Direct addition of BimL to mitochondria does not lead to cytochrome *c* release

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Abstract Pro-apoptotic members of the Bcl-2 family can be subdivided in two classes according to their structure: a group including Bax, Bak, and Bok that display Bcl-2 homology (BH) 1, BH2 and BH3 domains and a second group including Bid (BH3 interacting domain death agonist), Bad, Bim (Bcl-2 interacting mediator of cell death) and several others that contain only a BH3 domain, the BH3-only proteins. The BH3-only proteins have been proposed to activate pro-apoptotic members of the Bax subfamily to trigger a mitochondrial pathway that leads to the release of cytochrome *c* and other apoptogenic factors. Here we report that the mechanism of action of Bim is different from that of Bid. Although overexpression of Bid or Bim in cells leads to cytochrome *c* release, only Bid is able to trigger the release of cytochrome *c* through Bax activation when added directly to isolated mitochondria. Bim_L, although unable to activate Bax, can directly inhibit Bcl-2 or Bcl-x_L. Our data suggest two functional classes of BH3-only proteins: those such as Bid which directly activate Bax-like proteins leading to mitochondrial membrane permeability and apoptosis and those such as Bim which inhibit anti-apoptotic proteins and render the cells more susceptible to apoptogenic stimuli.

Key words: Apoptosis; Mitochondrion; Bcl-2 family; Bim; Bid; BH3-only; Cytochrome *c*

1. Introduction

In many apoptotic responses, mitochondria play a major role in coordinating caspase activation through the release of apoptogenic factors, such as cytochrome *c*, Smac/DIABLO, AIF (apoptosis-inducing factor) (for review, see [1]), endonuclease G [2] and HtrA2 [3]. Release of these proteins is controlled by members of the Bcl-2 family. The anti-apoptotic members of the Bcl-2 family, such as Bcl-2 or Bcl-x_L, inhibit the efflux of these proteins whereas the pro-apoptotic members, Bax or Bak, trigger their release. The activity of these proteins appears to be controlled by a class of proteins called Bcl-2 homology (BH) 3-only proteins because they only display one of the three or four domains contained in either Bcl-2/Bcl-x_L or Bax and Bak. The BH3-only subfamily presently includes mammalian Bid (BH3 interacting domain death agonist), Bad, Bik/Nbk, Hrk/DP5, Bim (Bcl-2 interacting mediator of cell death), Bmf, Blk, Noxa, Puma and *Caenorhabditis elegans* EGL-1 [4–6]. Different groups have recently demonstrated that at least some of the BH3-only proteins undergo post-translational modifications, which determine their active conformation following death signals (for review [7]). For example, Bid has been shown to undergo cleavage by caspase-8 and myristoylation during Fas-mediated apoptosis. These structural changes allow cleaved Bid (tBid) to translocate to mitochondria, to induce insertion and oligomerization of Bax and/or Bak into the outer mitochondrial membrane (OMM), and trigger the release of cytochrome *c* [8–12]. Phosphorylation of Bid by casein kinases I and II was found to regulate its cleavage by caspase-8 [13]. In many cell types, Bad is normally phosphorylated and, as such, interacts with 14-3-3 scaffold proteins. During apoptosis, Bad is dephosphorylated and interacts with Bcl-2 or Bcl-x_L, thereby inhibiting the anti-apoptotic activity of these proteins [14–16].

Alternative splicing generates three isoforms of Bim: Bim_S, Bim_L, and Bim_{EL} [17]. All three isoforms are potent inducers of apoptosis, Bim_S being considerably more cytotoxic than Bim_L or Bim_{EL}. The additional regions present in the longer isoforms (Bim_L and Bim_{EL}) attenuate its activity, by allowing Bim to be sequestered to the microtubule-associated dynein motor complex through binding to dynein light chain LC8/DLC1 [18]. Upon receipt of damage signals, Bim is released (together with LC8/DLC1) to activate the cell death machinery. In addition to the post-translational control, Bim may also be transcriptionally regulated in response to signaling by growth factors in some cell types [19–21]. Gene targeting in mice revealed that Bim is required for hematopoietic homeostasis and its deficiency causes a systemic lupus erythematosus-like autoimmune disease. $Bim^{-/-}$ lymphocytes are resistant to apoptotic stimuli such as cytokine deprivation, calcium flux or microtubule perturbation [22].

