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Review

BH3-Only Proteins: the Lords of Death

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Abstract. Although the mechanisms by which Bcl-2 family proteins control the apoptotic machinery of the cell are not fully understood, it becomes clear that the role of BH3-only proteins consists in serving as sensors or sentinels of cellular damage, transducing the apoptotic stimuli to the mitochondria. For this reason, mammalian cells have developed several strategies for their strict regulation throughout evolution. This review aims to highlight the different ways by which BH3-only proteins are controlled, including transcriptional regulation, post-translational modifications and subcellular localization.

Key words: apoptosis; Bcl-2 family; BH3-only proteins; transcriptional regulation; post-translational modifications.

Introduction

Apoptosis, or programmed cell death (PCD), is essential for a wide variety of normal processes, ranging from embryonic development to adult tissue homeostasis. It functions as a genetically regulated program to eliminate cells in an ordered manner[48], e.g. in self-renewing tissues, such as gut, skin and bone marrow[46]. Deregulation of the apoptotic machinery can lead to many diverse diseases. Irreversible loss of cells in the brain and heart can have dramatic consequences for the organism, whereas potently dangerous cells that show resistance to apoptotic responses are thought to be the origin of many types of cancer. Deregulation in the suicide program has been experimentally demonstrated in AIDS, Alzheimer's, Parkinson's, autoimmunity, cancer, heart failure, inflammation, and osteoporosis[62, 87, 111].

Apoptotic cells usually exhibit morphological and biochemical features such as plasma membrane blebbing, cell shrinkage, exposure of phosphatidylserine on the extracellular side of the plasma membrane, chromatin condensation and nuclear fragmentation[48, 101]. Although PCD can be induced by a wide range of death stimuli, the execution phase of the apoptotic process is uniformly carried out by an evolutionarily conserved family of cysteine proteases, the caspases, that cleave target proteins at certain aspartate residues, leading to the morphological changes described above[88].

There are two major pathways that lead to PCD, depending on the apoptotic stimuli cells are exposed to. The activation of death receptors, such as Fas- or TNF--receptors, directly induces caspase-8 activation, which takes on the execution part in PCD[40]. Other apoptotic signals, such as UV and ionizing radiation, anticancer drugs, overexpression of some oncogenes or tumor suppressor genes and growth-factor withdrawal, activate the "mitochondrial pathway", leading to the release of cytochrome *c* and other apoptogenic factors, such as

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Smac/Diablo, AIF or endonuclease G, from the mitochondrial intermembrane space into the cytosol[26, 52, 83, 90]. The exit of cytochrome *c* from the mitochondrial intermembrane space is considered as the "point of no return" and triggers the activation of caspase-9, which activates the caspase cascade resulting in cell death. Crosstalk between both pathways exists, based on the fact that death receptor-induced cell death converges on the mitochondrial level by activating Bid, a pro-apoptotic member of the Bcl-2 family[51, 55].

Proteins of the Bcl-2 family play a pivotal role in the cell-death scenario by regulating the permeabilization of the outer mitochondrial membrane[59]. This family comprises both anti-apoptotic and pro-apoptotic members. The prosurvival members of the Bcl-2 family share three or four regions of similarity (BH1-BH4: Hcl-2 Bomology). The pro-apoptotic Bcl-2-like proteins can be divided into two subclasses: the Bax-like subgroup, such as Bax itself, Bak and Bok/Mtd, contains three BH domains (BH1–BH3), but lacks the BH4 domain, whereas the second subgroup is formed by the BH3-only proteins (Bad, Hrk/DP5, Bim/Bod, Blk, Bik/Nbk, Bid, Nip3, Nix and $ITM2B_S$) that only share homology in a stretch of 9–16 amino acids called the BH3 motif[82], suggesting that this domain has arisen through convergent evolution[33]. Many Bcl-2 members also possess a conserved C-terminal transmembrane region that mediates their localization to different organelles, including the nuclear envelope, endoplasmatic reticulum and, especially, the outer mitochondrial membrane[50, 61, 73, 105].

