BH3-DOMAINS OTHER THAN BIM AND BID CAN DIRECTLY ACTIVATE BAX/BAK

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Running title: Low-grade direct activation of Bax/Bak by BH3-only proteins

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Bcl-2 family proteins regulate a critical step in apoptosis referred to as mitochondrial outer membrane permeabilization (MOMP). Members of a subgroup of the Bcl-2 family, known as the BH3-only proteins, activate pro-apoptotic effectors (Bax and Bak) to initiate MOMP. They do so by neutralizing pro-survival Bcl-2 proteins and/or directly activating Bax/Bak. Bim and Bid are reported to be direct activators, however, here we show that BH3 peptides other than Bim and Bid exhibited various degrees of direct activation of the effector Bax or Bak, including Bmf and Noxa BH3s. In the absence of potent direct activators, such as Bim and Bid, we unmasked novel direct activator BH3 ligands capable of inducing effector-mediated cytochrome C release and liposome permeabilization, even when both Bcl-xL- and Mcl-1-type anti-apoptotic proteins were inhibited. The ability of these weaker direct activator BH3 peptides to cause MOMP correlated with that of the corresponding full-length proteins to induce apoptosis in the absence of Bim and Bid. We propose that, in certain contexts, direct activation by BH3-only proteins other than Bim and Bid may significantly contribute to MOMP and apoptosis.

Regulation of mitochondrial outer membrane permeabilization (MOMP) is one of the most critical steps in apoptosis pathways, as the release of apoptogenic proteins from the mitochondrial intermembrane space commits the cell to death - either by a caspase-dependent or -independent mechanism (1,2). Bcl-2 family proteins regulate MOMP (3-5), and the molecular mechanisms underlying MOMP have been a focus of intensive studies. The Bcl-2 family includes both pro- and anti-apoptotic members. The pro-apoptotic members are divided into multi-domain (Bax, Bak and perhaps Bok in a limited number of tissues) and BH3 domain-only members (Bim, Bid, Bad, Bik, Bmf, Hrk, Puma, Noxa etc.) (5). Loss-of-function studies have established that the multi-domain pro-apoptotic members, Bax and Bak, are the effectors of MOMP (6,7), and that the BH3-only proteins effect the activation of Bax and Bak. To initiate MOMP, Bax and Bak change their conformation (8-12) and form homo-oligomers in the membrane (11-16). These events are referred to as Bax/Bak (for Bax and/or Bak) ‘activation’. As a consequence of this activation, the membrane is permeabilized and apoptogenic molecules are released from the intermembrane space.

The neutralization model postulates that Bax and Bak are activated, when they are liberated from anti-apoptotic Bcl-2 proteins by BH3-only proteins (17,18), whereas the direct activation model posits that, in addition to inhibiting pro-survival proteins, select BH3-only proteins need to directly activate Bax/Bak (19-21). It has also been shown that certain BH3-only proteins displace activator BH3-only proteins (Bim, cleaved Bid or Puma in some instances) from anti-apoptotic proteins, which will then directly activate
Bax/Bak. We investigated the membrane permeabilization by Bcl-2 family proteins in a defined liposome system, using BH3 peptides (13,22). It is reasonable to assume that the BH3 peptides mimic the corresponding full-length proteins, for the BH3 domain acts as a ligand (14). In support of this assumption, by NMR spectroscopy, the Bid BH3 region was identified as the sole contributor to Mcl-1 binding by the cleaved full-length Bid (23). Further, when Bid BH3 is made α-helical by chemical stapling, as in the full-length protein, it becomes more potent in Bax activation (24). We found that Bim and Bid BH3 peptides are the most effective direct activators, promoting Bax-mediated membrane permeabilization, and that all BH3 peptides acted as de-repressors, or sensitizers, contributing to MOMP by stably binding to and inhibiting the anti-apoptotic members, Bcl-xL and/or Mcl-1 (22). These findings were consistent with the direct activation model. However, they did not explain why Bid and Bim double knockout (Bim/Bid DKO) mice do not have a phenotype similar to that of Bax/Bak DKO mice, or how some BH3 peptides other than Bim and Bid BH3 induce MOMP in the absence of Bax and Bim proteins (17,18).

A number of BH3 peptides other than Bim and Bid BH3s showed weak direct activation above the background level (22), so we postulated that redundancy is at work to mask the apoptotic defect in Bim/Bid DKO mice. We set out to re-examine direct activation by these BH3 peptides of Bax or Bak, for which we used a truncated version of Bak (25), cBak, that mimicked native Bak. We report that 1) BH3 peptides other than Bim and Bid BH3 exhibited varying degrees of direct activation in the liposome system on both Bax and cBak and 2) neutralization was found necessary to cause liposome permeabilization and cytochrome C release, but not sufficient.

**Experimental procedures**

**Cytochrome C release assay on digitonin permeabilized Bim/Bid DKO MEFs**

MEFs were grown in DMEM, harvested and washed with PBS supplemented with 1mM CaCl$_2$ and 1mM MgCl$_2$. Cells were then permeabilized by resuspension into a buffer containing 20mM HEPES, pH 7.4, 250mM sucrose, 100mM KCl, 5mM MgCl$_2$, 1mM EDTA, 1mM EGTA and 100 µg/ml digitonin at 3.5 x10$^7$ cells/ml. After incubation on ice for 5min, 1% BSA was added to remove digitonin, and the cells were pelleted and resuspended in the same volume of buffer without digitonin. This suspension of permeabilized cells was incubated with N/C-Bid or BH3 peptides at 30°C for 1h, and was spun down at 1000xg for 5min to separate the supernatant and the pellet. Equivalent amounts of the supernatant and the pellet were loaded onto 15% SDS-PAGE gels for cytochrome C immunoblotting, using an anti-cytochrome C antibody (BD Pharmingen, 556433). We used mouse BH3 peptides for Bid and Bmf and the rest were human BH3s in this experiment.

