BCL-2 Selectively Interacts with the BID-induced Open Conformer of BAK, Inhibiting BAK Auto-oligomerization*

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Caspase-8 cleaves BID to tBID, which targets mitochondria and induces oligomerization of BAX and BAK within the outer membrane, resulting in release of cytochrome c from the organelle. Here, we have initiated these steps in isolated mitochondria derived from control and BCL-2-overexpressing cells using synthetic BH3 peptides and subsequently analyzed the BCL members by chemical cross-linking. The results show that the BH3 domain of BID interacts with and induces an “open” conformation of BAK, exposing the BAK N terminus. This open (activated) conformer of BAK potently induces oligomerization of non-activated (“closed”) conformers, causing a cascade of BAK auto-oligomerization. Induction of the open conformation of BAK occurs even in the presence of excess BCL-2, but BCL-2 selectively interacts with the open conformer and blocks BAK oligomerization and cytochrome c release, dependent on the ratio of BID BH3 and BCL-2. This mechanism of inhibition by BCL-2 also occurs in intact cells stimulated with Fas or expressing tBID. Although BID BH3 interacts with both BCL-2 and BAK, the results indicate that when BCL-2 is in excess it can sequester the BH3-induced activated conformer of BAK, effectively blocking downstream events. This model suggests that the primary mechanism for BCL-2 blockade targets activated BAK rather than sequestering tBID.

Recent genetic analysis of mammalian programmed cell death indicates that many death signals depend on the multidomain pro-apoptotic members of the BCL-2 family, BAX and BAK, to effect the mitochondrial apoptotic pathway, including release of the Apaf-1 co-factor, cytochrome c, to the cytosol, initiating a caspase cascade (1). BAX and BAK, however, differ in their initial cellular location, with BAK primarily constitutively integrated into the lipid bilayer of membranes including the mitochondrial outer membrane. BAX, on the other hand, typically resides in the cytosol or is loosely associated with membrane surfaces (2, 3). After a death stimulus, BAX undergoes conformational changes including exposure of its N terminus and functional activation of its C-terminal membrane anchor, which correlate with integration of the protein into the mitochondrial outer membrane (3–8). In this location, stimulated BAX assumes higher order oligomeric structures, resulting in cytochrome c release from the organelle (9). Although lacking the initial step of regulated insertion into mitochondrial membrane, constitutively integrated BAK likewise responds to multiple death stimuli by forming oligomers in the membrane (10). BAX and BAK likely cooperate with lipid components of the outer membrane to effect protein egress from the organelle (11–13).

A second pro-apoptotic sub-group of the BCL-2 family, the BH3 domain-only members, couples upstream death signals to downstream activation of BAX/BAK (for review, see Ref. 14). Genes for some of these BH3 only proteins respond to transcriptional cues, yielding the active entity (e.g. Noxa, PUMA, and BIK) (15–18). Others, however, are constitutively expressed and undergo an activating conformational change in response to a death stimulus (e.g. BAD, BIM, and BID) (19–23), which is predicted to make available the BH3 helix. BID is cleaved by caspase-8 to tBID after death receptor stimulation and moves to mitochondria, where it interacts with and induces oligomerization of BAX and BAK (9, 10, 24). Although direct interactions between most other BH3 domain-only members and either BAX or BAK have not been reported, BH3-only members such as BAD also stimulate BAX/BAK as the death effectors (25, 26) by binding BCL-2 and sensitizing BAX/BAK to other stimuli, including tBID itself (27). Multidomain BCL proteins contain a deep groove formed by BH1 and BH2, which accepts the BH3 domain of a binding partner (28). In instances where anti-apoptotic BCL-2/BCL-3, in excess, one model holds that these members bind to and sequester BH3-only proteins via their exposed BH3 helix, preventing them from stimulating the BAX/BAK effectors (25). Another model suggests that BH3-only proteins bind anti-apoptotic BCL-2 proteins, preventing them from inhibiting the BAX/BAK effectors (26). A third model, proposed on the basis of the anti-apoptotic function of adenovirus E1B 19-kDa protein, argues that the protein preferentially binds and inhibits a conformationally altered form of BAX in cells after receipt of a death stimulus (24). Which of these pathways dominates may in fact depend on the 3-way relative expression levels of anti-apoptotic BCL members, pro-apoptotic BAX, BAK, and the active BH3-only entities that initiate mitochondrial apoptosis.

