

Chapter 2

The Bcl-2 Family Proteins

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Abstract The Bcl-2 family proteins are a group of evolutionarily conserved molecules that regulate apoptosis mainly at the site of mitochondria. This family of proteins consists of both antideath and prodeath molecules. The latter are also composed of multidomain prodeath molecules and the BH3-only prodeath molecules. While the BH3-only molecules act at the distal, receiving the death signals, the multidomain prodeath and antideath molecules regulate the mitochondrial outer membrane's permeability to control apoptosis. Protein interactions among the family members are important for their functions and have been explored for therapeutic purposes, as illustrated by the development of the BH3-only mimetics. Recent studies have also indicated that these molecules can act in other subcellular locations and their functions are beyond apoptosis regulation. Thus, the Bcl-2 family proteins also play important roles in autophagy, cell proliferation, and many other cellular functions.

Keywords Bcl-2 family proteins · Multidomain prodeath molecule · BH3-only molecule · BH3 mimetics · Mitochondria

Introduction

Apoptosis is an active process of cellular destruction with distinctive morphological and biochemical features. Two major apoptotic pathways have been defined in the mammalian cells: the death receptor pathway and the mitochondrial pathway. The Bcl-2 family proteins are the most important regulators of the mitochondrial pathway. Signals from the death receptor pathway could be also bridged to the mitochondrial pathway via the Bcl-2 family proteins. This family of proteins consists of both antiapoptosis and proapoptosis members.

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While the proapoptosis members serve as sensors to death signals and executors of the death program, the antiapoptosis members inhibit the initiation of the death program. The Bcl-2 family proteins are evolutionarily conserved but may accomplish these tasks by different mechanisms. In addition, multiple cellular signals can modify the activities and locations of these proteins, thus forming an intracellular signaling network that sets the delicate balance between cell death and survival.

Evolutionary Conservation of Bcl-2 Family Proteins

Bcl-2, the prototype of the Bcl-2 family proteins, was the first defined molecule involved in apoptosis. It was initially cloned from the t(14;18) breakpoint in human follicular lymphoma (1–3). Although its role as a proto-oncogene was quickly realized, its biological function as an antiapoptosis gene was not realized until some years later (4, 5). A number of proteins were soon discovered, which share sequence homology with Bcl-2, but only some of those possess the antiapoptosis activities. Others actually promote apoptosis (Table 2.1).

Notably, this family of proteins is evolutionarily conserved. A number of viruses encode Bcl-2 homologues, including most, if not all, gamma herpes viruses (6). Most of these viral homologues are antiapoptotic, probably because viruses need to keep the infected cells alive for the latent and persistent infection (6). Bcl-2-related genes can also be found in the sponge, sea urchin, and zebra fish (7). The nematode *C. elegans* has its sequence and functional homologues for a death antagonist, CED-9 (8), and a BH3-only death agonist, EGL-1 (9). On the other hand, both prodeath homologue (dBorg-1/Drob-1/Debcl/dBok) and antideath homologue (dBorg-2/Buffy) have been described in *Drosophila* (10). These homologues are discussed in details in Chapters 13 and 14, respectively.

One of the key features of the Bcl-2 family proteins is that members share sequence homology in four domains, the BH1, 2, 3, and 4 domains, although not all members have all the domains (11–14), (Table 2.1, Fig. 2.1). Mutagenesis studies have revealed that these domains are important for function as well as for protein interactions among the family members. The BH1, BH2, and BH4 domains are necessary for the death repression function of antideath molecules, whereas the BH3 domain is required for the death promotion function of prodeath molecules (15, 16).

Thus, the antideath members contain all four BH domains, whereas the prodeath molecules can be further divided into those with the BH3 domain only and those with BH1, BH2, and BH3 domains. It seems that the so-called BH3-only molecules, such as Bid, Bim, and Bad, are sensors for the peripheral death signals and are able to activate the “multidomain” prodeath molecules, Bax or Bak, either directly or indirectly, which in turn activate the mitochondria apoptosis program.

Table 2.1 Major functions of the mammalian Bcl-2 family proteins

Category	Molecule	Function	Refs.
Antideath: Multidomain (BH1, BH2, BH3, BH4)	Bcl-2 (1G5M)	Inhibits apoptosis, autophagy, and proliferation. Genetic deletion causes major phenotypes in the lymphoid system, the kidney, the melanocytes, and other cells.	(7, 16, 146)
	Bcl-xL (1R2D)	Inhibits apoptosis, autophagy, and proliferation. Genetic deletion causes embryonic lethality, abnormalities in fetal erythroid, and neuronal development.	(7, 16, 143)
	Bcl-w (1MK3)	Inhibits apoptosis. Genetic deletion causes male sterility.	(150)
	Mcl-1 (1WSX)	Important for the development of trophectoderm and hematopoietic stem cells.	(144, 145)
	Bfl-1/A1	Important for the survival of granulocytes and mast cells.	(148, 149)
	Bcl-B/Bcl-2L10/Nrh	Interacts with Bax, but not Bak, and suppresses only Bax-mediated cell death.	(34, 36)
Prodeath: Multidomain (BH1, BH2, BH3)	Bax (1F16)	Genetic deletion causes male sterility and abnormal neuronal death. Together with Bak, controls all mitochondria-mediated apoptosis. Also involved in ER-related function and cell proliferation.	(125, 151)
	Bak (2IMS)	No obvious defects in knockout mice, but combined deletion with Bax blocks all mitochondrial apoptosis.	(125)
	Bok/Mtd	Apoptotic effects could be suppressed by Mcl-1, BHRF-1, and Bfl-1, but not Bcl-2 or Bcl-xL, consistent with the heterodimerization pattern.	(37, 190)
Prodeath: BH3-only (classical)	Bad	A potential target in growth factor-mediated suppression of apoptosis. Involved in glucose-stimulated insulin resistance in pancreatic beta cells.	(160)
	Bid (2BID)	Is cleaved by proteases and links the death signals mediated by these proteases, such as those during death receptor activation, to the mitochondria.	(101, 158, 159)

Table 2.1 (continued)

Category	Molecule	Function	Refs.
BH3-only (nonclassical)		Genetic deletion causes resistance of hepatocyte to Fas-mediated apoptosis and disturbed myeloid homeostasis.	
	Bik/Nbk/Blk	Single genetic deletion does not cause any obvious defect. Concomitant loss of Bik and Bim in mice causes defective spermatogenesis.	(7)
	Bim/Bod	Important for apoptosis in the immune system; important for apoptosis induced by ER stress and many other agents.	(140, 156)
	Bmf	Not required for apoptosis induced by developmental process and UV irradiation, or for anoikis. Participates in glucocorticoid or HDAC inhibitor-induced apoptosis in lymphocytes.	(161)
	Hrk/DP5	May participate in neuronal death caused by NGF deprivation, but genetic deletion does not result in obvious phenotypes.	(7)
	Noxa/APR	Participate in cell death induced by irradiation, particularly by UV irradiation.	(152, 154, 155)
	PUMA/BBC3	Important for apoptosis induced by DNA damage, cytokine deprivation, and several other agents.	(152–155)
	Bnip3/Nip3	Mainly induces autophagic death, which requires its TM domain, not the BH3 domain. Participates in mitophagy during erythrocyte maturation.	(167)
	Nix/Bnip3L	Required for mitophagy during erythrocyte maturation.	(165, 166)
	Beclin1	Participates in autophagy induction, which may be suppressed by its interaction with Bcl-2, Bcl-xL, and Mcl-1. Does not induce apoptosis.	(18)
	Apolipoprotein L1 (ApoL1)	Induces autophagic death that requires the BH3 domain.	(169)
	Spike	Does not interact with any other Bcl-2 family proteins but the BH3 domain required for its	(191)

Table 2.1 (continued)

Category	Molecule	Function	Refs.
		killing activity. Resides at ER and interacts with Bap31 to suppress the interaction of the latter with Bcl-xL.	
	MULE/ARF-BP1	An E3 ligase that can bind to and ubiquitinate Mcl-1 and p53 for degradation.	(177, 178)

Notes: Protein Data Bank (PDB) identifiers are included in the parentheses for those molecules whose structures have been resolved. Other much less characterized Bcl-2 homologues include Boo/Diva, Mil-1/Bcl-RAMBO, Bcl-G, Bflk, and MOAP-1/MAP-1 (7, 43, 192, 193). These molecules have atypical BH domains, a nonconventional combination of different BH domains, or a controversial function in regulating cell death. For discussion of the Bcl-2 family proteins in *C. elegans* and *Drosophila*, see Chapters 13 and 14. The nonclassical BH3-only molecules differ from the classical ones in that the former either do not induce apoptosis or do not interact with the antideath members.

