Regulation of Bcl-2 Family Proteins by Posttranslational Modifications

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Abstract: Like many proteins, function and abundance of Bcl-2 family proteins are influenced by posttranslational modifications. These modifications include phosphorylation, proteolytic cleavage, ubiquitination, and proteosomal degradation. These modifications, depending on cellular context and the proteins involved, can result either in a promotion of inhibition of apoptosis. Many of these modifications are governed by the activity of enzymes. As modulation of enzymatic activity can be achieved fairly efficiently using small molecules, understanding the effects of posttranslational modifications may allow for the therapeutic inhibition or promotion of apoptosis.

Keywords: BCL-2, apoptosis, BH3-only, BAX, BIM, BID, BAD, phosphorylation, kinase, proteosome, MCL-1.

INTRODUCTION

Abnormal regulation of apoptosis can be involved in the pathogenesis of human diseases, such as cancer, in which there is insufficient apoptosis, or neurodegenerative disorders or myocardial infarction, in which there is excessive apoptosis. The Bcl-2 proteins are crucial regulators of apoptosis signaling pathways [1-3]. Antiapoptotic members of the Bcl-2 protein family include Bcl-2, Bcl-xL, Bcl-w, Mcl-1 and Bfl-1. They control mitochondrial outer membrane permeabilization (MOMP) through sequestering proapoptotic Bcl-2 protein family members [4]. The proapoptotic members of the Bcl-2 protein family include BH3-only proteins Bid, Bim, Bad, Noxa, Puma, Bmf, Bik, Hrk and multi-domain proapoptotic proteins Bak and Bax. BH3-only proteins are classified into two groups; activator BH3-only proteins (Bid, Bim) act through direct induction of Bax/Bak oligomerization and sensitizer BH3-only proteins (Bad, Noxa, Puma, Bmf, Bik, Hrk) promote apoptosis by displacing activators from antiapoptotic Bcl-2 proteins. A model for the functional regulation of apoptosis by Bcl-2 proteins is summarized in Fig. (**1**).

The signaling pathways that regulate cell death and cell survival are complex. The cellular apoptotic or survival response is often dictated by the expression levels, protein-protein interaction patterns and posttranslational modifications (PTMs) of Bcl-2 protein family members. Multi-site posttranslational modifications of proteins modulate protein activity, folding and conformation, stability and half-life, subcellular localization and protein-protein interactions [5]. Most PTMs are covalent attachments of chemical moieties to amino acid residues of proteins. The functional cooperation between multiple PTMs within distinct proteins orchestrates the overall cellular response and may facilitate the fine-tuning of protein-protein interactions. Proteolytic processing may lead to activation or inhibition of a protein by selective cleavage in the context of a specific sequence motif. Polyubiquitination may mark proteins for proteasome-mediated protein degradation. PTMs target specific amino acids at specific recognition motifs in the protein structure and they are often dynamic in nature which enables rapid and effective response to different cellular conditions.

In this review, we discuss some of the posttranslational modifications of the Bcl-2 protein family members that govern apoptosis signaling pathways, highlighting their role on disease pathogenesis and therapy response. There have been many different posttranslational modifications noted in Bcl-2 family proteins. The effects of such modifications can be hard to remember. As a convenient reference, therefore, we provide Table **1**, which summarizes the many modifications discussed in this review.

PHOSPHOREGULATION OF BCL-2 PROTEINS

Phosphoregulation of Antiapoptotic Bcl-2 Proteins

Protein phosphorylation on serine, threonine and tyrosine residues is a fundamental mechanism for cellular regulation, metabolism, growth and signaling. Nearly 30% of all eukaryotic proteins are phosphorylated and chromosomal mapping indicate that 244 of 518 kinase genes are disease- or cancer-related [6]. Reversible phosphorylation and dephosphorylation cascades modulated by kinases and phosphatases are one of the main mechanisms that directly regulate the function and conformation of both antiapoptotic and proapoptotic members of the Bcl-2 protein family.

Bcl-2 operates as a binding protein for proapoptotic Bcl-2 proteins; its posttranslational modifications may influence the function of both Bcl-2 and its binding partners. Therefore, alteration of the upstream signaling pathways leading to Bcl-2 phosphorylation or dephosphorylation in cancer cells may play a role in malignant transformation or modulate their sensitivity to chemotherapeutics. The functional significance of the

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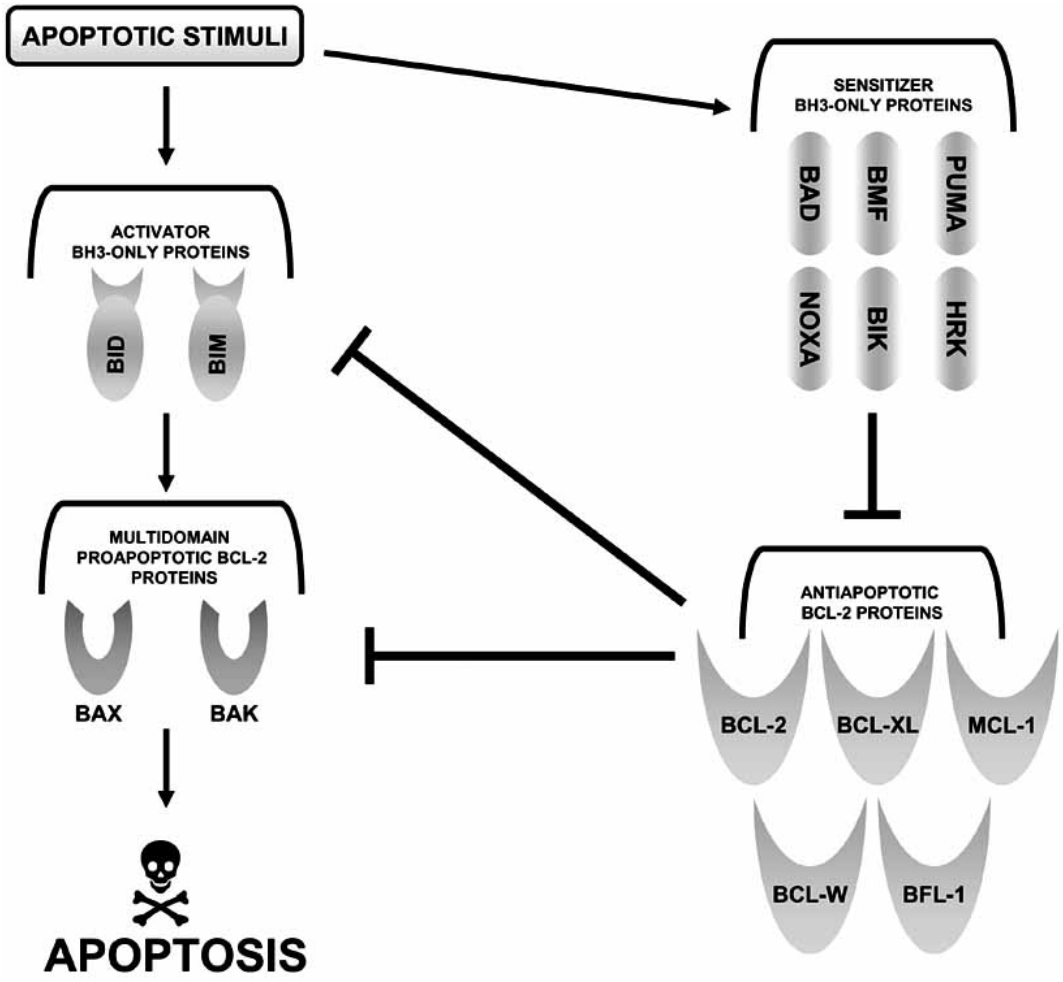

Fig. (1). A model for the regulation of apoptosis by Bcl-2 proteins. In response to apoptotic stimuli including chemotherapy, radiation, growth factor withdrawal, activator BH3-only proteins Bid and Bim trigger Bax and Bak oligomerization to promote cytochrome *c* release and apoptosis. Antiapoptotic Bcl-2 proteins sequester activator BH3-only proteins, thus prevents Bax and Bak activation. Antiapoptotic Bcl-2 proteins could also directly capture monomeric Bax and Bak to inhibit apoptosis. Sensitizer BH3-only proteins competitively displace either activator BH3-only proteins or Bax/Bak to activate apoptosis.

phosphorylation of Bcl-2 in regulation of apoptotic response remains controversial. Phosphorylation of Bcl-2 in its flexible loop domain has been designated as an essential mechanism for modulation of Bcl-2 function in response to either pro-apoptotic or survival stimuli [7-10]. This flexible loop domain is between BH3 and BH4 domains of Bcl-2 and conserved among Bcl-2, Bcl-xL and Mcl-1 underscoring its functional consequence. Paradoxically, the deletion of the flexible loop domain was demonstrated to either enhance or abrogate the survival function of Bcl-2 against paclitaxel-induced apoptosis [7,9,11]. Phosphorylation of Bcl-2 at Ser-70 at the flexible loop domain by PKC-α has been reported to be required for inhibition of apoptosis in IL-3-deprived or etoposide-treated myeloid cells [12,13]. PKC-α was also shown to localize to mitochondria with increased Bcl-2 phosphorylation and increased resistance to etoposide-induced cell death. Bcl-2 can be phosphorylated either at the Ser-70 alone or at multiple sites (S70, T69, S87) simultaneously [14,15]. Furthermore, both monosite (Ser-70) and multisite (S70, T69, S87) phosphorylations of Bcl-2 in the flexible loop domain were demonstrated to protect against paclitaxel-induced apoptosis and augment the stability of Bcl-2 protein to proteolytic degradation [14]. NGF-mediated phosphorylation of Bcl-2 in PC12 pheochromocytoma cells was associated with increased survival and dephosphorylation of Bcl-2 was linked to apoptosis in response to Angiotensin Type 2 receptor activation [16]. These results are compatible with the recent reports which proposed that the dephosphorylation of Bcl-2 at Ser-87 facilitates its ubiquitin-dependent 26S proteasome-mediated degradation [17]. Moreover, ERK1/2 MAP kinases were shown to colocalize with Bcl-2 and induce Bcl-2 phosphorylation at Ser-70 in response to IL-3 treatment in NSF/N1.H7 cells, which promotes the IL-3-mediated survival signaling [15].