In this report, we have compared the molecular mechanisms whereby tBid and Bim trigger apoptosis. Although overexpression of both proteins triggers apoptosis in HeLa cells, only tBid activates Bax and triggers cytochrome *c* release from isolated mitochondria. Bim appears to act through inhibition of Bcl-2 or Bcl-x_L.

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Abbreviations: BH, Bcl-2 homology; Bid, BH3 interacting domain death agonist; Bim, Bcl-2 interacting mediator of cell death; OMM, outer mitochondrial membrane; Z-VAD-fmk, Z-Val-Ala-Asp(OMe)-CH2F

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2. Materials and methods

2.1. Materials

Mouse monoclonal anti-Bax antibodies were purchased from Alexis (Alexis Corporation). Goat anti-Bim monoclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal anti-Flag M2 antibodies were purchased from Sigma. Mouse monoclonal antibodies against mitochondrial heat shock protein 70 (mt-hsp-70) were purchased from ABR (Laufelfingen, Switzerland) and monoclonal anti-COX antibodies were from Molecular Probes (Eugene, OR, USA). Antigen–antibody complexes were detected by using horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G and enhanced chemiluminescence detection reagents (ECL system: Amersham Pharmacia Biotech).

2.2. Immunochemistry

For immunochemistry analysis, cells were seeded onto glass coverslips. HeLa cells were cultured in the presence or absence of 100 μM Z-Val-Ala-Asp(OMe)-CH2F (Z-VAD-fmk). Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min and permeabilized with 0.5% Triton X-100 in PBS at room temperature for 15 min. After washing, the cells were incubated for 1 h with anti-Bim polyclonal antibodies, anti-Bax polyclonal antibodies, anti-cytochrome *c* monoclonal antiboby, anti-mt-hsp-70, all diluted in PBS+5% normal goat serum, washed twice in PBS, and developed with fluorescein and Texas red-labeled goat anti-rabbit and goat anti-mouse antibodies, respectively. In the last wash, 1 μg/ml of Hoechst 33258 was added to the cells. Coverslips were placed on a glass slide in Vectashield mounting medium (Vector) and observed by conventional fluorescence microscopy.

2.3. Recombinant proteins

Recombinant truncated Bid (mouse Bid residues 60–195) (rtBid) with a tag of six histidines at the N terminus, His-tagged human Bcl-x_L (rBcl-x_L) and human Bcl-2 both lacking the C-terminus hydrophobic domain (rBcl-2) were expressed in the pET23d vector in *Escherichia coli* and purified as previously described [11,23–25].

Flag-tagged Bim_L was expressed as a glutathione *S*-transferase (GST) fusion protein in the pGEX-2T vector in *E. coli*. The GST-Flag-Bim_L fusion proteins were recovered in the soluble bacteria fraction and were bound to glutathione-Sepharose. Flag-Bim_L was released by thrombin cleavage (100 U; 12 h) and further purified by chromatography on Q-Sepharose followed by Mono Q-Sepharose (see Fig. 2).

The purified proteins which were >95% pure were stored in 25 mM Tris–HCl, 0.1 mM dithiothreitol (DTT), 30% glycerol, pH 7.5, at −80°C.

2.4. Cell cultures and isolation of mitochondria

HeLa cells were grown at 37°C in 5% CO_2, in RPMI 1640 medium (Gibco, BRL), supplemented with antibiotics, glutamine and 10% fetal bovine serum.

Mitochondria were isolated from HeLa cells as previously described [11,26]. In brief, HeLa cells were harvested in isotonic mitochondrial buffer (MB) (210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM HEPES (pH 7.5)) supplemented with complete protease inhibitor cocktail (Boehringer Mannheim). The cells were broken by six passages through a 25G1 0.5- by 25-mm needle fitted on a 5-ml syringe, and the suspension was centrifuged at 2000×*g* in an Eppendorf centrifuge at 4°C. This procedure was repeated twice, and supernatants from each step were pooled before being subjected to centrifugation at 13 000×*g* at 4°C for 10 min. The supernatant was further centrifuged at 100 000×*g* for 10 min at 4°C to yield the light membrane pellet (not analyzed) and the final soluble fraction (S100). The heavy membrane material was pooled and resuspended in MB-EGTA (MB with 0.5 mM EGTA instead of EDTA) and centrifuged at 500×*g* for 3 min at 4°C to eliminate residual nuclei. The resulting supernatant was centrifuged at 13 000×*g* for 10 min at 4°C to further purify the mitochondrial fraction. Protein concentration was estimated by the method of Bradford [27] with bovine serum albumin as the standard.

