Epothilone B Analogue (BMS-247550)-mediated Cytotoxicity through Induction of Bax Conformational Change in Human Breast Cancer Cells[1]

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ABSTRACT

Epothilone B is a novel nontaxane antimicrotubule agent that is active even against paclitaxel (Taxol)-resistant cancer cells. The present study further explores the mechanisms underlying epothilone B-mediated cytotoxicity in human breast cancer cells. We show that BMS-247550 (EpoB), a novel epothilone B analogue, induces cell cycle arrest at the G_2-M phase transition and subsequent apoptotic cell death of MDA-MB-468 (468) cells. Treating cells with EpoB triggers a conformational change in the Bax protein and its translocation from the cytosol to the mitochondria, which is accompanied by cytochrome *c* release from the inter-membrane space of mitochondria into the cytosol. Overexpression of Bcl-2 delays Bax conformational change, cytochrome *c* release, and apoptosis induced by EpoB. Conversely, the Bcl-2 antagonist Bak-BH3 peptide or HA14-1 compound abrogates the antiapoptotic effects of Bcl-2 and enhances apoptosis of 468 cells pretreated with EpoB (to induce mitotic arrest). In synchronized 468 cells, EpoB is more potent in inducing Bax conformational change and apoptosis at G_2-M phase compared with G_1-S phase of the cell cycle. Taken together, these findings demonstrate that EpoB induces apoptosis through a Bcl-2-suppressible pathway that controls a conformational change of the proapoptotic Bax protein. The enhanced cytotoxicity of EpoB by blocking Bcl-2 at mitochondria implies a potential application of the combination of EpoB and Bcl-2 antagonists in the treatment of human breast cancer.

INTRODUCTION

Epothilone B is a novel nontaxane microtubule-targeting agent that induces tubulin polymerization and stabilizes microtubules. Similar to paclitaxel (Taxol), epothilone B also blocks the cell cycle at the G_2-M phase transition and subsequently induces apoptosis (1, 2). Although Taxol is a highly effective drug against breast, ovarian, head and neck, and non-small cell lung cancer (3, 4), epothilone B or its lactam analogue BMS-247550 (EpoB) is more potent and active even against Taxol-resistant cancer cells (1, 2, 5). However, the precise mechanisms underlying the cytotoxicity of these antimicrotubule agents have yet to be elucidated.

Mitochondria play a central role in the signaling pathway for apoptosis under many physiological and pathological conditions (6, 7). Cellular damage transduces intrinsic death signals to mitochondria, resulting in the disruption of the mitochondrial potential ($\Delta\psi$) across the inner membrane and leading to the release of several proapoptotic molecules including cytochrome *c*, AIF, Smac, endonuclease G, and HtrA2 into the cytosol (8–12). Once released from mitochondria, cytochrome *c* forms a multiprotein complex with Apaf-1 and procaspase-9, leading to activation of this initial caspase and induction of downstream apoptotic protease cascade (13). The Bcl-2 family proteins are critical mediators of the mitochondrial pathway of apoptosis

that control the release of apoptogenic molecules from the mitochondria (14, 15). The antiapoptotic members of the Bcl-2 family such as Bcl-2, Bcl-x_L, and Mcl-1 prevent the release of cytochrome *c* from the mitochondria, whereas the proapoptotic proteins like Bax, Bak, Bid, Bad, and Bim promote it. Although the biochemical mechanism underlying this process is still controversial, the relative ratios of pro- and antiapoptotic Bcl-2 family proteins decide the fate of the cell (16).

Bax is a proapoptotic member of the Bcl-2 protein family that is predominantly localized in the cytosol of healthy cells and translocates to mitochondria after a variety of death stimuli (17). During apoptosis, Bax undergoes a conformational change, leading to exposure of its N- and C-termini that appears to be required for the mitochondrial targeting of the Bax protein (17, 18). Once translocated to the outer membranes of the mitochondria, Bax forms oligomers or clusters that cause mitochondrial dysfunction and apoptotic cell death (19, 20). The proapoptotic Bcl-2 family member Bid promotes the mitochondrial translocation of Bax, whereas the antiapoptotic proteins Bcl-2 and Bcl-x_L inhibit Bax conformational change and mitochondria-associated Bax cluster formation (20, 21). Thus, Bax conformational change is a critical step in the Bax-mediated signaling pathway for apoptosis.

In this paper, we investigated the role of Bax and Bcl-2 in the novel epothilone B analogue BMS-247550 (EpoB)-induced apoptosis of human breast cancer cells. We found that EpoB triggers a conformational change in the NH_2 terminus of the Bax protein and subsequently induces apoptotic cell death in MDA-MB-468 cells through a Bcl-2-suppressible mechanism.

MATERIALS AND METHODS

Materials. BMS-247550 (EpoB) was kindly provided by Bristol-Myers Squibb (Princeton, NJ). Anti-Bax 6A7 monoclonal antibody was purchased from Sigma Chemical Co. (St. Louis, MO). Anti-Bid polyclonal antibody, anti-cytochrome *c* monoclonal antibody, and anti-Bcl-2 4D7 monoclonal antibody were purchased from PharMingen (San Diego, CA). Anti-COX IV monoclonal antibody was purchased from Molecular Probes (Eugene, OR). Antihuman Bax, Bcl-2, and Bcl-x_L polyclonal antibodies were described previously (22). Cellular membrane-permeable Bak-BH3 peptide, and monoclonal antibodies against caspase-3 and cleaved D4-GDI were kindly provided by Imgenex (San Diego, CA). Caspase inhibitor Z-VAD-FMK was purchased from Alexis (San Diego, CA). Bcl-2 inhibitor HA14-1 was described previously (23). Human breast cancer cell line MDA-MB-468 (468) was obtained from the American Type Culture Collection (Manassas, VA).

Cell Culture and Transfection. The 468 cells were maintained in RPMI 1640 medium supplemented with 10% FCS and 1% penicillin-streptomycin. To establish Bcl-2-stable transfectants, pRC/CMV-Bcl-2 plasmid DNA was transfected into 468 cells by TRANSIT transfection reagent (PanVera, Madison, WI), and positive clones were selected with 1 mg/ml G418.

MTT Assay. Cells were seeded at 2×10^4 cells per well of 96-well plates in 0.1 ml of complete medium and cultured for 12 h. At the indicated time points after drug treatment, 10 μl of 5 mg/ml MTT[3] solution (MTT Thiazolyl blue) was added into the cell cultures and incubated for 3 h at 37°C. The reaction was stopped by adding 0.1 ml of 10% SDS in 10 mM HCl. After an

[3] The abbreviations used are: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

additional incubation at 37°C for 12 h, the absorbance at 570 nm was measured using a microplate reader, and the cell viability was expressed as percentage relative to time 0.

