The Bcl-2 homology (BH) 3-only pro-apoptotic Bcl-2 family protein Bim plays an essential role in the mitochondrial pathway of apoptosis through activation of the BH1–3 multidomain protein Bax or Bak. To further understand how the BH3-only protein activates Bax, we provide evidence here that BimEL induces Bax conformational change and apoptosis through a Bcl-XL-suppressible but heterodimerization-independent mechanism. Substitution of the conserved leucine residue in the BH3 domain of BimEL for alanine (M1) inhibits the interaction of BimEL with Bcl-XL but does not abolish the ability of BimEL to induce Bax conformational change and apoptosis. However, removal of the C-terminal hydrophobic region from the M1 mutant (M1ΔC) abolishes its ability to activate Bax and to induce apoptosis, although deletion of the C-terminal domain (ΔC) alone has little if any effect on the pro-apoptotic activity of BimEL. Subcellular fractionation experiments show that the Bim mutant M1ΔC is localized in the cytosol, indicating that both the C-terminal hydrophobic region and the BH3 domain are required for the mitochondrial targeting and pro-apoptotic activity of BimEL. Moreover, the Bcl-XL mutant (mt1), which is unable to interact with Bax and BimEL, blocks Bax conformational change and cytochrome c release induced by BimEL in intact cells and isolated mitochondria. BimEL or Bak-BH3 peptide induces Bax conformational change in vitro only under the presence of mitochondria, and the outer mitochondrial membrane fraction is sufficient for induction of Bax conformational change. Interestingly, native Bax is attached loosely on the surface of isolated mitochondria, which undergoes conformational change and insertion into mitochondrial membrane upon stimulation by BimEL, Bak-BH3 peptide, or freeze/thaw damage. Taken together, these findings indicate that BimEL may activate Bax by damaging the mitochondrial membrane structure directly, in addition to its binding and antagonizing Bcl-2/Bcl-XL function.

Mitochondrion is the crucial regulatory organelle for the signaling pathway of apoptosis (1–4). Many death signals cause irreversible dysfunction of mitochondria, leading to the release of several mitochondrial intermembrane space proteins such as cytochrome c, AIF, Smac/DIABLO, Endo G, and Omi/HtrA2 into the cytosol or nucleus where they are actively involved in the process of apoptotic cell death (5–14). The Bcl-2 family of proteins plays a pivotal role in the regulation of mitochondrial integrity and response to apoptotic signals (1, 15). Anti-apoptotic Bcl-2 family proteins prevent the release of apoptogenic molecules from the intermembrane space of mitochondria, whereas pro-apoptotic members of the Bcl-2 family induce those events. In addition, the Bcl-2 family proteins have been reported to control the mitochondrial function such as the permeability transition pore opening through adenine nucleotide translocator or voltage-dependent anion channel, ADP/ATP exchange, or oxidative phosphorylation during apoptosis (1, 16–18). Although the relative ratio of pro- and anti-apoptotic Bcl-2 family members is known to affect the mitochondrial homeostasis, the molecular mechanism underlying the release of mitochondrial intermembrane proteins remains elusive.

Bcl-2 family proteins contain up to four evolutionarily conserved domains called Bcl-2 homology (BH)1 domains 1 to 4 (15, 19). The anti-apoptotic Bcl-2 family proteins such as Bcl-2 and Bcl-XL share all the conserved BH domains (BH1–4) and can interact with pro-apoptotic Bcl-2 family proteins. In contrast, the pro-apoptotic members of the Bcl-2 protein family are divided into the BH1–3 multidomain and BH3-only subgroups. It has been shown that the pro-apoptotic BH1–3 proteins Bax and Bak have redundant function and are required for the induction of apoptosis in response to a variety of death signals (20, 21). Bax is localized predominantly in the cytosol of healthy cells and translocates to mitochondria after apoptotic stimulation (22). It has been reported that death signals induce a conformational change in the N and C termini of Bax, leading to its mitochondrial translocation, oligomerization or cluster formation, and integration into the mitochondrial membranes (23–33). These events have been implicated in the process of cytochrome c release, although the precise biochemical mechanism by which Bax induces cytochrome c release is still controversial (17, 31, 34–37).

