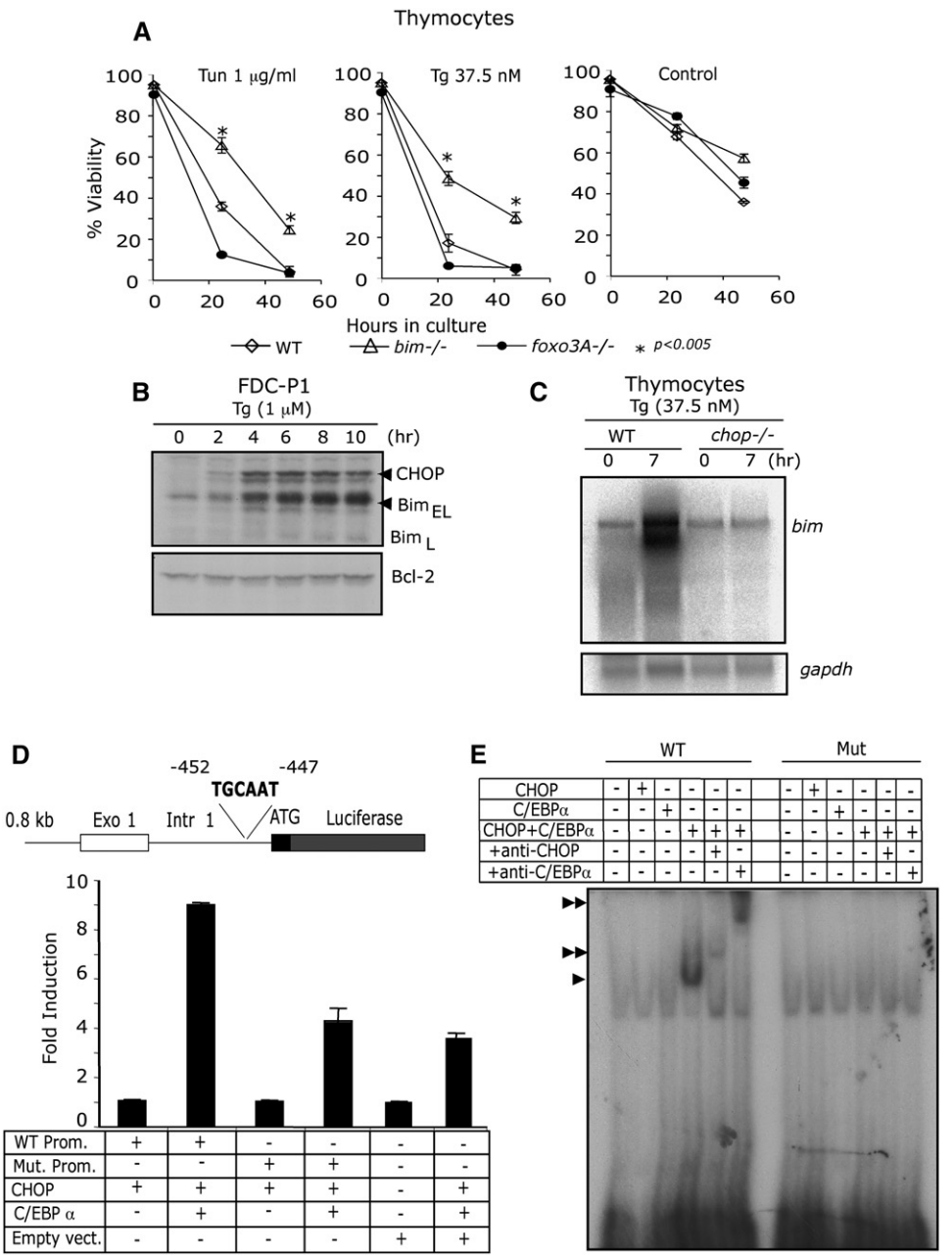

Figure 5. *Bim* Is Transcriptionally Upregulated during ER Stress by the Heterodimeric Transcription Factor CHOP-C/EBPα

(A) Thymocytes from WT, *bim*$^{-/-}$ and *foxo3A*$^{-/-}$ mice were treated for 24 or 48 hr with tunicamycin (1 μg/mL, left panel) or thapsigargin (37.5 nM, right panel) or were left untreated (control). Cell survival, determined as in Figure 2A is presented as means ± SD from three mice of each genotype.

