Natural Diterpenoid Compound Elevates Expression of Bim Protein, Which Interacts with Antiapoptotic Protein Bcl-2, Converting It to Proapoptotic Bax-like Molecule*§

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Overwhelming evidence indicates that Bax and Bak are indispensable for mediating cytochrome c release from mitochondria during apoptosis. Here we report a Bax/Bak-independent mechanism of cytochrome c release and apoptosis. We identified a natural diterpenoid compound that induced apoptosis in bax/bak double knock-out murine embryonic fibroblasts and substantially reduced the tumor growth from these cells implanted in mice. Treatment with the compound significantly increased expression of Bim, which migrated to mitochondria, altering the conformation of and forming oligomers with resident Bcl-2 to induce cytochrome c release and caspase activation. Importantly, purified Bim and Bcl-2 proteins cooperated to permeabilize a model mitochondrial outer membrane; this was accompanied by oligomerization of these proteins and deep embedding of Bcl-2 in the membrane. Therefore, the diterpenoid compound induces a structural and functional conversion of Bcl-2 through Bim to permeabilize the mitochondrial outer membrane, thereby inducing apoptosis independently of Bax and Bak. Because Bcl-2 family proteins play important roles in cancer development and relapse, this novel cell death mechanism can be explored for developing more effective anticancer therapeutics.

A apoptotic cytochrome c release from mitochondria is largely governed by interactions among proteins in the Bcl-2 family, which is divided into three functionally distinct groups (1, 2). Proteins of the first group, such as Bcl-2 and Bcl-xL, inhibit apoptosis, whereas proteins of the second group, which includes Bax and Bak, promote apoptosis. Despite the opposite functions, the two groups of proteins share a similar structural fold related to the bacterial pore-forming toxins (3–5). Proteins of the third group, such as Bad and Bim, are divergent and only share a short sequence, termed the Bcl-2 homology 3 (BH3) motif, with the proteins in the other two groups that contain additional BH1, BH2, and/or BH4 motifs (6). Most of the BH3-only proteins are largely unstructured in solution and folded when binding to proteins or membranes (7–9). In particular, binding to the multi-BH family members may induce an α-helical conformation in the BH3 region of Bim.

Permeabilization of mitochondrial outer membrane (MOM) by Bax or Bak is required for the release of proapoptotic molecules, including cytochrome c, from mitochondria, a concept that was supported by the fact that bax/bak double knock-out

Background: Bcl-2-related proteins regulate mitochondrial membrane permeabilization during apoptosis.

Results: A natural compound increases Bim/Bcl-2 interaction, permeabilizing the membrane independently of Bax and Bak.

Conclusion: Bim converts Bcl-2 to a Bax- or Bak-like molecule.

Significance: We revealed a novel apoptotic mechanism that can be explored for developing therapeutics to treat cancers in which dysregulation of Bcl-2 family proteins is common.
mice and cells are resistant to many death cues that can induce MOM permeabilization (MOMP) and apoptosis in the presence of Bax or Bak (10). Most of the previous data also support that Bcl-2-like or BH3-only proteins function to inhibit or activate the membrane-permeabilizing activity of Bax and Bak, respectively. However, how they fulfill the role has been debated and regarded as the “holy grail” of apoptosis research (2). According to the derepression model, Bcl-2-like proteins inhibit Bax and Bak, which are constitutively active, and BH3-only proteins repress the inhibitory activity of Bcl-2-like proteins on Bax and Bak (11, 12). In contrast, the direct activation model suggests that a subset of BH3-only proteins, referred to as activators, is required to activate Bax and Bak; Bcl-2-like proteins sequester and inhibit the BH3-only activators that can be displaced from the antiapoptotic proteins by another subset of BH3-only proteins, referred to as sensitizers (13–16).

Because data supporting and against both models exist, we recently postulated the embedded together model to reconcile both models (17). In this model, structural reorganization of Bcl-2 family proteins after their interaction in MOM is a common feature and is required for their function. The BH3-only protein–induced conformational alterations and oligomerization of Bax/Bak are required for MOMP (18–20). Similarly, BH3-only proteins interact with Bcl-2/Bcl-xL and induce conformational alterations (21, 22). The conformation–altered Bcl-2/Bcl-xL have the potential to form pores in MOM. Indeed, we have shown that Bcl-2 permeabilizes a model MOM after conformational alterations induced by truncated Bid (tBid) (23). However, the Bcl-2 pore is too small to release cytochrome c because the tBid-activated Bcl-2 only forms small oligomers in the membrane unlike the tBid-activated Bax, which forms large oligomers (24, 25). The small oligomeric Bcl-2 pore may function to maintain the normal permeability of the MOM as suggested previously (26, 27). In fact, we found that the conformation–altered Bcl-2 was able to inhibit the extensive oligomerization and large pore formation of Bax in both model and native mitochondrial membranes, thereby preventing apoptosis (23, 24). Therefore, although interaction with tBid changes Bcl-2 to an active Bax-like conformation, it fails to convert Bcl-2 to a proapoptotic protein. On the other hand, conversion of Bcl-2 to a proapoptotic protein has been observed in other circumstances. Upon binding to Nur77 protein or peptide, Bcl-2 undergoes conformational changes that convert it to a proapoptotic molecule with an exposed BH3 motif that neutralizes Bcl-xL and initiates apoptosis in a Bax- or Bak-dependent manner (28, 29). We observed that treatment with gossypol triggered similar conformational changes in Bcl-2 that induced apoptosis in cells lacking both Bax and Bak, but the mechanism was unclear (30).

