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Review

Alternative splicing of Bcl-2-related genes: functional consequences and potential therapeutic applications

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Abstract. Apoptosis is a morphologically distinct form of cell death. It is executed and regulated by several groups of proteins. Bcl-2 family proteins are the main regulators of the apoptotic process acting either to inhibit or promote it. More than 20 members of the family have been identified so far and most have two or more isoforms. Alternative splicing is one of the major mechanisms providing proteomic complexity and functional diversification of the Bcl-2 family proteins. Pro- and anti-apoptotic Bcl-2 family members should function in harmony for the regulation of the apoptosis machinery, and their relative levels are critical for cell fate. Any mechanism breaking down this harmony by changing the relative levels of these antagonistic proteins could contribute to many diseases, including cancer and neurodegenerative disorders. Recent studies have shown that manipulation of the alternative splicing mechanisms could provide an opportunity to restore the proper balance of these regulator proteins. This review summarises current knowledge on the alternative splicing products of Bcl-2-related genes and modulation of splicing mechanisms as a potential therapeutic approach.

Key words. Apoptosis; Bcl-2 family; alternative splicing; antisense.

Programmed cell death and Bcl-2 family of proteins

Programmed cell death (PCD), a term currently used synonymously with apoptosis, is defined as the removal of individual cells after their fragmentation into membrane-bound particles which are then phagocytosed by specialised cells. During this process there is no loss of membrane integrity of the apoptotic cell and so no spillage of cytoplasmic contents as the cell dies. Apoptosis, in contrast to necrosis, is genetically controlled and is an internally mediated response to external stimuli. It appears to be an evolutionarily conserved process with similar sets of controlling proteins in a variety of organisms.

Many physiological, pharmacological and environmental factors can induce apoptosis [1]. Despite the huge structural and functional diversity of these initiating factors, the downstream pathways of apoptosis are often shared, indicating the presence of common death-execution pathways. Recent studies have indicated that the critical components of the apoptosis process are the caspases, the Bcl-2 family proteins whose activities are controlled by complex signal-transducing proteins.

Bcl-2-related proteins are key regulators of apoptosis, either inhibiting or promoting it. Bcl-2 was the first family member to be reported in mammalian cells, and since

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its discovery a number of structurally and functionally related proteins have been identified based on sequence similarities. These include the anti-apoptotic family members Bcl-2, Bcl-X_L, Mcl-1, Bfl-1 and Bcl-w, and the pro-apoptotic members Bax, Bcl-X_S, Bad, Bak, Bik, Bid, Bim, Bcl-G, Bok, Puma and Noxa [2, 3]. All these proteins share homology in one to four regions designated as Bcl-2 homology domains (BH1, BH2, BH3, BH4). Whilst all four domains are present in some members, including Bcl-2 and Bcl-X_L, others lack one or more of these domains. Some pro-apoptotic members of the family including Bad, Bid, Bik and Bim contain only the BH3 domain and are called 'BH3-only' proteins [3]. Most of the Bcl-2 family members also contain a hydrophobic membrane anchor domain in their C-terminal region that targets them predominantly to the mitochondrial membrane, nuclear envelope and endoplasmic reticulum [2]. Most of the anti-apoptotic members contain at least three of the BH domains (BH1, 2, 3) as well as the membrane anchor domain. Only one of these domains, BH3, is essential for the dimerisation and pro-apoptotic activity of death-promoting members of the family [3].

Although there is still much debate about how these proteins regulate the apoptotic machinery, analysis of their structural and functional properties reveals potential mechanisms of action. The most striking feature of Bcl-2 family proteins is their ability to form homodimers or heterodimers with each other. The initial discovery of Bcl-2/Bax heterodimerisation has been a key to understanding the functions of the Bcl-2 family of proteins [4]. Later, the relative levels of the anti-apoptotic and the pro-apoptotic proteins of the family were shown to determine the relative sensitivity or resistance of cells to apoptotic stimuli [5].

Bcl-2 and Bax have intrinsic and independent functions. However, dimerisation among family members still provides an attractive mechanism for the regulation of apoptosis; for example, heterodimerisations between anti-apoptotic and pro-apoptotic members may neutralise their respective intrinsic functions [2]. This view is supported by the finding that most of the pro-apoptotic members of the family contain only a BH3 domain and operate as transdominant inhibitors of functional anti-apoptotic proteins. These BH3-only proteins cannot homodimerise or heterodimerise with each other and they do not possess any apparent intrinsic function on their own [2, 3].

Most of the Bcl-2 family proteins have been shown to be targeted to organelle membranes, predominantly to the mitochondrial membrane via their C-terminal membrane anchor domain. These observations indicate that Bcl-2 family proteins might, at least in part, regulate the apoptosis machinery by regulating the functions of mitochondria [6]. The release of apoptotic factors, including cytochrome c, from mitochondria has been proposed as a possible reason for the involvement of mitochondria in the apoptosis machinery. The anti-apoptotic proteins might function, somehow, to retain these factors sequestered within the mitochondria. Investigation of the three-dimensional (3-D) structure of Bcl-X_L has indicated that it shares some structural similarities to the pore-forming domain of some bacterial toxin, leading to the prediction that membrane-associated Bcl-2 family proteins might function as a gateway to some critical components of the apoptosis machinery [6, 7].