(B) FDC-P1 myeloid cells were treated for the times indicated with 1 μM thapsigargin and analyzed by Western blotting for Bim, CHOP and Bcl-2 (loading control).

(C) Thymocytes from WT or *chop*$^{-/-}$ mice were treated for 7 hr with 37.5 nM thapsigargin and polyA+ RNA was purified and analyzed by Northern blot analysis for *bim* expression levels (3 μg polyA+ RNA/lane). Probing for *gapdh* was used as a loading control.

(D) Upper panel: the stick diagram shows the position of the CHOP-C/EBPα binding site within the first intron of the *bim* gene. The conventional 0.8 kb promoter, the first noncoding exon and the first intron fused to luciferase reporter are also indicated. The bottom panel shows the results from luciferase reporter assays using this reporter or a mutant version (in which the CHOP-C/EBPα binding site was mutated from TGCAAT to TAAGGT). Note that significant luciferase activity was observed upon cotransfection of the mutant vector with CHOP and C/EBPα . The same fold increase was also seen with empty vector, most likely due to the presence of cryptic CHOP-C/EBPα binding site(s) in the vector backbone.

(E) Electrophoretic mobility shift assays (EMSA) were performed with oligos containing either the WT CHOP-C/EBPα binding site (TGCAAT) or a mutant version (TAAGGT) together with either CHOP, C/EBPα or both. The single arrowhead indicates the mobility shift induced by CHOP-C/EBPα heterodimers and double arrowheads indicate the bands super-shifted by antibodies to CHOP or C/EBPα.

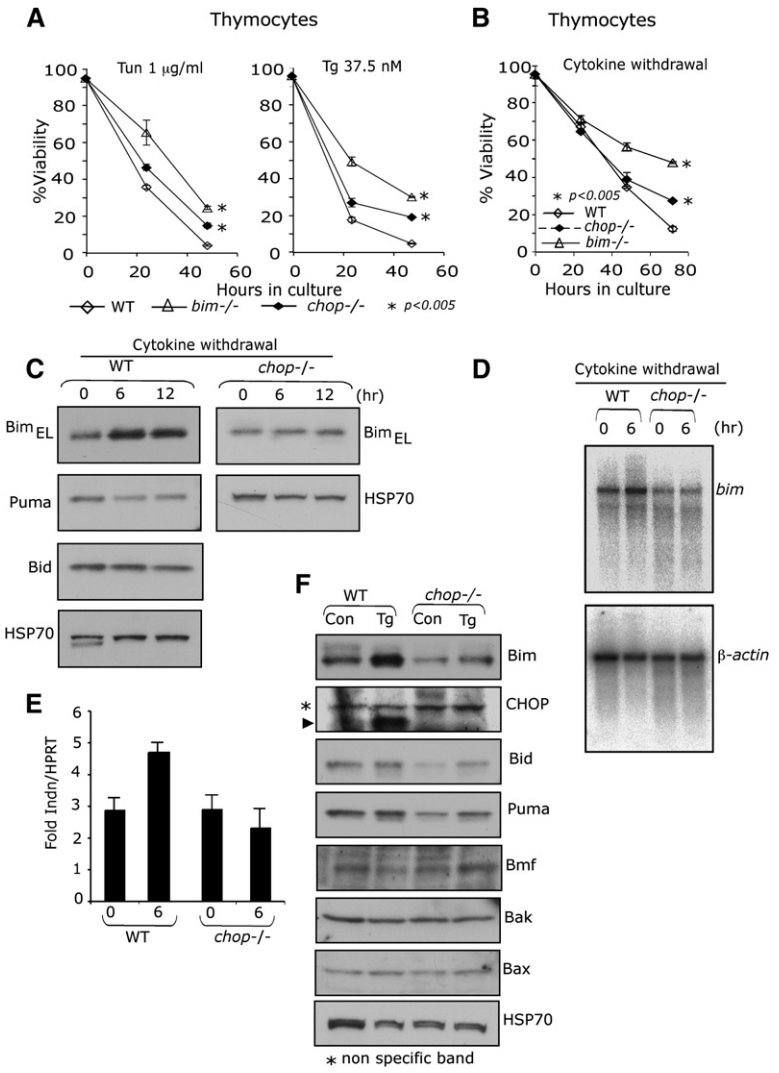

Figure 6. CHOP Is Required for ER Stress and Cytokine Withdrawal-Induced Bim Upregulation and Apoptosis in Thymocytes

(A and B) Thymocytes from WT, *bim*$^{-/-}$, or *chop*$^{-/-}$ mice were treated for 24, 48, or 72 hr with either 1 μg/mL tunicamycin (A, left panel) or 37.5 nM thapsigargin (A, right panel) or cultured in simple medium (B) and cell survival determined as indicated in Figure 2A. Data represent means ± SD from three mice of each genotype.