In contrast, paclitaxel was shown to induce JNK-mediated monosite (Ser-70) and multisite (S70, T69, S87) phosphorylations of Bcl-2 in the unstructured flexible loop domain and attenuate its antiapoptotic effect in MCF-7, MDA-MB-231, Jurkat human T cells and WEHI-231 murine B cells [9,18]. In addition to taxanes, Bcl-2 monosite or multisite phosphorylation has been

Table 1. Regulation of Bcl-2 Family Proteins by Posttranslational Modifications

Protein	Modification	Residue	Outcome	Reference
Bcl-2	Phosphorylation	S70	Inhibition of apoptosis	[12,13,15]
Bcl-2	Phosphorylation	S70 or S70, T69, S87	Inhibition of apoptosis, increased Bcl-2 stability	[14,15]
Bcl-2	Phosphorylation	S70 or S70, T69, S87	Promoting apoptosis, inhibition of Bcl-2 function	[9,18-21]
Bcl-2	Dephosphorylation	S87	Proteasome-mediated degradation of Bcl-2	[17]
Bcl-2	Dephosphorylation	n/a	Promoting apoptosis, inhibition of Bcl-2 function	[22-24]
Bcl-2	Dephosphorylation	n/a	Inhibition of apoptosis, increased Bcl-2 stability	[25]
Bcl-xL	Phosphorylation	S62	Promoting apoptosis, inhibition of Bcl-xL function	[26]
Bcl-xL	Phosphorylation	T47, T115	Promoting apoptosis, inhibition of Bcl-xL function	[28]
Mcl-1	Phosphorylation	S121, T163	Promoting apoptosis, inhibition of Mcl-1 function	[38]
Mcl-1	Phosphorylation	T163	Inhibition of apoptosis, increased Mcl-1 stability and half-life	[39]
Mcl-1	Phosphorylation	S159	Promoting apoptosis, increased Mcl-1 degradation	[41]
Mcl-1	Phosphorylation	S155, S159 and T163	Promoting apoptosis, increased Mcl-1 degradation	[43]
Mcl-1	Phosphorylation	S64	Inhibition of apoptosis	[44]
Bad	Phosphorylation	S112, S136, and S155	Inhibition of apoptosis	[46-51]
Bad	Phosphorylation	S170	Inhibition of apoptosis	[52]
Bad	Phosphorylation	S128	Promoting apoptosis	[53,54]
Bad	Phosphorylation	T201	Inhibition of apoptosis	[55]
Bad	Dephosphorylation	S112, S155	Promoting apoptosis	[56,58]
BimEL	Phosphorylation	S69 (human BimEL)	Inhibition of apoptosis, increased BimEL degradation	[60-62]
BimEL	Phosphorylation	S55, S65 and S100 (mouse BimEL)	Inhibition of apoptosis	[65,66]
BimEL	Phosphorylation	S65 (mouse BimEL)	Inhibition of apoptosis	[67]
BimEL	Phosphorylation	S65 (mouse BimEL)	Promoting apoptosis	[69,70]
BimEL	Phosphorylation	S87 (human BimEL)	Inhibition of apoptosis	[71]
BimL	Phosphorylation	T56 (human BimL)	Promoting apoptosis	[68]
BimEL	Dephosphorylation	n/a	Promoting apoptosis	[72]
Bik	Phosphorylation	T33, S35	Promoting apoptosis	[75,76]
Bid	Phosphorylation	T59 (human Bid)	Inhibition of apoptosis, attenuates caspase-8-mediated cleavage of Bid	[77]
Bid	Phosphorylation	S61, S64 (mouse Bid)	Inhibition of apoptosis, attenuates caspase-8-mediated cleavage of Bid	[78]
Bid	Phosphorylation	S61, S78 (mouse Bid), S78 (human Bid)	Inhibition of apoptosis (S61) and induction of intra-S replication arrest (S78)	[80,81]
Bax	Phosphorylation	S184	Inhibition of apoptosis	[82,83,85]
Bax	Phosphorylation	S163	Promoting apoptosis	[86]
Bax	Phosphorylation	T167	Promoting apoptosis	[87]
Bax	Dephosphorylation	n/a	Promoting apoptosis	[84]

(Table 1). Contd…..

Protein	Modification	Residue	Outcome	Reference
Mcl-1	Ubiquitination	K5, K40, K136, K194 and K197 (human Mcl-1)	Promoting apoptosis, increased Mcl-1 degradation	[89]
Bcl-2	Ubiquitination	n/a	Promoting apoptosis, increased Bcl-2 degradation	[101]
Bcl-xL	Ubiquitination	n/a	Promoting apoptosis, increased Bcl-xL degradation	[91]
Bax	Ubiquitination	n/a	Inhibition of apoptosis, increased Bax degradation	[92, 93]
Bid (tBid)	Ubiquitination	K144, K146, K157 and K158 (human Bid)	Inhibition of apoptosis, increased Bid (tBid) degradation	[94]
Bid (tBid)	N-myristoylation	G60 (mouse Bid), G61 (human Bid)	Promoting apoptosis	[96]
Bcl-2	S-nitrosylation	C158, C229	Inhibition of apoptosis, decreased Bcl-2 ubiquitination and degradation	[101,102]
Bcl-2	Carbonylation	n/a	Promoting apoptosis, increased Bcl-2 degradation	[104,105]
Bcl-xL	Deamidation	N52, N66	Promoting apoptosis, inhibition of Bcl-xL function	[106-108]
Bcl-2	Cleavage	D31, D34	Promoting apoptosis, formation of a proapoptotic Bcl-2 fragment	[19,111-115]
Bcl-xL	Cleavage	D61, D76	Promoting apoptosis, formation of a proapoptotic Bcl-xL fragment	[116-119]
Mcl-1	Cleavage	D127, D157	Promoting apoptosis, formation of a proapoptotic Mcl-1 fragment	[120,125,127]
Bfl-1/A1	Cleavage	n/a	Promoting apoptosis, formation of a proapoptotic Bfl-1/A1 fragment	[128]
Bid	Cleavage	D59, D75, D98 and G70	Promoting apoptosis, formation of a proapoptotic tBid fragment	[130-133,136]
BimEL	Cleavage	D13	Promoting apoptosis	[143]
Bad	Cleavage	D56, D61 (mouse Bad), D57,D72 (human Bad)	Promoting apoptosis	[144-146,148]
Bax	Cleavage	Q28, G29	Promoting apoptosis	[151]

reported to be involved in induction of apoptosis in cells treated with proteasome inhibitors, diallyl trisulfide and α-tocopherol succinate [19-21].

Bcl-2 phosphoregulation is a consequence of the dynamic balance between active kinases and phosphatases targeting Bcl-2. Similar to phosphorylation of Bcl-2, the net effect of dephosphorylation of Bcl-2 in apoptosis regulation is not clear. Protein phosphatase 2A (PP2A), which is a serine/threonine phosphatase, has been demonstrated to colocalize with Bcl-2 at mitochondrial membrane and to dephosphorylate Bcl-2 when activated by C2-ceramide [22,23]. This dephosphorylation of Bcl-2 by PP2A was also required for C2-ceramide-induced apoptosis. Inhibition of PP2A activity and increased phosphorylation of Bcl-2 by polyamine depletion in intestinal epithelial cells was shown to increase Bcl-2 protein phosphorylation and expression levels along with prevention of cytochrome *c* release, caspase activation and apoptosis [24]. In contrast, dephosphorylation of Bcl-2 by PP2A has also been reported to increase the antiapoptotic potential of Bcl-2. In a recent study, PP2A was shown to localize at endoplasmic reticulum (ER) membrane and to stabilize Bcl-2 against proteasomal degradation by dephosphorylation, which enhances protection against apoptotic stimuli including ER stress [25]. In summary, based on the apparently contradictory results summarized above, it cannot be definitively stated whether phosphorylation of the flexible loop in Bcl-2 plays a net proapoptotic or antiapoptotic role.

Another antiapoptotic member of the Bcl-2 protein family, Bcl-xL, was demonstrated to be phosphorylated at Ser-62 by JNK in response to taxol or 2-methoxyestradiol treatment, which abrogated the antiapoptotic function of Bcl-xL and sensitized prostate cancer cells to apoptosis [26]. In HeLa cells treated with a small-molecule inhibitor of the kinesin spindle protein, Bcl-xL phosphorylation was detected before caspase activation and this phosphorylation was shown to be required for apoptosis pathway activation [27]. JNK has been shown to localize to mitochondria and phosphorylate Bcl-xL on Thr-47 and Thr-115 residues

upon exposure to ionizing radiation, contributing to induction of apoptosis [28]. On the contrary, treatment of prostate cancer cells with tea polyphenols or epigallocatechin-3-gallate induced the downregulation of phosphorylated Bcl-xL and apoptosis [29]. Collectively, these controversial results highlight the complexity of different signaling pathways, including distinct classes of kinases and phosphatases, responsible for regulation of Bcl-2 or Bcl-xL function in apoptosis mechanisms. The possibility that Bcl-2 or Bcl-xL may be phosphorylated by kinases or dephosphorylated by phosphatases which are not necessarily related to apoptosis pathways further complicates the interpretation of these data.

