Cell. Mol. Life Sci. 64 (2007) 318 – 330
1420-682X/07/030318-13
DOI 10.1007/s00018-006-6364-4

Cellular and Molecular Life Sciences

Review

BH3-only proteins in tumorigenesis and malignant melanoma

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Received: 12 August 2006; received after revision: 2 October 2006; accepted 13 November 2006
Online First 27 December 2006

Abstract. BH3-only proteins are a subset of the Bcl-2 family of apoptotic regulators. BH3-only proteins function as 'damage sensors' in the cell; they are activated in response to cellular stress or DNA damage, whereupon they initiate apoptosis. Apoptosis is the primary mechanism by which the body rids itself of genetically defective cells and is critical for preventing the accumulation of cells with tumorigenic potential. Therefore, dysregulation of BH3-only proteins may promote tumorigenesis. Furthermore, functional apoptosis pathways are required for the success of most cancer treatments, including chemotherapy. Resistance to chemotherapy, as seen with malignant melanoma, often reflects an inability of tumor cells to undergo apoptosis. By deciphering the roles of BH3-only proteins in tumorigenesis, we may learn how to manipulate cell death pathways to overcome apoptotic resistance. This review summarizes the current knowledge of BH3-only proteins and how they contribute to tumorigenesis, with particular attention given to studies involving melanoma.

Keywords. BH3-only protein, Bcl-2 family, apoptosis, tumorigenesis, melanoma.

Introduction

It is now recognized that cancer is not simply a disease of excessive cell proliferation. More accurately, tumor development reflects an *imbalance* between cell production and cell elimination [1]. Over-production may result from the mutation of cell cycle control genes or the hyperactivation of cell proliferation pathways. Ineffective cell elimination, on the other hand, is thought to arise from the deregulation of cell death pathways, such as apoptosis. Apoptosis (programmed cell death) is the primary mechanism by which the body rids itself of damaged, genetically defective, or superfluous cells [2], and is therefore critical for preventing the accumulation of cells with tumorigenic potential. Cancer cells often acquire defects in genes regulating apoptosis, allowing them to evade cell death. Furthermore, conventional cancer treatments such as chemotherapy and radiation work primarily by inducing apoptosis in tumor cells and thus require functional apoptotic pathways [3]. Chemotherapeutic resistance, in many cases, may actually reflect an underlying resistance to apoptosis.

Malignant melanoma is a notoriously apoptotic-resistant tumor type that responds poorly to both chemotherapy and radiation treatment [4]. Melanoma tumors have been shown to exhibit low rates of spontaneous apoptosis compared with other tumor types [5]. It is generally thought that melanocytes acquire a resistance to apoptosis during their transformation from normal to melanoma cells [6]. In accordance with this notion, melanocytic nevus cells show greater resistance to apoptosis than melanocytes

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when grown in collagen gels [7]. Furthermore, melanoma cell lines are usually resistant to drug-induced apoptosis [8]. Melanoma patients with advanced disease have essentially no treatment options and face a dismal prognosis: only 16% of metastatic melanoma cases survive for 5 years [9]. There is an urgent need to develop effective therapies for this disease. Part of the solution lies in understanding why melanoma cells are so resistant to DNA damage-induced apoptosis and how we can manipulate apoptotic pathways to overcome this resistance.

Apoptosis is a complex process, controlled by many different genes and proteins. A particular subset of proteins, known as BH3-only proteins, have recently emerged as critical effectors of apoptosis in mammalian cells. Deciphering the roles of BH3-only proteins in melanoma cell death is critical to understanding how melanoma cells can be induced to undergo cell death. In this review we summarize the current knowledge of BH3-only proteins and how they contribute to tumorigenesis, with particular attention given to studies involving melanoma.

Mechanisms of BH3-only protein-induced cell death

BH3-only proteins function as the "damage sensors" of the cell. They are activated in response to cellular stress or DNA damage, whereupon they initiate apoptosis (Fig. 1) [10]. BH3-only proteins are activated to induce apoptosis by a diverse range of stimuli including cytokine withdrawal, loss of adhesion to the extracellular matrix, DNA damage (by chemotherapeutic drugs or radiation), and oncogene activation [11]. Distinct BH3-only proteins are induced depending on the nature of the cytotoxic stimulus and the tissue type involved.

BH3-only proteins are a subgroup of the Bcl-2 protein family, all of whose members either promote or inhibit apoptosis. The Bcl-2 family can be divided into three functional groups: *(i)* Pro-survival Bcl-2-like members (Bcl-2, Bcl-X_L, Bcl-w, Mcl-1, and A1), *(ii)*pro-apoptotic Bax-like members (Bax, Bak and Bok), and *(iii)* Pro-apoptotic BH3-only members (Puma, Noxa, Bid, Bim, Bmf, Bad, Bik, and Hrk) [2]. The cell controls apoptosis by finely balancing the expression and activities of pro-survival proteins with those of pro-apoptotic proteins. In terms of protein structure, all Bcl-2 family proteins possess one or more Bcl-2 homology (BH) domains, BH1, BH2, BH3, and BH4. Pro-survival members share all four BH domains (except Mcl-1, which does not contain BH4), Bax-like members have three BH domains (BH1, BH2, BH3), and BH3-only members share just the BH3 domain, as their name suggests (Fig. 2) [11].