(C) Thymocytes from WT or *chop*$^{-/-}$ mice were cultured in simple medium for 0, 6, or 12 hr and analyzed by Western blotting for the expression of Bim, Puma, Bid, and HSP70 (loading control).

(D and E) Thymocytes from WT or *chop*$^{-/-}$ mice were cultured in simple medium for 0 or 6 hr, polyA+ RNA isolated and analyzed by Northern blotting (D) or Q-PCR (E) for *bim* expression levels. The Northern blot was reprobed for *β-actin* (loading control) (D). In the Q-PCR analysis, *bim* mRNA expression was normalized against *hprt* mRNA (E).

(F) Thymocytes from WT or *chop*$^{-/-}$ mice were treated for 7 hr with 37.5 nM thapsigargin and analyzed by Western blotting for the expression of Bim, CHOP (indicated by an arrowhead), Bax, Bak, Bmf, Bid, Puma, and HSP70 (loading control).

CHOP-Dependent Induction of *bim* Is Required for ER Stress-Mediated Apoptosis In Vitro and In Vivo

Since CHOP together with C/EBPα can bind to a regulatory site within the *bim* gene to increase its transcription, we investigated whether this pathway to Bim activation was critical for ER stress-induced apoptosis. Upon treatment with thapsigargin or tunicamycin, WT thymocytes died very rapidly, whereas *bim*$^{-/-}$ cells were markedly resistant. Interestingly, thymocytes from CHOP-deficient mice exhibited intermediate sensitivity (Figure 6A). In addition, *chop*$^{-/-}$ thymocytes also survived significantly better than WT thymocytes in simple medium (no added growth factors), although they were not as protected as those from *bim*$^{-/-}$ mice (Figure 6B). Consistent with the notion that CHOP is critical for transcriptional upregulation of *bim*, Western blot, Northern blot, and Q-PCR analyses all revealed a significant increase in Bim protein and *bim* mRNA levels in WT thymocytes grown in simple medium but no such increase was seen in *chop*$^{-/-}$ thymocytes (Figures 6C–6E). Moreover, Western blot analysis showed that while Bim protein was upregulated in WT thymocytes treated with thapsigargin, this was markedly attenuated in *chop*$^{-/-}$ thymocytes (Figure 6F). Neither growth factor withdrawal nor treatment with thapsigargin caused a marked increase in Bid, Puma, Bmf, Bax, or Bak protein levels in WT or *chop*$^{-/-}$ thymocytes (Figures 6C and 6F). This indicates that these death stimuli specifically upregulate Bim through a CHOP-dependent process.

Peritoneal macrophages from *chop*$^{-/-}$ mice were reported to be abnormally resistant to apoptosis induced by the ER stressors indomethacin or thapsigargin (Tsutsumi et al., 2004). Interestingly, *bim*$^{-/-}$ peritoneal macrophages were also significantly resistant to treatment with indomethacin or thapsigargin (Figure 7A). Moreover, Western blot analysis demonstrated that in contrast to WT macrophages, those from *chop*$^{-/-}$ mice failed to upregulate Bim in response to treatment with thapsigargin (Figure 7B).

