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How do BCL-2 proteins induce mitochondrial outer membrane permeabilization?

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Abstract

The mitochondrial pathway of apoptosis proceeds when molecules sequestered between the outer and inner mitochondrial membranes are released to the cytosol by mitochondrial outer membrane permeabilization (MOMP). This process is controlled by the BCL-2 family, which is composed of both pro- and anti-apoptotic proteins. Although there is no disagreement that BCL-2 proteins regulate apoptosis, the mechanism leading to MOMP remains controversial. Current debate focuses on what interactions within the family are crucial to initiate MOMP. Specifically, do the BH3-only proteins directly engage BAX and/or BAK activation or do these proteins solely promote apoptosis by neutralization of anti-apoptotic BCL-2 proteins? We describe these models and contend that BH3-only proteins must perform both functions to efficiently engage MOMP and apoptosis.

Introduction: where we agree – the BCL-2 family and mitochondria

The mitochondrial pathway of apoptosis is dependent upon the BCL-2 (B-cell CLL/Lymphoma 2) family of proteins for the efficient release of pro-apoptotic factors [e.g. cytochrome c, SMAC/DIABLO (Second mitochondria-derived activator of caspase/Direct IAP-bind protein with low pI)] from the mitochondrial intermembrane space (for more information, refer to [1]). These factors cooperate with cytosolic adaptor proteins [e.g. APAF-1 (Apoptotic protease activating factor 1)] to induce caspase activation, which is necessary to elicit the phenotypes associated with apoptosis (e.g. DNA laddering, nuclear condensation, and cellular blebbing; for more information, refer to [2]). The BCL-2 family of proteins is divided into three groups, based on the presence of up to four BCL-2 homology domains (BH1–4 domains) (Figure 1; for more information, refer to [3]). Anti-apoptotic BCL-2 proteins [e.g. BCL-2, BCL-w, BCL-xL (BCL-2 related gene, long isoform), A1 and MCL-1 (Myeloid cell leukemia 1)] contain BH domains 1–4 and are generally integrated within the outer mitochondrial membrane, but there are instances of cytosolic, endoplasmic and nuclear membrane localizations. These proteins function within the apoptotic pathway to directly bind and inhibit the pro-apoptotic BCL-2 proteins. The pro-apoptotic members are functionally divided into two classes: the effector molecules [e.g. BAK (BCL-2 antagonist killer 1) and BAX (BCL-2 associated x protein)], which contain BH1–3 domains and permeabilize the outer mitochondrial membrane by creating the proteolipid pore responsible for cytochrome c release (for more information, refer to [4]); and the BH3-only proteins [e.g. BAD (BCL-2 antagonist of cell death), BID (BCL-2 interacting domain death agonist), BIK (BCL-2 interacting killer), BIM (BCL-2 interacting mediator of cell death), BMF (BCL-2 modifying factor), bNIP3 (BCL-2/adenovirus E1B 19-KD protein-interacting protein 3), HRK (Harakiri), Noxa and PUMA (p53-upregulated

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modulator of apoptosis)], which function in distinct cellular stress pathways and, by protein–protein interactions with other BCL-2 family members (i.e. anti-apoptotic BCL-2 proteins and/or the effector molecules), signal that a cellular stress has occurred. The combined signaling within the BCL-2 family dictates the immediate fate of the affected cell – to induce mitochondrial outer membrane permeabilization (MOMP) or not. What remains highly controversial in the apoptosis field is the exact nature of protein–protein interactions among members of the BCL-2 family that are the key mediators of the signal required to engage MOMP and apoptosis.

To understand the basis of this debate, we discuss both the historical and the current hypotheses (the 'anti-apoptotic protein neutralization' and 'direct activation of BAX and BAK' models) relating to the function of pro-apoptotic BCL-2 proteins. We present a model based on current evidence that MOMP occurs through multiple protein–protein interactions within the BCL-2 family. As reference, several aspects of BCL-2 family function are defined in the glossary, along with important concepts related to this review.

Revisiting the rheostat model

The realization that BCL-2 blocked the activation of caspases was central to our understanding of how the BCL-2 family proteins function within the apoptotic cascade. Investigations using *Xenopus* oocyte extracts led to the first observations that incubation of cytosol promoted caspase activation through a mitochondria-dependent mechanism that was inhibited by the addition of BCL-2 [5]. This placed BCL-2 upstream of mitochondria in vertebrate model systems. This is in contrast to the situation in *Caenorhabditis elegans*, wherein the BCL-2 homolog (ced-9) functions by directly inhibiting the adaptor protein responsible for caspase activation (ced-9 inhibited ced-4, the APAF-1 homolog) [6–8]. Several groups then screened for BCL-2-interacting proteins and identified BAX and BAD, which led to the idea that the cellular decision to die centered on tipping the balance of total BCL-2 family expression from an anti-apoptotic (or pro-survival) to a pro-apoptotic position (Figure 2a) [9,10].

