Cell Death and Differentiation (2006) 13, 1268–1271

www.nature.com/cdd

News and Commentary

BH3-only proteins: orchestrating cell death

JI Fletcher[1] and DCS Huang*,[1]

[1] The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, Victoria, Australia 3050
* Corresponding author: D Huang, The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, Victoria, Australia 3050.
Tel: +61 3 9345 2555; Fax: +61 3 9347 0852;
E-mail: huang_d@wehi.edu.au

Cell Death and Differentiation (2006) **13,** 1268–1271.
doi:10.1038/sj.cdd.4401995; published online 9 June 2006

Our recent progress in understanding how cellular life and death decisions are made is heavily indebted to the numerous contributions made by the late Dr. Stanley Korsmeyer and his colleagues.[1] From their early characterization of the t(14;18) chromosomal translocation involving *bcl-2* in human follicular lymphoma, through to elucidating the biochemical and molecular processes governing programmed cell death (apoptosis), the impact of his laboratory has been enormous, imprinted upon current thinking and influencing future progress. Although much of this work concerned the Bcl-2 family of proteins, many fundamental questions remain unanswered and part of Stan's legacy is the challenge for us to resolve these. This is critical not only for understanding the fundamental biological process of apoptosis, but has broader implications as we harness our knowledge of cell death mechanisms to develop novel therapeutics for treating human diseases such as cancer.[2]

Studies from the Korsmeyer and Thompson laboratories established a central role for the Bcl-2 family members Bax and Bak in mediating apoptotic cell death. With the notable exception of cell death during early development, which remains unperturbed in mice lacking Bax and Bak, these molecules are essential for apoptosis to proceed. Otherwise, their combined absence abolishes cell death induced by developmental cues and by many forms of stress signals.[3–5] Bax/Bak probably act by triggering release from the mitochondria of proapoptogenic factors such as cytochrome *c*.[6] Their action is under the control of two other factions of the Bcl-2 family: proapoptotic BH3-only proteins and prosurvival Bcl-2-like proteins. Killing by the BH3-only proteins, such as Bim or Puma, depends on Bax/Bak,[7,8] whereas Bcl-2 and its close relatives (Bcl-x_L, Bcl-w, Mcl-1, A1) counter this process. This article will focus on how the balance between these two factions determines if Bax/Bak is activated, and hence if cell death proceeds.

Proapoptotic BH3-only proteins monitor cellular well-being and are activated in response to stress signals. The multiple mammalian BH3-only proteins are coupled to distinct upstream controls, making them ideal stress sensors. Expression of Puma, for example, is transcriptionally induced by the tumor suppressor p53 in response to certain types of DNA damage.[9,10] Consistent with this observation, loss of *puma*, like p53-deficency, renders thymocytes resistant to apoptosis caused by γ-irradiation.[11,12] On the other hand, Bim is required for the deletion of autoreactive thymocytes during negative selection,[13] thereby preventing autoimmunity. These observations are consistent with the idea that BH3-only proteins signal for cellular damage.[14] Once activated by such damage signals, what is the molecular target(s) of their action?

Bax exists predominantly as a cytosolic monomer in healthy cells.[15,16] Damage signals trigger Bax to undergo a conformational change, mitochondrial translocation and oligomerization. This series of events transforms the normally inert molecule into an agent of destruction.[17,18] Some BH3-only proteins have been found to bind Bax,[19–24] suggesting that they may directly trigger Bax activation, although other candidates have been suggested.[25] Several other lines of evidence give weight to this argument. Killing by BH3-only proteins requires Bax or Bak, placing them upstream of Bax activation.[7,8] In addition, despite limited sequence identity, and opposing functions, the solution structure of Bax[26] closely resembles that of its prosurvival relatives such as that of Bcl-x_L.[27] It is therefore plausible that the hydrophobic groove targeted by BH3-only proteins in Bcl-x_L[28,29] is similarly targeted in Bax. Even more compellingly, reconstitution studies using purified components suggest that peptides spanning the BH3 regions of Bim or Bid cooperate with Bax to permeabilize mitochondria or synthetic liposomes, causing release of their contents.[30,31] Taken together, these studies mount a strong case that at least some BH3-only proteins, such as Bim, Bid or Puma,[23,30–32] activate Bax directly.

