Cell Death and Differentiation (2002) 9, 505–512

www.nature.com/cdd

Review

Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins

H Puthalakath[1] and A Strasser*,[1]

[1] The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia
* Corresponding author: A Strasser, The Walter and Eliza Hall Institute of Medical Research, Post Office Royal Melbourne Hospital, 3050 VIC, Australia. Tel: +61-3-9345-2555; Fax: +61-3-9347-0852; E-mail: strasser@wehi.edu.au

Received 25.10.01; revised 15.11.01; accepted 29.11.01
Edited by RA Knight

Abstract

BH3-only proteins are structurally distant members of the Bcl-2 protein family that trigger apoptosis. Genetic experiments have shown that these proteins are essential initiators of programmed cell death in species as distantly related as mice and *C. elegans*. BH3-only proteins share with each other and with the remainder of the Bcl-2 family only a nine amino acid BH3 (*B*cl-2 *H*omology) region. Mutational analyses have demonstrated that this domain is required for their ability to bind to Bcl-2-like pro-survival proteins and to initiate apoptosis. So far only one BH3-only protein, EGL-1, has been identified in *C. elegans* and it is required for all developmentally programmed death of somatic cells in this species. In contrast, mammals have at least 10 BH3-only proteins that differ in their expression pattern and mode of activation. Studies in gene targeted mice have indicated that different BH3-only proteins are required for the initiation of distinct apoptotic stimuli. The pro-apoptotic activities of BH3-only proteins are stringently controlled by a variety of mechanisms. *C. elegans egl*-1 as well as mammalian *hrk/dp5*, *noxa*, *puma/bbc*3 and *bim/bod* are regulated by a diverse range of transcription factors. Certain BH3-only proteins, including Bad, Bik/Nbk, Bid, Bim/Bod and Bmf, are restrained by post-translational modifications that cause their sequestration from pro-survival Bcl-2 family members. In this review we describe current knowledge of the functions and transcriptional as well as post-translational control mechanisms of BH3-only proteins.

Cell Death and Differentiation (2002) **9,** 505–512. DOI: 10.1038/sj/cdd/4400998

Keywords: apoptosis; programmed cell death; BH3-only proteins; Bcl-2; signal transduction

Abbreviations: BH domain, Bcl-2 Homology domain; TNF-R, tumour necrosis factor receptor, FADD, Fas associated death domain protein; Apaf-1, apoptosis activating factor 1; CED, cell death abnormal mutations in *C. elegans*; EGL-1, egg laying defective mutations in *C. elegans*; gf, gain of function mutations; lf, loss of function mutations; HSN, hermaphodite specific neurons in *C. elegans*; CES, cell death specification mutations in *C. elegans*; Aβ, amyloid β; SAPK, stress activated protein kinase; JNK, Jun N-terminal kinase; SAGE, serial analysis of gene expression; IRF, interferon response factor; NGF, nerve growth factor; FKHR-L1, forkhead related transcription factor L1; MAPK, mitogen activated protein kinase; IL, interleukin; PI3 kinase, phosphatidylinositol 3′ kinase; PKB, protein kinase B; PKA, protein kinase-A; PP2A, protein phosphatase 2A; DLC, dynein light chain; UV, ultraviolet; CDK, cyclin dependent kinase

Introduction

Mammals have two distinct apoptosis signalling pathways that utilise different 'initiator caspases' with their adapters and are controlled by different sets of regulators.[1] 'Death receptors', members of the tumour necrosis factor receptor (TNF-R) family that have an intra-cellular 'death domain', trigger apoptosis through FADD adapter protein-mediated recruitment and activation of the 'initiator caspase' caspase-8 (and in humans also caspase-10).[2] In contrast, cytokine deprivation and many other apoptotic stimuli, such as DNA damage, anoxia or cytotoxic drugs, kill cells through activation of different 'initiator caspases' and their adapters.[3–5] Caspase-9 and its activators, Apaf-1 and cytochrome *c*, are part of this pathway but additional apoptosis initiation mechanisms must exist since many cell types from Apaf-1-, caspase-9- or cytochrome *c*-deficient mice are still vulnerable to the aforementioned death-inducing stimuli (V Marsden, JM Adams, DCS Huang and A Strasser, unpublished observations).[6,7]

Pro-survival members of the Bcl-2 protein family

Members of the Bcl-2 protein family are critical regulators of those pathways to programmed cell death that are not activated by 'death receptors'.[3,8,9] Bcl-2 and related anti-apoptotic family members, mammalian Bcl-x_L, Bcl-w, A1, Mcl-1, Boo/DIVA/Bcl2-L-10, Bcl-B and *C. elegans* CED-9, share 3 or 4 regions of structural homology (*B*cl-2 *H*omology or BH regions) (Figure 1). All of these proteins are localised on the cytoplasmic aspect of the nuclear envelope, endoplasmic reticulum and the outer mitochondrial membrane.[10–12] Bcl-2-like proteins protect cells against a broad range of apoptotic stimuli and are essential for cell survival but their biochemical action is still controversial. Since Bcl-2 prevents release of cytochrome

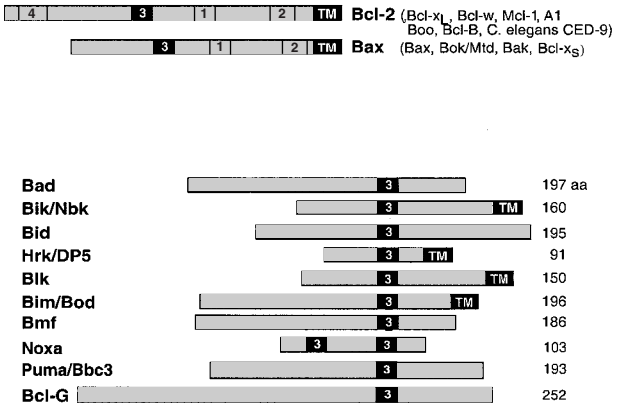

Figure 1 Primary structures of known mammalian BH3-only proteins and *C. elegans* EGL-1. For comparison, the structures of one pro-survival Bcl-2 family member, Bcl-2, and one multi-BH domain pro-apoptotic Bcl-2 family member, Bax, are also represented

c into the cytoplasm, thereby blocking its ability to promote Apaf-1-mediated caspase-9 activation, it has been speculated that Bcl-2 and its homologues function to keep mitochondrial membranes intact.[9,13] The alternative view is based on the discovery that pro-survival *C. elegans* CED-9 prevents CED-3 caspase activation by blocking its adapter CED-4.[1] Since Bcl-2 and its pro-survival relatives do not bind to Apaf-1,[14,15] mammalian Bcl-2-like molecules would have to interact with other adapters that activate 'initiator caspases' other than caspase-9. Support for this idea comes from the observation that Bcl-2 over-expression inhibits apoptosis of haematopoietic cells in mice more potently than loss of Apaf-1 or caspase-9 (V Marsden, JM Adams, DCS Huang and A Strasser, unpublished observations).