The BH3 motif constitutes structurally an amphipathic helix capable of binding to the hydrophobic cleft formed by the BH1, BH2 and BH3 domains of prosurvival members[64, 77]. Mutational analysis of the BH3 region indicates that substitution along the hydrophobic face with charged residue influences the potential to dimerize and to exert their killing function[96]. Despite the heterogeneity and the poor definition of the BH3 motif, two conserved residues can be identified on closer inspection, namely the leucine and the aspartic acid residues, which represent the residues most responsible for mediating the proapoptotic activity of the BH3 domain[38, 56, 68, 70, 106].

So far, ten distinct BH3-only proteins have been described in mammals. The question about this redundancy is certainly justified. It has been suggested that BH3-only members function as death sensors, where various death stimuli seem to activate different BH3--only proteins. Most probably, they could exert their pro-apoptotic activity in a cell-type-specific manner, transducing the apoptotic signal to the mitochondria[10].

How Do Bcl-2 Family Proteins Function?

Bcl-2 family proteins seem to act at the mitochondrial level, regulating the exit of apoptogenic factors that are sequestered within these organelles, especially cytochrome *c*[1, 33]. Several studies performed on $bax^{-/-}$ $bak^{-/-}$ cells have shown that the BH3-only proteins Bad, Bid, Bim and Noxa are unable to induce cytochrome *c* exit and apoptosis in the absence of these multidomain proapoptotic proteins, whereas cells expressing only Bax or Bak remain sensitive[13, 99, 110]. These findings suggest that BH3-only proteins activate multidomain proapoptotic members to trigger the mitochondrial pathway.

In healthy cells, Bax is located in the cytosol, and after an apoptotic stimulus translocates to the mitochondria, where it forms homodimers or oligomerizes with Bak and induces cytochrome *c* exit[28, 43, 98]. Bax/Bak could mediate this process by forming a pore in the mitochondrial membrane[2, 27, 67] or by binding to VDAC or ANT, components of the mitochondrial permeability transition pore (PTP), thus controlling its activity[60, 66]. The antiapoptotic members of the Bcl-2 family localized at the mitochondria could prevent integration and aggregation of Bax-like proteins[2]. Furthermore, ion channel activity has been demonstrated for the BH3--only protein Bid, which can interact with ANT[78, 104].

A model that fits these observations would be as follows: an apoptotic signal activates BH3-only proteins by one of the strategies discussed later in this review. Activated BH3-only proteins move to the mitochondria, and also to other intracellular membranes, such as the endoplasmic reticulum[32], where they will bind and inactivate prosurvival members of the Bcl-2 family. On the other hand, it is possible that they will collaborate with Bax/Bak aggregation[28, 98]. In this way, antiapoptotic members could prevent membrane integration and aggregation of Bax-like multidomain proapoptotic proteins, until they are antagonized by activated BH3-only proteins[10].

Regulation of BH3-Only Proteins

Recent studies have shed light on the mechanisms by which BH3-containing proteins are controlled. Depending on the stimulus, such as death signals or survival factors, they will be activated or deactivated, respectively. BH3-only proteins are regulated by a wide range of mechanisms at the mRNA as well as at the protein level, including post-translational modifications and sequestration in cellular structures.

Transcriptional regulation of BH3-only proteins

The fact that expression of the BH3-only protein EGL-1, a core element in the cell-death pathway of the nematode *Caenorhabditis elegans*, lies under the control of different transcription factors or repressors[17, 18] has prompted scientists to search for transcription regulated BH3-only proteins in mammalian cell systems[56, 97]. In addition, it has been shown that *de novo* synthesis of specific mRNA molecules and their translated products can play an important role in the induction of the apoptotic process. Indeed, PCD can be sometimes suppressed by inhibiting transcription or translation, which provides evidence that apoptosis is mediated by intrinsic cellular components[31, 41].

Several studies have been performed to identify genes that are induced by p53 and mediate apoptosis[72]. Recently, Puma/Bbc3 and Noxa have been described as p53-inducible genes[34, 65, 68, 102] that appear to transduce apoptotic signals resulting from DNA-damage. Puma is localized in the mitochondria, where it associates with Bcl-2, thereby inducing cytochrome *c* release as well as caspase-9 and -3 activation. In contrast, another group has demonstrated that Puma can also be upregulated by p53-independent apoptotic stimuli, such as dexamethasone treatment of thymocytes and serum deprivation of cancer cells, suggesting that this protein represents a common target in diverse cell-death signaling pathways[34].