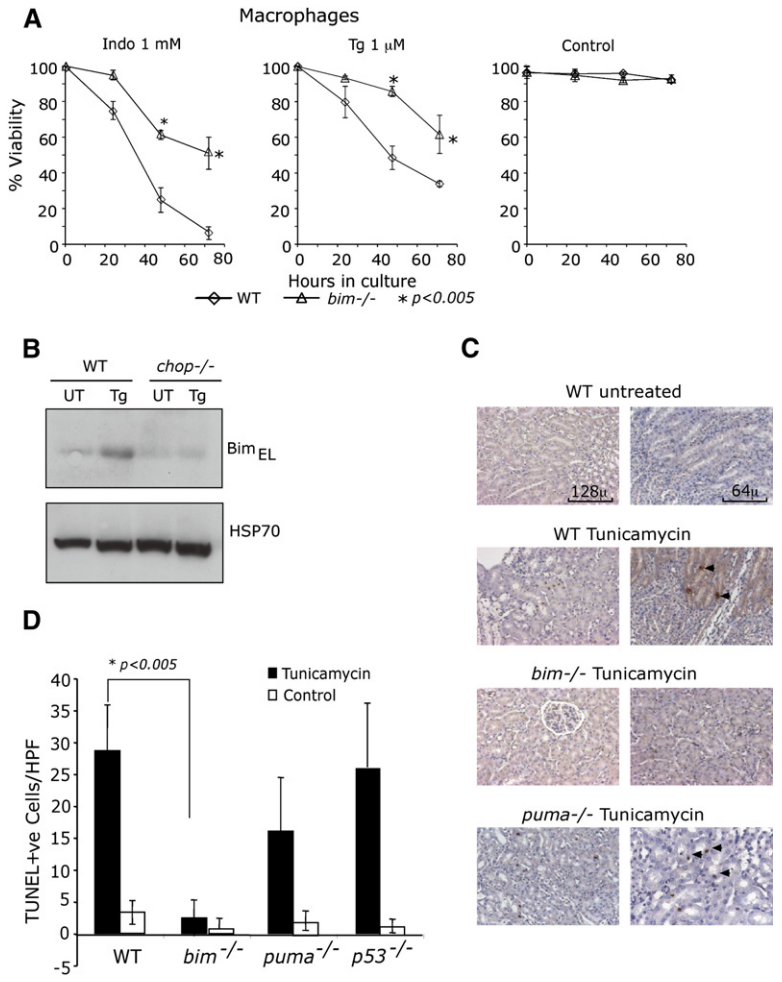

Figure 7. Bim Is Essential for CHOP-Dependent ER Stress-Induced Apoptosis in Macrophages In Vitro and in Kidney Tubular Epithelial Cells within the Whole Mouse

(A) Peritoneal macrophages from WT or *bim*$^{-/-}$ mice were treated for 24, 48, or 72 hr with 1 mM indomethacine or 1 μM thapsigargin or were left untreated, and cell survival was measured by phase contrast microscopy. Data represent means ± SD from three mice of each genotype.

(B) Peritoneal macrophages from WT or *chop*$^{-/-}$ mice were left untreated or exposed for 24 hr to 1.5 μM thapsigargin and analyzed by Western blotting for Bim and HSP70 (loading control).

(C and D) Mice of the indicated genotypes were injected i.p. with tunicamycin (at 1 μg/g body weight) or with carrier (150 mM dextrose). Kidneys were harvested after 96 hr and apoptotic cells identified by TUNEL staining (C). Apoptotic cells in kidney sections were counted under high power magnification (40× magnification) (D). The values represent the mean ± SEM from the averages of 20 fields from 3 mice of each genotype. p value was calculated by 2-tail Student's t test.

Intraperitoneal injection of tunicamycin causes extensive apoptosis of kidney epithelial cells in WT mice and this pathology can be prevented by loss of CHOP (Marciniak et al., 2004). Consistent with the notion that CHOP-mediated Bim activation is critical for ER stress-induced apoptosis in vivo, renal epithelial cells in *bim*$^{-/-}$ mice were refractory to tunicamycin (Figures 7C and 7D). This protection was specific, because mice lacking the BH3-only protein Puma or one of its activators, p53, were normally sensitive to this treatment (Figures 7C and 7D). Collectively, these results demonstrate that CHOP-dependent transcriptional activation of *bim* is critical for ER stress-induced apoptosis in diverse cell types, both in culture and within the whole animal.

DISCUSSION

ER stress constitutes a physiological as well as pathophysiological stress stimulus, which when overwhelming, can lead to apoptotic death of the damaged cell. This study demonstrates that the proapoptotic BH3-only family member Bim is essential for ER stress-induced apoptosis in diverse cell types in culture and in renal tubular cells within the whole animal. Moreover, it demonstrates how Bim is regulated by transcriptional and posttranslational mechanisms during ER stress.