Bim is required for apoptosis of autoreactive thymocytes and neurons (31). There are several isoforms of Bim, including BimEL, BimL, and BimS (32). BimS is more toxic than other Bim isoforms, may become active immediately after translation, and is subject to rapid protein turnover (33). It has been established that Bim can induce apoptosis by engaging both anti- and proapoptotic family members at mitochondria; however, the consequence of these interactions is debatable (12, 15, 34–36).

Here we identified a diterpenoid derivative that dramatically up-regulated Bim expression and effectively induced cytochrome c release and caspase-dependent apoptosis in bax/bak double knock-out cells. Bim interacted with and embedded Bcl-2 in membranes. Unexpectedly, the Bcl-2 and Bim formed oligomers that permeabilized the membranes to release cytochrome c or molecules of equivalent size. Therefore, antiapoptotic Bcl-2 could be converted to a proapoptotic Bax-like molecule via interaction with Bim.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Reagents**—E1A/K-Ras-transformed bax<sup>−/−</sup>/bak<sup>−/−</sup> mouse embryonic fibroblast (MEF) cells and bim<sup>−/−</sup> and the wild type (WT) MEF cells were cultured in DMEM supplemented with 10% heat-inactivated FBS and antibiotics. Antibodies against the following proteins were used (catalog numbers and company names are listed in parentheses): β-actin (A5441) and Bim (B7929) (Sigma); Bcl-2 (610539), cytochrome c (556433), and Bcl-x<sub>L</sub> (556361) (BD Transduction Laboratories); Bid (2003), Mcl-1 (5453), cspase-9 (9504), cytochrome c oxidase subunit IV (COX IV) (4844), and VDAC (4661) (Cell Signaling Technology); BH3 motif of Bcl-2 (AP1303a) (Abgent); and Bad (sc942) and Bok (sc19302) (Santa Cruz Biotechnology). Fluorescein isothiocyanate (FITC)–conjugated goat anti-mouse and anti-rabbit antibodies were from Santa Cruz Biotechnology, and horseradish peroxidase (HRP)–labeled goat anti-mouse and anti-rabbit IgG antibodies from Kirkegaard & Perry Laboratories. Enhanced chemiluminescence (ECL) reagents and disuccinimidyl suberate (DSS) were from Thermo Scientific, MitoTracker Red CMXRos and bismaleimidohexane (BMH) were from Invitrogen, and Z-VAD-fmk was from BioMol. Other chemicals were purchased from Sigma unless otherwise specified.

**Detection of Apoptotic Activities**—Externalization of phosphatidylserine or caspase activity in cells treated with 20 μM Z-VAD-fmk and/or 10 μM S-3 (supplemental experimental procedures) was quantified by using an Annexin V apoptosis detection kit (PharMingen) or the CaspACE<sup>™</sup> FITC—VAD-fmk kit (Promega), respectively, and flow cytometry according to the manufacturers’ instructions. Flow cytometry data were obtained with FACSscan and analyzed with CellQuest software (BD Biosciences).

**Determination of Subcellular Location of Cytochrome c and Bim**—Cells grown on glass coverslips were treated with 10 μM S-3 and stained with 50 nM MitoTracker Red CMXRos for 15 min at 37 °C. After fixation in 3.7% formaldehyde, the cells were permeabilized with 0.2% Triton X-100. Cytochrome c- or Bim-specific antibody was added to the cells followed by incubation at room temperature for 1 h. FITC-conjugated goat anti-mouse or anti-rabbit secondary antibody was then added to the cells followed by incubation at room temperature for 1 h. Fluorescence from MitoTracker Red and FITC in the cells was then measured by confocal microscopy using a Zeiss LSM 510 META microscope.

**Analyses of Expression, Subcellular Localization, and Interaction of Bcl-2 Family Proteins**— Immunoblotting was performed as described previously (37). Briefly, cells were treated with 20 μM Z-VAD-fmk and/or 10 μM S-3 and lysed in buffer A.
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(150 mM NaCl, 25 mM HEPES, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EGTA, 1 mM DTT, 50 μg/ml trypsin inhibitor, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin). Proteins from the cell lysate were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with milk and then probed with the indicated antibodies overnight at 4 °C. The resulting immunocomplexes were detected with HRP-conjugated secondary antibody and ECL. To determine subcellular localization of the proteins, cells were fractionated as described previously (30, 37). The cytosolic and mitochondrial fractions were subjected to immunoblotting as described above. To detect the Bim/Bcl-2 interaction, cells were lysed in buffer A. 500 μl of lysate containing 500 μg of total protein were incubated with 2 μg of Bcl-2- or Bim-specific antibody overnight at 4 °C. Immunocomplexes in the resulting sample were collected after incubation with 20 μl of protein A-coupled agarose for 2 h at 4 °C, washed with buffer A, and analyzed by SDS-PAGE and immunoblotting with Bim- or Bcl-2-specific antibody, respectively.

Stable Knockdown of Bim, Bcl-2, or Bad Expression by shRNA—The DNA sequence for mouse Bim shRNA (TGATGTAAGT-TCTGAGTGTG), Bcl-2 shRNA (GATCGTGATGAAATATTA), Bad shRNA (TGGGATCGAAACTTGGGCAA), or a scrambled shRNA was inserted between BamHI and HindIII sites of the pSilencer 2.1-CMV Hygro vector. The resulting plasmids (1.5 μg) were individually transfected into the bax−/−/bak−/− MEF cells using Lipofectamine 2000 (Invitrogen). Transfectants were selected in a culture medium containing 100 μg/ml hygromycin.