DNA Fragmentation Assay. Cells in 6-cm culture dishes were harvested and incubated in 100 μl of lysis buffer (100 mM NaCl, 100 mM Tris-HCl, pH 8.0, 25 mM EDTA, 0.5% SDS, 100 μg/ml proteinase K) at 50°C overnight, followed by ethanol precipitation. The pellets were dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) containing 1 μg/ml RNase A and incubated for 1 h at 37°C. The DNA samples were subjected to 1.5% agarose gel and visualized with ethidium bromide staining.

Detection of Bax Conformational Change. Cells were lysed with Chaps lysis buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 1% Chaps) containing protease inhibitors as described (24). The cell lysates were normalized for protein content and 500 μg of total protein were incubated with 2 μg of anti-Bax 6A7 monoclonal antibody in 500 μl of Chaps lysis buffer for 3 h at 4°C. Then, 25 μl of protein G-agarose were added into the reactions and incubated at 4°C for an additional 2 h. After three washings in Chaps lysis buffer, beads were boiled in Laemmli buffer, and the conformationally changed Bax protein in the immunoprecipitates was subjected to SDS-PAGE immunoblot analysis with anti-Bax polyclonal antibody as described (24).

Subcellular Fractionation. Cells were harvested and resuspended in 3 volumes of isotonic lysis buffer (210 mM sucrose, 70 mM mannitol, 10 mM HEPES, pH7.4, 1 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 0.7 μg/ml pepstatin. After homogenization with a Dounce homogenizer, cell lysates were centrifuged at 1,000 $\times$ g to discard nuclei. The postnuclear supernatant was centrifuged at 10,000 $\times$ g to pellet the mitochondria-enriched heavy membrane fraction. The supernatant was further centrifuged at 100,000 $\times$ g to obtain cytosolic fraction. The membrane fractions were resuspended in Triton X-100 lysis buffer containing protease inhibitors (24). Protein concentration was determined by BCA assay (Pierce Chemical, Rockford, IL).

Cell Cycle Synchronization. Cells were treated with 2 mM thymidine for 17 h to synchronize the cell cycle at early S phase. After washing three times in RPMI 1640 medium, cells were shifted to complete medium to release the thymidine block. Cell cycle status was determined by DNA content analysis of propidium iodide-stained cells with a Becton Dickinson FACSort flow cytometer as described previously (22).
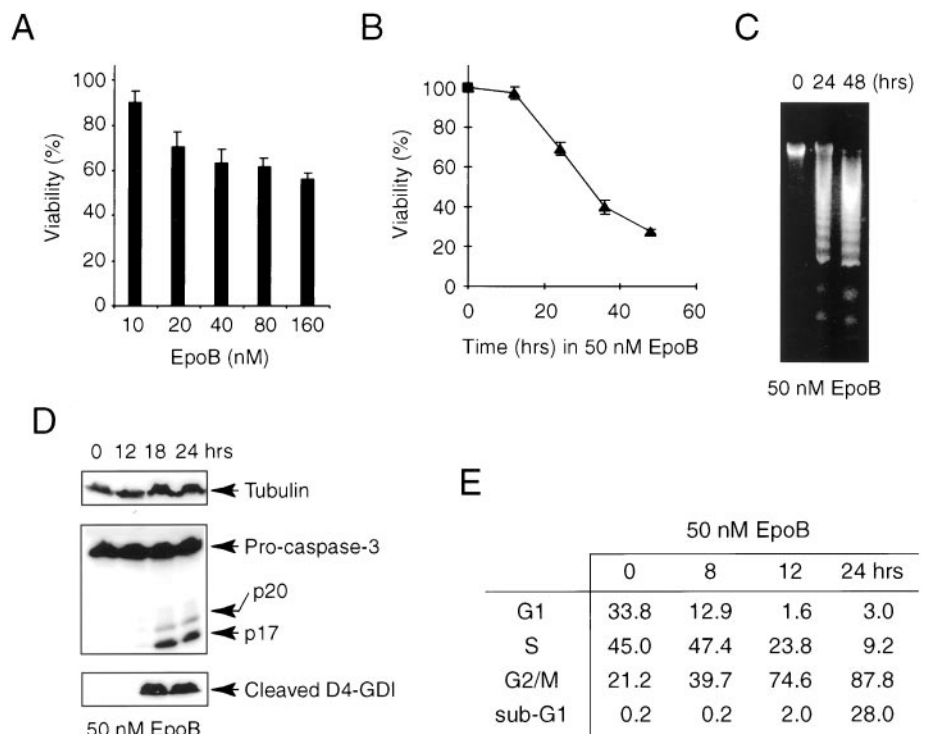
RESULTS

EpoB Induces Mitotic Arrest and Apoptosis of Human Breast Cancer Cells. BMS-247550 (EpoB) is a lactam analogue of epothilone B developed by Bristol-Myers Squibb, which is highly active *in vitro* and *in vivo* even against human cancers that are resistant to paclitaxel (5). To further understand the mechanisms of the antineoplastic effects of epothilone B, human breast cancer cells MDA-MB-468 (468) were treated with different concentrations of EpoB for 24 h or with 50 nM EpoB for various periods. The cell viability was assessed by MTT assay. As shown in Fig. 1, *A* and *B*, EpoB induced cell death of 468 cells in a dose- and time-dependent manner. To confirm that this cell death was apoptosis, we examined DNA ladder formation (one of the characteristics of apoptosis) in EpoB-treated 468 cells. A clear DNA fragmentation was observed at 24 h after treatment with 50 nM EpoB, indicating that EpoB induces apoptotic cell death in these human breast cancer cells (Fig. 1*C*). Apoptosis is caused by the activation of a family of cysteine-dependent aspartate-specific proteases known as caspases. Thus, we examined the activation of caspase-3, an executioner caspase, in 468 cells exposed to 50 nM EpoB. Caspase-3 activation was evaluated by immunoblot analysis with anti-caspase-3 monoclonal antibody that recognizes both pro- and activated (p20 and p17) caspase-3. As shown in Fig. 1*D*, caspase-3 was apparently activated around 18 h after EpoB treatment. Cleavage of D4-GDI (a caspase-3 substrate) demonstrated further caspase-3 activation within 468 cells treated with EpoB.

