Role of the BH3 (Bcl-2 homology 3) domain in the regulation of apoptosis and Bcl-2-related proteins

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Abstract
The Bcl-2 family of proteins play a prominent role in the regulation of apoptosis. From the initial identification of bcl-2 as an oncogene in follicular lymphoma through genetic studies in Caenorhabditis elegans to recent functional studies focusing on the importance of mitochondrial events in cell death signalling, the members of this protein family continue to be implicated in pivotal decision points regarding the survival of the cell. The family can be divided into two classes: those such as Bcl-2 and Bcl-xL that suppress cell death, and others, such as Bak and Bax, that appear to promote apoptosis. The Bcl-2 family is characterized by specific regions of identity termed Bcl-2 homology (BH1, BH2, BH3, BH4) domains, which are critical to the function of these proteins, including their impact on cell survival and their ability to interact with other family members and regulatory proteins. The identification of the BH3 domain as a potent mediator of cell death has led to the emergence of an additional family of pro-apoptotic proteins (such as Bad, Bik, Bid and Hrk) that share identity with Bcl-2 only within this death domain. These BH3-only proteins may be part of a regulatory network serving to integrate cell survival and death signals, an assertion that is supported by the identification of a BH3-only protein, Eg1-1, as a part of the central core of cell death signalling in C. elegans. While the mechanism of action of the BH3-only proteins remains unclear, recent studies on the regulation of critical protein–protein interactions and activity of Bad by phosphorylation in response to growth factor signalling suggest that the active state of BH3-only proteins may be regulated by post-translational modification. Additional modes of regulation, such as transcriptional, translational and subcellular localization, are also likely to be important.

Introduction
The ultimate fate of a cell is the outcome of an interplay between survival signals and signals that activate the cell death pathway. How are these signals integrated? What are the mechanisms that tip the balance from survival to death? The Bcl-2 family of cell death regulatory proteins appears to occupy a pivotal position in the control of this critical process [1]. The Bcl-2 family itself is composed of proteins with opposing functions. Certain family members, such as Bcl-2 and Bcl-xL, can suppress cell death induced by diverse death stimuli, whereas other members of the family, such as Bak and Bax, act to promote cell death. An additional property that is shared by these two groups is their propensity to heterodimerize with other family members. The Bcl-2 family is defined by conserved regions of identity called Bcl-2 homology (BH) domains. Family members share one to four of these BH domains, which in most cases have been shown to be critical to the regulation and activation of their function.

The importance of the BH3 domain as a potent mediator of cell death and protein binding function was first identified in studies aimed at determining the molecular requirements for the interaction of the pro-apoptotic Bcl-2 family member Bak with the death suppressor Bcl-xL [2]. The BH3 domain of Bak was uniquely required for its cell killing activity and its interaction with Bcl-xL. Furthermore, expression of small truncated derivatives of Bak containing the BH3 domain was sufficient for these activities [2]. Sequence analysis of other pro-apoptotic proteins
known at the time revealed that a homologous domain was present in Bax and in an otherwise unrelated protein, Bik (known initially as Bip1) [3], two proteins that also interact with Bcl-xL. Bad, another pro-apoptotic Bcl-xL-binding protein which had been identified earlier [4], was later recognized to have this important functional domain as well [5–7]. Therefore, unlike Bax and Bak, which have extensive identity with Bcl-2, Bik and Bad are members of a subfamily of Bcl-2-related proteins that share identity with Bcl-2 only within the conserved BH3 domain (Figure 1). All of the members of this family identified thus far are pro-apoptotic and have been shown to interact with one or more of the death suppressor family members.

The functional capabilities of the BH3-only proteins suggest that they may act as regulators or transducers of cell death signals. This hypothesis was strengthened by the identification of the BH3-only protein Egl-1 as a core element in the cell death pathway in Caenorhabditis elegans [8]. Genetic analyses in the nematode C. elegans had previously identified three genes that play a critical role in the control of programmed cell death [9] (Figure 2). Ced-9, a Bcl-2 homologue, is important for cell survival in the worm, while Ced-3 and Ced-4 are required for the induction of cell death. Ced-3 is homologous to the cysteine proteases of the caspase family [10], and Ced-4 serves as an activator of Ced-3. Ced-9 is thought to exert its protective effect by binding to Ced-4 and preventing the activation of the Ced-3 caspase. The BH3-only protein Egl-1 is pro-apoptotic, and a loss-of-function egl-1 mutation blocks most programmed cell deaths in the nematode, suggesting that egl-1 is part of the core cell death machinery in C. elegans [8]. Egl-1 interacts directly with Ced-9 [8] and this interaction disrupts Ced-9–Ced-4 complexes, resulting in the activation of the Ced-3 caspase and cell death [11]. The loss-of-function mutant of Egl-1, which deletes five amino acids in the BH3 domain, is impaired in both cell death induction and Ced-9 binding activity [8].