In healthy cells, replete with essential growth factors, Bim is phosphorylated by ERK1/2, thereby priming it for ubiquitination and proteasomal degradation (Ley et al., 2005). We found that ER-stress activates the phosphatase PP2A causing Bim dephosphorylation, which prevents its ubiquitination and proteasomal degradation (see model in Figure S13). While loss of phosphorylation and the resulting loss of ubiquitin/proteasome-dependent degradation of Bim was shown to be critical for growth factor withdrawal-induced apoptosis of osteoclasts and fibroblasts (Ley et al., 2005), ER-stress mediated increase in Bim through dephosphorylation is a novel finding. This dephosphorylation occurs in spite of sustained ERK activation, which is known to phosphorylate Bim and thereby prime it for degradation (Ley et al., 2005). We attempted to verify that PP2A-mediated Bim dephosphorylation is critical for ER stress-induced apoptosis but this was not feasible since PP2A inactivation (either pharmacologically or

through gene targeting) is highly toxic to cells, presumably because this phosphatase is required for several vital cellular processes (Kong et al., 2004).

Although we found that Bim is essential for ER stress-induced apoptosis in a broad range of cell types, including thymocytes, macrophages and epithelial cells from breast or kidney, it is possible that different BH3-only proteins are required for this process in other tissues. For example, gene expression profiling showed that *puma* is transcriptionally induced in neuroblastoma cells undergoing ER stress, and RNAi-mediated suppression of Puma protected HCT116 cells against thapsigargin-induced apoptosis, albeit not completely (Reimertz et al., 2003). Moreover, Puma as well as Noxa were reported to be critical mediators of ER stress-induced apoptosis in MEF (Li et al., 2006). We confirmed that loss of Puma rendered MEF partially resistant to ER stress (E.M. and A.S., unpublished data), but thymocytes and mature T lymphocytes from $puma^{-/-}$ mice were only marginally protected. Although loss of Bim greatly inhibited ER stress-induced apoptosis of hemopoietic and several types of epithelial cells, protection was incomplete. It is therefore conceivable that Bim cooperates with Puma in this apoptotic pathway.

Interestingly, others reported that *bim* was not transcriptionally induced in response to ER stress in a neuroblastoma line (Reimertz et al., 2003). We found that while thapsigargin induced *bim* transcription in FDC-P1 myeloid cells, MCF-7 breast carcinoma cells as well as in mouse thymocytes, macrophages and fibroblasts (MEF), the level of induction was not uniform and there was no apparent transcriptional induction in 293T kidney epithelial cells. These findings suggest that there exists cell type specificity in terms of ER stress-activated induction of BH3-only genes and this also underlines the importance of post-translational modifications in regulating the levels and activity of Bim. Regardless, our cell survival assays demonstrated that Bim is the most critical initiator of ER stress-induced apoptosis, at least in thymocytes, macrophages, renal tubular epithelial cells, breast cancer-derived MCF-7 cells and Vero cells.

Bim phosphorylation has been observed in many different cell types, including lymphocytes, fibroblasts, neurons and osteoclasts. The kinase(s) implicated in Bim phosphorylation include ERK1/2, activated by growth factors and JNK, which in turn is activated by NGF deprivation or UV-irradiation (Ley et al., 2005). Remarkably, although ERK and JNK phosphorylate the same site (S69) in BimEL, ERK promotes survival and JNK triggers apoptosis (Ley et al., 2005). This may indicate that additional modifications of Bim, such as those caused by the prolyl isomerase Pin1 (Becker and Bonni, 2006), determine whether it is degraded (and hence inactivated) or activated. Our 2D gel electrophoresis indicated the presence of approximately 8 phosphorylation sites within BimEL that are affected by ER stress. Thus, it is possible that apart from ERK and JNK, additional kinases, such as PKA, may be involved in the regulation of Bim function, possibly in a cell type- and/or cell death stimulus-specific manner.

CHOP is a transcription factor that is induced by ER stress and many other stress stimuli, such as amino acid starvation, DNA damage or nitric oxide (Marciniak et al., 2004), but how CHOP mediates apoptosis has so far been unclear. Although CHOP-induced cell death was associated with the downregulation of Bcl-2 (McCullough et al., 2001), we have not seen any change in Bcl-2 levels in cells subjected to ER stress despite readily detectable induction of CHOP (Figures 4A and 5B). Simultaneous induction of CHOP and Puma was observed in MEF undergoing ER stress (Li et al., 2006), but whether CHOP can directly induce *puma* was not examined. Our observation that Puma levels did not increase in thapsigargin-treated thymocytes, macrophages and MCF-7 cells, despite induction of CHOP and Bim, indicates that at least in these cells Puma is not regulated by CHOP. Thus, the present report demonstrates for the first time a direct link between the transcription factor CHOP and a proapoptotic Bcl-2 family member.