2.5. In vitro assay for Bax insertion and cytochrome c release

Mitochondria were incubated in the presence or absence of various recombinant proteins in 200 μl of KCl buffer (125 mM KCl, 4 mM $MgCl_2$, 5 mM $NaHPO_4$, 5 mM succinate, 0.5 mM EGTA, 15 mM HEPES-KOH, pH 7.4, 5 μM rotenone) for 15 min at 30°C and then centrifuged for 5 min at 13 000×*g* and 4°C. The supernatants and the pellets were used for the determination of cytochrome *c* release and were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. For alkali extraction, the mitochondrial pellets were resuspended (1 mg of protein/ml) in freshly prepared 0.1 M Na_2CO_3 (pH 11.5) and incubated for 20 min on ice. The membranes were then pelleted by centrifugation (100 000×*g* for 20 min at 4°C). Mitochondrial membrane pellets corresponding to supernatants were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

2.6. Gel filtration analysis

Gel filtration was performed at 4°C on a Superdex 200 (16/60) column (Amersham Pharmacia Biotech) equilibrated in 25 mM HEPES-NaOH, 300 mM NaCl, 0.2 mM DTT, 2% (w/v) CHAPS, pH 7.5, and run at a flow rate of 1 ml/min. The column was calibrated with gel filtration standard proteins from Amersham Pharmacia Biotech giving the following elution volumes: thyroglobulin (669 000 Da), 49.9 ml; ferritin (440 000 Da), 55.9 ml; catalase (232 000 Da), 65.5 ml; BSA (67 000 Da), 77.5 ml; ovalbumin (43 000 Da), 83.2 ml; chymotrypsinogen A (25 000 Da), 92.9 ml; ribonuclease A (13 700 Da), 97.4 ml. A 500-μl sample was loaded onto the column, and the eluate was monitored at 280 nm. After 20 min elution (20 ml), fractions of 2 ml were collected, and aliquots from the fractions were analyzed by Western blotting. The proteins were detected with the specific antibodies as indicated in the figures.

3. Results

3.1. Bim induces cytochrome c release of mitochondria in cultured HeLa cells

During apoptosis induced by overexpression of Bid, Bax undergoes a conformational change resulting in unmasking of the NH_2-terminal domain (5 and unpublished data). This change of Bax immunoreactivity, toward NH_2-terminal antibodies, allowed us to assess in vivo the activation of endogenous Bax by Bim. HeLa cells were transfected with pCI-His-Bim_L vector in the presence of 100 μM caspase inhibitor Z-VAD-fmk and localization of proteins was performed by microscopy (Fig. 1). Bim positive cells displayed a mitochondrial staining, clearly evidenced by co-localization with mt-hsp-70 (Fig. 1A-C). This is in agreement with the finding that Bim is associated with isolated mitochondria during apoptosis [20]. We also found cells in which Bim was clearly cytosolic. With an antibody raised against amino acids 1–21 of Bax, no staining was observed in non-transfected cells. In contrast, Bim overexpressing cells displayed a punctate Bax staining characteristic of Bax activation (Fig. 1D–F). In addition, double immunostaining revealed that Bim positive cells exhibited a diffused cytosolic pattern of cytochrome *c* (Fig. 1G–I). All Bim-transfected cells containing cytochrome *c*-depleted mitochondria displayed Bax immunoreactivity. Because cells had been cultured with the caspase inhibitor Z-VAD-fmk, no nuclear fragmentation was observed in Bim positive cells. In contrast, in the absence of Z-VAD-fmk, cells underwent caspase activation and died (data not shown). Taken together, these experiments show that overexpression of Bim in cells leads to Bax activation, cytochrome *c* release and apoptosis.

