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Apoptosis induction by Bcl-2 proteins independent of the BH3 domain

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ABSTRACT

Bcl-2 proteins, characterized by up to four Bcl-2 homology domains (BH1–BH4), are critical regulators of the mitochondrial proapoptotic pathway. Three major subgroups have been described, namely antiapoptotic proteins, proapoptotic multidomain and BH3-only proteins. These are basic for present models explaining the regulation of the mitochondrial outer membrane permeability. However, several Bcl-2 proteins have been described that do not fit into these models, due to their atypical domain structure or due to their ability to induce apoptosis independently of BH3. These proteins are indicators for new mechanisms in apoptosis control by Bcl-2 proteins, which may supply additional targets for novel therapeutic approaches.

1. Critical roles of apoptosis in cancer development and therapy

Programmed cell death (apoptosis) has been defined as counterbalance for cell proliferation in maintaining normal tissue homeostasis [1], and it provides fundamental safeguard mechanisms for protection against cancer, based on the elimination of altered and potentially harmful cells [2]. Thus, genomic aberration and cellular dysregulation, typical for cancer cells, trigger cell-intrinsic proapoptotic pathways [3,4], whereas aberrant protein expression may activate an immune response leading to the induction of extrinsic apoptosis pathways [5,6]. Further alternative cell death programs as in particular related to autophagy have been described in recent years. This is an evolutionary conserved mechanism, based on autophagosome formation, which primarily regulates the response of eucaryotic cells to starvation conditions, but can switch to a type II programmed cell death program [7].

For development of cancer, the different cell death-promoting programs are overcome leading to an accumulation of resistant tumor cells, which may later also reveal a therapy-resistant phenotype. Thus, new therapeutic strategies predominantly aim at the sensitization of cancer cells for proapoptotic regimens [8,9].

2. Intrinsic pathways to apoptosis

Most characteristic for a cell-intrinsic induction of apoptosis is the mitochondrial pathway characterized by increased mitochondrial outer membrane permeability (MOMP) and release of mitochondrial factors into the cytoplasm. This pathway is critically controlled by the family of pro- and antiapoptotic Bcl-2 proteins [3,10,11]. Intrinsic proapoptotic pathways are frequently initiated upon cellular damage via p53 activation/stabilization and a p53-mediated transcriptional activation of proapoptotic factors as in particular of proapoptotic Bcl-2 proteins [3,12].

Once a certain threshold of proapoptotic signals is exceeded, a rapid release of mitochondrial intermembrane factors is

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induced. Several of them exert characteristic proapoptotic functions in the cytoplasm, as reported for endonuclease G, AIF (apoptosis-inducing factor), Smac/DIABLO, HtrA2/Omi and cytochrome c [13]. Whereas Smac/Diablo and HtrA2/Omi have been described to augment caspase activation by antagonizing inhibitor of apoptosis proteins [14,15], the activities of AIF and endonuclease G appear as largely independent from caspase pathways. After translocation to the nucleus, their proapoptotic function depends on an endogeneous DNAse activity (Endo G) or on recruitment of downstream nucleases in case of AIF [16].

Cytochrome c triggers the formation of the apoptosome, a multiprotein complex enclosing each seven copies of the adaptor protein Apaf-1 (apoptosis-activating factor), cytochrome c, ATP and caspase-9 [17]. Here, initiator caspase-9 is activated, which sets up a subsequent caspase cascade starting with caspase-3. Caspase signaling cascades represent hallmarks in apoptosis [18,19], and downstream effector caspases cleave hundreds of different target proteins (death substrates). This leads to a complete reprogramming of the cell for apoptosis [20]. The mitochondrial membrane appears as a critical level for the integration of pro- and antiapoptotic signals. Here, the final decision about life and death is made, which is linked to the release of mitochondrial proteins. Proapoptotic Bcl-2 proteins support this release while antiapoptotic proteins try to block it.