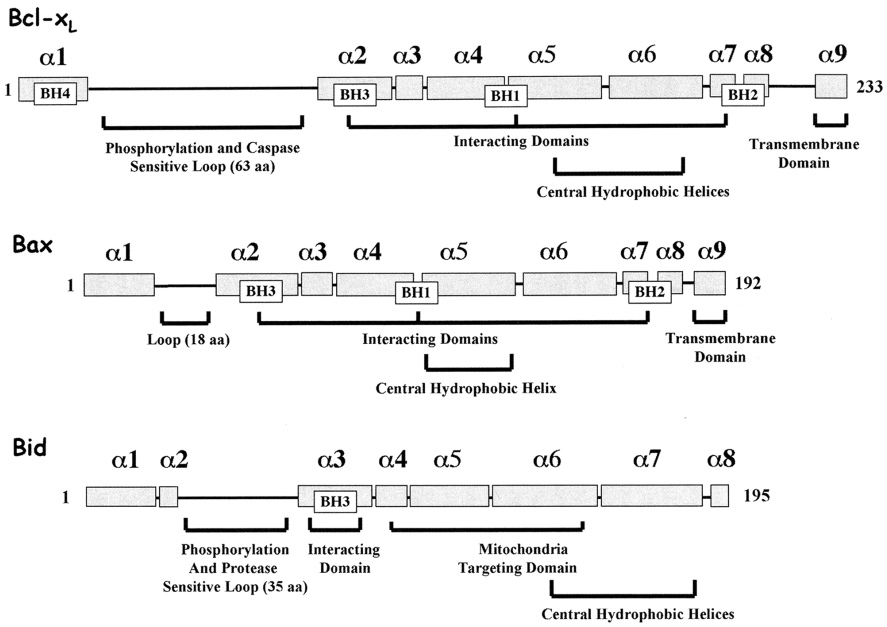


Fig. 2.1 Structural features of representative Bcl-2 family proteins. Bcl-xL, Bax, and Bid represent the three subgroups of the Bcl-2 family proteins: the multidomain antideath molecules, the multidomain prodeath molecules, and the BH3-only prodeath molecules (see Table 2.1). Bid is the only BH3-only molecule that has a structure similar to that of a multidomain protein. Other BH3-only molecules, such as Bim, Bad, and Bmf, are intrinsically unstructured. The structures of Bcl-2, Bax, and Bid are composed of multiple alpha helices with one or two central hydrophobic helices surrounded by six to eight amphipathic helices. The α7 helix of Bcl-xL (194) is also called the α6' helix (78). A large, flexible, unstructured loop is present between the α1 and α2 helices. The loop is known to be a regulatory domain, sensitive to protease or kinase effects. The Bcl-2 homology domains 1-4 are distributed

The definition of BH3 domain was initially based on the homology to a nine-amino-acid domain present in Bax, Bak, and Bik/NBK/Bip1 (12). Functionally, this domain is required for the interaction of the prodeath members with the antideath members and for their activity. Structural analysis indicated that it is contained within an amphipathic alpha helix (17) (Fig. 2.1). Thus, the definition of the BH3 domain has been broadly defined as a sequence that could form a four-turn amphipathic alpha helix containing the sequences motif A-X-X-X-A-X-X-A-B-C-X-A, where A represents hydrophobic residues, B represents a small residual, typically glycine, and C is Asp or Glu (18-22) (Fig. 2.1). The A and/or B residues form the hydrophobic side of the amphipathic helix. Detailed mutagenesis studies have indicated that residuals at the hydrophobic face have the greatest effects on heterodimerization and prodeath activity (17). This loose definition may contribute to the identification of several nonclassical BH3-only molecules because of the lack of invariant residues. As the result, some of these BH3 molecules cannot interact with other Bcl-2 family proteins, such as Spike. Some of them may not promote apoptosis, such as Beclin1 and Bnip3, while others may promote cell death via completely different biochemical mechanisms, such as via ubiquitination-mediated degradation, as in the case of MULE/ARF-BP1 (Table 2.1). Thus, it seems that a distinctly new category of BH3-only molecules is emerging that have diverse functions.

Bcl-2 Family Protein Interactions and Their Functional Significance

Protein Interactions Among Bcl-2 Family Members

The Bcl-2 family proteins can interact with each other and also with other proteins. In fact, the first proapoptosis Bcl-2 family protein, Bax, was cloned based on its interaction with Bcl-2 (23). Many other Bcl-2 family proteins were also cloned based on this type of interaction. A number of systems have been utilized to determine such interactions, including yeast two-hybrid, co-immunoprecipitation, phage expression library screening, and GST pull-down assay. More recently, peptides corresponding to the BH3 domain of various BH3-only molecules have been used to assess the variations in their interactions with the antideath members (19-22). Interpretation of the protein-protein interactions based on *in vitro* systems can sometimes be complicated. For example, it was found that the *in vitro* interaction of Bax with Bcl-2 occurred only in the presence of certain detergents, which caused a conformational change in Bax

Fig. 2.1 (continued) over one or two alpha helices. They are involved in protein-protein interactions. Other alpha helices are involved in membrane binding (the TM domain in Bcl-xL and Bax, and the alpha helices 4-6 in Bid) or pore forming (the central hydrophobic alpha helices). The TM domain of Bax, but not that of Bcl-xL, was included in the structural analysis, which was shown to bind to the hydrophobic pocket formed by the BH1, BH2, and BH3 domains. This feature is confirmed with Bcl-w, for which the TM domain was also included in the structural analysis

(24). However, the authenticity of such interactions may be verified by an *in vivo* interaction system, such as the yeast two-hybrids, that does not involve the use of detergent (25, 26). In addition, interactions among family members, such as Bcl-2 and Bax, likely occur on membranes *in vivo*, where a proper conformation may be assumed as a result of activation by death signals (24, 27–30).

Based on these analyses, several interaction patterns can be defined [Fig. 2.2(B)]. The most common is the interaction between the antideath and prodeath members, such as Bcl-2 versus Bax (23) or Bid (31). This can result in antagonistic action of the two types of molecules and thus can set a rheostat

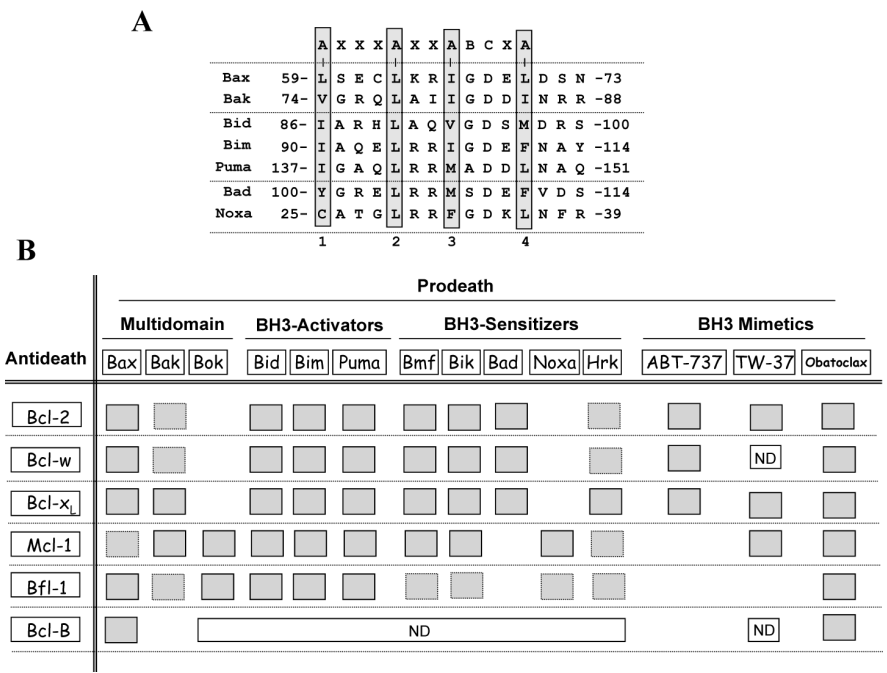


Fig. 2.2 Interactions between the antideath and prodeath Bcl-2 family proteins. (A) Sequence homology of the BH3 domain of representative prodeath molecules (human origin). Structurally, this domain forms an amphipathic α helix that has four turns, (17–22). The four hydrophobic residues are important for the interactions and are represented by A, whereas B denotes a small residue and C denotes Asp or Glu. These seem to be the only signature of the BH3 domain. (B) Illustrated are the interaction patterns of six antideath molecules with three multidomain antideath molecules and eight classical BH3-only molecules (19, 21, 22, 25, 33–35, 37, 132), which can be further divided into activators and sensitizers based on their ability to directly permeabilize mitochondria outer membranes. Gray squares indicate the interaction occurs between the selected members, such as that between Bak and Bcl-xL or Mcl-1. Squares with a dotted line indicate interactions observed in certain studies but not in others, or with some methods but not with other methods, thus suggesting the interactions could be weak. ND indicates that no data are publicly available at the moment. Small molecule BH3 mimetics have been developed, which can bind to different antideath members to competitively disrupt the endogenous interactions with the prodeath molecules to induce apoptosis (132, 180, 186–189). See the text for a discussion of the relative strength of interactions

control of the death program (32). Interestingly, not all antideath molecules can interact with all prodeath molecules. It seems that some members of one group will preferentially bind to some members of the other group. For the interaction between the multidomain prodeath molecules and the antideath molecules, Bax seems to be able to interact with all of them except Mcl-1 (33, 34). Previously, Bax had been found to interact with Mcl-1 in yeast two-hybrid system (25). On the other hand, Bak preferentially binds to Mcl-1 and Bcl-xL as a full-length molecule, although its BH3 peptide can bind to all antideath molecules except Bcl-B, in fluorescence polarization assay (FPA) (34, 35). Functional studies support the FPA result; thus, Bak-overexpression-induced apoptosis can be suppressed by overexpressed Bcl-2, Bcl-xL, Mcl-1, or Bfl-1/A1, but not Bcl-B (36). Finally, it has also been shown that Bok/Mtd binds to Mcl-1, BHRF-1, and A1, but not to Bcl-2 or Bcl-xL (37). Correspondingly, these molecules may only antagonize the function of those molecules to which they bind.