Reconstitution of BimEL, BimL, or BimS into bax−/−/bak−/− Bim Knockdown MEF Cells—The cDNA sequence for mouse BimEL, BimL, or BimS was inserted into the pcDNA4TO vector. The resulting plasmids or the vector (1.5 μg) was transfected into the bax−/−/bak−/−/Bim knockdown MEF cells using Lipofectamine 2000.

Effect of S-3 on Tumor Growth and Apoptosis—The bax−/−/bak−/− MEF cells (5 × 10^5) were injected into the backs of 4–5-week-old nude BALB/c mice. The mice were separated into two groups (10 mice/group). Seven days after injection, one group was treated with one dose of S-3 (30 mg/kg), and the other group was treated with vehicle control (50% (v/v) propylene glycol in 1.8% NaCl) every 3 days for 3 weeks. Both groups of mice were sacrificed at the end of treatment, and tumors were excised. Tumor length (a) and width (b) were measured by using a slide gauge to determine the tumor volumes (V) according to the following formula: \( V = \frac{a \times b^2}{2} \). Apoptotic cells in the tumors were visualized under fluorescence microscopy after terminal deoxynucleotidyltransferase-mediated nick end labeling (TUNEL) using the In Situ Cell Death Detection kit (Roche Applied Science) according to the manufacturer’s instructions. More than 2,000 cells in each of the four sections from three separate tumors were analyzed to determine the mean percentage of TUNEL-positive cells. Mice were maintained in specific pathogen-free conditions, and all studies were approved by the Animal Care and Use Committee of the Institute of Zoology, Chinese Academy of Sciences.
proteins as described above. The membrane-bound proteins were isolated by the CL-2B gel filtration chromatography described above and then solubilized by 2% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), a detergent that does not dissociate complexes formed by Bcl-2 family proteins in membranes (38). The resulting solution was subjected to Superdex 200 gel filtration chromatography as described (39). The Bcl-2 and/or BimS proteins in the eluted fractions were processed and analyzed as described above for the cross-linking experiments.

RESULTS

To explore Bax/Bak-independent cell death mechanisms, we screened over 300 natural compounds and derivatives for the capacity to kill E1A/K-Ras-transformed embryonic fibroblasts from bax/bak double knockout mice (bax<sup>-/-</sup>/bak<sup>-/-</sup> cells) (40). Results from a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay showed that several compounds significantly reduced the cell viability (supplemental Fig. S1A). One of these compounds, a Spiraea diterpenoid derivative, 15-oxospiramilactone (named S-3 hereafter, prepared as described in supplemental experimental procedures), was of particular interest because it induced nuclear condensation and fragmentation in bax<sup>-/-</sup>/bak<sup>-/-</sup> cells in a time-dependent manner (supplemental Fig. S1B).

To further characterize the death process induced by S-3 in bax<sup>-/-</sup>/bak<sup>-/-</sup> cells, we used Annexin V (AV) staining to measure the exposure of phosphatidylserine on the cell surface, a hallmark of apoptosis. S-3 treatment time-dependently increased the AV-positive population of bax<sup>-/-</sup>/bak<sup>-/-</sup> cells (Fig. 1A) to an extent comparable with E1A/K-Ras-transformed WT mouse embryonic fibroblasts treated with S-3 (supplemental Fig. S1C). We also used a FITC-conjugated caspase marker to detect caspase activity, another defining characteristic for apoptosis, in individual cells. S-3-induced caspase activation occurred in a time course parallel to phosphatidylserine exposure (Fig. 1B). Moreover, z-VAD-fmk, a pan-caspase inhibitor, potently blocked the phosphatidylserine exposure and caspase activation (Fig. 1, A and B).

To understand how S-3 activates caspases and induces apoptosis in the absence of Bax and Bak, we analyzed cytochrome <i>c</i> release from mitochondria in bax<sup>-/-</sup>/bak<sup>-/-</sup> cells treated with S-3. Cytochrome <i>c</i> appeared in the cytosolic fraction after 12-h exposure of the cells to S-3 (Fig. 1C). The amount of cytochrome <i>c</i> increased in the cytosolic fraction as the treatment time lengthened and was accompanied by a decrease of cytochrome <i>c</i> in the mitochondrial fraction. Immunostaining of the cells with a cytochrome <i>c</i>-specific antibody revealed a diffuse staining in S-3-treated cells compared with a punctate staining in untreated cells (Fig. 1D), which together with the fractionation data indicate the release of cytochrome <i>c</i> from mitochondria into the cytosol. Twelve hours after cytochrome <i>c</i> release was detected, procaspase-9 was cleaved as indicated by reduction of procaspase-9 in the cells; this could be blocked by z-VAD-fmk (supplemental Fig. S1D). Because z-VAD-fmk also blocked S-3-induced cytochrome <i>c</i> release at late time points (supplemental Fig. S1E), caspase-9 (supplemental Fig. S1D) and possibly other caspases that were activated by the initial cytochrome <i>c</i> release likely promoted more cytochrome <i>c</i> release through a feedback loop as observed previously (37, 41, 42). These data clearly demonstrate that S-3-treated bax<sup>-/-</sup>/bak<sup>-/-</sup> cells can undergo apoptosis with typical morphological and biochemical hallmarks. S-3 thus offers a new tool for investigating mechanisms of Bax/Bak-independent apoptosis.
Despite the absence of Bax and Bak, other Bcl-2 family members may still play a key role in regulating apoptosis. We thus used immunoblotting to analyze the level of other Bcl-2 family proteins in S-3-treated bax<sup>−/−</sup>/bak<sup>−/−</sup> cells. Expression of antiapoptotic Bcl-2 and Bcl-xL was not significantly changed up to 48 h after S-3 treatment, and Mcl-1 was increased initially and then decreased to a level similar to that in the untreated cells (Fig. 2A). Proapoptotic multi-BH protein Bok was not detectable until 36 h after S-3 treatment (supplemental Fig. S2A). Expression of BH3-only proteins Bid and Bad was elevated initially and then dropped to a level comparable with or lower than that in the untreated cells (Fig. 2A). Moreover, tBid, the active derivative of Bid, was not detected in the S-3-treated cells (data not shown). In contrast, expression of BimEL, BimL,
and BimS was elevated by 2–5-fold in these cells (Fig. 2A) and in WT cells (supplemental Fig. S2B). Because the mRNA levels for the Bim isoforms were increased only at certain times during the S-3 treatment and only for certain isoforms (supplemental Fig. S2C), up-regulation of their expression might occur at the level of transcription, translation, and/or stabilization of the protein(s). Of note, the expression of Bim proteins was elevated as early as 12 h after S-3 treatment, coincident with the cytochrome c release, suggesting that these Bim proteins may mediate MOMP.