3.2. Purification of LC8-free recombinant Bim_L

In an attempt to study the direct effect of Bim and Bid on isolated mitochondria, we purified to homogeneity recombinant Flag-Bim_L (rBim_L) (Fig. 2A, see Section 2). Protein immunoblotting with specific anti-Bim antibodies detected a

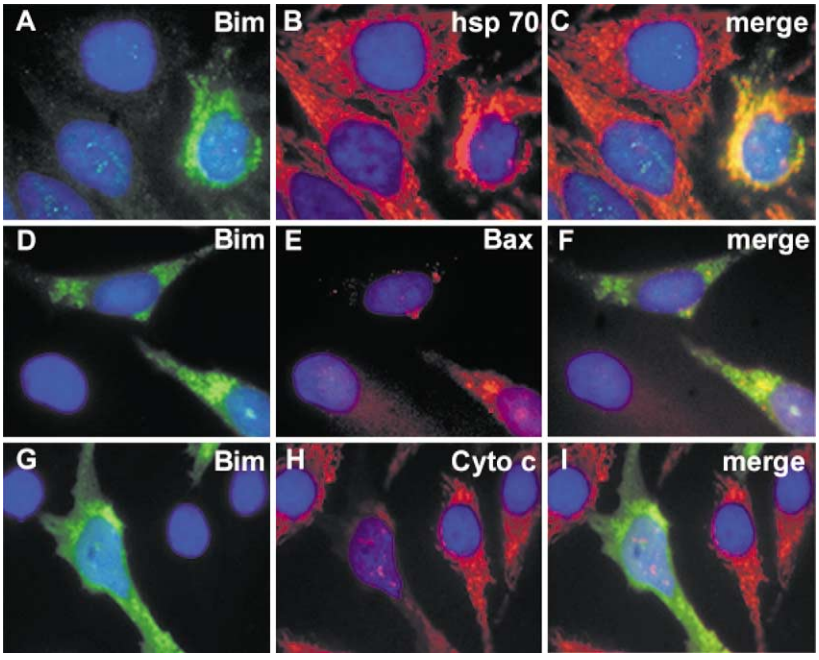

Fig. 1. Bax and cytochrome *c* immunostaining in Bim expressing cells. HeLa cells were transfected with a Bim_L expression vector, in the presence of 100 μM Z-VAD-fmk, and were immunostained for Bax, cytochrome *c*, Bim and mt-hsp-70, 24 h later. A–C: Bim and mt-hsp-70; D–F: Bim and Bax; G–I: Bim and cytochrome *c*. Nuclei were visualized by Hoechst 33258 staining.

single 18-kDa band (data not shown). Gel filtration analysis of the recombinant protein in 2% CHAPS extraction buffer showed that $rBim_L$ was eluted in the low molecular weight fractions corresponding to a relative molecular weight of 20–25 kDa (Fig. 2B, fractions 36–38), and indicating that it was purified as a monomer.

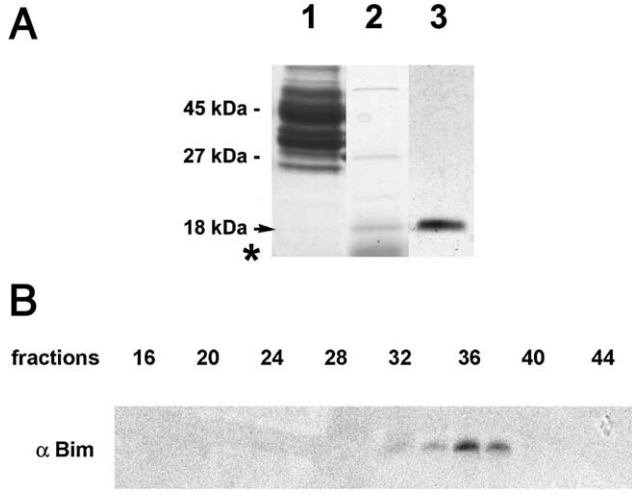

Fig. 2. Analysis of Bim recombinant protein on a Coomassie-stained gel. A: Coomassie gel of GST-Flag-tagged Bim_L proteins (45 kDa) recovered in the soluble bacteria fraction, treated with thrombin (100 U, 12 h). 1: glutathione column; 2: thrombin elution; 3: $rBim_L$ (1 μg) from Q- and Mono Q-Sepharose chromatography purification. Arrow indicates Flag-tagged Bim_L ($rBim_L$) (18 kDa). Note the elimination of the shorter contaminating elements (*) after elution. B: Analysis of purified $rBim_L$ in 2% CHAPS extraction buffer by gel filtration on Superdex 200. Fractions of 2 ml were collected and every second fraction was analyzed by Western blotting using the rabbit polyclonal anti-Bim antibody.