A recent study suggests a two-step mechanism for mitochondrial release of cytochrome c in response to tBID; that is, BH3-independent mobilization of intra-crystalline cytochrome c stores and a BH3-dependent egress of cytochrome c from the organelle associated with BH3-stimulated oligomerization of BAK (29). Documentation of this latter step accompanied by findings from mutagenesis in which the tBID mitochondrial targeting sequence was replaced with that of BCL-2 argue that simple concentration of the tBID BH3 domain at the surface of mitochondria is sufficient to initiate BAK oligomerization in the outer membrane (10). Furthermore, generation of tBID by caspase cleavage involves exposure of its BH3 domain (30, 31).
and a freely diffusing synthetic peptide corresponding to the tBID BH3 domain stimulates BAK oligomerization in the mitochondrial outer membrane and release of cytochrome c (27). Use of the isolated BH3 domain in the form of a pure oligopeptide offers certain advantages, such as biotinylation and directional cross-linking, for probing directly the molecular interactions that mediate these BH3-dependent events. Outstanding issues, for example, relate to the nature of the BAK oligomerization pathway itself and the opposing roles of tBID and BCL-2 in its regulation.

Here, we have utilized derivatized oligo-peptides corresponding to the entire helix 3 of BID to study these questions and find that this BH3-containing peptide binds BAK, resulting in exposure of the BAK N terminus. This open conformer of BAK egresses from mitochondria, does not interfere with binding of BID BH3 to BAK or induction of the open conformer of BAK, but it selectively interacts with the tBID BH3-stimulated (open) BAK conformer, inhibiting BAK oligomerization and cytochrome c release.

**EXPERIMENTAL PROCEDURES**

**General—Isolation of cytosol and mitochondria, SDS-PAGE of whole cell lysates or cell fractions, transfer of proteins to nitrocellulose filters, development of blots with antibodies and detection by enhanced chemiluminescence, and assays to measure the release of cytochrome c from mitochondria have been documented in earlier publications (3, 32, 38). Alkali treatment of mitochondria was performed as described in Ruffolo et al. (32).**

**Peptides—** The wt and mt BID BH3 peptides were generated by the Alberta Peptide Institute (University of Alberta, Edmonton, Alberta, Canada) and represent amino acids 79–101 of BID. Both are biotinylated at their N termini and contain an Arg to Lys substitution at position 88 and an amide in place of a carbonyl at the C terminus. The mutant peptide also contains a Leu to Gln substitution at position 90. The corresponding wild-type BAD and BIK BH3 peptides were also synthesized. All peptides were purified (>95%) by high performance liquid chromatography.

**BAK Translation Products—** Two separate BAK constructs were engineered for *in vitro* assays, both of which contain a FLAG epitope at their N termini. FLAG-BAK ΔN(C to A) has a deletion of the N-terminus and a substitution of its single Cys to Ala at position 146. FLAG-BAK (C to A) comprises full-length BAK with substitutions of their N termini. FLAG-BAK-N(C to A) has a deletion of the N-terminal 36 amino acids and a substitution of its single Cys to Ala at position 166. FLAG-BAK(C to A) comprises full-length BAK with substitutions of all of its Cys residues at positions 14 and 166 to Ala. The constructs were cloned into pCDNA3.1 and *in vitro* transcribed and translated using the TNT® rabbit reticulocyte lysate system (Promega) as per the manufacturer’s protocol for non-radioabeled translations.

**Release of Cytochrome c from Mitochondria—** Mitochondria (50 μg of protein in 25 μl of cMRR (250 mM sucrose, 10 mM HEPES, 1 mM ATP(K+), 5 mM sodium succinate, 0.08 mM ADP, 2 mM K3HPO4) isolated from control (HA-BCL-2) KB or from KB cells overexpressing HA-BACL-2 (39) were incubated with 20 μl of HIM (200 mM mannitol, 70 mM sucrose, 10 mM HEPES-KOH, 1 mM EDTA, pH 7.5) or 20 μl of HeLa cytosolic extract in HIM (4 mg protein/ml) as described (3, 32) plus or minus recombinant caspase-8 (100 ng in 1 μl of Tris-HCl). Alternatively, the mitochondria were incubated with recombinant tBID (in 5 μl of phosphate-buffered saline (PBS) (22), synthetic BH3 peptides (in 5 μl of 0.5 μM Pipes, pH 6), BAK translation product, or control buffer (0.5 μM Pipes, pH 6), adjusted to a final volume of 50 μl with HIM, and incubated for 1 h at 37°C (3, 32). The reaction mixtures were then centrifuged at 9000 rpm (Servall MC 12V) and, equivalent aliquots of both the supernatants and pellets (resuspended to the same volume as the supernatant) were analyzed by SDS-PAGE and immunoblot using the mouse monoclonal anti-pig-cytochrome c antibody 7H8.2C12 (Pharmingen).