Noxa was discovered by mRNA differential display as a p53-target gene[68]. Increased expression of Noxa mRNA and protein in response to x-ray irradiation has been demonstrated in wild-type thymocytes, but not in p53-deficient thymocytes. Noxa translocates to the mitochondria and interacts with Bcl-2 and Bcl-x_L, leading to a decrease in mitochondrial membrane potential and cytochrome *c* release.

Another mammalian BH3-only protein, DP5, is transcriptionally regulated upon nerve growth factor (NGF) deprivation in rat sympathetic neurons or following exposure to the amyloid β peptide[44, 45]. Optic nerve transection also causes increased DP5 levels in the adult rat and was stably expressed at high levels for one week[94]. Moreover, the human homolog of DP5, Hrk, shows rapid upregulation in hematopoietic progenitors after growth factor deprivation or treatment with chemotherapeutic drugs[75], probably by regulation of intracellular calcium stores. By contrast, SANZ et al.[76] described a transcriptional repression mechanism triggered by IL-3 that blocks the expression of Hrk. The 3' untranslated region of the *hrk* gene contains a downstream regulatory element (DRE) to which the calcium-regulated protein DREAM can bind and inhibit the transcription of Hrk in the presence of IL-3. The activity of DREAM is controlled by calcium mobilization and direct phosphorylation through a PI3-kinase (PI3K)-dependent pathway.

Recent studies have suggested that Hrk and Bim are induced in neuronal cells undergoing apoptosis following trophic or growth-factor withdrawal. This induction seems to be dependent on the JNK signaling pathway, since addition of the inhibitor of JNK activation CEP-1347 blocks the upregulation of these BH3-only proteins in hyperpolarized granule neurons[37, 100]. Bim deletion in NGF-deprived or K^+-deprived neurons leads to protection against cytochrome *c* release and neuronal apoptosis[69]. Elevated Bim levels through activation of the Forkhead transcription factor FKHR-L1 have also been observed in lymphocytes upon IL-3 withdrawal. PI3K/PKB-mediated inhibition of FKHR-L1 can therefore contribute to the survival of hematopoietic cells[23, 24]. Another Forkhead transcription factor, FoxO3, has been implicated in the upregulation of Bim[81]. This group has proposed a model in which FoxO transcription factors are inactivated by IL-2, resulting in T cell proliferation and survival. Recently, several groups have identified some novel isoforms of Bim, indicating that alternative splicing may play a crucial role in Bim regulation, but the function of the different isoforms is still unclear[53, 58, 89]. Bimgamma inhibits clonal growth and promotes apoptosis in prostate cancer cells. Bim_{AD} and Bim_S are able to interact with the pro-apoptotic multidomain member Bax, leading to the latter's conformational change required for its killing activity on mitochondria.

The BH3-only protein Bid, known to be regulated by post-translational modifications as described below, has been identified by DNA microarray analysis as a target of the transcription factor Ets-1, involved in the PCD of serum-deprived HUVECs[86]. A different stress stimulus, high glucose levels, causes increased levels of Bid and Bik in cultured human pancreatic islets of Langerhans[29]. A further DNA microarray study suggested that Bik/Nbk is transcriptionally upregulated in E1A-induced apoptosis that is mediated via p53[61]. Increased expression of Bik has also been observed after sIgM ligation in human B104 B lymphoma cells. Sustained Bik expression correlates with B cell apoptosis, as calcineurin or PI3K inhibitors are able to diminish Bik transcription and block the apoptotic process. Furthermore, Bik seems to sequester the anti-apoptotic Bcl-2 member Bcl-x_L, thus allowing a change of the balance in favor of the proapoptotic members of the Bcl-2 family[47].

Recently, Nip3 has been proposed to play an important role in cell death triggered by hypoxia. The transcription factors PlagL2 and HIF1α appear to mediate Nip3 transactivation via a functional hypoxia-responsive element located in the Nip3 promoter. Therefore, Nip3 may have an important function in hypoxia-induced apoptosis, as observed after ischemic injury[11, 63]. Nix, closely related to Nip3, has been described as a mitochondrial death protein that is upregulated in cardiac hypertrophy, leading to cytochrome *c* release, activation of caspase-3 and apoptotic cell death. Nix may represent a key protein implicated in the development of heart failure[103].