This hypothesis was widely accepted because it was supported by various observations. First, genetic deletion of anti-apoptotic BCL-2 members (e.g. *bcl-2* and *bcl-x*) resulted in enhanced incidence of apoptosis (as was true in *C. elegans ced-9* deficient animals); likewise, deletion of pro-apoptotic members (e.g. *bax*) prevented some forms of cell death [11–13]. Also, it was revealed that the tumor suppressor p53 was able to *trans*-activate the human *bax* gene by directly binding to p53-responsive elements, and this substantiated the notion that a pro-apoptotic event (i.e. p53 stabilization) was converted to a death signal by increasing pro-apoptotic protein expression [14]. Essentially, the model described that the stoichiometry between the pro- apoptotic and anti-apoptotic BCL-2 proteins dictated cellular commitment to apoptosis.

The rheostat model became the foundation for our understanding of mammalian BCL-2 protein family function, and some aspects continue to be time-honored (this will be pointed out throughout our discussion). Nevertheless, it does not explain the more recently discovered complexities within the BCL-2 family. For example, how does a cell tolerate relatively high levels of BAX and/or BAK expression without these proteins being constitutively bound to anti-apoptotic members? How do certain BH3-only proteins engage BAX and/or BAK activation and MOMP whereas others do not? These issues were the core of several recent publications and continue to fuel controversies and discussions on how the BCL-2 family proteins engage mitochondrial cell death. Below, we discuss the two major competing hypotheses regarding the activation of BAX and BAK.

The anti-apoptotic protein neutralization model

Healthy cells express all classes of BCL-2 proteins, and this model proposes that anti-apoptotic proteins must continually inhibit the function of BAX and BAK to ensure mitochondrial integrity and survival. The signal for MOMP is the moment that all anti-apoptotic proteins are functionally neutralized (i.e. the hydrophobic binding groove of the anti-apoptotic protein is occupied by the BH3 domain of a BH3-only protein) by activated (either transcriptional or post-translational) BH3-only proteins. This indirectly promotes BAX and/or BAK liberation, homo-oligomerization into proteolipid pores, and cytochrome c release (this mechanism is also referred to as the 'indirect' model).

This model was primarily established by determining the affinities of BH3 domain peptides for the anti-apoptotic BCL-2 proteins. Once compiled, it was hypothesized what combination of BH3-only proteins were required for complete neutralization of anti-apoptotic proteins and MOMP [15–17]. For example, a hypothetical cell that expresses BCL-2 and MCL-1 would require a different combination of BH3-only proteins to engage MOMP than would another cell expressing BCL-2 and BCL-xL. The difference arises because several of the BH3-only proteins have a restricted ability to bind anti-apoptotic proteins (Figure 2b). BID, BIM and PUMA BH3 domain peptides can bind to all anti-apoptotic proteins [15–19]. By contrast, for example, BAD and Noxa BH3 domain peptides selectively bind BCL-2/BCL-xL/BCL-w and MCL-1/A1, respectively. The first type of hypothetical cell — that expressing BCL-2 and MCL-1 –would engage MOMP with BID, BIM or PUMA alone, or with a combination of BAD and Noxa. The second type of hypothetical cell – that expressing BCL-2 and BCL-xL–would not require Noxa, because BID, BIM, PUMA or BAD neutralize BCL-2 and BCL-xL, and all effector molecules should be free to induce MOMP. In all cases, it is assumed that the anti-apoptotic proteins equally inhibit BAX and BAK, and that neutralizing a subset of anti-apoptotic proteins causes the newly liberated effectors to quickly be inhibited by any available anti-apoptotic proteins.