3. Major groups of Bcl-2 proteins

The large family of Bcl-2-related proteins is characterized by one to four structural motifs termed Bcl-2 homology domains (BH1–BH4) as well as a transmembrane domain (TM). Most antiapoptotic Bcl-2 proteins share all five domains as Bcl-2, Bcl-x_L, Bcl-w, Bcl-B and Nrh/Nr-13. On the other hand, proapoptotic Bcl-2 homologs typically lack one or several BH domains and further subdivide into multidomain proteins sharing BH1, BH2, BH3 and TM (Bax, Bak and Bok/Mtd) as well as BH3-only proteins as Bad, Bik/Nbk, Bim, Bmf, Bnip3, Hrk, Noxa, Puma, Spike and tBid [10,21]. BH3-only proteins may have or may lack the TM (Fig. 1). The structure of Bcl-2-related proteins will be addressed in more detail in other articles of this issue. Of importance for the function, Bcl-2 proteins may heterodimerize with each other, and the balance between pro- and antiapoptotic Bcl-2 proteins may decide about the cell death response [22,23].

The conformational change of proapoptotic multidomain Bcl-2 proteins Bax and Bak and their association or deeper integration into the outer mitochondrial membrane has been tightly related to induction of apoptosis [24,25]. However, the mechanism how Bax and Bak trigger MOMP is still controversial. The direct pore formation by Bax and Bak themselves allowing the release of mitochondrial proteins has been suggested, and several cell-free data are consistent with this hypothesis [26]. However, the biochemical nature of Bax/Bak pores is still elusive and its presence in cells could so far not be demonstrated [27]. Alternatively, MOMP has been suggested as dependent on the permeability transition pore (PTP), which may be blocked by antiapoptotic Bcl-2 proteins [24,25].

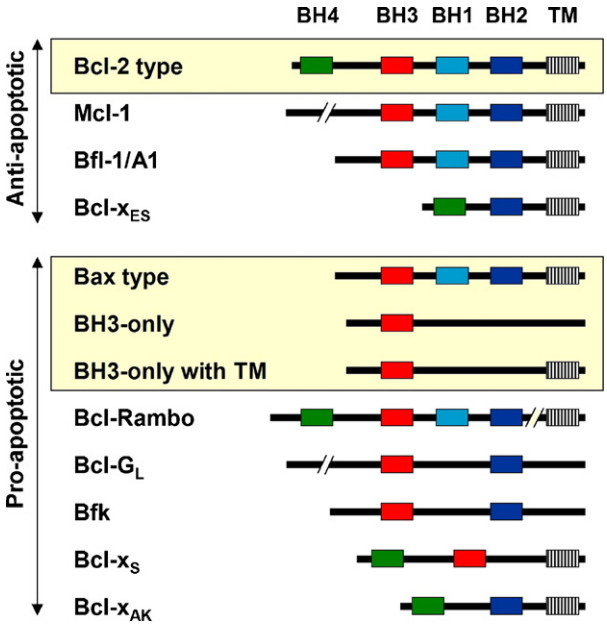

Fig. 1 – Distribution of Bcl-2 homology domains in Bcl-2 proteins. Typical domain structures of antiapoptotic as well as proapoptotic proteins are shown in boxes, atypical proteins are shown below. The presence of up to four Bcl-2 homology domains (BH1–4) as well as the transmembrane domain (TM) is shown. Modified according to Ref. [21] and further enclosing data on Bfk [71] and Bcl-x_{AK} [76].

In the following, we will briefly introduce present models explaining the mutual regulation of Bcl-2 proteins and the understanding on Bcl-2 homology domain functions. In particular, antiapoptotic Bcl-2 proteins without BH4, proapoptotic Bcl-2 proteins which trigger cell death independently of BH3 or reveal atypical domain structures and finally splice products of bcl-x with opposing functions will be discussed.

4. Models for mutual regulation of Bcl-2 proteins

Since the first identification of Bcl-2 in 1984 by the *t*(14;18) translocation in acute B-cell leukaemia [28], a large body of data has been collected on their function and regulation. Different models for explaining their mutual regulation have been suggested.