The anti-apoptotic Bcl-2 family members such as Bcl-2 and Bcl-XL can inhibit BH1–3 protein activation, whereas the BH3-only proteins promote it. It has been suggested that BH3-only proteins induce the activation of BH1–3 proteins by direct binding to these multidomain pro-apoptotic molecules or indirect inhibition of anti-apoptotic Bcl-2 family proteins (38).

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BH3-only proteins such as Bad, Bid, Noxa, Puma, and Bmf have been shown to absolutely require Bax or Bak to induce mitochondrial dysfunction and cell death as demonstrated in Bax and Bak double knock-out cells (21, 39). Evidence has accumulated that the BH3-only proteins transduce diverse proximal apoptotic signals to the BH1–3 multidomain proteins by a variety of post-translational modifications or transcriptional regulation (38, 40–42). For example, Bid is phosphorylated and inactivated by several survival protein kinases, whereas Bid is activated through proteolysis by caspase-8, Granzyme B, or lysosomal proteases. Noxa and Puma are induced by p53 in response to DNA damage, whereas Bmf is activated by release from the cytoskeleton compartment after anoikis stimulation. Bim is another BH3-only member of the Bcl-2 protein family whose expression is induced by growth factor withdrawal or T-cell receptor-CD3 stimulation (43–47). Several isoforms of Bim such as BimEL, BimL, BimS, and BimAD have been characterized (48, 49). BimEL and BimL are expressed in a variety of tissues and cell types (50), normally associated with the microtubule-dynein complex, and released after death stimulation (51).

We have reported previously that the survival protein kinase Akt inhibits Bax conformational change and its translocation to mitochondria (29). Akt has been shown recently to phosphorylate and inhibit Forkhead transcription factors that induce several pro-apoptotic proteins including Bim (46, 52). These findings suggest that Bid might have a pivotal role in activation of the pro-apoptotic Bax protein, which is regulated by Akt. In this study, therefore, we investigated the possible involvement of Bim in Bax conformational change and its redistribution to the mitochondrial membranes. We show that BimEL triggers Bax conformational change and cytochrome c release, which can be suppressed by Bcl-XL but is independent from its ability to heterodimerize with this anti-apoptotic Bcl-2 family protein.

EXPERIMENTAL PROCEDURES

Materials—Anti-Bax (N-20) polyclonal antibody was purchased from Santa Cruz Biotechnology. Anti-Bim, HA, and FLAG polyclonal antibodies, anti-Bax (6A7) monoclonal antibody, anti-FLAG M2 monoclonal antibody, and anti-FLAG M2-conjugated agarose were purchased from Sigma. Anti-HA monoclonal antibody was purchased from BabCO. Anti-cytochrome c monoclonal antibody was obtained from BD PharMingen. Anti-COX IV monoclonal antibody was purchased from Molecu- lar Probes. Cellular membrane-permeable Bak-BH3 peptide was described previously (33).

Cell Culture and Transfection—293T and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum and 1% penicillin/streptomycin. 293T cells were transfected with various combinations of Bim and Bcl-XL expression plasmids, with 10 ng of pGL3 encoding luciferase under the control of β-actin promoter. For 24 h, cells were washed, harvested, and subjected to luciferase assay with luciferase assay system (Promega) according to the manufacturer’s protocol.