3.3. *In contrast to Bid, Bim neither induces Bax insertion in OMM nor induces cytochrome c release from isolated mitochondria*

Bid-induced insertion/oligomerization of Bax in the OMM is induced before the release of cytochrome *c* [12]. To assess whether the release of cytochrome *c* induced by Bim in cells was a direct effect of Bim on mitochondria, we tested whether addition of $rBim_L$ to mitochondria was able to induce Bax insertion and cytochrome *c* release. As shown in Fig. 3, we assessed Bax insertion into the outer membrane with alkali extraction of freshly isolated mitochondria from HeLa cells, which contain loosely associated Bax [11,12]. As expected,

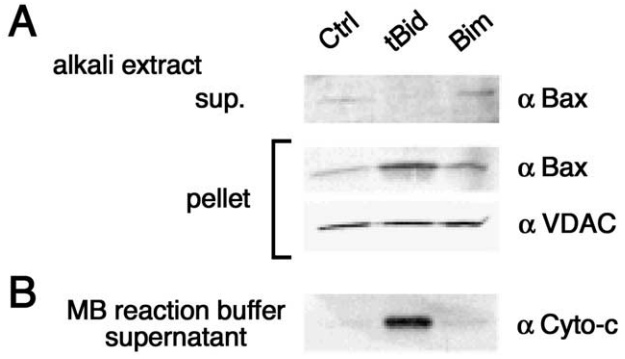

Fig. 3. Insertion of Bax in mitochondrial membranes. A: Mitochondria were isolated from HeLa cells, incubated with 5 nM of tBid or 0.5 μM of Bim_L and treated with alkali (0.1 M Na_2CO_3 pH 11.5). Mitochondrial pellets were analyzed by Western blotting for the presence of Bax. Voltage-dependent anion channel protein, inserted into mitochondrial membranes, was used as a gel-loading control. B: Cytochrome *c* was analyzed by Western blotting in the mitochondrial suspension following incubation with either Bid or Bim.

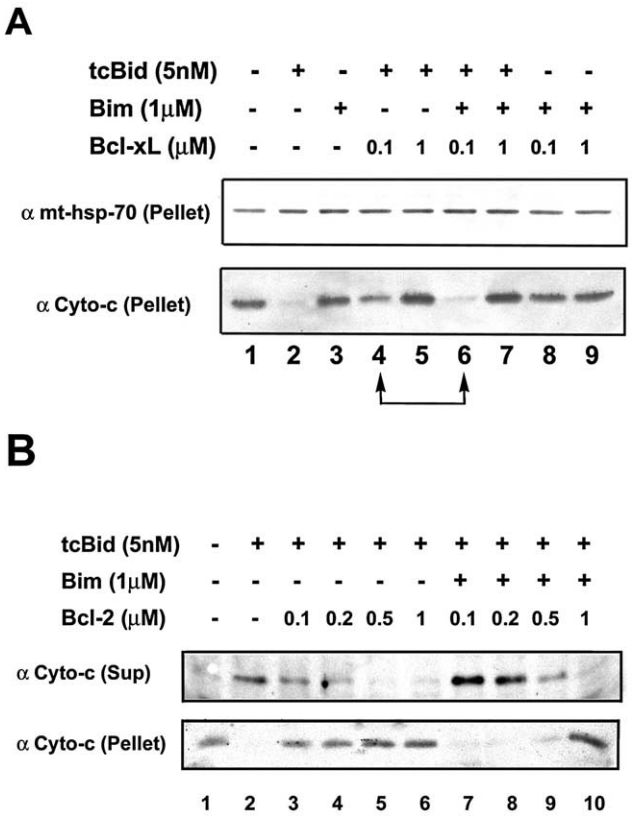

Fig. 4. Bim_L counters the inhibition of Bid-induced cytochrome *c* release by Bcl-x_L or Bcl-2. Mitochondria from HeLa cells were isolated and incubated with 5 nM of tBid or 1 μM of Bim_L in the presence of Bcl-x_L. A: Cytochrome *c* levels in mitochondrial pellets were analyzed by Western blot. Mt-hsp-70 was used as loading control. The counteracting effect of Bim on Bcl-x_L protection against Bid-induced cytochrome *c* release is clearly apparent when comparing lane 6 with lane 4. B: Bim inhibits Bcl-2 in a dose-dependent manner. Mitochondria were incubated with 5 nM of tBid, 1 μM of Bim_L and 0.1–1 μM of Bcl-2. Cytochrome *c* levels were determined by Western blot in both the pellet and in the supernatant. Data shown in A and B are representative of three different experiments.

