

## Mechanisms of Bax/Bak activation: Is there any light at the end of the tunnel?

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### ABSTRACT

In this review we summarize the current knowledge on the mechanisms by which the pore-forming Bcl-2 family members Bax and Bak are activated on the mitochondrial outer membrane (MOM) in apoptotic cells. We compare the two major working models and conclude that they are both insufficient to explain reality. Instead, we need to identify novel binding partners and posttranslational modifications of Bcl-2 family proteins to fully understand their *modi operandi* in healthy and apoptotic cells.

**Keywords:** Bax/Bak activation, Bcl-2, apoptosis.

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### INTRODUCTION

Programmed cell death, or apoptosis, is a crucial process for the proper embryonic development and tissue homeostasis of multicellular organisms. Too little cell death can lead to autoimmune diseases, arthritis and cancer, whereas excessive cell death may provoke degenerative diseases of the heart and the immune and nervous systems. Knowing the exact molecular mechanism of apoptosis will therefore not only help to understand organogenesis and tissue turnover, but will provide us with better tools to therapeutically interfere with many debilitating human diseases.

Apoptosis was first molecularly defined by studying programmed cell death during the development of the model organism *Caenorhabditis elegans* (1). In living cells, a

survival factor called CED-9 holds an ATPase, CED-4, in check and thereby prevents the latter from activating the cysteine aspartic acid protease (caspase) CED-3 (2-7). At a defined point during development when cell death is launched, a protein called EGL-1 is transcriptionally induced (8). Through high affinity binding to CED-9, EGL-1 allosterically releases CED-4 which in turn activates the effector caspase CED-3 leading to the proteolytic dismantling of the cell (9-11). Subsequently, it was found that CED-9 shared homology with mammalian Bcl-2 (12), the first oncogene identified to provoke enhanced survival instead of mitogenesis (13), and which was highly overexpressed in follicular lymphoma due to a t(14;18) chromosomal translocation (14). However, in contrast to nematodes, mammalian Bcl-2 belongs to a large family of both pro- and anti-apoptotic regulators which do not simply inhibit and activate CED-3-like caspases via the binding and release of a CED-4-like ATPase

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(15,16). Instead, during evolution to higher eukaryotes, mitochondria have been placed between Bcl-2 proteins and the activation of caspases because it turned out that to activate caspases, the mammalian CED-4 homolog Apaf-1 requires mitochondrial cytochrome c (17,18). The release of cytochrome c from the intermembrane space of mitochondria into the cytoplasm is controlled by Bcl-2 family members. Just how exactly these family proteins perform their task has remained enigmatic up to date.

### **The Bcl-2 family**

The Bcl-2 family can be divided into pro- and anti-apoptotic proteins (15,16). They contain one or more homologous Bcl-2 homology (BH) domains, which are important for homo- and heterodimeric interactions among family members. The anti-apoptotic members Bcl-2, Bcl-x<sub>L</sub>, Mcl-1 and A1 contain BH domains 1-4. Structurally, these domains form a hydrophobic binding groove into which the BH3-domain of another family member (basically an extended alpha helix) binds with high affinity (19). An attractive class of binding partners to this groove are the so-called BH3-only proteins. As their name says, they contain only a BH3 domain. As seen for nematodal EGL-1, which is the archetype of this subgroup, BH3-only proteins act as upstream sentinels of cellular damage and derangement (15,16). They can be activated by many apoptotic stimuli - including the lack of growth factors, cell-cell or cell-matrix interactions or the exposure to DNA damaging chemodrugs or irradiation, ER stress agents, viruses, bacteria or TNF-like cytokines - via transcriptional upregulation (like EGL-1), changes in subcellular localization, altered protein stability or posttranslational modifications. For example, Puma and Noxa are transcriptionally induced by p53 in response to DNA damage (20,21), Bim is transcriptionally induced by ER stress via the