Pro-Apoptotic members of the Bcl-2 family

One thing is now generally accepted: pro-apoptotic members of the Bcl-2 family are essential for the initiation of those pathways to apoptosis that are regulated by their pro-survival relatives. There are two structurally distinct subgroups of pro-apoptotic members of the Bcl-2 family. Mammalian Bax, Bak, Bok/Mtd, Bcl-x_S (a splice variant of the *bcl-x* gene) and Bcl-G_L have two or three BH regions (Figure 1) and appear to be structurally very similar to their pro-survival relatives.[8,9,16] In contrast, mammalian Bik/Nbk, Blk, Bad, Hrk/DP5, Bid, Bim/Bod, Noxa, Puma/Bbc3, Bcl-G_s (a splice variant of the *bcl-g* gene), Bmf and *C. elegans* EGL-1 share with each other only the short nine amino acid BH3 region but are otherwise unique (Figure 1).[17] These so-called BH3-only proteins bind via their α-helical BH3 domain to a groove formed by the BH1 and BH2 domains on the surface of pro-survival Bcl-2 family members and this interaction is required for their ability to kill cells.[17] Bid has also been shown to bind to Bax and Bak,[18] but it is currently unclear whether the interaction of Bid with pro-survival Bcl-2-like proteins or that with Bax/Bak-like proteins is of higher affinity and which one is critical for its pro-apoptotic activity.

Function of BH3-only proteins

Genetic studies have shown that BH3-only proteins are essential initiators of programmed cell death in *C. elegans* and mice.[17] In *C. elegans* hermaphrodites, a gain-of-function mutation causing inappropriate expression of the BH3-only protein EGL-1 causes abnormal death of a group of neurons.[19,20] This death can be prevented by a (single amino acid) gain-of-function (gf) mutation in the Bcl-2 homologue, CED-9, that abolishes its interaction with EGL-1, or by loss-of-function (lf) mutations of the caspase CED-3 or its adapter, CED-4. Conversely, EGL-1-deficiency prevents all developmentally programmed death of somatic cells.[19] This phenotype is similar to that caused by the CED-9 gain-of-function (gf) mutation or by loss of the caspase CED-3 or its adapter, CED-4.[21] These results indicate that the BH3-only protein EGL-1 activates the death effector machinery by binding to pro-survival CED-9, thereby blocking its ability to inhibit CED-4-mediated CED-3 caspase activation.

DNA damage-induced apoptosis of *C. elegans* germ cells can be blocked by the *ced*-9 gf as well as by *ced*-3 and *ced*-4 lf mutations but this cell death is only partially inhibited by an *egl*-1 lf mutation.[22] Although this might indicate that *C. elegans* has one or more additional BH3-only proteins, EGL-1 clearly plays a dominant and essential role in the induction of programmed death of somatic cells in this species.

Due to the existence of at least 10 BH3-only proteins, initiation of apoptosis is forcibly more complex in mammals, but gene targeting experiments have begun to demonstrate their essential roles in programmed cell death in mice (Figure 2). Bim-deficient mice accumulate 2–5-fold increased numbers of lymphoid and myeloid cells but have normal numbers of erythrocytes and megakaryocytes.[23] The $bim^{-/-}$ lymphocytes are resistant to certain apoptotic stimuli that can be antagonized by Bcl-2 over-expression, such as cytokine deprivation or calcium flux, but have near normal sensitivity to other Bcl-2-inhibitable death stimuli, including DNA damage or treatment with phorbol ester or glucocorticoids.[23] Mice lacking Bid have reduced sensitivity to Fas-induced hepatocyte destruction but their lymphocytes are normally sensitive to this treatment.[24] The latter observation is consistent with the notion that Bcl-2 and its pro-survival homologues do not regulate death receptor-induced apoptosis in haematopoietic cells.[3,25–27] Collectively, these results indicate that different cell types may require different BH3-only proteins to activate programmed cell death. The data also indicate that within a given cell type different BH3-only proteins may be required for activating the apoptosis machinery in response to different conditions of stress (Figure 2).

Functional interactions between mammalian BH3-only proteins and Bax/Bak-like proteins

Although mice lacking only Bax or Bak have relatively minor or no abnormalities in programmed cell death, those animals lacking both proteins ($bax^{-/-}bak^{-/-}$ mice) have substantial accumulation of extra cells in many tissues, causing