4.1. *Rheostat model*

According to the initial model, the balance of antiapoptotic and proapoptotic Bcl-2 proteins determines the cellular fate. It is based on the assumption that the activities of proapoptotic Bcl-2 proteins are kept in check by the antiapoptotic Bcl-2 family members, which are often permanent constituents of the mitochondrial membrane and which can bind and neutralize Bax and Bak. Upon a stress situation, proapoptotic Bcl-2 proteins are induced or

activated to trigger MOMP. On the other hand, growth factors promote cellular survival by increasing the amount of antiapoptotic Bcl-2 proteins or decreasing proapoptotic members [29]. For the interaction of Bcl-2 proteins, the BH3 domain seems to be required and binds into a hydrophobic pocket of antiapoptotic Bcl-2 proteins that is formed by BH1, BH2 and BH3 [30].

4.2. *Antiapoptotic protein neutralization model*

Subsequent models particularly tried to explain the proapoptotic activities of BH3-only proteins, which are regarded as triggers in apoptosis control. Their expression is induced or they are activated by post-translational modification in response to diverse apoptotic stimuli. Thus, Bid is activated by proteolytic cleavage through caspase-8, Bad is activated upon dephosphorylation, and Bim is released from cytoskeletal structures [31–33].

According to the neutralization model, which is similar to the indirect activation model, BH3-only proteins bind to antiapoptotic Bcl-2 proteins thus neutralizing their activity and leading to a release of Bax and Bak [10,34]. When several antiapoptotic Bcl-2 proteins are expressed, different BH3-only proteins are required to promote apoptosis, due to their different binding specificities to antiapoptotic Bcl-2 proteins (e.g. BAD can neutralize Bcl-2, Bcl-x_L and Bcl-w, whereas Noxa binds Mcl-1 and A1) [35].

4.3. *Direct activation model*

Also a direct interaction between proapoptotic multidomain proteins and BH3-only proteins has been suggested [36]. According to this model, BH3-only proteins are functionally subdivided into activators and sensitizers. Activation of Bax and Bak require direct association with activator BH3-only proteins. However, these proteins are normally bound and kept in check by antiapoptotic Bcl-2 proteins. Bax and Bak are activated when activator BH3-only proteins are released from antiapoptotic Bcl-2 proteins through their binding to sensitizer BH3-only proteins. These sensitizers cannot directly activate Bax and Bak [37]. The main statement of the last model is however still controversial as Bax and Bak were recently shown to mediate apoptosis also without association to BH3-only proteins [38].

4.4. *Indications for other Bcl-2 protein functions*

Besides these models, Bcl-2 proteins are also involved in control of other types of cell death. Thus, Bcl-2 has been shown to bind the autophagy regulator Beclin-1 resulting in a downregulation also of autophagy, in parallel to downregulation of apoptosis [39]. Furthermore, BH3-independent ways of Bax activation and induction of MOMP have been suggested, as through interaction of tBID with cardiolipin in mitochondrial membranes leading to distortion of the lipid bilayer [40]. Finally, several Bcl-2 proteins do not fit into the above detailed models due to their unusual domain structure (Fig. 1). Nevertheless, they exert typical pro- or antiapoptotic functions, but their roles in the present models are largely elusive. Deviations from the typical structures of pro- and antiapoptotic proteins are mainly concerning the presence or absence of BH4 and BH3 domains.

5. Bcl-2 homology domain function

5.1. *BH3*

The critical role of the BH3 domain as a mediator of cell death was identified in studies on the molecular interaction between Bak and Bcl-x_L, which revealed a unique requirement of BH3 for the interaction with Bcl-x_L as well as for cell killing [41]. Sequence comparison with other proapoptotic proteins then resulted in the identification of homologous domains in otherwise unrelated proapoptotic proteins, as initially described for Bik/Nbk and Bad [42,43], which resulted in the definition of the subfamily of BH3-only proteins.