**BAK Oligomerization—** Reactions were conducted as for release of cytochrome c from mitochondria, except that the mitochondria (50 μg) were resuspended at the end of the reaction were resuspended in 47.5 μl of HIM. Subsequently, 2.5 μl of MeSO (control) or 2.5 μl of a solution of bismaleimidoehexane (BMH) (Pierce) in MeSO were added and incubated at room temperature for 30 min. The reactions were centrifuged at 12,000 rpm (Servall MC 12V), and the pellets were analyzed by SDS-PAGE and immunoblot using a rabbit polyclonal antibody directed against amino acids 29–37 of human BAK (Upstate).

**Treatment of BAK with Trypsin—** 5 μl of MRM (250 mM sucrose, 10 mM HEPES, pH 7.5) containing trypsin (0.125 mg/ml, Sigma) and 5 μl of MRM with (mock) or without chymotrypsin-trypsin inhibitor (1.25 mg/ml, Sigma) were added directly to reaction mixtures, which were then incubated on ice for 20 min. The trypsin digestion was stopped by the subsequent addition of 5 μl of the inhibitor, then the reactions were recovered at 9000 rpm (Servall MC 12V) for 5 min through a cushion of MRM, and BAK was analyzed by SDS-PAGE and immunoblot as above.

**Directional Cross-linking—** Biotinylated peptides were incubated in the presence of excess succinimidyl-4-(N,N-dimethylamino)cyclohexane-1-carboxy-(6-amidocaproate) (LC-SMCC, 10 mM stock in MeSO, Sigma), an amine/sulphydryl hetero-bifunctional cross-linker, for 1 h at room temperature. The amine reactive group on any excess cross-linker was then quenched by the addition of Tri-HCl, pH 8, to a final concentration of 350 mM followed by incubation at room temperature for 15 min. The derivatized peptides were then added to 50-μl standard reaction mixtures containing mitochondria. After 1 h at 37°C, the mitochondria were recovered and solubilized in 200 μl of RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% deoxycholate, and 0.1% SDS). 20 μl of a 50% slurry of Immunopure® immobilized streptavidin beads (Pierce) were then added, and the incubations were incubated overnight at 4°C. The beads were washed, washed, resuspended in SDS-PAGE loading buffer, and analyzed for BAK or BCL-2 by SDS-PAGE and immunoblot using anti-BAK or the monoclonal hamster anti-human BCL-2 antibody 8CS (Biomol).

**Immunoprecipitation—** Mitochondria from HA-BCL-2-overexpressing cells were incubated in standard cross-linking reactions in the absence of detergent, recovered, and solubilized in 200 μl of RIPA buffer. The monoclonal mouse anti-HA antibody 16B12 (Berkeley Antibody Co.) was then added, and incubated overnight at 4°C. A 50% slurry of protein G-Sepharose (Amersham Biosciences) were added to the samples and incubated at 4°C for 1 h. The beads were washed and analyzed for the presence of BAK and BCL-2 as above.

**Analysis of BCL-2 and BAK Interaction in Fas-stimulated KB Cells—** Human KB epithelial cells overexpressing HA-BCL-2 were treated with Fas-activating anti-Fas antibody (0.5 μg of protein/ml) (Upstate, CH11), as described (38). At the indicated times, mitochondria were isolated, aliquots (100 μg protein) were treated with the cross-linker BMH, HA-Bcl-2 was recovered after protein dissolution in RIPA buffer with anti-HA, and precipitates were analyzed with antibodies against BCL-2 and BAK as described above.

**Immunofluorescence and Immunofluorescence—** Control and HA-BCL-2-overexpressing KB cells were plated to 50% confluency on 12-mm coverslips in 24-well plates and transfected overnight with a combination of pEGFP, pLND-tBID-GFP, and pVGRRX (22) in a 2:4:4 ratio using LipofectAMINE PLUS™ (Invitrogen) as per the manufacturer’s protocol. The expression of tBID was then induced by treating the cells with 1 μM panobinostat A (Invitrogen) in growth medium. At the indicated times, the cells were fixed, and immunofluorescence was performed as described in Ruffolo et al. (32) using either a mouse monoclonal anti-cytochrome c antibody (BD Biosciences, 6H2 B4) or a mouse monoclonal BAK confirmation-specific antibody directed to the N terminus of BAK (Oncogene, TC100) as the primary antibody. Alexa Fluor® 594 goat antibody (Molecular Probes) was used as a secondary antibody. BAK immunofluorescence was visualized, and the reactive BAK conjugation. Transfected cells expressing EGFP were scored for the release of cytochrome c from mitochondria to cytosol and for staining of the confirmation-specific, exposed BAK N terminus.