These observations have led to the division of BH3-only proteins into two subclasses (Figure 1a). Bim, Bid and perhaps Puma are thought to be the 'activators' of Bax and presumably Bak.[23,30–32] In this scenario, the prosurvival proteins act to promote cell survival by diverting such 'activators' away from Bax and Bak. The other BH3-only proteins (such as Bad, Noxa), which only bind to the prosurvival proteins, but not Bax, have been termed 'sensitizers'[32] or 'derepressors'.[31] They do not have the capacity to directly activate Bax, but rather counteract the protection imposed by prosurvival proteins by displacing the activators. Consistent with this idea, the sensitizer/depressor class cannot themselves damage membranes, but conspire with the activators to do so.[31,32] Of note, the role of Puma appears conflicting, some classify it in the sensitizer/depressor class,[31,32] whereas others ascribe an activator function to it.[23]

Regardless, this prevailing model for direct Bax/Bak activation by BH3-only proteins (Figure 1a) makes a number of predictions. It might be anticipated that the activators bind Bax in dying cells, but the results of these studies are somewhat conflicting.[17,24,33,34] As Bax is prone to detergent-induced conformational changes,[15] it is also unclear whether conditions used for solubilizing the proteins accurately represent their physiological states or are artifactual. The absence of BH3-only proteins in oligomeric Bax complexes

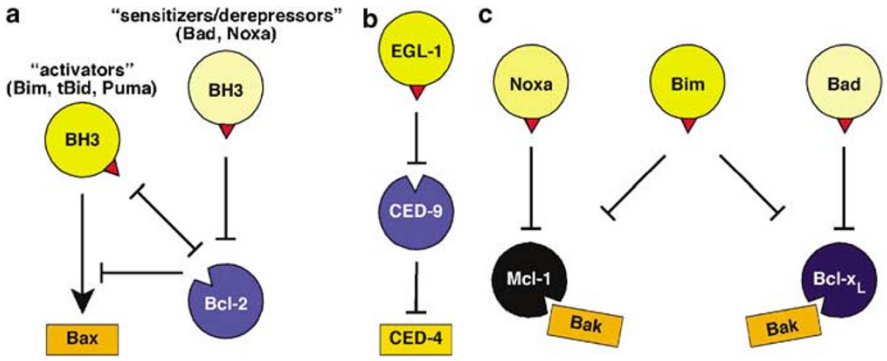

Figure 1 Models for the activation of Bax/Bak by BH3-only proteins. (**a**) Some BH3-only proteins (Bim, tBid, Puma) have been proposed[23,30–32] to activate Bax/Bak directly, whereas others (Bad, Noxa) lower the threshold for activation by neutralizing prosurvival Bcl-2 proteins. Here, the default state is cell survival, as loss of prosurvival proteins alone should not lead to killing. (**b**) In the worm *C. elegans*, the BH3-only protein EGL-1 induces apoptosis by displacing the adapter CED-4 from CED-9, the Bcl-2 ortholog in this organism. (**c**) Control of proapoptotic Bak by Mcl-1 and Bcl-x_L[45]. The proapoptotic activity of Bak is held in check by direct binding to Mcl-1 and to Bcl-x_L. Freeing Bak either from Mcl-1 (with its selective ligand, Noxa) or from Bcl-x_L (with Bad) does not induce apoptosis, unless it is freed from both. A BH3-only protein such as Bim is a potent killer because it binds to diverse prosurvival proteins including Mcl-1 and Bcl-x_L, thereby freeing Bak from its sequestration. The consequences of losing Mcl-1 and Bcl-x_L would be severe as Bak is now unconstrained, the default state being cell death

has previously been attributed to a 'hit-and-run' mechanism,[21,35] a difficult hypothesis to confirm experimentally. In addition, although it might also be anticipated that binding of the activators to prosurvival proteins is attenuated in dying cells, many studies instead report increased binding.[34]

The direct activation model also suggests that mice deficient in activator BH3-only proteins would phenocopy *bax*/*bak* knockout mice. This is not observed for individual knockouts as cells derived from *bim*-,[36] *bid*-[37] or *puma*-[11,12] deficient mice are only defective in their responses to a limited range of damage signals. However, no definitive conclusions about their role to directly activate Bax/Bak can be drawn until mice with combined deficiencies are examined. It might be anticipated that the complete absence of the activators would mimic the combined absence of Bax and Bak.