Two major pathways leading to apoptosis have been defined in a number of cell types. The first involves the death receptor pathways, initiated by TNFRs, TRAIL receptors and Fas that can activate a caspase cascade via activation of caspase-8 as an initiator caspase [8]. As this pathway requires the binding of an extracellular 'death signal' to its cognate receptor, this is often termed the 'extrinsic pathway'. The second is termed the intrinsic apoptosis pathway, and involves both mitochondria and the Bcl-2 family of proteins. This results in activation of a caspase cascade via activation of caspase-9 as an initiator caspase [9]. We now recognise that there may be some interplay between these two pathways. For example, whilst the Bcl-2 family of proteins do not directly control the death receptor pathway, caspase-8 that is activated by a death receptor can in turn activate the pro-apoptotic protein Bid, which then translocates to mitochondria and induces cytochrome c release. Caspase-8-mediated Bid activation is the best-characterised cross-talk between the two apoptosis pathways involving death receptors and mitochondria [10].

Caspases constitute the central components that execute the apoptosis process. These proteolytic enzymes are involved in a cascade that is triggered by apoptotic signals and results in the cleavage of a specific set of target proteins. The Bcl-2 family proteins thus constitute the central control stage of apoptosis and function upstream of caspase activation. Cytochrome c and Apaf-1 are the key elements for the activation of pro-caspase-9 [9]. Whilst membrane-bound anti-apoptotic Bcl-2 family proteins keep cytochrome c sequestered in the mitochondria, Bcl-X_L has also been shown to interact with Apaf-1 and inhibit Apaf-1 dependent caspase activation [11]. The pro-apoptotic Bcl-2 family proteins, on the other hand, heterodimerise with the anti-apoptotic proteins and antagonise survival functions [2, 3]. This interaction might displace the pro-survival proteins from their Apaf-1 complex and the released Apaf-1, together with cytochrome c and ATP, may subsequently activate pro-caspase-9 [12].

Thus, the Bcl-2 family proteins play key roles in regulating whether a cell dies or survives in response to external signals (such as death-inducing ligands, survival factors or growth factor withdrawal). However, there are a variety of mechanisms by which the levels or function of

these proteins can be regulated to affect survival or apoptosis. These include regulation by transcription, translation, posttranslational control, alternative splicing and intracellular redistribution [2].

The function of many Bcl-2 family members can be regulated by their phosphorylation status. For example, Bcl-2 is phosphorylated at Ser^{70} and this phosphorylation enhances its anti-apoptotic activity. However, Bcl-2 can be hyper-phosphorylated by drugs such as Taxol at the Thr^{69} and Ser^{87} within the flexible loop region and this level of phosphorylation can inhibit its function and enhance its proteolysis via the proteasome [13–15]. Bcl-X_L function is also regulated via its phosphorylation status [16] and Akt/PI3-K-dependent phosphorylation of Bad on Ser^{112}, Ser^{136} and Ser^{155} promotes its association with 14-3-3 protein in the cytoplasm, thereby releasing it from Bcl-X_L. After this release it can then heterodimerise with pro-apoptotic proteins to promote cell survival [17–22].

Mcl-1 has several potential phosphorylation sites and it has also been shown in Hek292 cells that Mcl-1 can be phosphorylated on Ser^{121} and Thr^{163} via a JNK-dependent pathway in response to oxidative stress [23]. In the Burkitts lymphoma cell line BL41-3, Mcl-1 is phosphorylated at two levels: phorbol esters induce a phosphorylation via Erk that does not result in a band shift, whereas Taxol and oxadaic acid can induce Mcl-1 hyper-phosphorylation that does result in a band shift on SDS-PAGE [24].

In addition to these posttranslational changes in Bcl-2 family proteins, alternative splicing can apparently greatly increase the diversity of proteins that can be expressed from the Bcl-2 family genes. In some instances the alternatively spliced products have altered sub-cellular locations, whereas in others profound and sometimes antagonising changes in function arise from alternative splicing.

Alternative splicing as a way to proteomic diversity: mechanisms and significance

At the start of the Human Genome Sequencing Project, it was widely anticipated that the human genome would contain around a hundred thousand genes. However, as the project neared completion, it gradually became obvious that only 30000–40000 genes would be identified [25]. As there are many more expressed sequence tags (EST-s) and proteins than there are genes, many mechanisms must exist to increase proteome diversity from the available number of genes.