To test this hypothesis, we stably knocked down expression of the three Bim isoforms by transfecting bax−/−/bak−/− cells with an shRNA specific to all the isoforms and not affecting other Bcl-2 family members (Fig. 2B). For comparison, expression of Bcl-2 or Bad was also knocked down by the corresponding shRNA (Fig. 2, C and D). S-3-induced apoptosis was potently inhibited when the Bim isoforms were knocked down but not when Bad was knocked down or when a scrambled shRNA was used in the transfection (Fig. 2E). The Bim-specific shRNA also effectively prevented S-3–induced Bim expression in the transfectants (supplemental Fig. S2D). Surprisingly, Bcl-2 knockdown inhibited S-3–induced apoptosis as potently as Bim knockdown. As expected, S-3–induced cytochrome c release was greatly inhibited in the Bim or Bcl-2 knockdown cells but not in the scrambled shRNA transfectants (Fig. 2F). To evaluate the function of the three Bim isoforms in S-3–treated bax−/−/bak−/− cells, the isoforms were individually reconstituted into the Bim shRNA transfectants (Fig. 2G). S-3–induced apoptosis and cytochrome c release were restored by reconstitution of each Bim isoform with BimS being the most effective (Fig. 2H and supplemental Fig. S2E). These results suggest that the three Bim isoforms and Bcl-2 play a critical role in S-3–induced apoptosis in bax−/−/bak−/− cells. Of note, apoptosis was also induced by reconstitution of each Bim isoform in the untreated cells, consistent with the correlation between Bim up-regulation and apoptosis in the S-3 treated cells (Figs. 1A and 2A). To further substantiate the role of Bim in S-3–induced apoptosis, we compared phosphatidylserine exposure and caspase activation in embryonic fibroblast cells from WT or bim knock-out mouse after S-3 treatment. Both apoptosis readouts were significantly reduced in bim−/− cells compared with WT cells (supplemental Fig. S2, F and G), although Bax and Bak, which were expressed in the bim−/− cells (data not shown), could be the effectors of Bim in addition to Bcl-2.

To determine the effect of S-3 on growth of bax−/−/bak−/− cells in vivo, we implanted the cells into nude mice, which were then treated with S-3 or vehicle control. The size and weight of the tumors from the S-3–treated group were significantly reduced compared with the control group at the end of treatment (Fig. 3, A–C). Similar results were obtained for the implanted bax−/−/HCT116 colon cancer cells (data not shown). As expected, a TUNEL assay revealed more apoptotic cells in the tumors from the S-3–treated mice than from the controls (Fig. 3, D and E). Interestingly, expression of BimEL was up-regulated in these tumors by S-3 treatment compared with the control, whereas expression of Bcl-2 and Bcl-xL was not affected (Fig. 3F). The S-3–treated mice did not show any adverse effects in their livers and kidneys and did not have obvious weight loss compared with the control mice (supplemental Fig. S3, A and B). Given the tolerable toxicity and ability to up-regulate Bim and induce apoptosis in the Bax- and/or Bak-deficient tumors, S-3 may serve as a lead for anticancer drug development as well as a tool to explore mechanisms of Bax- and/or Bax-independent apoptosis in vivo.

As knockdown of either Bim or Bcl-2 inhibited S-3–induced cytochrome c release in bax−/−/bak−/− cells, we reasoned that Bim might engage Bcl-2 at mitochondria to induce MOMP when both Bax and Bak were absent. To test this scenario, we first determined the location and interaction of Bim in S-3–treated bax−/−/bak−/− cells. Subcellular fractionation showed that most of the three Bim isoforms generated by S-3 treatment were in the mitochondrial fraction (Fig. 4A). Immunostaining of the cells with Bim-specific antibodies confirmed the mitochondrial localization of Bim (Fig. 4B). Reciprocal co-immunoprecipitation revealed that BimL interacted with Bcl-2, and the amount of BimL:Bcl-2 complex was increased during the course of S-3 treatment (Fig. 4C). This is likely due to the increased levels of BimL at mitochondria (Fig. 4A) where Bcl-2 is localized. We failed to detect BimEL and BimS in the Bcl-2 immunoprecipitate due to co-localization of BimEL with the light chain of Bcl-2 antibody on the immunoblot and the insufficient amount of BimS in the cells (Fig. 4C). However, chemical cross-linking in the S-3–treated cells detected homo- and/or hetero-oligomers of Bcl-2 and the Bim isoforms (Fig. 4D), suggesting that they interact directly in the cells.