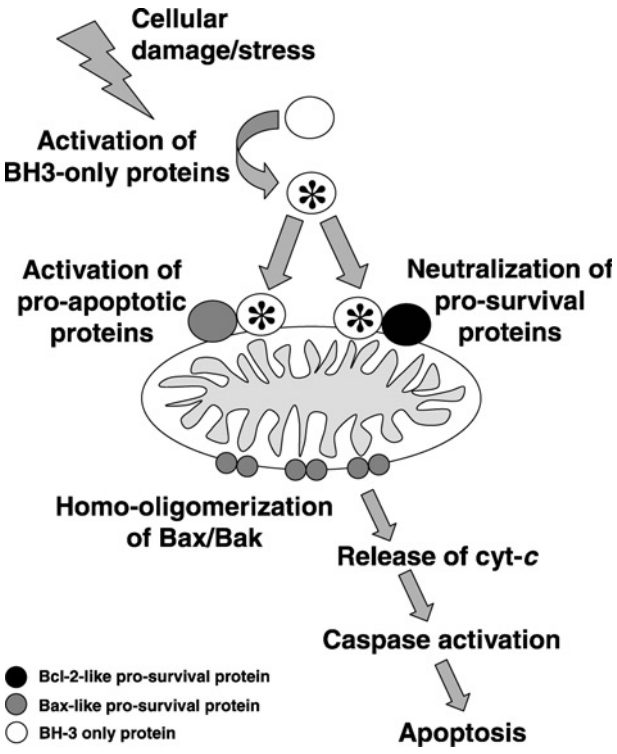

Figure 1. Induction of apoptosis by BH3-only proteins. BH3-only proteins are activated in response to DNA damage or other cellular stress via either transcriptional upregulation (Puma, Noxa, Bim, Hrk) or posttranslational modification (Bid, Bim, Bmf, Bad, Bik). Activated BH3-only proteins interact with Bcl-2-like proteins at the mitochondrial membrane, neutralizing their pro-survival function and "priming" the cell for apoptosis. Activated BH3-only proteins are also thought to activate pro-apoptotic Bax and Bak proteins. Activated Bax/Bak form homo-oligomers that lead to permeabilization of the mitochondrial outer membrane, release of cytochrome c, caspase activation, and, ultimately, apoptotic cell death.

There are two other pro-apoptotic Bcl-2 family proteins, Bcl-X_S and Bcl-X_{AK}, that do not fall into the aforementioned categories. Bcl-X_S contains a BH3 and BH4 domain, while Bcl-X_{AK} contains a BH2 and BH4 domain. Both proteins are alternative splice products of the *bcl-x* gene that codes for Bcl-X_L. The abilities of both Bcl-X_S and Bcl-X_{AK} to induce apoptosis have been described in melanoma cells [12, 13], but their mechanisms of action so far remain unclear.

Multidomain Bax-like proteins appear to possess intrinsic cell death-inducing ability while BH3-only proteins act more indirectly, by engaging other Bcl-2 family members to either suppress pro-survival proteins (Bcl-2, Bcl-X_L) or to activate pro-apoptotic multidomain proteins (Bax, Bak) [14]. BH3-only protein activity is tightly controlled via transcriptional upregulation (Puma, Noxa, Bim, Hrk) or posttranslational modification (Bid, Bim, Bmf, Bad, Bik) [15]. Once activated, BH3-only proteins translocate to the