Mcl-1 is another antiapoptotic Bcl-2 protein, initially identified in differentiating myeloid cells. It also localizes to the mitochondria and other intracellular membranes [30]. The function of Mcl-1 is tightly regulated at the posttranslational level in addition to transcriptional and post-transcriptional control of its expression. Increased Mcl-1 expression has been recognized to be associated with drug resistance and poor therapeutic response in various human malignancies [31-34]. In particular, phosphorylation plays a significant role for regulation of Mcl-1 protein function. Mcl-1 has a shorter half-life (~2-4 hours) compared to Bcl-2 and Bcl-xL, due to the presence of a PEST region upstream of its BH3 domain [35-37]. In response to oxidative stress (H_2O_2 treatment), JNK has been shown to phosphorylate Mcl-1 at Ser-121 and Thr-163 and inactivate Mcl-1 functionally [38]. The expression of unphosphorylatable (S121A/T163A) Mcl-1 resulted in an increased antiapoptotic potential of Mcl-1 against H_2O_2-induced apoptosis, even though there was no alteration in the interaction of Bax and Mcl-1 in response to phosphorylation and dephosphorylation of Mcl-1. Although both Ser-121 and Thr-163 are located in the PEST domain of Mcl-1, H_2O_2-induced JNK-mediated phosphorylation did not alter the half-life of wild type and S121A/T163A mutant of Mcl-1 [38]. In contrast, phorbol ester-triggered ERK-dependent Mcl-1 phosphorylation at Thr-163 was shown to prolong the half-life of Mcl-1 and to promote cell viability *via* reducing Mcl-1 protein turnover [39]. In the same experimental model, paclitaxel and protein serine/threonine phosphatase inhibitor okadaic acid induced Mcl-1 phosphorylation at distinct phosphoacceptor sites in an ERK-independent manner and promoted cell death, indicating the existence of multiple phosphorylation mechanisms targeting Mcl-1 with different outcomes.

Glycogen synthase kinase-3 (GSK-3) is a principal component of the metabolic signaling machinery and plays an essential role in embryogenesis and carcinogenesis [40]. Mcl-1 has been recently described as one of the downstream targets of GSK-3 as a potential mechanism to modulate apoptotic response. Growth factor stimulation and viral oncogenic proteins activate upstream kinases such as those in the PI3K/Akt pathway, which phosphorylate N-terminal serines (Ser-21 for GSK-3α, Ser-9 for GSK-3β) and inactivate GSK-3, thereby inhibiting proteasome-mediated Mcl-1 degradation [32]. Mcl-1 protein levels decreased in IL-3-deprived FL5.12 cells and inhibition of GSK-3 by a small molecule GSK inhibitor (CHIR-611) attenuated Mcl-1 decrease, cytochrome *c* release and apoptosis [41]. GSK phosphorylates Mcl-1 at an evolutionarily conserved phosphorylation site at Ser-159, leading to enhanced ubiquitination and accelerated degradation of Mcl-1 upon IL-3 withdrawal. The expression of the constitutively active myristoylated-Akt abolished Ser-159 phosphorylation of Mcl-1 and provided similar protection against IL-3 withdrawal-induced apoptosis as inhibition of GSK-3 [41]. Moreover, a S159A mutant of Mcl-1 increased the stability and half-life of Mcl-1 suggesting a proteasomal degradation mechanism is mediated by this GSK-3 phosphorylation site. Indeed ubiquitination of Mcl-1 was only detected in wild type Mcl-1, but not in S159A mutant protein upon PI3K inhibition by LY294002 [41]. Similarly, increased glucose metabolism was demonstrated to repress the ubiquitination and degradation of Mcl-1 *via* phosphorylation and inhibition of GSK-3α and -3β and to prevent apoptosis in growth factor-deprived cells [42]. PKC activation was also shown be required for this effect of glucose-induced alteration of GSK-3-Mcl-1 pathway. Additionally, GSK-3 was reported to interact with Mcl-1, phosphorylating and promoting the ubiquitin-mediated degradation of Mcl-1 by the E3 ligase, β-TrCP [43]. Moreover, three phosphorylation sites (Ser-155, Ser-159 and Thr-163) on Mcl-1 were identified as GSK-3 targets and S155A/S159A/T163A Mcl-1 mutant was found to be more stable than wild-type Mcl-1 in mouse embryonic fibroblasts expressing constitutively active GSK-3. Using an orthotopic mammary tumor mouse model, GSK-3-mediated tumor suppression and chemosensitization was demonstrated to be attenuated by the expression of the mutated Mcl-1 (S155A/S159A/T163A) resistant to β-TrCP-mediated degradation [43]. Despite the findings that GSK-3 enhances Mcl-1 degradation through phosphorylation, Ser-64 phosphorylation of Mcl-1 by TRAIL treatment in the human cholangiocarcinoma cell line KMCH-1 was shown to enhance the antiapoptotic potential of Mcl-1 without affecting its ubiquitination and half-life [44]. The possible kinases involved in this mechanism were identified as CDK-1, -2 and JNK using *in vitro* phosphorylation assays. The S64A mutant failed to protect against TRAIL-induced apoptosis in contrast to the phosphomimetic S64E mutant which augmented TRAIL resistance in KMCH-1 cells. The increased antiapoptotic potency of S64E mutant Mcl-1 was associated with enhanced binding of this mutant to proapoptotic Bcl-2 protein Bak, Bim and Noxa [44]. Small molecule CDK inhibitors (SU9516, olomoucine and roscovitine) and a JNK inhibitor (SP600125) reduced Mcl-1 Ser-64 phosphorylation and sensitized KMCH-1 cells to TRAIL-induced apoptosis, which emphasized the role of this posttranslational modification in TRAIL-resistance. In summary, most phosphorylation events on Mcl-1 have a net proapoptotic effect, either by shortening the half-life of Mcl-1 or by compromising its antiapoptotic function. An exception to this rule maybe found in the

phosphorylation at Ser64, which apparently improves the antiapoptotic function of Mcl-1.

Phosphoregulation of Proapoptotic Bcl-2 Proteins

BH3-Only Proteins

Like antiapoptotic Bcl-2 protein family members, BH3-only proapoptotic Bcl-2 proteins are regulated by phosphorylation. Several mechanisms have been proposed to describe how BH3-only proteins conduct cell death signals [45]. A number of survival signals, such as growth factors and cytokines, have been reported to inhibit the proapoptotic function of Bad by phosphorylation on multiple serine residues (Ser-112, Ser-136, and Ser-155). These phosphorylating events result in the cytoplasmic sequestration and inactivation of Bad by 14-3-3 proteins by preventing its interaction with antiapoptotic proteins Bcl-2 and Bcl-xL [46-49]. IGF-1R activation followed by PI3K/Akt activation was shown to phosphorylate Bad on Ser-136, which was proposed to be the dominant serine residue in determining 14-3-3 binding [50], whereas activation of the 90 kDa ribosomal S6 kinases (RSK) resulted in phosphorylation of Bad at Ser-112 and Ser-136 [51], or Ser-112 and Ser-155 [47], suppressing the proapoptotic potential of Bad. Moreover, phosphorylation of Ser-170 was also demonstrated to negatively regulate proapoptotic activity of Bad without any effect on its heterodimerization with Bcl-xL [52]. A novel Cdc2- or JNK-mediated phosphorylation site at Ser-128 of Bad was mapped recently and this phosphorylation was demonstrated to inhibit sequestration of Bad by members of 14-3-3 family and promote its proapoptotic effect [53,54]. In contrast, the expression of a constitutively active JNK was shown to inhibit IL-3 withdrawal-induced apoptosis in FL5.12 cells by phosphorylating Bad at Thr-201 and inhibiting Bad interaction with Bcl-xL [55]. A phospho-site mutated Bad (T201A) significantly enhanced IL-3-induced apoptosis, highlighting the involvement of Thr-201 in IL-3-mediated cell survival signaling. In addition to multiple kinases which have been proposed to phosphorylate Bad such as Akt/PKB, RSK and PKA, phosphorylation of Bad was also shown to be mediated by protein phosphatases. Protein phosphatase type 2C (PP2C) was reported to colocalize and to dephosphorylate Bad at critical serine residues with a significant preference for Ser-155, triggering the proapoptotic function of Bad [56]. Ca^{2+} -dependent protein phosphatase type 2B (PP2B) or calcineurin was also shown to interact with and to dephosphorylate Bad after stimulation with Ca^{2+}-mobilizing agents, inducing Bad translocation to mitochondria, dimerization with Bcl-xL and BAD-dependent apoptosis [57]. Furthermore, in FL5.12 cells, PP2A was demonstrated to be the principal Ser-112 phosphatase and to advance IL-3-withdrawal-induced apoptosis by displacing Bad from 14-3-3 proteins to bind Bcl-xL [58]. These findings suggest that both phosphorylation and dephosphorylation signaling pathways work cooperatively on Bad to regulate its function as a proapoptotic protein in different cellular contexts, depending on the nature of cell death or survival stimuli. In the case of Bad, moreover, one can make the general comment that phosphorylation has generally antiapoptotic effects, primarily by promoting Bad's sequestration by 14-3-3.