Alternative splicing of primary transcripts is now widely accepted to constitute the major mechanism for generating protein diversity from the genome of many species, including humans [26]. There are numerous examples in eukaryotes, where a particular pre-mRNA can give rise to more than one type of mature mRNA via alternative splicing. A recent genome-wide analysis has indicated that alternative splice forms exist for about half of the human genes and these have distinct biological functions [26]. The splicing of pre-mRNA requires accurate removal of introns and rejoining of exons to form a mature mRNA. Essential, in this process, is that consensus sequences in exon-intron junctions are recognised and bound by trans-acting splicing factors constituting a splicesome. Whilst exon-intron boundaries are critical in defining the splicing sites, other proximal enhancer and inhibitor sequence elements can also contribute to the splicing process [27].

Expression of proteins with distinct biological functions from a single gene may be important for generating tissue-specific isoforms or for developmental stage-specific isoforms. Thus, in these instances, isoforms arise not from gene diversity but via alternative splicing. Physiological factors and environmental stimuli may be involved in determining which splicing variant is needed. Multiplication of function from a single genetic unit through alternative splicing can potentially take place via one or more mechanisms as depicted in figure 1. Several examples of genes whose expression is controlled by these alternative splicing patterns have been reported [27–29]. The following section summarises the processes and consequences of alternative splicing seen in the expression of *Bcl-2* family genes, and the distinct biological functions of these splicing variants.

Alternative splicing in *Bcl-2*-related genes and modulation of protein function

Alternative splicing of a primary transcript is one of the major mechanisms in the production of proteomic diversity of higher organisms and many genes are expressed as multiple splice variants ranging in number from two to several thousand [29, 30]. Thus, whilst the sequencing of the human genome has been a major breakthrough in understanding gene structure and genetic regulation, major challenges now exist to determine how this genetic complexity can be further increased by alternative splicing mechanisms.

The discovery of multiple splice variants of almost every member of the Bcl-2 family adds a further layer of complexity to the control of apoptosis. Table 1 summarises the alternative splice products of the *Bcl-2*-related genes that have been reported. Bcl-2, the founding member of the family, has been reported to have two isoforms produced by alternative splicing [31]. These two splicing variants, designated as α and β, can potentially encode two proteins that differ only in their carboxyl tails. The longer protein, Bcl-2α, contains a hydrophobic trans-