ER stress is an important patho-physiological stimulus for apoptosis induction and has been implicated in several human diseases, such as ''idiopathic pancreatitis'' in cystic fibrosis patients, liver cirrhosis caused by α_1-anti-trypsin deficiency, congenital goitre in thyroglobin deficiency, diabetes insipidus and tyrosinase deficient albinism (Rutishauser and Spiess, 2002). From the data presented here, particularly the resistance of Bim-deficient cells to ER stress, it is tempting to speculate that Bim or its regulators might be targets for therapeutic intervention in some of these diseases.

EXPERIMENTAL PROCEDURES

Mice

All experiments with mice were performed according to the guidelines of the Melbourne Health Research Directorate Animal Ethics Committee and are described in detail within the Supplemental Data.

Cell Culture and RNA Interference

Cell culture was performed as previously reported (Puthalakath et al., 1999) and is described in detail within the Supplemental Data section. The RNAi construct for stable repression of Bim expression has previously been described (Bouillet et al., 2005).

Protein Extraction, Sucrose Gradient Fractionation, Western Blotting, and 2D Gel Electrophoresis

Protein extractionWestern blotting, 2D gel electrophoresis and sucrose gradient fractionation were performed as previously described (Puthalakath et al., 1999).

DMP Crosslinking of Antibodies to Protein G Sepharose

Immunoprecipitations of Bim were carried out after crosslinking monoclonal anti-Bim antibody 3C5 to protein-G sepharose according to a published protocol (Schneider et al., 1982). Bound proteins were eluted from the antibody-coupled beads using 0.1 M glycine-HCl (pH 2.7) and were then precipitated using chilled methanol (80% final concentration) and dissolved in Laemmli sample buffer for Western blot analysis.

In Vitro Ubiquitination Assay

In vitro ubiquitination assays were performed according to a published procedure (Etlinger and Goldberg, 1977) using as the reaction mix the

ubiquitin-protein conjugation kit (Alexis) plus lysates from MCF-7 *bim* RNAi cells to make sure that all Bim protein was derived from the substrate (see below). For substrate preparation, 10^8 MCF-7 cells were harvested, washed in PBS, lysed in 1.5 volumes of 1 mM DTT by homogenisation. The lysate was centrifuged at 10,000 × g for 5 min to remove debris. The supernatant was centrifuged at 100,000 × g for 1 hr and then dialyzed against 500 volumes of 8 mM KCl, 4 mM $MgCl_2$ and 0.5 mM DTT for 20 hr with one change of dialysis solution. The resulting supernatant was aliquoted and stored at −70°C until use. The assay mixture (60 μL) contained 20 mg of the MCF-7-*bim*RNAi cell extract, 60 μg of ubiquitin, 6 μl of 10× buffer (80 mM KCl, 40 mM $MgCl_2$, and 5 mM DTT), 6 μl of energy solution (Alexis Biochemicals), 3 μl of ubiquitin aldehyde (50 mM), 2 μl of proteosome inhibitor PS-341 (100 μl) and the respective substrates, i.e., Bim protein purified from either healthy or thapsigargin-treated MCF-7 cells. The assay mixture was incubated for 5 min at 37°C before the substrate was added. Samples were removed after 0, 1.5, and 12 hr and analyzed by SDS-PAGE and Western blotting.

Poly-A+ RNA Extraction, Northern Blot, and Q-PCR Analysis

Poly-A+ RNA extraction, Northern blot and Q-PCR analysis were performed as described in detail within the online Supplemental Experimental Procedures section.

Supplemental Data

Supplemental Data include thirteen figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://www.cell.com/cgi/content/full/129/7/1337/DC1/.

ACKNOWLEDGMENTS

We are grateful to Drs. J. Adams, S. Cory, C. Vandenberg, D. Huang, J. Visvader, and D. Vaux for advice, assistance, and gifts of gene-targeted mice. We thank K. Vella, G. Siciliano, A. Naughton, K. Pioch, N. Iannarella, and J. Allen, S. Shehata, and E. Loza for expert animal care; B. Helbert and M. Robati for genotyping; Dr. F. Battye, V. Lapatis, C. Tarlinton, C. Young, and J. Garbe for cell sorting and Dr. S. Mihajlovic, E Tsui, and A. Hasanein for histology. This work was supported by fellowships and grants from the National Health and Medical Research Council (Australia; program #257502), the Anti-Cancer Council of Victoria, the Leukemia and Lymphoma Society (New York; SCOR grant #7015), and the National Cancer Institute (NIH, US; CA 80188 and CA 43540).