Bim is another BH3-only proapoptotic member of the Bcl-2 protein family. Three major splice variants of Bim have been described; BimEL, BimL and BimS [59]. Bim activity and expression is also regulated strictly by transcriptional and posttranslational mechanisms. Phosphorylation and ubiquitin-mediated degradation of Bim is recognized as a fundamental mechanism regulating cellular Bim levels. ERK 1/2 has been identified as an important kinase involved in the control of the proteasomal degradation of Bim. BimEL is preferentially targeted by ERK due to the presence of a unique ERK photoacceptor site on BimEL encoded by exon 3. BimL and BimS were reported not to be phosphorylated by ERK *in vitro* [60,61]. Ser-69 of human BimEL (Ser-65 of mouse BimEL) was shown to be phosphorylated by ERK 1/2 and this phosphomodification was found to be sufficient and necessary for degradation of BimEL *via* the proteasome pathway in Raf:ER transfected 293 fibroblast cells treated with tamoxifen [62]. However, neither BimL nor BimS were phosphorylated in similar conditions. BimEL Ser-69 mutant (S69G) was also shown to be resistant to ERK 1/2-mediated degradation and to promote apoptosis more efficiently than wild-type BimEL in K562 CML cells with constitutively active ERK 1/2 due to Bcr-Abl expression [62]. Additionally, activation of ERK 1/2 pathway either by FBS or by expression of tamoxifen-inducible Raf-1 was shown to target BimEL for degradation and accelerate the turnover of BimEL protein from ~8 hours to ~3 hours, thereby contributing to cell survival [63]. Accordingly, inactivation of ERK 1/2 pathway by withdrawal of growth factors or serum resulted in dephosphorylation and stabilization of BimEL and induction of cell death [61]. Consistent with these observations, Ser-65 (Ser-69 of human BimEL) was also defined as the primary ERK 1/2 phosphorylation site and mutation of Ser-65 to alanine abrogated the phosphorylation of BimEL by ERK 1/2 *in vitro* [61]. The direct interaction of ERK 1/2 with BimEL led to the identification of two putative DEF (docking for ERK) domains distinct from phosphoacceptor sites on BimEL [64]. These docking domains, DEF1 and DEF2 are unique to BimEL and located between amino acids 70-97. Interestingly, mutation of DEF2 domain located at amino acids 70-87 of BimEL abrogated the serum-induced Bim turnover and promoted BimEL-induced apoptosis. Furthermore, IL-3-mediated ERK 1/2-dependent phosphorylation of three serine residues (Ser-55, Ser-65 and Ser-100 of mouse BimEL) were reported to mediate the survival of FL5.12 cells and expression of a non-phoshorylatable BimEL mutant (S55A, S65A and S100A) augmented IL-3 withdrawal-induced apoptosis [65]. An interesting finding of this study was the persistence of BimEL protein following its phosphorylation. Instead, ERK-dependent phosphorylation of BimEL prevented its interaction with the Bax and eliminated its proapoptotic effect. In contrast, TLR stimulation of macrophages was shown to induce

Bim phosphorylation at Ser-55, Ser-65 and Ser-100 residues through ERK 1/2 activation, accelerating Bim degradation by the proteasome [66]. Moreover, Bim -/- MEFs and BMK epithelial cells were resistant to serum withdrawal-induced cell death in comparison to wild-type cells. In response to serum withdrawal, BimEL was shown to interact with Bcl-xL and Mcl-1, which resulted in the dissociation of Bax from these proteins to facilitate its proapoptotic function [67]. Nevertheless, activation of ERK 1/2 by serum stimulation resulted in phosphorylation of BimEL at Ser-65 and detachment from Bcl-xL and Mcl-1 and inhibition of serum withdrawal-induced cell death.

Bmf and Bim have also been reported to be targets for JNK-mediated phosphorylation within their conserved dynein light chain (DLC) binding motif. JNK phosphoacceptor sites were mapped in BimL (Ser-44, Thr-56 and Ser-58 of human BimL) and phosphorylation of BimL at Thr-56 by UV-activated JNK was demonstrated to inhibit its interaction with DLC1 and to enhance its Bax-dependent apoptotic activity [68]. Recent reports suggested that JNK- and p38-mediated phosphorylation of BimEL at Ser-65 potentiates the apoptotic effect of BimEL in neuronal cells without altering its subcellular localization [69,70]. The differential outcome of Ser-65 phosphorylation by JNK and p38 or ERK 1/2 could suggest additional phosphorylation targets on BimEL or additional target proteins of these kinases that determine the enhancement or inhibition of apoptotic response. In addition to MAP kinases, Akt was shown to phosphorylate BimEL at Ser-87 upon IL-3 stimulation and to attenuate the proapoptotic function of BimEL by promoting its association with 14-3-3 proteins similar to Bad [71]. Surprisingly, S87A mutant of BimEL was found to be a more potent inducer of apoptosis in comparison to wild-type or S69A mutant of human BimEL. In a recent study, a novel mechanism by which PP2A-mediated dephosphorylation of Bim prevents its ubiquitination and proteasomal degradation in ER stress-induced apoptosis has been delineated [72]. As is the case for phosphorylation of Bcl-2, no simple generalization can be made about the effect of phosphorylation of Bim, whether it is proapoptotic or anti-apoptotic. The net effect may well depend upon specific combinations of phosphorylation events that are yet to be determined. It does seem, however, that ERK1/2 mediated phosphorylation reduces the proapoptotic effect of Bim, either by reducing Bim protein levels or reducing Bim's ability to interact with Bax.

Bik is another BH3-only Bcl-2 protein, originally identified as a binding partner of E1B 19K and highly expressed in pancreas and kidney tissue [73,74]. Bik was reported to be phosphorylated at Thr-33 and Ser-35 residues which are required for its apoptotic potency, but the kinase responsible for posttranslational phosphorylation of Bik is still unclear [75]. The expression of T33A and S35A mutants of Bik was shown to reduce its apoptotic functionality without a drastic effect on its heterodimerization capacity with Bcl-2. Interestingly, T33D and/or S35D phospho-mimicking mutants of Bik exhibited increased binding affinity with Bcl-2 and Bcl-xL and enhanced cell death more potently than wild-type Bik in various human cancer cell lines and in an orthotopic nude mouse tumor model [76].

The BH3-only member Bid serves to engage the mitochondrial apoptotic pathway during death receptor-activated apoptosis. The cleavage of Bid by caspase-8 at Asp-60 results in exposure and myristoylation of an N-terminal glycine residue, which promotes its mitochondrial translocation to result in permeabilization of the mitochondrial outer membrane. The initially identified potential phosphorylation sites of Bid are located adjacent to the cleavage sites on Bid. The phosphorylation of Thr-59 of human Bid was shown to attenuate but not completely abolish the cleavage of Bid by caspase-8, although phosphorylation of Ser-65 did not exert any notable effect [77]. The dephosphorylation of Bid by potato acid phosphatase significantly enhanced its cleavage by both caspase-3 and caspase-8. Bid was reported to be phosphorylated by both casein kinase I and II, which protected against Fas-induced apoptosis and prevented the cleavage of Bid by caspase-8 *in vitro* [78]. Additionally, a non-phosphorylatable mutant of Bid (S61A/S64a of mouse Bid) was found to be more effective at inducing spontaneous apoptosis in HeLa cells in comparison to wild-type Bid. Ser-64 phosphorylation of Bid was shown to be the major phosphorylation site for the regulation of cleavage by caspase-8, but it could only be phosphorylated if Ser-61 was already phosphorylated [78]. Even though phosphorylation of Bid prevented caspase-8-mediated cleavage of Bid, it did not affect granzyme B-mediated cleavage of Bid. This was further confirmed by the increased cleavage of S61A/S64A mutant of Bid compared with wild-type Bid in cisplatin- or UV-treated HeLa cells undergoing apoptosis [78]. In a chronic liver disease mouse model, activation of Fas receptor by monoclonal anti-mouse Fas-activating antibody Jo2 led to dephosphorylation of Bid followed by its subsequent cleavage, while no dephosphorylation and cleavage of Bid was detected in hepatic tissues of apoptosis resistant chronic cholestatic mice [79]. In these studies, therefore, phosphorylation of Bid was generally found to inhibit the caspase cleavage necessary for Bid's full proapoptotic function.

Recently, two seminal reports identified a novel regulatory role of Bid in DNA-damage response regulation as a substrate for ATM and the related DNA repair kinases [80,81]. ATM kinase was shown to phosphorylate Bid on two consensus sites (Ser-61 and Ser-78 of mouse Bid) in response to double-strand breaks in DNA induced by etoposide and ionizing radiation [81]. Human Bid only exhibits the Ser-78 as an ATM kinase target residue. In contrast to wild-type Bid, the expression of a non-phosphorylatable (S61A/S78A) mutant in Bid -/- MEF cells did not reestablish the arrest of cells in the S-phase following etoposide treatment, but resulted in an enhanced sensitivity to etoposide-induced apoptosis. This effect was unique to agents inducing double strand breaks in DNA and was not observed in UV- or TNF-α-treated cells and ATM-mediated phosphorylation of Bid did not alter Bid cleavage susceptibil-

ity and apoptotic response in response to TNF-α treatment. Indeed, Bid was shown to localize to nucleus even in untreated healthy MEF cells and to be essential for apoptosis induction by Adriamycin. These findings strongly suggest that the phosphorylation of Bid may act as a molecular switch between ATM-dependent cell cycle arrest and activation of apoptotic machinery in response to double-strand break DNA damage [81]. On the contrary, Bid was reported to be phosphorylated at Ser-61 and Ser-78 in response to treatment with hydroxyurea, aphidicolin and mitomycin c in addition to etoposide and ionizing radiation, which was completely abrogated in ATM -/- MEF cells [80]. Furthermore, the expression of S78A mutant of Bid in Bid -/- myeloid progenitor cells was incapable of restoring S-phase arrest in response to aphidicolin treatment and displayed a defective intra-S phase checkpoint in IR-treated cells. However, the expression of the S61A mutant of Bid resulted in extensive apoptotic response. In summary, phosphorylation of Bid at Ser-78 is crucial for intra-S replication arrest induced by DNA damage and ATM is the key kinase mediating this posttranslation phosphorylation [80].