Interestingly, even truncated peptides of Bak containing the BH3 domain were shown to trigger apoptosis [41], which was later also shown for other BH3 domain peptides. This finally resulted in the identification of new therapeutic tools, namely the BH3 mimetics [44–46]. The critical role of BH3 for apoptosis induction by BH3-only proteins has been demonstrated by deletion mutants. Thus, point mutation or deletion of the BH3 of Spike almost completely abrogated its proapoptotic activity [47]. Also, deletion of BH3 in Hrk strongly diminished its killing activity and abolished its ability to interact with Bcl-2 and Bcl-x_L [48].

5.2. *BH4*

The BH4 domain is typical for most antiapoptotic Bcl-2 proteins, whereas it is mostly lacking in proapoptotic family members (except for Bcl-x_S and Bcl-x_{AK}; Fig. 1). It is directly involved in heterodimerization, however it may be decisive for the distinction between anti- and proapoptotic functions. Indeed, caspase-mediated cleavage of BH4 from Bcl-2 and Bcl-x_L, thus the conversion of Bcl-2-like proteins to Bax-like proteins, has been previously shown to result in Bcl-2- and Bcl-x_L-derived proapoptotic forms [49,50].

Deletion and substitution mutants have been constructed for the BH4 of Bcl-2 and Bcl-x_L, which clearly demonstrated its antiapoptotic function. Its deletion rendered Bcl-2 and Bcl-x_L inactive, although the binding ability for proapoptotic members seemed not to be impaired [51]. In other studies, the BH4 domain of Bcl-x_L was shown to be required for stabilization of the mitochondrial membrane potential ($\Delta\Psi$) and for preventing cytochrome *c* release.

Beyond this, BH4 alone revealed an antiapoptotic function. Thus, isolated BH4 oligopeptides of Bcl-2 and of Bcl-x_L as well as constructs with BH4 linked to HIV-1 TAT protein blocked $\Delta\Psi$ loss even in the presence of Bax [52]. The antiapoptotic, protective activities of BH4-TAT constructs have been proven in several experimental systems, as in sepsis-induced apoptosis in lymphocytes [53] or beta-amyloid peptide-induced apoptosis in endothelial cells [54]. These investigations indicated a BH4-mediated antiapoptotic activity that was independent from BH3-mediated heterodimerization.

6. Antiapoptotic Bcl-2 proteins with and without BH4

Most antiapoptotic members of the Bcl-2 family are highly homologous to Bcl-2 and Bcl-x_L enclosing all four Bcl-2 homology domains and TM. The domains BH1 and BH2, together with BH3, are involved in formation of a hydrophobic groove thus necessary for suppression of apoptosis based on heterodimerization with proapoptotic members. The BH4 domain seems to mediate the binding of even other proteins involved in apoptosis [55], thus also mediating antiapoptotic effects independent of heterodimerization. Antiapoptotic members with only BH1–BH3 should lack these additional activities and may thus block apoptosis particularly by inactivating multidomain proapoptotic Bcl-2 proteins.

Two well-known examples, which lack BH4, are Mcl-1 and Bfl-1/A1 (Fig. 1). Mcl-1 is rapidly degraded in response to cell death signals and is immediately re-induced by survival stimuli, thus indicating a critical pro-survival role in the development and maintenance of tissues [56]. Coimmunoprecitation experiments revealed for both BH4-lacking proteins (Mcl-1 and Bfl-1/A1) binding to Bak and tBid but not to Bax, whereas antiapoptotic Bcl-2 proteins with a BH4 were less selective [57–59].

7. Proapoptotic Bcl-2 proteins that trigger cell death independently of BH3

According to present models, the BH3 domain is considered as largely essential for heterodimerization of Bcl-2 proteins as well as for the proapoptotic capability itself. However, increasing evidence indicates that proapoptotic functions of Bcl-2 proteins can also be independent of a functional BH3.

7.1. *Bok/Mtd*

This protein is a structural homolog of Bax and Bak and predominantly detected in brain, liver, and lymphoid tissues. Bok contributed in neuroblastoma and breast cancer cells to p53-dependent, DNA damage-induced apoptosis, while Bax was not essential [60]. Unlike to Bax and Bak, Bok/Mtd revealed selective binding to Mcl-1 and Bfl-1 but not to Bcl-2 or Bcl-x_L [61]. Most strikingly, apoptosis through Bok appeared as independent of BH3, as demonstrated by a mutant with a substituted BH3 that retained the proapoptotic activity [62].