It has been shown that epothilone B has an action similar to that of Taxol, which binds to and stabilizes microtubules leading to mitotic arrest of the cell cycle and subsequent apoptosis in several cell lines (1, 2). To determine whether EpoB induces mitotic arrest in human breast cancer cells, we performed cell cycle analysis of 468 cells after EpoB treatment. As summarized in Fig. 1*E*, exposure to 50 nM EpoB triggered cells to accumulate in G_2-M phase of the cell cycle, beginning at 8 h and reaching a maximum at 24 h after treatment. The population of sub-G_1 cells (apoptotic cells) was significantly increased

Fig. 1. EpoB treatment causes G_2-M phase arrest and subsequently induces apoptosis in 468 cells. *A*, 468 cells were treated with different concentrations of EpoB for 24 h, and cell viability was evaluated by MTT assay (mean $\pm$ SD; $n = 3$). *B*, 468 cells were treated with 50 nM EpoB for various periods, and cell viability was determined by MTT assay (mean $\pm$ SD; $n = 3$). *C*, 468 cells were treated with 50 nM EpoB, and DNA ladder was detected as described in "Materials and Methods." *D*, 468 cells were treated with 50 nM EpoB for 0, 12, 18, or 24 h, followed by preparation of cell lysates and SDS-PAGE/immunoblot assay with monoclonal antibodies specific for caspase-3, cleaved D4-GDI, or α-tubulin as control. *E*, 468 cells were treated with 50 nM EpoB and subjected to DNA content analysis by flow cytometry at the indicated time points.

| | 50 nM EpoB | | | |
	0	8	12	24 hrs
G1	33.8	12.9	1.6	3.0
S	45.0	47.4	23.8	9.2
G2/M	21.2	39.7	74.6	87.8
sub-G1	0.2	0.2	2.0	28.0

at 24 h after EpoB treatment, suggesting that EpoB blocks cell cycles at the G_2-M phase and subsequently induces apoptosis in 468 cells.

EpoB Induces Bax Conformational Change and Mitochondrial Translocation. Because the Bcl-2 family proteins are known to regulate the mitochondrial pathway of apoptosis, we next examined the expression of several Bcl-2 family proteins in 468 cells treated with EpoB. Immunoblotting analysis revealed that the protein levels of Bcl-2, Bcl-x_L, Bax, and Bid were not significantly affected by 50 nM EpoB treatment for up to 24 h (Fig. 2A), although a slight proportion of Bid was cleaved after 18 h of treatment (Fig. 2A). In addition, treating 468 cells with EpoB slightly induced a mobility shift of endogenous Bcl-2 protein in SDS-polyacrylamide gels (Fig. 2A), similar to those observed in Taxol-treated cells (25), indicating that Bcl-2 is phosphorylated by EpoB treatment.

It has been reported that Bax undergoes a conformational change and translocation to mitochondria during apoptosis (17, 26). To examine Bax conformation, 468 cells were lysed in 1% Chaps (a condition that retains the Bax protein in its native conformation), and immunoprecipitation was performed with anti-Bax 6A7 monoclonal antibody that only recognizes conformationally changed Bax (24, 27). As shown in Fig. 2B, conformationally changed Bax was
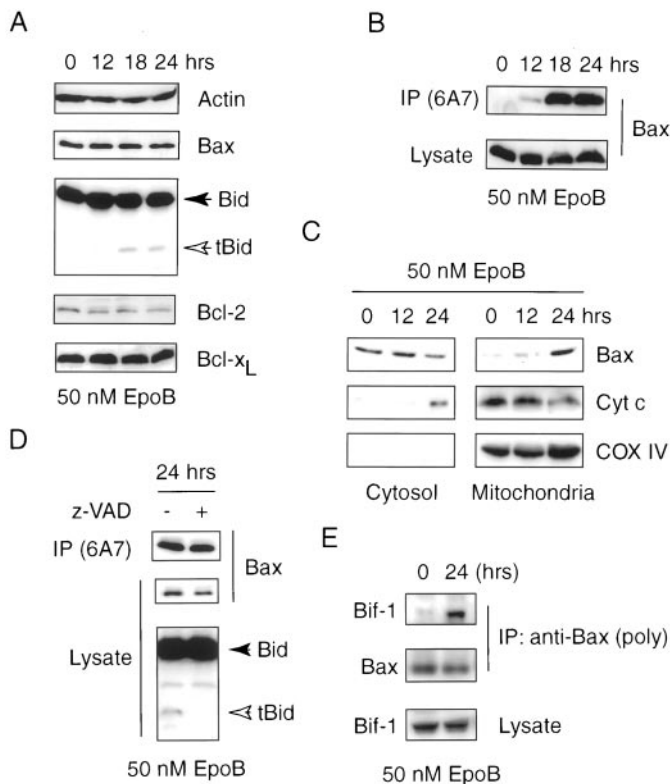

Fig. 2. EpoB induces Bax conformational change and cytochrome *c* release. *A,* 468 cells were treated with 50 nM EpoB for various times and subjected to SDS-PAGE/immunoblot analysis with the indicated antibodies. *B,* 468 cells were treated with 50 nM EpoB and applied to immunoprecipitation with anti-Bax 6A7 antibody for detection of conformationally changed Bax protein. *C,* 468 cells were exposed to 50 nM EpoB for 0, 12, or 24 h and subjected to subcellular fractionation. The mitochondria-enriched heavy membrane fraction (*Mitochondria*) and cytosolic fraction (*Cytosol*) were analyzed by immunoblotting (100 μg of protein per lane) with antibodies specific for Bax, cytochrome *c,* or mitochondrial protein COX IV for control. *D,* 468 cells were treated with 50 nM EpoB in the presence (+) or absence (−) of 100 μM Z-VAD-FMK (*z-VAD*) for 24 h. Cell lysates were prepared in Chaps lysis buffer and subjected to immunoprecipitation with anti-Bax 6A7 antibody or applied directly to SDS-PAGE/immunoblot analysis with anti-Bid or anti-Bax antibody. *E,* 468 cells were incubated with 50 nM EpoB for 0 or 24 h before preparation of cell lysates in Chaps lysis buffer. Immunoprecipitation was performed using anti-Bax polyclonal serum, followed by SDS-PAGE/immunoblot analysis with antibodies specific for Bax or Bif-1. In addition, the total lysates (30 μg of protein per lane) were analyzed directly.

detectable in 468 cells from 12 h after treatment with 50 nM EpoB, indicating that EpoB could induce Bax conformational change after mitotic arrest of the cell cycle. Moreover, subcellular fractionation experiments (Fig. 2C) revealed that the protein level of Bax decreased in the cytosolic fraction and increased in the mitochondria-enriched heavy membrane fraction of 468 cells during EpoB treatment. This indicates that EpoB induces Bax translocation from the cytosol to the mitochondria. In addition, immunoblot analysis of the fractionated cell lysates with anti-cytochrome *c* antibody showed that cytochrome *c* was released from the mitochondria into the cytosol at 24 h after EpoB treatment (Fig. 2C). The mitochondrial protein COX IV was used as a control for the fractionation procedure.