To determine whether targeting to mitochondria and interaction with Bcl-2 are required for Bim to induce apoptosis in S-3–treated bax−/−/bak−/− cells, we used the cells that ectopically express WT BimS or the mutant that loses one of these activities as shown previously (supplemental experimental procedures) (33). Compared with the vector-transfected cells, expression of BimS enhanced the S-3–induced apoptosis (supplemental Fig. S5). In contrast, expression of BimS(1–88) that lacks the C-terminal hydrophobic sequence required for mitochondrial targeting (33) failed to enhance the S-3–induced apoptosis. Addition of the mitochondrial targeting sequence of TOM5 to BimS(1–88), which restored the mitochondrial targeting (33), also restored the proapoptotic activity. Therefore, mitochondrial targeting is required for the proapoptotic activity of BimS. Interaction with Bcl-2 is also required for the proapoptotic activity because expression of BimSΔΔ that lacks Leu64 and Ile67 in the BH3 motif and does not interact with Bcl-2 (33) did not increase the S-3–induced apoptosis. By comparing the extent of apoptosis in vector- and BimSΔΔ–transfected cells, BimSΔΔ seemed to act dominant negatively as it reduced the S-3–induced apoptosis that was mediated by endogenous WT Bim. This may be due to the interaction between the mutant and WT Bim that may block the interaction of WT Bim with Bcl-2 as discussed below.

To assess the consequence of S-3–induced Bim/Bcl-2 interaction, we monitored the conformational change of Bcl-2 in S-3–treated bax−/−/bak−/− cells by flow cytometry after using the antibody that recognizes Bcl-2 in the BH3 motif–exposed conformation (28, 29) to immunostain the cells. S-3 treatment induced exposure of the BH3 motif in a time-dependent manner (Fig. 4E and supplemental Fig. S4). Consistent with the pro-
The proposal that the Bcl-2 conformational change is largely due to its interaction with Bim, knockdown of Bim greatly inhibited the conformational change. Together, these results indicate that S-3 induces Bim/Bcl-2 interaction at mitochondria in \( \text{bax}^{-/-} \), \( \text{bak}^{-/-} \), \( \text{bax}^{-/-}/\text{bak}^{-/-} \) cells, altering the Bcl-2 conformation.

The above data suggest that Bim and Bcl-2 may cooperate to permeabilize MOM. To directly test this, we investigated the interaction and activity of purified Bim and Bcl-2 proteins in biochemically defined membranes. We purified full-length BimS (data not shown) and the cytosolic domain of Bcl-2 (25, 43). In cells, full-length Bcl-2 is anchored in MOM by the C-terminal hydrophobic sequence. However, full-length Bcl-2 was not soluble when expressed in bacteria and hence could not be purified. We therefore reconstituted the membrane-bound
FIGURE 4. Bim isoforms induced by S-3 in bax<sup>−/−</sup>/bak<sup>−/−</sup> cells are localized to mitochondria where they interact with Bcl-2, altering its conformation.

A, immunoblotting of the cytosolic and mitochondrial fractions of the bax<sup>−/−</sup>/bak<sup>−/−</sup> cells, which were treated with 10 μM S-3 for the indicated times, with antibody specific for Bim, β-actin, or VDAC. The data are representatives from three independent experiments. In all the experiments, β-actin or VDAC was not detected in the mitochondrial or cytosolic fraction, respectively (data not shown). B, confocal microscopy images of the bax<sup>−/−</sup>/bak<sup>−/−</sup> cells that were treated with 10 μM S-3 for the indicated times and stained with Bim-specific antibody, the FITC-conjugated secondary antibody, and MitoTracker Red. The data are representatives from three independent experiments. The zoomed-in images on the right are from the boxed areas. Scale bars are 5 μm in the zoomed-in images and 20 μm in the other images. C, co-immunoprecipitation analysis of the lysate from the bax<sup>−/−</sup>/bak<sup>−/−</sup> cells that were treated with 10 μM S-3 for the indicated times. The data from the experiments using the indicated antibodies for immunoprecipitation (IP) and immunoblotting (IB) are shown in the top two blots. The data from immunoblotting of the indicated proteins in the total cell lysate are shown in the bottom four blots. The data are representatives from three independent experiments. D, immunoblotting of the chemically cross-linked Bim and Bcl-2 proteins in the bax<sup>−/−</sup>/bak<sup>−/−</sup> cells treated with 10 μM S-3 for the indicated times using the Bim- (IB: Bim) or Bcl-2 (IB: Bcl-2)-specific antibody. The data are representatives from three independent experiments. The positions of protein standards on each blot are indicated on the left with their relative molecular mass in kDa. On the right, monomeric Bim isoforms and Bcl-2 are indicated by their names, and the homo- and/or hetero-oligomeric Bim and Bcl-2 proteins are indicated by a bracket in the Bim blot and a brace in the Bcl-2 blot. The assignment for these products was based on their relative molecular mass and that of monomeric Bcl-2 (~25 kDa), BimEL (~25 kDa), BimL (~18 kDa) and BimS (~15 kDa) and the immunoblotting by which they were detected. Immunoblotting of β-actin from each cross-linked sample is shown at the bottom. E, flow cytometric analysis of the bax<sup>−/−</sup>/bak<sup>−/−</sup> cells that were stably transfected with Bim-specific or scrambled shRNA, treated with 10 μM S-3 for the indicated times, and stained with antibodies against Bcl-2 with exposed BH3 motif and the FITC-conjugated secondary antibody. The percentages of FITC-positive (FITC<sup>+</sup>) cells are indicated in parentheses. The data are representatives from three independent experiments.
Bim-2 by adding His$_6$ tag to the C terminus of the cytosolic domain that could bind to Ni$^{2+}$-chelating phospholipid analog incorporated into liposomal membranes containing the MOM characteristic lipids. The resulting membrane-bound Bcl-2 topographically and functionally mimics the native Bcl-2 as demonstrated before (23, 25). To determine whether BimS can cooperate with the Bcl-2 to permeabilize the membrane, we encapsulated fluorophore-labeled dextran on the inside of the liposome and placed the fluorophore-specific antibody on the outside so that the membrane permeabilization could be quantified by fluorescence quenching as described before (23). Although the dextran molecule has a molecular mass $\sim$10 kDa, which is less than that of cytochrome $c$ ($\sim$12 kDa), it displayed a larger hydrodynamic size than cytochrome $c$ in our gel filtration chromatographic analysis as it eluted earlier from the column (supplemental Fig. S6A), consistent with a previous study showing that polydisperse dextrans exhibit in vitro Stokes-Einstein radii that are larger than those for compact spherical molecules of equivalent molecular masses (44). BimS and Bcl-2 together, but not individually, permeabilized the liposome (Fig. 5A). This result suggests that BimS can cooperate with Bcl-2 to release cytochrome $c$ from mitochondria. Addition of S-3 did not affect their membrane-permeabilizing activity (Fig. 5A), suggesting that the proapoptotic activity of S-3 in cells is not mediated by its interaction with the two proteins; instead, S-3 elevates the level of Bim, which interacts with Bcl-2 to permeabilize MOM. Consistent with this mechanism, the level of BimS was elevated in the S-3-treated cells (Figs. 2A and 4C), and the extent of membrane permeabilization was increased as more BimS proteins were added to the liposomes (Fig. 5A).