**Immunoprecipitation of Mcl-1, Bcl-xL and Bcl-2 in Bim/Bid DKO MEFs**

Following incubation with BH3 peptides, an aliquot of the pellet of digitonin-permeabilized cells was dissolved in lysis buffer (10mM HEPES, pH 7.4, 10mM KCl, 0.1mM EGTA, 200mM NaCl, 1% CHAPS or 0.5% NP-40) containing Complete Protease Inhibitor (Roche, #11873580001) and 1mM PMSF. After incubation for 30min on ice, cells were spun down at 13,000g for 15min and the supernatant was collected. Antibodies (0.5-1 µg) (anti-Bcl-2; Santa Cruz, sc-23960 and BD, 554087, anti-Mcl-1; Rockland, 600-401-394, anti-Bcl-xL; Cell Signaling, 2762) and 20 µl Protein A/G PLUS-agarose (Santa Cruz) were added to this cell lysate (450 µg protein) and incubated overnight at 4°C. The beads were washed in lysis buffer and added directly to the loading buffer for SDS-PAGE, together with the depleted lysate. Mcl-1, Bcl-2 and Bak were detected by immunoblotting.

**Transient over-expression in Bim/Bid DKO MEFs using retrovirus**

293T cells were transfected with pMSCV-IRES-GFP with/without the gene of interest together with an equal amount of AdVAntage™ Vector (Promega, Part# TB207) and ectopic helper virus DNA using Lipofectamine 2000 (Invitrogen, 11668-019) according to the manufacturer's instructions. The media containing retroviral pseudotypes were collected at 16, 24, 40 and 48h.
post-transfection individually and added to growing MEFs in 6-well plates after each viral harvest in the presence of 8µg/ml polybrene (Sigma, H9268). Separately prepared viruses containing Bad or Noxa genes were used for simultaneous overexpression of both genes. MEFs were infected with the first virus for 2-3 h and then with the second virus overnight. For both single and double gene over-expressions, MEFs were harvested after 50h post-transfection, and cell death was measured by FACS-based analysis of Annexin V staining, using the AnnexinV-Cy5 apoptosis detection kit (Biovision Inc. #K103-100). Transfection efficiency was determined as FACS-based measurement of GFP expression, and was >80% in all transfectants.

**Recombinant proteins and BH3 peptides**

Recombinant full-length human Bax, N/C-Bid (cleaved Bid), Bcl-xL, Mcl-1 and cBak were produced in *E.coli* according to the published procedures (13,22,25). All these proteins showed more than 90% purity, as assessed by Coomassie staining. BH3 peptides were obtained from AnaSpec at >95% purity. We used human sequences for the BH3 peptides. We did not detect significant difference in activity between mouse and human Bid or Bmf in terms of cytochrome C release and liposome permeabilization.

**Liposome assays**

Liposomes containing 7% cardiolipin were generated by extrusion, and the end-point assay was performed at RT for 2.5 h, as previously described (13,22). The release was normalized to Bax and N/C-Bid induced release as 100%. We adjusted the concentrations of Bax, cBak and N/C-Bid in order to achieve ~20% release by the effector alone and ~70%, the effector plus N/C-Bid between different liposome and protein preparations. The concentrations of Bcl-xL or Mcl-1 used in Fig. 4 were expected to interact with Bax or cBak (see Fig. 5A). We averaged 3-5 independent experiments for each graph, or chose representative of 4-6 independent experiments.

The time course assay measured the released fluorescein-dextran through the quenching by anti-fluorescein antibodies (Invitrogen #A889) added to the external solution (26). N/C-Bid (45 nM) and BH3 peptides (25 µM) were added 2-3 min prior to the addition of Bax (120 nM). The fluorescence measurement was taken every 30 seconds in a 96-well plate by a microplate fluorescence reader, FL600 (Bio-Tek). The experiments were performed more than 5 times to ensure the reproducibility.

**cBak association with the liposomes and oligomerization in the liposome**

cBak (5 µM), N/C-Bid (190 nM) and Bcl-xL (4.6 µM) were incubated with the liposomes (~4 times more than the regular liposome assay) at RT for 2.5 h. At the end of the incubation, the sample was divided into two aliquots to measure dextran release and to carry out the association assay as described previously (22). The liposome suspension in the running buffer was loaded on a Superdex 200HR(10/30)(GE) as previously described (13). Each fraction was concentrated using a microcon filter and loaded on an SDS-PAGE gel. cBak was detected by immunoblotting.

**Cytochrome C release assay with mouse liver mitochondria**

Mouse liver mitochondria were prepared and the cytochrome C release assay was performed as described previously (27). Cytochrome C was detected by immunoblotting. Mouse liver mitochondria deficient for both Bax and Bak were prepared as described in Cassidy-Stone et al. (28).