Multidomain Proapoptotic Proteins

Bax is a multidomain proapoptotic Bcl-2 protein and plays an essential role in the execution of apoptosis in response to various stimuli. The exposure of the C-terminal transmembrane domain of Bax initiates the insertion and oligomerization of Bax on mitochondria, where it mediates the release of apoptogenic factors, such as cytochrome *c*. GM-CSF was shown to induce Akt-mediated Bax phosphorylation at Ser-184 residue located in the C-terminal hydrophobic domain, inactivating Bax and preventing apoptosis in neutrophils [82]. The phosphorylation of Bax could not be detected in PLB-985 human myelomonocyte leukemia cells expressing S184A/S184E Bax mutants upon stimulation with GM-CSF. Furthermore, inhibition of PI3K/Akt pathway resulted in diminished phosphorylation of Bax and apoptotic neutrophils were found to express reduced levels of Ser-184-phosphorylated Bax with increased Bax mitochondrial translocation [82]. The phosphorylated form of Bax could only be detected in cytoplasm and the phosphorylation of Bax was shown to promote the heterodimerization of Bax with antiapoptotic Mcl-1, Bcl-xL and A1 proteins [82]. Moreover, in A549 human lung cancer cells nicotine was demonstrated to induce Bax phosphorylation at Ser-184 *via* activation of Bax-colocalized Akt , which abrogated the proapoptotic function of Bax [83]. Treatment of A549 cells with PI3K inhibitor LY294002 or siRNA-mediated silencing of Akt was shown to prevent nicotine-induced Bax phosphorylation and to enhance cisplatin-induced apoptosis. Nicotine-induced phosphorylation of Bax not only inhibited cisplatin-induced translocation and insertion of Bax into mitochondria, but also reduced the half-life of Bax protein from ~9-12 hours to less than 6 hours. The S184E/S184A Bax mutants were not phosphorylated by nicotine treatment in H157 cells and only S184A mutant could be detected at mitochondria by subcellular fractionation analysis [83]. Accordingly, treatment of lung cancer cells with PP2A inhibitor okaidic acid or inhibition of PP2A activity by SV40 expression was reported to increase Bax phosphorylation and to protect against cisplatin-induced apoptosis. In contrast, activation of PP2A by C2-ceramide suppressed nicotine-induced Bax phosphorylation and sensitized A549 cells to cisplatin-induced apoptosis [84]. PP2A was shown to colocalize and to interact with Bax, directly dephosphorylating Bax to disrupt Bax-Bcl-2 interaction and to facilitate Bax insertion and oligomerization in mitochondria. Recently, PKCζ was identified as a Bax kinase which phosphorylates Bax at Ser-184 [85]. Phosphorylation of Bax by PKCζ was shown to promote the sequestration of Bax in cytosol, the abrogation of the proapoptotic function of Bax and the inhibition of cisplatin-induced Bax conformational change and apoptosis. Morevoer, treatment of H460 lung cancer cells, which express high levels of Bcl-2, Bax and PKCζ, with nicotine led to increased PKCζ-Bax and attenuated Bcl-2-Bax interaction. Thus, phosphorylation of Bax at Ser-184 represses the proapoptotic potency of Bax.

By contrast, GSK-3β-induced phosphorylation of Bax at Ser-163 was shown to enhance the conformational activation and the mitochondrial localization of Bax in cerebellar granule neurons undergoing apoptosis [86]. Expression of a constitutively active GSK-3β mutant promoted the phosphorylation of wild-type Bax, but not the S163A Bax mutant, which was also defective in mitochondrial localization. Additionally, phosphorylation of Thr-167 of Bax by JNK and p38 MAP kinases in response to treatment with staurosporine, H_2O_2, etoposide and UV was demonstrated to facilitate the activation and mitochondrial translocation of Bax and apoptotic response [87]. T167D mutant of Bax was not phosphorylated and could not translocate to mitochondria after staurosporine treatment in MEF cells. Briefly, phosphorylation of individual serine or threonine residues on Bax protein may regulate its localization, conformation and stability to control its apoptotic function.

In summary, both antiapoptotic and proapoptotic members of the Bcl-2 protein family are widely regulated by upstream protein kinase and phosphatase enzymes. In specific circumstances, phosphorylation appears to play a critical role in controlling apoptotic function, either to enhance or repress apoptosis. Lack or excess of apoptosis can be important in human pathology, and kinase inhibition is a feasible medical intervention. It may be, therefore, that kinase inhibition in defined cases may be useful for the selective promotion or opposition of apoptosis in the clinic.

ADDITIONAL POSTTRANSLATIONAL MODIFICATIONS REGULATING BCL-2 PROTEINS

Ubiquitination

The regulation of protein stability is an essential mechanism for controlling cell survival and apoptosis in physiologic and pathologic conditions. Ubiquitin is a 76

aa polypeptide which is covalently attached to target proteins by a ubiquitinating enzyme complex formed by a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin ligase (E3) [88]. Selective ubiquitination of lysine residues by isopeptide bond formation tags proteins for ubiquitin-dependent degradation by the intracellular proteasome complexes, such as 26S proteasome [88]. The ubiquitin-proteasome system sensitively regulates the stability of both antiapoptotic and proapoptotic Bcl-2 family proteins. Any abnormal regulation of the stability of Bcl-2 family proteins may promote the development of apoptosis resistance in cancer cells. As discussed in phosphoregulation of Bcl-2 proteins section, differential modification of individual residues on Bcl-2, Mcl-1 and Bim either favor their ubiquitin-proteasome-mediated degradation or protect them by increasing their stability. The degradation of Mcl-1 was shown to be mediated by Mule (Mcl-1 ubiquitin ligase E3) that is a member of HECT domain family of E3 ligases [89]. Interestingly, Mule contains a BH3-like domain which is involved in its selective interaction with Mcl-1 as confirmed by pull-down experiments using a 26 amino acid Mule BH3 peptide and coimmunoprecipitation experiments. Human Mcl-1 contains 13 lysine residues and five of them (Lys-5, -40, -136, -194 and -197) were reported to be ubiquitinated. Among these five lysines, Lys-136, -194 and -197 are conserved in human, mouse and rat. Mutation of these five lysines or siRNA-mediated knockdown of Mule was demonstrated to prolong the half-life of Mcl-1 in the absence or presence of an apoptotic stimulus [89]. Moreover, in U2OS human ostesosarcoma cells, knockdown of Mule resulted in accumulation of Mcl-1 and attenuated apoptosis induced by DNA-damaging treatments such as UV radiation, etoposide and cisplatin. An added complexity of the interdependence of proapoptotic and antiapoptotic proteins in the context of ubiquitination is that Mcl-1 and Bim were found to be decreased in a concomitant manner [90]. Additionally, multiubiquitinated forms of Mcl-1 or Bim were found to be increased in Bim and Mcl-1 knockdown cells, indicating a reciprocally related regulation of ubiquitination and degradation process.

Bcl-xL protein levels were also shown to be regulated by ubiquitination in rat cardiomyocytes [91]. Overexpression of Hsp10 and Hsp60 or doxorubicin treatment in these cells led to decreased Bcl-xL ubiquitination and increased Bcl-xL levels, but the lysine residues targeted by ubiquitin-proteasome system on Bcl-xL remain to be identified.

Bax was initially proposed to be degraded by ubiquitin-mediated proteasomal pathway in HeLa cells [92]. In addition, when the proteasome activity was inhibited in Bcl-2 overexpressing Jurkat T cells, Bax was shown to accumulate at both cytoplasm and mitochondria and increased levels of ubiquitinated Bax protein was detected, preceding cytochrome *c* release and caspase-mediated apoptosis [93]. In an *in vitro* ubiquitination assay, Bax was reported to be degraded by MCF-7 protein extract which could be abrogated by utilization of a proteasome inhibitor or immunodepletion of the proteasome. Intriguingly, decreased levels of Bax protein due to increased degradation correlated well with increased Gleason score of human prostate adenocarcinoma samples which underscores ubiquitin-mediated Bax degradation as an exclusive cancer cell survival mechanism.

The carboxy-terminal cleaved product of Bid, tBid, stimulates the release of cytochrome c upon localization to mitochondria. tBid was found to be ubiquitinated and successively degraded by 26S proteasome [94]. The inhibition of proteasome-mediated tBid degradation by MG-132 and lactacystatin enhanced tBid-induced apoptosis. Furthermore, the mutation of Lys-144, -146, -157 and 158 human tBid to arginine resulted in stabilization of tBid protein as confirmed by pulse chase analysis. When this lysine-free stabilized tBid was overexpressed in HeLa cells, it induced an augmented cytochrome c release and apoptotic response in comparison to wild-type tBid. Of interest, the cleavage of Bid was shown to be regulated by phosphorylation, but the involvement of phosphorylation in the regulation of tBid ubiquitination and degradation remains elusive. However, ubiquitin-proteasome-mediated degradation of tBid may be a novel survival mechanism employed by cancer cells to promote tumorigenesis and chemotherapy resistance.

N-Myristoylation

N-myristoylation is an irreversible protein modification, which involves the covalent attachment of a myristate moiety to N-terminal glycine residue of a polypeptide. N-myristoyl transferase (NMT) is the enzyme which catalyzes myristoylation *via* recognition of MGXXXS/T myristoylation consensus motif. N-myristoylation of proteins generally modulates signal transduction and protein trafficking and along with other lipid modification of proteins, such as palmitoylation and isoprenylation, plays important roles in membrane anchorage and binding [95]. Bid is the only Bcl-2 protein family member known to be modified by myristoylation. Upon binding of FasL to its receptor triggers the formation of death-inducing signaling complex (DISC), recruitment of FADD and activation of caspase-8. Active caspase-8 cleaves p22 Bid into p15 Bid and p7 Bid fragments which creates an N-terminal myristoylation consensus site (GSQASR) on p15 Bid [96]. The truncated p15 Bid relocates to mitochondria in complex with p7 and induces cytochrome *c* release and apoptosis. In fact, p15 Bid (tBid) but not p22 Bid was shown to be myristoylated in Jurkat cells labeled with [^{3}H] myristic acid. Gly-60 of p15 Bid was identified as the myristoylation site using an *in vitro* myristoylation assay and G60A mutation of p15 Bid completely abrogated its myristoylation [96]. Moreover, myristoylation of p7/p15 complex was found to promote mitochondrial targeting, enhanced membrane insertion and efficient cytochrome *c* release. Hence, Fas activation-mediated mitochondrial localization of Bid and apoptosis was significantly defective in MCF-7 cells expressing Bid G60A

mutant in comparison to wild-type Bid, highlighting the significance of myristoylation in consistent functioning of Bid in response to death receptor signaling.

