tBID Homooligomerizes in the Mitochondrial Membrane to Induce Apoptosis*

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Activation of the tumor necrosis factor R1/Fas receptor results in the cleavage of cytosolic BID to truncated tBID. tBID translocates to the mitochondria to induce the oligomerization of BAX or BAK, resulting in the release of cytochrome c (Cyt c). Here we demonstrate that in tumor necrosis factor α-activated FL5.12 cells, tBID becomes part of a 45-kDa cross-linkable mitochondrial complex that does not include BAX or BAK. Using fluorescence resonance energy transfer analysis and co-immunoprecipitation, we demonstrate that tBID-BID interactions occur in the mitochondria of living cells. Cross-linking experiments using a tBID-GST chimera indicated that tBID forms homotrimers in the mitochondrial membrane. To test the functional consequence of tBID oligomerization, we expressed a chimeric FKBP-tBID molecule. Enforced dimerization of FKBP-tBID by the bivalent ligand FK1012 resulted in Cyt c release, caspase activation, and apoptosis. Surprisingly, enforced dimerization of tBID did not result in the dimerization of either BAX or BAK. Moreover, a tBID BH3 mutant (G94E), which does not interact with or induce the dimerization of either BAX or BAK, formed the 45-kDa complex and induced both Cyt c release and apoptosis. Thus, tBID oligomerization may represent an alternative mechanism for inducing mitochondrial dysfunction and apoptosis.

The BCL-2 family members are major regulators of the apoptotic process. The cell death regulatory activity of these molecules is unknown, although it is thought that their function depends mostly on their ability to modulate mitochondrial function (1). This family is comprised of both pro-apoptotic (e.g. BAX and BAK), as well as anti-apoptotic (e.g. BCL-2 and BCL-XI) molecules. Most family members share homology in three domains (BH1–3; “multidomain” members) and carry a C-terminal 59 amino acids would potentially expose the BH3 domain (2–4). Moreover, enforced dimerization of an FKBP-BAX chimera by the bivalent ligand FK1012 induces apoptosis (2). In addition, using fluorescence resonance energy transfer (FRET) analysis, BCL-2 and BAX have been demonstrated to interact with each other in the mitochondrial membrane (5).

The three-dimensional structures of several family members show similarities to the structure of the pore-forming region of bacterial toxins (6). Based on this structural similarity and on in vitro electrophysiological data (7), it is suspected that BCL-2 family members may function as pore-forming proteins. Their tendency to oligomerize and their prominent subcellular location at intracellular membranes may add further support to the “pore hypothesis.”

Caspases are the major executors of the apoptotic process (8). The BH3-only molecule BID serves as one of the links between BCL-2 family members and caspases. Following TNF-R1/Fas receptor activation, cytosolic p22 full-length BID is cleaved by caspase-8 (9–11). The truncated product, p15 tBID, translocates to the mitochondria to induce the release of cytochrome c (Cyt c). Cyt c activates Apaf-1, which in turn activates caspase-9 (12). Most importantly, BID is an essential component of the TNF-R1/Fas-death receptor pathway in hepatocytes, because Bid-deficient mice are resistant to the lethal effect of anti-Fas antibody injection (13).

Full-length BID was initially identified as the only BCL-2 family member that can bind both pro-apoptotic (e.g. BAX) as well as anti-apoptotic (e.g. BCL-2) molecules (14). It was also demonstrated that full-length BID does not homodimerize. The identification of the binding partners led to the assumption that BID acts as a “death ligand”: it receives a death signal in the cytosol and translocates to the mitochondria to transfer the signal to “death receptors” (e.g. BAX or BCL-2). Recently, it was indeed demonstrated that following its cleavage and translocation to the mitochondria, tBID induces the oligomerization of BAX or BAX, which results in Cyt c release (3, 4). Strikingly, murine embryonic fibroblasts lacking both BAX and BAK are resistant to tBID-induced apoptosis (15).

Based on the three-dimensional structure of BID, it was proposed that cleavage by caspase-8 and removal of the N-terminal 59 amino acids would potentially expose the BH3 domain.
domain together with a large hydrophobic domain (6). This hydrophobic domain, which includes two central hydrophobic core helices, constitutes the potential to form channels in membranes (16). The exposure of this hydrophobic core may also lead to association with new partners.