Of note, more BimS was required to induce significant membrane permeabilization in the presence of Bcl-2 than in the presence of Bax (Fig. 5A), suggesting that BimS would induce MOMP via Bax if it is present in cells. In fact, Bim is an activator of Bax (15, 35), and tBid, another Bax activator, binds to Bax in membranes, promoting Bax oligomerization and consequent membrane permeabilization (20, 21). To determine whether BimS and Bcl-2 would use the same mechanism to permeabilize membranes, we examined their interaction in liposomes. To correlate the membrane-permeabilizing activity with the physical interaction between BimS and Bcl-2, we also examined BimS and Bcl-2 with mutations that abolish their interaction. As expected, BimS$\Delta$, which does not bind Bcl-2 (33), could not induce membrane permeabilization in the presence of Bcl-2 even at concentrations higher than that of BimS (Fig. 5B). In contrast, Bcl-2-G145A, which loses its ability to interact with several Bcl-2 proapoptotic family members, including Bim (23, 24, 45), only partially lost the BimS-induced membrane-permeabilizing activity. To verify whether these mutations indeed inhibit the interactions between BimS and Bcl-2, we performed chemical cross-linking experiments using a sulfhydryl- or amide-specific bifunctional chemical cross-linker, BMH or DSS, respectively, because Bcl-2 and BimS contain both cysteine and lysine residues. The resulting adducts were analyzed by SDS-PAGE and immunoblotting with either Bcl-2- or Bim-specific antibody. Whereas BMH cross-linking only captured BimS homodimer, DSS cross-linking captured not only BimS homodimer/oligomer but also BimS/Bcl-2 heterodimer/oligomer (Fig. 5C). When Bcl-2-G145A was used, it still formed a dimer/oligomer with BimS albeit less efficiently, suggesting that the mutation in Bcl-2 only partially inhibits the interaction with BimS in membranes. When BimS$\Delta$ was used, the amount of Bcl-2 associated with membranes was too low to generate detectable adducts (data not shown), suggesting that the mutation in BimS greatly inhibits the interaction with Bcl-2, thereby reducing the stable association of Bcl-2 with membranes as discussed below. The cross-linking data therefore provide a rationale for the mutational effects observed in the membrane permeabilization and apoptosis assays (Fig. 5, A and B and supplemental Fig. S5) and suggest that the interaction between BimS and Bcl-2 is required for their membrane-permeabilizing activity.

Interaction with BimS may change the conformation of Bcl-2 from the membrane-anchored form to a multispanning form with $\alpha$-helix 5 (a5) embedded in the membrane, similar to the Bcl-2 activated by Bim BH3 peptide (22). If so, the reconstituted membrane-tethered Bcl-2 would still bind to the membrane even in the presence of EDTA that breaks the Ni$^{2+}$–His$_6$ bond. Indeed, addition of BimS resulted in EDTA-resistant membrane-bound Bcl-2 (Fig. 5D). In contrast, addition of either BimS$\Delta$ to Bcl-2 or BimS to Bcl-2-G145A generated no or less EDTA-resistant membrane-bound Bcl-2, respectively, suggesting that this form of Bcl-2 is generated by interaction with BimS. Of note, the amount of membrane-bound Bcl-2 in the absence of EDTA was increased by BimS. This is likely because BimS can insert into the membrane and recruit soluble Bcl-2 to the membrane through interaction with Bcl-2, particularly when the membrane contains limited Ni$^{2+}$-lipids and hence a limited number of membrane-tethered Bcl-2. Consistent with this possibility, no or less increase of membrane-bound Bcl-2 was observed in the absence of EDTA when BimS$\Delta$ or Bcl-2-G145A was used, respectively. To determine whether the EDTA-resistant Bcl-2 is embedded in the membrane, we used a chemical labeling approach (23, 24). Bcl-2 contains a cysteine residue (Cys$^{158}$) in a5; if it is embedded in the membrane, it will not be labeled by IASD, a membrane-impermeant sulfhydryl-specific reagent (supplemental experimental procedures). As shown in supplemental Fig. S6B, $\sim$10% of the membrane-bound Bcl-2 was labeled by IASD. The IASD labeling was increased to $\sim$40% by addition of Triton X-100, which solubilizes the membrane, indicating that Cys$^{158}$ was buried in the membrane and thereby inaccessible to IASD. In contrast, $\sim$60% of the soluble Bcl-2 was labeled even in the absence of Triton X-100, and addition of Triton did not significantly increase the percentage of IASD-labeled Bcl-2 ($p > 0.1$; Student’s t test). These results thus show that Cys$^{158}$ in the membrane-bound Bcl-2 was switched from a water-exposed to a membrane-embedded location in the presence of BimS, indicating that BimS induced a conformational change in Bcl-2, embedding a5 into the membrane to permeabilize the membrane.