Bid is a BH3-only proapoptotic member of the Bcl-2 family that undergoes proteolysis during apoptosis (28, 29), and the resulting truncated Bid (tBid) induces Bax conformational change and subsequent translocation to mitochondria (26, 30). Therefore, we examined the role of Bid cleavage in EpoB-induced Bax conformational change. Treating 468 cells with the caspase inhibitor Z-VAD-FMK blocked Bid cleavage (Fig. 2D) and apoptosis (not shown), but had no effect on Bax conformational change (Fig. 2D) induced by EpoB. These results suggest that Bax conformational change is an early event upstream of caspase activation and Bid cleavage in EpoB-mediated apoptosis.

Bif-1 is a novel Bax-binding protein that promotes interleukin-3 deprivation-induced Bax conformational change and apoptotic cell death, when overexpressed in murine pre-B hematopoietic FL5.12 cells (24). Interleukin-3 withdrawal from cultured FL5.12 cells results in increased association of Bax with Bif-1, which may play a role in subsequent Bax conformational change (24). Consistent with this, coimmunoprecipitation analysis revealed that EpoB also induced Bax association with Bif-1 in 468 cells (Fig. 2E), implying that Bif-1 may be involved in EpoB-mediated Bax conformational change.

Bcl-2 Decreases EpoB-induced Bax Conformational Change and Apoptosis. Overexpression of Bcl-2 protects cells from apoptosis induced by multiple chemotherapeutic drugs (31), but it still remains controversial whether Bcl-2 blocks cell death induced by microtubule-damaging agents including Taxol (25, 32–34). To determine the effect of Bcl-2 on EpoB-induced apoptosis, we stably transfected 468 cells with human Bcl-2. Several independent clones of 468 transfectants with a variety of Bcl-2 expression levels were obtained and used to determine the relationship between Bcl-2 expression and EpoB resistance (Fig. 3A). As shown in Fig. 3, *B* and *C,* Bcl-2 significantly delayed EpoB-induced apoptosis of 468 cells, which had a correlation with the higher expression levels of this antiapoptotic protein. The inhibitory effect of Bcl-2 against EpoB-mediated cytotoxicity was comparable with that observed in cells treated with etoposide or staurosporine (Fig. 3C). However, overexpression of Bcl-2 in 468 cells had no significant effect on EpoB-induced mitotic arrest of the cell cycle (not shown).

To elucidate the mechanism underlying Bcl-2 protection against EpoB-induced apoptosis, we determined the ability of Bcl-2 to retain Bax conformation after EpoB treatment. The 468/Bcl-2 or control 468 cells were treated with 50 nM EpoB for various times before preparation of cell lysates in 1% Chaps. The conformationally changed Bax protein was detected by immunoprecipitation with anti-Bax 6A7 monoclonal antibody. Compared with control cells, overexpression of Bcl-2 apparently delayed Bax conformational change after EpoB treatment (Fig. 4A). In addition, subcellular fractionation analysis revealed that Bax translocation to mitochondria and cytochrome *c* release were also delayed in 468 cells overexpressing Bcl-2 compared with control cells after EpoB treatment (Fig. 4B).

Inhibition of Bcl-2 Enhances the Cytotoxicity of EpoB. If Bcl-2 has a pivotal role in the EpoB-mediated mitochondrial pathway for
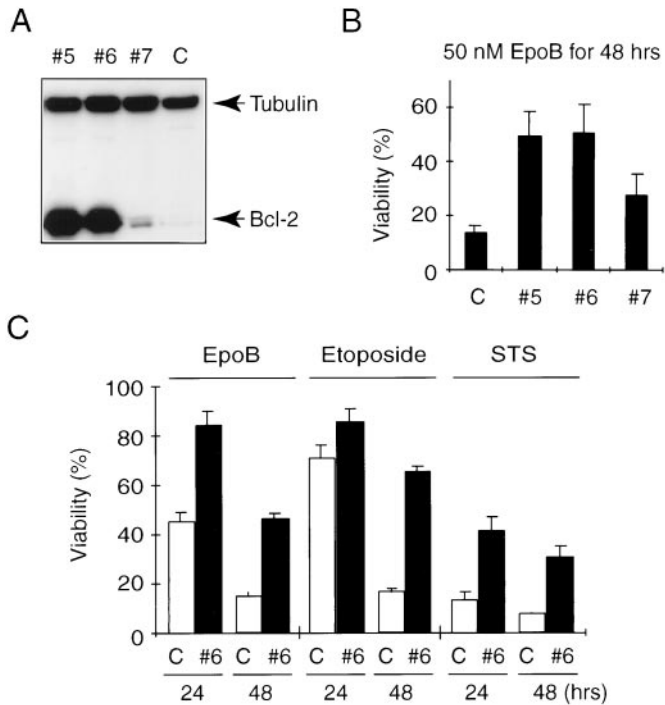
Fig. 3. Bcl-2 protects 468 cells from apoptosis induced by EpoB. *A,* 468 cells were transfected with plasmid DNA encoding human Bcl-2 protein and selected by G418 to obtain stable clones. Three independent clones (*#5, #6,* and *#7*) and control 468 (*C*) cells were analyzed by SDS-PAGE/immunoblotting with antibodies specific for Bcl-2 or control protein α-tubulin. *B,* stably transfected 468 clones were treated with 50 nM EpoB, and cell viability was determined 2 days later by MTT assay (mean $\pm$ SD; $n = 3$). *C,* 468/Bcl-2 (clone *#6*) and control 468 (*C*) cells were treated with either 50 nM EpoB, 12.5 μg/ml etoposide, or 1 μM staurosporine (*STS*) for 24 or 48 h, and cell viability was evaluated by MTT assay (mean $\pm$ SD; $n = 3$).