Here we examine the binding partners of tBID in the mitochondrial membrane following its translocation. We note that following a TNFα death signal, tBID becomes part of a 45-kDa cross-linkable complex in the membrane, which most likely represents a tBID homotrimer. Using a chimeric FKBP-tBID protein, we demonstrate that enforced dimerization of tBID is sufficient to induce Cyt c release, caspase activation, and apoptosis. However, these tBID dimers did not induce the dimerization of BAX or BAK. Thus, tBID oligomers may act in an alternative pathway to induce mitochondrial dysfunction and apoptosis.

EXPERIMENTAL PROCEDURES

TNFα/CHX Treatment and Subcellular Fractionation—FL5.12, an immortalized murine early hematopoietic cell line, was maintained in 10% fetal bovine serum, supplemented with 10% WEHI-3B conditioned medium as a source of interleukin-3. FL5.12 cells were treated with recombinant mouse TNFα (40 ng/ml; Sigma) and cycloheximide (1 μg/ml; Sigma) for 6 h, suspended in isotonic HIM buffer (200 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM HEPES, pH 7.5) and homogenized either using a polytron homogenizer (Brinkmann Instruments) at setting 6.5 for 10 s or by passing the cells 20 times through a 25 G (0.5 × 16) needle. Nuclei and unbroken cells were removed by centrifugation at 120 × g for 5 min. The supernatant was centrifuged at 10,000 × g for 10 min to collect the mitochondria-enriched fraction (mitochondria) and the supernatant (cytosol).

Transient Transfection System—293T, an embryonic kidney cell line, HeLa, a human cervical adenocarcinoma cell line, and Cos-7, a simian kidney cell line, were maintained in 10% fetal bovine serum. Transient transfections were performed by the calcium phosphate method (17) or with LipofectAMINE (Invitrogen). For the FKBP/FK1012 experiments, the cells were treated with 0.3 μM FK1012/EZ alone (1 μM stock solution in 50% ethanol, 50% dimethyl sulfoxide) or in combination with 1 μM FK506. FK1012/EZ and FK506 were generous gifts from P. Clemons and S. L. Schreiber (Harvard University).

For propidium iodide staining experiments, the cells were allowed to grow to ~60% confluence in 10-cm plates before transfection. The cells were transfected with 15 μg of each of the indicated plasmids. Twenty-four hours post-transfection, the cells were collected, washed once in phosphate-buffered saline (PBS), and fixed with methanol at −20 °C. The cells were recovered by centrifugation at 1,000 × g for 5 min, washed once in 488-labeled goat anti-rabbit Abs (dilution 1:150, Molecular Probes), and the cells were viewed under a Nikon fluorescence microscope. For immunostaining experiments, Cyt c, the caspase activation substrates, and Anti-mBID Ab and anti-Cyt c H6.2 H4 mAb (PharMingen) were treated briefly with trypsin and seeded into eight-chamber glass slides (Lab-Tek). The cells were then allowed to settle on the slide for 5 h. Single cells were excited at 430 nm using Polychrome II (TILL Photonics, Martinsried, Germany) and detected by first subtracting the dark noise (average of 258 counts/collection time) from the signal and then dividing the signal at 535 nm (YFP emission) by the signal at 490 nm (CFP emission).

RESULTS

BID Becomes Part of a 45-kDa Mitochondrial Complex in TNFα-activated FL5.12 Cells—To reveal the mechanism by which BID executes its function, we utilized a series of chemical cross-linkers to identify proteins that closely associate with tBID at the mitochondrial membrane following its translocation. Treatment of FL5.12 hematopoietic cells with TNFα in the presence of CHX induces the cleavage of cytosolic full-length BID to generate p15 tBID that translocates to the mitochondria (Fig. 1A and Ref. 11). Mitochondria-enriched fractions prepared from healthy cells or from cells pretreated with TNFα/CHX were treated with sulfo-BSOCOES, a 13-μM, primary

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Expression Plasmids—Wild-type p15 tBID and tBID mIII-4 were amplified by PCR from wild type p22 BID (14). Human tBID (12) and a series of chemically crosslinkable complexes in the membrane, which most likely represents a tBID homotrimer. Using a chimeric FKBP-tBID protein, we demonstrate that enforced dimerization of tBID is sufficient to induce Cyt c release, caspase activation, and apoptosis. However, these tBID dimers did not induce the dimerization of BAX or BAK. Thus, tBID oligomers may act in an alternative pathway to induce mitochondrial dysfunction and apoptosis.