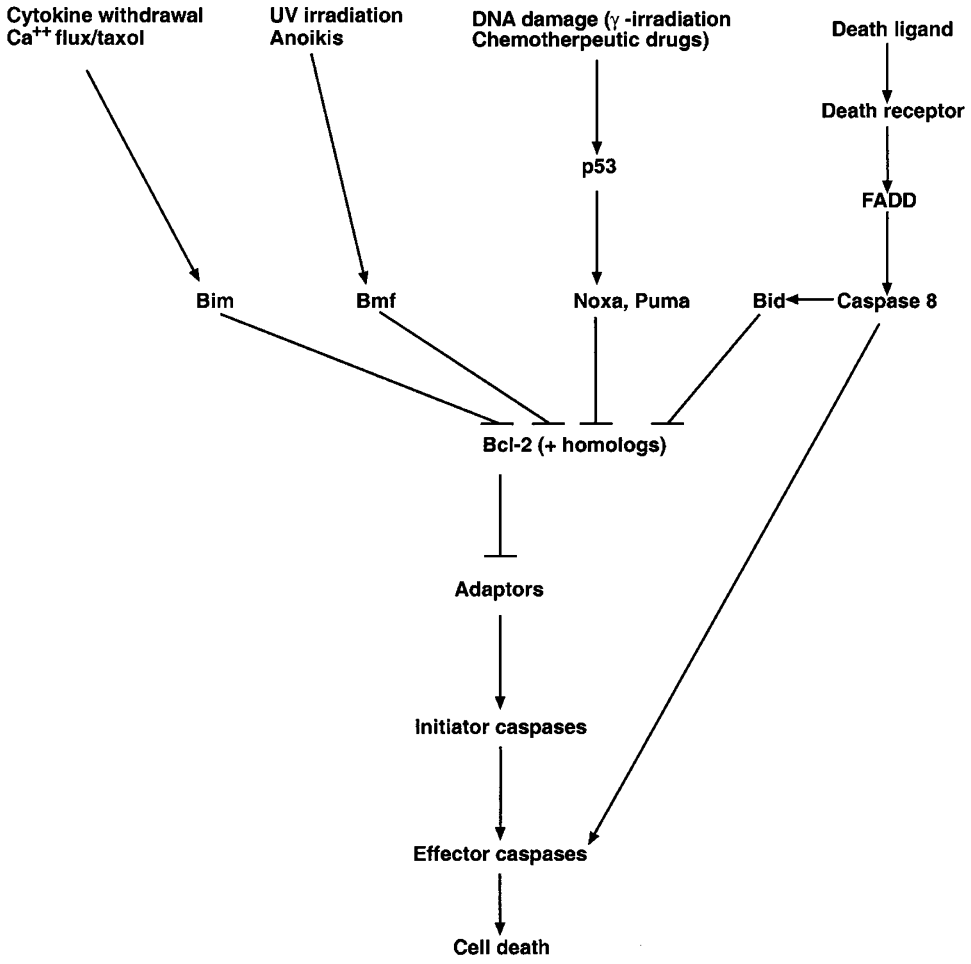

Figure 2 Different stress conditions require different BH3-only proteins to activate the apoptosis effector machinery. Some of the signalling pathways that are indicated are based on results from studies of knock-out mice (Bim, Bid). The others are based on biochemical and expression analyses but have not yet been proven by gene targeting in mice

persistence of interdigitating webbing and progressive lymphadenopathy.[28] Intriguingly, the lymphoid phenotype of $bax^{-/-}bak^{-/-}$ mice[28] is remarkably similar to that caused by Bim-deficiency,[23] indicating that Bim and Bax/Bak might act in the same pathway. Recent experiments with transfected cells have shown that over-expression of Bim or other BH3-only proteins (Bad, Bid, Noxa) can only kill cells that contain either Bax or Bak.[29,30] Since Bim, Bad and Noxa only bind to Bcl-2 and its pro-survival relatives but not to Bax/Bak-like proteins,[17] we propose the following mechanism (Figure 3). When BH3-only proteins become activated through transcriptional or post-translational processes (see below), they bind to Bcl-2 and its homologues, thereby changing their conformation. We speculate that in their 'switched' state Bcl-2-like molecules resemble Bax/Bak and that this forms the nucleation for homotypic 'prion'-like aggregation of Bax/Bak and 'switched' Bcl-2-like molecules.[1] This idea is supported by the extensive structural similarity observed between Bax and Bcl-x_L.[16,31] Since Bax/Bak-like proteins have not been found in *C. elegans*, we believe that CED-9 can function as either, a Bcl-2 or a Bax/Bak-like protein. Wild-type CED-9 enhances (rather than suppresses) programmed cell death in *C. elegans* carrying a mutation in *ced*-3 that reduces but does not abolish caspase activity.[32] This observation provides evidence that, depending on its conformation and/or other events in the cell, CED-9 can inhibit or promote cell killing. We further propose that Bax/Bak and 'switched' Bcl-2-like molecules create a platform that promotes recruitment of CED-4-like adaptors thereby promoting aggregation and autocatalytic activation of 'initiator caspases' (Figure 3).[1]

Transcriptional control of BH3-only genes

The pro-apoptotic activity of BH3-only proteins is subject to stringent control, both at the transcriptional and the post-translational level.[17] In *C. elegans*, expression of EGL-1 in hermaphrodite specific neurons (HSN) is normally repressed by the transcription factor TRA-1A in hermaphrodites, but not in males.[20] EGL-1 expression must also be regulated by other factors since the *egl*-1 (gf) mutation, which prevents binding of the TRA-1A repressor to a regulatory region within the *egl*-1 gene, does not cause abnormal death of other cell types.[20] CES-1, a member of the Snail family of zinc finger containing transcription

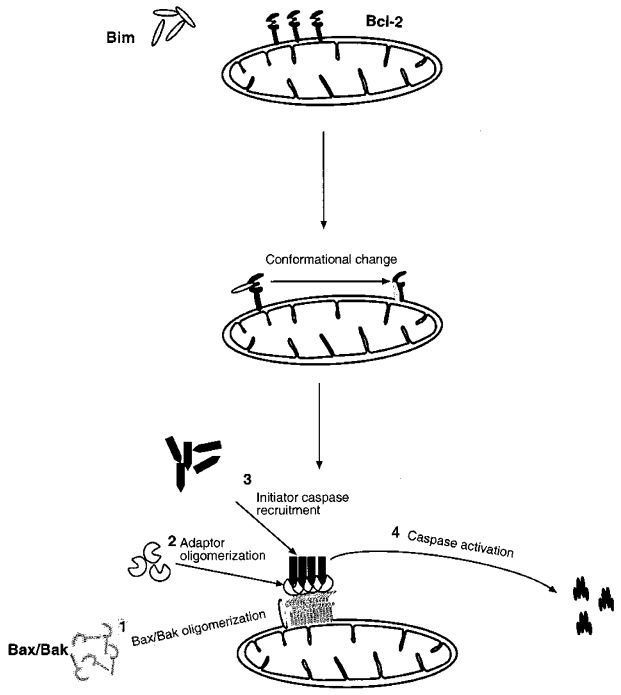

Figure 3 Speculative model for the biochemical action of BH3-only proteins and their functional interaction with Bax/Bak-like proteins and Bcl-2-like pro-survival proteins. The protein interactions that are shown here to occur on the outer mitochondrial membrane may also occur on the cytoplasmic aspect of the nuclear envelope and the ER

factors, and CES-2, a member of the PAR family of transcription factors, are possible candidates for regulating EGL-1 expression. A group of neurons in the pharynx survive inappropriately in *ces*-1 (gf) mutants and *ces*-2 (lf) mutant worms.[33,34] Genetic studies indicate that CES-2 represses the pro-survival function of CES-1, but it is presently unclear whether CES-1 promotes survival by repressing EGL-1 expression, by enhancing CED-9 expression or by a different mechanism.