Commmunoprecipitation/Immunoblot Analysis—Cells were lysed in Nonidet P-40 lysis buffer (1% Triton X-100, 50 mM KCl, 5 mM MgCl2, 1 mM EGTA, 0.5% Nonidet P-40, and 10 mM Hepes, pH 7.5) or Chaps lysis buffer (29) supplemented with protease inhibitors (29). Cell lysates were normalized for protein content and subjected to immunoprecipitation by incubation of 500 μg of total proteins with 20 μl of anti-FLAG M2 beads or protein G-agarose preadsorbed with anti-HA monoclonal antibody in and the same lysis buffer at 4 °C for 2 h. After immunoprecipitation with the same lysis buffer, the beads were resuspended in Laemmli sample buffer, and the eluted proteins were subjected to SDS-PAGE immunoblot analysis with the indicated antibodies.

Detection of Bax Conformational Change—Active Bax was detected by communoprecipitation with anti-Bax 6A7 antibody, which recognizes only the conformationally changed Bax protein in Chaps lysis buffer (53). Briefly, cells were lysed in Chaps lysis buffer containing protease inhibitors (29), and 500 μg of total protein was incubated with 2 μl of anti-Bax 6A7 monoclonal antibody in 500 μl of Chaps lysis buffer at 4 °C for 2 h. Then, 20 μl of protein G-agrose was added into the reactions and incubated at 4 °C for an additional 2 h, followed by elution of protein washing in the same lysis buffer and SDS-PAGE/immunoblot analysis with anti-Bax polyclonal antibody (33).

Subcellular Fractionation—The subcellular fractionation experiments were performed as described previously (29, 33). In brief, cells were homogenized in isotonic mitochondrial buffer (210 mM sucrose, 70 mM mannitol, 10 mM Hepes, pH 7.4, 1 mM EGTA) containing protease inhibitors and centrifuged at 1,000 × g for 10 min to discard nuclei and unbroken cells. The resulting supernatant was centrifuged at 10,000 × g for 15 min to pellet mitochondria-enriched heavy membrane fraction, and the supernatant was centrifuged further at 100,000 × g for 30 min to obtain cytosolic (S-100) fraction.

In Vitro Bax Conformational Change and Cytochrome c Release Assay—Cells were lysed in isotonic mitochondrial buffer with a Dounce homogenizer. After centrifugation at 1,000 × g for 10 min, the supernatant was centrifuged further at 10,000 × g for 15 min. The pellet was resuspended with isotonic mitochondrial buffer and centrifuged again at 1,000 × g for 5 min to remove residual nuclei. The resulting supernatant was then centrifuged at 8,000 × g for 15 min to obtain purified mitochondria. The mitochondrial pellet was resuspended in mitochondria assay buffer (210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 4 mM MgCl2, 5 mM KH2PO4, 5 mM succinate, pH 7.4), and the protein concentration was measured by BCA assay (Pierce). To obtain S-100 for in vitro assay, the cells were homogenized in mitochondria assay buffer and centrifuged at 10,000 × g for 10 min, followed by further centrifugation at 100,000 × g for 30 min. The resulting supernatant (S-100) was adjusted to 10 mg/ml total proteins and kept at −80 °C for future assays. The purified mitochondria (300–500 μg of total protein) were incubated with His6-BimEL recombinant proteins or Bak-BH3 peptide in 100 μl of mitochondria assay buffer or S-100 at 30 °C for the indicated times and subjected to the following assays. For cytochrome c release assay, the samples were centrifuged at 15,000 × g for 10 min, and 20 μl of the resulting supernatant was subjected to SDS-PAGE/immunoblot analysis with anti-cytochrome c antibody. For detection of Bax conformational change, 10% Chaps, 5 mM NaCl, and 1 mM Hepes, pH 7.5, were added to the samples to a final concentration of 1% Chaps, 150 mM NaCl, and 1 mM Hepes in a total volume of 200 μl. After centrifugation at 13,000 × g for 30 min, the supernatant was subjected to immunoprecipitation with anti-Bax 6A7 antibody to determine the conformationally changed Bax protein. For alkali lysis assay, the samples were centrifuged at 10,000 × g for 10 min, and the pellets (mitochon-dria) were resuspended in 1 ml of freshly prepared 0.1 M Na2CO3, pH 11.5. After 30 min on ice incubation, the samples were centrifuged at 13,000 × g for 30 min. The resulting supernatant was resuspended in Nonidet P-40 lysis buffer and subjected to SDS-PAGE/immunoblot analysis.