addition of 5 nM rtBid to the mitochondria induced Bax insertion in the OMM. Consequently, Bax levels decrease in the supernatant fraction (Fig. 3A). In clear contrast, when mitochondria were incubated with Bim_L, even at a concentration up to 1 μM, Bax was not inserted. We next checked the release of cytochrome *c* (Fig. 3B). Addition of 5 nM rtBid released cytochrome *c* whereas 1 μM $rBim_L$ had no effect. Thus, these data showed that Bim, unlike Bid, cannot induce endogenous Bax insertion in the OMM, and cannot release cytochrome *c* from isolated mitochondria.

3.4. Unlike Bid, Bim does not trigger release of cytochrome c but overcomes Bcl-2 inhibition of Bid-induced cytochrome c release in vitro

Recombinant tBid and Bim_L were then tested for their ability to trigger the release of cytochrome *c* in the presence of recombinant anti-apoptotic proteins (Fig. 4). Whereas 5 nM tBid was able to trigger the release of cytochrome *c* from mitochondria, Bim, even at a concentration of 1 μM, had no effect (Fig. 4A). The cytochrome *c* release induced by 5 nM rtBid was partially inhibited by 0.1 μM rBcl-x_L (lane 4) whereas a complete inhibition was obtained with 1 μM rBcl-x_L (lane 5). Equivalent results were obtained when we used rBcl-2 instead of rBcl-x_L (Fig. 4B). Because Bim has previously been shown to bind to anti-apoptotic Bcl-2 ([17,18] and our unpublished results), we tested whether $rBim_L$ could counteract the rBcl-2 or rBcl-x_L effect. Addition of 1 μM $rBim_L$ overcame the inhibitory effect obtained with 0.1 μM rBcl-x_L on rtBid-induced cytochrome *c* release (lane 6). However, addition of 1 μM $rBim_L$ appeared insufficient to block the protective effect conferred by 1 μM rBcl-x_L (lane 7). Similar results were obtained when rBcl-x was replaced by rBcl-2 (Fig. 4B). As for Bcl-x_L, addition of an equimolar concentration of $rBim_L$ to rBcl-2 was insufficient to inhibit rBcl-2 control over rtBid-induced cytochrome *c* release. However, an excess of rBim over rBcl-2 abrogated rBcl-2 inhibition of cytochrome *c* release. Together, these results demonstrate that Bim does not release directly cytochrome *c*, but is able to facilitate rtBid-induced cytochrome *c* release by blocking rBcl-2 and/or rBcl-x_L and probably by inhibiting endogenous Bcl-x inserted in the OMM of mitochondria [12,28].

4. Discussion

We have previously shown that recombinant tBid efficiently renders the OMM permeable to cytochrome *c* [12]. This potent pro-apoptotic BH3-containing fragment, produced by caspase cleavage of Bid, probably acts by directly activating Bax [11] or Bak [9]. In favor of this hypothesis, tBid interacts directly with either protein in yeast two-hybrid or in co-immunoprecipitation experiments [9,29]. The addition of Bid to mitochondria was also found to trigger insertion and oligomerization of both Bax [12] and Bak [9]. Furthermore, Bax and Bak are essential for the release of cytochrome *c* induced by tBid [30].

In contrast to Bid, addition of another BH3-only protein Bim_L, even at a 200-fold higher concentration, did not cause cytochrome *c* release from isolated mitochondria, or directly trigger the membrane insertion and oligomerization of Bax