In mammals, at least four BH3-only genes are subject to transcriptional control.[17] These include *hrk/dp5*,[35–37] *noxa*,[38] *puma/bbc3*[39–41] and *bim*.[42–46] During embryogenesis, Hrk/DP5 expression is induced in those neuronal tissues that contain a relatively large number of apoptotic cells, including the trigeminal and dorsal root ganglia and the anterior horn of the spinal cord.[36] In cultured neurons, Hrk/DP5 expression is up-regulated upon NGF withdrawal or treatment with amyloid β protein (Aβ) and its levels peak at the time when these cells are committed to die.[36] Studies with chemical inhibitors have demonstrated that transcriptional activation of the *hrk/dp5* gene requires stress-activated/c-jun N-terminal (SAPK/JNK) kinase.[45,47]

Noxa and Puma/Bbc3 are both regulated by the tumour suppressor p53.[38–41] Noxa was cloned by mRNA differential display of normal and p53/IRF-1 doubly-deficient mouse embryo fibroblasts.[38] Puma/Bbc3 was cloned by three different methods: microarray analysis[39] or serial analysis of gene expression (SAGE) analysis[40] comparing control cells with cells over-expressing p53 and by yeast two-hybrid library screening using Bcl-2 as bait.[41] In response to DNA damage, Noxa and Puma/Bbc3 mRNA and protein expression levels are induced in a p53-dependent manner not only in thymocytes but also in fibroblasts.[38–41] Why then do only thymocytes activate the apoptotic machinery in response to DNA damage, while fibroblasts undergo cell cycle arrest? Perhaps other pro-apoptotic molecules that are only expressed in thymocytes must also be present for cell death to occur. Alternatively, fibroblasts but not thymocytes might express sufficient levels of anti-apoptotic proteins to be able to survive transient induction of Noxa and Puma/Bbc3. It is noteworthy that Puma/Bbc3 expression is also induced in thymocytes by treatment with dexamethasone,[41] which kills lymphoid cells in a p53-independent manner.[48–50] It is therefore possible that Puma/Bbc3 is critical for apoptosis induced by a range of stress signals that act through a variety of transcriptional regulators.

Transcriptional control of Bim appears to be complex, as different groups have reported different modes of regulation. These discrepancies may have resulted from the analysis of different types of tissues, perhaps indicating cell type-specific transcriptional regulation of the *bim* gene. Growth factor withdrawal was shown to cause increased Bim expression in various populations of neuronal[42,43] and hematopoietic cell types.[44,46] Interestingly, in NGF-deprived neurons apparently only one isoform of Bim, Bim_{EL}, is up-regulated.[42,43] Since all three isoforms of Bim (Bim_{EL}, Bim_L and Bim_S) are synthesised from the same transcript,[51] preponderance of the Bim_{EL} form may represent an additional level of regulation at the level of pre-mRNA splicing in addition to transcriptional induction. Growth factor withdrawal-induced up-regulation of Bim expression was shown to require JNK activation in neurons[42,43,45] but depended on the forkhead transcription factor FKHR-L1 in hematopoietic cells.[44] Other studies with haematopoietic cell lines indicated that cytokine (IL-3) stimulation represses Bim expression through activation of the MAPK pathway and/or phosphatidylinositol 3′ (PI3) kinase.[46] These conclusions are based on experiments using either chemcial inhibitors or over-expression of signalling molecules. A better understanding of the role of JNK, FKHR-L1, the MAPK pathway and PI3-kinase in the control of Bim expression will only be gained through studies of cells from knock-out mice that lack these transcription factors or signal transducers.

Post-translational regulation of BH3-only proteins

Several BH3-only proteins are present in healthy cells at levels that are relatively easily detectable. These proteins are regulated by post-translational modifications that result in conformational changes to cause release from an inactive complex and increased affinity or accessibility to anti-apoptotic Bcl-2 homologues (Figure 4). Pro-apoptotic activity of different BH3-only proteins is unleashed by different post-translational mechanisms.[17]

In cells that are stimulated by growth factors, Bad is phosphorylated at several serine residues and this allows

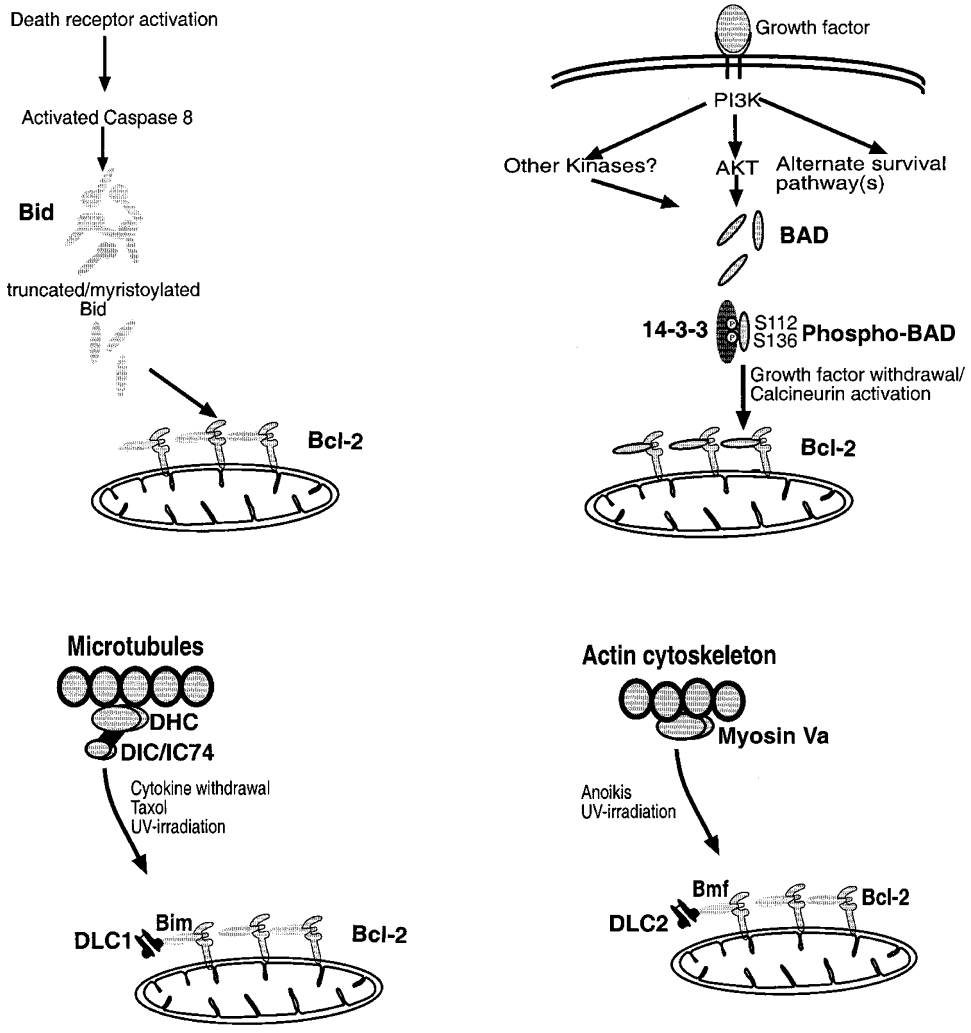

Figure 4 Models for post-translational regulation of the BH3-only proteins Bad, Bid, Bim and Bmf