7.2. *BNIP3*

This protein is structurally related to the BH3-only subfamily [63]. In healthy cells, it loosely associates with mitochondria and integrates deeper upon induction of cell death, while its N-terminus remains in the cytoplasm [64]. Interestigly, deletion mapping excluded its BH3 but identified the N-terminus and the TM domain as critical for the heterodimerization with Bcl-2 and Bcl-x_L as well as for the proapoptotic activity. Complete removal of the BH3 could not diminish the killing activity of BNIP3, clearly distinguishing it from other BH3-only proteins [65]. Apoptosis induced by BNIP3 appeared as largely independent of Apaf-1, caspase activation or cytochrome *c* release [64] but was correlated with an opening of the mitochondrial PTP, followed by chromatin condensation and DNA fragmentation [66,67]. Furthermore, BNIP3 appears as critical for hypoxia-induced autophagy, and its expression and induction of autophagy upon hypoxia was blocked in HIF-1-deficient cells [68].

7.3. *Spike*

Also this novel BH3-only protein revealed specific characteristics different to other proteins of this group. Thus, it was not associated with mitochondria but with the ER, where it showed interaction with Bap31 protein. In contrast, no interaction with other Bcl-2 family members was found, thus again indicating alternative proapoptotic pathways, that are independent of a BH3-mediated interaction [47].

8. Atypical domain structures of proapoptotic Bcl-2 proteins

8.1. *Bcl-rambo*

This atypical protein encloses all four conserved BH motifs as well as the TM but was interestingly described as a proapoptotic factor [69]. Unlike other Bcl-2 proteins, the BH domains in Rambo are separated from TM by a unique 250-aa-long sequence with no relation to BH domains. Deletion analysis revealed that the proapoptotic function of Rambo was dependent on both this inserted sequence and the TM. Interestingly, also a construct enclosing only the inserted sequence and TM retained the proapoptotic activity, thus acting independently of all BH domains. Further separating Rambo from other Bcl-2 proteins, no interaction of Bcl-rambo with other members of the family was found [69].

8.2. *Bcl-G*

The *bcl-G* gene encodes for two proteins resulting from alternative splicing. Whereas Bcl-G_S (short) resembles typical BH3-only proteins, Bcl-G_L (long) is characterized by a novel combination of BH3 with BH2. Both proteins exert proapoptotic activities, but only Bcl-G_S was found to bind to Bcl-x_L. Deletion of the BH2 domain in Bcl-G_L resulted in a recovered Bcl-x_L binding capacity and an even enhanced proapoptotic activity, thus suggesting a repressing function of the BH2 domain in Bcl-G_L [70].

8.3. *Bfk*

This protein had been identified by database screening and represents a second proapoptotic member of the Bcl-2 family that contains BH2 and BH3. Unlike to Bcl-G_L, it was not associated with organelles but localized to the cytoplasm, which may correlate to the lack of TM. As Bcl-G_L, Bfk did not bind other Bcl-2 family members. This was however not due to a non-functional BH3 domain, as replacement of the BH3 in BimL by the BH3 of Bfk resulted in a chimeric protein that was capable to bind to Bcl-2, Bcl-w and Bcl-x_L [71]. Thus again, the

presence of BH2 was associated with a suppression of the BH3 proapoptotic function.

9. Splice products of Bcl-x with deviating functions

Several proteins of the Bcl-2 family arise from alternative splicing, and many splice products still await their characterization. Thus besides the already characterized splice variants Bim_S, Bim_L and Bim_{EL}, the *bim* gene encodes for even further splice products with so far undetermined function [72], and up to eight splice variants with partly deviating domain structures have been reported for Bax [73]. A well-known example is the *bcl-x* gene, which is expressed in at least four reported isoforms of different activities. While Bcl-x_L (long) and Bcl-x_{ES} (extra short) are antiapoptotic [74,75], Bcl-x_S (short) and Bcl-x_{AK} (alternative killer) exert proapoptotic functions [74,76].