Isolation of Mitochondrial Outer Membrane—The outer membrane fraction of mitochondria was prepared as described (54). Briefly, the purified mitochondria from Du145 cells were resuspended in 10 mM Mops/KOH buffer, pH 7.4, and stirred on ice for 1 h. The resulting suspension was homogenized gently with a Dounce homogenizer and centrifuged twice at 13,000 × g for 10 min. The supernatants were centrifuged further at 100,000 × g for 1 h to obtain mitochondrial outer membrane (pellet fraction).
RESULTS

BimEL Requires Both BH3 Domain and C-terminal Hydrophobic Region for Its Full Mitochondrial Localization and Pro-apoptotic Activity—To elucidate the mechanisms by which Bim activates Bax, we first prepared Bim mutant expression plasmids by deletion of the C terminus hydrophobic region (ΔC), substitution of Leu152 in BH3 domain for alanine (M1), or mutation of both (M1ΔC) in BimEL (Fig. 1A) and expressed these Bim proteins in HeLa cells. The effects of these Bim mutants on cell viability was determined by luciferase assay (Fig. 1B), Bax conformational change was examined by immunoprecipitation with anti-Bax 6A7 antibody (Fig. 1C), and Bax translocation to mitochondria was detected by subcellular fractionation/immunoblot analysis (Fig. 1D). Overexpression of wild type BimEL exhibited the highest pro-apoptotic activity and most efficiently induced Bax conformational change and its translocation to mitochondria compared with the Bim mutants. The ΔC or M1 mutant also induced cell death and Bax activation, but their activities appeared to be a little weaker than that of the wild type BimEL protein. In contrast, the M1ΔC mutation completely abrogated the ability of BimEL to trigger apoptosis and to induce Bax conformational change and mitochondrial redistribution. These results indicate that the pro-apoptotic activity of the BH3-mutated BimEL protein requires its C-terminal hydrophobic region, although deletion of this hydrophobic domain alone does not much affect BimEL to induce apoptosis and Bax conformational change. It has been supposed that the hydrophobic region of BH3-only proteins may function as membrane targeting sequences (40). As shown in Fig. 1D, BimEL was localized in the mitochondria-enriched heavy membrane fraction as reported previously (48, 55). The BH3 mutant of BimEL (M1) was still localized predominantly on mitochondria compared with the ΔC mutant that resided in both cytosolic and heavy membrane fractions. However, the intracellular localization of M1ΔC was only found in the cytosol, indicating that both the C terminus hydrophobic region and the BH3 domain are required for the mitochondrial localization of BimEL, which may be critical for BimEL-mediated Bax activation and apoptosis.

BimEL Induces Bax Conformational Change and Apoptosis in a Heterodimerization-independent Manner—It has been reported that Bid, a member of the BH3-only subfamily, triggers Bax conformational change and subsequent oligomerization and insertion into mitochondrial membranes by direct binding of Bid to Bax (24, 56). Similar to Bid, BimS and BimAD have also been shown to be capable of interacting with Bax (49).
Therefore, we confirmed Bax association with BimEL or Bcl-XL by co-immunoprecipitation in the presence of Nonidet P-40 versus Chaps; it has been shown that Nonidet P-40 causes Bax conformational change whereas Chaps keeps Bax in its native conformation (53). In agreement with previous reports (49, 55), BimEL failed to interact with Bax in any conformation (Fig. 2A), suggesting that BimEL induces Bax conformational change through a heterodimerization-independent mechanism. In contrast, Bcl-XL was coimmunoprecipitated with Bax under the presence of Nonidet P-40 but not Chaps (Fig. 2A), consistent with previous reports (25, 53). Anti-apoptotic Bcl-2 family proteins suppress the pro-apoptotic function of Bax and Bak, whereas BH3-only proteins bind to Bcl-2/Bcl-XL and abrogate their pro-survival function. To investigate the association between Bcl-XL and BimEL, 293T cells were co-transfected with HA-Bcl-XL and FLAG-tagged BimEL or mutants, and co-immunoprecipitation was performed with anti-FLAG antibody. As shown in Fig. 2B, both wild type and ΔC mutant BimEL bound to Bcl-XL, but a single point mutation in the BH3 domain of BimEL (M1 or M1ΔC) dramatically abrogated the association of BimEL with Bcl-XL. These results indicate that the conserved leucine residue in the BH3 domain of BimEL is important for its association with Bcl-XL but is not absolutely required for its pro-apoptotic activity. Thus, the BH3-only protein BimEL may have a new pathway for induction of Bax conformational change and apoptosis, which is independent from its properties of heterodimerization with the anti-apoptotic Bcl-XL protein.