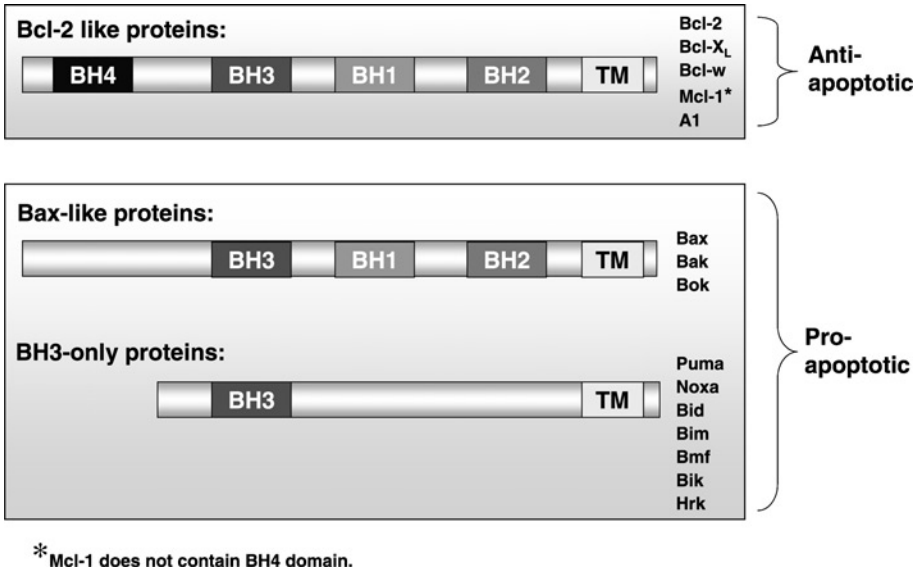

Figure 2. Schematic diagram of the Bcl-2 protein family. Bcl-2 proteins are divided into three functional groups: (i) Bcl-2-like, (ii) Bax-like, and (iii) BH3-only proteins. Anti-apoptotic Bcl-2-like proteins functionally oppose Bax-like and BH3-only proteins, which are both pro-apoptotic. Most Bcl-2-like proteins contain four Bcl-2 homology (BH) domains; BH1, BH2, BH3, and BH4. Bax-like proteins contain two or three BH domains. BH3-only proteins contain only a BH3 domain. Most Bcl-2 family members also express a trans-membrane (TM) domain that facilitates mitochondrial membrane localization.

mitochondria, where they bind to their Bcl-2-like and Bax-like relatives [10]. Binding of BH3-only proteins to Bcl-2-like proteins is thought to neutralize the pro-survival function of the latter and 'prime' the cell for apoptosis [2]. This antagonizing interaction is mediated by the BH3 domain, an amphipathic α helix, which inserts into a hydrophobic groove formed by the BH1, BH2, and BH3 domains on the surface of the Bcl-2-like protein [16]. Changes induced by BH3-only proteins are also thought to activate pro-apoptotic Bax and Bak proteins [17, 18]. Bax or Bak activation is required for apoptosis to proceed [17, 18]. Activated Bax/Bak form homo-oligomers that either directly or indirectly permeabilize the mitochondrial outer membrane [17, 18]. Disruption of the mitochondrial membrane results in the leakage of pro-apoptotic factors (cytochrome c, Smac/DIABLO, AIF) into the cytosol, subsequent apoptosome formation, activation of caspases and, ultimately, cell death [19].

Systematic studies of BH3-only peptide binding affinities suggest that most BH3-only proteins bind selectively, not promiscuously, to Bcl-2-like pro-survival proteins [20–22]. Although Puma, Bim, and t-Bid (the truncated form of Bid) can bind and neutralize all five Bcl-2-like proteins (Bcl-2, Bcl-X_L, Bcl-w, Mcl-1, and A1), other BH3-only members cannot. Specifically, Bad and Bmf bind only to Bcl-2, Bcl-X_L, and Bcl-w; Bik and Hrk bind only to Bcl-X_L, Bcl-w, and A1; and Noxa binds only to A1 and Mcl-1 [2]. Thus, some BH3-only proteins (e.g. Puma and Bim) appear to play broader, more dominant roles in the apoptotic program, whereas others act with more narrow situational specificity.

Models of BH3-only protein-induced Bax/Bak activation

As previously mentioned, BH3-only proteins require Bax and/or Bak activation in order to induce apoptosis [17, 18]. However, the exact mechanism underlying such activation has not been fully elucidated. Two models have been postulated to account for the role of BH3-only proteins in Bax/Bak activation. The first, suggested by Letai et al. [23] and expanded upon by Certo et al. [22], proposes that all BH3-only proteins are either 'activators' or 'sensitizers'. Activators (t-Bid and Bim) interact directly with Bax/Bak, activating them and inducing their oligomerization. However, they are prevented from doing so under normal conditions because they are held inactive by pro-survival Bcl-2-like proteins. Sensitizers (Bad and Bik) are activated following cytotoxic stress, whereupon they bind to Bcl-2-like proteins and cause them to release bound activators [23]. An alternative model proposed by Willis et al. [24] asserts that under normal conditions, Mcl-1 and Bcl-X_L sequester an active form of Bak at the mitochondrion. Following cytotoxic stimuli, activated BH3-only proteins must displace Bak from both Mcl-1 and Bcl-X_L in order to achieve Bak activation. Currently, there is experimental evidence to support both models [2]. Therefore, further study is needed to determine which model, if either, is correct.

Roles of BH3-only proteins in tumorigenesis

Bcl-2-like pro-survival proteins have long been implicated in tumorigenesis. The most well known example is the deregulation of Bcl-2 expression in human follicular lymphomas. This disease is characterized by a t(14;18) chromosomal translocation that places the Bcl-2 gene under control of an immunoglobulin heavy-chain promoter, resulting in constitutive expression of Bcl-2 protein [25, 26]. Bcl-2 and Bcl-X_L overexpression have also been linked to several other malignancies, including acute promyelocytic leukemia, breast cancer, and pancreatic β cell cancer [27]. Meanwhile, the absence of pro-apoptotic Bax-like proteins is associated with tumor progression. For example, loss or mutation of Bax may contribute to colon cancer [28], mammary tumors [29], and brain tumors [30]. However, it is only in recent years that researchers have begun to examine the roles of pro-apoptotic BH3-only proteins in tumorigenesis. Because BH3-only proteins are critical for initiating apoptosis following cellular damage, their proper regulation is a crucial component of tumor suppression. If BH3-only protein expression is lost or altered, apoptosis may be suppressed, leading to the accumulation of damaged cells with tumorigenic potential. Absence of BH3-only protein expression may also promote apoptotic resistance. In the context of cancer treatment, this creates a major therapeutic obstacle.