9.1. *Bcl-x_L and Bcl-x_S*

Whereas *Bcl-x_L* appears as a typical antiapoptotic protein with all four BH domains and the TM, *Bcl-x_S* has an extraordinary combination and exclusively encloses BH3, BH4 and TM, thus being clearly distinct from Bax or Bak. BH1 and BH2 are required for inhibition of apoptosis and heterodimerization with proapoptotic family members [77,78], thus ruling out this function for Bcl-x_S. Correspondingly, high induction of apoptosis was seen when sarcoma cells were treated with a Bcl-x_S adenovirus [79], and also in melanoma cells, its exogenous expression resulted in a strong increase of apoptosis, both for in vitro and in vivo models [80].

Concerning the unique domain structure, a critical contribution of the BH3 for the proapoptotic activity of Bcl-x_S has been clearly demonstrated. However, also deletion of the TM resulted in a significant drop in its proapoptotic activity, in parallel to findings for BNIP3 and Bcl-Rambo. Immunoprecipitation showed binding of Bcl-x_S to Bcl-x_L but not to Bcl-2, and overexpression of Bcl-x_L but not of Bcl-2 attenuated Bcl-x_S-induced apoptosis. The Bcl-x_L interaction required the BH3 domain of Bcl-x_S, whereas deletion of other sequences did not affect heterodimerization [79].

In contrast, deletion of BH4 did not diminish the proapoptotioc function [79]. As even isolated BH4 domains of Bcl-x_L were able to suppress apoptosis [51], the same BH4 in Bcl-x_S might be involved in suppression or modulation of the apoptotic response.