**Immunoblotting for Bcl-2 family proteins**

MEFs or digitonin-permeabilized cell pellets (25 µg protein) were loaded onto 12% SDS-PAGE gels and transferred to nitrocellulose membrane for immunoblotting using antibodies against Bcl-2 family members; anti-Bcl-2 (Santa Cruz, sc-23960), anti-Bcl-xL (Santa Cruz, sc-8392), anti-Bcl-w (Calbiochem, 197209), anti-Mcl-1 (Rockland, 600-401-394), anti-Bax (Santa Cruz, sc-493), anti-Bak (Upstate, 06-536), anti-Bid (R & D, AF860), anti-Bmf (Lifespan, LS-C4126) and anti-Bim (Sigma, B7929 or Stressgen, AAP330). An anti-actin antibody was obtained from Sigma (ac-40). Secondary antibodies used were; antimouse IgG (BioRad, 170-6516), anti-rabbit IgG (BioRad, 170-6515), anti-rat IgG (Santa Cruz, sc-2006) and anti-goat IgG (Santa Cruz, sc-2350).

**Circular dichroism (CD)**
All the BH3 peptides except for BikBH3 were dissolved in 10 mM phosphate buffer (pH 7.0) at 25 µM. Bik BH3 was dissolved at 15 µM in buffer containing 0.1% DMSO. CD spectra were obtained at 25°C on an AVIV model 202-01 spectropolarimeter; four scans were averaged using 1 nm bandwidth and 1 mm path length quartz cell (Hellma). Molar ellipticity was calculated as CD/[#of amino acids x concentration (mol/L) x path length (0.1 cm) x 10 (conversion factor to decimoles)].

RESULTS

Some BH3 peptides induce cytochrome C release from Bim/Bid DKO MEFs

We first examined the abilities of various BH3 peptides (Fig. 1A) to induce cytochrome C release in the absence of the known direct activators, Bim and Bid. In digitonin-permeabilized Bim/Bid DKO MEFs, not only Bim and Bid BH3 peptides, but also Puma and Bmf BH3 peptides triggered the release of cytochrome C from mitochondria (Fig. 1B). The peptides alone at 50 µM did not cause mitochondrial permeabilization in digitonin-permeabilized Bax/Bak DKO MEFs, confirming that cytochrome C release at the concentration range tested was Bax/Bak dependent (Fig. 1C).

MOMP in Bim/Bid DKO cells may be induced by 1) the liberation of Bax/Bak from anti-apoptotic Bcl-2 proteins by these peptides as the neutralization model predicts, 2) displacement of yet unknown direct activators and/or 3) BH3 peptides themselves acted as direct activators as well as de-repressors.

Approximately 10% of Bak was bound to Mcl-1 in Bim/Bid DKO MEFs

The neutralization model hypothesizes that Bax and Bak are sequestered by anti-apoptotic Bcl-2 proteins until BH3-only proteins liberate them. We immunoprecipitated Bcl-2, Bcl-xL and Mcl-1 from non-apoptotic Bim/Bid DKO MEFs to the extent that the amount left in the cytosol was minimal. The immunoprecipitation was performed in CHAPS, a detergent known not to induce effector conformational changes associated with activation and oligomerization. We found that Bak was not co-immunoprecipitated with any of the anti-apoptotic proteins and only ~10-15% Bak was found associated with Mcl-1, but not at all with Bcl-2 or Bcl-xL (Fig. 1D). It appears that most Bax and Bak molecules were not sequestered by binding to anti-apoptotic Bcl-2 proteins. These cells do not express A1 (29) and only trace amounts of Bcl-w (Fig. 1E), making it unlikely that the rest of the effectors are bound to these anti-apoptotic proteins. We conclude that the majority of Bax and Bak are not bound to anti-apoptotic Bcl-2 proteins and, yet, not inducing MOMP in healthy cells.

cBak associates with the membrane, oligomerizes and permeabilizes the membrane.

We were also interested to know whether Bak is activated by a different or the same set of BH3-only proteins as with Bax. In order to investigate the activation of Bak by BH3 peptides in the liposome system, we used recombinant cBak. cBak was generated by calpain cleavage from a C-terminally truncated species, and lacks 15 amino acids at the N-terminus and 21 at the C-terminus (Fig. 2A) (25). cBak was soluble and monomeric in solution, became associated with the liposome membrane and formed oligomers in the membrane in the presence of cleaved Bid (Figs. 2B and C). Furthermore, it was capable of synergizing with BH3 peptides to promote the release of cytochrome C from mouse liver mitochondria devoid of Bax and Bak. The mitochondrial permeabilization correlated with cBak oligomer formation (Fig. 2D). Cytochrome C release in the presence of BH3 peptides was also tested in mitochondria isolated from wild type mouse livers that are known to contain Bak only. The release profile with native Bak and that with cBak were in good agreement (Figs. 2E and D upper panel). We conclude that cBak mimics native Bak, although ~10 times more cBak than Bax was required to produce the same level of membrane permeabilization.

A number of BH3 peptides other than Bim and Bid BH3 directly activate Bax and cBak

Liposome permeabilization is influenced by differences in recombinant protein and liposome preparations. In order to remedy this problem and obtain representative data, we selected and averaged the experiments in which we saw that the
release by Bax alone was 20-30% that of Bax plus N/C-Bid (that typically gives the maximum release in our system). BH3 peptides alone did not permeabilize liposomes at 25µM (Fig. 3A). We examined direct activation of Bax or cBak by each BH3 peptide and found that the direct activation profile of cBak in response to various BH3 peptides was similar to that for Bax; Bim BH3 exhibited the highest degree of activation, Bid, Bmf and Noxa BH3 constituted the next highest group, then, the group of Bik, Hrk and Puma BH3, and Bad BH3 showed the lowest activation (Figs. 3B and C). Most peptides exhibited some degrees of direct activation. Particularly, the previously unrecognized Bmf and Noxa BH3 peptides were notable. We confirmed that there was no significant false direct activation over a range of Bax-alone release by Bad BH3 (Fig. 3D). In order to characterize direct activation by BH3 peptides other than Bim and Bid BH3, we performed a time course assay. In this assay, as the membrane permeabilizes and the dextrans are released, anti-fluorescein-antibodies in the external milieu quench the fluorescence instantaneously (26). Two representative data are shown in Fig. 3E. The permeabilization induced by the weak direct activator BH3 peptides was slower than the strong direct activators.