Puma

Puma (*p*53 *u*pregulated *m*odulator of *a*poptosis) is a highly potent pro-apoptotic protein that induces the rapid and complete death of many malignant cell types, including colorectal cancer, lung cancer, osteosarcoma, glioma, head and neck cancer, and melanoma cells [31–35]. Puma was discovered by three independent groups, two of which were searching for p53-inducible target genes [31, 32], and one of which was screening for Bcl-2-binding partners [36]. Puma contains two consensus p53 binding sites within its promoter region, indicating that it is a direct transcriptional target of p53 [31, 32, 36]. Puma is not known to undergo posttranslational modifications, but upon translation, localizes to mitochondria where it antagonizes pro-survival members of the Bcl-2 family [11]. Puma expression is strongly and rapidly upregulated in response to DNA-damaging agents, such as adriamycin, 5-fluorouracil (5-FU), actinomycin D, etoposide, and ionizing radiation, in a p53-dependent manner [31, 32, 36].

There is abundant experimental evidence to support the notion that Puma is a critical mediator of p53-dependent apoptosis. For example, mouse embryonic fibroblasts (MEFs) transduced with the adenoviral oncoprotein E1A are normally rendered sensitive to p53-mediated apoptosis [37], but Puma$^{-/-}$ MEFs remain strongly resistant to DNA-damaging agents under the same conditions [38]. Furthermore, colorectal and lung cancer cells lacking wild-type p53 are unable to upregulate Puma following DNA damage [31, 32]. Puma expression can also be induced by p53-independent apoptotic stimuli, including glucocorticoid treatment, serum starvation, and hypoxia [31, 32, 36]. However, less is known about the p53-independent mechanisms of Puma induction.

Puma is not required for normal development, and Puma$^{-/-}$ mice suffer no developmental abnormalities. However, thymocytes and myeloid progenitors from these animals are resistant to apoptosis induced by various stimuli, including anticancer drugs, γ-irradiation, cytokine withdrawal, glucocorticoid treatment, staurosporine, and the phorbol ester PMA [39, 40].

Loss of Puma alone has not been shown to cause cancer, but inhibition of Puma expression does promote certain malignant phenotypes. For example, Hemann et al. [41] reported that short hairpin RNA (shRNA)-mediated knockdown of Puma potently induces transformation of MEFs co-expressing the oncogenes E1A and ras. It is well established that p53 mutations cooperate with E1A and ras to promote oncogenic transformation [38, 42, 43]. Thus, Puma loss mimics p53 mutation in this context. Puma suppression can also accelerate lymphomagenesis in an Eμ-myc transgenic mouse model [41]. Eμ-myc transgenic mice express the c-myc oncogene from an Ig heavy-chain enhancer, causing them to develop B cell lymphomas [44]. Hematopoietic stem cells (HSCs) from these mice give rise to lymphomas when transferred into healthy recipient mice, and deletion of p53 accelerates this process [37, 45]. To determine whether deletion of Puma would have a similar effect, Hemann et al. [41] used shRNA against Puma to stably suppress its expression in Eμ-myc HSCs. Upon transferring these cells to normal mice, they observed dramatically accelerated lymphomagenesis with reduced latency in all recipient animals. Thus, Puma loss can mimic the effects of p53 loss/mutation and Puma is a bona fide tumor suppressor.

Considering the essential role of Puma in apoptosis and its 'p53-like' tumor-suppressing effects, it seems intuitive that Puma loss may contribute to human cancer. Accordingly, Puma's chromosomal locus, 19q13.3, is frequently lost in human gliomas [46], neuroblastomas [47], and certain B cell lymphomas [48]. Deletion of chromosomal arm 19q has also been reported in head and neck squamous cell carcinomas (HNSCCs) [49–51] and lung cancers [52–54]. Only

one study, conducted by Hoque et al. [34], has focused specifically on the mutational status of Puma in cancer. Loss of heterozygosity (LOH) analysis of 30 primary HNSCCs and lung tumors detected LOH at 19q in 56% of HNSCCs and 27% of primary lung cancer samples. However, sequence analysis of Puma in 10 cell lines and 30 primary tumors revealed no gene mutations. The only study of Puma expression in melanoma was conducted by our own laboratory [35]. In a tissue microarray analysis of melanoma tumor biopsies, we found that Puma protein expression was reduced in primary malignant melanomas ($n=107$) compared to non-malignant dysplastic nevi ($n=64$) ($p<0.0001$). Puma expression was even further decreased in metastatic melanomas ($n=51$) ($p=0.001$). Puma expression level also inversely correlated with 5-year survival ($p<0.001$) and was deemed to be an independent prognostic factor by Cox regression analysis ($p=0.05$), indicating that Puma expression in melanoma tissue has a protective effect in terms of patient survival [35]. Whether reduced Puma expression is a causative factor in melanoma or is simply a consequence of melanoma-associated molecular changes is not clear. One also cannot rule out the possibility that epigenetic changes, such as promoter methylation, may be responsible for Puma down-regulation in melanoma.