The anti-apoptotic protein neutralization scenario is a modern interpretation of the rheostat model, because it is based on the hypothesis that the pro-apoptotic protein function overcomes inhibition by the anti-apoptotic proteins (Figure 2). Certainly, as the pro-apoptotic stimulation of a cell is converted into BH3-only protein activation and function, the amount of pro-apoptotic protein activity increases and MOMP ensues. One caveat of this hypothesis is that not all endogenous effector molecules appear to be actively sequestered by anti-apoptotic proteins [9,20–23]. Isolation of endogenous BAX and/or BAK does not generally stoichiometrically co-purify anti-apoptotic proteins (actually, in many cases, very little seems to be bound, although these interactions can be observed with non-ionic detergents [21]), which suggests that further changes in the effector molecules are required for their interaction or permeabilization activity. Also, the majority of data concerning the interactions between BH3-only protein and anti-apoptotic members is based on synthetic BH3 domain peptides and their interaction with immobilized, recombinant anti-apoptotic proteins by Bia-core analysis or overexpression [15,18]. Furthermore, several lines of evidence suggest that BH3-only proteins rarely bind effector proteins in the absence of a lipid environment and that unique binding surfaces are created by anti-apoptotic and pro-apoptotic protein interactions. Various examples exist: (i) caspase-8-cleaved BID does not readily bind anti-apoptotic BCL-2 proteins in solution (although the anti-apoptotic BCL-2 protein–BID BH3 domain peptide interaction is easily detected), but they functionally interact in the presence of mitochondria or lipid vesicles [24–26]; and (ii) BCL-2 proteins have been shown to bind to non-BCL-2 family members, and such interactions can be disrupted by only a specific BH3-only protein. For example, PUMA and BAD uniquely disrupt complexes between cytosolic p53 and BCL-xL or between Beclin-1 and BCL-2, respectively [27,28]. These examples hint that the calculated *in vitro* affinity between two

BCL-2 proteins does not directly translate into activity, because both environment (e.g. a cytoplasmic versus a hydrophobic membrane) and binding partners dictate the cellular response.

The direct activation of BAX and BAK model

The effector molecules BAX and BAK are essential for MOMP, because combined deletion renders cells resistant to multiple pro-apoptotic stimulation [29,30]. These proteins undergo marked conformational changes during apoptosis, through interactions with the outer mitochondrial membrane and BH3-only proteins, which are probably involved both in stable mitochondrial insertion of the effector and in proteolipid pore formation. This was first demonstrated with BID, which induced intramembrane oligomerization of BAK [31]. Using an inclusive panel of BH3 domain peptides, it was determined that only a subset of BH3 domains could directly induce conformational changes within BAX and BAK, and cytochrome c release [18,32]. These BH3-only proteins (i.e. BID and BIM) were classified as ‘direct activators’, and all other BH3-only proteins (e.g. BAD and Noxa) were termed ‘de-repressors’ or ‘sensitizers’, owing to their ability to increase mitochondrial sensitivity to BID and BIM (Figure 3cd). This division of BH3-only protein activity was recapitulated using recombinant proteins and large unilamellar vesicles (LUVs—A liposome comprised of defined mitochondrial lipids used to test the function of recombinant proteins in the absence of mitochondrial proteins. For example, the synergy of an effector molecule with a BH3-only protein, such as BAX and BID, on liposome permeabilization). This demonstrated that BAX-dependent pore formation could be induced by an interaction with LUVs and BID protein (and extended to the BID and BIM BH3 domain peptides) [18,25]. Likewise, an *in vitro* de-repression LUV system (LUVs in the presence of BID, BCL-xL, BAX and an additional BH3-only protein or BH3 domain peptide) validated the division of BH3-only protein function [18].

The key event necessary to engage MOMP in this scenario is the interaction between direct activator BH3-only proteins and the effector molecules (e.g. BID or BIM interacting with BAX or BAK) at the outer mitochondrial membrane [25,31]. Preventing this interaction is crucial for mitochondrial integrity and cellular survival, because recovery after MOMP is unusual. To ensure that BAX and/or BAK activation only occurs when appropriate, the cell expresses a variety of anti-apoptotic BCL-2 proteins that sequester the occasionally activated BID or BIM molecules. Under conditions of cellular stress and transformation, direct activator proteins are induced to engage BAX and/or BAK activation [19]. Yet a cell can overcome a death signal by sequestering the direct activator BH3-only proteins onto its repertoire of anti-apoptotic BCL-2 proteins (Figure 3c, often referred to as ‘primed for death’). Although this temporarily preserves cellular survival, it presents the cell with the duty of increasing anti-apoptotic proteins to counteract future stress-induced direct activator BH3-only protein expression – this challenge is also referred to as ‘BCL-2 addiction’ and is thought to occur during tumor initiation [19,33].

The cooperation between effector molecules and the direct activator BH3-only proteins has been described, but the affinity appears to be weak and is not readily detectable [25,31,34,35]. This mechanism is referred to as the ‘hit and run activation model of BAX and/or BAK’ and is substantiated by the LUV model system previously described. However, the weak association between BH3-only activators and effectors has led to speculation that the interaction is not essential for apoptosis to proceed and that the crucial step towards MOMP is in fact the robust interaction between BH3-only proteins and anti-apoptotic proteins. Consequently, mutant forms of BIM that fail to interact with BAX, yet maintain anti-apoptotic interactions, are potent inducers of MOMP, and their effects equal that of wild type BIM; furthermore, combined genetic deletion of *bid* and *bim* failed to yield a major

apoptotic phenotype comparable to *bax*$^{-/-}$;*bak*$^{-/-}$ animals, suggesting that these molecules are not the sole mediators of MOMP [17].