**RESULTS AND DISCUSSION**

BAK is a 211-amino-acid protein that contains a predicted membrane-anchor sequence toward its C terminus and three of the four BH domains that define membership in the BCL family of apoptosis regulators, BH1–BH3 (Fig. 1A). The protein is constitutively integrated in the outer membrane of mitochondria, facing the cytoplasm, and undergoes oligomerization in response to tBID, which correlates with tBID-induced release of cytochrome c from the organelle (10). As a base line for

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1 The abbreviations used are: wt, wild type; mt, mutant; Pipes, 1,4-piperazinediethanesulfonic acid; BMH, bismaleimidoehexane; LC-SMCC, succinimidyl-4-(N,N-dimethylamino)cyclohexane-1-carboxy-(6-amidocaproate); RIPA, radioimmune precipitation assay buffer; HA, hemagglutinin; EGFP, enhanced green fluorescent protein.
BAK Auto-oligomerization

FIG. 1. tBID-initiated release of cytochrome c from mitochondria is mimicked by an engineered peptide representing the BID BH3 domain. A, amino acid sequence of BAK with BH1, -2, and -3 domains highlighted, the two Cys residues at positions 14 and 166 indicated with asterisks, deletion of amino acids 1–36 represented by the arrowhead, and the predicted transmembrane segment underlined. B, sequence of biotinylated wt and mt BID BH3 peptides, with the BH3 domain (highlighted), loss of function Leu to Gly point mutation (white), and silent Arg to Lys substitution (asterisk) indicated. C, HeLa cell cytosolic extracts were incubated with or without recombinant caspase-8 (100 ng) and then combined with isolated mitochondria from control or BCL-2-overexpressing KB cells. The mitochondria were recovered, and the presence of the alkaline-resistant cleaved product of BID (tBID) was determined by SDS-PAGE and immunoblot. D, mitochondria were isolated as in C and incubated with either recombinant caspase-8 (100 ng), recombinant BID (0.06 μM), or the indicated concentrations of wt or mt BID BH3 domain peptides in the presence of HeLa cell cytosolic extract. After centrifugation, the supernatants (Sup.) and mitochondrial pellets, derived from an equivalent cell number, were analyzed for cytochrome c by SDS-PAGE immunoblot. Pellets were also probed with antibody against the outer membrane marker, TOM20. E, mitochondria isolated from control and HA-BCL-2-overexpressing KB cells were incubated with increasing amounts of the BID BH3 peptide in the absence of cytosol. Cytochrome c release to the supernatant was analyzed by immunoblotting, and the bands were quantified by NIH Image version 1.61 and plotted as relative cytochrome c release versus peptide concentration. Inset, SDS-PAGE and immunoblot of extracts from control (minus HA-BCL-2) and HA-BCL-2-overexpressing KB cells probed with anti-BCL-2 and anti-γ-secretin (38). F, as in E, except that BID, BAD, and BIK BH3 peptides were analyzed with mitochondria derived from control KB cells.

establishing an in vitro system for studying the responses of mitochondrial BAK to a synthetic BID BH3 peptide (Fig. 1B), we first combined a high speed cytosolic fraction derived from HeLa cells with a heavy membrane fraction enriched in intact mitochondria isolated from KB epithelial cells (3). Treatment of the cytosol with caspase-8 generated p15 tBID, which was recovered in the alkaline-insoluble mitochondrial pellet fraction (Fig. 1C). As previously noted (21), BCL-2/BCL-XL covered, and the presence of the alkaline-resistant cleaved product of caspase-8 (100 ng) and then combined with isolated mitochondria from control or BCL-2-overexpressing KB cells. The mitochondria were recovered, and the presence of the alkaline-resistant cleaved product of BID (tBID) was determined by SDS-PAGE and immunoblot. D, mitochondria were isolated as in C and incubated with either recombinant caspase-8 (100 ng), recombinant BID (0.06 μM), or the indicated concentrations of wt or mt BID BH3 domain peptides in the presence of HeLa cell cytosolic extract. After centrifugation, the supernatants (Sup.) and mitochondrial pellets, derived from an equivalent cell number, were analyzed for cytochrome c by SDS-PAGE immunoblot. Pellets were also probed with antibody against the outer membrane marker, TOM20. E, mitochondria isolated from control and HA-BCL-2-overexpressing KB cells were incubated with increasing amounts of the BID BH3 peptide in the absence of cytosol. Cytochrome c release to the supernatant was analyzed by immunoblotting, and the bands were quantified by NIH Image version 1.61 and plotted as relative cytochrome c release versus peptide concentration. Inset, SDS-PAGE and immunoblot of extracts from control (minus HA-BCL-2) and HA-BCL-2-overexpressing KB cells probed with anti-BCL-2 and anti-γ-secretin (38). F, as in E, except that BID, BAD, and BIK BH3 peptides were analyzed with mitochondria derived from control KB cells.