Noxa

Noxa was the first BH3-only protein to be identified as a p53 transcriptional target [55]. Noxa is similar to Puma in many respects; it contains a p53 response element and is directly upregulated by p53 following DNA damage [55]. Noxa can also function independently of p53. For example, hypoxia-inducible factor (HIF)-1α is reported to directly induce Noxa transcription [56]. Posttranslational modifications of Noxa have not been described so far. Like Puma, Noxa protein is thought to translocate to mitochondria, where it antagonizes Bcl-2 family pro-survival proteins.

Like Puma$^{-/-}$ mice, Noxa$^{-/-}$ mice exhibit no developmental abnormalities. Although MEFs from these animals exhibit drug resistance, Noxa$^{-/-}$ thymocytes readily undergo apoptosis in response to both p53-dependent stimuli (etoposide, γ-irradiation) and p53-independent stimuli [cytokine withdrawal, the glucocorticoid treatment, calcium flux, and the phorbol ester 12-myristate 13-acetate (PMA)] [39, 57]. Noxa$^{-/-}$ mice are resistant to gastrointestinal (GI) epithelial cell death following whole body irradiation, indicating that Noxa also plays a role in the maintenance of GI tract tissues [57].

Studies of Noxa$^{-/-}$ MEFs suggest that Noxa is important for oncogene-induced apoptosis. Specifically, Noxa$^{-/-}$ MEFs transduced with the adenovirus oncoprotein E1A are protected against apoptosis induced by etoposide, adriamycin, and X-ray irradiation [39, 57]. Unlike Puma, exogenous Noxa expression in wild-type MEFs does not induce apoptosis. However, Noxa sensitizes MEFs to etoposide and UV irradiation regardless of p53 status, suggesting that Noxa cooperates with p53-independent apoptotic pathways [16, 51]. Discrepancies in the abilities of Puma and Noxa to induce apoptosis may be explained by a model postulated by Chen et al. [20]. They assert that Puma binds promiscuously to all pro-survival Bcl-2 family proteins whereas Noxa associates with only a subset (Mcl-1 and A1), making Noxa a less potent apoptotic effector. Alternatively, Puma and Noxa may simply be subject to tissue-specific regulation that differs between thymocytes and MEFs.

In melanoma, Noxa appears to mediate the apoptotic effects of certain drugs. For example, the γ-secretase tripeptide inhibitor GSI (z-Leu-Leu-Nle-CHO) has been shown to induce apoptosis in melanoma cell lines (but not in normal melanocytes) via upregulation of Noxa protein [58]. GSI can induce Noxa in melanoma cells despite the absence/mutation of p53, low Apaf-1 levels, and an abundance of Bcl-2, Bcl-X_L, and Mcl-1 protein expression. This suggests that GSI-mediated apoptosis is p53-independent and may be effective in treating apoptosis-resistant melanoma cells.

Proteasome inhibitors have also been shown to induce melanoma-specific cell death and, so far, two studies have identified Noxa as a key mediator of this effect [59, 60]. Bortezomib (Velcade) can induce apoptosis in melanoma cell lines expressing low levels of Apaf-1 and high levels of anti-apoptotic factors (Bcl-2, Bcl-xL, Mcl-1, Survivin), as well as melanoma cells harboring mutations of p53, Ras, BRAF, or INK4/ARF [60]. It is thought that rapid accumulation of Noxa antagonizes Bcl-2 at the mitochondrial membrane, thus promoting intrinsic cell death. Bortezomib treatment has also inhibited the growth of melanoma xenotransplants *in vivo*, with excised tumors showing evidence of Noxa protein induction [59, 60]. These studies suggest that the proteasome helps to maintain the malignant phenotype of melanoma cells by blocking the expression of Noxa and thus hampering the activation of apoptosis.

Studies of Noxa in human cancer have so far provided no evidence that Noxa loss contributes to tumorigenesis. The most comprehensive study of Noxa in human tumors was conducted by Lee et al. [61]. They examined the mutational status of Noxa in a large panel of tumors, including colon adenocarcinomas ($n=78$), advanced gastric adenocarcinomas ($n=53$),

non-small-cell lung carcinomas (n = 86), breast carcinomas (n = 76), urinary bladder transitional cell carcinomas (n = 33) and heptocellular carcinomas (n = 90). Only one Noxa mutation missense was discovered in the entire sample set (in a bladder carcinoma), and the mutation had no functional impact *in vitro*.