We also performed circular dichroism on our peptides in order to test whether the peptide’s helicity determines the potency of direct activation. The spectra show that all the peptides were equally unstructured (Fig. 3F), therefore, eliminating the possibility of the helical propensity of free peptides exclusively correlating with the observed degree of direct activation.

**Low-grade direct activation influences membrane permeabilization**

In order to confirm direct activation by BH3 peptides other than Bim and Bid, we performed the liposome assay in the presence of anti-apoptotic proteins. In our previous work, de-repression was examined in the presence of a cleaved form of Bid protein (N/C-Bid) that was ready to activate Bax. This obscured the BH3 peptides’ ability to directly activate Bax. Therefore we performed the assay without N/C-Bid. BH3 peptides were consistently used at 25 µM, except for titrations. As shown in Fig. 4A, Bim, Bid, Bmf, Hrk and Puma BH3 were capable of removing Bcl-xL inhibition as well as directly activating Bax. Hrk and Puma BH3 peptides appear to have more pronounced Bax activation in the presence of Bcl-xL (compare to Fig. 3B). Bcl-xL might somehow change the conformation of the peptides, which enhanced their activities, as discussed below in the context of Mcl-1. Noxa BH3 can activate Bax directly (Fig. 4A), but in the presence of Bcl-xL, it could not do so. We hypothesized that there was direct inhibition of Bax by Bcl-xL, which Noxa BH3 could not remove. When we added Bad BH3 to the system to inhibit Bcl-xL, now direct activation of Bax by Noxa BH3 was revealed (Fig. 4B). This data also demonstrates that neutralization is necessary, but not sufficient, and direct activation is required to achieve full membrane permeabilization. We performed the same experiment with cBak and found that Bim, Bmf and Puma BH3 could directly activate cBak, but not Bid and Noxa BH3 (Fig. 4C). We reasoned that Bid and Noxa BH3 could not remove Bcl-xL inhibition, so added Bad BH3. As shown in Figs. 4D and E, their direct activation capabilities manifested when Bcl-xL was inhibited. Bid BH3 is known to inhibit Bcl-xL, however, we suspect that the interaction between cBak and Bcl-xL is stronger than Bid BH3 and Bcl-xL at the concentrations used. We performed the same experiments with Mcl-1 instead of Bcl-xL and the results show that Bim, Bid, Bmf, Puma and Noxa BH3 directly activated Bax or cBak in the presence of Mcl-1 (Figs. 4F and G). It is somewhat surprising that we did not see significant levels of permeabilization by Noxa BH3 in the presence of Mcl-1 (Fig. 4G, the last bar). On the other hand, it appears inconsistent that Puma BH3 very modestly activates Bax or cBak (Figs. 3B and C), and yet, in the presence of Mcl-1, its direct activation was more pronounced than would have been anticipated (Figs. 4F and G). We do not have good explanations for these discrepancies regarding Puma and Noxa BH3s, however, the presence of Mcl-1 might somehow alter the folding state of some BH3 peptides and their activities.

**Direct inhibition of Bax or cBak by anti-apoptotic proteins and its removal by BH3 domain**
Since neutralization of Bax/cBak by anti-apoptotic Bcl-xL was suspected in the experiments described above, we investigated the neutralization in the liposome system. We increased Bax or cBak to the levels at which they significantly permeabilize liposomes on their own, and found that they were directly inhibited by Bcl-xL or Mcl-1, in a dose dependent manner (Fig. 5A). cBak was more easily inhibited than full-length Bax, possibly because its truncation renders it less potent. The inhibition of Bax or cBak by the anti-apoptotic Bcl-xL was reversed by applications of Bad BH3 peptide (Fig. 5B) or a Bad-like BH3 mimetic compound, ABT-737 (30). Direct demonstration of binding between the proteins by immunoprecipitation was impossible, as the proteins bound to the beads non-specifically even in the presence of carrier proteins. We conclude that BH3-only proteins can dissociate Bax/Bak from anti-apoptotic proteins, much like they displace direct activator BH3-only proteins from the anti-apoptotic proteins (20,21).

Low-grade direct activation by Bmf, Puma or Noxa BH3 can induce MOMP

As shown in Fig. 1B, Bmf and Puma BH3 peptides released cytochrome C from mitochondria in the absence of Bim and Bid. Previous reports also demonstrated that the combination of Bad and Noxa BH3 led to the release of cytochrome C (17,18). We treated the digitonin-permeabilized cells with the peptides and performed immunoprecipitation experiments using these cell pellets to directly compare cytochrome C release and Bak dissociation from Mcl-1 (Figs. 6A-C). Even though only a small portion of Bak appears to be bound to Mcl-1 in these cells, Bak dissociation from Mcl-1 correlated well with cytochrome C release (Figs. 6A and C). Digitonin permeabilization lost ~70% of Bax from these cells, and yet, we observed that remaining Bax accumulated in the pellet fraction, when MOMP occurred (data not shown). Thus, both Bax and Bak were involved in inducing MOMP. Notably, the level of Bak association with Mcl-1 decreased to the same level in Noxa BH3 only-added sample as in both Noxa and Bad BH3-added one (Fig. 6C, compare the lanes for Noxa BH3 and Noxa/Bad BH3). Nevertheless, cytochrome C release was not observed, when only Noxa BH3 was added, presumably because the cells also express Bcl-xL and Bcl-2 that would sequester activated (?) Bax/Bak, which cannot be removed by Noxa. Therefore, we conclude that Bak dissociation occurs during MOMP, but this is insufficient to induce MOMP, consistent with the direct activation model. Cell death induced in these cells by over-expression of the full-length BH3-only proteins (Fig. 6D) correlated well with the cytochrome C-release data, supporting the idea that the BH3-peptides mimic the full-length proteins regarding MOMP.