fore, the amount of tBID that binds to mitochondria that were derived from cells that do or do not overexpress BCL-2 (see the inset, Fig. 1E) was the same (Fig. 1C).

FIG. 2. BID BH3 domain peptide induces a conformational change in BAK. A, mitochondria isolated from control and HA-BCL-2-overexpressing cells were incubated (minus cytosol) in the presence of the indicated peptides. The mitochondria were isolated, subsequently treated with 0.5 mM BMH, and then analyzed by SDS-PAGE and immunoblotting with anti-BAK. The positions of monomeric BAK (arrow), monomeric BAK harboring an intramolecular cross-link (double asterisk), and BAK oligomers (bracket and asterisk) are indicated. Probing the blot with an antibody against the mitochondrial protein TOM20 indicated equivalent loading of mitochondrial protein across all lanes (not shown). DMSO, MeSO. B, mitochondria from control cells and cells overexpressing HA-BCL-2 were incubated in the absence (−) or presence of 3.75 μM wt or mt BID BH3 peptide or with 0.15 μM recombinant tBID, treated with trypsin (Tryp) or trypsin plus trypsin inhibitor (mock), and analyzed for BAK by SDS-PAGE and immunoblotting with antibody directed against amino acids 23–37 of BAK (top panel). The immunoblot membrane was also probed with antibody recognizing mitoplast (inner membrane + matrix) proteins (bottom panel).

BID BH3 Peptide Recapitulates tBID or the Treatment of Cytosol with Caspase-8—When the combined cytosol-mitochondria reaction mixtures were subsequently centrifuged to pellet the organelle, cytochrome c was observed in the supernatant only if the cytosol had been treated with caspase-8 (Fig. 1D, lanes 3 and 4). Mitochondria isolated from KB cells overexpressing HA-BCL-2, on the other hand, resisted this caspase-8-induced release of cytochrome c (Fig. 1D, lanes 1 and 2). As expected, supplementing the HeLa cell cytosol with 0.06 μM recombinant tBID (22, 32) bypassed the requirement for treatment of the cytosol with caspase-8, and the control mitochondria (HA-BCL-2) effectively released cytochrome c (lane 5). An equivalent 0.06 μM concentration of synthetic peptide corresponding to the 23-amino acid helix 3 of BID, which contains the 15-amino acid BH3 domain (Fig. 1B), also stimulated release of cytochrome c from control mitochondria (lane 6), but concentrations of peptide that were 5–10-fold higher than the equivalent concentration of recombinant tBID were required for a comparable amount of cytochrome c release (compare lane 5 with lanes 6 and 7). This differential is consistent with a
membrane-targeting sequence present in tBID but lacking in the synthetic peptide that serves to concentrate tBID at the mitochondria (10). Of note, a mutant peptide in which the highly conserved Leu present in all BH3 domains examined to date (amino acid 90 in BID) was changed to Gly completely abrogated the ability of the BID BH3 peptide to cause cytochrome c release from the organelle (Fig. 1D, lanes 9–11). Also, as expected, cytosol had a negligible effect on the ability of the wt BID BH3 peptide to induce cytochrome c release in this assay (not shown).

As anticipated, mitochondria from HA-BCL-2 overexpressing cells (inset, Fig. 1E) resisted the induction of cyt c release from the organelle both by recombinant tBID (not shown) and by the synthetic BH3 peptide (Fig. 1E, conducted in the absence of cytosol). This protection by HA-BCL-2, however, could be overcome by increasing the concentration of BH3 peptide (a representative experimental result is shown in Fig. 1E). The ratio of BCL-2 to BH3 peptide, therefore, appears to determine the sensitivity of the mitochondria to cyt c release (see also Letai et al. (27)). Mitochondria from cells overexpressing HA-BCL-2 were able to resist the effects of BID BH3 up to about 10 μM peptide (Fig. 1E) and of tBID up to about 1 μM (not shown). A corresponding resistance was also observed toward the ability of the BID BH3 peptide to induce BAK oligomerization in the mitochondrial outer membrane (Fig 2A, compare lanes 3–5 with lanes 10–12), which was visualized as high molecular weight anti-BAK immunoreactive products detected by SDS-PAGE after chemical cross-linking of mitochondria with the sulfhydryl-specific homo-bifunctional BMH (10). Interestingly, at the peptide concentrations used, both BAK oligomerization (Fig. 2A) and cyt c release from mitochondria (Fig. 1F) were much more responsive to the BID BH3 peptide compared with BH3 peptides of similar size and design derived from BAD and BIK (see also Letai et al. (27)). This may reflect the observation that among BH3-only proteins, tBID is

