Post-transcriptional and post-translational regulation of Bcl2

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Abstract
Bcl2 is an important pro-survival protein that has an essential function in normal immunity and whose constitutive expression leads to the development of lymphomas. Although transcriptional control of Bcl2 has been reported, increasing evidence suggests an important component of Bcl2 regulation is post-transcriptional. Phosphorylation of Bcl2 has been shown to enhance activity to allow response to extracellular growth-factor-mediated signals. Bcl2 mRNA contains regulatory elements in both its 5′- and 3′-UTRs (untranslated regions). An IRES (internal ribosome entry sequence) in the 5′-UTR permits continued translation in the presence of cellular stresses that reduce cap-dependent translation. The 3′-UTR of Bcl2 mRNA is 5.2 kb in length and contains multiple predicted miRNA (microRNA) and RNA-BP (RNA-binding protein)-binding sites. mir-15a and mir-16-1 have been found to inhibit Bcl2 expression in B-cells, whereas the RNA-BP nucleolin has been shown to increase Bcl2 expression by binding to the 3′-UTR and enhancing mRNA stability. Both decreased expression of mir-15a and mir-16-1 and increased nucleolin have been shown to be associated with increased Bcl2 expression and resistance to apoptosis in the common human disease, chronic lymphocytic leukaemia. miRNA-based therapeutic approaches to treat cancer are emerging. Bcl2 is highly regulated by miRNAs and is therefore an excellent candidate for such approaches.

Introduction
Bcl2 and its related family members play a central role in the regulation of apoptosis, a form of cell death that is required for normal development and which is defective in many malignant diseases. Apoptosis can be recognized by several morphological features, which include chromatin condensation and fragmentation, cell membrane blebbing and the formation of apoptotic bodies.

The morphological features of apoptosis are the result of activation of cysteine proteases (caspases), which can be activated by one of two complementary pathways. The extrinsic pathway is activated by trimerization of cell-surface ‘death receptors’, members of the TNF (tumour necrosis factor) family, which leads to recruitment of the adaptor protein FADD (Fas-associated death domain) and activation of caspase 8 (and caspase 10 in humans). The intrinsic pathway (also termed the ‘mitochondrial’ pathway) is initiated by release of cytochrome c from the mitochondria to cause activation of caspase 9 on the scaffold protein Apaf-1 (apoptotic protease-activating factor 1). Caspases 8, 9 and 10 (called the initiator caspsases) activate the effector caspsases (caspases 3, 6 and 7) that, in turn, mediate apoptosis.

Bcl2 family proteins contain both pro-survival and pro-apoptotic members and their function is to regulate mitochondrial integrity. The five major pro-survival proteins, Bcl2, Bcl2A1, Bcl-XL, Mcl1 and Bcl-w, consist of BH (Bcl2 homology) domains BH1–BH4. Together, these domains form a globular structure with a surface hydrophobic groove into which a further subset of the Bcl2 family, BH3-only proteins, bind and neutralize the pro-survival function of the pro-survival proteins. In turn, this is believed to lead to oligomerization of the pro-apoptotic proteins Bax and Bak on the outer mitochondrial membrane and the consequent loss of mitochondrial membrane integrity.

Bcl2 is the archetypal pro-survival protein (Figure 1a). It was discovered by cloning of chromosomal translocations from cases of the human B-cell non-Hodgkin’s lymphoma, follicular lymphoma [1,2]. Early work showed that Bcl2 conferred growth-factor-independent growth on cell lines [3] and that constitutive expression of Bcl2 in mice inhibited apoptosis in T-cells and produced prolonged antibody responses [4,5]. Mice bearing an Eμ-Bcl-2-myc transgene develop B-cell tumours [6], providing an experimental demonstration that Bcl2 overexpression is sufficient for the development of malignant disease.