**Mechanism of BH3-mediated cell death**

The displacement of Ced-4 from Ced-9 by Egl-1 may serve as a model for the mechanism of death induction by mammalian BH3-only proteins. BH3-domain-containing proteins and peptides can block the interaction between Ced-4 and the human Ced-9 homologue Bcl-xL [12,13]; furthermore, the interaction between Bcl-xL and a mammalian Ced-4 homologue, Apaf-1 [14,15], is disrupted by co-expression of Bax and Bak [15]. However, the functional significance of the Bcl-xL–Ced-4 interaction has yet to be demonstrated. Nonetheless, BH3-only proteins may induce death by binding to a Bcl-2-related death suppressor such as Bcl-2 or Bcl-xL and disrupting the complex of the death suppressor with a caspase activator such as Apaf-1, which in turn results in the activation of Ced-3-like caspases (such as caspase 9) and the death of the cell (Figure 2).

This model, however, does not consider the complexity added by (1) the presence of pro-apoptotic Bcl-2 family members such as Bax and Bak, which may be activated directly or indirectly by the BH3-only proteins (C. elegans has no known counterpart to these pro-apoptotic Bcl-2 homologues), and (2) the role of the Bcl-2 death suppressors in blocking the release of cytochrome c from the mitochondria, a hallmark of apoptosis in mammalian cells (and absent from C. elegans). The release of cytochrome c leads to the activation of Apaf-1 downstream of the Bcl-2 block. Furthermore, uncertainty remains as to whether these pro-apoptotic proteins act as negative regulators of death suppressors and survival factors, or as positive death effectors engaging downstream components of the cell death pathway. The answer may be that they do both.

In the case of the BH3-only protein Bad, it appears that targeting of anti-apoptotic proteins such as Bcl-xL and Bcl-2 is essential to its pro-apoptotic activity. Bad was identified due to its ability to bind to Bcl-xL and antagonize the death repressor activity of Bcl-xL and Bcl-2 [4]. The BH3 domain of Bad is essential for its interaction with Bcl-xL and its death-inducing activity [5–7]. Importantly, expression of Bad no longer had any

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**Figure 1**

BH3-only sequence identity of the BH3-only proteins

<table>
<thead>
<tr>
<th>BH3-ONLY PROTEIN</th>
<th>BH3 SEQUENCE HOMOLOGY</th>
<th>REFERENCES</th>
</tr>
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<tbody>
<tr>
<td>BAD</td>
<td>AQRDSRGERPFSDF</td>
<td>4-7</td>
</tr>
<tr>
<td>BIK</td>
<td>SDAKNLCAKDEGTVSL</td>
<td>3</td>
</tr>
<tr>
<td>BAD</td>
<td>IRNDLACGKORSI</td>
<td>13</td>
</tr>
<tr>
<td>HMR/DFS</td>
<td>AQRDSRGERPFSDF</td>
<td>30,37</td>
</tr>
<tr>
<td>BM</td>
<td>EINRDEGFYVAYY</td>
<td>40</td>
</tr>
<tr>
<td>EGL-1 (C. elegans)</td>
<td>QYEDAPSHAGM</td>
<td>8</td>
</tr>
</tbody>
</table>

*(Human sequence shown except where indicated)*
effect on the function of Bcl-xL mutants that had lost the ability to heterodimerize with Bad, yet retained the ability to protect cells [7].

Another mechanistic model comes from the mutational analysis of Bid. Bid is a pro-apoptotic BH3-only protein that was identified by interactive cloning with Bcl-2 [16]. Mutational analysis, however, separated the ability of Bid to heterodimerize with Bcl-2 from its cell-death-inducing activity. In fact, cytotoxicity was correlated with the ability of Bid to interact with Bax [16]. The interaction of Bid with Bax is associated with a change in the conformation of Bax, leading to the release of cytochrome c from mitochondria [17].

Studies examining the activity of small BH3 peptides may also shed some light on the mechanism of action of this pro-apoptotic domain. BH3 peptides can bind directly to death suppressors such as Bcl-xL [18] and block their interaction with pro-apoptotic family members such as Bax and Bak in vitro [6,19]. This supports the hypothesis that the BH3 domain may induce death by interfering with the function of the death suppressors in vivo. In Xenopus egg extracts, BH3 peptides derived from Bax and Bak were sufficient to induce the release of cytochrome c from mitochondria, resulting in the activation of caspases. Bcl-2 was able to block the BH3-induced response; however, excess BH3 peptide could overcome this block and trigger caspase activation even in the presence of Bcl-2 [20]. Bax- and Bak-derived BH3 peptides were also able to induce the release of cytochrome c in isolated rat mitochondria [21]. A mutant BH3 peptide which cannot bind Bcl-xL and lacks pro-apoptotic activity had no effect. However, the ability of BH3-containing peptides to induce cytochrome c release in isolated mitochondria has not been observed in all cases [22].