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Immunocytochemistry and Confocal Microscopy—For immunocytochemistry, the cells were grown on fibronectin-coated glass coverslips. Twelve hours post-transfection the cells were fixed with 3% paraformaldehyde in PBS for 6 min and permeabilized with 0.2% Triton X-100 in PBS for 5 min. For blocking, the cells were incubated in PBS containing 0.1% Triton and 3% BSA for 1 h at room temperature. To immunostain both Cyt c, the caspase activation substrates, and Anti-mBID Ab and anti-Cyt c H6.2 H4 mAb (PharMingen) diluted 1:200 in blocking solution. After three washes with PBS containing 0.1% Triton, the cells were stained for 30 min at room temperature with Cy3-labeled goat anti-mouse (dilution 1:100, Jackson Immunoresearch) and Alexa 488-labeled goat anti-rabbit Abs (dilution 1:150, Molecular Probes), followed by 5 min of 4’,6-diamidino-2-phenylindole dihydrochloride staining (diluted 1:500 in PBS). For staining of mitochondria, the cells were incubated with 100 nM mitotracker red (MTR; Molecular Probes) for 30 min at 37 °C prior to fixation. The coverslips were mounted with elvanol, and the cells were viewed under a Nikon fluorescence microscope at a magnification of 400×. The pictures were taken with a 1310 digital camera (DVC). Confocal microscopy was performed using a Zeiss Axiovert 100 TV microscope (Oberkochen, Germany) attached to the Bio-Rad Radiance 2000 laser scanning system and operated by LaserSharp software.

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2 N. Grammatikakis, paper in preparation.
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Fig. 1. BID becomes part of a 45-kDa mitochondrial complex in TNFα-activated FL5.12 cells. A, FL5.12 cells were grown in the absence or in the presence of TNFα/CHX for 6 h. Cytosol (cyto) and mitochondrial membranes (mito) suspended in isotonic buffer were treated with either the sulfo-BSOCOES cross-linker (+) or with MeSO as a control (−). Western blot analysis was performed with the indicated antibodies. IB, immunoblot. B, BID is not part of the BAX-immunoreactive cross-linkable complexes. FL5.12 cells were grown in the presence of TNFα/CHX for 6 h and treated as above. Following cross-linker treatment of the mitochondrial fraction, BAX was immunoprecipitated using anti-mBAX (4D2) monoclonal antibodies. Western blot analysis was performed with either anti-mBAX (651) (left panel) or anti-BID (right panel) polyclonal antibodies. Lane 1 is a direct Western blot of the mitochondrial fraction. As a control, the mitochondrial fraction was incubated only with the secondary antibody (cont; lanes 3 and 5). IB, immunoblot; IP, immunoprecipitation.

It was previously demonstrated that tBID heterodimerizes with BAK or BAX to induce their oligomerization in the mitochondrial membrane (3, 4). Therefore we checked whether BAX or BAK were part of the 45-kDa complex. Western blot analysis with anti-BAK antibodies did not detect any new bands (Fig. 1A, lane 9), indicating that BAK is not part of the 45-kDa BID complex. On the other hand, Western blot analysis with anti-BAX antibodies detected two additional BAX-immunoreactive bands slightly below the 46-kDa marker (Fig. 1A, lane 13). To determine whether one of these bands represents a BAX-BID heterodimer, BAX was immunoprecipitated (using the 4D2 monoclonal antibody) from mitochondria following cross-linker treatment, and the immunocomplexes were subjected to Western blot analysis using either anti-BAX (651) or anti-BID polyclonal antibodies. As shown in Fig. 1B, anti-BAX antibodies recognized these two upper bands in the BAX immunoprecipitate (lane 2), but anti-BID antibodies did not (lane 4). Based on these results, it is likely that these bands represent a BAX homodimer and not a BAX-BID heterodimer. Western blot analysis with a variety of other antibodies against BCL-XL, BCL-2, porin/VDAC, adenine nucleotide translocator, and Cyt c indicated that none of these molecules were part of the 45-kDa BID complex (not shown).

Fig. 2. Truncated BID is part of the 45-kDa mitochondrial complex. A, kinetics of tBID targeting to mitochondria and formation of the 45-kDa complex. 35S-Labeled in vitro transcribed and translated tBID was incubated with purified intact mitochondria from mouse liver in a standard protein import reaction for 2.5–20 min. At the indicated time points, mitochondria were separated from the soluble fraction by centrifugation, and both fractions were treated with sulfo-BSOCOES. After treatment, membranes and soluble fractions were lysed and analyzed by autoradiography (lanes 1–7) or by Western blot with anti-BID antibodies (lanes 8 and 9). The asterisk indicates protein bands in the mitochondrial and soluble fractions that migrate at ~40 kDa but do not include tBID. B, recombinant tBID forms homooligomers in solution in the absence of mitochondria and cross-linker. Recombinant tBID was suspended in Laemmli sample buffer in the presence (lanes 1 and 2) or absence (lanes 3 and 4) of 50 mM dithiothreitol (DTT) and incubated for 3 min at 100 °C (lanes 1 and 3). The samples were analyzed by Western blot with anti-BID Ab.