Bcl2 expression levels vary widely during the course of normal B-cell development: Bcl2 is highly expressed in naive B-cells, down-regulated in germinal centre B-cells (where Bcl-XL becomes the major pro-survival protein) and then re-expressed on exit from the germinal centre. Bcl2 is therefore expressed in a lineage- and temporal-specific manner. Although transcriptional control of Bcl2 in B-cells
Bcl2 and the Bcl2 family pro-survival proteins

(a) Modular structure of Bcl2 protein. BH1, BH2, BH3 and BH4 domains are shown together with the FLD, which is the site of phosphorylation. TM, transmembrane domain. (b) mRNAs of the five major Bcl2 family pro-survival proteins drawn to scale. The 5′-cap site is shown together with the 5′-UTR, coding region, 3′-UTR and poly(A) tail. The lengths of the 5′- and 3′-UTRs are indicated, together with the presence or absence of a proven IRES site. Bcl2 and Bcl-xL, which have IRES sites, have longer 5′-UTRs than the other proteins. Bcl2 has a 3′-UTR of 5.2 kb, whereas that of Bcl2A1 is <0.2 kb. AREs (found using sequence data in [51]) are indicated by arrowheads superimposed on the 3′-UTRs.

has been reported [6], others [7–9] have found expression of Bcl2 mRNA in normal germinal centre B-cells from which Bcl2 protein is absent, suggesting an important component of post-transcriptional control.

The purpose of the present review is to outline the current state of knowledge and recent advances in the area of post-transcriptional regulation of Bcl2, and to discuss whether the post-transcriptional mechanisms that have been highlighted may be appropriate targets for future cancer therapies.

Post-translational modification by phosphorylation

Phosphorylation of Bcl2 enhances cell survival, whereas dephosphorylation leads to apoptotic induction [10,11]. The phosphorylation of Bcl2 appears to be dynamically regulated during the cell cycle, and both protein kinases and phosphatases have been identified as regulating Bcl2 activity. Phosphorylation of Bcl2 on Ser70 stabilizes Bcl2, inhibits Bcl2 degradation and blocks p53 binding [12,13]. Ser70 is located within an FLD (flexible loop domain) of Bcl2, which is a sequence of approx. 50 amino acids between the BH3 and BH4 domains at the N-terminus of Bcl2 (Figure 1a). Several growth-factor-activated protein kinases can accomplish phosphorylation of the FLD of Bcl2, and include MAPK (mitogen-activated protein kinase), ERK1/2 (extracellular-signal-regulated kinase 1/2), JNK1 (c-Jun N-terminal protein kinase 1) and PKC (protein kinase C) [12,14,15]. Multisite phosphorylation of Bcl2 at Ser70, Thr69 and Ser87, has also been reported; however, the effect of this on survival have not been clearly defined [10,11]. A more recent suggestion is that phosphorylated Bcl2 may exist for lengthy periods after microtubule damage and represent a pre-apoptotic phase, and that subsequent dephosphorylation initiates apoptosis [16]. Protein phosphatase 2A has been shown to dephosphorylate Bcl2, which causes enhanced p53 binding and reduced pro-survival ability [17].

Control of translation by an IRES (internal ribosome entry sequence)

Translation of mRNA requires assembly of a protein complex at the 5′-cap site to allow binding to the ribosome. The formation of the translation initiation complex requires, in most cases, signalling through Akt and mTOR (mammalian target of rapamycin) [18], which results in phosphorylation of 4E-BP [eIF (eukaryotic initiation factor) 4E-binding protein]. When phosphorylated, 4E-BP dissociates from the cap-binding complex eIF4E, allowing formation of the heterotrimeric cap binding complex of which the other components are a scaffold protein (eIF4G) and a DEAD (Asp-Glu-Ala-Asp) box RNA helicase (eIF4A) [19]. The cap-binding complex recruits the 40S ribosomal subunit and ultimately allows translation initiation [20].

An alternative mechanism of translation initiation involves recruitment of the 40S ribosomal subunit directly to a structured cis-element located in the 5′-UTR (untranslated region) of an mRNA, called an IRES [21,22]. During apoptosis, there is a large reduction in the overall rate of protein synthesis [23–25], and this is due to caspase cleavage of proteins of the cap complex and increased mRNA degradation [26–28]. However, protein can continue to be produced from mRNAs that contain an IRES element. IRES activity is controlled by ITAFs (IRES trans-activating factors), that bind to the IRES to allow translation under specific conditions such as DNA damage or apoptosis [29]. IRESs in different mRNAs have a common secondary structure, but do not share a common primary base pair sequence.