DISCUSSION

BH3-only proteins, Bim and Bid, are distinguished to be "direct activators" of Bax and Bak and are required for MOMP. If this model is correct, Bim and Bid DKO mice should have a phenotype close to Bax/Bak DKO mice. However, Bim/Bid DKO mice and cells derived from them show no obvious apoptotic defect. We found that BH3 peptides other than Bim and Bid can directly activate Bak. This redundancy could explain the lack of phenotype in Bim/Bid DKO mice, although there might be other factors involved, such as physical insults to Bax/Bak, including heat and pH changes (31,32), or non-Bcl-2 protein direct activators like p53 (33).

From the liposome studies, we propose that direct activation is not an all-or-none phenomenon, but most BH3-only proteins possess this activity in various degrees. Our analysis suggests a hierarchy of direct activation based on the activities of the BH3 regions in isolation with Bim and Bid (high), followed by Bmf and Noxa (medium), Puma, Bik and Hrk (low) and Bad (no activity). Nevertheless, we do not exclude possibilities of deviations from this hierarchy in full-length proteins, amenable to fine-tuning by post-translational modification in pathophysiology. cBak was similarly activated, suggesting that, in terms of direct activation, BH3-only proteins do not discriminate between Bax and Bak. Although cBak is dually truncated, we found that its behavior upon encounter with a direct activator (e.g., N/C-Bid) is identical to that of Bax in both liposomes and isolated mitochondria; it associates with the membrane, oligomerizes and permeabilizes the membrane by synergizing with
Neutralization of Bax/Bak was not demonstrated in the liposome system in our previous work (22). However, now we have revealed the roles of neutralization and direct activation in this system. We took advantage of the fact that Bad BH3 does not directly activate Bax/Bak, and Noxa BH3 does not bind to Bcl-xL, but directly activates Bax/Bak. Our data show that direct activator BH3 peptides are not able to act on Bax/Bak, unless pro-survival proteins are neutralized by them, and that neutralization is not sufficient for full membrane permeabilization. The importance of direct activation is further supported by the result with the digitonin-permeabilized cell system that Noxa BH3 alone was sufficient to dissociate Bak from Mcl-1, and yet cytochrome C was not released. Our conclusion on Bcl-2 family protein interactions is summarized in Fig. 7.

Our liposome data are consistent with the part of the neutralization model that postulates that Bax and Bak are dissociated from anti-apoptotic proteins by BH3-only proteins. We suggest that BH3-only proteins not only displace direct activator BH3-only proteins as reported by the Letai and Cheng laboratories (19-21), but that they also liberate Bak/Bax from pro-survival proteins. The neutralization could also play a role downstream of Bax/Bak activation in preventing "activated" Bax/Bak from being sequestered by pro-survival proteins, as it has been suggested that anti-apoptotic Bcl-2 proteins bind an "active conformant" of Bax/Bak (38). The molecular mechanism of direct activation has been suggested by Gavathiotis et al. (39), and together with the recent reports (34,40,41), we envisage that the direct activator BH3 would interact with α1 and α6 helices of Bax/Bak transiently to induce conformational change of the effector, which would lead to membrane association (in Bax case) and subsequent oligomerization (Bax and Bak). In the liposome system, we postulate that there are two conformations of Bax/Bak; one is "half-activated" and the other, "fully" activated. Both forms can bind anti-apoptotic proteins, but the half-activated species can be further activated by direct activator BH3-only proteins. Anti-apoptotic proteins could interact with the half-activated Bax/Bak species before the direct activation takes place and this interaction is mediated by the canonical BH3 groove, as Bad BH3 removes the

N/C-Bid. It also mimics the native Bak in mitochondria. Although eBak expression in Bax/Bak DKO MEFs has recently been reported not to lead to cross-linkable oligomers and cell death in response to UV (34), we suspect that the level of expression achieved in those cells was not high enough to lead to this outcome.

We noticed that the peptides that induced MOMP in Bim/Bid DKO cells are capable of inhibiting both Bcl-xL- and Mcl-1-type pro-survival Bcl-2 proteins, suggesting that both types of anti-apoptotic Bcl-2 proteins must be inhibited to pave the way for direct activation, and subsequently MOMP. This is consistent with the in vivo finding that Mcl-1/A1 must be inhibited in order for a Bad BH3-like BH3 mimetic, ABT-737, to induce apoptosis in tumors (35,36). Our in vitro data show that Bim, Bid, Bmf, Puma and the Noxa/Bad combination all inhibit both types of pro-survival proteins as well as directly activate Bax/Bak. Therefore, they are capable of inducing cytochrome C release from mitochondria, as well as apoptosis in cells lacking Bim and Bid. Hrk and Bik BH3 also elicit weak direct activation, but only inhibit Bcl-xL-type anti-apoptotic proteins, which is likely the reason why they fail to trigger cytochrome C release in these cells. We could not test whether their low direct activation activity is sufficient to induce MOMP in these cells or membrane permeabilization in liposomes, as there is no known factor that inhibits Mcl-1 but does not directly activate Bax/Bak. The stapled BH3 domain of Mcl-1 was recently described to specifically and potently antagonize Mcl-1, and when the technology becomes affordable and accessible, we will test its effect in our system (37). Noxa BH3 cannot release cytochrome C for the same reason that it only inhibits Mcl-1. We were unable to rule out the possibility that these weak direct activators induced MOMP by displacing yet unknown direct activators. These MEFs do not express detectable levels of Bmf (Fig. 1E) and Puma (27), although the endogenous Noxa level is unknown as there are not suitable antibodies for the detection of mouse Noxa. With this caveat in mind, we propose that weak direct activation plays a role in MOMP and that it is important to inhibit both types of anti-apoptotic proteins for MOMP to occur.
inhibition. It remains to be investigated whether the half-activated species is generated before or after the association with the membrane, and whether the direct activation has any effect on the established binding between anti-apoptotic proteins and Bax/Bak.