transcription factor CHOP (22) or by growth factor deprivation via FOXO transcription factors (23). Additionally, the protein levels of Bim are controlled by JNK (to stabilize it in apoptotic cells) (24) and ERK1/2 (to destabilize it in surviving cells) (25). Bid is activated by caspase-8-mediated cleavage to form tBid in response to TNF-like cytokines (26) whereas Bad is activated by dephosphorylation in response to growth factor deprivation (27). If anti-apoptotic Bcl-2 proteins are highly abundant in a cell (as is often the case in chemoresistant cancer cells), the BH3-only sentinels are effectively sequestered to their hydrophobic binding pocket and thereby inhibited in their pro-apoptotic action (15,16). This reveals one mechanism by which anti-apoptotic Bcl-2 proteins can act as potent survival factors. But how do BH3-only proteins induce apoptosis under normal conditions, i.e. when the expression of Bcl-2 survival factors is low? It turns out that for this effector function another pro-apoptotic subclass of Bcl-2 family members is needed, namely Bax and Bak (15,16). These two proteins are responsible for the increased mitochondrial outer membrane permeability (MOMP) and the subsequent release of cytochrome c. This is absolutely essential for apoptosis as Bax/Bak double knockout mice are embryonic lethal, and cells from these mice are highly resistant to apoptosis by all stimuli which trigger the mitochondrial death signaling pathway (28,29). So it seems that Bax/Bak activation is a crucial, if not the most important commitment step for apoptosis induction in mammalian cells. Yet despite intense research for the last two decades, we still have no clue as to how the BH3-only sentinels trigger Bax/Bak activation. Why has this been so difficult to achieve?

Bax is merely a cytoplasmic protein in healthy cells and needs translocation to mitochondria to become a pro-apoptotic, pore forming factor (30). Although about a dozen inhibitory binding partners of Bax have been published (15,16), most

of them have not withstood the acid test to act as such in viable cells (S. Vogel, unpublished). The structure of Bax closely resembles that of Bcl-2 and Bcl-x<sub>L</sub> although it contains only three BH domains (BH1-3) (19,31). These domains also form a hydrophobic binding pocket to which BH3 peptides could theoretically bind. In healthy cells, Bax is kept in the cytoplasm by having its C-terminal mitochondrial targeting region tucked into its own hydrophobic pocket (31,32). At least in eosinophils, we have recently shown that this conformation is stabilized by the binding of the peptidyl-prolyl isomerase Pin1 to Pro168, an essential, surface exposed residue just in front of the C-terminal targeting sequence (33). Pin1 binding requires the phosphorylation of Thr167 by ERK/MAPK, a protein kinase activated in healthy, surviving eosinophils. In response to apoptotic stimuli, both the C-terminal and the N-terminal parts of Bax unfold (34). While the N-terminal part becomes accessible to a conformation-specific antibody called 6A7, and therefore serves as a marker for Bax activation, the C-terminal part is hydrophobic and inserts into the MOM. It is yet unknown if posttranslational modifications or the binding of specific pro-apoptotic proteins such as BH3-onlies trigger the N-/C-terminal unfolding and conformational change of Bax during apoptosis. In eosinophils, we assume that dephosphorylation of Thr167 in apoptotic cells releases Pin1, thereby allowing the C-terminus to disengage for mitochondrial targeting.

In contrast to Bax, Bak is constitutively inserted into the MOM (35). It therefore does not require unfolding of its hydrophobic C-terminus but still shows N-terminal exposure upon activation. For both Bax and Bak, N-terminal changes are thought to be a prerequisite for their oligomerization and their capacity to form pores in the MOM (30,34). Bak is thought to be kept inactive by its interaction with VDAC-2 (36) or anti-apoptotic members of the Bcl-2 family (15,16). Indeed, initially Bax and Bak were