apoptosis, blockade of Bcl-2 should sensitize cells to apoptosis induced by EpoB. It has been shown that the membrane-permeable Bak-BH3 peptide (fused to the internalization domain of the Antennapedia homeoprotein) as well as the small compound HA14-1 kills cells through binding to and antagonizing the antiapoptotic proteins Bcl-2 and Bcl-x_L (23, 35). Thus, we treated 468 cells with EpoB and/or Bcl-2 inhibitor Bak-BH3 peptide or HA14-1. As shown in Fig. 5, treatment with either Bak-BH3 peptide or HA14-1 induced cell death in 468 cells, indicating that Bcl-2/Bcl-x_L is essential for the survival of this human breast cancer line. Moreover, these Bcl-2 antagonists could overcome the cytoprotective effects of the over-expressed Bcl-2 protein (Fig. 5). When EpoB was added to the cell cultures with the Bcl-2 inhibitors at the same time, there was no additive effect of EpoB on apoptosis induced by Bak-BH3 peptide or HA14-1 (Fig. 5A). On the contrary, when the cells were exposed to Bcl-2 inhibitors after pretreatment with EpoB for 12 h, an additive or supra-additive effect of the Bcl-2 inhibitors on EpoB-induced cell death was observed in both 468/Bcl-2 and control 468 cells (Fig. 5B).

EpoB Is More Active in Inducing Bax Conformational Change and Apoptosis in G_2-M Phase Cells. To determine whether the EpoB-mediated cytotoxic effect is cell cycle dependent, we synchronized 468/Bcl-2 and control 468 cells in early S phase of the cell cycle by thymidine treatment. DNA content analysis revealed that cells were in S phase (85–91%), G_2-M phase (58–65%), and G_1 phase (67%) at 2, 6, and 10 h, respectively, after release of cells from thymidine block (Fig. 6A). At various times after release from thymidine block, cells were treated with 50 nM EpoB for 12 h, and the cell viability was determined by MTT assay. As shown in Fig. 6B, a maximal cytotoxicity of EpoB was observed at 4–6 h after thymidine release, when ~65% of cells were in G_2-M phase of the cell cycle.

Bcl-2 protected 468 cells from EpoB-induced apoptosis throughout the cell cycle, but it was less effective in G_2-M cells (Fig. 6B). In contrast, Bak-BH3 peptide killed 468 cells in a cell cycle-independent manner (Fig. 6C). Furthermore, immunoprecipitation experiments with anti-Bax 6A7 antibody showed that EpoB rapidly induced Bax conformational change in cells that were released from thymidine block for 6 h (at this time, a majority of cells were in G_2-M phase) compared with G_1-S phase (time 0) cells (Fig. 6D).

DISCUSSION

The findings reported here demonstrate that BMS-247550 (EpoB), a lactam analogue of epothilone B, kills cells through a mitochondrial pathway of apoptosis controlled by Bcl-2 and Bax. Similar to Taxol, EpoB causes cell cycle arrest at the G_2-M transition and secondarily induces apoptosis of human breast cancer cells. In agreement with this, cells synchronized in G_2-M phase of the cell cycle are more sensitive to EpoB-induced cytotoxicity. Upon EpoB treatment, Bax undergoes a conformational change and subsequently translocates to mitochondria. This conformational change of Bax occurs upstream of Bid cleavage, cytochrome *c* release, and caspase activation. In addition, overexpression of Bcl-2 decreases EpoB-induced Bax conformational change, cytochrome *c* release, and apoptosis, whereas blockade of Bcl-2 by Bak-BH3 peptide or HA14-1 increases EpoB-mediated cytotoxicity.

In healthy cells, Bax exists predominantly in the cytosol, despite the presence of a typical membrane-anchoring sequence near its COOH-terminus (17, 36). It has been reported that the putative transmembrane domain is hidden in the inactive cytosolic form of Bax and becomes accessible to mitochondrial targeting following apoptotic signals (17, 37). Early during apoptosis, Bax undergoes a conformational change and translocates to mitochondria where it participates in
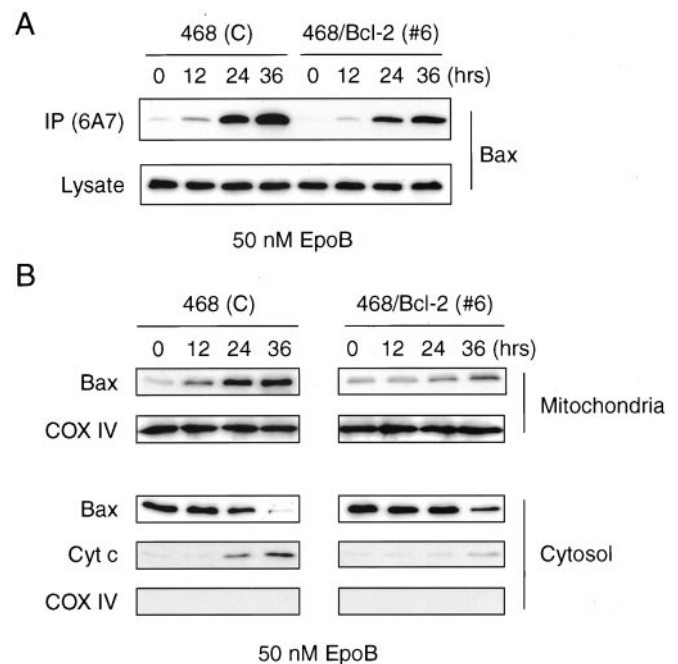