Bid

Whether loss of Bid expression promotes tumorigenesis or contributes to drug resistance in humans is not clear. A large study of Bid protein expression in prostate, ovarian, colorectal, and brain cancers, and B cell non-Hodgkin's lymphomas showed that Bid expression sometimes increases during tumor progression but that Bid expression is not related to chemotherapeutic responsiveness [78]. Likewise, a recent study of Bid expression in cervical cancer specimens indicated no relationship between protein level and sensitivity to radiation treatment [79]. Low rates of inactivating Bid mutations have been reported in gastric cancers [80] and reduced Bid expression is observed in hepatocellular carcinomas [81], but the significance of these findings is unclear. Bid functions as a cell death signal amplifier that links various peripheral death pathways to intrinsic, mitochondrial-mediated apoptosis [62]. Cytosolic Bid protein is quite stable until it is cleaved, usually by caspase-8, to form truncated Bid (t-Bid). This cleavage step is thought to induce a conformational change that fully exposes the Bid BH3 domain, thus facilitating activating interactions with Bax or Bak proteins [63-66]. T-bid then translocates to mitochondria and activates Bax or Bak to induce apoptosis. Caspase-8 is a component of the extrinsic apoptosis pathway and is typically activated following the engagement of the death receptors such as Fas, TNF-R1 and TRAIL [62]. Consequently, Bid was initially thought to be specific to death receptor-mediated apoptosis. However, subsequent studies revealed that Bid is also subject to cleavage by other proteases, such as granzyme B [67, 68], calpain [69, 70], and lysosomal enzymes [71, 72], which are activated in response to a broad range of cell injury stimuli. $Bid^{-/-}$ mice spontaneously develop a myeloid hyperplasia that progresses to a malignancy resembling chronic myelomonocytic leukemia (CMML). Thus, Bid functions as a tumor suppressor in myeloid homeostasis [73]. However, Bid does not mediate p53-dependent apoptosis [74–77].

In melanoma, Bid appears to be important for some types of drug-induced apoptosis. For example, cisplatin-induced apoptosis of melanoma cells seems to depend significantly on calpain-mediated Bid cleavage [70]. The antitumor effects of the flavanoid compound propolin-C also appear to be mediated by Bid in melanoma cells [82].

Bim

Bim functions to monitor the integrity of cytoskeletal structures by inducing apoptosis in response to microtubule disruption. Bim contains a highly conserved N-terminal dynein light chain (DLC)-binding motif, allowing it to associate with DLC1, a component of the microtubular dynein motor complex [83]. Upon cellular damage, such as UV irradiation, Bim is activated through phosphorylation by Jun N-terminal kinase (JNK) [84]. Phosphorylated Bim is released from the cytoskeleton and translocates to mitochondria, where it antagonizes pro-survival Bcl-2 proteins. Bim protein stability is reportedly regulated by extracellular signal-regulated kinase (ERK), which phosphorylates Bim to promote its ubiquitination and subsequent degradation via the proteasome pathway [85–87]. Bim is also subject to transcriptional upregulation by the forkhead transcription factor FKHR-L1 [88] and c-myc [89, 90].

Knockout mouse studies indicate that the major physiologic function of Bim is to help regulate lymphoid cell populations, as $Bim^{-/-}$ mice exhibit a range of hematopoietic abnormalities [91–93]. Bim loss has also been shown to promote malignancies on tumor-prone backgrounds. In one study, Bim suppressed the tumorigenic growth of epithelial cells transformed with E1A and dominant negative p53 [94]. In another study, loss of a single Bim allele greatly accelerated B cell lymphoma development in c-myc transgenic mice [89]. Consistent with its role as a microtubule damage sensor, Bim protein rapidly accumulates in cells treated with the anti-microtubule drug paclitaxel and $Bim^{-/-}$ tumors are resistant to the drug [94]. This may explain why H-ras-induced tumors are paclitaxel resistant; over-expression of H-ras constitutively activates the MAPK signaling cascade, which in turn promotes continuous Bim protein degradation via the ubiquitin-proteasome pathway [85]. Accordingly, the proteasome inhibitor Velcade (bortezomib) restores paclitaxel sensitivity and promotes Bim-dependent tumor regression in epithelial solid tumors over-expressing H-ras [94].

Studies of Bim in melanoma indicate that it can mediate specific types of cell death. For example, Bim may initiate the melanoma-specific apoptosis induced by certain histone deacetylase inhibitors. A study by Zhang et al. [95] demonstrated that the histone deacetylase inhibitor suberic bishydroxamate (SBHA) induced apoptosis in melanoma cell lines,

but not in melanocytes. Cytosolic Bim protein translocated to mitochondria and associated with Bcl-2 following SBHA treatment, suggesting that Bim is a key initiator of SBHA-induced apoptosis in melanoma cells. Bim is also reported to be a key mediator of anoikis in epithelial cells [96] and may suppress anchorage-independent melanoma cell growth. A study by Jorgenson et al. [97] showed that PMA promotes proliferation and anchorage-independent survival of melanoma spheroids, in part by inactivating Bim and Bad. PMA activates the PKCs, a family of kinases involved in signal transduction induced by extracellular stimuli (e.g., growth factors) and the regulation of cell growth, differentiation, apoptosis, malignant transformation, and metastasis [98]. Jorgensen et al. [97] reported that PMA protected melanoma spheroids against anoikis by activating ERK1/2 (via a MEK-independent mechanism), which in turn inactivated the downstream BH-3 only proteins, Bim and Bad.