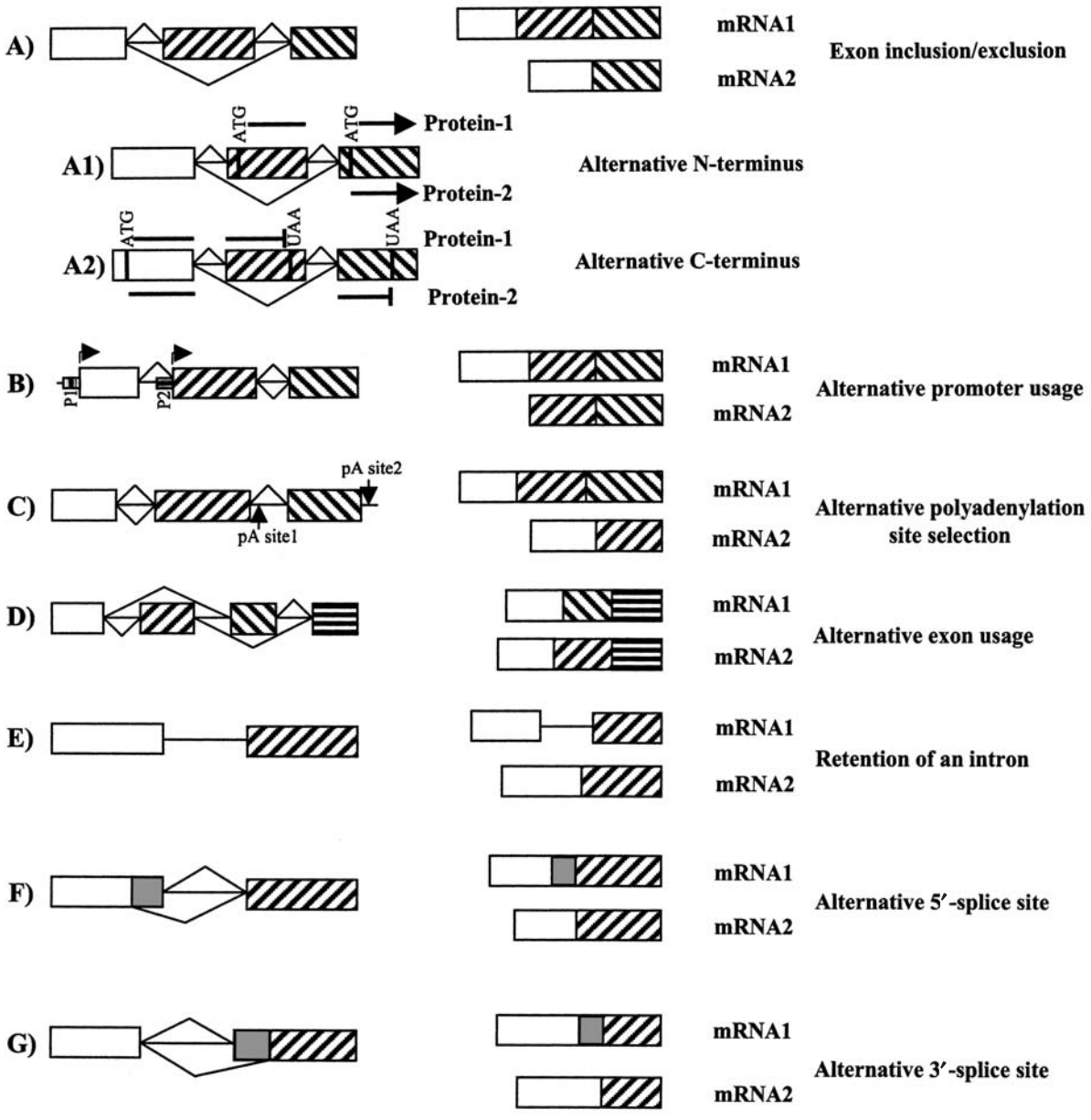

Figure 1. Potential alternative splicing mechanisms. (*A*) Inclusion or exclusion of an exon could provide inclusion or exclusion of a functional domain or alternative translation initiation/termination sites. (*B, C*) Alternative promoters or polyadenylation site (pA) selection could change the exon arrangements and produce proteins with distinct N or C termini. (*D*) Alternative combinations of exons could be employed to encode two protein isoforms. (*E*) An intron could be retained in the mature mRNA and used in translation. (*F, G*) Use of alternative 5′ or 3′-splice sites could change downstream reading frames or include/exclude functional residues [26, 27, 30].

membrane domain within its C terminus, thereby targeting it to mainly the mitochondrial membrane, and this form functions to prolong cell survival. In contrast, Bcl-2β that contains no transmembrane domain resides predominantly in the cytosol and fails to prolong either cell survival or apoptosis under the experimental conditions examined [32].

Three alternative splicing variants of the *Bcl-X* gene are known [33, 34]. Bcl-X_L, the best-characterised family member, contains all four BH domains plus a C-terminal transmembrane domain, which is responsible for its mitochondrial localisation. This isoform has an anti-apoptotic function. The carboxy-terminal 63 amino acids encoding BH1 and BH2 domains are deleted by use of an alternative 5′ splice site in the first coding exon to produce the shorter splicing variant, Bcl-X_S (figs. 1F, 2A). In contrast to the anti-apoptotic activity of Bcl-X_L, Bcl-X_S antagonises cell survival and induces apoptosis [33]. Bcl-Xβ is the least-studied isoform of Bcl-X and results from an unspliced first coding exon that introduces a new stop

Table 1. General characteristics of the alternative splice variants expressed from *Bcl-2*-related genes.

Gene	Alternative splice Variants[a]	Amino acid residues	Protein size (kDa)[b]	BH, TM domains[c]	Protein activity[d]	Subcellular localisation[e]	References[f]
Bcl-2	Bcl-2α	239	26	BH1, 2, 3, 4, TM	AA	mitochondria	31, 32
	Bcl-2β	205	22	BH1, 2, 3, 4	?	cytoplasm	31, 32
Bcl-X	Bcl-X_L	233	31	BH1, 2, 3, 4, TM	AA	mitochondria	33
	Bcl-X_S	170	19	BH3, 4, TM	PA	mitochondria	33
	Bcl-Xβ	227	26	BH1, 2, 3, 4, TM(?)	NS	NS	34
Mcl-1	Mcl-1_L	350	38	BH1, 2, 3, TM	AA	mitochondria	35, 36, 37
	Mcl-1_S	271	30–35	BH3	PA	NS	38, 39
Bfl-1	Bfl-1_L	175	20	BH1, 2, 3, 4, TM	AA	mitochondria	40, 41
	Bfl-1_S	163	19	BH1, 2, 3, 4	AA	nucleus	42
Bax	Baxα	192	21	BH1, 2, 3, TM	PA	mitochondria	4, 47
	Baxβ	218	24	BH1, 2, 3	PA	cytosol	4
	Baxγ	41	5	–	NS	NS	4
	Baxδ	143	16	BH1, 2, TM	NS	NS	45
	Baxε	164	18	BH1, 3	PA	NS	46
	Baxω	221	24	BH1, 2, 3	PA	NS	44
	Baxσ	179	20	BH1, 2, 3, TM	PA	mitochondria	43
	Baxψ	172	19	BH1, 2, 3, TM	PA	mitochondria	82
Bim	Bim_L	140	19	BH3, TM	PA	mitochondria	48
	Bim_S	110	15	BH3, TM	PA	mitochondria	48
	Bim_{EL}	196	23	BH3, TM	PA	mitochondria	48
	Bimα_1	169	19	BH3	PA	mitochondria	49
	Bimα_2	109	12	BH3	PA	mitochondria	49
	Bimβ_1	135	14	–	?	cytosol	49
	Bimβ_2	135	14	–	?	cytosol	49
	Bimβ_3	75	8	–	?	cytosol	49
	Bimβ_4	45	5	–	?	cytosol	49
	Bimγ	112	15	BH3	PA	intracellular	50
PUMA	PUMA-α	193	25	BH3	PA	mitochondria	53
	PUMA-β	131	16	BH3	PA	mitochondria	53
Bcl-G	Bcl-G_L	327	37	BH2, 3	PA	cytosol	54
	Bcl-G_S	252	28	BH3	PA	mitochondria	54
Bcl-rambo	Bcl-rambo	485	85	BH1, 2, 3, 4, TM	PA	mitochondria	55
	Bcl-ramboβ	104	12	BH4	PA	cytosol	56
Bid	Bid_L	195	22	BH3B, BH3	PA	cytosol	52
	Bid_S	137	15	BH3B, *	AA	cytosol	52
	Bid_{ES}	99	11	–	AA/PA	mitochondria	52
	Bid_{EL}	242	27	BH3B, BH3, #	PA	golgi/mitochondria	52