The Bcl2 IRES was discovered using bicistronic reporter assays, and was confirmed by transfecting Bcl2 mRNA directly into cells [30]. The authors went on to show that apoptosis induced by chemotherapeutic agents caused a 3–6-fold induction of IRES activity. It therefore appears that cells have a mechanism for maintaining production of Bcl2 despite the onset of apoptosis. IRES-mediated translation may represent a means by which apoptosis can be delayed or by which the cell can be rescued if the apoptotic stress is removed. Of the other Bcl2 family pro-survival proteins, Bcl-xL has also been proved to contain an IRES [31], but Bcl2A1, Mc1 and Bcl-w are unlikely to possess this element because they all have short 5′-UTRs.
Nucleolin, an RNA-BP (RNA-binding protein), binds to an ARE (AU-rich element) in the 3′-UTR of Bcl2 to enhance mRNA stability

AREs are typically found in mRNAs that encode proteins regulating either cell growth or the response of an organism to external factors such as micro-organisms, inflammatory stimuli and environmental factors. AREs are targeted by ARE-binding proteins and these trigger rapid mRNA degradation by initiating exonucleolytic decay of the poly(A) tail [32]. Nucleolin is a multifunctional protein that is a member of the RNP (ribonucleoprotein)-containing family of RNA-BPs. Recent studies in HL60 cells have identified nucleolin as a Bcl2 mRNA-stabilizing protein [33,34]. Nucleolin binds specifically to the ARE-1 instability element in the 3′-UTR of Bcl2 mRNA and inhibits ribonuclease degradation [35,36]. Nucleolin overexpression increases Bcl2 mRNA stability, and nucleolin knockdown reduces Bcl2 mRNA and protein levels. Nucleolin is overexpressed in the common B-cell lymphoproliferative disorder CLL (chronic lymphocytic leukaemia).

Knock down of nucleolin by antisense oligonucleotides may reduce Bcl2 levels in leukaemic cells. However, one problem might be that the role of nucleolin in global mRNA regulation in different cell types is not known. One of the characteristics of nucleolin overexpression in CLL is that it is predominantly expressed in the cytoplasm. Thus one strategy to exploit this would be to specifically inhibit cytoplasmic nucleolin [36]. AS1411, a DNA aptamer, i.e. an oligomer with a specific three-dimensional structure, bound with high affinity to nucleolin and is currently entering clinical trials for acute myeloid leukaemia [36].

miR-15a and miR-16-1 inhibit Bcl2 translation

miRNAs (microRNAs) are, on average, 22-nt-long oligonucleotides of which approx. 700 are encoded in the human genome. In metazoans, miRNAs regulate specific sets of mRNAs by binding to seed sequences in their 3′-UTRs and enhancing degradation or preventing translation [37]. The chromosomal deletion 13q14.3 occurs in more than 50% of CLL cases, and is also common in myeloma, mantle cell lymphoma and prostate cancer. miR-15a and miR-16-1 have been mapped to this chromosomal region, thus suggesting that their deletion may have a role in CLL development. Deletion of miR-15a and miR16-1 in mice results in a lymphoproliferative disease similar to human CLL, thus providing strong evidence that deletion of these miRNAs is a cause of CLL [38,39]. Potential miR-15a and miR-16-1 mRNA targets have been compiled from studies involving cell lines, human cancer cells, mice [40] and bioinformatics [41,42], and a large number of mRNAs have been found to be targets of miR-15a and miR-16-1, including the cell cycle-related genes CCND1 (encoding cyclin D1) and WNT3A [42] and Bcl2 [41]. In CLL cells, Bcl2 expression is 2–5-fold higher than in normal B-cells, and this correlates with significantly reduced levels of miR-15a and miR-16-1 compared with normal counterparts [41]. Overexpression of a genomic fragment containing miR-15a and miR-16-1 in a leukaemic cell line expressing Bcl2 [41] significantly reduced Bcl2 expression, thus providing direct proof that miR-15a and miR-16-1 regulate Bcl2 expression. Reporter assays in which fragments of the 3′-UTR of Bcl2 were cloned downstream of a luciferase gene demonstrated that the effects of these miRNA was through direct interaction with Bcl2 mRNA.