Fig. 4. Bcl-2 delays EpoB-induced Bax conformational change and cytochrome *c* release. The 468/Bcl-2 (clone *#6*) and control 468 (*C*) cells were treated with 50 nM EpoB for various times and subjected to the following analysis. *A,* cells were lysed in Chaps lysis buffer, and the conformationally changed Bax was detected by immunoprecipitation with anti-Bax 6A7 antibody. *B,* subcellular fractionation was performed to produce cytosolic (*Cytosol*) and heavy membrane (*Mitochondria*) fractions, which were subjected to SDS-PAGE/immunoblot analysis (100 μg of protein per lane) with the indicated antibodies.
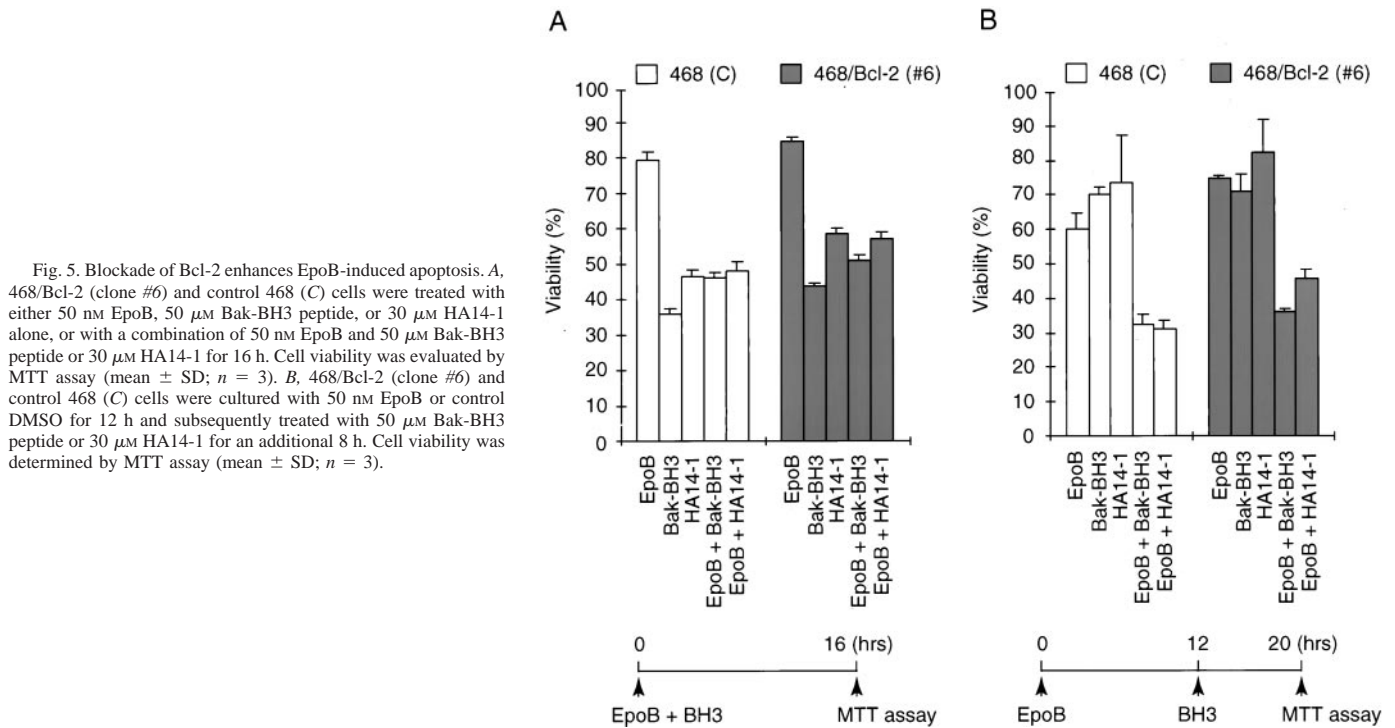
Fig. 5. Blockade of Bcl-2 enhances EpoB-induced apoptosis. *A*, 468/Bcl-2 (clone #6) and control 468 (*C*) cells were treated with either 50 nM EpoB, 50 μM Bak-BH3 peptide, or 30 μM HA14-1 alone, or with a combination of 50 nM EpoB and 50 μM Bak-BH3 peptide or 30 μM HA14-1 for 16 h. Cell viability was evaluated by MTT assay (mean ± SD; *n* = 3). *B*, 468/Bcl-2 (clone #6) and control 468 (*C*) cells were cultured with 50 nM EpoB or control DMSO for 12 h and subsequently treated with 50 μM Bak-BH3 peptide or 30 μM HA14-1 for an additional 8 h. Cell viability was determined by MTT assay (mean ± SD; *n* = 3).

mitochondrial disruption and the release of cytochrome *c* (6, 38). Although exactly how mitochondrial apoptogenic molecules escape during apoptosis remains unclear, one possibility is that Bax may form selective channels for cytochrome *c* release from the inter-membrane space of mitochondria into the cytosol (6).

The mechanism by which EpoB treatment causes a conformational change of the Bax protein remains to be elucidated. It is possible that EpoB induces a rapid increase in intracellular pH, which has been suggested to trigger Bax conformational change and subsequent translocation to mitochondria (39). In addition, some factor(s) may become activated after mitotic arrest induced by EpoB, which directly triggers Bax conformational change. One of the potential candidates is the proapoptotic protein Bid, which undergoes proteolysis during apoptosis, and the cleaved product (tBid) promotes Bax conformational change (26, 30). Although Bid has been shown to be important for Bax conformational change induced by extrinsic death signals such as Fas and tumor necrosis factor α (26, 40), it is not required for cellular damage (intrinsic death signal)-mediated conformational change of the Bax protein (41, 42). Consistent with this, our results indicate that Bid cleavage is not necessary for EpoB-induced Bax conformational change in human breast cancer cells. Another potential activator for Bax conformational change is Bif-1, which binds to native/inactive Bax during apoptosis that may have a role in the Bax-mediated pathway of apoptosis (24). Indeed, treating cells with EpoB induces increased association between Bax and Bif-1. Moreover, some unidentified factors may exist in mitochondria, because Bcl-2 prevents Bax conformational change that is mainly localized at the outer membranes of mitochondria. This is supported by our results that blocking of Bcl-2 by Bak-BH3 peptide or HA14-1, which directly binds to and antagonizes the antiapoptotic protein Bcl-2/Bcl-x$_L$ (23, 35), promotes apoptosis induced by EpoB. In addition, the BH3-only proapoptotic protein Bim has recently been shown to function upstream of Bax-mediated cytochrome *c* release (43). It will be interesting to see whether EpoB induces Bim translocation from microtubules to mitochondria where it binds to and abrogates the antiapoptotic activity of Bcl-2, thus promoting Bax conformational change and inducing apoptosis.
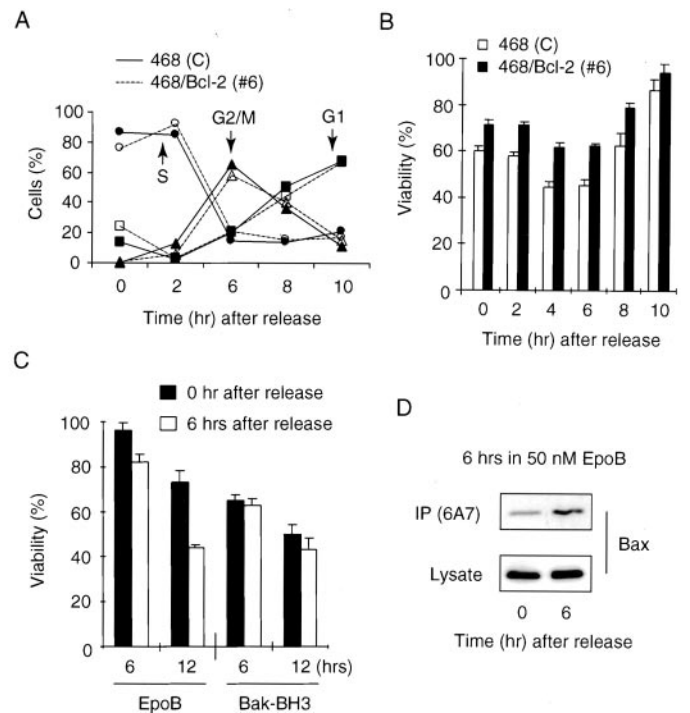