isolated as Bcl-2 binding partners in co-immunoprecipitates (15,16,37). Later it was shown by the group of R. Youle that the interactions between Bcl-2 and Bax/Bak were partially artificial because they mainly occurred in the presence of the non-ionic detergent Triton-X-100, the agent required to solubilize membrane-bound Bcl-2 (38). Unfortunately, this detergent triggers a similar conformational change in Bax (and also Bak) as seen upon their activation in apoptotic cells. As recently shown for Bak, the conformational change does not only unfold N- and C-terminal parts but also exposes the BH3-domain of Bak (revealed by a specific anti-BH3 Bak antibody) (39). This leads to Bak and Bax oligomerization, most likely due to multiple BH3:groove interactions, but also allows the binding of Bax and Bak to the hydrophobic groove of anti-apoptotic Bcl-2 proteins. This reflects a second mechanism by which these latter proteins can act as potent survival factors, namely by sequestering structurally altered Bax/Bak during apoptosis and thereby preventing their oligomerization and pore formation.

But are Bax and Bak also kept in check by anti-apoptotic Bcl-2 proteins in normal cells which typically contain low levels of Bcl-2, Bcl-x<sub>L</sub>, Mcl-1, Bcl-w or A1? We and others have shown that Bak can indeed be co-immunoprecipitated with Bcl-x<sub>L</sub> and Mcl-1 (and in some cells also with Bcl-2) under endogenous conditions from healthy mitochondrial extracts in the presence of CHAPS or digitonin (40,41), both detergents which do not trigger a conformational change in Bax and Bak. However, the amount of Bak that interacted with Bcl-2 survival factors was very low (40) and the interaction was quite weak (i.e. it can be abolished by high salt washes in co-immunoprecipitations, T. Kiefer, unpublished) indicating that most of the Bak was bound to other cellular proteins in healthy cells. Indeed, gel filtration chromatography and blue native gels revealed that Bak is already part of high molecular

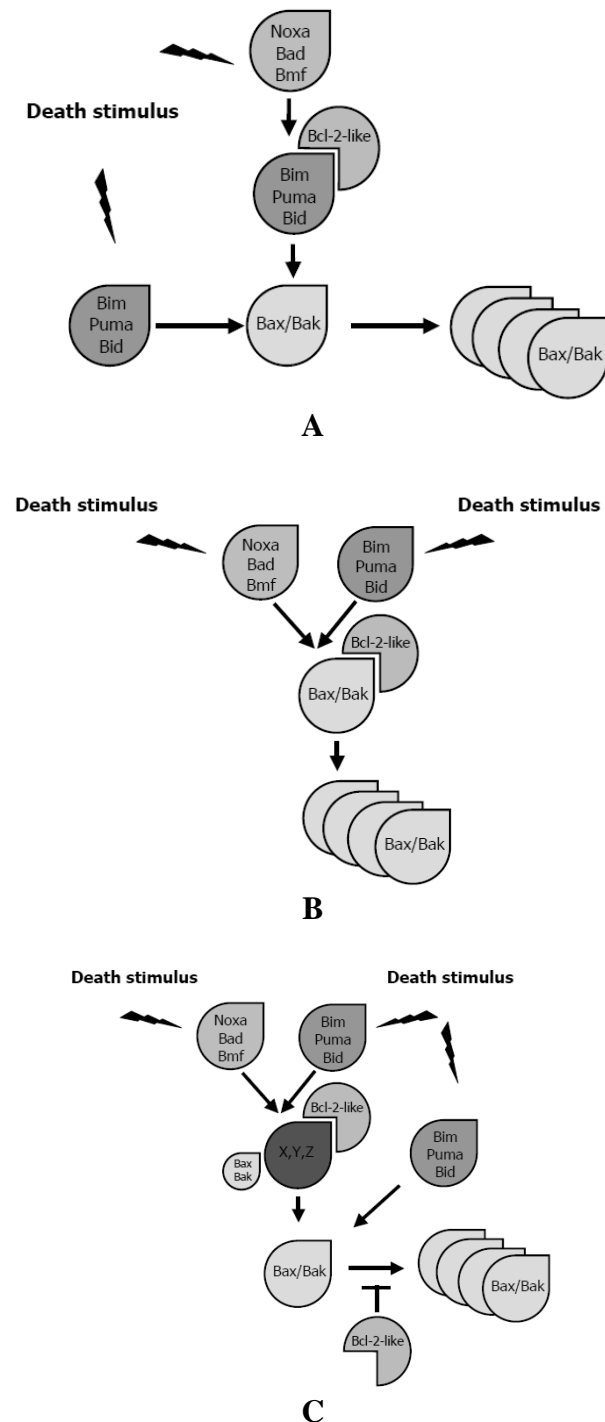
mass complexes in healthy cells and only a few Bak complexes were found in the molecular weight range of Bak/Bcl-2, Bak/Bcl-x<sub>L</sub> or Bak/Mcl-1 dimers (50-70 kD) (T. Kiefer and N. Raulf, unpublished). We therefore propose that Bak is bound to and inhibited by either a large protein, several small proteins or a mitochondrial platform of oligomerized or dimerized proteins. The situation with Bax is even more complex. Its cytosolic form cannot interact with anti-apoptotic Bcl-2 proteins because some of them, like Bcl-2, are never cytosolic (42). Moreover, the binding groove and the BH3 domain of Bax are not accessible or exposed for binding to Bcl-2 survival factors. In some established cell lines, Bax is already found loosely attached or even inserted in the MOM (S. Vogel, unpublished). This has never been seen in primary cells or cells/tissues *in vivo* (43) and most likely reflects an adaptation to cell culture conditions. This form of membrane associated Bax is still inactive and does not have its N-terminus exposed. It is therefore unlikely that it has its BH3-domain accessible for Bcl-2 binding. Indeed, we and others have not succeeded to co-immunoprecipitate Bax with Bcl-2, Bcl-x<sub>L</sub> or Mcl-1 in cytosolic or CHAPS/digitonin solubilized membrane fractions under endogenous conditions (40,41,44) (S. Vogel and A. Manoharan, unpublished). We therefore propose that endogenous, inactive Bax and Bak do not (or only minorly) interact with Bcl-2-like survival factors under healthy conditions. Only in response to apoptotic stimuli when both undergo a conformational change, they may bind to Bcl-2 survival proteins via a BH3-pocket interaction until the latter are saturated. The rest of Bax/Bak oligomerizes and triggers MOMP. Our view calls for the identification of novel Bax and Bak binding partners and posttranslational modifications in order to understand how these two proteins are kept inactive via Bcl-2 protein independent mechanisms.