The postulated role for Bid in Bax/Bak activation is of particular interest. It is now appreciated that full-length Bid is inert and that the bioactive truncated form, tBid is generated by caspase cleavage.[38,39] Whereas the Bcl-2 family of proteins plays a key role in committing cells to die when the intrinsic (mitochondrial) pathway is triggered, caspases are generally thought to be activated downstream of this commitment point.[1,40] These executioners, caspases, then act to cleave multiple cellular substrates, including Bid. In this scenario, tBid is a product of caspase cleavage and presumably acts to amplify rather than initiate the process. This conundrum with Bid illustrates some of the challenges in unraveling pathways with multiple checks and balances, both positive and negative.

Given the evolutionary conservation of the cell death pathways, it is also worth comparing the function of *Caenorhabditis elegans* CED-9 with its mammalian ortholog, Bcl-2. Elegant genetic studies from the Horvitz laboratory, satisfyingly complemented by structural studies,[41] revealed that the key function of CED-9 is to prevent the adapter protein CED-4 from binding to and activating the caspase CED-3, thereby preventing cell death (Figure 1b). This delicate balance is upset when the BH3-only protein EGL-1 is induced by signals such as those from developmental cues. EGL-1 binds tightly to induce a conformational change in CED-9, causing CED-4 release. Thus, worms lacking CED-9 are not viable, reverting to the default state of cell death. Is the function of its mammalian Bcl-2-like counterparts primarily to buffer the action of BH3-only proteins, or like CED-9, do they act instead to control downstream mediators? Whereas mammalian prosurvival proteins do not appear to interact directly with the mammalian CED-4 homolog Apaf-1,[42,43] specific interactions with Bax[44] and Bak[45] are observed. In a manner akin to EGL-1, which indirectly triggers CED-4 activation by neutralizing CED-9, might mammalian BH3-only proteins also activate Bax/Bak indirectly by neutralizing the prosurvival Bcl-2 proteins, rather than directly as proposed (Figure 1a)?

Recently, a systematic study of the affinities of the eight mammalian BH3-only proteins for their five prosurvival relatives[46] (Chen and DCS Huang, unpublished observations) has provided further insights into the interplay between Bcl-2 family members. Although these interactions were assumed to be promiscuous, only certain BH3-only proteins (Bim, tBid, Puma) were revealed to bind tightly to each of the prosurvival proteins, whereas others (Bad, Noxa) show surprising selectivity. Interestingly, a dichotomy again emerges in the BH3-only proteins, although now based on selectivity for prosurvival proteins rather than for Bax. These observations suggest an alternative hypothesis for Bax and Bak activation, with the potent induction of apoptosis by Bim, tBid and Puma being accounted by their ability to target a wide range of prosurvival proteins, whereas BH3-only proteins, such as Bad and Noxa, are poor killers because they only bind a selected subset of prosurvival proteins.

Such considerations have prompted a re-evaluation of whether Bax, and by implication Bak, is directly activated by BH3-only proteins.[47] Unlike the predominantly cytosolic Bax, Bak is exclusively located on intracellular membranes,[48] implying either that its conformation promotes membrane targeting or that a membrane component targets it there.[49] There is also little evidence for direct binding to Bak by the BH3-only proteins. As some damage signals preferentially utilize Bax[50–53] or Bak[45,54,55] to signal for cell death, the

checks imposed upon activation of the latter may well be distinct.

By using BH3-only proteins that selectively antagonize some of the prosurvival Bcl-2-like proteins[46] and cells derived from various knockout mice, it is apparent that Bcl-x_L and Mcl-1 are the key controls on Bak activation.[45] Inactivation of either Bcl-x_L or Mcl-1 alone does not cause Bak-mediated apoptosis, but when both are neutralized, efficient killing occurs, even when Bcl-2 is highly expressed. Furthermore, the protection afforded by Bcl-x_L or Mcl-1 mirrors their ability to bind Bak, whereas Bcl-2 or the other prosurvival proteins, which bind Bak poorly, have no role in controlling Bak. These studies would be most consistent with a model in which Bak is normally kept in check by being bound to Bcl-x_L or Mcl-1, akin to CED-4 sequestration by CED-9 (Figure 1b). In this model (Figure 1c), BH3-only proteins trigger Bak activation indirectly by displacing it from Bcl-x_L and Mcl-1. In healthy cells, the critical function of Bcl-x_L or Mcl-1 is to keep Bak in check. Apoptotic stimuli trigger the activation of BH3-only proteins, which bind to the prosurvival proteins, displacing and thus activating Bak. By implication, the constitutive absence of Bcl-x_L and Mcl-1 would lead to uncontrolled Bak activation and cell death. Indirect activation via release from prosurvival proteins seems a plausible model for Bak, which exists constitutively on membranes. However, the extrapolation of such a model to Bax is problematic, as Bax exists predominantly as a cytosolic monomer before an apoptotic stimulus, implying that some initial activation may be required.