![Fig. 3. BID BH3 interacts with BAK and BCL-2.](http://www.molcell.org/)

**A**, biotinylated wt and mt BID BH3 peptides were preconjugated with LC-SMCC. **B**, the conjugated peptides were incubated with mitochondria isolated from control and HA-BCL-2-overexpressing cells. After directional chemical cross-linking, the mitochondria were then isolated and solubilized in RIPA buffer, and the peptides were pulled down using streptavidin immobilized on Sepharose beads and analyzed for the presence of peptide-BAK cross-links by SDS-PAGE and immunoblotting with antibody against BAK. The input BAK (25%) total for each lane is also shown. C, as in **B** except that blots were developed with anti-BCL-2.

![Fig. 4. BID BH3 promotes binding of BCL-2 to BAK, which otherwise has the intrinsic capability of auto-oligomerization.](http://www.molcell.org/)

**A**, mitochondria from HA-BCL-2-overexpressing KB cells were incubated in the absence (−) or presence of 3.75 μM indicated peptide and either vehicle (MeSO) or vehicle plus 0.1 mM BMH. After cross-linking, the mitochondria were solubilized in RIPA buffer and subjected to immunoprecipitation (**IP**) with anti-HA. The precipitates were resolved by SDS-PAGE and probed with anti-BAK to detect HA-BCL-2/Bak heterodimers (**upper panel**). Input monomeric HA-BCL-2 after cross-linking was detected by immunoblotting with anti-BCL-2 (**lower panel**). **DMSO**, MeSO. **B**, FLAG-tagged in vitro translated wt and N-BAK containing Cys to Ala mutations were incubated with mitochondria isolated from KB cells. After centrifugation the supernatants (**Sup.**) were analyzed for cyt c by immunoblot (**bottom panel**), whereas the mitochondrial pellet was treated with BMH, subjected to SDS-PAGE, and immunoblotted with anti-BAK to visualize cross-linked BAK oligomers (**upper panel**, bracket and asterisk) or with anti-FLAG to visualize input translation product (**middle panel**). The full-length translation product was subjected to trypsin digestion at concentrations of the protease 1× and 10× that used in Fig. 2B (right panel).
**BAK Auto-oligomerization**

unique in that it yields detectable interactions with pro-apoptotic BAX and BAK (33).

**BID BH3 Interacts with BAK and Induces an Open Conformation, Exposing the BAK N Terminus**—Inspection of the BAK sequence reveals the presence of two cysteine residues, the first lying toward the N terminus at amino acid position 14, and the second, at position 166, within the BH2 domain (Fig. 1A). As noted by Wei et al. (10), treatment of non-induced mitochondria with BMH results in intramolecular cross-linking of these two cysteine moieties in BAK, generating a BAK species that migrates in SDS-PAGE with a faster mobility than unmodified BAK (double asterisk in Fig. 2A). This intramolecular cross-linked species was lost in mitochondria that were stimulated with the BID BH3 peptide, whereas high molecular weight cross-linked BAK oligomers appeared (Fig. 2A, lanes 3–5). A similar pattern was observed in intact cells after Fas stimulation (not shown). Although one explanation is that the cysteine residues in BH3-stimulated BAK preferentially engage in intermolecular cross-linking, this explanation does not account for the observation that in the presence of excess BCL-2, BAK oligomerization was inhibited, yet the loss of intramolecular cross-linking was still observed after treatment with the BID BH3 peptide (Fig. 2A, lanes 10–12). Another explanation, therefore, is that BH3-stimulated BAK undergoes a change that precludes intramolecular cross-linking between Cys-14 and Cys-166, even in the presence of BCL-2.