To determine whether BimS can induce Bcl-2 oligomerization like tBid does to Bax, the membrane-bound BimS and Bcl-2 were treated with CHAPS, a detergent that solubilizes the membrane proteins but preserves their complexes (20, 21, 24). The resulting sample was subjected to gel filtration chromatography, and the eluted fractions were analyzed by SDS-PAGE.
FIGURE 5. **BimS and Bcl-2 cooperate to permeabilize liposomal membranes containing MOM characteristic lipids.** A and B, release of fluorescent dextrans from the liposomes by the indicated proteins and/or S-3. The concentration of S-3 is 10 μM, and the concentration of all proteins is 50 nM unless otherwise indicated (in nM). The extent of release was quantified by fluorescence quenching and expressed as percentage of the complete release caused by Triton X-100. Data shown are the means from three to seven independent experiments with S.D. indicated by error bars. C, immunoblotting (IB) of the liposome-bound BimS and Bcl-2 or Bcl-2-G145A after they were treated with cross-linker BMH or DSS and separated by reducing SDS-PAGE. The data are representatives from three independent experiments. The positions of protein standards on each blot are indicated on the left with their relative molecular mass in kDa. Other labels are as follows: #, Bcl-2 or Bcl-2-G145A monomer; *, BimS monomer; arrowhead, BimS homodimer; arrow, BimS homo-oligomer; dot, Bcl-2/BimS heterodimer; and circle, Bcl-2/BimS hetero-oligomer. The dimers and oligomers were assigned according to their relative molecular mass and that of monomeric Bcl-2 (∼25 kDa) and BimS (∼15 kDa) and the immunoblotting(s) by which they were detected. Other products are not labeled because either they are not chemical adducts or their molecular composition cannot be determined by the above criteria. D, immunoblotting of Bcl-2 or Bcl-2-G145A that was associated with the liposomes in the absence or presence of BimS or BimSΔΔ. The proteoliposomes analyzed in the + EDTA lanes were treated with EDTA before they were isolated, whereas those in the − EDTA lanes were not. The data are representatives from three independent experiments. E, immunoblotting of Bcl-2 or BimS that was eluted in fractions 22–34 from the Superdex 200 HR10/30 gel filtration column after BimS was associated with the liposomes in the absence (bottom blot) or presence (top two blots) of Bcl-2 and then solubilized by CHAPS. The elution positions from protein standards are indicated at the top with their relative molecular mass in kDa. The data are representatives from two independent experiments. F, a model for how S-3 induces apoptosis in the bax−/−/bak−/− cells. S-3 treatment increases Bim expression (step i). Bim migrates to mitochondria and is inserted into MOM via the C-terminal hydrophobic sequence (step ii) where it uses the BH3 motif (green cylinder) to interact with the BH1–3 groove of Bcl-2 that is anchored in MOM via the C-terminal hydrophobic sequence (black cylinder) (step iii). The interaction changes the Bcl-2 conformation, embedding α5 and α6 (gray cylinders) in MOM and exposing the BH3 motif to the cytosol (orange cylinder) (step iv). The resulting Bim:Bcl-2 complexes form oligomeric pores in MOM that release cytochrome c (Cyt C) to initiate caspase activation and apoptosis (step v).
and immunoblotting with Bcl-2- or Bim-specific antibody. Some Bcl-2 and BimS co-eluted in fractions 24–27 (Fig. 5E). Comparing the relative molecular mass of Bcl-2 and BimS monomer (~25 and 15 kDa, respectively) with those of protein standards that co-eluted with them, we concluded that Bcl-2 and BimS formed a homo- or heterodimer/oligomer in membranes, a conclusion that was further supported by the cross-linking data (Figs. 5C and 4D). In the absence of Bcl-2, most BimS was eluted in fractions 25–27, indicating that BimS homo-oligomerizes in membranes, consistent with the cross-linking data. When excess BimSΔΔ was added together with BimS, it inhibited the membrane-permeabilizing activity of Bcl-2 induced by BimS (Fig. 5B). BimSΔΔ may block the interaction of BimS with Bcl-2 by binding to BimS, a scenario that would explain why BimSΔΔ functions dominantly-negatively to inhibit the S-3-induced apoptosis in bax−/−/bak−/− cells that express BimS (supplemental Fig. S5).