[a] The table only includes human Bcl-2 family members with multiple splice variants. Whilst multiple alternative splicing products for Bad, Bod, Bak and Bok have been identified in rat and mouse tissues [63–66], their human homologues have not been reported.
[b] Given protein sizes are either predicted or determined by SDS-PAGE.
[c] Bcl-2 homology domains (BH) and C-terminal hydrophobic transmembrane domains (TM) in each splice variant are indicated. * Distinct C terminus, # distinct N terminus.
[d] Proteins have either anti-apoptotic (AA) or pro-apoptotic (PA) functions when they expressed in various cell lines. ? Indicates that studies have been done to determine activities of splice isoforms, but no clear function was assigned to these isoforms.
[e] Given subcellular compartments are the main sites in which the proteins are located using various visualisation techniques. However, other minor intracellular localisations have been reported in indicated references[f]. Protein translocations upon external stimuli as in the case of Bax, Bid, etc. should also be taken into consideration [47, 52]. NS, These isoforms have been described genetically but their activities and subcellular localisations have not been studied.

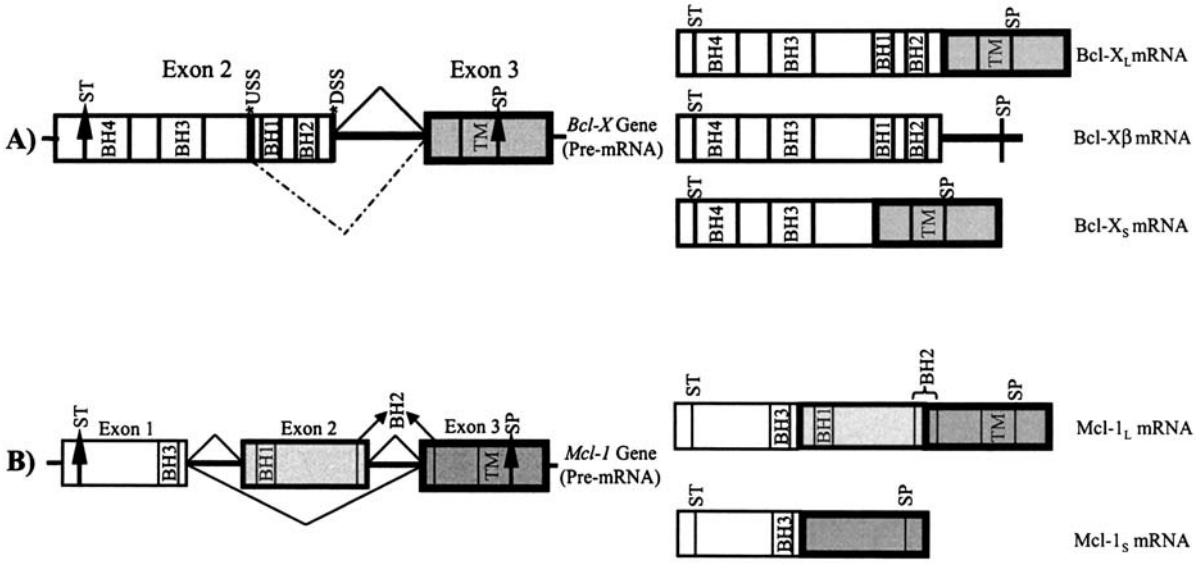

Figure 2. Alternative splicing mechanisms of Bcl-X and Mcl-1 producing protein isoforms with opposing functions. Rectangles represent exons, straight lines between exons represent introns. ST; start codon, SP; stop codon. Regions encoding the homology domains of the proteins are shown on the genes and on mRNA sequences. The pictures are not drawn to scale. (*A*) Bcl-X is alternatively spliced to produce three distinct mRNAs encoding three protein isoforms. The Bcl-X gene has a three-exon structure and two 5′-alternative splice sites in the first coding exon. When the upstream 5′-splice site (USS) is used, Bcl-X_S mRNA is produced. However, the use of the downstream site (DSS) produces Bcl-X_L mRNA without changing the downstream reading frame. When the intron between two coding exons is not spliced, it yields an mRNA for Bcl-Xβ [33, 34]. (*B*) The full-length Mcl-1 (Mcl-1_L) is encoded by three coding exons. A short splice variant (Mcl-1_S) results from the skipping of the second coding exon and joining of the first and third exons by a process shifting the downstream reading frame. Since the second and in-frame third exons encode the BH1, BH2 and TM domains, only the BH3 domain remains intact in Mcl-1_S, making it a pro-apoptotic BH3-only protein antagonising the anti-apoptotic functions of Mcl-1_L [35–39].