As shown in Fig. 2B, the addition of wt but not mt BID BH3 peptide to mitochondria caused BAK to undergo a conformational change, in which trypsin-resistant BAK became susceptible to the protease and resulted in loss of reactivity of BAK toward an antibody directed toward the BAK N terminus (amino acids 27–35). This indicates that in its unstimulated form, BAK is in a closed conformation with its N terminus inaccessible (34, 35), and this corresponds to the form of BAK whose N-terminal Cys is susceptible to intramolecular cross-linking by BMH to the Cys residue within the BH2 helix (Figs. 1A and 2A). Importantly, HA-BCL-2 in excess did not inhibit the ability of either 3.75 μM BID BH3 peptide or 0.15 μM recombinant tBID to induce exposure of the BAK N terminus (Fig. 2B), whereas BCL-2 did inhibit both reagents from inducing BAK oligomerization (Fig. 2A, lanes 10–12; not shown) and cytochrome c release (Fig. 1E, not shown). Therefore, BCL-2 blocks tBID-induced cytochrome c release from mitochondria downstream of tBID induction of the BAK open (trypsin-sensitive) conformation.

The BID BH3-dependent change at the N terminus of BAK correlated with a direct interaction between BAK and the BID BH3 peptide. Such interaction was demonstrated by first covalently attaching the hetero-bifunctional (amino/sulfhydryl) cross-linker, LC-SMCC, to the single Lys residue in the BH3 peptide (Fig. 1A). This Lys was introduced in place of Arg-88, which normally resides at this position in the wt sequence. This Lys was therefore, is that BH3-stimulated BAK undergoes a conformational change, in which trypsin-resistant BAK became susceptible to the protease and resulted in loss of reactivity of BAK toward an antibody directed toward the BAK N terminus (amino acids 27–35). This indicates that in its unstimulated form, BAK is in a closed conformation with its N terminus inaccessible (34, 35), and this corresponds to the form of BAK whose N-terminal Cys is susceptible to intramolecular cross-linking by BMH to the Cys residue within the BH2 helix (Figs. 1A and 2A). Importantly, HA-BCL-2 in excess did not inhibit the ability of either 3.75 μM BID BH3 peptide or 0.15 μM recombinant tBID to induce exposure of the BAK N terminus (Fig. 2B), whereas BCL-2 did inhibit both reagents from inducing BAK oligomerization (Fig. 2A, lanes 10–12; not shown) and cytochrome c release (Fig. 1E, not shown). Therefore, BCL-2 blocks tBID-induced cytochrome c release from mitochondria downstream of tBID induction of the BAK open (trypsin-sensitive) conformation.

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**Fig. 5.** BAK conformational change and interaction with BCL-2 in intact cells overexpressing HA-BCL-2. A and B, control and HA-BCL-2-expressing cells were transiently co-transfected with EGFP and tBID under the control of an inducible promoter. After 4 and 6 h of treatment with the inducer, ponasterone A, the cells were fixed to coverslips, and cytochrome c release to the cytosol and (i) selective reactivity with antibody recognizing BAK with an exposed N terminus (ii) were scored in EGFP-positive (green) cells as described under “Experimental Procedures.” The averages from three independent deter-
of endogenous BAK in isolated mitochondria was assessed. Because oligomerization is assayed by cross-linking with the Cys-specific homo-bifunctional BMH cross-linker, both the full-length and ΔN-BAK constructs that were used as the “donor” proteins were further modified to change their constituent Cys residues to Ala (Fig. 1A). In this way, the donor BAK molecules cannot be visualized as part of the oligomeric complex, whereas oligomerization of endogenous “acceptor” BAK molecules can. As documented in Fig. 4B, the transcription-translation product of FLAG-ΔN-BAK(C to A) stimulated oligomerization of endogenous mitochondrial BAK and release of cytochrome c from the organelle, whereas full-length FLAG-BAK(C to A) did not. The latter construct, as expected, was resistant to degradation of its N terminal by trypsin (Fig. 4B, right panel). Furthermore, mitochondria containing excess HA-BCL-2 (Fig. 1, C–E, and 2A) resisted BAK oligomerization and cytochrome c release in response to FLAG-ΔN-BAK(C to A) (not shown). Because the antibody that was used to detect BAK oligomers recognizes the BAK N terminus, which is lacking in the FLAG-ΔN-BAK(C to A) translation product, the observed oligomers must have derived from the endogenous population of mitochondrial BAK.