The BH3-only molecules also show different binding capability to the different prosurvival members (19–22) [Fig. 2.2(B)]. In general, Bim and PUMA bind avidly to all the prosurvival proteins, whereas the other BH3-only molecules demonstrate a range of selectivity. Noxa may engage only Mcl-1, whereas Bad and Bmf bind mainly to Bcl-2, Bcl-xL, and Bcl-w. Bid, Bik, and HRK prefer Bcl-xL and Bcl-w over Bcl-2 or Mcl-1. Bad only binds to Bcl-2, Bcl-xL, and Bcl-w. Bim, PUMA, and Bid are consistently potent killers, as they are able to neutralize all prosurvival molecules, whereas a combination of Noxa and Bad is required to achieve maximal prodeath effects. These findings indicate that efficient apoptosis requires the neutralization of multiple prosurvival proteins.

The second type of interaction occurs between two members of the same functional group. In the prodeath group, one “BH3-only” molecule can interact with one “multidomain” molecule, such as Bid to Bax (31) or Bak (38), and Bim_S or Bim_{AS} to Bax (39). This can be important for the activation of the multidomain executioner molecules Bax or Bak by the “BH3-only” molecules (20–22, 29, 38) (see the section entitled “Activation of the Multidomain Bax and Bak at the Mitochondria”). Interactions between two members of the antideath group have also been reported, such as between Bcl-B and Bcl-2 or Bcl-xL (36), although the significance is not clear. The third type of interaction is dimerization or oligomerization of the same molecule. This has been observed in both antideath molecules, such as Bcl-2 or Bcl-xL (11, 25, 26, 40), and prodeath molecules, such as Bax and Bak (38, 41). Oligomerization of Bax or Bak has been considered important for releasing mitochondrial apoptotic factors, such as cytochrome c (38, 41).

The Importance of BH Domains in Bcl-2 Family Protein Interactions

BH domains are critically involved in protein interactions among family members, whereas the structures involved in homotypic interactions are less clear. In general, although the hydrophobic pocket formed by the BH1, BH2, and BH3

domains of the antideath molecules interact with the BH3 domain of the prodeath molecules, with the latter serving as the donor for the former's "pocket site," as suggested by structural studies (7, 11, 12, 31, 42). Similarly, the domains involved in the interactions of two prodeath proteins, such as Bid and Bax, are the BH3 domain of the BH3-only molecule and likely the hydrophobic pocket formed by the BH1, BH2, and BH3 domains of the multidomain molecules (31). Mutations at one of the domains can usually disrupt such interactions. Critical amino acids have been defined in each BH domain, such as Gly¹⁴⁵ in the BH1 domain of Bcl-2, Trp¹⁸⁸ in the BH2 domain of Bcl-2, and Gly⁹⁴ in the BH3 domain of Bid (11, 31). However, it may be necessary to introduce mutations in all BH domains of a particular antideath molecule to disrupt its interaction with a prodeath molecule (43). At other times, regions outside the BH domain may be required for interactions, such as the N-terminal region and the transmembrane domain in the case of Bnip3 (44).

Interestingly, there is selective use of certain amino acids in one molecule when it interacts with different partners, which is of functional significance. For example, while Bcl-xL can bind to both BH3-only molecules, such as Bid and Bad, and multidomain molecules, such as Bax and Bak, certain amino acids (Phe¹³¹ and Asp¹³³) seem to be important for binding to the BH3-only molecules, but not to Bax (45, 46). The mutant (F131V, D133A) will bind to Bid, Bad, or Bim_L, but not to Bax. However, it retains the antideath function and indicates that it may block the activity of the BH3-only molecules (46). In addition, variations in certain key amino acids may result in different affinities in binding to the same molecule, as indicated in two Bcl-2 isoforms for the binding to Bak- or Bad-derived BH3 peptides (47).

The binding of antideath Bcl-2 family members to the prodeath death members may require the latter's BH3 domain to be exposed. This may not be an issue for binding to BH3-only molecules, such as Bid, which exposes its BH3 domain following protease cleavage (48, 49), or Bim, which does not seem to have a structural confinement (30). However, this could be an issue for binding to the multidomain prodeath molecules Bax and Bak. The nonactivated conformer does not seem to bind to the antideath molecules (22). The BH3 domain may need to be exposed in an activated conformation for the binding (50) (see ahead).

Interactions Between Bcl-2 Family Proteins and Other Molecules

Regulation of the Bcl-2 Family Proteins by Nonfamily Molecules

The Bcl-2 family proteins can also interact with many other proteins. While some of the interactions relate to their functions in cell death, others lead to different physiological activities. A number of proteins can sequester the prodeath Bcl-2 molecules to prevent them from activation. For example, 14-3-3 ϵ binds to phosphorylated Bad and prevents it from translocation to the

mitochondria (51). Similarly, Bax can be sequestered by 14-3-3 ζ in the cytosol, which is liberated when the latter is phosphorylated by JNK (52), or sequestered by Ku-70 (53), which is liberated when the latter is acetylated (54), a process that could be inhibited by SIRT1 deacetylase and stimulated by histone deacetylase inhibitors (HDAC). Other proteins that have similar functions include humanin, ARC, HSP70, and crystallins (50). On the other hand, the dynein light chain complex 1 or 2 retains Bim_{EL}/Bim_L or Bmf in the microtubular dynein motor complex or actin filamentous myosin V motor complex, respectively (55, 56). The mechanism of liberation is less clear in these cases.

Bak can be sequestered on the mitochondria by the antideath Bcl-2 members Mcl-1 and Bcl-xL (35) or by the nonfamily member VDAC2 (22, 57). The displacement of these molecules by BH3-only molecules allows Bak to be activated. Similarly, Bak interaction with insulin-like growth factor-binding protein 1 (IGFBP1) may prevent its interaction and activation by p53 (58).

Interactions with some of the other proteins can lead to direct activation of the prodeath Bcl-2 members. One example is the interaction of Bax and Bak with Bif-1, which seems to promote their oligomerization upon apoptotic stimulation (59). Bax can also be activated by MOAP-1/MAP-1, which has a BH3-like domain that binds to Bax (43). p53 is another well-studied activator. The tumor repressor p53 is able to promote apoptosis through its transcriptional activity to upregulate a number of prodeath molecules, such as PUMA, Noxa, Bid, Bad, and Bax (see Chapter 9). However, cytoplasmic p53 can also directly participate in the mitochondrial apoptosis pathway by binding to Bax (60) or Bak (61) to dissociate their interactions with the prosurvival molecules Bcl-xL and Mcl-1, respectively, leading to their oligomerization and cytochrome c release. In this capacity, p53 acts like an “activator” BH3-only molecule (see ahead). Interestingly, like Bax or Bak, p53 may also be “sequestered” and thus suppressed by the prosurvival Bcl-2 family molecules, such as Bcl-xL, and liberated by a BH3-only molecule, such as PUMA, via competitive binding to Bcl-xL (62). Interestingly, PUMA is a transcriptional target of p53 and also promotes apoptosis by engaging in dissociating Bax/Bcl-xL interaction, thus allowing p53/Bax interaction.

As mentioned above, the binding of p53 with Bak can be blocked by a non-Bcl-2 family protein, IGFBP1, in hepatocytes due to competitive binding (58). It is thought that this mechanism may explain why hepatocytes are, in general, insensitive to p53-mediated apoptosis following genotoxic stress. Remarkably, IGFBP1 is also a transcriptional target of p53, thus constituting a differential mechanism to selectively block the apoptosis function of p53 without affecting its other functions. The coordinated actions of the nuclear and cytoplasmic p53 are also well illustrated in the case of Bad, which is a transcription target of p53 (63). But Bad can also bind to cytoplasmic p53 to prevent it from entering the nucleus. Instead, the complex is redirected to the mitochondria, where Bak is then activated by the complex under genotoxic stress.

One dramatic effect caused by the nonfamily binding partner was the functional conversion of the family member. An orphan nuclear receptor,

Nur77/TR3, can convert Bcl-2 or Bcl-B into a prodeath molecule upon interaction, perhaps by inducing conformational change that leads to exposure of the BH3 domain (64, 65).

Participation in Multiple Functions via Binding to Different Molecules

Bcl-2 family proteins may bind to other proteins to regulate their activity, thus indirectly regulating cell death, or to affect cell death. For example, CED-9 binds to CED-4 to prevent it from activating the caspase CED-3 (66). Bcl-xL may bind to BAR to regulate the activity of caspase-8 (67) and to Aven to regulate the activity of Apaf-1 (68). Bcl-2 or Bcl-xL may also bind to Bap 31 at the site of the endoplasmic reticulum to regulate the activity of caspase-8 (69). Recently, Bcl-2 family proteins have been found to affect the mitochondrial morphology associated with apoptosis, which can be related to protein-protein interactions, such as that between Bak and Mfn1 or Mfn2 (70), or between protein-lipid interactions, such as that between Bid and cardiolipin (71).

Interaction of Bcl-2 with calcineurin may be responsible for Bcl-2-mediated inhibition of cell cycle progression (72). In addition, some Bcl-2 molecules can reside in the nucleus, which can be increased following ionizing radiation treatment. Nuclear Bcl-2 can interact with both Ku70 and Ku86 via its BH1 and BH4 domains to inhibit the nonhomologous end-joining pathway, which may lead to an accumulation of DNA damage and genetic instability (73). Bcl-2 can interact with IP3 receptor 1 to affect calcium homeostasis in the ER (74), and Bax and Bak can interact with IRE-1 α to regulate the unfolded protein response (UPR) following ER stress (75). Finally, Bcl-2 and Bcl-xL can interact with NALP1 to suppress NALP-1-mediated caspase-1 activation, which is required for the generation of the inflammatory cytokine interleukin-1 (76). Thus, Bcl-2 and Bcl-xL can regulate the inflammation process. In addition, this relationship among Bcl-2/NALP-1/caspase-1 is analogous to that of CED-9/CED-4/CED-3 in which the Bcl-2 family proteins (Bcl-2, CED-9) are connected to the activation of caspases (caspase-1, CED-3) via the link of a molecule that has a CARD domain and a nucleotide-binding oligomerization domain (NALP-1, CED-4), indicating an evolutionary conservation in the mechanism.