codon before the third exon, as depicted in figure 1E and figure 2A [34]. Whilst a stretch of 18 highly hydrophobic residues in its C-terminal region resembles the membrane anchor region found in Bcl-2α and Bcl-X_L, its function in the apoptosis machinery of the cell has not been defined clearly.

Mcl-1, an anti-apoptotic Bcl-2 family member, was first identified as an early induction gene during myeloblastic leukaemia cell differentiation [35], shares sequence homology with Bcl-2 in its BH1, 2 and 3 domains and possesses a C-terminal membrane anchor domain that targets it to mitochondria [36]. Mcl-1 pre-mRNA contains three coding exons and two introns [37]. Whilst full-length Mcl-1 is encoded by all three coding exons, a short splicing variant (Mcl-1_S) of Mcl-1 with an altered C terminus results from the joining of the first and third coding exons (figs. 1A, 2B) [38, 39]. Since the skipping of the second exon shifts the downstream reading frame, BH1, BH2 and the C-terminal membrane anchor domains are deleted, whilst the BH3 domain encoded by the first exon remains intact. This BH3-only protein has the characteristics of other BH3-only proteins and functions to induce apoptosis by dimerising specifically with the long form of Mcl-1 (Mcl-1_L) [38, 39]. Relative amounts of these two isoforms with opposing functions are regulated by alternative splicing and suggest this process could decide cell fate in Mcl-1-expressing cells [38].

Bfl-1 (A1) possesses pro-survival activity and has a tissue-specific expression pattern, contains all BH domains and a membrane anchor domain [40]. Whilst Bfl-1 colocalizes, at least in part, with the mitochondria and functions as a pro-survival factor, its splicing variant, Bfl-1_S, localizes to the nucleus via its distinct C terminus created by alternative splicing [41, 42]. Bfl-1_S like Bfl-1 acts as an anti-apoptotic protein when coexpressed with Bax. However, the functional relevance of these two isoforms requires further investigation.

Bax was the first pro-apoptotic Bcl-2 homologue to be identified and it was discovered by virtue of its coimmunoprecipitation with Bcl-2 [4]. Expression analysis showed that it is widely expressed in various tissues as multiple splice variants [4, 43–46]. Baxα is the best-characterised isoform, contains BH1, 2, and 3 and membrane anchor domains and colocalizes with mitochondria upon stimulation of apoptosis [47]. Baxβ lacks the transmembrane region, localises in the cytoplasm, but still induces apoptosis [4]. Baxγ is a very short protein of 4.5 kDa without any BH domains or membrane anchor domain, and its functional significance remains unknown [4]. Although the structural properties of these and other Bax splice variants have been defined (table 1), their functional properties are still unclear and we do not know if they regulate the apoptosis machinery in different ways [43–46].

A screen of a cDNA expression library with a Bcl-2 protein probe identified a novel BH3-containing protein, Bim [48]. At least ten alternative splice variants of Bim have been reported so far and activity-guided studies have shown that they all promote apoptosis, albeit with differing potency [48–51]. Bim_L, Bim_S and Bim_{EL} all contain membrane anchor domains in addition to the BH3 domains, and they colocalise mainly with mitochondria [48]. Whilst the BH3 domain is necessary for induction of apoptosis in all isoforms with known pro-apoptotic activity, this domain is also required for mitochondrial localisation of Bimα_1 and Bimα_2 and Bimγ [48–50]. Four β isoforms without any BH domains are found in the cytosol and they are neither pro- nor anti-apoptotic [49].

Bid is a 22-kDa BH3-only protein that is cleaved by caspase-8 to release its C-terminal region yielding a truncated form (tBid) that translocates to the mitochondria and interacts with other Bcl-2 family proteins to promote cytochrome c release. Recently, a number of novel Bid isoforms have been identified that arise from alternative splicing [52]. These have been termed: Bid_{EL}, which corresponds to full-length Bid plus an additional N-terminal sequence; Bid_S, which contains the N-terminal regulatory domains of Bid minus the BH3 domain; Bid_{ES}, which contains only the Bid sequence downstream of the BH3 domain. These isoforms have different functions in apoptosis, and appear distinct in their patterns of expression and cellular localization [52] as summarised in table 1.