Our group has recently demonstrated that the BH3--only protein ITM2B$_S$ is induced in T lymphocytes undergoing apoptosis following IL-2 deprivation[30]. Mutation of the critical leucine and aspartic acid residues within the BH3 motif abolishes the ability of ITM2B$_S$ to promote apoptosis. Interestingly, the alternative splice form ITM2B$_L$ was not upregulated. Indeed, this variant did not show any pro-apoptotic activity. Further studies confirmed that ITM2B$_S$ regulates apoptosis by loss of mitochondrial membrane potential, cytochrome *c* release and caspase activation (unpublished data, manuscript in preparation).

Post-translational modifications

Control by phosphorylation. The proapoptotic function of Bad is regulated by its phosphorylation status. First of all, phosphorylation of Bad at serine 112 (ser112) and ser136 (all the positions given correspond to mouse Bad) has been described upon stimulation of cells with different growth factors, such as PDGF, NGF, IL-3, IL-2 or IL-4[5, 6, 9, 19, 21]. Akt is the major kinase responsible for ser136 phosphorylation[9, 19, 21], although other protein kinases, such as PAK1, PKCθ and p70S6K, were also shown to phosphorylate this residue, depending on the stimuli[35, 79, 92]. PKA and RSK phosphorylate Bad at ser112 upon IL-3 or TPA stimulation[8, 36, 85]. The result of these phosphorylation events is the binding of Bad to the cytoplasmic protein 14-3-3, avoiding its interaction with Bcl-2 and Bcl-x$_L$ and allowing survival of the cells[42, 107]. Recently, some works have been published in which the 14-3-3/Bad interaction cannot be recovered when studied with the endogenous proteins[4, 5]. One explanation could lie in the differences between the growth factors used to stimulate cell survival.

Later, several groups described Bad phosphorylation at ser155 by PKA[20, 54, 93, 109] or RSK[84]. This phosphorylation also avoids the interaction between Bad and Bcl-x$_L$, blocking the proapoptotic function of Bad. Some of these works proposed that Bad phosphorylated at ser112 and ser136 binds to 14-3-3, and then is phosphorylated at ser155[20, 84]. Recently, two more phosphorylation sites have been identified: ser128 and ser170. The phosphorylation at ser170 blocks the proapoptotic activity of Bad[25], but does not avoid Bad/Bcl-x$_L$ interaction. Interestingly, ser128 is phosphorylated in cerebellar granule neurons upon apoptotic stimuli by cdc2, inhibiting the interaction of Bad with 14-3-3 and promoting the apoptotic activity of Bad[49].

With the exception of ser128, growth factors seem to inhibit Bad proapoptotic function by inducing the phosphorylation at one or more of these serine residues. Withdrawal of growth factors or exposition to other apoptotic stimuli induces Bad dephosphorylation, which migrates to mitochondria and promotes apoptosis. Up to now, three phosphatases have been described as Bad-phosphatases: PP1α, PP2A and PP2B or calcineurin, all belonging to the super-family of serine/threonine phosphatases[6, 14, 74, 80, 95]. In the case of PP1α, our group has performed further studies concerning the regulation of this phosphatase, as PP1 is a holoenzyme that requires a regulatory subunit to access its substrates (reviewed in reference[15]). We have shown that the regulatory subunits that allow PP1α to dephosphorylate Bad are Bcl-2, Bcl-w or Bcl-x$_L$, depending on the cytokine used to stimulate the cells, IL-2 or IL-4[3, 4]. In this way, these antiapoptotic members of the Bcl-2 family directly control the phosphorylation status of Bad and, in consequence, its function. Moreover, Bad activity is also regulated by its subcellular localization and cleavage by caspases (see below).