The 45-kDa Mitochondrial Complex Appears Immediately following the Mitochondrial Targeting of tBID—To confirm that tBID is part of the 45-kDa complex and to assess the time course of its formation in the mitochondrial membrane, we performed a standard protein import reaction using tBID. 35S-Labeled in vitro transcribed and translated tBID was incubated with purified intact mouse liver mitochondria, followed by centrifugation to separate the mitochondria from the soluble fraction. Both fractions were treated with the sulfo-BSOCOES cross-linker and analyzed by autoradiography. Ten minutes after adding tBID to mitochondria, a substantial amount of tBID was incorporated into the mitochondrial membrane (Fig. 2A, lane 4). As soon as tBID appeared in the mitochondria, the 45-kDa cross-linked complex also appeared. Moreover, as more tBID incorporated into the membrane, the intensity of the 45-kDa band increased (Fig. 2A, lane 7). We have also probed the blot with anti-BAX antibodies against BAX, BAK, BCL-XL, BCL-2, porin/VDAC, adenine nucleotide translocator, and Cyt c and found that none of these molecules were part of the mitochondrial 45-kDa complex assembled from 35S-labeled tBID.

Recombinant tBID Homooligomerizes in Solution—We have performed a similar protein import assay using recombinant tBID (rtBID). rtBID also became part of a 45-kDa protein complex in the mitochondrial membrane following cross-linker
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FIG. 3. tBID-tBID interactions occur in the mitochondrial membrane but not in the cytosol. A, 293T cells were transiently co-transfected with both HA-tBID and FLAG-tBID and subfractionated 24 h post-transfection. FLAG-tBID was immunoprecipitated from the mitochondrial fraction (mito) using anti-FLAG Ab, followed by Western blot with the indicated Ab. As a control, the mitochondrial fraction was incubated only with the secondary antibody (cont). B, FLAG-tBID was immunoprecipitated (IP) from both mitochondrial and cytosolic fractions with anti-FLAG Ab and analyzed by Western blot with the indicated Ab. IB, immunoblot.

tBID-tBID Interactions Occur in the Mitochondrial Membrane but Not in the Cytosol—Based on our previous findings with tBID, we suspected that the 45-kDa complex might represent a tBID homotrimer. To assess whether tBID-tBID interactions occur in the mitochondrial membrane, we have performed co-immunoprecipitation experiments in cells transiently transfected with tBID tagged with either an HA or a FLAG epitope. It was previously demonstrated that non-ionic detergents (e.g. Nonidet P-40) artificially induce the dimerization of BCL-2 family members, whereas zwitterionic detergents (e.g. CHAPS) do not (20). Therefore, we have used CHAPS in these experiments to extract tBID from the mitochondrial membrane. Mitochondria-enriched fractions purified from transfected cells were incubated in isotonic buffer containing 0.1% CHAPS for 1 h, followed by centrifugation and separation of the mitochondrial pellet from the soluble fraction. FLAG-tBID was immunoprecipitated from the soluble fraction by anti-FLAG antibodies followed by Western blot with either anti-FLAG antibodies (Fig. 3A, left panel) or anti-HA antibodies (right panel). The results indicate that HA-tBID interacts with FLAG-tBID in the mitochondrial membrane. Next, we addressed the question of whether tBID-tBID interactions occur only in the mitochondrial membrane or can also occur in the cytosol. Using differential centrifugation, we have purified the cytosolic and mitochondria-enriched fractions from transfected cells and immunoprecipitated FLAG-tBID from each fraction. As shown in Fig. 3B, the amount of HA-tBID that was co-immunoprecipitated from the mitochondria-enriched fraction, was ~10-fold higher than the amount co-immunoprecipitated from the cytosolic fraction (right panel). Similar levels of FLAG-tBID were immunoprecipitated from both fractions (Fig. 3B, left panel). Thus, the major site for tBID-tBID interactions is the mitochondrial membrane.