Clearly, many issues remain unresolved. In contrast to the relatively simple linear pathway in *C. elegans*, multiple overlapping Bcl-2 family members in mammals pose greater difficulties for validating the models by genetic tests. Whereas a significant body of evidence suggests that Bax may be directly activated by certain BH3-only proteins,[19–24] an indirect activation mechanism appears more consistent with Bak activation.[45,55] Whether they will indeed turn out to be regulated by distinct mechanisms remains to be seen.

How can we best study interactions between the members of this protein family in order to understand normal physiology? For example, is the interaction between prosurvival Bcl-2 and Bax[44,56,57] physiologically relevant, or simply a consequence of detergent-induced conformational changes? If not, which prosurvival proteins control Bax? Perhaps prosurvival proteins control Bax indirectly.[58] How do we distinguish the initiation and amplification steps of the process? The absence of the effector caspase-3 and -7 markedly attenuates key biochemical changes thought to be characteristic of cell death commitment, including Bax translocation.[59] However, caspase activation is generally believed to be an event downstream of Bax activation. Similarly, only limited Bax activation and translocation is observed during apoptosis in Bak-null-activated T cells, with substantial activation and translocation only seen after cell death.[60] These observations suggest that Bax translocation and cytochrome *c* release may be considerably amplified following caspase activation.

The realization that there is a high level of specificity in many of the interactions between the Bcl-2 family proteins[46] will provide some of the tools we need to resolve these issues. We also need to study the biologically relevant forms of the key players. The interactions of Bid with Bax, for example, have to be reanalyzed as previous studies were carried out with the inert full-length molecule[19] rather than the active moiety.[38,39] Furthermore, although much is known of the tertiary structures of soluble Bcl-2 family proteins and their complexes,[61,62] we have only very limited knowledge of their conformations when located on their main site of action, intracellular membranes, and no structural information on the biologically active presumably oligomeric states of Bax and Bak.

Although deregulation of apoptosis can promote malignancies, BH3-only proteins can still kill tumor cells downstream of some of the most common genetic alterations in tumorigenesis, such as loss of the tumor suppressor p53. This advantage has stimulated substantial interest in the development of BH3 mimetics as anticancer agents.[2,63] As we begin to exploit these approaches, it is increasingly important to understand the precise mechanism by which BH3-only proteins kill. If Bax and Bak require direct activation by BH3-only proteins (Figure 1a), resistance to 'sensitizer'-type BH3 mimetics might readily develop if tumors acquire mutations that inactivate genes that encode for their Bax/Bak 'activators'. Perhaps, targeting Bax/Bak with 'activator' BH3 mimetics might be a more fruitful strategy. On the other hand, would such compounds be intolerably toxic? These are some of the many challenges that lie ahead as we contemplate the key implications of the discoveries made by Stan Korsmeyer and his team.