## Two highly debated models on how Bax/Bak are activated

Two models of Bax/Bak activation have been proposed. In the „direct activation“ model, some BH3-only proteins such as Bim, Bid and Puma directly interact with and induce conformational changes in Bax and Bak (Fig. 1A) (45-49).

A direct interaction between endogenous Bim, Bid or Puma with Bax/Bak has been difficult to detect biochemically, probably due to the transient nature of this interaction (a so-called „hit-and-run“ mechanism). However, studies using overexpressed full-length proteins, lipid membrane and real-time fluorescence resonance energy transfer (FRET) analysis have provided some evidence of such an interaction and its role in MOMP (50). In addition, very recent results proposed a novel binding site between a stapled BH3-domain of Bim and Bax, which was not the hydrophobic binding pocket but rather an area distal from it (49). Unfortunately, the structure of this complex has not been determined *de novo*, but only modeled from NMR data. Moreover, it is unclear if a stapled BH3-domain reflects the mode of action of a natural BH3-domain in the context of the entire Bim protein under physiological conditions. In the „direct activation“ model, BH3-only proteins are divided into activators (Bim, Bid, Puma) and sensitizers (the rest) (52,53). Sensitizers cannot activate Bax and Bak directly, but bind anti-apoptotic Bcl-2 proteins with high affinity (although Bim, Bid and Puma can do so as well). One idea is that once these sensitizers are produced/activated in apoptotic cells, they cause the release of activator BH3-only proteins from Bcl-2 survival factors, leading to activation of Bax and Bak (Fig. 1A). The problem with this model is that in most normal cells, the levels of Bim, Bid and Puma are too low to detect their interaction with Bcl-2 survival factors. An exception might be cancer cells where these proteins have been found to be elevated and to be sequestered by high levels

of Bcl-2, a mechanism which the group of A. Letai called „addiction“ (53).