To determine whether the membrane-permeabilizing activity of BimS and Bcl-2 can be generalized to other family members, we tested purified full-length Bcl-xL and BimL and found that Bcl-xL was unable to permeabilize liposomes in the presence of BimS (supplemental Fig. S6C), whereas BimL was slightly less effective than BimS to permeabilize liposomes in the presence of Bcl-2 (supplemental Fig. S6D). Therefore, BimS and likely BimL can interact with Bcl-2, embedding its α5 into the membrane to form an oligomeric pore, which is a potential mechanism used by the two Bim isoforms and Bcl-2 in the S-3-treated bax−/−/bak−/− cells to induce MOMP (Fig. 5F). Moreover, Bcl-2 is most likely unique among the antiapoptotic family members to have this proapoptotic activity. Unlike BimS and BimL, we could not purify BimEL and test it in the membrane permeabilization assay. However, BimEL may use the same mechanism as BimS and BimL to induce apoptosis because knock-in of each isoform restored the apoptosis in the bax−/−/ bak−/− cells in which all three endogenous Bim isoforms were knocked down (Fig. 2H). Because Bid was increased at an early stage in the S-3-treated bax−/−/ bak−/− cells (Fig. 2A), we tested its membrane-permeabilizing activity. Unlike Bim proteins, purified full-length Bid protein was unable to release the fluorescent dextrans from liposomes even at concentrations higher than those for Bim and in the presence of Bcl-2 (supplemental Fig. S6E). Bid was also unable to significantly increase the EDTA-resistant binding of Bcl-2 to liposomes (supplemental Fig. S6F). In contrast, purified tBid at a concentration higher than those used in our previous studies (23, 25) released the dextrans, particularly in the presence of Bcl-2 (supplemental Fig. S6E). However, tBid would not be the reason for cytochrome c release in the S-3-treated cells because it was not detected in the cells (data not shown). Whether other BH3-only proteins in the cells were involved in the MOM permeabilization requires further studies.

DISCUSSION

Antiapoptotic Bcl-2/Bcl-xL and proapoptotic Bax/Bak appear to have evolved from the same ancestor (46). Their structures are similar to the pore-forming domain of bacterial toxins (4, 5). Accordingly, both groups of proteins display membrane-permeabilizing activity (13, 23). Previous studies have generated overwhelming evidence that Bcl-2 is an antiapoptotic protein (1–3). However, Bcl-2 promotes apoptosis after caspase cleavage (47) or after interaction with Nur77 (28, 29). Although these findings show that antiapoptotic Bcl-2 can be converted to a proapoptotic molecule, the conversion either requires caspase activation that occurs downstream of Bax- or Bak-mediated MOMP or generates a molecule whose apoptotic activity depends on Bax or Bak. In contrast, the S-3-induced apoptosis was independent of Bax and Bak but dependent on Bcl-2 and Bim (Figs. 2 and 4 and supplemental Figs. S2 and S5). Mechanistically, interaction of Bcl-2 and Bim in membranes was required for permeabilizing the membranes (Fig. 5). The interaction induced conformational alterations in Bcl-2 in both biologically relevant cells and biochemically defined membranes (Figs. 4 and 5 and supplemental Figs. S4 and S6). Moreover, mutations that inhibit the interaction and the conformational alterations also impaired the membrane-permeabilizing activity (Fig. 5 and supplemental Fig. S6). Therefore, when its expression was up-regulated by S-3, Bim physically interacted with Bcl-2 in the MOM, changing its conformation to form large pores that release cytochrome c and induce apoptosis independently of Bax and Bak (Fig. 5F).

Following interaction with Bim or tBid, the conformation of Bcl-2 is altered from a tail-anchoring state to a multispanning state that forms small pores in membranes, an activity that is directly correlated with the antiapoptotic activity in Bax/Bak-expressing cells (22–24). These results can be reconciled with our current findings. Thus, although interaction with Bim can change Bcl-2 to the “pore” conformation, Bim is mostly sequenced by microtubules in the cytosol and hence is not able to interact with Bcl-2 at mitochondria (48). When the number of Bim molecules localized to mitochondria is limited, the number of Bcl-2 molecules in the pore conformation is also limited and insufficient to form large oligomeric pore to releases cytochrome c (23). Indeed, Bim expression alone was not sufficient to kill cells in the presence of Bcl-2 (40), and BimS, which activated Bcl-2 to form large pores only at high concentrations (Fig. 5A), is the least abundant Bim isoform in cells (49). When Bim expression was dramatically increased by S-3 treatment, more Bim would escape from the cytosolic microtubules and migrate to mitochondria where it interacted with Bcl-2 to form more and larger pores via mass action. Indeed, the mutation that abolished BimS targeting to mitochondria also abolished the proapoptotic activity, which could be restored by adding back a mitochondrial targeting sequence (supplemental Fig. S5). Of note, Bim may preferentially interact with Bax/Bak when they are present. Therefore, the Bim-dependent thymic selection (31) was significantly impaired by Bax/Bak deficiency (50). However, Bcl-2 may become the default target for Bim in the absence of both Bax and Bak.

Eviding apoptosis is absolutely required for cancer development (51), and most cancers do so by altering the expression and activity of Bcl-2 family proteins. They may increase the expression of Bcl-2 and other antiapoptotic proteins and/or reduce or eliminate the expression or activation of BH3-only proteins and/or Bax and Bak (52–54). Treatment of these cancers with S-3 may have the following advantages over the conventional anticancer therapies that rely on the proapoptotic
Bcl-2 family proteins to eradicate cancers. First, S-3 can increase the expression of Bim, which can activate Bax/Bak if Bax/Bak are present. Second, if Bax/Bak are eliminated or mutated, Bim can convert Bcl-2 to a Bax-like molecule. Third, overexpression of Bcl-2 in the cancers would enhance, instead of inhibit, the S-3-induced, Bim-mediated, and Bax/Bak-independent apoptosis. Fourth, S-3 displayed no adverse effect on the animals yet induced apoptosis in the bax−/−/bak−/−/ tumor implanted in mice (Fig. 3 and supplemental Fig. S3). Therefore, our findings have important implications for the development of cancers and its mutants plasmids. Y. Liu for operating the confocal microscope, and J. Wang for operating the flow cytometer.

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