tBID-CFP Interacts with tBID-YFP in Living Cells—We also wanted to test whether tBID-tBID interactions occur in living cells. For this purpose we have used cyan and yellow fluorescence proteins (CFP and YFP) and performed FRET analysis (21). To perform these experiments with tBID, we constructed chimeras of tBID with either CFP (tBID-CFP) or YFP (tBID-YFP), and transfected cells with both tBID-CFP and tBID-YFP, only tBID-CFP, only tBID-YFP, or both unfused CFP and YFP as a control. Western blot analysis of whole cell lysates with anti-BID antibodies indicated that both fusion proteins were expressed at the expected sizes (not shown). To assess the cellular localization of both chimeras, transfected cells were incubated with MTR (to label mitochondria) and were analyzed by confocal microscopy. These studies demonstrated that a major part of both chimeric molecules co-localized with MTR, suggesting mitochondrial localization (Fig. 4A). To assess whether both chimeric proteins were inducing apoptosis, we measured the percentage of cells displaying a sub-G1 DNA content. Both chimeras were found to be fully functional, because transfection with either of the chimeras induced apoptosis to levels that were similar to the levels induced with wild type tBID (Fig. 4B). Next, we performed FRET analysis on cells 14 h post-transfection. Single intact cells were excited at 430 nm, and the emission spectrum between 450 and 700 nm was recorded. As expected, cells transfected with only tBID-CFP showed an emission peak at 490 nm, whereas cells transfected with only tBID-YFP showed an emission peak at 535 nm (Fig. 4C). A significant difference in the emission ratio (535/495 nm) was seen between cells co-transfected with both tBID-CFP and tBID-YFP compared with cells co-transfected with CFP and YFP, with emission ratios of 1.39 ± 0.04 (n = 20) and 0.98 ± 0.04 (n = 17) (p < 0.005), respectively (Fig. 4D). These results indicate that there is a significant FRET between the tBID-CFP and tBID-YFP in living cells, suggesting physical interaction between the tBID molecules.

tBID Forms Homotrimers in the Mitochondrial Membrane—The results presented in Figs. 3 and 4 indicate that tBID-tBID interactions occur in mitochondria of living cells. To assess whether tBID forms homotrimers in mitochondria, we have constructed a tBID-human GST chimera and performed cross-linking experiments. We have transiently transfected tBID, tBID-GST, or unfused GST into cells. Mitochondria-enriched fractions prepared from these cells were treated with cross-linker followed by Western blot analysis with anti-BID or anti-GST antibodies. Cross-linking treatment of mitochondria prepared from cells transfected with tBID resulted in the appearance of the 45-kDa complex (Fig. 5A, lane 4). Cross-linking treatment of mitochondria from these cells also resulted in the appearance of an additional ~30-kDa band, which may represent a tBID homodimer. Next we analyzed the mitochondria-enriched fractions prepared from cells transfected with either tBID-GST or unfused GST. Because tBID-GST is a ~40-kDa protein, a tBID-GST homotrimer would appear as a ~120-kDa protein band. However, if the 45-kDa original complex represented a complex between a tBID monomer and a 30-kDa protein or a complex between a tBID homodimer and a 15-kDa protein, cross-linking of tBID-GST would result in the appearance of a 72-kDa protein band or a 99-kDa protein band, respectively. Expression of tBID-GST in cells and treatment of the mitochondria-enriched fraction with the sulfo-BSOCOES cross-linker resulted in the appearance of one new band, above
the 100-kDa marker, that correlates with a tBID-GST homotrimer (Fig. 5B, lane 2). Stripping and reprobing the blot with an anti-GST antibody confirmed that tBID-GST is part of this cross-linked complex (lane 4). Analysis of the mitochondrial-enriched fraction from cells transfected with unfused GST indicated that most of the GST localized to the cytosolic fraction (Fig. 5B, lanes 5–8). Cross-linking treatment of the cytosolic fraction resulted in the appearance of one new 50-kDa band that correlates with a GST dimer (Fig. 5B, lane 8). Thus, the ability of tBID-GST to trimerize in the mitochondrial membrane relies on tBID and not on GST.

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ment of the mitochondrial-enriched fraction prepared from cells transfected with FKBP-tBID resulted in the appearance of several bands that probably represent FKBP-tBID dimers and trimers (Fig. 6A, lane 2). Treatment with FK1012 resulted in a clear enhancement in FKBP-tBID dimers and possibly trimers (lane 4), and co-treatment with FK506 significantly inhibited the effect of FK1012 (lane 6).