**Figure 1.** Models of Bax/Bak activation. A: Direct activation model; B: Displacement model; C: Our model

How widespread this mechanism is has yet to be shown. It is certainly not present in most normal, untransformed cells. Another caveat of this model is that a combined knock-out of Bid and Bim together with a downregulation of PUMA by siRNA resulted in relatively minor defects in apoptosis (54). Therefore, in addition to Bim, Bid and Puma, other Bax/Bak activators have to exist, which may not even be members of the Bcl-2 family. One scenario could be that anti-apoptotic Bcl-2 proteins do not have BH3-only sensitizers bound in normal cells but so far unknown Bax/Bak activators (Fig. 1C). These activators are then displaced by any BH3-only protein bound to the pocket of Bcl-2 survival factors upon apoptosis. Indeed, our preliminary data indicate that Bcl-2 is part of high molecular mass complexes (> 150 kD) that are higher than those predicted from interactions of Bcl-2 with BH3-only proteins and/or Bax/Bak (T. Kiefer, N. Raulf unpublished).

The second model, called the „displacement“ model is based on published data showing interactions between anti-apoptotic Bcl-2 proteins and Bax/Bak (40,41). In this scenario, Bax and Bak are held in check by Bcl-2, Bcl-x<sub>L</sub>, Bcl-w, Mcl-1 or A1 until BH3-only proteins displace them by binding to the hydrophobic pocket of the Bcl-2 survival factors (Fig. 1B). Different BH3-only proteins exert different affinities/specificities for Bcl-2, Bcl-x<sub>L</sub>, Bcl-w, Mcl-1 and A1 (55), but in principle all would work in the same way. Again, there are a couple of problems with this model. As discussed above, although there is no doubt that active, conformationally changed Bax and Bak can interact with and be neutralized by Bcl-2 survival factors in apoptotic cells, it is difficult to imagine that such interactions occur in healthy cells due to different subcellular localizations and structural constraints between Bax/Bak and the Bcl-2 survival proteins. A structure of a dimer between Bax/Bak and any

anti-apoptotic Bcl-2 protein has not yet been solved. Thus, we do not know if they interact via the hydrophobic pocket (BH3 peptide of Bax/Bak nestled into the binding pocket of Bcl-2) or via other surface areas. Moreover, although we and others found low amounts of Bak co-precipitating with Bcl-2 survival proteins (40,41), most of the Bak was bound to other proteins which make up a rather high molecular weight complex (T. Kiefer, unpublished). Thus, both the „direct activation“ and the „displacement“ model seem to only partially reflect reality.

### **What is the real mechanism of Bax/Bak activation? Do we need new/other models to see the light at the end of the tunnel?**

Based on published data by us and others, we agree that BH3-only proteins sensitize apoptotic signals and most likely act by binding to the hydrophobic groove of Bcl-2 survival factors in apoptotic cells with different affinities/specificities. Some such as Puma, Bim or Bid may also directly activate Bax and Bak. However, we do not think that the major role of BH3-only proteins is to displace Bax and Bak from anti-apoptotic Bcl-2 proteins. Rather, we propose that other, yet unknown proteins (X, Y, Z) are bound to the survival factors in healthy cells and are then freed upon BH3-only binding in apoptotic cells (Fig. 1C). These proteins may contribute to Bax/Bak activation, but could also trigger other cellular processes regulated by Bcl-2 family proteins such as autophagy, DNA recombination, cell cycle, etc. Importantly, we invoke that Bax/Bak activators do not necessarily have to physically interact with Bax and/or Bak. They may also change the lipid microenvironment or some other crucial component on the MOM in order to facilitate the membrane insertion and oligomerization of Bax and Bak. We hope that the real mechanisms of Bax/Bak activation will soon

be discovered through novel proteomics and genetic screens, so that we can leave the darkness of this mysterious topic.

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