The association of the BH3-only molecule Bad with a non-Bcl-2 family protein, glucokinase (hexokinase IV), on the mitochondria actually promotes a survival function by enhancing its activity. Notably, this activity is associated with glucose-mediated survival signaling that leads to Bad phosphorylation and inactivation of its prodeath function (77). This activity seems important for normal glycolysis and glucose homeostasis. Another BH3-only molecule, Beclin1, while not participating in apoptosis, is important for autophagy induction through its interactions with a large number of proteins, including the Class III PI-3 kinases VPS34, UVRAG, and Ambra-1, in addition to its interaction with the prosurvival Bcl-2 and Bcl-xL (see Chapter 29).

The Crystal and Solution Structures of the Bcl-2 Family Proteins

The crystal and solution structures of several Bcl-2 family proteins (i.e., Bcl-xL, Bcl-2, Bcl-w, Mcl-1, Bax, Bak, and Bid) have been defined [see the review (7) and also Chapter 4]. One of the common structural features is that these proteins are all composed of alpha helices and assume an overall similar conformation. These alpha helices consist of two central hydrophobic helices surrounded by multiple amphipathic ones. Such an arrangement of alpha helices is similar to that of the membrane translocation domain of bacterial toxins, in particular diphtheria toxin and the colicins, and suggests that the Bcl-2 family proteins may be capable of forming pores. Indeed, Bcl-xL, Bcl-2, Bax, and Bid have been shown to possess ion channel activities *in vitro* on lipid bilayers or liposomes. This activity may relate to the function of these molecules on regulating mitochondrial permeability. In addition, the BH1, BH2, and BH3 domains of the multidomain proteins form a hydrophobic pocket that is the binding site for the BH3 domain of another molecule. The hydrophobic pocket may be further stabilized by the BH4 domain.

Some viral proteins, such as Ks-Bcl-2, M11L, and N1L, have a helical fold similar to that of Bcl-xL (78, 79). Interestingly, only Ks-Bcl-2 has some sequence homology to Bcl-2; neither M11L nor N1L has sequence similarity to Bcl-2 family proteins. However, they can all inhibit apoptosis by binding to Bax/Bak. These studies indicate that structural homology may be the most important feature conserved in evolution.

Despite the similarities, differences do exist. For example, for the solution structures of Bcl-2 and Bcl-xL, differences in the structural topology and electrostatic potential of the hydrophobic pocket can be detected, consistent with the finding that the two molecules have different affinities to various interacting molecules (42, 47, 78). Such a difference even exists between two different isoforms of human Bcl-2 (47), and between the human Bcl-2 and its viral homologue, KSHV Bcl-2 (78). These variations within an overall conserved structure are compatible with the conserved antiapoptosis function but are also indicative of an emphasis on different strategies to achieve this function.

The multidomain prodeath molecules Bax and Bak have an overall structure very similar to that of the prosurvival molecules Bcl-2, Bcl-xL, Bcl-w, and Mcl-1. As of this publication, it is not clear whether and how their opposite function is affected by their structural similarity. Alternatively, these two groups of proteins may not differ in their fundamental activity in engaging the membranes, instead differing in the consequence depending on the activity of other molecules, such as the BH3-only molecules, the exposure of the BH3 domain, or the status of oligomerization during the membrane-dependent conformational change (27).

Structures of Bax and Bcl-w include the C-terminal transmembrane domain. Interestingly, they adopt a conformation similar to that of C-terminal truncated

Bcl-xL binding to a Bak BH3 peptide (7, 42) (see also Chapter 4). Here the transmembrane domain of these molecules has actually occupied its own hydrophobic pocket formed by the BH1, BH2, and BH3 domains. Bax needs to be activated for its proapoptotic function through conformational change (24, 29). It is likely that the solution structure of an activated Bax would be quite different from that of a quiescent Bax. The transmembrane domain of Bax may be released when Bax changes its conformation, thus freeing the hydrophobic pocket for interaction with other Bcl-2 family proteins, such as Bid, and/or exposing the BH3 domain to exercise the prodeath function. Similarly, the interaction of a BH3-only molecule with the prosurvival molecule may allow the displacement of the transmembrane domain, which becomes exposed and targets the molecule to the membrane location. The reverse process may also be possible, in which conformational changes related to membrane translocation trigger the release of the transmembrane domain, making the hydrophobic pocket available for binding to the BH3-only molecules. Interestingly, cytosol to mitochondria translocation occurs for Bcl-xL, Bcl-w, Mcl-1, and Bax upon apoptosis induction (7).

Among the BH3-only molecules, only Bid retains a conserved structure similar to that of the multidomain molecules (48, 49). This feature may confer to Bid some activities that may not be shared with other BH3-only molecules, such as the pore-forming activity (80), which may in turn contribute to its apoptotic function. However, one major structural difference between the multidomain proteins and Bid is that the hydrophobic pocket formed by the BH1, BH2, and BH3 domains is not present in Bid. This may permit a quick exposure of the BH3 domain upon activation, such as by proteolysis to remove the N-terminus (15, 48). On the other hand, Bad, Bmf, and Bim are intrinsically unstructured but are subjected to localized conformation upon interaction with Bcl-2 (30). For example, when Bim binds to the prosurvival Bcl-2 members, only the BH3 element becomes structured, while most residues remain disordered. Together, these observations indicate that the BH3 domain of Bid or any other BH3-only protein may function as a “donor” in its interaction with the multidomain proteins, whose hydrophobic pocket can serve as an “acceptor.” Structural studies of antideath members interacting with a BH3 domain peptide support this notion (7, 42) (see also Chapter 4).

It is not clear how the multidomain prodeath molecules Bax and Bak will change their conformation to allow their BH3 domain to be exposed, which seems to be “locked” with their BH1 and BH2 domains in the pocket site. The conformational change may be achieved upon membrane insertion (27). It is interesting to note that if the BH3 domain is made freely accessible, the molecules may acquire active killing ability via the engagement of this domain. It may very likely be the case that when Bcl-2 or Bcl-xL is cleaved by caspase-3 to remove the N-terminal region (81, 82), or when Bcl-2 or Bcl-B undergoes conformational change upon interaction with Nur77/TR3 (64, 65), they are endowed with apoptotic activity.

The differences in the structure as well as sequence of the various BH3-only molecules may suggest a diverse origin and evolution of these molecules and indicate that they may be further divided into the core group (Bcl-2, Bcl-xL, Bcl-2, Mcl-1, Bax, Bak, and Bid) that shares both the sequence and structure homology, and the group that differs either in the structure, such as Bim and Bad, or in the sequence, such as the viral proteins M11L and N1L. This may further imply that nonconserved functions may be expected among these molecules (see the upcoming section entitled “The Physiological Roles of the Bcl-2 Family Proteins”).

Regulation of the Bcl-2 Family Proteins

Regulation of Expression

Because of the potent effects of Bcl-2 family proteins on the balance between life and death, cells impose strict regulations on the expression and activity of these molecules. While certain antideath or prodeath molecules are expressed constitutively in cells, others are expressed only following death stimuli. This is particularly true for a number of proapoptosis molecules. For example, DNA damage can induce the expression of PUMA, Noxa, Bid, and Bad in a p53-dependent manner (**63, 83–86**) (see also Chapter 9). The upregulation of prodeath molecules can also be developmentally regulated, such as EGL-1, which is required for the death of the HSN neurons in the male *C. elegans* (**9**) (see Chapter 13). Similarly, deprivation of nutritional factors can also induce the expression of proapoptosis molecules. For example, Hrk/DP5 or Bim_{EL} can be induced in cultured sympathetic neurons following NGF withdrawal (**87**). While the death signals for the upregulation of the BH3-only molecules can be specific, those that can upregulate the multidomain molecule Bax are often more diverse, indicating the central position of this prodeath molecule (see the upcoming section entitled “Molecular Mechanisms of the Control of Apoptosis by the Bcl-2 Family Proteins”).

The expression of antiapoptosis molecules can be induced by survival signals or inflammatory signals, which may occur in a cell-specific or time-specific manner. For example, Mcl-1 can be upregulated by GM-CSF in myeloid cells (**88, 89**) and Bfl-1/A1 can be induced in endothelial cells or neutrophils in response to the phorbol esters LPS, TNF α , IL-1, or G-CSF (**90–92**). The expression of Bcl-xL and Bcl-2 in thymocytes and matured T cells is a good example of how homeostasis can be maintained by differential expression of these genes in a temporal-specific manner (**93**). Thus, Bcl-xL, but not Bcl-2, is preferentially expressed in the immature CD4/CD8 double positive cells. On the other hand, Bcl-2, but not Bcl-xL, is expressed in the matured CD4 or CD8 single positive cells. However, the expression of Bcl-xL is upregulated in the activated matured T cells. This probably allows the activated cells to survive for their immune functions (**94**).

Regulation Through Alternative Splicing

A puzzling fact of the regulation of the Bcl-2 family proteins is the alternative splicing. A number of these proteins, including pro- and antideath members, can be expressed in different forms. For some the differentially spliced forms can have opposite functions, such as Bcl-xL versus Bcl-xS (95) and Mcl-1L versus Mcl-1S (96). For others, alternative splicing does not alter the prodeath or antideath nature of the product, but their potency. The longer form of Bcl-2, Bcl-2 α , is more potent than the short form of Bcl-2 β (97), whereas the short form of Bim, Bim_S, is much more potent than the long form, Bim_L, or the extra-long form, Bim_{EL} (98). Bim_S and another newly defined Bim splicing variant, BimAD, may also activate the mitochondria by different mechanisms (see the upcoming section “Activation of the Multidomain Bax and Bak at the Mitochondria”). It is not clear how the alternative splicing is regulated. Tissue-specific or signal-specific mechanisms may be involved. For example, Bcl-GL is widely expressed, but Bcl-GS is only found in the testis (99). Thus, it is possible that splicing variants could regulate apoptosis in a temporally and spatially specific way.