Taken together, there are several points for important consideration. First, although the interaction between effector molecules and direct activators can be weak *in vitro*, the conditions in which this occurs in the cell are not well understood and their affinity might be higher *in vivo*. This leads one to question what structural and cellular components are essential for effector-direct activator BH3-only functional association; for example, perhaps a component(s) of the outer mitochondrial membrane. Also, are there essential residues outside of the direct activator BH3 domain that might facilitate the interaction? Second, there might be additional direct activator proteins that regulate BAX and/or BAK activation; this might explain the lack of a major apoptotic phenotype in the *bid*$^{-/-}$; *bim*$^{-/-}$ animals. There are already several non-BCL-2 family proteins known to regulate MOMP. These include cytosolic p53, Nur77, and ATG5. Very little is known about how these proteins interact within the BCL-2 network [27,36–38]. Understanding the biochemical and structural requirements for effector molecule and direct activator protein interactions within the cell will help to solve these fundamental issues.

How much controversy is there?

To begin addressing this question, let us first discuss this in the context of a simplified cellular BCL-2 network comprising only one protein for each subtype – anti-apoptotic, effector and BH3-only. For example, a hypothetical cell that only expresses BCL-xL, BAX and BID is treated with a death receptor agonist that promotes BID cleavage and activation. Where in this restricted BCL-2 network is the essential BID interaction? Is it between BID and BAX or between BID and BCL-xL? First, we must determine what, if any, interaction is present before BID cleavage. This is fundamental to understanding how the effector molecules behave before BH3-only protein induction, and how the anti-apoptotic BCL-2 proteins preserve mitochondrial integrity. Let us consider that BAX is indeed constitutively active and must always be inhibited by BCL-xL; under this hypothesis, as molecules of cleaved BID accumulate, they will displace BAX from BCL-xL to promote MOMP. (It should be noted that this will only occur if physiological levels of cleaved BID can displace BAX, which is currently unknown). This scenario is complementary to the rheostat and anti-apoptotic protein neutralization models; pro-apoptotic activity overcomes anti-apoptotic function and MOMP ensues. On the contrary, the direct activation model would contend that BCL-xL and BAX do not constitutively interact; but, as cleaved molecules of BID accumulate, these can either induce BAX activation or become inhibited by BCL-xL. Eventually, BCL-xL will become saturated with cleaved BID, and it will be able to activate BAX at the mitochondrial membrane, because further BID sequestration is not possible. This also highlights the sensitizer function of BH3-only proteins, which is possible with any BH3-only peptide or, in theory, protein. In this situation, BH3-only proteins inhibit cellular anti-apoptotic proteins, and any further direct activator BH3-only activity is free to induce MOMP (Figure 3d) [18,19,32]. Of particular note, if the actively sequestered BH3-only protein is a direct activator, then further sensitizer/de-repressor BH3-only expression will displace the direct activator and promote MOMP (Figure 3c). This is referred to as ‘primed for death’ – the death signal is actively inhibited but easily revealed with additional de-repressor proteins (e.g. PUMA) or BH3-only mimetics [e.g. ABT-737 (Abbott compound 737)] [19,27,33,39]. This scenario is highlighted by recent reports of leukemia cells actively sequestering BIM, which can be induced to undergo BIM-dependent MOMP by a combination of ABT-737 treatment (to inhibit BCL-2, BCL-xL and BCL-w) and MCL-1 downregulation. This combination is necessary because ABT-737 can release BIM from BCL-2, BCL-xL and BCL-w and forces a BIM–MCL-1 interaction to preserve survival [19,33,40,41].

The use of ABT-737 in cellular and animal models of tumor initiation and treatment has suggested that targeting the BCL-2 family of proteins is an attractive chemotherapeutic modality; however, it has not clarified the molecular mechanisms of these proteins [39]. Long-term systemic administration of ABT-737 produced no pathophysiologic consequences except for enhanced platelet-depletion, suggesting that generalized BCL-2-, BCL-xL- and BCL-w-dependent sequestration of effector molecules is not present in primary tissues [42]. In the case of platelets, an interaction between BCL-xL and BAK was suggested to be crucial to maintain cellular survival, as disruption of this complex by ABT-737 promoted MOMP (akin to the rheostat and anti-apoptotic protein neutralization models) [42]. One point that has not been addressed, but which the direct activation model implies, is that ABT-737 might displace a covert direct activator protein to promote BAK-dependent MOMP. Another pharmacological inhibitor of the anti-apoptotic proteins, TW-37, is suggested to neutralize all members. However it does not induce MOMP in the absence of additional pro-apoptotic stimulation (which probably yields direct activator BH3-only protein expression and/or activation) [43]. Fundamentally, it is becoming clear that uncovering the molecular behavior – constitutively active or not – of the effector molecules in the absence of BH3-only proteins will yield a greater understanding of the entire BCL-2 family; do anti-apoptotic BCL-2 proteins inhibit MOMP by blocking BH3-only proteins or effector function? Do BH3-only proteins engage MOMP by neutralizing anti-apoptotic proteins or by activating BAX and BAK? Intriguingly, are the requirements the same for every cell and do they change during a cell's lifespan?