Our data show that there are both weak and strong direct activators, just as there are weak and strong binders of anti-apoptotic proteins. Direct activation is not an all-or-none event, and whether a particular BH3-only protein acts as a direct activator depends on the context as well as the system employed. A whole-animal study has demonstrated that a form of Bim in which the BH3 domain is replaced by its Puma counterpart does not completely compensate for wild-type Bim (42). This could be explained by the fact that Bim is a stronger direct activator than Puma, although we do not yet know what determines the potency of direct activation. Walensky et al. reports that increasing α-helicity in Bid BH3 peptide enhanced its direct activation capability, however, the same treatment on Bad BH3 does not convert it to a direct activator (24). The circular dichroism data on our peptides also does not support the idea that the peptide's helicity determines the potency of direct activation.

Future studies will test the model proposed in this study in more specific and physiological contexts. BH3 peptide mimetics such as ABT-737 and Obatoclax (19,30,36,43,44) have already shown to have some desirable effects in animal tumor models and in clinical trials, therefore understanding the details of the molecular mechanisms that underlie MOMP will allow us to develop more potent and specific chemotherapeutics.
REFERENCES

FOOTNOTES

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The abbreviations used are: DKO, double knockout; MEF, mouse embryonic fibroblast; MOMP, mitochondrial outer membrane permeabilization

FIGURE LEGENDS

Fig. 1. Cytochrome C release by BH3 ligands from Bim/Bid DKO MEFs. A) Amino acid sequences of BH3 peptides (human) used in this study. B) Bim/Bid DKO MEFs were permeabilized with digitonin and incubated with cleaved Bid protein (N/C-Bid) or the BH3 peptides. The Bim BH3 peptide had the most potent cytochrome C releasing activity; the Bid, Bmf and Puma BH3 peptides also had some activity at higher concentrations. Blots are representative of three independent experiments. C) BH3 peptides at 50 µM did not release cytochrome C in digitonin-permeabilized Bax/Bak DKO MEFs, therefore even at a relatively high concentration of 50 µM, cytochrome C release by BH3 peptides shown in A was Bax/Bak-dependent. Digitonin permeabilization was confirmed by cytochrome C release observed in the control in which Bax (480 nM) and N/C-Bid (45 nM) were added. D) Bcl-xL, Bcl-2 and Mcl-1 were immunoprecipitated from CHAPS or NP-40-solubilized Bim/Bid DKO MEFs and probed for Bax or Bak. L, lysate or supernatant; B, beads. Bax and Bak were found not associated with Bcl-xL or Bcl-2 in these cells with either detergent. Bax was not associated with Mcl-1, whereas approximately 10-15% of Bak was bound to Mcl-1, estimated from the fact that 7.5% lysate and 50% beads samples were loaded on the gel (see also Figs. 6B and C). E) Bcl-2 proteins detected in WT MEFs and Bim/Bid DKO MEFs. Protein was loaded at 50 µg per lane, and was detected by immunoblotting using specific antibodies. Equal loading was confirmed by immunoblotting for actin (bottom panels). Puma is not detectable in either WT MEFs or DKO MEFs (data not shown) (26). Bmf was barely detectable in the DKO s. There are no antibodies suitable for detection of mouse Noxa. Bad was not detectable in these cells by a few different antibodies (data not shown). The mRNA of A1, another anti-apoptotic Bcl-2 protein, is not present in MEFs (28). WT MEFs express similar levels of Bax and Bak, as estimated based on standards using recombinant Bax and cBak, respectively. The Bak and Bcl-2 levels in Bim/Bid DKO MEFs were found to be lower than those in the WT.

Fig. 2. Recombinant cBak permeabilizes the liposome membrane and mitochondria. A) Schematic diagrams of native Bak and cBak. Recombinant cBak lacks the N-terminal 15 amino acids and the C-terminal 25. B) cBak, N/C-Bid and Bcl-xL were incubated with fluorescein (F)-dextran-loaded liposomes,
and dextran release was measured as reported previously (13). The liposomes were recovered by float-up centrifugation and Bak associated with the membranes was detected by immunoblotting. cBak association with the membrane was promoted by N/C-Bid, and this effect was blocked by Bcl-xL. Liposome permeabilization correlated with the amount of cBak in the membrane. Blots shown are representative of 5 experiments. C) N/C-Bid induced oligomerization of cBak in the membrane. cBak and N/C-Bid-incubated liposomes from the middle lane in Fig. 2B were solubilized in 2% CHAPS and fractionated by gel-filtration (N/C-Bid treated). As a control, total cBak that was incubated with liposomes in the absence of cleaved Bid was fractionated and found to be monomeric (Non treated). D) cBak was incubated with mouse liver mitochondria devoid of Bak and Bak. The Bim, Bid and Bmf BH3 peptides stimulated robust cytochrome C release, and the Hrk, Puma and Bik BH3 showed a modest release (upper panel). cBak oligomers were detected (lower panel). Note that cytochrome C release correlated well with the formation of cBak oligomers. E) BH3 peptides (25 µM) were added to mitochondria isolated from wild type mouse livers. The profiles of cytochrome C release in response to native Bak in combination with BH3 peptides was similar to those for cBak with BH3 peptides; Bim and Bid BH3 were highly potent, and Bmf and Puma BH3 also triggered moderate release consistently. We used mouse BH3 peptides for Bid and Bmf and the rest were human BH3s.