Fig. 6. The ability of EpoB to induce apoptosis and Bax conformational change is G$_2$-M dependent. 468/Bcl-2 (clone #6) and control 468 (*C*) cells were incubated with 2 mM thymidine for 17 h to synchronize cells in early S phase. *A*, cell cycle status was determined by DNA content analysis at various time points after the release of cells from thymidine block. *B*, synchronized cells were cultured in fresh medium to release thymidine block for various times and then treated with 50 nM EpoB for 12 h. The cell viability was evaluated by MTT assay (mean ± SD; *n* = 3). *C*, synchronized cells were released from thymidine block for 0 or 6 h before treatment with 50 nM EpoB or 50 μM Bak-BH3 peptide for 6 or 12 h, followed by MTT assay (mean ± SD; *n* = 3). *D*, after release of cells from thymidine block for 0 or 6 h, cells were treated with 50 nM EpoB for 6 h and subjected to immunoprecipitation with anti-Bax 6A7 antibody to detect conformationally changed Bax protein. In addition to immune complexes, total lysates were also applied directly to SDS-PAGE/immunoblot analysis.

It has been proposed that the mechanism by which Taxol exerts its cytotoxic effect involves mitotic arrest, resulting in JNK activation and Bcl-2 phosphorylation and inactivation leading to apoptosis (44). EpoB also causes a slower migration of the Bcl-2 protein in gels, similar to what occurred in Taxol-treated cells (not shown), indicating a post-translational modification of Bcl-2. However, Bcl-2 can significantly delay EpoB-induced apoptosis when overexpressed in human breast cancer cells, suggesting that this post-translational modification may not have an essential effect on the cytoprotective activity of Bcl-2. Until further evidence is provided, we do not exclude the possibility that EpoB-mediated phosphorylation of Bcl-2 does inactivate this antiapoptotic protein, because there is a significant proportion of Bcl-2 without post-translational modification in Bcl-2 transfected cells after EpoB treatment (not shown).

High levels of *bcl-2* gene expression have been documented in a wide variety of human cancer (31). Overexpression of Bcl-2 contributes not only to the origins of cancer but also to treatment failures, because the relative levels of Bcl-2 protein control the sensitivity of tumor cells to cell death induced by nearly all chemotherapeutic drugs as well as radiation (31). Thus, inhibition of Bcl-2 may put tumor cells into a more vulnerable state with reference to apoptosis induction by currently available anticancer drugs. The present study demonstrates that the Bcl-2 inhibitor Bak-BH3 peptide or HA14-1 enhances EpoB-induced cell death of human breast cancer cells. These findings may lead to the development of additional supplementary and even synergistic strategies for treating breast cancer patients.

ACKNOWLEDGMENTS

REFERENCES

1. Bollag, D. M., McQueney, P. A., Zhu, J., Hensens, O., Koupal, L., Liesch, J., Goetz, M., Lazarides, E., and Woods, C. M. Epothilones, a new class of microtubule-stabilizing agents with a Taxol-like mechanism of action. Cancer Res., *55:* 2325–2333, 1995.

2. Kowalski, R. J., Giannakakou, P., and Hamel, E. Activities of the microtubule-stabilizing agents epothilones A and B with purified tubulin and in cells resistant to paclitaxel (Taxol^R). J. Biol. Chem., *272:* 2534–2541, 1997.

3. Rowinsky, E. K., and Donehower, R. C. Paclitaxel (Taxol). N. Engl. J. Med., *332:* 1004–1014, 1995.

4. Crown, J., and O'Leary, M. The taxanes: an update. Lancet, *355:* 1176–1178, 2000.

5. Lee, F. Y., Borzilleri, R., Fairchild, C. R., Kim, S. H., Long, B. H., Reventos-Suarez, C., Vite, G. D., Rose, W. C., and Kramer, R. A. BMS-247550: a novel epothilone analog with a mode of action similar to paclitaxel but possessing superior antitumor efficacy. Clin. Cancer Res., *7:* 1429–1437, 2001.

6. Green, D. R., and Reed, J. C. Mitochondria and apoptosis. Science (Wash. DC), *281:* 1309–1312, 1998.

7. Desagher, S., and Martinou, J. C. Mitochondria as the central control point of apoptosis. Trends Cell Biol., *10:* 369–377, 2000.

8. Liu, X., Kim, C. N., Yang, J., Jemmerson, R., and Wang, X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. Cell, *86:* 147–157, 1996.

9. Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Snow, B. E., Brothers, G. M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., *et al*. Molecular characterization of mitochondrial apoptosis-inducing factor. Nature (Lond.), *397:* 441–446, 1999.

10. Du, C., Fang, M., Li, Y., Li, L., and Wang, X. Smac, a mitochondrial protein that promotes cytochrome *c*-dependent caspase activation by eliminating IAP inhibition. Cell, *102:* 33–42, 2000.

11. Li, L. Y., Luo, X., and Wang, X. Endonuclease G is an apoptotic DNase when released from mitochondria. Nature (Lond.), *412:* 95–99, 2001.

12. Suzuki, Y., Imai, Y., Nakayama, H., Takahashi, K., Takio, K., and Takahashi, R. A serine protease, htra2, is released from the mitochondria and interacts with xiap, inducing cell death. Mol. Cell, *8:* 613–621, 2001.

13. Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell, *91:* 479–489, 1997.

14. Wang, H-G., and Reed, J. C. Mechanisms of Bcl-2 protein function. Histol. Histopathol., *13:* 521–530, 1998.

15. Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, I-I., Jones, D. P., and Wang, X. Prevention of apoptosis by Bcl-2: release of cytochrome *c* from mitochondria blocked. Science (Wash. DC), *275:* 1129–1132, 1997.