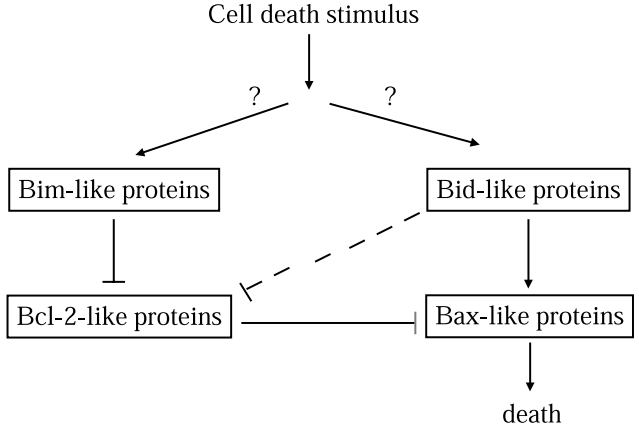

Fig. 5. Two classes of BH3-only proteins. This model proposes two classes of BH3-only proteins: those, like tBid, that trigger apoptosis through direct activation of Bax or Bak and others such as Bim that contribute to apoptosis through inhibition of the anti-apoptotic activity of Bcl-2, Bcl-x_L and other anti-apoptotic members of this family. Apoptosis only occurs if Bcl-2-like anti-apoptotic proteins are inhibited by Bim-like proteins and if at the same time Bax-like proteins are activated by Bid-like proteins.

(Fig. 3 and data not shown). The failure of Bim to activate Bax directly probably reflects its inability to interact with Bax since none of the Bim isoforms binds Bax (or Bak) in yeast two-hybrid or in co-immunoprecipitation experiments ([17,18] and data not shown). By contrast, the killing activity of Bid appears to be closely coupled to its ability to bind Bax since those mutations in its BH3 domain affecting Bax binding also abrogated biological activity [11,29].

Although $rBim_L$ cannot activate Bax/Bak directly, it can, in a dose-dependent manner, antagonize the ability of rBcl-2 or rBcl-x_L to block tBid-induced cytochrome *c* release from purified mitochondria (Fig. 4) [12,31,32]. The mutual neutralization of Bim by Bcl-2 is reflected in the recent data reporting that the cellular attrition caused by Bcl-2 deficiency can be prevented in mice lacking both Bim and Bcl-2 [33]. However, a complete inhibition was only observed when Bim_L was in excess to rBcl-2/rBcl-x_L. If we assume that one molecule of Bid interacts with one molecule of Bcl-2 or Bcl-x_L then it is unclear why an excess of Bim_L is required to restore rtBid-induced cytochrome *c*. This may reflect higher affinity of Bcl-2 (or Bcl-x_L) for Bid than for Bim, or that, to be fully active, Bim requires post-translational modifications such as phosphorylations [19], which are not present in the recombinant Bim protein. Even though $rBim_L$ can antagonize rBcl-x_L, its addition did not appear sufficient to trigger the release of cytochrome *c* from mitochondria isolated from HeLa cells [12]. This appears inconsistent with the observation that Bim_L overexpression in HeLa cells was cytotoxic and caused significant mitochondrial damage (Fig. 1). These data suggest that overexpression of Bim in cells generates an unknown signal that leads to activation of Bax-like proteins. It is reasonable to think that BH3-only proteins able to interact directly with Bax, as for example Bid, could represent such a signal. Though the anti-apoptotic proteins such as Bcl-2 or Bcl-x_L may sequester and thereby inhibit BH3-only proteins such as Bim [34], they may also act to directly inhibit Bax or Bak. Indeed, we have previously shown that a mutant of Bcl-x_L that cannot interact with Bax or Bak but interacts with Bid was less efficient in blocking Bax activation and cytochrome *c* release [12]. In such a scenario, binding of Bim may release Bax/Bak from inhibition by Bcl-2/Bcl-x_L thereby allowing Bax/Bak to be activated by a second class of BH3-only proteins, like Bid.

In conclusion, we have shown that two BH3-only proteins of the Bcl-2 family, namely Bid and Bim, act predominantly through different mechanisms. Bid probably triggers apoptosis by directly activating Bax/Bak, whereas Bim sensitizes cells to apoptosis by inhibiting Bcl-2, Bcl-x_L and other anti-apoptotic members of this family (Fig. 5). Depending on their ability to interact with either anti-apoptotic proteins (Bcl-2, Bcl-x_L) or with pro-apoptotic proteins such as Bax or Bak, BH3-only proteins fall into functionally distinct subclasses that act at different steps during cell death signaling. Following a death stimulus, apoptosis would occur if anti-apoptotic proteins of the Bcl-2 family are inhibited by Bim-like proteins and at the same time Bax-like proteins (Bax, Bak and Bok) are activated by Bid-like proteins (for a model see Fig. 5).

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