In intact cells, whereas microinjection of recombinant Bcl-xL could suppress Fas-induced death in HeLa cells, co-injection of a BH3 peptide blocked the activity of Bcl-xL and resensitized the cell to Fas-mediated killing [23]. Furthermore, the sustained delivery of synthetic BH3 peptides as fusion proteins with the Antennapedia homeo-protein internalization domain (Ant) resulted in the rapid induction of apoptosis in the absence of additional death stimuli [23]. Ant–BH3 peptides were able to antagonize the function of Bcl-xL and block its ability to suppress apoptosis induced by Fas ligation. Mutant BH3 peptides that failed to bind Bcl-xL in vitro were unable to induce cell death, suggesting that the cytotoxicity of BH3 peptides may be due to their inhibitory interaction with endogenous Bcl-2-related death suppressors. In contrast with the situation in cell-free assays, Ant–BH3-induced death did not cause a significant release of cytochrome c into the cytosol,
although it did depend on the activation of caspases [23].

These studies suggest that the BH3 domain may be sufficient to trigger cell death by (1) antagonizing the function of death suppressors such as Bcl-xL or activating pro-apoptotic proteins such as Bax, resulting in the release of cytochrome c from the mitochondria and subsequent activation of caspases, and/or (2) the activation of caspases in a cytochrome c-independent manner, perhaps through the activation of caspase-activating factors such as Apaf-1.

**Regulation of BH3-only proteins**

While the mechanism of BH3-mediated cell death is still unclear, recent studies have demonstrated that the active state of BH3-only proteins may be regulated by a variety of specific regulatory mechanisms, including post-translational modification, regulated expression and sequestration. These regulatory mechanisms appear to be engaged in response to an array of different signalling molecules, such as survival factors, growth factors and death inducers.

**Phosphorylation of Bad**

One example of post-translational regulation is the control of Bad function by phosphorylation. In the interleukin-3 (IL-3)-dependent haematopoietic cell line F15.12, IL-3 induces the phosphorylation of Bad, and this phosphorylation was mapped to two serine residues at positions 112 and 136 (S112 and S136) [24]. Only unphosphorylated Bad was able to bind Bcl-xL, while phosphorylated Bad was found to complex with the signal transducer protein 14-3-3 [25], suggesting that phosphorylation may alter the pro-apoptotic activity of Bad by blocking its interaction with death suppressor proteins. Indeed, mutants that cannot be phosphorylated on S112 and S136 show increased cytotoxicity compared with wild-type Bad. Bad phosphorylation is induced by other survival factors as well, such as platelet-derived growth factor, nerve growth factor (NGF) and insulin-like growth factor-I [26]. Phosphorylation of Bad induced by these factors can be blocked by inhibitors of phosphoinositide 3-kinase [26,27]. Akt, a survival-promoting kinase which is activated in response to these survival factors in a phosphoinositide 3-kinase-dependent manner, was found to be sufficient to promote phosphorylation of Bad in vivo and in vitro [26,27]. These data suggest that the phosphoinositide 3-kinase/Akt pathway is an important regulator of Bad function.

It is likely that other kinase pathways may be important as well. Granulocyte/macrophage colony-stimulating factor promotes the survival of MC/9 cells in the absence of phosphoinositide 3-kinase activity and stimulates phosphorylation of Bad even when Akt activation is completely blocked [28]. In fact, even in IL-3-treated F15.12 cell extracts, the majority of Bad kinase activity remained in the Akt-depleted fraction following immunoprecipitation of Akt [29]. A mitochondria-associated Bad S112 kinase was isolated from IL-3-stimulated cells and was identified to be protein kinase A. Protein kinase A inhibitors blocked Bad phosphorylation by this kinase in vitro and inhibited IL-3-induced phosphorylation of endogenous Bad by approx. 50%. The Raf-1 kinase is also able to phosphorylate Bad [24,29,30] and can co-operate with Bcl-2 in suppressing apoptosis [30].

Dephosphorylation of Bad would be expected to lead to enhanced apoptosis. The calcium-activated protein phosphatase calcineurin is capable of dephosphorylating Bad and inducing apoptosis [31]. Calcium-mobilizing agents induced the dephosphorylation of Bad, resulting in its dissociation from 14-3-3 and translocation from the cytosol to the mitochondria, where it associated with Bcl-xL. These calcium-induced changes were suppressed by expression of a dominant negative calcineurin mutant [31].