To further investigate the role of Bcl-XL in BimEL-induced Bax activation and apoptosis, we examined the effects of Bcl-XL mutant 1, which cannot bind Bax but retains anti-apoptotic function (57) on Bax conformational change and cell death induced by BimEL. As reported previously (57), this Bcl-XL mutant failed to interact with Bax, regardless of the presence of nonionic detergent (Fig. 3A). Coimmunoprecipitation experiments showed that BimEL was associated with wild type but not mutant 1 Bcl-XL in cells (Fig. 3B). However, both wild type and mutant 1 of Bcl-XL protected cells from apoptosis induced by BimEL overexpression (Fig. 3C). Moreover, overexpression of Bcl-XL m1 blocked Bim-induced Bax conformational change similar to wild type Bcl-XL (Fig 3D). These results further support a model that BimEL induces Bax conformational change and apoptosis through a Bcl-XL-suppressible but heterodimerization-independent pathway.

**BimEL Induces Bax Conformational Change and Membrane Insertion, as Well as Cytochrome c Release, in Vitro**—To further examine Bax activation by BimEL, we next employed an *in vitro* system using isolated mitochondria and recombinant BimEL proteins. The isolated mitochondria from MDA-MB-468 cells were incubated with different amounts of purified His$_{6}$-tagged BimEL protein without the C-terminal hydrophobic region (ΔC) for 60 min or with 100 ng of His$_{6}$-BimEL (ΔC) for various periods. Cytochrome c release from mitochondria was detected by immunoblot analysis, and Bax conformational change was examined by immunoprecipitation with anti-Bax 6A7 antibody. As shown in Fig. 4A, His$_{6}$-BimEL (ΔC) induced cytochrome c release from isolated mitochondria and triggered Bax conformational change in a dose- and time-dependent manner. Unlike His$_{6}$-BimEL (ΔC) and Bak-BH3 peptide, the His$_{6}$-tagged M1ΔC BimEL mutant failed to induce Bax conformational change and cytochrome c release from isolated mitochondria in *in vitro* (Fig. 4B), consistent with the results obtained in cultured cells. It has been shown that Bax becomes resistant to alkali extraction once integrated into mitochondrial membrane (58). Therefore, we treated mitochondria at alkaline pH after incubation with recombinant BimEL proteins or Bak-BH3 peptide to determine membrane-integrated Bax protein. As shown in Fig. 4C, treating mitochondria with His$_{6}$-BimEL (ΔC) protein or Bak-BH3 peptide dramatically increased the residence of Bax to alkaline extraction compared with M1ΔC BimEL mutant or phosphate-buffered saline control treatment, suggesting that the functional BimEL protein triggers Bax integration into the outer mitochondrial membrane. Moreover, mitochondria isolated from MDA-MB-468 cells expressing either wild type or mt1 mutant Bcl-XL were resistant to His$_{6}$-BimEL (ΔC)-induced cytochrome c release and Bax conformational change *in vitro* (Fig. 4D).