PUMA is another BH3-only protein and is expressed as two alternative splice variants termed PUMA-α and PUMA-β. Both are associated with mitochondria and induce apoptosis by binding to Bcl-2 thereby mediating p53-induced cell death [53]. Bcl-G_L and Bcl-G_S are two pro-apoptotic isoforms produced via alternative splicing [54]. The long isoform is expressed widely in adult tissues whereas the short form is expressed only in the testis. Whilst they both contain BH3-death domains, only the long form Bcl-G_L possesses a BH2 domain. Bcl-G_S partially localises to the mitochondrial membranes whereas Bcl-G_L is diffusely distributed throughout the cytoplasm [54]. Bcl-rambo and Bcl-ramboβ are two alternative splice variants expressed from the *Bcl-rambo* gene. Unlike Bcl-rambo, the beta isoform lacks the BH1, 2, and 3 and membrane anchor domain and becomes the first BH4-only protein to be identified in the family [55, 56]. The beta isoform is located in the cytosol of a variety of tissues such as heart, lymph nodes and cervix, but is absent from brain. Both isoforms induce apoptosis despite the differences in their structural elements and subcellular localisations [55, 56].

Whilst the primary transcripts of Bcl-2 family members are conserved across many species, their splice variants have also been identified in different organisms. Bcl-2α, Bcl-X_L, Bcl-X_S, Mcl-1_L and Bid_L are commonly expressed in a variety of tissues of many species, but other splice variants so far only identified in certain tissues are: Bcl-2β in mouse and chicken [57, 58]; Bcl-XΔTM in mouse [59]; Bcl-Xβ in rat and mouse [60, 61]; Bcl-Xγ in mouse [61]. Mcl-1_S and three minor Bid splice variants have, so far, only been identified in human tissues [38, 39, 52]. Baxα is the major transcript expressed in many species, and whilst Baxβ, Baxγ, Baxδ, Baxε, Baxω, Baxσ and Baxψ have been identified in human tissues, Baxκ has been identified in rat tissues [62]. Bcl-rambo, PUMA, Bcl-G transcripts and the splice variants shown in table 1 have only been reported in human tisssues.

In addition to human alternative splice variants summarised in table 1, splice variants of some other Bcl-2 family members have been reported in other species. N-Bak is an isoform of Bak generated by alternative splicing in mouse brain [63] and N-Bak transcripts are expressed in neurons but are absent in non-neuronal cells. When over-expressed in neuronal cells, N-Bak is anti-apoptotic, while it is pro-apoptotic when over-expressed in other cell types. Neuron-specific expression of N-Bak is conserved in human, mouse and rat species [63]. Two splice variants of the rat pro-apoptotic protein Bad differ in their carboxy-terminal regions and both interact with the anti-apoptotic molecule Bcl-w [64]. Bok-S is a splicing variant of Bok that has a truncated BH3 domain, and has been identified in reproductive tissues of rat. The truncated version was shown to retain its pro-apoptotic activity but has lost the ability to dimerise with anti-apoptotic proteins in vitro [65]. Three splicing variants of pro-apoptotic BOD (BOD-L, BOD-M, BOD-S) containing only the BH3 domains have been identified in rats and were shown to interact with anti-apoptotic Bcl-2 proteins [66]. Sequence comparisons indicated that rat BOD-L and BOD-M are the orthologues of the Bim splicing variants Bim-EL and Bim-S, respectively [48, 65].

Since the molecular pathways that direct the alternative splicing of these genes are largely undefined, the individual components involved and the physiological conditions that favour the expression of specific splicing variants are not well characterised. A much more defined understanding of the function of each splice variant and of the factors that regulate their expression is necessary to exploit this proteomic diversity originating from *Bcl-2*-related genes for the treatment of disease. The factors that regulate the differentiation specificity of tissue specificity of these variants also needs to be determined. This knowledge is accumulating fast and a great impetus already exists to find new ways to correct defective cell suicide programmes by interfering with alternative splicing mechanisms to treat a number of diseases, including cancer.

Directed alternative splicing of the Bcl-2 family genes and future perspectives

Cancers and neurodegenerative disorders often show an impaired cell death program [28], the former arising from too little apoptosis and the latter arising from too much cell death. About 15% of genetic diseases are believed to result from mutations in splice sites [67] and many cancer-related genes can give rise to alternatively spliced variants [28]. Whilst the determination of the functions of each splice variant is one of the major challenges facing molecular cell biologists in the postgenomic era, a shift in the relative ratios of specific splice variants of the same gene clearly may lead to some forms of cancer. For example, Bcl-X_L has been reported to be expressed predominantly in many lymphomas [68] whilst Bcl-X_S is down-regulated in transformed cells. Therefore, elucidation of mechanisms regulating the splicing events might provide new therapeutic approaches to manipulate the splicing mechanisms in favour of a particular splicing variant [28, 69–72].

The Bcl-2 family of proteins are involved in many types of cancer. Whilst the Bcl-X_L splicing variant has an anti-apoptotic function and is over-expressed in several common types of cancer, Bcl-X_S is a pro-apoptotic protein and antagonises the survival functions of Bcl-X_L [73]. The Bcl-X_L/Bcl-X_S ratio can be quite high in vivo. However, several physiological conditions and external stimuli can alter this ratio by modulating the splicing machinery [74]. Therefore, increasing the ratio of Bcl-X_S/Bcl-X_L would not only lower cell resistance to chemotherapy, it would also sensitise the cells to undergo apoptosis [28, 69–71].