its sequestration in the cytoplasm by binding to 14-3-3 scaffold proteins (Figure 4).[52] The phosphorylation of conserved residues serine 112 and serine 136 has been attributed to different kinases, including AKT/PKB,[53,54] a signal transducer within the PI3-kinase pathway, protein kinase A (PKA), which is located at mitochondria,[55] and Raf-1 which was reported to be targeted to mitochondria by binding to Bcl-2.[56] PKA has also been shown to phosphorylate serine 155 within the BH3 domain of Bad, thereby reducing its affinity for pro-survival Bcl-2 family members.[57] It therefore appears that reprieve from a Bad-imposed death sentence can occur at several locations within the cell. An intriguing conundrum is how Bad can be modulated by PKA or Raf-1 on mitochondria without being bound to any of the anti-apoptotic Bcl-2 family members. Perhaps Bad has higher affinity for these kinases than for Bcl-2-like molecules? Alternatively, during translocation inside cells, Bad may be bound to a chaperone that directs it primarily to these kinases rather than to Bcl-2-like molecules. Withdrawal of essential growth factors results in accumulation of de-phosphorylated Bad. Moreover, under certain stress conditions (at least in some cell types), Bad was shown to be actively de-phosphorylated by calcineurin.[58] De-phosphorylated Bad is released from 14-3-3 and becomes free to interact with anti-apoptotic Bcl-2 family members,[52] thereby activating the apoptotic effector machinery (Figure 4). Although it is widely believed that Bad is critical for growth factor withdrawal-induced apoptosis, there is so far no evidence for this from gene knock-out studies in mice. Accumulation of lymphocytes, macrophages and granulocytes in *bim*$^{-/-}$ mice[23] and resistance of *bim*$^{-/-}$ lymphocytes[23] and neurons[42,43] to cytokine withdrawal in culture indicates that Bim is an essential initiator of this pathway to apoptosis. Since Bim-deficient lymphocytes and neurons are not completely resistant to cytokine withdrawal and since many cell types (e.g. erythrocytes) do not accumulate abnormally in *bim*$^{-/-}$ mice,[23] it is possible that several BH3-only proteins cooperate in growth factor deprivation-induced apoptosis. We therefore anticipate that interesting phenotypes will emerge in mice lacking two or more BH3-only proteins, such as Bim and Bad.

Bik is another BH3-only protein whose activity can be regulated by phosphorylation (residues threonine 33 and serine 35), possibly by a casein kinase II-related enzyme.[59] Opposite to the case of Bad, phosphorylation increases the pro-apoptotic potency of Bik by a presently unknown mechanism that does not affect its affinity for Bcl-2. Since casein kinase II is ubiquitously expressed and constitutively active,[60] how is Bik kept inactive in healthy cells? Perhaps casein kinase activity increases in response to certain stress conditions, but so does the activity of the phosphatase PP2A that can negate casein kinase II function.[61]

Bid is an unusual BH3-only protein since it has been shown to bind not only to Bcl-2-like pro-survival proteins but also to the Bax/Bak-like proteins.[18,62] However, these conclusions are based on over-expression studies and the affinities of Bid-Bcl-2 and Bid-Bax interaction have not yet been reported. It is therefore unclear which of these interactions is likely to dominate in a physiological setting and it remains possible that activation of Bid (and other BH3-only proteins) causes oligomerisation of Bax/Bak-like proteins indirectly by binding to and changing the conformation of Bcl-2-like molecules (Figure 3). Ligation of 'death receptors' activates caspase-8, which processes the inactive cytosolic form of Bid into a truncated fragment (tBid) that translocates to mitochondria (Figure 4).[63,64] Targeting of tBid to mitochondria is facilitated by N-myristoylation at a site that becomes available for modification after caspase-mediated processing (Figure 4).[65] The truncated tBid also appears to have increased affinity for anti-apoptotic Bcl-2 as well as for Bax/Bak-like proteins and it is thought that this causes Bax/Bak oligomerisation and cytochrome *c* release.[62,66] Recently it was shown that caspase-8-mediated cleavage of Bid is attenuated by casein kinase I- or II-mediated phosphorylation at serine 61 and serine 64 (Figure 4).[60] This implies that caspase-induced Bid processing must be preceded by activation of a phosphatase that can de-phosphorylate these sites. The Ser/Thr phosphatase 2A (PP2A) could carry out this function because these sites (serine 61 and serine 64) are potential substrates for PP2A and because PP2A activity is greatly increased in Jurkat cells undergoing Fas-induced apoptosis.[61] Since Bid-deficiency renders only hepatocytes but not lymphocytes resistant to anti-Fas antibodies and since Bid is activated downstream of caspase-8,[63,64] it appears that Bid forms part of an amplification mechanism that is critical for 'death receptor'-induced apoptosis in only certain cell types. In fact, since caspases other than caspase-8 can also process Bid,[63,64] Bid may function as part of a general amplification mechanism and not be restricted to 'death receptor' signalling.

Bim and Bmf represent two BH3-only proteins that are regulated by sequestration to cytoskeletal structures inside cells (Figure 4).[67,68] Although Bim is subject to transcriptional regulation (see above), many cell types express this protein, particularly the Bim_{EL} isoform, at relatively easily detectable levels under normal conditions.[69] In healthy cells, Bim_{EL} an Bim_L are sequestered to the microtubular dynein motor complex through interaction with DLC1/LC8 (Figure 4).[67] Bim_S does not bind to DLC1 and the dynein motor complex and this appears to be the reason why it has greater killing potency than Bim_{EL} and Bim_L, because mutations in Bim_L that abolish its interaction with DLC1, behave like Bim_S. Bim_S has not yet been demonstrated in normal cells,[69] perhaps indicating that this protein may only be synthesised in cells that need to be killed very rapidly without possibly for reprieve from the death sentence by post-translational regulation. Certain stress conditions, such as UV-irradiation or treatment with taxol, cause release of Bim_{EL} and Bim_L (still bound to DLC1) from the dynein motor complex, enabling them to translocate and bind to Bcl-2 or its homologues (Figure 4). Bim release takes place in the absence of caspase activation,[67] demonstrating that it constitutes an upstream event in apoptosis signalling and is not part of an amplification mechanism. Since Bim is released together with DLC1, we suspect that post-translational modifications of components of the dynein motor complex that normally bind DLC1 unleash the killer Bim. Enzymes that are known to affect dynein motor function, such as cyclin dependent kinase CDK5, are candidate regulators of this process.

The BH3-only protein Bmf on the other hand is sequestered to the actin cytoskeleton-based myosin V motor complex through its interaction with dynein light chain DLC2 (Figure 4).[68] Bmf is released together with its partner DLC2 from the myosin V motor complex in response to stress stimuli, such as detachment of adherent cells from their substratum (anoikis) or exposure to actin depolymerising drugs (Figure 4). In contrast, treatment with taxol, which causes release of Bim, has little effect on the cellular distribution of Bmf.[68] The molecular events that lead to release of Bmf from the myosin V complex are unknown, but since Bmf is released together with DLC2, we speculate that it involves modifications on components of the myosin V motor to which DLC2 is docked in healthy cells. A number of enzymes that affect myosin V function, such as calmodulin kinase or the cysteine protease calpain, are candidate regulators of this process.