Regulation Through Posttranslational Modifications

Posttranslational modification is probably the most significant mechanism to regulate the activities of the Bcl-2 family proteins. This is particularly important for those prodeath molecules that are normally expressed in healthy cells. These modifications often occur in response to death or survival signals and mainly include proteolytic cleavage and phosphorylation. In addition, protein conformational changes or degradation could be also induced as a posttranslational event.

Change of Subcellular Locations Resulting from Posttranslational Modifications

One of the main outcomes of the posttranslational modifications is the translocation of the modified death agonists to the mitochondria, as in the case of Bax, Bid, Bim, and Bad. In these cases, the Bcl-2 family proteins serve as sensors to the external death signals and transmit those signals to the mitochondria.

The first type of posttranslational modifications is conformational change, which, for Bax, is the first step in response to death signals (24, 28). This change may be due to an elevated cytosolic pH (100). Cellular alkalinization may alter the ionization of key amino acid residues at the N- and C-termini, thus breaking the intramolecular interactions maintained by the ionic force. The conformational change allows the exposure of the two termini, and the availability of the hydrophobic C-terminus now gives the molecule the ability to target the mitochondrial membrane (100). Translocation of Bax can also be

regulated by several Bax binding partners, such as 14-3-3 ζ (**52**) and Ku70 (**53**, **54**), upon phosphorylation or acetylation (see the earlier section on interactions between Bcl-2 family members and other molecules). The insertion of Bax seems to be also greatly facilitated by the presence of another prodeath molecule, Bid, which may induce further conformational change of the molecule (**101**).

Translocation of Bid is dependent on caspase cleavage, which is the second type of posttranslational modification (**101**). Bid can be activated by multiple proteases in various apoptosis scenarios (**101**). The cleavage occurs at the so-called loop region (aa 43-77) (Fig. 2.1), which is also susceptible to cleavage by granzyme B (Asp⁷⁵) and lysosomal enzymes (Arg⁶⁵). The 15-Kd carboxy-terminal cleaved fragment of Bid (aa 60-195), called tBid, can be further myristoylated at Gly⁶⁰ near the N-terminus (**102**). The modified Bid can now efficiently target mitochondria. This newly acquired ability may be due to the appearance of a large hydrophobic surface, which was previously buried, but revealed by the protease cleavage (**15**, **48**). The changes in hydrophobic exposure and the related surface charges, together with the myristoylation, contribute to the translocation and integration of tBid into the mitochondrial membranes.

The third type of posttranslational modification that results in subcellular translocation is phosphorylation and dephosphorylation. For example, phosphorylated Bcl-2 is largely present in the ER and correlates with the change in Bcl-2's ability to regulate the calcium balance in the ER (**103**) (see the upcoming section entitled "Regulation of Apoptosis at the Endoplasmic Reticulum"). However, Bad is the best studied for this regulatory event. In the presence of growth factor, such as IL-3, Bad can be phosphorylated (**51**). Bad has several phosphorylation sites, but phosphorylation at Ser¹³⁶ and Ser¹¹² regulates its subcellular location. While Ser¹³⁶ seems to be mainly phosphorylated by Akt/PKB (**104**, **105**), Ser¹¹² is phosphorylated by a cAMP-dependent kinase (**106**). When phosphorylated, Bad binds to a cytosolic protein, 14-3-3, and is trapped in the cytosol. Subsequent to a death stimulus, such as IL-3 deprivation or calcium influx, dephosphorylation occurs through certain phosphatases, such as calcineurin (**107**). Dephosphorylated Bad disassociates from 14-3-3 and translocates to the mitochondria, contributing to cell death (**51**). Phosphorylation of Bad at other sites, Ser¹⁵⁵ and Thr²⁰¹, can affect other aspects of Bad's function (see the next section).

There are other mechanisms to induce translocation of Bcl-2 family proteins. For example, translocation of Bim_{EL}/Bim_L from the microtubule-associated dynein motor complex to the mitochondria can be induced by cytokine withdrawal, taxol, or UV irradiation (**56**). Another BH3-only molecule, Bmf, can be activated by anoikis or UV irradiation. It is released from the myosin V motor complex and translocated to the mitochondria (**55**). In both cases, it seems that some noncaspase proteases are involved to release these molecules from their normal location in cells.

Change of Functions Resulting from Posttranslational Modifications

Both Bcl-2 and Bcl-xL can be phosphorylated by death stimuli. The chemotherapeutic drug taxol is well known for its ability to inactivate Bcl-2 by inducing its phosphorylation (108, 109). The phosphorylation occurs on serine residuals in the loop region between the first and second alpha helices (108–110) (Fig. 1.1). The phosphorylation results in decreased antiapoptotic activities of Bcl-2 and Bcl-xL (109), because their ability to interact with Bax (103, 111) and/or to regulate ER calcium balance is suppressed (103). Phosphorylation of Bcl-2 also inactivates its antiautophagy function. Bcl-2 phosphorylation by JNK dissociates its interaction with the proautophagy molecule Beclin1 and reduces its ability to inhibit Beclin1-mediated autophagy (112). Consistent with other findings, phosphorylation happens to Bcl-2 at the endoplasmic reticulum (ER), where its ability to regulate apoptosis (103) or autophagy (113) is affected by this modification.

Both Bcl-2 and Bcl-xL may also be subject to caspase cleavage (81, 82). Caspase-3 is the main caspase that cleaves these molecules, again at the loop region. The cleavage does more than just inactivate the function of these molecules; it actually bestows apoptotic activity to them. Thus, the C-terminal fragment of Bcl-2 or Bcl-xL (tBcl-2 or tBcl-xL) is able to induce apoptosis. Furthermore, cleavage-resistant molecules, which are engineered through site-directed mutagenesis to delete the caspase-3 recognition site, possess stronger antiapoptotic activity (81, 82). It seems that the altered tBcl-2 or tBcl-xL may not contribute to cell death at the early initiation stage, since the modification occurs only after caspase activation. However, these truncated molecules can further accelerate the death process.

In another case, phosphorylation of Bad can cause the molecule not only to be trapped in the cytosol by 14-3-3 (see the preceding section), but also to be inactivated. The latter was accomplished through phosphorylation at Serine¹⁵⁵, which is in the middle of the BH3 domain, by protein kinase A (114). This event is simulated by growth factors and requires the prior phosphorylation at Ser¹³⁶, which leads to Bad being trapped by 14-3-3 (see above). However, Ser¹⁵⁵ phosphorylation suppresses Bad-Bcl-xL interaction, thus further restricting it from engaging in apoptosis (114, 115). Interestingly, growth factor deprivation, while causing Bad dephosphorylation at the three serine sites (Ser¹¹², Ser¹³⁶, Ser¹⁵⁵), can also activate JNK, which in turn phosphorylates Bad at Thr²⁰¹ (116). This phosphorylation event has the same effect as Ser¹⁵⁵ phosphorylation to suppress Bad-Bcl-xL interaction, providing another way to inhibit apoptosis. Notably, phosphorylation at Ser¹⁵⁵ or Thr²⁰¹ can engage Bad in nonapoptotic function related to glycolysis (77, 117) (see the upcoming section entitled “Role in Other Physiological Functions”), thus serving as a functional switcher.

Bid is another BH3-only molecule that can be regulated by phosphorylation. It can be phosphorylated by Casein Kinase I or II, which results in the Bid's resistance being cleaved by caspase-8 (118). Phosphorylation-resistant mutant (S61A, S64A) was more cytotoxic than the wild-type molecule, reflecting that

this type of phosphorylation could probably be physiologically relevant. Phosphorylation of Bid at the same sites by ATM during DNA damage or replication stress could allow Bid to perform a function at the intra-S phase checkpoint and cause S phase arrest (119, 120). This function of Bid's has yet to be more firmly established because disputes have arisen (121).

Molecular Mechanisms of the Control of Apoptosis by the Bcl-2 Family Proteins

The Bcl-2 family proteins regulate apoptosis mainly via their effects on mitochondria. They can also be found at the endoplasmic reticulum and thus can regulate the contributions of these organelles to apoptosis. The activation of the mitochondrial pathway is signified by the release of mitochondrial apoptotic proteins and by mitochondrial dysfunction (7, 15, 16, 122–124). Both processes are inhibited by the death antagonists (Bcl-2, Bcl-xL, etc.) but are promoted by the death agonists (Bax, Bak, Bid, Bim, etc.). A detailed discussion of the mitochondria's activation and mechanisms can be found in Chapter 6. Briefly, the release of the mitochondrial apoptotic proteins results from an increase in the outer membrane's permeability, which may be due to opening of the pore formed by Bax or Bak, the mitochondrial permeability transition pore, or a pore made from components of the two. The mitochondrial dysfunction is often characterized by the mitochondrial depolarization and ROS generation, which are in part contributed by the loss of cytochrome c. Recent studies have also defined the significant impact of the Bcl-2 family proteins on mitochondrial morphology by regulating the fission and fusion processes, therefore affecting the mitochondrial apoptosis process, such as cytochrome c release (see Chapter 6).