Received: October 11, 2006
Revised: January 23, 2007
Accepted: April 10, 2007
Published: June 28, 2007

REFERENCES

Becker, E.B.E., and Bonni, A. (2006). Pin1 mediates neural-specific activation of the mitochondrial apoptotic machinery. Neuron *49*, 655–662.

Bouillet, P., Metcalf, D., Huang, D.C.S., Tarlinton, D.M., Kay, T.W.H., Köntgen, F., Adams, J.M., and Strasser, A. (1999). Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. Science *286*, 1735–1738.

Bouillet, P., Purton, J.F., Godfrey, D.I., Zhang, L.-C., Coultas, L., Puthalakath, H., Pellegrini, M., Cory, S., Adams, J.M., and Strasser, A. (2002). BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes. Nature *415*, 922–926.

Bouillet, P., Robati, M., Bath, M.L., and Strasser, A. (2005). Polycystic kidney disease prevented by transgenic RNA interference. Cell Death Differ. *12*, 831–833.

Bouillet, P., Zhang, L.C., Huang, D.C., Webb, G.C., Bottema, C.D., Shore, P., Eyre, H.J., Sutherland, G.R., and Adams, J.M. (2001). Gene structure alternative splicing, and chromosomal localization of pro-apoptotic Bcl-2 relative Bim. Mamm. Genome *12*, 163–168.

Dijkers, P.F., Medema, R.H., Lammers, J.J., Koenderman, L., and Coffer, P.J. (2000). Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. Curr. Biol. *10*, 1201–1204.

Enders, A., Bouillet, P., Puthalakath, H., Xu, Y., Tarlinton, D.M., and Strasser, A. (2003). Loss of the pro-apoptotic BH3-only Bcl-2 family member Bim inhibits BCR stimulation-induced apoptosis and deletion of autoreative B cells. J. Exp. Med. *198*, 1119–1126.

Etlinger, J.D., and Goldberg, A.L. (1977). A soluble ATP-dependent proteolytic system responsible for the degradation of abnormal proteins in reticulocytes. Proc. Natl. Acad. Sci. USA *74*, 54–58.

Favre, B., Turowski, P., and Hemmings, B.A. (1997). Differential inhibition and posttranslational modification of protein phosphatase 1 and 2A in MCF7 cells treated with calyculin-A, okadaic acid, and tautomycin. J. Biol. Chem. *272*, 13856–13863.

Hildeman, D.A., Zhu, Y., Mitchell, T.C., Bouillet, P., Strasser, A., Kappler, J., and Marrack, P. (2002). Activated T cell death in vivo mediated by pro-apoptotic Bcl-2 family member, Bim. Immunity *16*, 759–767.

Hitomi, J., Katayama, T., Eguchi, Y., Kudo, T., Taniguchi, M., Koyama, Y., Manabe, T., Yamagishi, S., Bando, Y., Imaizumi, K., et al. (2004). Involvement of caspase-4 in endoplasmic reticulum stress-induced apoptosis and Abeta-induced cell death. J. Cell Biol. *165*, 347–356.

Huang, D.C.S., and Strasser, A. (2000). BH3-only proteins – essential initiators of apoptotic cell death. Cell *103*, 839–842.

Jeffers, J.R., Parganas, E., Lee, Y., Yang, C., Wang, J., Brennan, J., MacLean, K.H., Han, J., Chittenden, T., Ihle, J.N., et al. (2003). Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. Cancer Cell *4*, 321–328.

Kaufman, R.J. (2002). Orchestrating the unfolded protein response in health and disease. J. Clin. Invest. *110*, 1389–1398.

Kong, M., Fox, C.J., Mu, J., Solt, L., Xu, A., Cinalli, R.M., Birnbaum, M.J., Lindsten, T., and Thompson, C.B. (2004). The PP2A-associated protein alpha4 is an essential inhibitor of apoptosis. Science *306*, 695–698.

Lewis, R.S. (2001). Calcium signaling mechanisms in T lymphocytes. Annu. Rev. Immunol. *19*, 497–521.

Ley, R., Ewings, K.E., Hadfield, K., and Cook, S.J. (2005). Regulatory phosphorylation of Bim: sorting out the ERK from the JNK. Cell Death Differ. *12*, 1008–1014.