miRNAs can act as tumour suppressors or oncopgenes. miR-15a and miR-16-1 which are down-regulated in CLL appear to be tumour suppressors. There is interest in using another type of non-coding RNA, siRNAs (small interfering RNAs), as well as miRNA as the basis for therapies. Inhibition of miRNA expression can be achieved with antisense oligonucleotides (antagomirs) or by inhibiting the processing of immature forms [43,44]. Other strategies include the use of miRNA sponges [45] and miRNA ‘masks’, i.e. morpholino oligonucleotides to protect miRNA-binding sites in mRNA [46]. Alternatively, restoring miRNA expression can be achieved by targeting miRNA mimics to cancer cells. However, to be an effective strategy, efficient targeted delivery systems need to be developed [47]. These may include viral vectors or exosomes [48].

Conclusions

Bcl2 is a critical survival gene controlling the intrinsic mitochondrial pathway of apoptosis. Post-transcriptional mechanisms of regulation allow for rapid changes in activity in response to changes in the extra- or intra-cellular environments. Bcl2 can be phosphorylated by kinases that are themselves activated by signals from growth factor receptors. Bcl2 protein expression can also be regulated by its mRNA 3′-UTR. The RNA-BP nucleolin slows mRNA decay, whereas expression of miR-15a and miR-16-1 is inversely correlated with Bcl2 protein expression. Interestingly, in the common leukaemia CLL, increased amounts of nucleolin and decreased expression of miRNA operate in a co-ordinated way to produce levels of Bcl2 higher than those in normal B-cells. Finally, Bcl2 and another pro-survival protein, Bcl-XL, are two examples of a small group of mammalian proteins that have been proved to have a functional IRES site in their 5′-UTRs. The function of these IRES sites is not clear, but they may represent a mechanism to maintain protein production in the face of an apoptotic environment that reduces cap-dependent translation.

There are five major pro-survival Bcl2 family members, and it is believed that they all act similarly to maintain mitochondrial integrity. They have different transcriptional controls, but it is striking that they have very different 5′- and 3′-UTRs (Figure 1b). Bcl2 and Bcl-XL have longer 5′-UTRs than the other pro-survival proteins, which is consistent with their containing IRES sites. There are also very different 3′-UTRs. Bcl2A1 has a 3′-UTR of 200 bp, whereas that of
Bcl2 is over 5 kb, with the other three members having 3'-UTRs of intermediate length. This suggests that each of these proteins is regulated uniquely in order to protect the outer mitochondrial membrane from diverse threats.

**Future perspectives**

Bcl2 is overexpressed in CLL and is a target for chemotherapy in other malignancies, including lung cancer. Rationally designed small-molecule inhibitors (ABT-737 and ABT-263, Abbott Laboratories) [49] and antisense oligonucleotides against Bcl2 (Oblimersen, Genta) [50] have proven to be highly effective at killing cancer cells in vitro. Oblimersen has shown some efficacy in Phase III clinical trials, and ABT-263 is currently being trialled. Novel strategies to inhibit Bcl2 expression in cancer would complement the current range of agents. Bcl2 is heavily post-transcriptionally regulated through multiple mechanisms, suggesting targets for future therapies.

miRNA-based therapeutic strategies involve the selective transfection of cancer cells with miRNA mimics or antagonists to block translation of functionally important proteins. Bcl2 mRNA has a 5.2 kb 3'-UTR and multiple predicted miRNA-binding sites, making it an excellent candidate target gene for future miRNA-based chemotherapies. The effectiveness of an miRNA-based chemotherapeutic strategy against Bcl2 will rely heavily on finding miRNAs that are highly effective at blocking Bcl2 mRNA translation, and finding novel ways to specifically target miRNA mimics and antagonists to cancer cells. New miRNAs against Bcl2 will undoubtedly be discovered in the near future, and recent technical advances suggest that targeting miRNAs to cancer cells is possible. Thus an miRNA-based approach to block Bcl2 expression in cancer cells may be an effective therapeutic strategy.

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**References**


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