Fig. 3: Bax and cBak activation by BH3 ligands reveal low-grade direct activation. A) BH3 peptides alone do not trigger liposome permeabilization. Bax (120 nM), N/C-Bid (45 nM) or BH3 peptides (25 µM) were added to liposomes containing fluorescein (F)-dextran (10 kD). B, C) Direct activation of Bax or cBak by a panel of BH3 peptides. The potency of direct Bax activation ranked Bim BH3 as the strongest activator, followed by Bid, Bmf and Noxa BH3 as moderate activators, Bik, Hrk and Puma BH3 as weak activators and Bad BH3 as non-activating. D) Difference in background release (Bax-alone release; black bars) does not lead to false direct activation. Bad BH3 peptide was consistently unable to directly activate Bax (gray bars) over a range (8-42%) of Bax alone-induced permeabilization. E) A time course of dextran release demonstrates low-grade direct activation by Bmf and Noxa BH3 (upper panel) and Bik, Hrk and Puma BH3 (lower panel). Bim and Bmf BH3 peptides were included in both assays in order to show the relative potency between the peptides. Permeabilization induced by weak direct activators was slow, but notably higher than the Bax-alone background, which is consistent with the endpoint assay. F) Circular dichroism (CD) confirms that all the BH3 peptides used in this study were intrinsically disordered at 10 mM phosphate buffer of pH 7.0. The poor solubility of Bik BH3 in aqueous solutions required the use of 0.1% DMSO in the CD sample, which introduced significant noise in the data below 200 nm. Although the Bim and Noxa BH3 peptides apparently dissolved in water, they may be less soluble in the CD buffer, illustrated by a less pronounced ellipticity drop at 200 nm.

Fig. 4: BH3 ligands show direct activation when anti-apoptotic proteins are inhibited. A) In the presence of Bcl-xL, Bax was activated by the same set of BH3 peptides as in the absence of Bcl-xL, with the exception that Noxa BH3 was ineffective. B) Bad BH3 peptide presumably removed Bcl-xL inhibition, revealing direct activation of Bax by Noxa BH3. C) In the presence of Bcl-xL, cBak was only activated by Bim and Bmf BH3. D and E denote the fact that these situations were addressed in the following figures, D and E, respectively. D) Bad BH3 interfered with Bcl-xL, revealing direct activation of cBak by Bid BH3. E) Bad BH3 inhibited Bcl-xL, uncovering direct activation of cBak by Noxa BH3. F, G) In the presence of Mel-1, Bax and cBak were directly activated by the same peptides that directly activated them in the absence of Mel-1, except for Puma BH3 exhibiting more potent direct activation in both cases and Noxa BH3 failing to activate cBak.

Fig. 5: BH3 ligands overcome effector inhibition by anti-apoptotics in liposomes. A) High levels of Bax (left panel) or cBak (right panel) led to significant permeabilization of liposomes, and this permeabilization was inhibited in a dose-dependent manner by Bcl-xL or Mcl-1. B) Both Bad BH3 and its mimetic, ABT 737, were capable of interfering with the inhibition of Bax or cBak by Bcl-xL or Mcl-1.
**Fig. 6.** Bak dissociation from Mcl-1 correlates with cytchrome C release in permeabilized cells. A) Cytochrome C release from mitochondria in Bim/Bid DKO MEFs, as in Fig. 1. Various BH3 peptides were added at 50 µM to digitonin-permeabilized Bim/Bid DKO MEFs for immunoprecipitation studies whose outcomes are shown in B and C. The combination of Noxa and Bad BH3 peptides triggered cytchrome C release, as previously reported. B) Effects of Mcl-1 and Bak in the supernatant after immunoprecipitation with anti-Mcl-1 antibody. The cell pellet in A after the incubation with the peptide was dissolved in 1% CHAPS, and immunoprecipitation was performed. Mcl-1 was depleted from the lysate. The decrease in Bak levels was not detectable on the blot, presumably because only ~10-15% of Bak was co-immunoprecipitated. C) Bak co-immunoprecipitation with Mcl-1 was inversely correlated to cytchrome C release; the less Mcl-1 associated with Bak, the more cytchrome C was released. D) Apoptosis induced by over-expressing BH3-only protein correlated with cytchrome C release induced by BH3 peptides. We used mouse genes except for Bmf, which was human. The transfection efficiency across samples was comparable based on the FACS-based measurement of the expression of IRES-driven GFP expression from the same plasmid. All the data in this figure are representative of 3-7 experiments.