Conclusions and perspectives

It is now firmly established that BH3-only proteins are essential initiators of programmed cell death in species as distantly related as *C. elegans* and mice. These proteins trigger apoptosis by binding to Bcl-2 or its homologs and, at least in mammals, the presence of Bax/Bak-like proteins is also required for their ability to kill cells. Certain BH3-only proteins are regulated at the transcriptional level while others function as sentinels for intra-cellular stress and damage by sequestration to autoimmune disease. Many questions remain and we anticipate that research on BH3-only proteins over the coming years will provide interesting insight. Experiments with gene knock-out mice will determine which developmental cell deaths are activated by which BH3-only proteins. Insight into developmental cell death may also come from the identification and functional studies of BH3-only genes in other model organisms, such as flies, frogs and zebra fish. It will be important to further define the transcriptional and post-translational control mechanisms that

regulate the pro-apoptotic activity of BH3-only proteins. It will be challenging but particularly important to determine which of the mechanisms that have been identified in over-expression experiments are significant under physiological conditions. This will require generation of 'knock-in' mice in which sequences implicated in BH3 protein regulation (e.g. sites for phosphorylation, myristoylation, caspase cleavage, protein-protein interaction, transcription factor binding) have been specifically mutated. Finally, it will be important to test which diseases, in addition to autoimmunity,[23] can be caused by mutations in BH3-only genes. For example, the human *bmf* gene is located on chromosome 15q14,[68] a site lost in a sizeable fraction metastatic breast, lung, pancreatic and colon carcinomas but not primary tumours from these tissues.[70] Since the Bmf protein is activated by anoikis, which is thought to function as a barrier against mis-localisation of cells and metastatic spread of tumours,[71] it may be regarded as a candidate tumour suppressor. It will also be important to investigate whether BH3-only proteins are involved in killing cells in degenerative disorders. Intriguingly, loss of only one allele of *bim* prevents polycystic kidney degeneration in mice deficient for Bcl-2.[72] Further studies of these critical apoptosis initiators may therefore ultimately lead to the development of drugs that mimic or activate BH3-only proteins for treatment of cancer or autoimmune diseases, or for generating drugs that block BH3-only protein function to alleviate degenerative diseases.

Acknowledgements

We wish to thank all of our past and present colleagues, particularly D Huang, J Adams, L O'Reilly, A Villunger, S Cory, L O'Connor, L Cullen, K Scalzo, L Tai, M Pellegrini, L Coultas, K Newton, V Marsden, A Harris, S Bath, P Bouillet and D Vaux, for their help with our research and for discussions. Work in our laboratory is supported by grants and fellowships from the NHMRC (Canberra), the NIH, the Dr. Josef Steiner Cancer Research Foundation, the Leukemia and Lymphoma Society of America and the Anti-Cancer Council of Victoria.

References

1. Strasser A, O'Connor L and Dixit VM (2000) Apoptosis signaling. Annu. Rev. Biochem. 69: 217 – 245
2. Ashkenazi A and Dixit VM (1998) Death receptors: signaling and modulation. Science 281: 1305 – 1308
3. Strasser A, Harris AW, Huang DCS, Krammer PH and Cory S (1995) Bcl-2 and Fas/APO-1 regulate distinct pathways to lymphocyte apoptosis. EMBO J. 14: 6136 – 6147
4. Newton K, Harris AW, Bath ML, Smith KGC and Strasser A (1998) A dominant interfering mutant of FADD/Mort1 enhances deletion of autoreactive thymocytes and inhibits proliferation of mature T lymphocytes. EMBO J. 17: 706 – 718
5. Varfolomeev EE, Schuchmann M, Luria V, Chiannilkulchai N, Beckmann JS, Mett IL, Rebrikov D, Brodianski VM, Kemper OC, Kollet O, Lapidot T, Soffer D, Sobe T, Avraham KB, Goncharov T, Holtmann H, Lonai P and Wallach D (1998) Targeted disruption of the mouse *Caspase 8* gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. Immunity 9: 267 – 276
6. Haraguchi M, Torii S, Matsuzawa S, Xie Z, Kitada S, Krajewski S, Yoshida H, Mak TW and Reed JC (2000) Apoptotic protease activating factor 1 (Apaf-1)-independent cell death suppression by Bcl-2. J. Exp. Med. 191: 1709 – 1720
7. Li K, Li Y, Shelton JM, Richardson JA, Spencer E, Chen ZJ, Wang X and Williams RS (2000) Cytochrome *c* deficiency causes embryonic lethality and attenuates stress-induced apoptosis. Cell 101: 389 – 399
8. Adams JM and Cory S (1998) The Bcl-2 protein family: arbiters of cell survival. Science 281: 1322 – 1326
9. Gross A, McDonnell JM and Korsmeyer SJ (1999) Bcl-2 family members and the mitochondria in apoptosis. Genes Dev. 13: 1899 – 1911
10. Lithgow T, van Driel R, Bertram JF and Strasser A (1994) The protein product of the oncogene *bcl-2* is a component of the nuclear envelope, the endoplasmic reticulum and the outer mitochondrial membrane. Cell Growth Differ. 5: 411 – 417
11. Krajewski S, Krajewska M, Shabaik A, Wang HG, Irie S, Fong L and Reed JC (1994) Immunohistochemical analysis of *in vivo* patterns of Bcl-X expression. Cancer Res. 54: 5501 – 5507
12. O'Reilly LA, Print C, Hausmann G, Moriishi K, Cory S, Huang DCS and Strasser A (2001) Tissue expression and subcellular localization of the pro-survival molecule Bcl-w. Cell Death Differ. 8: 486 – 494
13. Green DR and Reed JC (1998) Mitochondria and apoptosis. Science 281: 1309 – 1311
14. Moriishi K, Huang DCS, Cory S and Adams JM (1999) Bcl-2 family members do not inhibit apoptosis by binding the caspase-activator Apaf-1. Proc. Natl. Acad. Sci. USA. 96: 9683 – 9688
15. Hausmann G, O'Reilly LA, van Driel R, Beaumont JG, Strasser A, Adams JM and Huang DCS (2000) Pro-apoptotic apoptosis protease-activating Factor 1 (Apaf-1) has a cytoplasmic localization distinct from Bcl-2 or Bcl-x_L. J. Cell Biol. 149: 623 – 634
16. Suzuki M, Youle RJ and Tjandra N (2000) Structure of Bax: coregulation of dimer formation and intracellular localization. Cell 103: 645 – 654
17. Huang DCS and Strasser A (2000) BH3-only proteins – essential initiators of apoptotic cell death. Cell 103: 839 – 842
18. Wang K, Yin X-M, Chao DT, Milliman CL and Korsmeyer SJ (1996) BID: a novel BH3 domain-only death agonist. Genes Dev. 10: 2859 – 2869
19. Conradt B and Horvitz HR (1998) The *C. elegans* protein EGL-1 is required for programmed cell death and interacts with the Bcl-2-like protein CED-9. Cell 93: 519 – 529
20. Conradt B and Horvitz HR (1999) The TRA-1A sex determination protein of *C. elegans* regulates sexually dimorphic cell deaths by repressing the *egl-1* cell death activator gene. Cell 98: 317 – 327
21. Ellis RE, Yuan J and Horvitz HR (1991) Mechanisms and functions of cell death. Annu. Rev. Cell Biol. 7: 663 – 698
22. Gartner A, Milstein S, Ahmed S, Hodgkin J and Hengartner MO (2000) A conserved checkpoint pathway mediates DNA damage-induced apoptosis and cell cycle arrest in *C. elegans*. Mol. Cell 5: 435 – 443
23. Bouillet P, Metcalf D, Huang DCS, Tarlinton DM, Kay TWH, Köntgen F, Adams JM and Strasser A (1999) Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. Science 286: 1735 – 1738
24. Yin X-M, Wang K, Gross A, Zhao Y, Zinkel S, Klocke B, Roth KA and Korsmeyer SJ (1999) Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. Nature 400: 886 – 891
25. Huang DCS, Cory S and Strasser A (1997) Bcl-2, Bcl-X_L and adenovirus protein E1B19kD are functionally equivalent in their ability to inhibit cell death. Oncogene 14: 405 – 414
26. Huang DC, Hahne M, Schroeter M, Frei K, Fontana A, Villunger A, Newton K, Tschopp J and Strasser A (1999) Activation of Fas by FasL induces apoptosis by a mechanism that cannot be blocked by Bcl-2 or Bcl-x_L. Proc. Natl. Acad. Sci. USA. 96: 14871 – 14876
27. Villunger A, O'Reilly LA, Holler N, Adams JM and Strasser A (2000) Fas ligand, Bcl-2, G-CSF and p38 MAPK: regulators of distinct cell death and survival pathways in granulocytes. J. Exp. Med. 192: 647 – 657
28. Lindsten T, Ross AJ, King A, Zong W, Rathmell JC, Shiels HA, Ulrich E, Waymire KG, Mahar P, Frauwirth K, Chen Y, Wei M, Eng VM, Adelman DM, Simon MC, Ma A, Golden JA, Evan G, Korsmeyer SJ, MacGregor GR and Thompson CB (2000) The combined functions of proapoptotic Bcl-2 family members Bak and Bax are essential for normal development of multiple tissues. Mol. Cell 6: 1389 – 1399