Bim mutations in cancer have not been reported. However, loss of chromosomal region 2q13, containing Bim, has been observed in mantle cell lymphomas [99]. Bim protein expression is reportedly absent in many renal cell carcinomas and in some metastatic melanoma cell lines [59]. In a recent tissue microarray analysis of melanoma biopsies, we found that the Bim expression level declines with increasing stage of tumor progression ($p < 0.001$), and that Bim overexpression induces apoptosis of melanoma cells *in vitro* [unpublished data]. Zhang et al. [100] reported that TRAIL-resistant melanoma cells exhibit decreased levels of Bim and Bid expression. Incidentally, such cells are known to have high levels of activated Akt and ERK1/2, which may account for Bim suppression [101].

Bmf

Bmf is similar to Bim in that it is also associated with the cytoskeleton. In the case of Bmf, however, its N-terminal dynein light chain (DLC)-binding motif facilitates its sequestration by DLC2, a component of the myosin V actin motor complex [102]. Bmf is activated by damage signals such as UV irradiation, loss of adhesion signaling, and cell detachment (anoikis). Following these apoptotic stimuli, Bmf is phosphorylated and becomes detached from the actin cytoskeleton. Bmf then translocates to mitochondria where it binds to Bcl-2 survival proteins and promotes apoptosis. Bmf is expressed in hematopoietic cells and has also been shown to mediate granulocyte cell death upon cytokine withdrawal [103]. The chromosomal location that contains Bmf (15q14/15) is suspected to harbor a tumor suppressor gene, as loss of this region is associated with advanced breast cancer, lung, and colon carcinomas. However, the role of Bmf in cancer remains poorly defined and there are so far no studies of Bmf in melanoma [104, 105].

Bad

Bad was one of the first BH3-only proteins identified [106]. Bad is negatively regulated by phosphorylation on multiple serine residues in response to growth factor-induced signals [107]. Under normal conditions, when growth factors are present, Bad is kept phosphorylated by various protein kinases such as the 90-kDa ribosomal S6 kinase (RSK) [106, 108], Akt [109, 110], protein kinase A [111], Raf-1 [112], and p70S6 kinase [113]. Phosphorylated Bad is sequestered in the cytosol by 14-3-3 proteins [114]. Upon growth factor deprivation, Bad is dephosphorylated and allowed to translocate to mitochondria where it antagonizes the pro-survival Bcl-2 family members Bcl-2 and Bcl-X_L, and thus promotes apoptosis [115]. Knockout mouse studies highlight the role of Bad in maintaining the integrity of the hematopoietic system. $Bad^{-/-}$ mice spontaneously develop diffuse B cell lymphoma and exhibit an increased incidence of thymic lymphomas after γ-irradiation [116]. $Bad^{-/-}$ cells also exhibit increased resistance to a combination of serum deprivation plus any of the following: FasL, tumor necrosis factor (TNF), or etoposide [116].

Bad is reported to play a crucial role in melanoma-specific MAPK survival signaling. The Ras-Raf-MEK-ERK (MAPK) pathway, which promotes cell differentiation, proliferation, and survival, is frequently hyperactivated in human melanomas, but not in normal melanocytes [117–119]. Eisenmann et al. [120] showed that dephosphorylation of Bad is the critical step leading to melanoma cell death induced by MEK inhibition. They suggest that Bad is phosphorylated by RSK, a downstream effector in the MAPK cascade, and is thus maintained in an inactive state in melanomas. Accordingly, exogenous expression of a constitutively active RSK mutant conferred apoptotic resistance to melanoma cells. Conversely, exogenous expression of a constitutively active mutant form of Bad sensitized melanoma cells to apoptosis induced by MEK inhibition.

However, recent studies by Panka et al. [121] indicate that Bad does not protect melanoma cells from apoptosis induced by the raf inhibitor Sorafenib (BAY 43-9006). Although the drug suppressed Bad phosphorylation, siRNA-mediated knockdown of Bad did not protect most melanoma cell lines from the lethal effects of Sorafenib. Therefore, Bad de-

phosphorylation and mitochondrial translocation does not appear to mediate raf inhibitor-induced apoptosis of melanoma.