**BimEL Requires Mitochondria to Induce Bax Conformational Change**—To determine whether mitochondria are required for Bax conformational change, we incubated His$_{6}$-BimEL (ΔC) or Bak-BH3 peptide with cytosolic (Cyt) fraction *versus* mitochondria (Mt) or Mt plus Cyt isolated from healthy MDA-MB-468 cells. After incubation at 30 °C for 1 h, the conformationally changed Bax protein was detected by immuno-
precipitation with anti-Bax 6A7 antibody. As shown in Fig. 5A, both BimEL (ΔC) and Bak-BH3 peptide triggered Bax conformational change when incubated with Mt or Mt plus Cyt but not Cyt alone; immunoblot analysis however confirmed the presence of the Bax protein in all fractions, indicating that mitochondria are required for Bax conformational change. If Bax conformational change occurs on the surface of mitochondria, we reasoned that the native Bax molecules should be associated loosely with mitochondria in a concentration-dependent manner. Once conformationally changed, Bax becomes membrane-integrated and accumulated on mitochondria. To test this hypothesis, mitochondria isolated from Bax-deficient Du145 cells were incubated with a Cyt mixture of MDA-MB-468 (Bax+) and Du145 (Bax−) at different ratios for 1 h (Fig. 5B) or with 100 μM of MDA-MB-468 Cyt for various periods (Fig. 5C). After incubation, mitochondria were collected by centrifugation and subjected to immunoblot analysis with anti-Bax antibody. As shown in Fig. 5, B and C, Bax associated with mitochondria in a concentration- and time-dependent manner. However, this Du145 mitochondria-bound Bax protein was in native conformation as demonstrated by immunoprecipitation with anti-Bax 6A7 antibody (not shown). To determine whether the mitochondrial binding of nonintegrated native Bax is reversible, mitochondria from MDA-MB-468 cells were incubated in mitochondria assay buffer for up to 60 min. After centrifugation, the resulting supernatant and pellet (mitochondria) were subjected to SDS-PAGE/immunoblot analysis with anti-Bax antibody. As shown in Fig. 5D, Bax was released from mitochondria to supernatant in an incubation time-dependent manner. Interestingly, addition of purified recombinant His₆-BimEL (ΔC) protein (Fig. 5D) or synthesized Bak-BH3 peptide (not shown) prevented the dissociation of Bax from mitochondria. These results suggest that native Bax is associated loosely with mitochondria and undergoes a conformational change and integration into mitochondrial membranes upon apoptotic signals such as BimEL and Bak-BH3 peptide.