S-Nitrosylation/Carbonylation

Nitric oxide (NO) is recognized as a physiologic signaling messenger molecule involved in the regulation of numerous biological functions, including vascular smooth muscle relaxation and neurotransmission. The major source of NO is the synthesis by NO synthases from L-arginine and oxygen. NO has been shown to exert both antiapoptotic and proapoptotic properties, influenced by the redox homeostasis [97,98]. NO-mediated S-nitrosylation of protein cysteine residues emerged as a novel posttranslational protein modification to modulate the function of several proteins including kinases and transcription factors [99,100]. Bcl-2 was recently identified as an S-nitrosylation target [101]. In H460 human lung cancer cells, cisplatin induced formation of reactive oxygen species and Bcl-2 dephosphorylation at Ser-87, followed by ubiquitination and proteasomal degradation. Contrarily, treatment of cells with NO donors resulted in generation of NO and induced S-nitrosylation of Bcl-2, which abrogated its ubiquitination and proteasomal degradation without any effect on its phosphorylation. As expected, NO inhibitors enhanced cisplatin-induced Bcl-2 ubiquitination, degradation and cellular apoptotic response. Moreover, Bcl-2 was shown to be S-nitrosylated by endogenous NO in chromium (VI)-treated H460 cells which also inhibited its ubiquitination and degradation [102]. Cys-158 and Cys-229 of Bcl-2 were identified as targets for S-nitrosylation and mutation of these two cysteine residues (C158A/C229A) prevented chromium (VI)-induced S-nitrosylation of Bcl-2 and led to increased ubiquitination of Bcl-2. In conclusion, S-nitrosylation of Bcl-2 by NO may act as a negative regulator of apoptosis by increasing Bcl-2 stability.

Protein carbonylation generally occurs as a consequence of direct oxidation of Lys, Arg, Pro and Thr residues. The proteasomal system is the primary route for degradation of carbonylated proteins [103]. In addition to S-nitrosylation, Bcl-2 was also reported to be regulated through carbonylation. IL-1β stimulation of RINm5F rat insuloma cells was demonstrated to result in decreased Bcl-2 levels and apoptosis [104]. Additionally, carbonylation of Bcl-2 was detected in response to treatment with NO donors and IL-1β, preceding Bcl-2 downregulation and apoptosis. The mechanism by which protein carbonylation promote Bcl-2 degradation is not clear, but the ability of lipid peroxidation inhibitors and overexpression of MnSOD to inhibit Bcl-2 carbonylation and apoptosis provide a plausible link between protein carbonylation and regulation of cell death. This has been further supported by increased carbonylation and decreased levels of Bcl-2 in SOD1-deficient neuroblastoma cells along with impairment of mitochondrial membrane potential [105].

Deamidation

Glutaminyl and asparaginyl residues in proteins are deamidated, a process that involves the replacement of the amide functional groups of these amino acids by carboxyl groups at basic pH, which may result in alteration of the structure and function of proteins with potential biological consequences. Asparagine residues could be converted to aspartate or isoaspartate by nonenzymatic deamidation, introducing a negative charge into the protein structure. The deamidation of Bcl-xL at Asn-52 and Asn-66 was shown to be a critical component of apoptosis induced by DNA-damaging agents (cisplatin, etoposide and γ-radiation) [106]. These as asparagine residues are located in the unstructured loop of Bcl-xL, which is a key domain involved in the regulation of antiapoptotic function of Bcl-xL. In fact, mutation of Asn-52 and -66 to alanines significantly enhanced the antiapoptotic function of Bcl-xL. In growth-arrested fibroblasts, Bcl-xL deamidation was shown to be actively repressed, which provided resistance against cisplatin-induced apoptosis in these cells. In protumorigenic thymocytes isolated from a transgenic model of T cell lymphoma, increased activity of $P56^{lck}$ kinase resulted in inhibition of Bcl-xL deamidation, Bax conformational change and apoptosis induced by etoposide and ionizing radiation [107]. Recently Bcl-xL deamidation at Asn-52 and -66 was demonstrated to generate iso-Asp-52 and -66, which prevented Bcl-xL to sequester BH3-only proteins Bim and Puma [108]. DNA-damage induced increased expression of a Na/H antiport, NHE-1, was responsible for intracellular alkalinisation, Bcl-xL deamidation and apoptosis in these protumorigenic thymocytes. Furthermore, Bcl-xL deamidation was reported to mediate E1A-dependent sensitization of ovarian cancer cells to cisplatin treatment and the expression of a deamidated mutant of Bcl-2 protected against cisplatin-induced apoptosis in these E1A-transfected cells [109]. In human hepatocellular carcinomas, the rate of Bcl-xL deamidation was found to be considerably lower than adjacent nontumor or normal liver tissues [110]. Thereby, hepatocellular carcinoma cells were proposed to acquire resistance and gain a survival advantage by attenuating Bcl-xL deamidation, which might be involved in neoplastic transformation and carcinogenesis in liver tissue.

Cleavage of Bcl-2 Family Proteins: A Proapoptotic Modification

The sequence-specific posttranslational protein cleavage may regulate or alter the biological activity, cellular localization or turnover of proteins. Cleavage of antiapoptotic Bcl-2 family proteins converts them into proapoptotic molecules and cleavage of proapoptotic Bcl-2 family proteins increases their proapoptotic potency as an amplification loop of apoptosis. The unstructured loop domain of Bcl-2 was shown to be cleaved *in vitro*, in cells overexpressing caspase-3 and in response to apoptotic stimuli such as ionizing radiation, Fas ligation or IL-3 withdrawal [111,112]. A Bcl-2

mutant lacking the loop domain was resistant to caspase-dependent proteolysis, which suggested that the caspase cleavage site was localized in the loop domain. Asp-34 and Asp-64 of Bcl-2 were implicated as possible caspase cleavage sites and the mutation of D34A abrogated Bcl-2 cleavage by caspase-3 although D64A did not show any effect [112]. Caspase-3-mediated cleavage of Bcl-2 results in a 23 kDa proteolytic fragment due to loss of N-terminal BH4 domain critical for the antiapoptotic function of Bcl-2. The expression of D31A/D34A cleavage-deficient mutants of Bcl-2 improved the efficiency of its protective effect against IL-3-withdrawal-induced apoptosis in Ba/F3 cells. In contrast, N-terminal-cleaved form of Bcl-2, ΔN34 was demonstrated to act as a proapoptotic fragment and to enhance or induce apoptosis by stimulating cytochome *c* release [112]. Upon transfection into BMK cells, ΔN34 Bcl-2 was reported to localize mitochondria and trigger cytochrome *c* release. Furthermore, incubation of recombinant Bcl-2 and apoptotic extracts from 293 cells resulted in cleavage of Bcl-2, which could be inhibited by immunodepletion of caspase-3 [111]. In a myeloid leukemic cell line, etoposide treatment was shown to induce the generation of 23 kDa Bcl-2 fragment in a caspase-dependent manner in the early phases of apoptotic response [113]. Moreover, treatment of Me665/2/21 melanoma cells with either hydrogen peroxide or cisplatin and PC12 pheochromocytome cells with neocarzinostatin also resulted in Bcl-2 cleavage and generation of 23 kDa proapoptotic fragment, which is mediated by caspase-3 [114,115]. Interestingly, in H460 human lung cancer cells treatment with a proteasome inhibitor led to formation of two cleavage fragments at 23 kDa and 25 kDa, while treatment with chemotherapeutics induced only 23 kDa cleavage product [19]. Caspase inhibitors inhibited the generation of 23 kDa fragment, but did not show any effect on the formation of 25 kDa fragment, which implicated possible proteolytic pathways other than caspases. Another proof of the caspase-mediated cleavage of Bcl-2 was provided by the prevention of Bcl-2 cleavage in MEF cells deficient in Apaf-1 following UV, etoposide or Act-D treatment, indicating the requirement of effector caspases in this process [19].

Bcl-xL was also shown to be cleaved at its N-terminal by caspases *in vitro* and in cells undergoing apoptosis upon Sindbis virus infection or IL-3 withdrawal [116]. The transfection of cleaved Bcl-xL induced apoptosis in BHK cells and the expression of D61A Bcl-xL mutant augmented its antiapoptotic activity. IL-2-deprivation-mediated apoptosis of CTTL-2 mouse T lymphocytes was accompanied by Bcl-xL cleavage into two 18 kDa fragments and overexpression of Bcl-2 or treatment with caspase inhibitors drastically attenuated the cleavage of Bcl-xL [117]. Asp-61 and Asp-76 were identified as the caspase-3-mediated cleavage sites of Bcl-xL and the transfection of the C-terminal 18 kDa cleaved Bcl-xL fragment augmented sensitivity of 293 cells to etoposide. Correspondingly, ΔN61 and ΔN76 Bcl-xL were reported to exert pore forming activity and to permeabilize lipid vesicles [118]. Additonally, ΔN61 and ΔN76 Bcl-xL facilitated cytochrome *c* release when transfected into BHK cells or incubated with isolated rat mitochondria. Similarly, the exposure of squid stellate ganglia to hypoxia resulted in the cleavage of Bcl-xL by cysteine proteases, which was shown to be blocked by pancaspase inhibitor Z-VAD-FMK [119]. Overexpression of N-terminal cleaved Bcl-xL lacking amino acids 2-76 (ΔN76 Bcl-xL) was demonstrated to trigger the loss of mitochondrial potential, cytochrome c release and apoptosis in various cell lines. Hence, injection of this cleaved fragment into the presynaptic terminal of the squid giant synapse significantly attenuated synaptic transmission accompanied with formation of large multiconductance channels in the outer mitochondrial membrane. This effect of ΔN76 Bcl-xL was attenuated by the VDAC inhibitor NADH and ΔN76 Bcl-xL could not form NADH-sensitive channels in yeast mitochondria lacking VDAC [119].