The PI3-kinase/Akt signaling pathway also regulates Bad activity. Akt is commonly hyperactivated in human cancers, due to loss or mutation of PTEN, the phosphatase that negatively regulates PI3-kinase [122]. Constitutively active Akt has been detected in a large proportion (43–67%) of malignant melanomas [123] and is thought to contribute significantly to melanoma progression and invasion [123–125]. Despite the importance of Akt in melanoma, and the fact that active Akt phosphorylates Bad [109, 110], Bad has not been shown to play a contributing role in this disease. Infrequent mutations of the Bad gene have been described in colon adenocarcinoma [80], but so far no studies have implicated Bad dysregulation, mutation, or loss as a factor in melanoma development.

Bik

Bik (also called Nbk) expression is induced by several stress stimuli including genotoxic stresses [126]. Bik is regulated by phosphorylation (on residues Thr-33 and Ser-35), which enhances its pro-apoptotic potency [127]. Blk (the murine orthologue of Bik) is expressed in a range of hematopoietic cells, including lymphocytes, myeloid cells and nucleated erythrocytes. However, it appears to play a redundant role in apoptosis, as $Blk^{-/-}$ lymphocytes show no impairment of apoptosis induced by various stimuli: cytokine withdrawal, the glucocorticoid dexamethasone, phorbol ester, ionomycine, the topoisomerase II inhibitor etoposide, or B cell receptor [128].

Loss of Bik protein expression, associated with allelic loss and/or methylation, has been reported in renal cell carcinomas (RCCs) and may be a factor in RCC drug resistance [129]. Bik mutations have also been found in B-cell lymphomas [130].

There has been one study of Bik in melanoma, conducted by Oppermann et al. [131]. Examination of Bik expression in 17 melanoma cell lines revealed that Bik protein was weakly upregulated in melanoma compared to normal human melanocytes, in which Bik is undetectable. Exogenous Bik expression induced apoptosis and enhanced chemosensitivity of SKM13 melanoma cells to etoposide, doxorubicin, and pamidronate, as well as CD95 antibody-mediated apoptosis. Bik also inhibited the growth of human melanoma xenografts by 45% in a nude mouse model. However, the mechanism of Bik-induced growth inhibition and apoptosis in melanoma has not identified. Although Bik has been reported to activate caspases [126], Opperman et al. found no evidence of cytochrome *c* release or caspase activation in melanoma cells. Presently, the importance of Bik in cancer is not fully understood.

Hrk/DP5

DP5 was first cloned as a neuronal apoptosis-inducing gene in rats [132]. It is widely expressed in embryogenesis, during which massive destruction of neurons occurs to sculpt the developing nervous system. DP5 is thought to mediate the β-amyloid-induced formation of neuronal plaques in the brain, associated with neurodegenerative disorders such as Alzheimer's disease [133]. Harakiri (Hrk) (the human homologue of DP-5) was isolated from a HeLa cell cDNA library, based on its ability to bind with Bcl-2 [134]. Hrk is transcriptionally induced by nerve growth factor (NGF) deprivation, following which it strongly induces neuronal apoptosis [132, 135]. The role of Hrk in melanoma has not yet been studied. However, Hrk gene inactivation by methylation has been reported in gastric and colorectal cancers [136] and by LOH in astrocytomas and glioblastomas [137].

Conclusion

In summary, BH3-only proteins are key mediators of the apoptotic response to various forms of cytotoxic stress. Loss or aberrant expression of BH3-only proteins (like Puma) may contribute to malignancy in certain contexts. However, the relative importance of BH3-only proteins in tumorigenesis, and specifically malignant melanoma, remains to be clarified.

Despite these unknowns, current knowledge of BH3-only protein function provides us with some novel therapeutic possibilities. For example, gene therapy vectors may be used to forcibly express potent BH3-only proteins (such as Puma or Bim) and induce apoptosis in tumor cells. Alternatively, drugs designed to upregulate specific BH3-only proteins may be used to sensitize apoptotic-resistant tumors to DNA-damaging agents. Another promising therapeutic option is the development of BH3-only mimetic compounds. A recent study by Oltersdorf et al. [138] describes a small organic molecule, ABT-373, that fits into the BH3 domain-binding pocket present on the surface of Bcl-2-like proteins. This compound inhibits Bcl-2, Bcl-X_L, and Bcl-w proteins, sensitizes cancer cells to DNA-damaging agents, and causes regression of certain tumors *in vivo*. It will be exciting to see whether a new class of BH3-only mimetics emerges and whether such

compounds will be effective in treating apoptosis-resistant tumors.

As we learn more about the roles of BH3-only proteins in apoptosis, we will gain a better understanding of how dysregulation of these proteins may contribute to tumorigenesis and how they may be manipulated towards a therapeutic benefit.

Acknowledgements. This work is supported by the National Cancer Institute of Canada, Canadian Melanoma Foundation, and Canadian Dermatology Foundation. A.M.K. is supported by scholarships from the Michael Smith Foundation for Health Research and the Canadian Institutes of Health Research. G.L. is a recipient of the Research Scientist Award from the National Cancer Institute of Canada supported with funds provided by the Canadian Cancer Society.

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