One way to disrupt expression of specific alternatively spliced transcripts is via RNAi. This can be achieved via antisense oligodeoxynucleotides that are targeted to specific sequences on mRNA molecules to create DNA-RNA heteroduplexes, thereby providing a substrate for RNAse H. Chemically modified and improved design of antisense oligodeoxynucleotides has recently been shown capable of regulating alternative splicing mechanisms by binding to target pre-mRNA in a sequence-specific fashion, sterically hindering specific splice sites and redirecting the splicing machinery to other available splice sites without inducing an RNAase H attack [69–71]. This technology has already been applied to regulate the alternative splicing of some *Bcl-2*-related genes. For example, chemically modified antisense oligonucleotides targeted to the downstream 5′ splice site in exon 2 of the *Bcl-X* gene have resulted in a shift in expression from the anti-apoptotic Bcl-X_L towards the pro-apoptotic splicing variant, Bcl-X_S, in human cancer cell lines [69–71]. Antisense-mediated manipulation of the Bcl-X splicing mechanisms in this way has resulted in increased sensitivity to chemotherapeutic agents [70]. Cells that express higher levels of Bcl-X_L are more sensitive to antisense-induced cell death and cells predominantly expressing other pro-survival proteins elicited a much weaker apoptotic response, indicating the specificity of the antisense molecules [69, 70]. Antisense-mediated regulation of splicing machinery to knockdown expression of a particular alternatively spliced variant, may be preferable to approaches that block expression of all transcripts from a *Bcl-2* family gene. For example, specific antisense down-regulation of *Bcl-X_L* mRNA to decrease expression of only the anti-apoptotic protein, without altering the expression of the pro-apoptotic variant (Bcl-X_S) can amplify any apoptotic effects of the treatment on a cell. This therapeutic strategy to modulate the splicing patterns of *Bcl-X* expression to favour specific isoforms can be applied to other disease-related genes and may find clinical relevance in the future, since many diseases result from alternatively spliced variants of these genes [67]. As shown in table 1, alternative splicing can alter not only the biological properties of the encoded protein, but also its sub-cellular location. Such localisation experiments of splice variants have generally been performed following transfection of exogenous genes. However, where these have been checked, endogenous proteins usually localize to the same compartment as the exogenous proteins. Furthermore, dimerisation and antagonism among splice variants are of physiological significance.

Mcl-1, like Bcl-X, is expressed as two alternatively spliced variants that have opposing functions in apoptosis [25, 26]. Of interest therefore, would be to determine if the splicing machinery could be manipulated to enhance expression of the pro-apoptotic isoforms to promote apoptosis in those cells in which Mcl-1 over-expression generates an apoptosis-resistant phenotype [75–79]. The *Bcl-2* gene can potentially encode two proteins that differ only in their carboxyl tails because of alternative splicing [31, 32]. Whilst the longer protein contains a hydrophobic transmembrane domain targeting it to mitochondrial membrane and prolongs cell survival, the shorter protein, Bcl-2β, mainly resides in the cytosol and has very little or no anti-apoptotic activity [32]. Over-expression of the α isoform is found in many types of cancers and appears to contribute to resistance to chemotherapy [80]. Antisense oligodeoxynucleotides targeted to the coding region of the *Bcl-2* gene have been developed to inhibit Bcl-2α expression and tumour progression [81]. G-3139 (Genasense), which is an antisense Bcl-2 oligonucleotide developed by Genta, has already been tested in a variety of clinical trials alone or in combination with classical chemotherapeutics and is showing promising results [81]. However, antisense manipulation of the Bcl-2 alternative splicing mechanisms in favour of the β isoform requires better characterisation of the processes regulating splicing before this approach can be used to treat cancers.

Better understanding of the molecular processes regulating alternative splicing of Bcl-2 family members could therefore provide subtle fine tuning of the therapeutic control of diseases in which impaired apoptosis underlies pathology.

Whilst antisense technology has been demonstrated to be effective in manipulating the alternative splicing mechanisms, its use is limited by a number of technical difficulties. For example, unspecific binding to non-target sites, inefficient cellular uptake and inaccessibility of specific target sites because of secondary structures or RNA-binding proteins are the main disadvantages to the use of antisense molecules as therapeutics. Alternative strategies, in addition to improving existing antisense technologies, will be needed to therapeutically control the expression of these genes in favour of a desired splicing variant for use in vivo. Antisense RNA molecules encoded from viral vectors have recently been used to redirect the splicing mechanisms of the β-globin gene [72]. Whilst antisense RNA molecules and oligodeoxynucleotides used in these studies serve similar purposes, the former have the advantage of providing a long-term gene therapy when antisense RNA molecules are expressed from viral expression vectors or when the constructs can be incorporated into the host genome.

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