Mcl-1 is another antiapoptotic Bcl-2 protein which is extensively expressed in B-cell malignancies and contributes to survival during lymphomagenesis [120,121]. Mcl-1 was demonstrated to be cleaved during cisplatin-induced apoptosis of Akata6 B-cell lymphoma cells and spontaneous apoptosis of primary B-cell lymphomas [120]. *In vitro* cleavage of Mcl-1 resulted in the generation of two cleavage products due to cleavage at Asp-127 and Asp-157, but only the 28 kDA fragment cleaved after Asp-127 was shown to accumulate in apoptotic cells and to induce apoptosis in NIH-3T3 cells when overexpressed. Treatment of human multiple myeloma cells with Melpahalan, Bortezomib or TRAIL-receptor monoclonal antibodies also resulted in formation of a ~24-26 kDa Mcl-1 cleavage products as a positive feedback loop of apoptosis, which could be blocked by pretreatment with caspase inhibitors [122-124]. On the other hand, Mcl-1 was reported to be cleaved at both conserved Asp-127 and Asp-157 residues that resulted in formation of 24, 19, 17 and 12 kDa fragments both *in vitro* and in drug-treated cells, which could be abolished by caspase inhibitors [125]. Of note, these cleaved fragments were shown to preserve BH1-3 domains of Mcl-1. Expression of D127A and D157A mutant Mcl-1 led to the disappearance of 24 and 12 kDa, and of 19 kDA and 17 kDa cleaved fragments, respectively. Although wild type and D127A/D157A mutant of Mcl-1 protected against BimEL-induced apoptosis, ΔN127 and ΔN157 Mcl-1 failed to exert anti-apoptotic potency. Intriguingly, both ΔN127 and ΔN157 Mcl-1 was found to show increased binding to Bim in comparison to wild-type Mcl-1, but they did not elicit any effect on apoptosis when expressed alone.

In addition to phosphomodification by ERK 1/2 and GSK kinases, N-terminal truncation of Mcl-1 was also reported to contribute to the regulation of protein stability of Mcl-1 [126]. Mcl-1 is synthesized as a 42 kDa protein and truncation at its N-terminal hydrophobic region between amino acids 9-27 was shown to result in generation of a 40 kDa product by means of mass spectrometric and mutational analysis. Treatment of ML-1

cells with proteasome inhibitors did not affect the formation of this N-terminal cleaved product, which maintained its antiapoptotic activity and protected against etoposide- and MG-132-induced apoptosis in FDC-P1 mouse myeloid cells. Moreover, TPA-induced ERK activation in BL-41 cells led to stabilization of 40 kDa Mcl-1 form, which exerted an increased stability compared to 42 kDA Mcl-1. Neither caspase or nor calpain inhibitors exerted any effect on the formation of 42/40 kDa doublet formation of Mcl-1. Importantly, 40 kDa Mcl-1 was also found to be the major form of Mcl-1 in lymphomas from transgenic mice and human B-cell tumor cells, designating 40 kDA Mcl-1 as preferential target for blocking of survival and enhancing cell death in these tumors. In Jurkat T leukemia cells, TRAIL receptor activation resulted in cleavage of Mcl-1 at Asp-127 and Asp-157 by a caspase-3-dependent mechanism [127]. Expression of a D127A/D157E mutant attenuated TRAIL-induced apoptosis. The C-terminal cleaved form of Mcl-1 exerted proapoptotic potential through direct interaction with tBid, Bak and VDAC1.

Bfl-1/A1 was also shown to be cleaved between its BH3 and BH4 domains by a calpain-like protease activity in response to TNFα-stimulation in FL5.12 cells, generating a proapoptotic ~32 kDa cleaved fragment [128]. Although expression of Bfl-1/A1 protected against staurosporine or IL-3 withdrawal-induced apoptosis, it was shown to sensitize FL5.12 cells to TNFα/CHX-induced apoptosis. In summary, conversion of antiapoptotic Bcl-2 proteins into prodeath factors *via* cleavage by proteases leads to amplification of apoptosis signals and ensures the progression of cell death.

Proapoptotic Bcl-2 proteins are also regulated by cleavage. Following activation of death receptors by their specific ligands, a signaling complex, DISC (death-inducing signaling complex) is formed by adaptor proteins such as FADD, TRADD and FLIP [129]. FADD-mediated recruitment of caspase-8 to DISC complex results is accompanied by oligomerization and autocatalytic activation of caspase-8. The cleavage of Bid by caspase-8 in response to death receptor activation was shown to integrate receptor-mediated apoptosis pathway into mitochondrial apoptosis signaling [130-132]. The full-length Bid is mainly localized in the cytosol and translocates to mitochondria upon cleavage, formation of 15 kDa tBid and N-myristoylation at Gly-60 [96]. Anti-Fas antibody treatment of Jurkat cells was demonstrated to give rise to produce a 15 kDa Bid fragment and a further 13 kDa fragment. Asp-59 and Asp-75 in the N-terminal region of Bid was mapped as caspase and granzyme B cleavage sites, respectively [130]. N-terminal cleavage of Bid exerted an increased binding to Bcl-2 compared to wild-type Bid. In a similar study, an additional cleavage site on p22 full-length Bid was mapped at Asp-98, which was shown to result in formation of an 11 kDa fragment in addition to 15 kDa and 13 kDa fragments [131]. 11 kDa and 13 kDa cleaved fragments of Bid exclusively localized to mitochondria. The presence of Bcl-2 or Bcl-xL was found to be incapable of preventing TNFα-induced Bid cleavage and generation of 15 kDa tBid.

In addition to receptor-mediated apoptosis signaling, Bid was reported to be cleaved by caspase-3 at Asp-59 downstream of mitochondrial apoptosis pathway in response to treatment with multiple cytotoxic drugs and UV radiation, acting as a positive feedback loop to promote cytochrome *c* release [133]. Pretreatment with a caspase-3 inhibitor or expression of D59A Bid mutant prevented Bid cleavage in this setting. Cleavage of Bid at Asp-59 was also found to be involved in caspase-2-mediated apoptosis in MEF cells [134]. Bid-null MEF cells were resistant to caspase-2-induced apoptosis and the expression of wild-type Bid but not D59E mutant restored their sensitivity to caspase-2.

Despite the evidence that cleavage of Bid at Asp-59 by caspases enhances its proapoptotic activity, expression of a non-cleavable mutant of Bid (D59A) was reported to induce apoptosis in Bid -/-, caspase-8 -/- and wild-type primary MEF cells, although less effective than tBid [135]. When expressed at non-apoptotic levels, both non-cleavable Bid and wild-type Bid sensitized Bid -/- MEF cells to several death stimuli, including cisplatin, etoposide, staurosporine, UV radiation and TNFα.

Non-caspase proteases also target and cleave Bcl-2 family proteins to execute apoptosis independently or in cooperation with caspases. Bid was shown to be cleaved by calpain at Gly-70 both *in vitro* and in a myocardial ischemia/reperfusion model [136]. This finding was further supported by the inhibition of cisplatin-induced apoptosis by a calpain inhibitor, calpeptin, in 224 melanoma cells [137]. Calpain-cleaved Bid was able to trigger cytochrome c release when incubated with mitochondria isolated from 224 cells. During cytotoxic T-lymphocyte-mediated granule killing, granzyme B was demonstrated to directly cleave and activate Bid, which induced a caspase-independent mitochondrial permeabilization [138]. The granzyme B cleavage site of Bid was mapped at Asp-75 and expression of D75E Bid mutant abrogated Bid cleavage by granzyme B [139]. Treatment of Jurkat cells with granzyme B stimulated Bid cleavage and loss of mitochondrial potential despite the presence of caspase inhibitors. Furthermore, treatment of IL-3-dependent FDC-P1 cells with granzyme B induced Bid cleavage, although no such effect was observed upon IL-3 withdrawal or staurosporine treatment. Similar to caspase-cleaved Bid, granzyme B-cleaved tBid was shown to localize to mitochondria and to become integrated into mitochondrial membrane [140]. Following mitochondrial translocation, Bid triggered the recruitment of Bax to mitochondria to facilitate cytochrome *c* release and apoptosis. Moreover, expression of Bcl-2 did not prevent granzyme B-mediated Bid cleavage, but abrogated Bax activation and cytochrome *c* release. Bid was also reported to be cleaved by lysosomal proteases and lysosomal extracts from Bid -/- mice failed to induce cytochrome c release when stimulated with lysosomal extracts [141]. Lysosomal membrane permeabilization and translocation of cathepsins to cytosol in response to stimulation of neutrophils with type 1-fimbriated E.

coli induced the cleavage of Bid and apoptosis [142]. Treatment of neurophils with a cysteine-cathepsin inhibitor, but not with cathepsin D or caspase inhibitor attenuated type 1-fimbriated E. coli-mediated Bid cleavage and mitochondrial damage.

BimEL was reported to undergo caspase-mediated cleavage at the initiation phase of both mitochondrial and extrinsic apoptotic pathways. Caspase-3 was shown to cleave BimEL at Asp-13 and mutation of Asp-13 to asparagine prevented caspase-3-mediated BimEL cleavage [143]. Furthermore, treatment of Jurkat cells with staurosporine, TNFα, UV radiation and paclitaxel promoted N-terminal cleavage of BimEL. N-terminal cleavage of BimEL resulted in increased interaction of BimEL with Bcl-2 and increased apoptosis in 293 cells.