Activation of the Multidomain Bax and Bak at the Mitochondria

The multidomain prodeath proteins Bax and Bak are responsible for the induction of the mitochondrial outer membrane permeabilization. Deletion of both Bax and Bak, but not one of them, renders the cell completely resistant to all major mitochondrial death signals, including DNA damage, growth factor deprivation, and endoplasmic reticulum stress, and to the extrinsic pathway signals mediated by Bid (46, 125–127). We must point out, however, that Bax and Bak may not completely overlap in their functions. In many cases, it seems that Bax is more sensitive to apoptotic stimuli than Bak. In the human colon carcinoma cell line HCT116, which has mismatch repair deficiency, apoptosis induced by nonsteroidal antiinflammatory drugs or TRAIL is dependent on Bax, but not Bak (128, 129). In addition, the genetic deletion of Bax alone is sufficient to render sympathetic neurons resistant to NGF-deprivation-induced

apoptosis (130). In other cases, Bak, but not Bax, is required for apoptosis induced by conditions such as protein synthesis inhibition (131). One may keep in mind that Bax is usually localized in the cytosol in healthy cells and translocated to the mitochondria in response to death stimuli. But Bak constitutively resides in the mitochondria. This distinction may contribute to the differential stimulation of Bax and Bak in certain cases. Another implication is that since Bax and Bak have a different interaction profile with anti-death molecules (Fig. 2.2), induction of Bax- or Bak-dependent apoptosis would have to be regulated by a distinct set of molecules. For example, Bcl-xL, Mcl-1, Noxa, and Bik would be particularly important for modulating Bak-mediated apoptosis (35, 131).

The BH3-only proteins clearly act upstream of Bax and Bak, because they cannot induce apoptosis in cells lacking both Bax and Bak (46, 126, 127). The different BH3 molecules serve as sentinels to different apoptosis signals (16). For example, PUMA and Noxa are mainly responsible for the DNA damage-induced apoptosis, Bid is responsible for protease signals, and Bad and Bim are engaged in apoptosis induced by growth factor deprivation and cytokine deprivation. How Bax and Bak are activated by the BH3-only molecules and how the anti-death molecules inhibit the activation are not entirely clear. Currently, two models, a direct activation model and an indirect activation model, have been proposed to explain how the BH3-only molecules can activate Bax or Bak, based on whether or not they interact with each other (7, 22, 33, 50, 132, 133) (Fig. 2.3).

In the direct model, the BH3-only molecules can directly activate the multidomain molecules Bax and Bak to initiate the mitochondrial events (20–22, 38, 41). They are thus termed *activators*, which includes Bid, Bim, and PUMA (20–22). Bid can interact with Bax, Bak, and Bcl-2 (29, 31, 38). But its apoptosis-inducing capability is dependent on its ability to interact with the pro-death multidomain molecules, but not the anti-death Bcl-2 or Bcl-xL (22, 31). Although Bcl-xL can interact with Bid, Bax, and Bak, its effects consistently seem to be more dependent on its binding to Bid rather than to Bax, based on the use of Bcl-xL mutants that can differentially bind to Bid and Bax (45, 46). Conversely, Bax or Bak mutants that could not bind to Bcl-2, Bcl-xL, or Mcl-1 are still susceptible to suppression by these molecules, indicating that these anti-death molecules function by not directly antagonizing Bax or Bak (22). Instead, they bind to Bid, Bim, or PUMA to prevent them from activating Bax and Bak. Other BH3-only molecules, such as Bad, Noxa, and Bmf, can bind to the anti-death molecules to liberate the bound Bim, Bid, or PUMA so that the latter can activate Bax or Bak (22). The latter group of BH3-only molecules is called *inactivators* (22) or *sensitizers* (132). Notably, the ability of the inactivator to dissociate the activator BH3-only molecules from the binding with the anti-death Bcl-2, Bcl-xL, or Mcl-1 is dependent on its affinity to interact with the latter. Thus, while Bad could liberate Bid, Bim, and PUMA from interaction with Bcl-2 or Bcl-xL, it cannot liberate them from interaction with Mcl-1. On the other hand, Noxa works just the opposite. Bik and BMF can only liberate

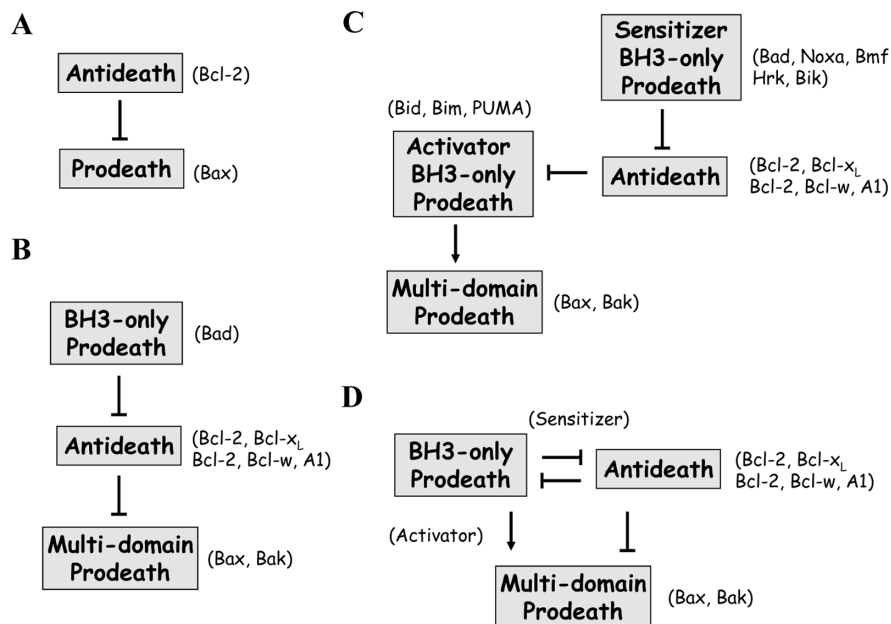


Fig. 2.3 Models for the activation of multidomain prodeath Bcl-2 family proteins. Genetic studies indicate that the multidomain prodeath Bcl-2 family proteins Bax and Bak are required for the mitochondria-mediated apoptosis, which is regulated by other family members. Since there are extensive and diverse interactions among the family members (see Fig. 2.2 and the text), how these molecules activate or suppress each other in response to different death signals is not entirely clear. The first proposed model (**A**) was based on two molecules, Bcl-2 and Bax (32). This rheostat model indicates that Bcl-2 suppresses apoptosis by heterodimerizing with Bax, whereas Bax homodimers kill the cell. Thus, the relative expression levels of Bcl-2 versus Bax dictate the cell's fate. With the discovery of more family members and in particular, the BH3-only molecules, the interaction pattern becomes convoluted and so is the mechanism of activation. Essentially, two models, the indirect model (**B**) and the direct model (**C**), have been proposed, which differ mainly in how Bax and Bak are activated and whether the multidomain or BH3-only prodeath molecules are sequestered by the antideath molecules (7, 22, 33, 50, 132, 133). There are experimental observations that could not be explained by either model alone. Therefore, it is possible to propose a model that combines the elements of the two (**D**). See the text for a detailed discussion

Bid from the interaction with Bcl-2 or Bcl-xL. These types of relationships, together with the different ability of the antideath molecules in suppressing apoptosis induced by different signals, signify the importance of understanding the specific relationship of the Bcl-2 family proteins in individual cases. A further distinction of the antideath molecules that are bound with the activator BH3-only molecules (called “primed”) from those without the binding partners (called “empty”) may have clinical significance in that only inactivation of the “primed” but not the “empty” antideath molecules, e.g., with the corresponding inactivator/sensitizer BH3-only molecules, may be expected to cause subsequent death due to the release of the activator BH3-only molecules (132, 134).

The direct model may not explain all the phenomena. Thus, the indirect model argues that once the BH3-only molecules have been translocated to the mitochondria, they may not affect Bax or Bak directly but rather bind to the antideath Bcl-2 family proteins to antagonize their function or to convert them to Bax- or Bak-like molecules for oligomerization with Bax or Bak (7, 33, 50) [Fig. 2.2(b)]. In this case, Bax or Bak is in check by the antideath molecules, which are deactivated by the BH3-only molecules. BH3-only molecules work as sensitizers only in this model. Perhaps the best evidence to support the activation of Bax or Bak by dissociation from the antideath molecules without the involvement of the activator BH3-only molecules is that cells deficient in Bid and Bim, and with reduced PUMA, are still susceptible to apoptosis (33). Another piece of evidence is that in *C. elegans*, the BH3-only molecule EGL-1 binds competitively to the antideath molecule CED-9 so that CED-4 is released from the binding with CED-9 to activate the caspase homologue CED-3 (66).

The major difference between the two models is whether Bax or Bak is directly activated by a subset of BH3-only molecules (Bid, Bim, and PUMA) (direct model) or indirectly activated due to the dissociation from the antideath molecules (indirect model). Conversely, they differ in whether the antideath Bcl-2, Bcl-xL, or Mcl-1 is sequestering/inactivating Bax and Bak (indirect model) or a subset of BH3-only molecules (Bid, Bim, and PUMA) (direct model). Finally, the sensitizer BH3-only molecules deactivate the antideath Bcl-2, Bcl-xL, and Mcl-1 in both models, but the resulting mechanisms are not considered the same in activating Bax and Bak. In reality, it is likely that events described in both models can occur. Direct activation of Bax and Bak by the activator BH3-only molecules and direct association of antideath molecules with Bax and Bak are both concrete findings. In addition, recent studies using BH3 mimetics demonstrate that both types of prodeath molecules can be desequestered from the complex with the antideath molecules from cancer cells. For example, ABT737 and GX15-070 (Obatoclax) (see the upcoming section entitled “Role of the Bcl-2 Family Proteins in Cancer Biology and the Development of BH3-Based Therapeutic Strategies”) could cause the release of Bim from the complex with Bcl-2 or Bcl-xL (134-136) and the release of Bak from Bcl-xL or Mcl-1 (135, 136). These results suggest that the antideath molecules are able to sequester both types of prodeath molecules and that activation of Bax or Bak can be caused either by the activator BH3-only molecules, such as Bim, or by the derepressed “spontaneous” oligomerization, depending on individual cellular context [Fig. 2.3(d)].