Bax Conformational Change Is Induced by Damaged Mitochondrial Membrane—The data presented above indicated that mitochondria are required for Bax conformational change induced by BimEL or Bak-BH3 peptide, suggesting a possibility that some factor(s) is released from mitochondria that is
involved in Bax conformational change. To investigate this possibility, Du145 mitochondria were treated with Bak-BH3 peptide or Me2SO control for 1 h and subjected to centrifugation. The resulting supernatant or pellet was incubated with MDA-MB-468 cytosolic fraction for 1 h, and the conformationally changed Bax protein was examined by immunoprecipitation with anti-Bax 6A7 antibody. As shown in Fig. 6A, the pellet (mitochondria) but not the supernatant induced Bax conformational change and cytochrome c release from isolated mitochondria. A, mitochondria from MDA-MB-468 cells were incubated with 100 ng of His6-BimEL (ΔC) protein at 30 °C for 1 h and analyzed for cytochrome c release and Bax conformational change. C, mitochondria from MDA-MB-468 cells expressing HA-tagged wild type (wt) or mutant 1 (mt1) Bcl-XL or control MDA-MB-468-Neo cells, incubated with 0, 50, or 100 ng of His6-BimEL (ΔC) protein at 30 °C for 1 h, and analyzed for cytochrome c (Cyt C) release and Bax conformational change. The total cell lysates were analyzed by immunoblotting to verify the expression of the transgenes.
Fig. 6. Damage of mitochondrial membranes causes Bax conformational change. A, Du145 mitochondria were incubated with 50 μM Bak-BH3 peptide or control Me2SO in 100 μl of mitochondria assay buffer at 30 °C for 1 h. After centrifugation, the resulting supernatant (S) or pellet (P) was incubated with 100 μl of MDA-MB-468 cytosol at 30 °C for 1 h, and the conformationally changed Bax protein was detected by immunoprecipitation with anti-Bax 6A7 antibody. In lanes 1 and 2, Du145 mitochondria were directly incubated with 100 μl of MDA-MB-468 cytosol in the presence of 50 μM Bak-BH3 peptide or control Me2SO for 1 h at 30 °C and analyzed for Bax conformational change. B, Du145 Mt or mitochondrial outer membranes (OM) were resuspended in 100 μl of MDA-MB-468 cytosol, incubated with 50 μM Bak-BH3 peptide or control Me2SO at 30 °C for 1 h, and subjected to analysis of Bax conformational change. C, MDA-MB-468 mitochondria in 100 μl of mitochondria assay buffer were subjected to three cycles of freeze-thaw (F/T) treatment (+) in liquid nitrogen and water bath or left untreated (–) and followed by incubation with 50 μM of Bak-BH3 peptide or control Me2SO at 30 °C for 2 h. The samples before (Pre-incubation) and after incubation were analyzed by immunoprecipitation with anti-Bax 6A7 antibody to detect conformationally changed Bax protein. D, mitochondria isolated from MDA-MB-468 cells expressing HA-tagged Bcl-XL or Neo control were subjected to freeze-thaw cycles and followed by incubation at 30 °C for the indicated times prior to analysis of Bax conformational change.

conformational change, implying that there is no Bax activator released from mitochondria after Bak-BH3 treatment. Moreover, the presence or absence of ATP, ADP, succinate, or alkaline pH did not alter Bax conformational change induced by Bak-BH3 peptide (not shown).

To determine whether the whole mitochondrial is required for Bax conformational change, Du145 mitochondria or outer mitochondrial membranes were incubated with MDA-MB-468 cytosol in the presence or absence of Bak-BH3 peptide, and the conformationally changed Bax protein was detected by immunoprecipitation with anti-Bax 6A7 antibody. As shown in Fig. 6B, the outer mitochondrial membrane fraction alone induced Bax conformational change. It is possible that the outer mitochondrial membrane structure was damaged during the process of isolation, which causes Bax conformational change. To test this possibility, mitochondria from MDA-MB-468 cells were damaged by freeze-thaw three times and incubated at 30 °C for 2 h prior to conformational change analysis with anti-Bax 6A7 antibody. As shown in Fig. 6C, there was no significant change in Bax conformation in samples after freeze-thaw cycles, but after 2 h of incubation at 30 °C the conformationally changed Bax apparently increased regardless of the absence of Bak-BH3 peptide. However, the cytosolic fraction did not exhibit any activity to induce Bax conformational change after freeze-thaw cycles and 2 h of incubation at 30 °C (not shown). Moreover, the Bax conformational change induced by freeze-thaw damage was inhibited significantly in mitochondria isolated from Bcl-XL-overexpressing MDA-MB-468 cells compared with Neo control cells (Fig. 6D).

**DISCUSSION**

It has been well examined that the BH1–3 proteins Bak and Bax are executors of the mitochondrial pathway of apoptosis whose activation can be prevented by anti-apoptotic Bcl-2 family proteins such as Bcl-2 and Bcl-XL (4, 38). Moreover, BH3-only proteins have been shown to be upstream regulators of Bax/Bak activation. However, the mechanisms by which BH3-only proteins regulate activity of BH1–3 multidomain proteins remain uncertain. Our findings reported here demonstrate that the BH3-only protein BimEL induces Bax conformational change and apoptosis through a Bcl-XL-suppressible but heterodimerization-independent mechanism.