Do these distinctions matter? Considering the prospects for the use of BH3 mimetic drugs in the treatment of neoplasia, the distinction might have life or death consequences. If apoptosis versus cellular survival depends on the extent to which anti-apoptotic BCL-2 family members are neutralized, then tumor cells should be more resistant to such drugs than their normal counterparts are, because tumors tend to have increased expression of anti-apoptotic BCL-2 proteins [44,45]. By contrast, if the direct activator model is correct, then there might exist a therapeutic window within which some tumors are more sensitive to apoptosis induction than are healthy cells, owing to a tumor cell's elevated levels of direct activators. As noted above, early studies with ABT-737 and TW-37 suggest the existence of such a window – at least for some tumor types. However, if the direct activators are limited to BID and BIM, as suggested [17,32], why are $bid^{-/-}$; $bim^{-/-}$ cells sensitive to MOMP and apoptosis? Will only tumors expressing BID and/or BIM be sensitive to BH3-mimetics?

Part of the difficulty in addressing these issues is our reliance on the assertion that only BID and BIM are able to activate BAX and BAK. It remains possible that other members of the BH3-only subfamily can perform this function. For example, it has been suggested that PUMA can be a direct activator of BAK, although whether or not this can happen at physiological concentrations is unknown [46]. It has also been suggested that BCL-2 interacts with the non-BCL-2 protein Nur77 and can, in turn, activate BAX and BAK [37]. Therefore, it is possible that non-BCL-2 proteins can function as direct activators of BAX and/or BAK. For example, cytosolic p53 was shown to activate BAX in an LUV system, and the participation of cytosolic p53 in a de-repressor/direct activator pathway has been demonstrated [27,36]. Finally, BAX and BAK can be activated under conditions that do not involve additional proteins, including detergent treatment and heat, and it has been suggested that alkaline pH can do this as well [47–49]. It is not known whether or not such activation of BAX and BAK occurs physiologically. Nevertheless, these observations make it clear that alternatives to BID and BIM as the only direct activators of BAX and BAK certainly exist. Another assumption is that all direct activator proteins share similar requirements and mechanisms to engage effector molecules, and, to date, only one study has begun to address potential differences between BID and BIM function [50].

Conclusion

In summary, we contend that the BH3-only proteins serve as both inhibitors to the anti-apoptotic proteins and direct activators of effector molecules, as suggested by the direct activator model. Aspects of the anti-apoptotic protein neutralization model must indeed be considered, because BH3-only proteins bind to anti-apoptotic BCL-2 members. But, in our opinion, solid evidence is still lacking to conclude that this is both necessary and sufficient to engage MOMP. Furthermore, as cellular direct activator function is generated, these proteins have the opportunity to either be absorbed by the anti-apoptotic proteins or engage BAX and/or BAK activation and MOMP. We suggest that identifying how the entire BCL-2 network interacts within a cell (e.g. which members of each BCL-2 subset are expressed? Which interactions do other members influence? What non-BCL-2 proteins influence BAX and/or BAK activation? See Box 1 for pertinent future investigations) to either promote survival or engage MOMP will be the next breakthrough in elucidating both BCL-2 protein family function and the mitochondrial pathway of apoptosis.

Box 1

Outstanding questions

1. Will our understanding of the BH3-only proteins, as determined by synthetic BH3 domain peptides, be corroborated following full-length BH3-only protein analyses? Or have the BH3 domain peptides yielded data (e.g. protein–protein interactions and protein function) that do not hold true for the intact proteins?
2. The BCL-2 effector proteins promote MOMP in the presence of direct activator proteins and a hydrophobic component (e.g. outer mitochondrial membrane or LUVs). What is the appropriate cellular milieu for BAX and/or BAK activation? Do these proteins activate before or after membrane insertion (i.e. does a BH3 stabilize insertion or activation)? Leber *et al.* discuss these issues at length [4].
3. Can the pharmacological modulation of BCL-2 family proteins to selectively promote MOMP in aberrant cells, while preserving healthy cell survival, be effectively applied as a therapeutic treatment for conditions such as cancer and inflammatory diseases?