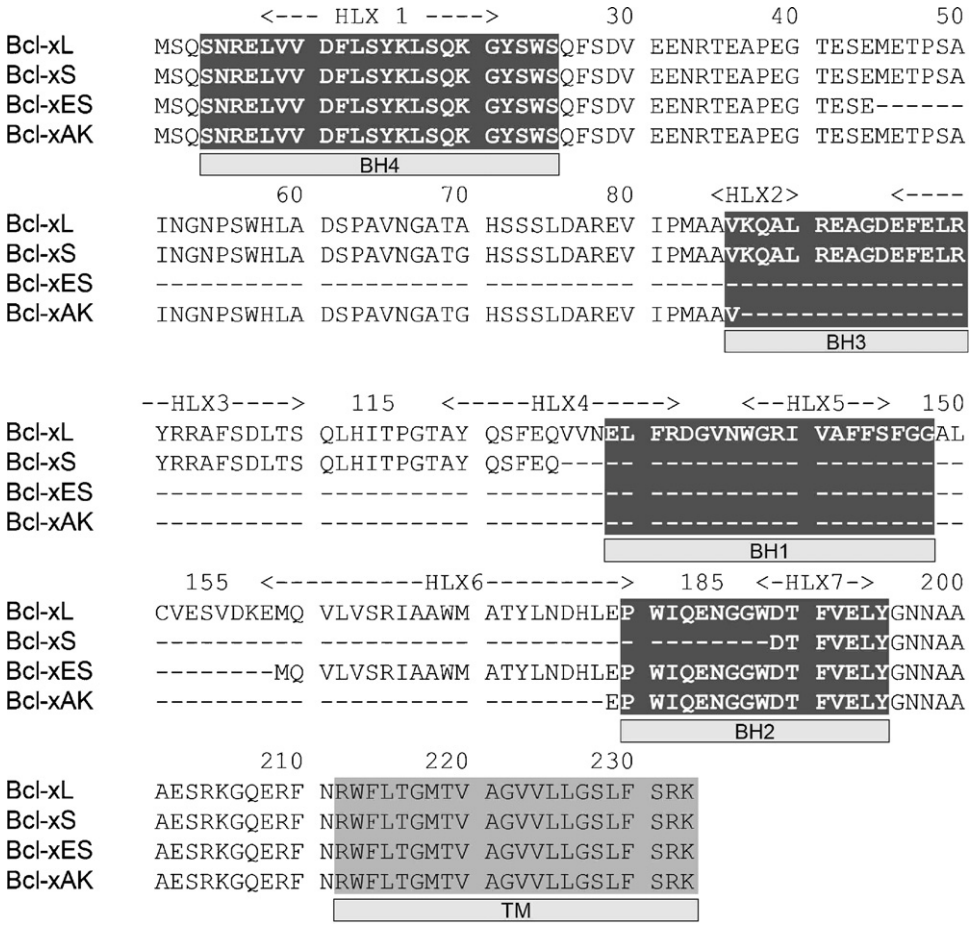

Fig. 2 – Aminoacid structure of bcl-x splice products. An aminoacid sequence alignment of the four described bcl-x splice products (Bcl-x_L, Bcl-x_S, Bcl-x_{ES} and Bcl-x_{AK}) is shown. Bcl-2 homology domains (BH1–4) and the TM are shown in boxes, and predicted α-helices (Hlx) are indicated above the sequence. Missing amino acids in splice products are indicated by hyphens. Modified according to Ref. [76].

9.2. *Bcl-x_{ES} and Bcl-x_{AK}*

Two more atypical Bcl-2 proteins (*Bcl-x_{ES}* and *Bcl-x_{AK}*) result from alternative splicing of bcl-x. Both share a common, but otherwise unique domain structure, namely BH4, BH2 and TM, but they lack BH3 (Fig. 2). Further complicating the situation, opposing effects have been reported for these two proteins: Whereas Bcl-x_{ES} was described as antiapoptotic in B-cell lymphoma cells [75] apoptosis was induced by Bcl-x_{AK} in melanoma cells after exogenous overexpression [76]. Sequence comparison reveals that they are distinguished by a lack of 41 AA of the BH4–BH3 interdomain region in Bcl-x_{ES} and a lack of 20 amino acids in front of BH2 in Bcl-x_{AK} (Fig. 2). Both proteins should be unable to form a hydrophobic groove, which requires BH1 and BH3 [30]. The antiapoptotic activity of Bcl-x_{ES} may thus be ascribed to the activity of BH4, resembling the antiapoptotic activities found for isolated BH4 domains of Bcl-x_L [52–54].

Bcl-x_{AK} is the first proapoptotic Bcl-2 protein that lacks the BH3 domain, which so far has been regarded as indispensable for the proapoptotic function. Induction of apoptosis by Bcl-x_{AK} can also not directly depend on TM, as possibly may apply to Bnip3 [65], because Bcl-x_{ES} shares the same TM. It may be rather based on the deviating sequence in the interdomain region or on the combination of both, thus resembling the situation in Bcl-Rambo. The examples of Bcl-x_{ES} and Bcl-x_{AK} clearly demonstrate a decisive role of the interdomain sequence for shifting the activity from anti- to proapoptotic. Here the lack of the central hydrophobic a-helix 6, which has been involved in channel formation [81,82] and which is lacking in both proapoptotic splice products (Bcl-x_{AK} and Bcl-x_S) but present in Bcl-x_{ES} and Bcl-x_L, may play a role (Fig. 2).

10. Conclusions

From a large number of studies it becomes clearly evident, that both pro- and antiapoptotic functions of Bcl-2 proteins are not restricted to the heterodimerization based on interaction between the BH3 domain and the hydrophobic groove. Especially BH4 appears to bear an independent antiapoptotic potential, and proapoptotic functions also occur independent of BH3, and may be attributed to the TM and interdomain sequences. These new functions may be particularly addressed by the atypical Bcl-2 proteins, which at present are not well classified.

The cancer mortality worldwide remains on a dramatically high level, particularly due to a lack of efficient therapies for metastatic disease [83,84], and apoptosis deficiency of cancer cells seems to be the major problem [8,9]. New apoptosis pathways and thus new possible targets are thus of particular interest. The better understanding of apoptosis regulation, and the full elucidation of all the roles of the most important Bcl-2 family in cancer cells, may finally help to overcome apoptosis deficiency and therapy resistance.

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