**Protein processing of Bid**

Another mode of regulation for BH3-containing proteins may be through activation by proteolytic processing, as has been reported for Bid. Although full-length Bid is capable of interacting with Bcl-2 and Bcl-xL, it does not co-localize with these death suppressors on mitochondria in healthy cells, but rather is found in the cytosol [32]. However, upon activation of Fas or the tumour necrosis factor receptor, Bid is cleaved by caspase 8 and the truncated Bid cleavage product translocates to the mitochondria and triggers the release of cytochrome c in a BH3-dependent manner [32,33]. Immunodepletion of Bid shows that it is required for caspase 8-induced cytochrome c release in cell extracts [33]. Bid cleavage may allow for a conformational change in truncated Bid that makes the BH3 domain more accessible for heterodimerization with Bax or Bcl-xL [34], or it may simply remove the N-terminus, which competes with Bcl-xL for binding to the BH3 helix [35]. Thus the proteolytic cleavage of Bid may be part of an activation cascade for signalling from death
receptors such as Fas and the tumour necrosis factor receptor.

Translocation of Bid from the cytosol to the mitochondria may not require proteolytic cleavage in all cases. Translocation of full-length Bid occurs during staurosporine-induced death in HeLa cells, and is associated with a change in the conformation of Bax and the release of cytochrome c from mitochondria [17].

Transcriptional regulation of BH3-only proteins

In addition to post-translational modification, BH3-containing proteins may be regulated at the level of expression in some cases. Genetic analysis in C. elegans demonstrated that Egl-1 acts downstream of or in parallel to two genes involved in specifying the fate of certain cells in the worm, ces-2 and ces-1 [8]. Ces-2 is a bZIP DNA-binding protein that may regulate the expression of Egl-1, either directly or through Ces-1. The bZIP domain of Ces-2 is similar to the PAR subfamily of mammalian bZIP proteins [36], which may prove to be a family of transcription factors involved in the regulation of cell death.

Neuronal cell death upon withdrawal of neurotrophic factors such as NGF can be blocked by inhibitors of RNA synthesis and protein synthesis, suggesting that this death is dependent on the regulated expression of some apoptotic factor(s). One candidate for such a factor is DP5, a BH3-only protein [37] that was identified by differential display upon NGF withdrawal in rat sympathetic neurons [38]. DP5 mRNA levels peak 15 h after NGF withdrawal, and this peak of expression is coincident with the onset of commitment to cell death. DP5 mRNA expression is also induced during neuronal death following exposure to the amyloid β peptide [37]. Agents that prevent amyloid β peptide-induced death also blocked DP5 induction. DP5-mediated death can be suppressed by co-expression of Bcl-2 [38]. DP5 can interact directly with Bcl-2 and Bcl-xL, and an intact BH3 domain is essential for this interaction [37]. Whether Hrk, the human homologue of DP5 [39], is regulated at the level of expression in response to specific death stimuli remains to be determined.

Localization/sequestration of Bim

While the BH3 domain appears to be a key functional domain for the BH3-only proteins, it is likely that other regions of these proteins may have regulatory significance. For example, the BH3-only protein Bim is a potent inducer of apoptosis [40], and in healthy cells is sequestered to the microtubule-associated dynein motor complex through a BH3-independent interaction with the dynein light chain, LC8 [41]. Localization to the motor complex probably attenuates the death-inducer activity of Bim. Mutants of Bim that cannot interact with LC8 are more potent inducers of cell death than the wild-type protein. After certain apoptotic stimuli, Bim and LC8 are released from the dynein motor complex and translocate to cytoplasmic membranes, where they bind to Bcl-2. Inhibition of caspase activation could block cell death, but had no effect on Bim relocalization. The mechanism that regulates this relocalization is not known.

Opportunities in disease

The hypothesis that the BH3-only proteins are key integrators of survival and death signals involved in the regulation of cell viability promotes the potential of these proteins as important targets in the development of therapeutics for the treatment of diseases where the regulation of cell survival has gone awry, such as cancer, neurodegenerative disease and heart disease. Pharmacological activation of BH3-only proteins may trigger cell death in tumour cells by overcoming inappropriate death-suppressor function in these cells. Inhibition of their activation may serve to protect neurons or cardiomyocytes from aberrant death stimuli. An understanding of how the cell regulates the activity of the pro-apoptotic BH3-only proteins may point the way.

References

GSH extrusion and the mitochondrial pathway of apoptotic signalling
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Abstract
New evidence suggests that physiological and damaging agents activate two different pathways of apoptotic signalling, which are mediated by protein–protein interactions and mitochondrial alterations respectively. The two pathways converge at the activation of caspase 3, the key effector of the execution phase of apoptosis, thus giving similar final results. The knowledge that different biochemical routes exist allows us to re-evaluate previous apparently contradictory results concerning the events occurring during apoptosis, and their respective roles. In particular, this applies to the role of oxidative stress and redox imbalance in the signal transduction events of apoptosis. It now appears that oxidative alterations are absent, or at least unnecessary, for the de-