The indirect activation model implies that Bax and Bak may be capable of “spontaneous” activation or may be preactivated by other molecules to convert into “primed conformation,” which is, however, suppressed by the antideath members until the BH3-only molecules inactivate the latter. This model suggests that Bax or Bak may have two conformers in cells, primed with their BH3 domain exposed and unprimed with their BH3 domain hidden. Antiapoptotic Bcl-2 members such as Bcl-2, Bcl-xL, and Mcl-1 bind the primed conformer, as

the BH3 domain of Bax or Bak is required for this interaction (50). Although the signal for priming is not known yet, it is possible that the membrane environment where these molecules reside could provide the condition required for the conformational change and interactions (27).

Regulation of Apoptosis at the Endoplasmic Reticulum

The Bcl-2 family proteins have a distinct role in regulating ER functions, which in turn affect cell death and other cellular functions. Only a brief discussion is given here, as detailed information can be found in Chapter 7. Bcl-2 family proteins, such as Bcl-2 and Bax, can be found in the ER (75, 103, 137–139). The most well-studied function of Bcl-2 family proteins related to ER physiology is regulation of the ER's calcium level. The balance between anti- and proapoptotic proteins at the ER may affect the steady-state ER calcium content and directly impacts the amount of calcium released after stimulation. Thus, cells deficient in both Bax and Bak exhibit decreased ER calcium content (138, 139), similar to the phenotype of the Bcl-2 overexpressing cells (103). This regulation may be mediated by the interaction of Bcl-2 with the IP3 receptor, which is antagonized by Bax and Bak (74). Calcium released from the ER can participate in cell death by activating the mitochondria's permeability transition pore. Thus, Bcl-2 family proteins could regulate the cross-talk between the ER and mitochondria to affect cell death.

A major cause of cell death is ER stress. A complicated cellular response called the *unfolded protein response* can be initiated in response to ER stress, which is mediated by the ATF6, PERK/eIF-2 α , and IRE-1 pathways, resulting in both protective and apoptotic effects. Bcl-2 family proteins can participate in this process on two different levels. First, Bim has recently been shown to be a transcriptional target of CHOP, downstream of the PERK/eIF-2 α pathway (140). This upregulation of Bim seems to be important for the ER stress-induced apoptosis, which is consistent with the general consensus that persistent activation of the PERK/eIF-2 α pathway can lead to cell death. Second, Bax or Bak could bind to IRE-1 α to promote stronger IRE-1 α -mediated signaling (75). Since this pathway is largely responsible for the protective effects of UPR, this finding suggests that Bax or Bak could have some prosurvival function in this setting. However, these findings have yet to be reconciled with other observations regarding the prodeath function of Bax and Bak at the ER site (via the regulation of calcium).

The Physiological Roles of the Bcl-2 Family Proteins

Because of the critical roles played by the Bcl-2 family proteins in the regulation of apoptosis, these proteins are important to organisms during embryonic development and in adult life. Bcl-2 family proteins also participate in functions not related to the classical apoptosis pathways.

Role in the Regulation of Apoptosis

Programmed cell death was initially defined by developmental biologists as describing the temporally and spatially controlled death of cells during development (141). In fact, the genetic pathway of programmed cell death was first characterized in the nematode *C. elegans* (see Chapter 13). The antideath molecule *ced-9* is essential to the normal development of the worm, so that the loss-of-function mutation of this molecule causes normally survived cells to die, which results in embryonic lethality. Such an essential role of antideath molecules has also been observed in mammals (7, 142) (Table 1) (also see Chapter 15). Inactivation of the mammalian antiapoptosis genes *bcl-xL* or *mcl-1* leads to embryonic lethality. While Bcl-xL seems to be important for the development of the neuronal and hematopoietic systems (143), Mcl-1 is critical to the development of trophectoderm, which is important for the implantation of embryos to the uterus (144) and to the development of hematopoietic stem cells (145). Although the deletion of Bcl-2 from the mouse genome only results in partial lethality, the survived mice nevertheless have significant developmental defects, including thymic atrophy, polycystic kidney disease, and melanocyte maturation arrest that leads to hypopigmentation (146). These deficiencies could be rescued by the concomitant deletion of Bim (147).

While Bak-deficient mice have no observable phenotype, a large fraction of *Bax* and *Bak* double-knockout mice die during embryogenesis or soon after birth (125). These mice have significant developmental defects that correlate with the deficiency in cell death. *Bax* and *Bak* doubly deficient cells are resistant to most forms of stress-induced apoptosis (126) or death caused by overexpression of the BH3-only molecules (127). These results demonstrate that *Bax* and *Bak* have a functional overlap but are essential for mitochondria-mediated apoptosis.

A number of nonlethal defects have also been observed in genetic models where other Bcl-2 family genes have been deleted. For example, mice efficient in one of the four A1 genes *A1A* are normal in development, but an abnormally rapid apoptosis occurs to their granulocytes and mast cells in culture (148, 149). Male mice deficient in *Bcl-w* or *Bax*, although alive, are infertile due to abnormal spermatogenesis (150, 151).

Mice deficient in different BH3-only molecules have various phenotypes, as these molecules serve as sentinels to different death signals. While not affected developmentally, mice deficient in *PUMA* or *Noxa* are resistant to p53-dependent DNA damage-induced apoptosis (152, 153). Interestingly, *PUMA* seems essential for γ radiation-induced apoptosis, while *Noxa* seems essential for UV radiation-induced apoptosis in embryonic fibroblasts (MEFs) (154). The *Noxa* and *PUMA* doubly deficient thymocytes cells are highly resistant to whole body γ -irradiation, equivalent to the loss of p53 (155). *PUMA* is also important in p53-independent apoptosis induced by cytokine deprivation, glucocorticoids, or phorbol ester (152, 153).

Bim-deficient mice mainly present a phenotype in the immune system, demonstrating a deficiency in the elimination of autoreactive and activated lymphocytes (156). These mice also have a deficiency in responding to other death stimuli. Together, Bim and PUMA are likely activated by the most apoptotic stimuli in multiple cell types, as deletion of both *Bim* and *PUMA* in mice seems to provide the same level of protection as the overexpression of Bcl-2 or the combined loss of Bax and Bak (157).

Bid is activated by protease, particularly by caspase-8 following death receptor activation. Thus, *Bid*-deficient mice are resistant to Fas-mediated apoptosis in hepatocytes, where mitochondrial activation is required for the death receptor-initiated apoptosis (158). In addition, old mice could develop abnormalities in myeloid cells (159). While Bad deletion does not seem to lead to any major deficiency in apoptosis, it causes a significant defect in glucose-stimulated insulin secretion, suggesting that its normal function in cells is more closely associated with glycolysis regulation (160). Mice lacking Bik, Hrk, and Bmf are essentially normal in development and in cellular response to apoptosis (7). Bmf-deficient thymocytes are resistant to glucocorticoids and histone deacetylase inhibitor-induced apoptosis and the mice developed B-cell restricted lymphadenopathy (161). Finally, double deletion of Bim and Bik leads to male sterility, which was not observed in the single-knockout mice (7), suggesting that these BH3-only molecules have overlapped or compensatory functions.

Role in the Regulation of Autophagy

Autophagy and apoptosis are intimately linked (see Chapter 29). Bcl-2 family proteins can be important for the regulation of autophagy. Several BH3-only molecules directly participate in autophagy activation. The best-studied molecule in this regard is Beclin1, which was originally identified as a Bcl-2-interacting protein (162), and it possesses a conserved BH3 domain that could also interact with Bcl-xL and Mcl-1 (163). Beclin1 is also the homologue of yeast Atg6 and can form a complex with multiple other molecules to promote autophagy. On the other hand, Beclin1 does not seem to cause apoptosis. Its interaction with the antideath Bcl-2 family protein leads to suppression of its proautophagy function. Thus, Bcl-2, Bcl-xL, and Mcl-1 also possess this antiautophagy function (163, 164). Interestingly, only the expression of Bcl-2 at the ER membrane could specifically interact and inhibit Beclin1, suggesting that signaling events originating from the ER are crucial for autophagy. The formation of a Bcl-2-Beclin1 or Bcl-xL-Beclin1 complex could be disrupted and thus suppressed by BH3-only proteins, like Bad, or BH3 mimetics, like ABT373, suggesting that extensive crosstalk can occur between apoptosis and autophagy (163).

Bnip3 and Nix/Bnip3L are two other well-defined BH3-only molecules that seem to be important for autophagy. Bnip3 and Nix share 53–56% amino acid sequence identity. They are important for the autophagic clearance of

mitochondria during the maturation of red cells (165–167). Bnip3-mediated autophagy in a nondevelopmental context could be either detrimental or protective, depending on the context (167). Unlike other BH3-only molecules, the TM domain of Bnip3 but not its BH3 domain is required for its function (44). Bnip3 is not ubiquitously expressed under normal conditions. Its expression is markedly increased in response to hypoxia and appears to be regulated by hypoxia-inducible factor 1 (HIF-1) (167).

Other BH3-only molecules that have been implicated in autophagy include Bik (168), EGL-1 (113), and ApoL1, a recently described lipid binding protein that possesses a BH3 domain (169). The expression of ApoL1 can be induced by p53. When overexpressed, it can cause autophagic death, which requires the BH3 domain and the classical autophagy machinery.