29. Zong WX, Lindsten T, Ross AJ, MacGregor GR and Thompson CB (2001) BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. Genes Dev. 15: 1481 – 1486
30. Cheng EH, Wei MC, Weiler S, Flavell RA, Mak TW, Lindsten T and Korsmeyer SJ (2001) Bcl-2, bcl-x(l) sequester bh3 domain-only molecules preventing bax- and bak-mediated mitochondrial apoptosis. Mol. Cell 8: 705 – 711
31. Muchmore SW, Sattler M, Liang H, Meadows RP, Harlan JE, Yoon HS, Nettesheim D, Chang BS, Thompson CB, Wong S-L, Ng S-C and Fesik SW (1996) X-ray and NMR structure of human Bcl-x_L, an inhibitor of programmed cell death. Nature 381: 335 – 341
32. Hengartner MO and Horvitz HR (1994) Activation of *C. elegans* cell death protein CED-9 by an amino-acid substitution in a domain conserved in Bcl-2. Nature 369: 318 – 320
33. Metzstein MM, Hengartner MO, Tsung N, Ellis RE and Horvitz HR (1996) Transcriptional regulator of programmed cell death encoded by *Caenorhabditis elegans* gene *ces*-2. Nature 382: 545 – 547
34. Metzstein MM and Horvitz HR (1999) The *C. elegans* cell death specification gene *ces-1* encodes a snail family zinc finger protein. Mol. Cell 4: 309 – 319
35. Inohara N, Ding L, Chen S and Núñez G (1997) *Harakiri*, a novel regulator of cell death, encodes a protein that activates apoptosis and interacts selectively with survival-promoting proteins Bcl-2 and Bcl-X_L. EMBO J. 16: 1686 – 1694
36. Imaizumi K, Tsuda M, Imai Y, Wanaka A, Takagi T and Tohyama M (1997) Molecular cloning of a novel polypeptide, DP5, induced during programmed neuronal death. J. Biol. Chem. 272: 18842 – 18848
37. Imaizumi K, Morihara T, Mori Y, Katayama T, Tsuda M, Furuyama T, Wanaka A, Takeda M and Tohyama M (1999) The cell death-promoting gene DP5, which interacts with the Bcl-2 family, is induced during neuronal apoptosis following exposure to amyloid β protein. J. Biol. Chem. 274: 7975 – 7981
38. Oda E, Ohki R, Murasawa H, Nemoto J, Shibue T, Yamashita T, Tokino T, Taniguchi T and Tanaka N (2000) Noxa, a BH3-only member of the bcl-2 family and candidate mediator of p53-induced apoptosis. Science 288: 1053 – 1058
39. Nakano K and Wousden KH (2001) *PUMA*, a novel proapoptotic gene, is induced by p53. Mol. Cell 7: 683 – 694
40. Yu J, Zhang L, Hwang PM, Kinzler KW and Vogelstein B (2001) PUMA induces the rapid apoptosis of colorectal cancer cells. Mol. Cell 7: 673 – 682
41. Han J, Flemington C, Houghton AB, Gu Z, Zambetti GP, Lutz RJ, Zhu L and Chittenden T (2001) Expression of bbc3, a pro-apoptotic BH3-only gene, is regulated by diverse cell death and survival signals. Proc. Natl. Acad. Sci. USA. 98: 11318 – 11323
42. Putcha GV, Moulder KL, Golden JP, Bouillet P, Adams JM, Strasser A and Johnson EMJ (2001) Induction of Bim, a proapoptotic BH3-only Bcl-2 family member, is critical for neuronal apoptosis. Neuron 29: 615 – 628
43. Whitfield J, Neame SJ, Paquet L, Bernard O and Ham J (2001) Dominant-negative c-Jun promotes neuronal survival by reducing BIM expression and inhibiting mitochondrial cytochrome *c* release. Neuron 29: 629 – 643
44. Dijkers PF, Medemadagger RH, Lammers JJ, Koenderman L and Coffer PJ (2000) Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. Curr. Biol. 10: 1201 – 1204
45. Harris CA and Johnson Jr EM (2001) BH3-only Bcl-2 family members are coordinately regulated by the JNK pathway and require Bax to induce apoptosis in neurons. J. Biol. Chem. 276: 37754 – 37760
46. Shinjyo T, Kuribara R, Inukai T, Hosoi H, Kinoshita T, Miyajima A, Houghton PJ, Look AT, Ozawa K and Inaba T (2001) Downregulation of Bim, a proapoptotic relative of Bcl-2, is a pivotal step in cytokine-initiated survival signaling in murine hematopoietic progenitors. Mol. Cell. Biol. 21: 854 – 864
47. Bozyczko-Coyne D, O'Kane TM, Wu ZL, Dobrzanski P, Murthy S, Vaught JL and Scott RW (2001) CEP-1347/KT-7515, an inhibitor of SAPK/JNK pathway activation, promotes survival and blocks multiple events associated with Abeta-induced cortical neuron apoptosis. J. Neurochem. 77: 849 – 863
48. Clarke AR, Purdie CA, Harrison DJ, Morris RG, Bird CC, Hooper ML and Wyllie AH (1993) Thymocyte apoptosis induced by p53-dependent and independent pathways. Nature 362: 849 – 852
49. Lowe SW, Schmitt EM, Smith SW, Osborne BA and Jacks T (1993) p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature 362: 847 – 849