**Mechanisms of Bax Activation by BimEL**—The BH3 domains have been shown to be essential for the toxicity of BH3-only proteins (19, 42). Most of the BH3-only proteins appear to function essentially as transdominant inhibitors by binding to anti-apoptotic Bcl-2 family proteins and antagonizing their pro-survival activity (19, 42). However, our results indicate that the BH3-only protein BimEL can also induce Bax activation and cell death in a heterodimerization-independent manner. Moreover, our findings suggest that BimEL requires the presence of mitochondria to trigger Bax conformational change and that the mitochondrial localization of BimEL seems to be critical for induction of Bax activation and apoptosis rather than its heterodimerization property. Indeed, it has been shown that α-helical peptide damages mitochondria and induces apoptosis (59). The Bad peptides with mutations in the BH3 domain are unable to bind to Bcl-2 but induce apoptosis by the retained α-helical structure (60). Therefore, BimEL may also exert its pro-apoptotic activity via its α-helical domain. Our results indicate that BimEL requires both the C terminus hydrophobic region and the BH3 domain for its mitochondrial localization. The BH3 domain may target BimEL to mitochondria not only through association with anti-apoptotic Bcl-2 family proteins but also by direct binding to mitochondrial membranes via its α-helical structure. This is supported by the findings that the BH3-mutated BimEL (M1) protein is unable to interact with Bcl-XL but can induce Bax conformational change and apoptosis. Moreover, deletion of the C-terminal hydrophobic region from the M1 BimEL mutant (M1ΔC) abrogates its mitochondrial targeting and pro-apoptotic activity.

Several observations indicate that the truncated Bim, another BH3-only protein of the Bcl-2 family, binds to cardiolipin...
Mechanisms of Bax Inhibition by Bcl-XL—It has been shown that anti-apoptotic Bcl-2 family proteins maintain mitochondrial integrity during apoptosis by controlling the release of pro-apoptotic mitochondrial proteins, dysfunction of ADP/ATP exchange, and oxidative phosphorylation, production of reactive oxygen species, and subsequent lipid peroxidation (2). Although many of the Bcl-2 family proteins can interact with each other, the significance of homo- and heterodimerization remains unclear (15). In this study, the Bcl-XL mutant (mt1) does not bind to Bim, but inhibits Bim-induced apoptosis. Moreover, the BimEL mutant (M1) does not associate with the anti-apoptotic Bcl-XL protein but induces apoptosis. It has also been shown that a Bid mutant, which can not bind to Bcl-2/Bcl-XL, induces Bax conformational change and apoptosis in a Bcl-2/Bcl-XL-suppressible manner (71, 72). Furthermore, Bcl-2 inhibits cytochrome c release induced by TRX or ceramide, which does not interact with Bcl-2 (67, 70).

The BH4 domain is conserved within the anti-apoptotic Bcl-2 family proteins and is implicated to be essential for their anti-apoptotic function but not heterodimerization with pro-apoptotic Bcl-2 family proteins (15, 73). The BH4-deleted Bcl-2 mutant can still interact with pro-apoptotic Bcl-2 family proteins including Bim but has no anti-apoptotic activity (74). In addition, it has been shown that BH4 peptide inhibits voltage-dependent anion channel activity and apoptosis (73). We also confirmed that mutation in the BH4 domain of Bcl-XL abolishes its cytoprotective activity against Bim-induced apoptosis (not shown), suggesting that anti-apoptotic Bcl-2 family proteins may also exert their anti-apoptotic effects through the conserved BH4 domain.

Interestingly, our results indicate that Bcl-XL can also inhibit Bax conformational change induced by freeze-thaw damage in vitro, suggesting that this anti-apoptotic protein has other roles in the regulation of mitochondrial integrity besides its previously described heterodimerization function. Clearly, further studies are required to identify the primary functions of Bcl-2/Bcl-XL and other players in the regulation of Bax conformational change.
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