Role in the Regulation of Cell Proliferation

It has been long known that the Bcl-2 family protein could regulate cell cycle progression from G₀/G₁ to the S phase, which can be inhibited by the antideath molecules Bcl-2, Bcl-xL, Bcl-w, and Mcl-1 and the adenoviral Bcl-2 homologue, E1B19K (16), but promoted by the prodeath molecules Bax (170), Bad (171), and Bid (172). This has been demonstrated in different cell types, including lymphocytes, hepatocytes, and fibroblasts.

The mechanism is not completely clear although several possibilities are present (16, 173). One possibility is the regulation of the classical cell cycle machinery, such as p27^{Kip1}, cyclin E, and the Rb family member p130. In Bcl-2-overexpressing quiescent T cells, the levels of p27^{Kip1} and the Rb family member p130 were increased following stimulation by growth factors or mitogens. In hepatocytes, while the deletion of Bid (172) or the overexpression of Bcl-2 (174) does not seem to affect the expression of p27^{Kip1}, they do change the kinetics of the expression of cyclin E following partial hepatectomy, which is delayed in both cases. Another possibility is the regulation of proliferation signaling along the calcium-calmodulin-calcineurin-NFAT pathway or the Raf-ERK pathway (173).

Notably, through a series of mutagenesis analyses of Bcl-2 and Bcl-xL, it was found that no mutant could segregate the function on cell death and the function on cell proliferation (175, 176), suggesting that Bcl-2 family proteins could operate through comparable mechanisms to regulate these two functions. In addition, it is possible that like their regulations on cell death, the prodeath and antideath Bcl-2 family proteins could act on common targets in an opposite way to regulate cell proliferation.

Role in Other Physiological Functions

As mentioned earlier (see the section entitled “Participation in Multiple Functions Through Binding to Different Molecules”), by interacting with nonfamily proteins, Bcl-2 family molecules could exert diverse functions, some of which are

further summarized here with additional perspectives. Bcl-2 family proteins could potentially affect the repair of DNA damage. For example, nuclear Bcl-2 could interact with both Ku70 and Ku86 via its BH1 and BH4 domains to inhibit the nonhomologous end-joining pathway, which may lead to an accumulation of DNA damage and genetic instability (73). Although still controversial, Bid may be phosphorylated by ATM to play a role in the intra-S phase checkpoint (119–121). This ability of Bid is not dependent on its BH3 domain (120).

The BH3-only molecule Bad has a unique role in reulating glycolysis via interaction with the mitochondrial glucokinase in a complex that also contains protein kinase A (PKA), protein phosphatase 1, and a PKA-anchoring protein (WAVE-1) (77). The glucokinase is responsible for phosphorylating glucose to produce glucose 6-phosphate, the first step in several pathways of glucose metabolism, including glycolysis and the storage of excess glucose as glycogen. A recent study further finds that BAD plays a physiological role in glucose-stimulated insulin secretion by beta cells (160). This function is also specifically dependent on the phosphorylation of the Ser¹⁵⁵ at the BH3 domain, which is important for Bad to interact with glucokinase. Interestingly, Bad can also interact with another key glycolysis enzyme, phosphofructokinase-1 (PFK-1), and phosphorylation of Bad at Thr²⁰¹ by JNK is required for the activation of PFK-1 by Bad (117).

Finally, MULE/ARF-BP1 is an E3 ligase that can ubiquitinate Mcl-1 for degradation during DNA damage-induced apoptosis (177). This E3 ligase has a BH3-only domain that is required for the binding with Mcl-1. Interestingly, MULE/ARF-BP1 can also ubiquitinate p53 for degradation, which is suppressed by ARF (178). Thus, this molecule may have different ways to regulate cell death under different contexts.

Role of the Bcl-2 Family Proteins in Cancer Biology and the Development of BH3-Based Therapeutic Strategies

Apoptosis, cancer development, and cancer therapy are closely associated (see Chapter 25). The ability of antideath members to maintain cell survival over a long period can be dangerous if it is not under tight control. Indeed, the abnormal expression of these molecules could lead to oncogenesis. A chromosomal translocation (14;18) that results in a deregulated expression of Bcl-2 is responsible for the etiology of 85% follicular lymphomas and 20% diffuse B-cell lymphomas in humans (179). The deregulated expression of both anti-death and prodeath molecules has been demonstrated in many types of cancers (50, 180). This finding leads to the definition of a new type of proto-oncogenes represented by *bcl-2* that mainly affect cell death (50, 181). While the overexpression of Bcl-2 could lead to increased tumorigenesis, the loss of any single prodeath molecule has not been found to cause tumor development (7, 50). The combined deletion of Bim and PUMA (157), or PUMA and Noxa (155), or Bax

and Bak (125) in mice has not conclusively demonstrated an increased spontaneous tumor development, although lymphoid hyperplasia is evident in these cases (125, 157). These findings may suggest that there is a significant redundancy in cell death molecules and pathways, including those not mediated by the Bcl-2 family proteins, which can work together to affect the survival of tumor cells. Alternatively, tumorigenesis may occur only when both cell death and cell cycle are deregulated, such as following p53 mutation. Notably, when the overexpression of Bcl-2 or the loss of a single prodeath gene, such as Bim, PUMA, or Bax, is combined with the overexpression of a classical oncogene, such as myc or SV40 large T antigen, tumor development could be significantly accelerated (50), indicating that cell death is important for tumor development in the context of oncogenic transformation, where other cellular functions, such as cell cycle progression and energy metabolism, are also deregulated.

The concept of cell death molecules serving a role in neoplasia is helpful in treating cancers that may have developed a resistance to chemotherapy. Bcl-2, Bcl-xL, and/or Mcl-1 are overexpressed in many cancers, which correlates with poor survival, progression of the disease, and resistance to therapy (180). Thus, they can be ideal targets for cancer therapy. Several strategies have thus been developed (50, 132, 180).

An early approach is to use antisense oligonucleotides to reduce the expression level of Bcl-2 proteins. Oblimersen (Genasense), an antisense oligonucleotide against *BCL2*, has been clinically tested for several types of cancers, but the results were not ideal (132, 180). This may be because Oblimersen does not inhibit Bcl-2 function, but merely reduces its level. There are no definite data available to address what level of reduction of Bcl-2 could be obtained *in vivo*. In addition, most cancer cells may also have an increased level of Bcl-xL and/or Mcl-1. Thus, simply targeting Bcl-2 may not be enough.

A more recent approach is based on the use of the BH3 domain to antagonize the prosurvival Bcl-2 molecules (Fig. 2.2). Synthetic BH3 peptides have been designed, which can be further chemically modified to stabilize their α -helical structure. These peptides have been shown to be cell-permeable, protease-resistant, and able to induce apoptosis (182). More extensively tested are small organic molecules that mimic the BH3 peptides. A well-characterized mimetic is ABT-737, developed based on a structure-activity relationship by NMR (183). ABT-737 can mimic the BH3 domain of Bad and bind to Bcl-2, Bcl-xL, and Bcl-w with subnanomolar affinity. It demonstrates synergistic cytotoxicity with chemotherapeutic drugs or radiation in various cell lines and patient-derived primary cells and in tumors xenotransplanted in mice (180). Several other BH3 mimetics are also under development (132).

Additional methods not based on known protein interactions of the Bcl-2 family molecules have also been employed, which include the standard high-throughput screening and structure-based virtual screening. These approaches have led to the discovery of several other molecules, including Tetrocarcin A, HA14-1, and antimycin (180). Both HA14-1 and antimycin seem to act like a

BH3-domain mimetic, inhibit the function of Bcl-2 and Bcl-xL, and activate the mitochondrial apoptosis pathway.

Several key issues may need to be emphasized in the use of these chemicals in treating cancers (132). First, it has to be determined in the first place whether Bcl-2 or Bcl-xL is actually important in suppressing cell death in the particular type of cancer to be targeted. As mentioned above, it seems that the BH3 mimetics will work only if the targeted antideath molecules have been primed, or in binding with a sequestered prodeath molecule. In such cases, the sequestered prodeath molecule can be released to activate the death process (132, 134–136). Second, targeting Bcl-2 or Bcl-xL may not be sufficient in certain cancers, as Mcl-1 could play a role in the resistance as well (184). Thus, it would be necessary to target Mcl-1 in cases that are resistant to ABT-737 or the like, which only targets Bcl-2, Bcl-xL, and Bcl-w. Although BH3 mimetics that selectively bind to Mcl-1 have not been described yet, Mcl-1 may be indirectly modulated by other agents, such as the proteasome inhibitors, which increase the level of Noxa that can inhibit Mcl-1, or a kinase inhibitor, Sorafenib, which can downregulate Mcl-1 expression (185). Alternatively, a pan-suppressor that can bind to all antideath molecules should work as well. BH3 mimetics that fulfill this requirement have been developed and are found to be effective in inactivating both Mcl-2 and Bcl-xL (GX15-070/Obatoclax and TW-37) (132, 136, 185–189).

Concluding Remarks

The Bcl-2 family proteins are important to a number of physiological functions that are beyond the regulation of apoptosis. Although they have been classically considered to function at the mitochondria, recent findings indicate that these molecules could work in other subcellular compartments, such as the ER and the nucleus, to fulfill their diverse roles. This family of proteins includes both antideath and prodeath members. Despite the significant sequence diversities, they share homology in certain BH domains. The prodeath and antideath members can directly or indirectly antagonize each other's activity in various functional scenarios. The significant understanding of their interactions and their fine structures in recent years has led to the development of novel therapeutic strategies and agents, which may have promising utilities in treating cancers.

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