To assess whether FKBP-tBID dimerization was inducing apoptosis of 293T cells, we measured the percent of cells displaying a sub-G1 DNA content. Treatment of cells with FK1012 for 12 h induced a 2-fold increase in apoptosis compared with untreated cells, and co-addition of FK506 significantly reduced this enhanced death (Fig. 6D). The addition of FK1012 to cells transfected with the empty vector had no effect on the viability of cells (not shown). The death of HeLa and Cos-7 cells expressing FKBP-tBID was also enhanced by FK1012 (not shown), indicating that this effect is not lineage-specific.

To assess whether caspases were activated following tBID dimerization, the cleavage of a specific fluorogenic peptide substrate DEVD-AMC for the caspase-3-like subset was measured. Enforced dimerization of FKBP-tBID induced a 2-fold increase in caspase-3 activity compared with untreated transfected cells (Fig. 6C). This enzymatic activity was blocked by pretreatment with the pan-caspase inhibitor, zVAD-fmk. Pretreatment of FK1012-treated cells with zVAD-fmk also completely blocked apoptosis (not shown), indicating that caspases were essential for tBID dimer-induced death.

To determine whether FKBP-tBID dimerization induces Cyt c release from mitochondria, the cells were treated with or without FK1012, and Cyt c release was monitored by immunocytochemistry 12 h post-transfection. For the immunofluorescence experiments, the cells were co-stained with an anti-BID and an anti-Cyt c antibody. The nuclei were stained by 4',6-diamidino-2-phenylindole dihydrochloride. Only cells that were positive for BID and showed intact nuclei were counted. As shown in Fig. 6D, FK1012 treatment induced a ~40% increase in the number of cells releasing Cyt c. Of note, the nuclear staining of FKBP-tBID (upper panel) does not represent its pattern of staining in all cells and does not seem to occur as a result of enforced dimerization because a similar pattern of staining is also seen in cells that were not treated with FK1012 (not shown).

It was previously demonstrated that BID induces the oligomerization of either BAK or BAX, resulting in the release of Cyt c (3, 4). Based on these studies and on the fact that enforced dimerization of tBID induces Cyt c release, we expected that enforced dimerization of tBID would induce the oligomerization of either BAX or BAK. To analyze this point, FKBP-tBID transfected cells were treated with either FK1012 alone or a combination of FK1012 together with FK506, and the oligomerization of BAX or BAK was assessed using specific cross-linkers. Mitochondria-enriched fractions prepared from these cells were treated with the sulfo-BSOCOES cross-linker. Western blot analysis demonstrated that tBID mIII-4 formed the 45-kDa complex with either wt tBID or tBID mIII-4, and mitochondria-enriched fractions prepared from these cells were treated with the sulfo-BSOES cross-linker. Western blot analysis demonstrated that tBID mIII-4 formed the 45-kDa complex as efficiently as wt tBID, indicating that an intact BH3 domain was not essential for forming this complex (Fig. 7B). The ability of tBID mIII-4 to form the 45-kDa complex was also observed in HeLa and Cos-7 cells (not shown), indicating that this phenomena is not lineage-specific.

To assess whether tBID mIII-4 can induce apoptosis, we measured the percentage of 293T, HeLa, and Cos-7 cells displaying a sub-G1 DNA content following transfection. In all three cell lines, tBID mIII-4 was capable of inducing apoptosis (Fig. 7C and data not shown). As shown in Fig. 7 (D and E), tBID mIII-4 was also capable of inducing caspase-3 activation and Cyt c release.