16. Oltvai, Z. N., and Korsmeyer, S. J. Checkpoints of dueling dimers foil death wishes. Cell, *79:* 189–192, 1994.

17. Wolter, K. G., Hsu, Y. T., Smith, C. L., Nechushtan, A., Xi, X. G., and Youle, R. J. Movement of Bax from the cytosol to mitochondria during apoptosis. J. Cell Biol., *139:* 1281–1292, 1997.

18. Nechushtan, A., Smith, C. L., Hsu, Y. T., and Youle, R. J. Conformation of the Bax C-terminus regulates subcellular location and cell death. EMBO J., *18:* 2330–2341, 1999.

19. Gross, A., Jockel, J., Wei, M. C., and Korsmeyer, S. J. Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis. EMBO J., *17:* 3878–3885, 1998.

20. Nechushtan, A., Smith, C. L., Lamensdorf, I., Yoon, S. H., and Youle, R. J. Bax and Bak coalesce into novel mitochondria-associated clusters during apoptosis. J. Cell Biol., *153:* 1265–1276, 2001.

21. Eskes, R., Desagher, S., Antonsson, B., and Martinou, J. C. Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. Mol. Cell. Biol., *20:* 929–935, 2000.

22. Komatsu, K., Miyashita, T., Hang, H., Hopkins, K. M., Zheng, W., Cuddeback, S., Yamada, M., Lieberman, H. B., and Wang, H. G. Human homologue of *S. pombe* Rad9 interacts with BCL-2/BCL-xL and promotes apoptosis. Nat. Cell Biol., *2:* 1–6, 2000.

23. Wang, J. L., Liu, D., Zhang, Z. J., Shan, S., Han, X., Srinivasula, S. M., Croce, C. M., Alnemri, E. S., and Huang, Z. Structure-based discovery of an organic compound that binds Bcl-2 protein and induces apoptosis of tumor cells. Proc. Natl. Acad. Sci. USA, *97:* 7124–7129, 2000.

24. Cuddeback, S. M., Yamaguchi, H., Komatsu, K., Miyashita, T., Yamada, M., Wu, C., Singh, S., and Wang, H. G. Molecular cloning and characterization of Bif-1: a novel SH3 domain-containing protein that associates with Bax. J. Biol. Chem., *276:* 20559–20565, 2001.

25. Haldar, S., Jena, N., and Croce, C. M. Inactivation of Bcl-2 by phosphorylation. Proc. Natl. Acad. Sci. USA, *92:* 4507–4511, 1995.

26. Murphy, K. M., Streips, U. N., and Lock, R. B. Bcl-2 inhibits a Fas-induced conformational change in the Bax N-terminus and Bax mitochondrial translocation. J. Biol. Chem., *275:* 17225–17228, 2000.

27. Hsu, Y. T., and Youle, R. J. Bax in murine thymus is a soluble monomeric protein that displays differential detergent-induced conformations. J. Biol. Chem., *273:* 10777–10783, 1998.

28. Luo, X., Budihardjo, I., Zou, H., Slaughter, C., and Wang, X. Bid, a Bcl2 interacting protein, mediates cytochrome *c* release from mitochondria in response to activation of cell surface death receptors. Cell, *94:* 481–490, 1998.

29. Li, H., Zhu, H., Xu, C. J., and Yuan, J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell, *94:* 491–501, 1998.

30. Desagher, S., Osen-Sand, A., Nichols, A., Eskes, R., Montessuit, S., Lauper, S., Maundrell, K., Antonsson, B., and Martinou, J. C. Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome *c* release during apoptosis. J. Cell Biol., *144:* 891–901, 1999.

31. Reed, J. C. Bcl-2: prevention of apoptosis as a mechanism of drug resistance. Hematol.-Oncol. Clin. N. Am., *9:* 451–474, 1995.

32. Ibrado, A. M., Liu, L., and Bhalla, K. Bcl-xL overexpression inhibits progression of molecular events leading to paclitaxel-induced apoptosis of human acute myeloid leukemia HL-60 cells. Cancer Res., *57:* 1109–1115, 1997.

33. Fang, G., Chang, B. S., Kim, C. N., Perkins, C., Thompson, C. B., and Bhalla, K. N. "Loop" domain is necessary for Taxol-induced mobility shift and phosphorylation of Bcl-2 as well as for inhibiting Taxol-induced cytosolic accumulation of cytochrome *c* and apoptosis. Cancer Res., *58:* 3202–3208, 1998.

34. Yamamoto, K., Ichijo, H., and Korsmeyer, S. J. BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G₂/M. Mol. Cell Biol., *19:* 8469–8478, 1999.

35. Holinger, E. P., Chittenden, T., and Lutz, R. J. Bak BH3 peptides antagonize Bcl-xL function and induce apoptosis through cytochrome *c*-independent activation of caspases. J. Biol. Chem., *274:* 13298–13304, 1999.

36. Hsu, Y-T., Wolter, K. G., and Youle, R. J. Cytosol-to-membrane redistribution of members of Bax and Bcl-X_L during apoptosis. Proc. Natl. Acad. Sci. USA, *94:* 3668–3672, 1997.

37. Suzuki, M., Youle, R. J., and Tjandra, N. Structure of bax. Coregulation of dimer formation and intracellular localization. Cell, *103:* 645–654, 2000.

38. Roucou, X., and Martinou, J. C. Conformational change of Bax: a question of life or death. Cell Death Differ., *8:* 875–877, 2001.

39. Khaled, A. R., Kim, K., Hofmeister, R., Muegge, K., and Durum, S. K. Withdrawal of IL-7 induces Bax translocation from cytosol to mitochondria through a rise in intracellular pH. Proc. Natl. Acad. Sci. USA, *96:* 14476–14481, 1999.

40. Perez, D., and White, E. TNF-α signals apoptosis through a bid-dependent conformational change in Bax that is inhibited by E1B 19K. Mol. Cell, *6:* 53–63, 2000.

41. Perkins, C. L., Fang, G., Kim, C. N., and Bhalla, K. N. The role of Apaf-1, caspase-9, and bid proteins in etoposide- or paclitaxel-induced mitochondrial events during apoptosis. Cancer Res., *60:* 1645–1653, 2000.

42. 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. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science (Wash. DC), *292:* 727–730, 2001.

43. Putcha, G. V., Moulder, K. L., Golden, J. P., Bouillet, P., Adams, J. A., Strasser, A., and Johnson, E. M. Induction of BIM, a proapoptotic BH3-only BCL-2 family member, is critical for neuronal apoptosis. Neuron, *29:* 615–628, 2001.

44. Blagosklonny, M. V. Unwinding the loop of Bcl-2 phosphorylation. Leukemia (Baltimore), *15:* 869–874, 2001.