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Glossary

Anti-apoptotic protein neutralization

Commitment to MOMP occurs when the cellular repertoire of anti-apoptotic BCL-2 proteins is fully inhibited by BH3-only proteins or peptides. This interaction promotes MOMP by blocking the constitutive interaction between the anti-apoptotic proteins and the active effector molecules. The BH3-only proteins BID, BIM and PUMA engage MOMP because they bind and neutralize all anti-apoptotic BCL-2 members. A combination of other BH3-only proteins is required to promote apoptosis, because each neutralizes only a subset of anti-apoptotic BCL-2 proteins; for example, BAD

	and Noxa neutralize BCL-2/BCL-xL/BCL-w and MCL-1/A1, respectively
BAX and BAK activation	This phrase is used throughout the BCL-2 literature, but there is very little understanding of what it actually defines. Activation is often associated with stable membrane insertion, oligomerization and cytochrome c release. Re-organization of the BAX molecule, such as amino terminal exposure (assayed by the association with an antibody that specifically recognizes amino terminal BAX residues; commonly referred to as clone '6A7 positive' BAX [21]) and several internal alpha helical rearrangements occur. Non-ionic detergents or heat can induce the activation phenotype in BAX and BAK, but association with a direct activator BH3-only protein does not seem to be sufficient. There is a hydrophobic requirement for a BH3-only protein to induce activation ; in cells, the outer mitochondrial membrane probably satisfies this requisite. To date, there is also no concrete evidence that any one feature of BAX and/or BAK activation produces pore-forming activity, or whether these phenotypes are consequences of an active molecule(s)
BCL-2 effector proteins	The proteins BAX and BAK are the pro-apoptotic 'effector' molecules of the BCL-2 family because they actively permeabilize the outer mitochondrial membrane and promote the release of intermembrane space proteins. In the direct activator model, these proteins activate and permeabilize in response to direct activator BH3-only protein stimulation. In the anti-apoptotic protein neutralization model, BAX and BAK are hypothesized to be constitutively active and sequestered, because they only require release from an anti-apoptotic BCL-2 protein to engage MOMP
De-repressor/ sensitizer BH3-only proteins	A subset of the BH3-only proteins (e.g., BAD, BIK, BMF, bNIP3, HRK, Noxa and PUMA) that can bind anti-apoptotic BCL-2 proteins, but which do not directly activate BAX or BAK. These proteins can displace direct activators from anti-apoptotic BCL-2 proteins, thus promoting MOMP (e.g. BID can be released from BCL-xL by PUMA, referred to as 'de-repressing' BID-induced MOMP). Also, these proteins can sensitize isolated mitochondria and cells to low levels of direct activator proteins. For example, a cell that expresses BAD (or which was transfected with BAD protein or BH3 peptide) is more sensitive to BID-induced MOMP because the BAD will bind and inhibit anti-apoptotic proteins from sequestering BID
Direct activator BH3-only proteins	A subset of the BH3-only proteins (e.g. BID and BIM) that transiently interact with a BAX and BAK to induce their activation. The cellular requirements for this association are not well defined, but a hydrophobic component – perhaps the outer mitochondrial membrane – is necessary. The direct activator proteins also bind and neutralize anti-apoptotic BCL-2 proteins, which subsequently inhibits their direct activator function until further BH3 stimulation (such as induction of a de-repressor/sensitizer BH3-only protein)

Mitochondrial pathway of apoptosis	A form of apoptosis that is engaged following cellular stress, such as DNA-damage or nutrient deprivation, and is inhibited by anti-apoptotic BCL-2 proteins. In this pathway, mitochondria release pro-apoptotic proteins (e.g. cytochrome c) that are required for the activation of caspases, thereby triggering cellular disassembly and clearance
Mitochondrial outer membrane permeabilization (MOMP)	This event occurs immediately downstream of BAX and/or BAK activation and is responsible for the release of intermembrane space proteins, such as cytochrome c, SMAC/Diablo, EndoG, Omi/HtrA2 and AIF (but any protein that resides between the outer mitochondrial membrane and IMM can be released, depending on its membrane association and solubility). Cytosolic proteins also gain access to the intermembrane space and alter mitochondrial function; for example, cytochrome c activated caspases can cleave intermembrane space proteins, which negatively impacts on ATP production [51]. Importantly, the anti-apoptotic BCL-2 proteins inhibit MOMP

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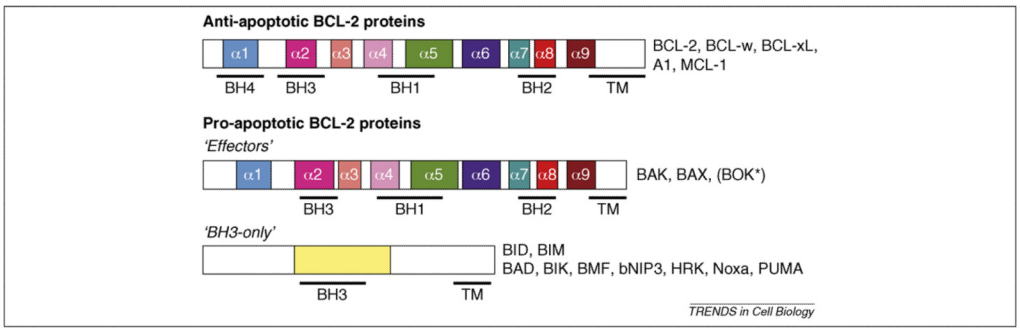

Figure 1.