**Fig. 7.** Summary of interactions governing the Bcl-2 family proteins. We propose that there are three-way interactions between BH3-only proteins, anti-apoptotic proteins and Bax/Bak. A) MOMP results when both the Bcl-xL- and Mcl-1-type anti-apoptotic Bcl-2 proteins are inhibited, and Bax/Bak are directly activated. BH3-only proteins displace activator BH3-only proteins and/or Bax/Bak. B) Even when all anti-apoptotic proteins are inhibited and Bax/Bak are liberated, Bax/Bak still need to be directly activated before MOMP occurs. C) Inhibiting only one type of pro-survival Bcl-2 protein is not sufficient to induce MOMP.
Figure 1

A

<table>
<thead>
<tr>
<th>BH3 Peptide</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Bim BH3</td>
<td>DMRPEIWIAQELRRIGDEFNAYAR</td>
</tr>
<tr>
<td>Bid BH3</td>
<td>EDIIRNIARHLAQVGDMSRDESFKK</td>
</tr>
<tr>
<td>Bad BH3</td>
<td>NLWAQRYGRELRRMSDEFVDSFKK</td>
</tr>
<tr>
<td>Bik BH3</td>
<td>CMEGSRALARLACIGDEMDVSLRA</td>
</tr>
<tr>
<td>Bmf BH3</td>
<td>LQHRAEVQIARKLQCIADQFHRLHT</td>
</tr>
<tr>
<td>Hrk BH3</td>
<td>LRSSAAQLTAARLKGDELHQRTM</td>
</tr>
<tr>
<td>Puma BH3</td>
<td>EEQWAREIGAVRLRMAADDNQAQYER</td>
</tr>
<tr>
<td>Noxa BH3</td>
<td>PAELEVECATQLRFFGDKLNFRQKLL</td>
</tr>
</tbody>
</table>

B

BH3 peptides; 5, 10, 25, 50 uM  
N/C-Bid; 11, 23, 45, 90 nM

C

BH3 peptide (50uM)  
bufer  DMSO  Bax/Bid

Cytochrome C  
Supernatant  Pellet
Estimate: 4 ng/25 ug
= 0.16 ug/mg cellular protein

Estimate: 6 ng/25 ug
= 0.24 ug/mg cellular protein
Figure 2

A

Bak

cBak

Calpain cleavage

Calpain cleavage

B

cBak (5 uM)
N/C-Bid (190 nM)
Bcl-xL (4.6 uM)

% dextran release
11 77 28

C

N/C-Bid treated
(membrane fraction)

Non treated
(soluble fraction)

D

WB: Cytochrome c

bax<sup>−/−</sup> bak<sup>−/−</sup>

E

cytochrome C

Supernatant

Pellet

 BH3 peptide (25 uM)
Figure 3

A

% F-dextran release

BimBH3
BidBH3
BadBH3
BikBH3
BmfBH3
HrkBH3
PumaBH3
NoxaBH3

B

Normalized % F-dextran release

Bax (120 nM)

BimBH3
BidBH3
BadBH3
BikBH3
BmfBH3
HrkBH3
PumaBH3
NoxaBH3

C

Normalized % F-dextran release

cBak (1.75 uM)

Bax alone
Bax+BadBH3

D

Normalized % F-dextran release

Bax (nM)

Bad BH3
(0, 10, 25 uM)

100
80
60
40
20
0

20
40
60
80
100

20
40
60
80
100

0
20
40
60
80
100

Bax alone
Bax+BadBH3

18

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Figure 4

(A) Normalized % F-dextran release

(B) Normalized % F-dextran release

(C) Normalized % F-dextran release

(D) Normalized % F-dextran release

(E) Normalized % F-dextran release
**E**

Normalized % F-dextran release

- cBak (1.2 uM)
- Bcl-xL (420 nM)
- Noxa BH3 (25 uM)
- Bad BH3 (uM)

**F**

Normalized % F-dextran release

- Bax (30 nM) + Mcl-1 (208 nM)

**G**

Normalized % F-dextran release

- cBak
- Bid
- Bad
- Bik
- Bmf
- Hrk
- Bim
- Bax

**Notes:**
- cBak (1.25 uM) + Mcl-1 (73 nM)
- Bax (30 nM) + Mcl-1 (208 nM)
Figure 5

A

Bax (240 nM) + Bcl-xL + Mcl-1

Bcl-xL: 45, 208, 625, 833, 1670 nM
Mcl-1: 27, 135, 270, 405, 540, 1080 nM

Bcl-xL + Mcl-1

Bcl-xL: 4, 20, 42, 208, 420, 833 nM
Mcl-1: 3, 14, 27, 135, 270, 540 nM

B

Bax (240 nM) + Bcl-xL (833 nM) + Bad BH3 + ABT-737

Bad BH3: 0.02, 0.1, 1 uM
ABT-737: 0.02, 0.1, 1 uM

Bcl-xL (208 nM) + Bad BH3 + ABT-737

Bad BH3: 10, 25, 50 uM
ABT-737: 1, 5, 10 uM
Figure 6

A

Digitonin permeabilized cell lysate (7.5% total loaded)

B

Beads (50% total loaded)

C
Figure 7

\[\text{Δ BH3-only} \quad \text{Bax/Bak} \quad \text{Bcl-xL-type} \quad \text{★ Direct activation} \quad \text{Mcl-1-type} \]

\[\begin{align*}
\text{A} & \rightarrow \text{MOMP} \\
\text{B} & \rightarrow \text{MOMP} \\
\text{C} & \rightarrow \text{No MOMP}
\end{align*}\]
BH3-domains other than Bim and Bid can directly activate BAX/BAK
Han Du, Jacob Wolf, Blanca Schafer, Tudor Moldoveanu, Jerry E. Chipuk and Tomomi Kuwana

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