**BCL-2 Selectively Interacts with Death-stimulated BAK in Intact Cells**—The results presented in Fig. 5 show that the key findings presented above utilizing isolated mitochondria *in vitro* can be recapitulated in intact human KB epithelial cells stimulated with tBID or Fas ligation. tBID-initiated changes in BAK conformation and cytochrome c distribution was achieved by co-transfecting a vector expressing EGFP and a vector conditionally expressing tBID under the control of an inducible promoter (22, 32). After induction of tBID expression with ponasterone A, EGFP-positive cells were probed by immunofluorescence using an antibody against cytochrome c and an antibody directed to the N terminal of BAK; the latter selectively recognizes a specific conformation of BAK with an exposed N terminus (34). As shown in Fig. 5, A and B, left panel, tBID induced the release of cytochrome c in control cells but not in cells overexpressing HA-BCL-2 (see the inset, Fig. 1E). Excess BCL-2 had no effect, however, on the ability of tBID to activate the conformationally altered BAK with the exposed N terminus (Fig. 5, A and B, right panel), consistent with the findings *in vitro* that BCL-2 functions downstream of the tBID-induced activated conformer of BAK to restrict loss of cytochrome c from the organelle (Fig. 2B). Moreover, the previous finding that BID BH3 peptide induces BAK/BCL-2 interaction in isolated mitochondria (Fig. 4A) argues that these interactions occur in the presence but not absence of cell death stimuli that are coupled to BID. To test this, KB cells overexpressing HA-BCL-2 were stimulated for 0, 2, or 3 h with agonistic anti-Fas antibody, and the mitochondria were isolated and subjected to cross-linking with BMH, after which proteins were dissolved in RIPA buffer and subjected to BCL-2 pull down with anti-HA, and the precipitates were probed by immunoblot with antibody against BCL-2 or BAK. HA-BCL-2/BAK heterodimers were observed after but not before stimulation of the cells by Fas ligation (Fig. 5C).

**Concluding Remarks**—Altogether, the findings presented here suggest a model (Fig. 6) in which the BAK N terminus represses the ability of unstimulated BAK to undergo spontaneous self-oligomerization in the mitochondrial outer membrane. By binding to BAK, the BID BH3 domain overcomes this barrier. Furthermore, the resulting open (N terminus exposed) BAK conformer can induce oligomerization of closed BAK conformers, indicative of a BID BH3-initiated cascade of BAK auto-oligomerization and cytochrome c release from mitochondria. BCL-2 preferentially interacts with the BID BH3-induced

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**Fig. 6.** Working model for stimulation of BAK oligomerization by BID BH3 in the outer membrane of mitochondria. See “Concluding Remarks” for details.

**A Cascade of BAK Auto-oligomerization**—Although tBID initiates BAK oligomerization, the mechanism for how this is accomplished is not known. The fact that BID BH3 caused BAK to adopt an open (i.e. trypsin accessible) conformation even in the presence of excess BCL-2 (Fig. 2B) suggests that BCL-2 functions downstream of this step to inhibit both BAK oligomerization (Fig. 2A) and release of cytochrome c from mitochondria (Fig. 1, D and E). One possibility is that the unstimulated (closed) conformation of BAK is incompetent for auto-oligomerization and that BID BH3, by inducing exposure of the N terminus, relieves this barrier. To test this idea, a BAK construct was generated that lacks amino acids 1–36 (FLAG-ΔN-BAK, see Fig. 1A), and its ability to induce oligomerization

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**Conclusion**

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**Conclusion**
open conformer of BAK, blocking this cascade. This model might also extend to BAX since the adenovirus BCL-2 member, the E1B 19-kDa protein, appears to selectively bind the BAX open conformer (8, 24, 36). By interacting with the BID BH3-stimulated open BAK conformer, BCL-2 can intercede and inhibit BCL-2 binding by selectively targeting the BH3-stimulated open conformation, BCL-2 can intercede open conformer (8, 24). By interacting with the BID BH3-stimulated open BAK conformer, BCL-2 can intercede open conformer (8, 24, 35, 36). By interacting with the BID BH3-stimulated open BAK conformer, BCL-2 can intercede open conformer (8, 24, 35, 36). By interacting with the BID BH3-stimulated open BAK conformer, BCL-2 can intercede open conformer (8, 24, 35, 36). By interacting with the BID BH3-stimulated open BAK conformer, BCL-2 can intercede open conformer (8, 24, 35, 36). By interacting with the BID BH3-stimulated open BAK conformer, BCL-2 can intercede open conformer (8, 24, 35, 36). By interacting with the BID BH3-stimulated open BAK conformer, BCL-2 can intercede open conformer (8, 24, 35, 36). By interacting with the BID BH3-stimulated open BAK conformer, BCL-2 can intercede open conformer (8, 24, 35, 36). By interacting with the BID BH3-stimulated open BAK conformer, BCL-2 can intercede open conformer (8, 24, 35, 36). By interacting with the BID BH3-stimulated open BAK conformer, BCL-2 can intercede open conformer (8, 24, 35, 36).