The BCL-2 family of proteins is divided into three functional groups based on their composition of BCL-2 homology domains. The anti-apoptotic members include BCL-2, BCL-xL, BCL-w, A1 and MCL-1 and contain four BCL-2 homology domains (designated BH1–4). The pro-apoptotic multi-domains (BAX, BAK and BOK*) contain BH1–3 domains (*there is little evidence that BOK is a functional effector molecule). The BH3-only proteins are structurally diverse and contain only one conserved region, the BH3 (e.g. leucine-x-x-x-x-aspartic acid, where x is any amino acid). Often, the BH3-only proteins are subdivided into direct activators (e.g. BID an d BIM) and de-repressors/sensitizers (e.g. BAD, BIK, BMF, bNIP3, HRK, Noxa and PUMA). The α helices of each protein are designated and the regions contained within each BH domain are illustrated by bold lines under each protein. The hydrophobic carboxyl terminal transmembrane domain (TM) of each protein is based on *in silico* predictions and/or structural data and is not necessarily present in each member. Also, a typical BH3 domain might not be absolutely required for every BH3-only protein to induce cell death; for example, deletion of the BH3 domain in bNIP3 does not alter its anti-apoptotic binding or pro-apoptotic activity [52].

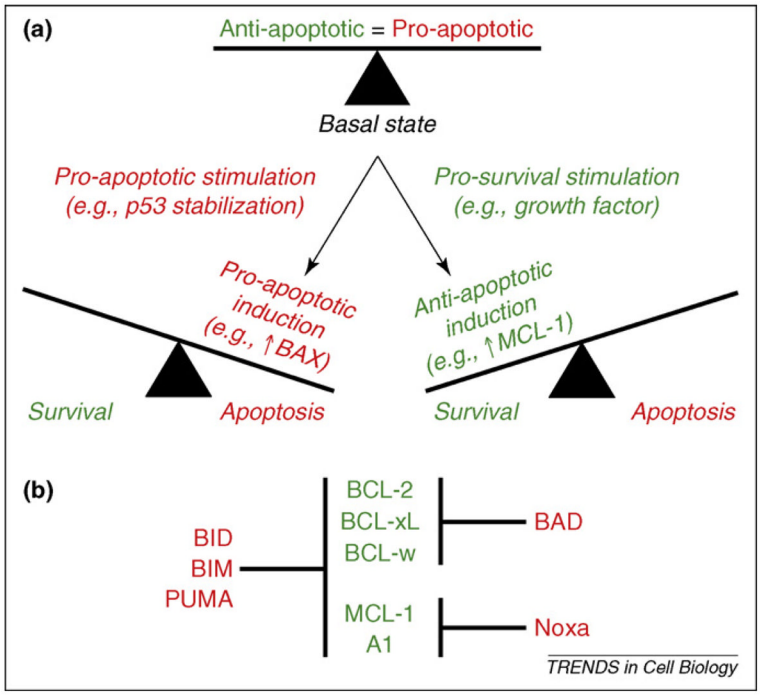

Figure 2.

The balance of anti-apoptotic and pro-apoptotic BCL-2 proteins dictates cellular fate. **(a)** The rheostat model. In a hypothetical basal state, the number of anti-apoptotic and pro-apoptotic molecules is equal; tipping this balance dictates cellular fate. If a stress (e.g. DNA damage) is applied, the induction of pro-apoptotic molecules provides the signal to engage MOMP. On the contrary, growth factor addition would promote cellular survival by increasing the amount of anti-apoptotic proteins. **(b)** The anti-apoptotic protein neutralization model. The BH3-only proteins BID, BIM and PUMA engage MOMP because they bind and neutralize all anti-apoptotic BCL-2 members. A combination of other BH3-only proteins is required to promote apoptosis because each neutralizes only a subset of anti-apoptotic BCL-2 proteins (e.g. BAD and Noxa neutralize BCL-2/BCL-xL/BCL-w and MCL-1/A1, respectively). This model contends that the BCL-2 effector molecules are sufficiently active to oligomerize and promote MOMP once anti-apoptotic proteins are neutralized.