DISCUSSION

In this study we demonstrate that tBID forms a 45-kDa complex in the mitochondrial membrane following a death signal. Our studies using FRET analysis, co-immunoprecipitation, and cross-linkers strongly suggest that the 45-kDa complex represents a tBID homotrimer. In addition, enforced dimerization of a chimeric FKBP-tBID molecule by the bivalent ligand FK1012 induces Cyt c release, caspase activation, and apoptosis. Strikingly, tBID dimers did not induce the dimerization of
Enforced dimerization of chimeric FKBP-tBID by FK1012 induces apoptosis. A, FK1012 induces dimerization of FKBP-tBID. FKBP-tBID was transiently expressed in 293T cells. Immediately post-transfection, the cells were treated for 12 h with FK1012/FK506 before extraction. The mitochondrial fractions were treated with sulfo-BSOCOES and Western blot analyzed with anti-BID Ab. The asterisk indicates a ~100-kDa band that may represent an FKBP-tBID trimer. B, FK1012 induces a 2-fold increase in apoptosis in cells expressing FKBP-tBID. 293T cells were transiently transfected with FKBP-tBID for 10 h, treated for 12 h with FK1012/FK506 as indicated, fixed, and stained with propidium iodide. The samples were then analyzed by FACS for sub-G1 DNA content. The data represent the means ± S.D. of three different experiments. C, FK1012 induces a 2-fold increase in activation of caspase-3 in cells expressing FKBP-tBID. 293T cells were transiently transfected as in A and treated with FK1012 for 12 h. Caspase-3 activity was measured using the fluorogenic substrate DEVD-AMC. Activities were blocked by pretreatment with 50 μM zVAD-fmk. The data represent the means ± S.D. of three different experiments. D, FK1012 treatment induces an increase in the number of cells that release Cyt c from mitochondria. Top panel, Cyt c release was monitored by immunofluorescence 12 h post-FK1012 treatment. The cells were immunostained for both BID (green) and Cyt c (red), and the nuclei were visualized by 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining (blue). The arrows indicate cells that highly express FKBP-tBID and released Cyt c (top row), whereas the arrowheads indicate cells that highly express FKBP-tBID but did not release Cyt c (bottom row). Bottom panel, a total of 150 cells expressing high levels of FKBP-tBID (treated or not with FK1012) were counted per field. The data represent the means ± S.D. of three different experiments. E, FK1012 treatment does not induce an increase in either BAX or BAK dimers. FKBP-tBID was transiently expressed in 293T cells in the presence or absence of HA-BAX. Immediately post-transfection, the cells were treated for 12 h with FK1012/FK506 as indicated and then extracted. Mitochondrial fractions were either treated with BMH (top panel) or BSS (bottom panel) and Western blot analyzed with the indicated Ab. The asterisk indicates the faster migrating inactive conformer of BAK (top panel). IB, immunoblot.
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**FIG. 7.** tBID mIII-4 forms the 45-kDa complex. A, tBID mIII-4 does not induce the dimerization of either BAX or BAK. wt tBID or tBID mIII-4 were transiently expressed in 293T cells in the presence or absence of HA-BAX. The cells were extracted 12 h post-transfection. Mitochondrial fractions were treated with BS3 (left panel) or BMH (right panel) and Western blot analyzed with the indicated Ab. The asterisk indicates the faster migrating inactive conformer of BAX. B, an intact BH3 domain is not essential for the formation of the tBID 45-kDa complex. 293T cells were transiently transfected with the indicated vectors. 12 h post-transfection, the mitochondrial fractions were treated with the sulfo-BSOCOES cross-linker, lysed, and analyzed by Western blot with anti-BID Ab. The asterisk indicates a cross-reactive, nonspecific band. C, tBID mIII-4 induces apoptosis in 293T cells. The cells were transiently transfected with the indicated vectors. 24 h post-transfection, the cells were fixed and stained with propidium iodide. The samples were analyzed by FACS for sub-G 1 DNA content. The data represent the means ± S.D. of three different experiments. D, tBID mIII-4 induces caspase-3 activation in 293T cells. Caspase-3-like activity was measured using the fluorogenic substrate DEVD-AMC. The data represent the means ± S.D. of three different experiments. E, tBID mIII-4 induces Cyt c release from mitochondria of 293T cells. Cyt c release was monitored as in Fig. 6. The data represent the means of two different experiments.

BAX or BAK, and a tBID BH3 mutant was capable of forming the 45-kDa complex and inducing apoptosis. It is currently believed that tBID acts as a monomer because yeast two-hybrid and in vitro binding assays showed that full-length BID does not homodimerize (14). Our results indicate that recombinant tBID forms homooligomers in solution (Fig. 2). To assess whether tBID-tBID interactions occur in cells, we have performed co-immunoprecipitation experiments using cells transfected with tBID tagged with either a FLAG or an HA epitope. These experiments led to the conclusion that tBID-tBID interactions occur in cells and that the major site of these interactions is the mitochondrial membrane (Fig. 3). To further confirm that tBID-tBID interactions occur in cells, we analyzed these interactions in living cells using FRET. Out data strongly suggest that tBID-CFP and tBID-YFP co-assemble to allow FRET (Fig. 4). The fact that both of these molecules localize to the mitochondria suggests that FRET between tBID-CFP and tBID-YFP occurs in the mitochondrial membrane.