Bad was shown to be cleaved at its N-terminal by caspases in 32Dcl3 murine myeloid cells upon IL-3 withdrawal to generate a 15 kDA truncated Bad fragment [144]. This truncated Bad form was found to be solely localized to mitochondria and to be a more potent apoptosis-inducer in comparison to wild-type Bad. Asp-56 and Asp-61 of mouse Bad were identified as caspase cleavage sites and IL-3 withdrawal-mediated Bad cleavage was blocked with the expression of D56E/D61E mutant Bad and caspase inhibitor pretreatment. Activation of receptor-mediated apoptosis pathway in Jurkat cells also resulted in the cleavage of human Bad. N-terminal cleaved fragments of both human and mouse Bad were more effective than their full-length counterpart at inducing cytochrome *c* release and apoptosis. TGF-β treatment of hepatoma cells and raloxifene treatment of human bladder cancer cells also promoted the cleavage of Bad in a caspase-dependent manner, which could be abrogated by the expression of D56E/D61E mutant Bad [145,146]. Two majors forms of murine Bad were defined (mBadL and mBadS), which are generated by alternative splicing [147]. In a recent report, mBadL was demonstrated to protect Sindbis virus-induced apoptosis of hippocampal neurons [148]. mBadL was also found to be cleaved at Asp-61, but not at Asp-56 by recombinant caspase-3 and-7 and to be converted into a proapoptotic 21 kDa protein upon N-terminal cleavage. Expression of D61A mutant mBadL abrogated its cleavage and enhanced the antiapoptotic potency of mBadL both *in vitro* and *in vivo* cell death models. Furthermore, ΔN61 mBadL acted as a more potent apoptosis-inducer compared to wild-type mBadL when transiently transfected into HEK293 cells. Caspase-mediated cleavage of human Bad was shown to promote but not to be necessary for its proapoptotic function. Human Bad was believed to exist only in one form corresponding to mBadS and lacks the caspase cleavage site at Asp-61. Caspase-3 was shown to cleave human Bad at both Asp-14 and Asp-29, which corresponds to Asp-56 and Asp-71 of mBadL. D14/29A double mutation of Bad was found to be necessary to block caspase-3-mediated cleavage of human Bad. A predicted human BadL (hBadL) was generated by using an engineered cDNA and transfected into CCL10-BHK cells. Expression of wild-type and D57A/D72A cleavage mutant of human BadL did not alter staurosporine- or Sindbis virus-induced apoptosis and no cleavage product of human BadL was detected as the critical Asp-61 residue of mouse BadL was not present. In contrast, D57A/D72A cleavage mutant of hBadL was reported to inhibit IL-3-induced apoptosis in Ba/F3 cells, although wild-type Bad did not exert such an effect. Cleavage of hBadL at Asp-57 either alone or in combination with Asp-72 was proposed to regulate IL-3 withdrawal-induced apoptosis [148].

Calpain-mediated cleavage of Bax was detected following staurosporine or etoposide treatment into an 18 kDa fragment in the execution phase of apoptotic response [149]. This cleaved 18 kDa form of Bax was found to be completely localized in the mitochondrial fraction. Calpain-cleaved Bax did not bind to Bcl-2 in contrast to full-length Bax and the transfection of 18 kDa Bax into multiple cancer cell lines triggered cytochrome *c* release and apoptosis, which could not be blocked by Bcl-2. On the contrary, Bcl-2 overexpression blocked etoposide-induced calpain activation and Bax cleavage. Bax cleavage site by calpain activity was mapped around amino acids 30-33 of human Bax and purified calpain enzymes were shown to cleave Bax in a calcium-dependent manner [150]. Pretreatment of cells with calpeptin effectively abrogated topoisomerase inhibitor-induced Bax cleavage. Recently calpain was shown to cleave Bax between Gln-28 and Gly-29 and ΔN28 Bax was found to elicit a more potent apoptotic function in comparison to wild-type Bax in 293 fibroblasts [151]. N-terminal cleaved Bax (ΔN33) was reported to have a reduced half-life compared to wild-type Bax due to increased sensitivity to cathepsin-mediated proteolytic cleavage [152]. As expected, treatment with cathepsin inhibitors enhanced the expression of 18 kDa Bax fragment and thereby amplified drug-induced apoptotic response in various cancer cells.

In contrast with other posttranslational modifications, proteolytic cleavage appears to have a uniformly proapoptotic affect, whether performed on proapoptotic or antiapoptotic Bcl-2 family proteins. The cleavage may well not be an initiating event in apoptosis, but rather part of a positive feedback loop in a cell that has already activated proteases need to commit the cell to death. This loop may ensure that a cell that has committed to apoptosis executes its cell death as quickly and efficiently as possible. Such rapid execution minimizes the organism's burden of damaged, dangerous, and useless cells.

CONCLUDING REMARKS

The ability of cancer cells to evade apoptosis either during oncogenesis or in response to therapy is an essential mechanism of tumor maintenance and Bcl-2 family proteins are key regulators of this process. The propensity of myocardial cells to die following infarction is likewise controlled by the Bcl-2 family, Posttranslational modifications of both antiapoptotic and proapoptotic Bcl-2 proteins govern their function in important

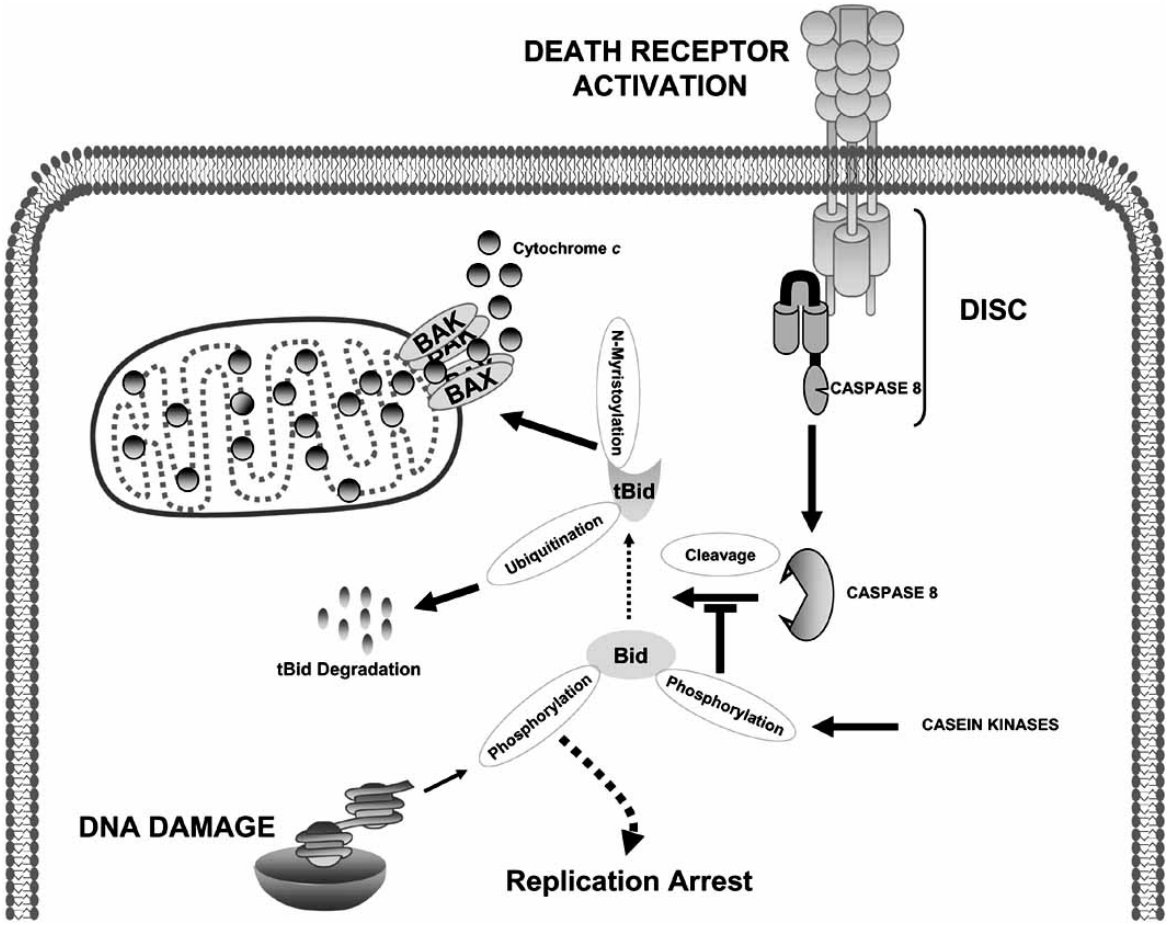

Fig. (2). Bid is regulated by various posttranslational modifications. Upon stimulation of death receptor apoptosis pathway and DISC formation, caspase-8 is activated and cleaves Bid into tBid. Cleavage of Bid at Asp-60 results in N-myristoylation of tBid, which promotes its mitochondrial translocation to induce the activation of multidomain proapoptotic Bax and Bak proteins and cytochrome *c* release. Phosphorylation of Bid by casein kinases inhibits caspase-8-mediated cleavage and its proapoptotic function. Moreover, phosphorylation of Bid by ATM kinases results in intra-S replication arrest in response to DNA damage. Ubiquitination of tBid results in degradation of tBid by 26S proteasome and inhibition of apoptosis.

ways. Accepting the fact that multiple posttranslational modifications could happen synchronously, identification of each kind of protein modification in a particular cellular context is required for exploiting specific responses. The interconnection of posttranslational modifications further complicates the situation, since phosphorylation of a residue may promote ubiquitination or cleavage may result in exposure of an N-myristoylation consensus motif in the same protein. As an example of a single protein in which multiple posttranslational modifications have differing affects on function, the regulation of Bid by posttranslational modifications is depicted in Fig. (**2**). Thereby, the generalization of experimental results on posttranslational modifications of Bcl-2 proteins is clearly a challenging approach and many controversial and apparently contradictory experimental studies demonstrating the effects of posttranslational modifications on Bcl-2 proteins exist.

Nonetheless, these continuing efforts to explain the role of posttranslational modifications on Bcl-2 proteins will allow us to better understand the myriad of the posttranslational modifications involved in functional organization of Bcl-2 proteins. Finally, these findings can help us to develop novel therapeutic strategies including pharmacological manipulation of Bcl-2 proteins, such as kinase inhibitors targeting upstream of Bcl-2 protein phosphorylation events. For instance, since BIM can be targeted for proteasomal degradation by phosphorylation by the MAPK pathway, it may be possible to increase death selectively by inhibiting proteasome function or MAPK phosphorylation. In fact, the former strategy has shown positive *in vitro* results [153]. In cases where pathologically excess death is taking place in a BID dependent fashion, the steps of myristoylation and caspase-8 cleavage may be most readily accessible to pharmacologic inhibition to reduce cell death.

The apoptosis field has gradually increased its appreciation of the significance of posttranslational modifications in the control of cell death. An important next step is linking individual posttranslational modifications to specific disease states. Once identified, such modifications offer promise as targets for therapeutic intervention *via* inhibition of critical enzymatic activities.

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