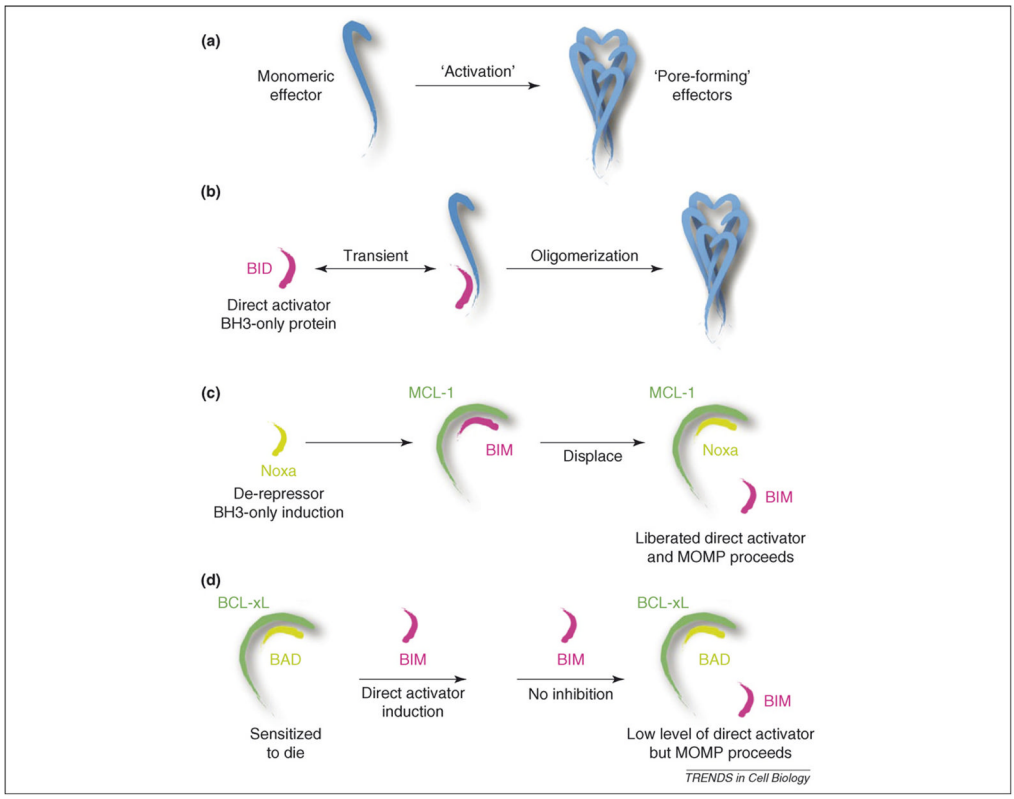

Figure 3.

The direct activation model: mechanisms of pro-apoptotic effector activation by BH3-only proteins. **(a)** Central to MOMP is the activation and oligomerization of BAX or BAK. These proteins, once activated by a BH3-only protein, create oligomeric proteolipid pores in the outer mitochondrial membrane that permit the release of intermembrane space proteins to the cytosol. **(b)** Direct activator BH3-only proteins (e.g. BIM and BID) induce the oligomerization and activation of BAX or BAK in the absence of other proteins. Through a transient interaction with BAX or BAK, the direct activator BH3-only proteins (BID is shown in this example), or peptides derived from the BH3 region, induce MOMP and cytochrome c release. This is often referred to as the 'hit and run' mechanism for effector activation. **(c)** A subset of BH3-only proteins, the de-repressors/sensitizers, cannot induce the activation of BAX or BAK alone. In this scenario, a direct activator BH3-only protein is sequestered by an anti-apoptotic BCL-2 protein. Following stress, a de-repressor/sensitizer BH3-only protein is induced, either by transcriptional up-regulation or by post-translat ional modification, and this protein then binds to an anti-apoptotic BCL-2 protein, promoting the release of a sequestered, direct activator BH3-only protein. In this example, B IM istonically sequestered by MCL-1, and the induction of Noxa enables the release of BIM to engage MOMP. If cells constitutively harbor a sequestered direct activator protein, they are referred to as being 'primed for death' or 'BCL-2 addicted'. Not shown in this figure is the potential influence of Noxa-induced MCL-1 degradation after binding, which might have important implications in maintaining anti-apoptotic levels to preserve outer mitochondrial membrane integrity [53]. **(d)** Cells are sensitized to undergo MOMP when de-repressor/sensitizer BH3-only proteins are constitutively inhibiting anti-apoptotic BCL-2 proteins, and any future induction of BID or BIM cannot be tolerated. This scenario is referred to as 'sensitized for death'. In this example, BCL-xL is inhibited by BAD, and the induction of BIM engages MOMP; in the absence of B AD expression, the MOMP signal would have been inhibited by BCL-xL.