TNFα treatment of FL5.12 cells, targeting of tBID to mouse liver mitochondria, or transfection of cells with tBID induces the formation of a 45-kDa cross-linkable mitochondrial complex that includes tBID (Figs. 1, 2, and 5). A tBID BH3 mutant, mIII-4, that does not interact with BAX, BAK, BCL-2, or BCL-X_L (14) could still form this 45-kDa mitochondrial complex (Fig. 7). Moreover, Western blot analysis with antibodies against the BCL-2 family members mentioned above or with antibodies against several mitochondrial proteins indicated that none of them were part of this complex (Fig. 1 and data not shown). Based on these data and on the fact that recombinant tBID was capable of forming homooligomers, we suspected that the 45-kDa complex represented a tBID homotrimer. To address this possibility, we transfected cells with a ~40-kDa tBID-GST chimera and performed cross-linking experiments with the mitochondrial-enriched fraction. Cross-linking treatment resulted in the appearance of a single ~120-kDa band, which most likely represents a tBID-GST homotrimer (Fig. 5). Similar experiments using unfused GST indicated that GST forms dimers but not trimers in the cytosolic fraction. Taken together, these data strongly suggest that the tBID 45-kDa complex represents a tBID homotrimer.

The 45-kDa complex seems to represent a very small percentage of the total tBID present in the mitochondrial fraction (Fig. 1). Similarly, the BAX dimer seems to represent a very small percentage of the total BAX present in the mitochondrial fraction (Fig. 1). Because the dimerization of BAX is a well-established phenomenon, the faint cross-linked bands of BAX and tBID may not represent the actual concentrations of these complexes in the mitochondrial membrane.

Enforced dimerization of tBID seems to enhance its trimerization (appearance of a ~100-kDa band; Fig. 6), suggesting that dimer formation may lead to trimer formation and that trimers may contribute to the cellular effects observed with the FKBP system. One possible explanation for this result is that dimerization of tBID is the rate-limiting step in the process of trimerization, and once dimerization is accelerated, then much more trimers can be formed. Nevertheless, the FKBP-tBID/FK1012 strategy argues that tBID dimers are active, because enforced dimerization of FKBP-tBID induces Cyt c release, caspase activation, and apoptosis (Fig. 6). A similar approach using FKBP-BAX demonstrated that enforced dimerization of BAX resulted in mitochondrial dysfunction and apoptosis (2). Based on these similar effects, we suspected that tBID dimers were acting through BAX dimers to induce apoptosis. Moreover, it was previously demonstrated that tBID translocates to the mitochondria to induce the oligomerization of BAX or BAK (3, 4) and that both molecules are essential for tBID to induce apoptosis in MEFs (15). Our results with the FKBP-tBID/FK1012 system indicate that tBID dimers are inducing Cyt c release and apoptosis without inducing the dimerization of BAX or BAK (Fig. 6). The fact that enforced dimerization of FKBP-tBID by FK1012 reduced the amount of BAX dimers further suggests that tBID dimers are inducing apoptosis in certain cells by an alternative mechanism/pathway that does not include the dimerization of either BAX or BAK. Supporting evidence for the existence of two separate pathways are the findings that in certain settings BID induces Cyt c release in the absence of mitochondrial depolarization, whereas BAX and BAK induce apoptosis with mitochondrial depolarization (23).

To further assess the involvement of multidomain pro-apoptotic molecules in the tBID oligomer death pathway, we have analyzed tBID mIII-4 in three different cell lines. This BH3 mutant was previously shown to be incapable of inducing the dimerization of BAX or of inducing the release of Cyt c from purified mouse liver mitochondria (4). In our experiments performed in intact cells, tBID mIII-4 did not induce the dimerization of either BAX or BAK but was capable of inducing Cyt c release, caspase activation, and apoptosis (Fig. 7). Thus, it seems that a BH3-independent pathway for inducing Cyt c release exists in intact cells. The fact that tBID mIII-4 was capable of inducing apoptosis and of forming the 45-kDa com-
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A proposed alternative pathway for tBID-induced Cyt c release. The known pathway, which is BH3-dependent and leads to BAX/BAK oligomerization and Cyt c release, and the proposed alternative pathway, which is BH3-independent and leads to tBID oligomerization and Cyt c release, are shown.

The known pathway, which is BH3-dependent and leads to caspase activation and apoptosis but not the dimerization of BAX or BAK. Thus, tBID oligomerization in response to a death signal appears to represent an alternative/additional mechanism for inducing mitochondrial dysfunction.

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