Another two BH3-only proteins have been shown to be regulated by phosphorylation. Bik is phosphorylated at serine 35 and threonine 33 by a kinase related to casein kinase II (CKII). These phosphorylations are required for an efficient Bik-mediated cell death, although they do not affect the interaction between Bik and Bcl-2[91]. Finally, several serine residues of Bid are phosphorylation targets for CKI and CKII, being then insensitive to caspase-8 cleavage[22]. The activation of Bid by cleavage will be discussed below.

Regulation by proteolysis. There are many proteins implicated in apoptosis that are activated or inactivated by proteolysis, including some BH3-only proteins. In healthy cells the protein Bid remains in the cytosol and different apoptotic signals induce its cleavage and translocation of the truncated protein (tBid) to the mitochondria, where it promotes the exit of cytochrome *c*. After the activation of cell-surface death receptors, such as Fas and TNF-α, caspase-8 is activated and

cleaves Bid, generating two fragments[51, 55]. The carboxyl terminal fragment (tBid) has a newly exposed glycine residue that is myristoylated, and targets tBid to the mitochondria[108].

Bid can also be proteolysed by granzyme B during granule-mediated cytotoxic T lymphocyte killing[7, 39], and its truncated product translocates to the mitochondria. The cleavage site of caspase-8 is different from that of granzyme B; the phosphorylation of Bid described above affects only caspase-8 cleavage, but not granzyme B cleavage[22]. Another protease, calpain, is able to cleave Bid in cisplatin-induced apoptosis and during myocardial ischemia/reperfusion, resulting also in cytochrome *c* release from the mitochondria[12, 57].

Recently it has been shown that Bad can be cleaved by caspase-3 after IL-3 deprivation, resulting in a more potent inducer of apoptosis. This truncated form is barely phosphorylated at ser112 and ser136 and interacts weakly with 14-3-3, but its binding to Bcl-x_L remains unaffected[16].

Control of subcellular localization

One strategy to modulate the activity of a protein is to control its subcellular localization in distinct organelles or cellular structures. The BH3-only member Bim is regulated transcriptionally and by alternative splicing, but two of its isoforms, Bim_L and Bim_{EL}, are also regulated by their interaction with the microtubule-associated dynein motor complex. In healthy cells, Bim_L associates with LC8 (a component of the minus end-directed dynein motor complex), thereby sequestered to this cytoskeletal structure. After apoptotic stimuli, both proteins are released together from the cytoskeletal structures and translocated to cytoplasmic membranes, where they bind to Bcl-2[70]. This process occurs independently of caspase activity, constituting an upstream signaling event in apoptosis.

Another BH3-only protein, Bmf, is controlled in a similar way, being sequestered in the myosin V actin motor by its binding to LC2 in healthy cells. Certain damage signals, such as anoikis (loss of cell attachment), induce the release of Bmf together with LC2 from the myosin actin motor, allowing them to interact with Bcl-2 at the mitochondria; this process is also caspase-independent[71]. The authors have proposed a model in which Bim and Bmf function as "sentinels" localized on the main cytoskeletal structures to sense intracellular damage.

In addition to phosphorylation and cleavage, the function of Bad is also regulated by subcellular localization. In IL-4 stimulated T cells, Bad does not interact with 14-3-3, but a major fraction of Bad is localized in the rafts, plasma membrane microdomains enriched in sphingolipids and cholesterol[5]. This pool of Bad localized in rafts is dephosphorylated (unpublished data), but it cannot exert its function because of its sequestration in these plasma membrane structures. The withdrawal of IL-4 induces the disorganization of lipid rafts, resulting in the segregation of dephosphorylated Bad from the plasma membrane and its translocation to the mitochondria, correlating with T lymphocyte death[5].

Conclusions and Future Prospects

Although many questions concerning the molecular mechanisms of action of Bcl-2 family proteins remain unanswered, BH3-only proteins appear as key integrators of survival and death signals in the cell. Up to now, ten different BH3-only proteins have been described that seem to have evolved to recognize and be activated after different stress stimuli. A failure in the regulation of these proteins could lead to the development of diseases, such as cancer, autoimmunity or neurodegenerative diseases. Further studies will be necessary to determine more exactly the biochemical mechanisms by which these molecules are so strictly controlled, thus helping in the development of therapeutics for diseases in which BH3-only protein deregulation is implicated.

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