Li, J., Lee, B., and Lee, A.S. (2006). Endoplasmic reticulum-stress induced apoptosis: Multiple pathways and activation of p53-up-regulated modulation of apoptosis (PUMA) and NOXA by p53. J. Biol. Chem. *281*, 7260–7270.

Marciniak, S.J., Yun, C.Y., Oyadomari, S., Novoa, I., Zhang, Y., Jungreis, R., Nagata, K., Harding, H.P., and Ron, D. (2004). CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. Genes Dev. *18*, 3066–3077.

McCullough, K.D., Martindale, J.L., Klotz, L.O., Aw, T.Y., and Holbrook, N.J. (2001). Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. Mol. Cell. Biol. *21*, 1249–1259.

Nakagawa, T., Zhu, H., Morishima, N., Li, E., Xu, J., Yankner, B.A., and Yuan, J. (2000). Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-β. Nature *403*, 98–103.

Okada, T., Haze, K., Nadanaka, S., Yoshida, H., Seidah, N.G., Hirano, Y., Sato, R., Negishi, M., and Mori, K. (2003). A serine protease inhibitor prevents endoplasmic reticulum stress-induced cleavage but not transport of the membrane-bound transcription factor ATF6. J. Biol. Chem. *278*, 31024–31032.

Puthalakath, H., Huang, D.C.S., O'Reilly, L.A., King, S.M., and Strasser, A. (1999). The pro-apoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. Mol. Cell *3*, 287–296.

Puthalakath, H., and Strasser, A. (2002). Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. Cell Death Differ. *9*, 505–512.

Puthalakath, H., Villunger, A., O'Reilly, L.A., Beaumont, J.G., Coultas, L., Cheney, R.E., Huang, D.C.S., and Strasser, A. (2001). Bmf: a pro-apoptotic BH3-only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis. Science *293*, 1829–1832.

Reimertz, C., Kogel, D., Rami, A., Chittenden, T., and Prehn, J.H. (2003). Gene expression during ER stress-induced apoptosis in neurons: induction of the BH3-only protein Bbc3/PUMA and activation of the mitochondrial apoptosis pathway. J. Cell Biol. *162*, 587–597.

Ron, D. (2002). Translational control in the endoplasmic reticulum stress response. J. Clin. Invest. *110*, 1383–1388.

Rutishauser, J., and Spiess, M. (2002). Endoplasmic reticulum storage diseases. Swiss Med. Wkly. *132*, 211–222.

Saleh, M., Mathison, J.C., Wolinski, M.K., Bensinger, S.J., Fitzgerald, P., Droin, N., Ulevitch, R.J., Green, D.R., and Nicholson, D.W. (2006). Enhanced bacterial clearance and sepsis resistance in caspase-12-deficient mice. Nature *440*, 1064–1068.

Schneider, C., Newman, R.A., Sutherland, D.R., Asser, U., and Greaves, M.F. (1982). A one-step purification of membrane proteins using a high efficiency immunomatrix. J. Biol. Chem. *257*, 10766–10769.

Tsutsumi, S., Gotoh, T., Tomisato, W., Mima, S., Hoshino, T., Hwang, H.J., Takenaka, H., Tsuchiya, T., Mori, M., and Mizushima, T. (2004). Endoplasmic reticulum stress response is involved in nonsteroidal anti-inflammatory drug-induced apoptosis. Cell Death Differ. *11*, 1009–1016.

Villunger, A., Michalak, E.M., Coultas, L., Müllauer, F., Böck, G., Ausserlechner, M.J., Adams, J.M., and Strasser, A. (2003). p53- and drug-induced apoptotic responses mediated by BH3-only proteins Puma and Noxa. Science *302*, 1036–1038.

Vousden, K.H., and Lu, X. (2002). Live or let die: the cell's response to p53. Nat. Rev. Cancer *2*, 594–604.

Wang, H.-G., Pathan, N., Ethell, I.M., Krajewski, S., Yamaguchi, Y., Shibasaki, F., McKeon, F., Bobo, T., Franke, T.F., and Reed, J.C. (1999). Ca2+-induced apoptosis through calcineurin dephosphorylation of BAD. Science *284*, 339–343.

Wei, M.C., Zong, W.X., Cheng, E.H., Lindsten, T., Panoutsakopoulou, V., Ross, A.J., Roth, K.A., MacGregor, G.R., Thompson, C.B., and Korsmeyer, S.J. (2001). Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science *292*, 727–730.