50. Strasser A, Harris AW, Jacks T and Cory S (1994) DNA damage can induce apoptosis in proliferating lymphoid cells via p53-independent mechanisms inhibitable by Bcl-2. Cell 79: 329 – 339
51. O'Connor L, Strasser A, O'Reilly LA, Hausmann G, Adams JM, Cory S and Huang DCS (1998) Bim: a novel member of the Bcl-2 family that promotes apoptosis. EMBO J. 17: 384 – 395
52. Zha J, Harada H, Yang E, Jockel J and Korsmeyer SJ (1996) Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not Bcl-x_L. Cell 87: 619 – 628
53. del Peso L, González-Garcia M, Page C, Herrera R and Nuñez G (1997) Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science 278: 687 – 689
54. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y and Greenberg ME (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 91: 231 – 241
55. Harada H, Becknell B, Wilm M, Mann M, Huang LJ, Taylor SS, Scott JD and Korsmeyer SJ (1999) Phosphorylation and inactivation of BAD by mitochondria-anchored protein kinase A. Mol. Cell 3: 413 – 422
56. Wang H-G, Rapp UR and Reed JC (1996) Bcl-2 targets the protein kinase Raf-1 to mitochondria. Cell 87: 629 – 638
57. Datta SR, Katsov A, Hu L, Petros A, Fesik SW, Yaffe MB and Greenberg ME (2000) 14-3-3 proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation. Mol. Cell 6: 41 – 51
58. Wang H-G, Pathan N, Ethell IM, Krajewski S, Yamaguchi Y, Shibasaki F, McKeon F, Bobo T, Franke TF and Reed JC (1999) Ca^{2+}-induced apoptosis through calcineurin dephosphorylation of BAD. Science 284: 339 – 343
59. Verma S, Zhao L and Chinnadurai G (2001) Phosphorylation of the pro-apoptotic protein bik. mapping of phosphorylation sites and effect on apoptosis. J. Biol. Chem. 276: 4671 – 4676
60. Desagher S, Osen-Sand A, Montessuit S, Magnenat E, Vilbois F, Hochmann A, Journot L, Antonsson B and Martinou J (2001) Phosphorylation of bid by casein kinases i and ii regulates its cleavage by caspase 8. Mol. Cell 8: 601 – 611
61. Santoro MF, Annand RR, Robertson MM, Peng YW, Brady MJ, Mankovich JA, Hackett MC, Ghayur T, Walter G, Wong WW and Giegel DA (1998) Regulation of protein phosphatase 2A activity by caspase-3 during apoptosis. J. Biol. Chem. 273: 13119 – 13128
62. Wei MC, Lindsten T, Mootha VK, Weiler S, Gross A, Ashiya M, Thompson CB and Korsmeyer SJ (2000) tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. Genes Dev. 14: 2060 – 2071
63. Luo X, Budlhardjo I, Zou H, Slaughter C and Wang X (1998) Bid, a Bcl-2 interacting protein, mediates cytochrome *c* release from mitochondria in response to activation of cell surface death receptors. Cell 94: 481 – 490
64. Li H, Zhu H, Xu C-J and Yuan J (1998) Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 94: 491 – 501
65. Zha J, Weiler S, Oh KJ, Wei MC and Korsmeyer SJ (2000) Posttranslational N-myristoylation of BID as a molecular switch for targeting mitochondria and apoptosis. Science 290: 1761 – 1765
66. Desagher S, Osen-Sand A, Nichols A, Eskes R, Montessuit S, Lauper S, Maundrell K, Antonsson B and Martinou J-C (1999) Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. J. Cell Biol. 144: 891 – 901
67. Puthalakath H, Huang DCS, O'Reilly LA, King SM and Strasser A (1999) The pro-apoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. Mol. Cell 3: 287 – 296
68. Puthalakath H, Villunger A, O'Reilly LA, Beaumont JG, Coultas L, Cheney RE, Huang DCS and Strasser A (2001) Bmf: a pro-apoptotic BH3-only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis. Science 293: 1829 – 1832
69. O'Reilly LA, Cullen L, Visvader J, Lindeman G, Print C, Bath ML, Huang DCS and Strasser A (2000) The pro-apoptotic BH3-only protein Bim is expressed in hemopoietic, epithelial, neuronal and germ cells. Am. J. Path. 157: 449 – 461
70. Wick W, Petersen I, Schmutzler RK, Wolfarth B, Lenartz D, Bierhoff E, Hummerich J, Muller DJ, Stangl AP, Schramm J, Wiestler OD and von Deimling A (1996) Evidence for a novel tumor suppressor gene on chromosome 15 associated with progression to a metastatic stage in breast cancer. Oncogene 12: 973 – 978
71. Ruoslahti E and Reed JC (1994) Anchorage dependence, integrins, and apoptosis. Cell 77: 477 – 478
72. Bouillet P, Cory S, Zhang L-C, Strasser A and Adams JM (2001) Degenerative disorders caused by Bcl-2 deficiency are prevented by loss of its BH3-only antagonist Bim. Developmental Cell 1: 645 – 650