1. Danial NN and Korsmeyer SJ (2004) Cell 116: 205–219.
2. Fesik SW (2005) Nat. Rev. Cancer 5: 876–885.
3. Lindsten T *et al.* (2000) Mol. Cell 6: 1389–1399.
4. Rathmell JC *et al.* (2002) Nat. Immunol. 3: 932–939.
5. Lum JJ *et al.* (2005) Cell 120: 237–248.
6. Jürgensmeier JM *et al.* (1998) Proc. Natl. Acad. Sci. USA 95: 4997–5002.
7. Zong WX *et al.* (2001) Genes Dev. 15: 1481–1486.
8. Cheng EH *et al.* (2001) Mol. Cell 8: 705–711.
9. Nakano K and Vousden KH (2001) Mol. Cell 7: 683–694.
10. Yu J *et al.* (2001) Mol. Cell 7: 673–682.
11. Villunger A *et al.* (2003) Science 302: 1036–1038.
12. Jeffers JR *et al.* (2003) Cancer Cell 4: 321–328.
13. Bouillet P *et al.* (2002) Nature 415: 922–926.
14. Huang DCS and Strasser A (2000) Cell 103: 839–842.
15. Hsu Y-T and Youle RJ (1998) J. Biol. Chem. 273: 10777–10783.
16. Hsu Y-T *et al.* (1997) Proc. Natl. Acad. Sci. USA 94: 3668–3672.
17. Nechushtan A *et al.* (2001) J. Cell Biol. 153: 1265–1276.
18. Wolter KG *et al.* (1997) J. Cell Biol. 139: 1281–1292.
19. Wang K *et al.* (1996) Genes Dev. 10: 2859–2869.
20. Desagher S *et al.* (1999) J. Cell Biol. 144: 891–901.
21. Wei MC *et al.* (2000) Genes Dev. 14: 2060–2071.
22. Marani M *et al.* (2002) Mol. Cell. Biol. 22: 3577–3589.
23. Cartron PF *et al.* (2004) Mol. Cell 16: 807–818.
24. Harada H *et al.* (2004) Proc. Natl. Acad. Sci. USA 101: 15313–15317.
25. Lucken-Ardjomande S and Martinou JC (2005) J. Cell Sci. 118: 473–483.
26. Suzuki M *et al.* (2000) Cell 103: 645–654.
27. Muchmore SW *et al.* (1996) Nature 381: 335–341.
28. Sattler M *et al.* (1997) Science 275: 983–986.
29. Liu X *et al.* (2003) Immunity 19: 341–352.
30. Kuwana T *et al.* (2002) Cell 111: 331–342.
31. Kuwana T *et al.* (2005) Mol. Cell 17: 525–535.
32. Letai A *et al.* (2002) Cancer Cell 2: 183–192.
33. Antonsson B *et al.* (2001) J. Biol. Chem. 276: 11615–11623.

34. Zhu Y *et al.* (2004) Proc. Natl. Acad. Sci. USA 101: 7681–7686.
35. Korsmeyer SJ *et al.* (2000) Cell Death Differ. 7: 1166–1173.
36. Bouillet P *et al.* (1999) Science 286: 1735–1738.
37. Yin X-M *et al.* (1999) Nature 400: 886–891.
38. Li H *et al.* (1998) Cell 94: 491–501.
39. Luo X *et al.* (1998) Cell 94: 481–490.
40. Adams JM (2003) Genes Dev. 17: 2481–2495.
41. Yan N *et al.* (2005) Nature 437: 831–837.
42. Moriishi K *et al.* (1999) Proc. Natl. Acad. Sci. USA 96: 9683–9688.
43. Conus S *et al.* (2000) Cell Death Differ. 7: 947–954.
44. Oltvai ZN *et al.* (1993) Cell 74: 609–619.
45. Willis SN *et al.* (2005) Genes Dev. 19: 1294–1305.
46. Chen L *et al.* (2005) Mol. Cell 17: 393–403.
47. Willis SN and Adams JM (2005) Curr. Opin. Cell Biol. 17: 617–625.
48. Zong WX *et al.* (2003) J. Cell Biol. 162: 59–69.
49. Cheng EH *et al.* (2003) Science 301: 513–517.
50. Eischen CM *et al.* (2001) Mol. Cell. Biol. 21: 7653–7662.
51. LeBlanc H *et al.* (2002) Nat. Med. 8: 274–281.
52. Gillissen B *et al.* (2003) EMBO J. 22: 3580–3590.
53. Dansen TB *et al.* (2006) J. Biol. Chem 281: 10890–10895.
54. Nijhawan D *et al.* (2003) Genes Dev. 17: 1475–1486.
55. Cuconati A *et al.* (2003) Genes Dev. 17: 2922–2932.
56. Yin X-M *et al.* (1994) Nature 369: 321–323.
57. Yang E *et al.* (1995) Cell 80: 285–291.
58. Mikhailov V *et al.* (2001) J. Biol. Chem. 276: 18361–18374.
59. Lakhani SA *et al.* (2006) Science 311: 847–851.
60. Zhu Y *et al.* (2006) J. Exp. Med 203: 1147–1152.
61. Hinds MG and Day CL (2005) Curr. Opin. Struct. Biol. 15: 690–699.
62. Petros AM *et al.* (2004) Biochim. Biophys. Acta 1644: 83–94.
63. Oltersdorf T *et al.* (2005) Nature 435: 677–681.