BCL-2 REGULATES PROAPOPTOTIC CALCIUM SIGNALS BY INTERACTING WITH THE INOSITOL 1, 4, 5- TRISPHOSPHATE RECEPTOR

by

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LIST OF ABBREVIATIONS

IP3R: Inositol 1, 4, 5-trisphosphate receptor

IP3: Inositol 1, 4, 5-trisphosphate

ER: Endoplasmic reticulum

BH domain: Bcl-2 homology domain

FRET: Förster resonance energy transfer

TCR: T cell receptor

BCR: B cell receptor

NFAT: Nuclear factor of activated T-cells

IL: Interleukin

PP1α: Protein phosphatase 1 α

TAT: Trans-activating transcriptional activator

VDAC: Voltage-dependent anion channel

CLL: Chronic lymphocytic leukemia
Bcl-2 Regulates Proapoptotic Calcium Signals by Interacting with the Inositol 1, 4, 5-Trisphosphate Receptor

Abstract

by

Yiping Rong

Bcl-2 is the founding member of a large family of apoptosis regulating proteins. The antiapoptotic protein Bcl-2 inhibits Ca\(^{2+}\) release from the endoplasmic reticulum (ER). One proposed mechanism involves an interaction of Bcl-2 with the inositol 1, 4, 5-trisphosphate receptor (IP3R) Ca\(^{2+}\) channel localized with Bcl-2 on the ER. Here we document Bcl-2-IP3R interaction within cells by FRET and identify a Bcl-2 interacting region in the regulatory and coupling domain of the IP3R. A peptide (peptide 2) based on this IP3R sequence displaced Bcl-2 from the IP3R and reversed Bcl-2-mediated inhibition of IP3R channel activity in vitro, IP3-induced ER Ca\(^{2+}\) release in permeabilized cells, and cell permeable IP3 ester-induced Ca\(^{2+}\) elevation in intact cells. This peptide also reversed Bcl-2's inhibition of T cell receptor-induced Ca\(^{2+}\) elevation and apoptosis. Furthermore, peptide 2 enhances ABT-737-induced cell death in chronic lymphocytic leukemia cells. The interaction of Bcl-2 with IP3R's contributes to the regulation of proapoptotic Ca\(^{2+}\) signals by Bcl-2. We also investigated the region of Bcl-2 responsible for interaction with the IP3R. Based on results of coimmunoprecipitation and
GST pull-down experiments the BH4 domain of Bcl-2 is necessary for interaction with the IP3R. A synthetic peptide corresponding to the BH4 domain of Bcl-2 interacts with the same IP3R domain as full length Bcl-2. TAT-BH4, formed by fusing a peptide corresponding to the BH4 domain of Bcl-2 with the protein transduction domain of HIV TAT, enters cells and functions like full length Bcl-2, to inhibit cytoplasmic Ca\textsuperscript{2+} elevation and apoptosis induced by T cell receptor (TCR) activation. Two experimental findings establish that these actions of TAT-BH4 are mediated through interaction with the IP3R. First, TAT-BH4 inhibits Ca\textsuperscript{2+} elevation induced by a cell permeant IP3 ester. Second, peptide 2 that blocks Bcl-2-IP3R interaction reverses the inhibitory effect of TAT-BH4 on both Ca\textsuperscript{2+} elevation and apoptosis following TCR activation. In summary, these findings indicate that the BH4 domain of Bcl-2 is both necessary and sufficient for interaction with the IP3R. The Bcl-2-IP3R interaction is a potential therapeutic target in diseases associated with Bcl-2's inhibition of cell death.
CHAPTER 1: AN INTRODUCTION TO APOPTOSIS, CALCIUM AND THE REGULATION OF IP3-MEDIATED CALCIUM AND APOPTOSIS BY BCL-2

Part of this Chapter was previously published in:

The Ca²⁺ ion: versatile mediator of life and death signals

Bcl-2 is the founding member of a large family of proteins that either promote or inhibit apoptosis (reviewed in [1, 2]). The family is typically divided into three groups according to structural homology and function. One group includes the anti-apoptotic proteins Bcl-2, Bcl-xL and Mcl-1; a second group includes the pro-apoptotic proteins Bax and Bak; the third group, also pro-apoptotic, encompasses the BH3-only proteins, including Bim, Bad and Bak. A common theme among virtually all members of this family is the ability to regulate the permeability of intracellular membranes to ions and proteins (reviewed in [3]). Well recognized is the enhancement of cytochrome c release from mitochondria by the pro-apoptotic family members Bax and Bak, and inhibition of cytochrome c release by anti-apoptotic family members Bcl-2 and Bcl-xL. The regulatory influence of Bcl-2 family members on cytochrome c release is of undoubted importance because of the central role of cytochrome c in mediating caspase activation during apoptosis. Not as widely recognized, but also of considerable importance, is the role of the endoplasmic reticulum (ER) in apoptosis and specifically of calcium (Ca²⁺) release from the ER and its regulation by Bcl-2 and other Bcl-2 family members in determining cell fate [4].

The concept that Bcl-2 represses apoptosis by inhibiting Ca²⁺ release from the ER originated over a decade ago [5, 6]. Through efforts of many investigators, it is now evident that anti-apoptotic and pro-apoptotic Bcl-2 family members have opposing effects on the Ca²⁺ permeability of both ER and mitochondrial membranes. This review summarizes evidence that pro-apoptotic Bcl-2 family members mediate apoptosis by releasing Ca²⁺ from the ER, and also by enhancing its uptake by mitochondria, whereas the anti-apoptotic family members have evolved the capacity to discriminate between
pro-apoptotic $\text{Ca}^{2+}$ signals and pro-survival $\text{Ca}^{2+}$ signals, inhibiting the former but enhancing the latter.

**Ca$^{2+}$ release from ER and Ca$^{2+}$ signaling**

Ca$^{2+}$ released into the cytoplasm from the ER functions as a second messenger that regulates many processes, including life and death decisions [7-10]. The ER is the major organelle involved in intracellular Ca$^{2+}$ homeostasis and signaling. Ca$^{2+}$ is sequestered in the ER through the action of SERCA (sarcoplasmic endoplasmic reticulum Ca$^{2+}$-ATPases) pumps located in the ER membrane, thus maintaining ER luminal Ca$^{2+}$ concentration at levels much higher than in the surrounding cytoplasm. Ca$^{2+}$ signals are generated when Ca$^{2+}$ is released from the ER via ER membrane-associated inositol 1,4,5-trisphosphate (IP3)-gated channels, or IP3Rs [11, 12]. These Ca$^{2+}$ release channels, of which there are three subtypes, play a central role in Ca$^{2+}$ signaling in cells and are therefore regulated by a number of different accessory factors and by post-translational modifications, including phosphorylation [12-15]. The cytoplasmic Ca$^{2+}$ elevation may be transient, sustained, or a more complex pattern of either repetitive spikes (oscillations) or waves [16]. Many physiological processes are mediated by Ca$^{2+}$ oscillations, in which information is encoded by the frequency and amplitude of repetitive spikes and this information is transmitted to a wide range of downstream effectors, including Ca$^{2+}$-sensitive phosphatases and kinases.

**Ca$^{2+}$ signals and cell fate determination**

The versatility of Ca$^{2+}$ as a second messenger and the manner in which information is encoded by different patterns of Ca$^{2+}$ elevation is illustrated by the responses of immature T cells to T cell receptor (TCR) activation [17-20]. Immature T cells, developing in the
cortex of the thymus gland, make the decision to live (positive selection) or die (negative selection) based on the strength of TCR activation. The former is in response to weak TCR activation by self-antigens presented in the thymus, while the latter is induced by strong TCR activation, also by self-antigens. Ca\textsuperscript{2+} signals mediate both processes [21] and distinct patterns of Ca\textsuperscript{2+} elevation are associated with each [22, 23]. As illustrated in Figure 1.1, weak TCR activation induces Ca\textsuperscript{2+} oscillations, whereas strong TCR activation induces sustained Ca\textsuperscript{2+} elevation. The former optimally activates NFAT (nuclear factor of activated T cells) and thereby up-regulates expression of the pro-survival cytokine interleukin-2, whereas the latter up-regulates the pro-apoptotic BH3-only protein Bim, thereby mediating apoptosis. Thus, Ca\textsuperscript{2+} signals can play dual roles in response to the same stimulus and in the same cells depending on the pattern and duration of Ca\textsuperscript{2+} elevation. This is just one example of the versatile role that Ca\textsuperscript{2+} plays in mediating both apoptosis and survival. Others are summarized in the next two sections.

**Role of IP3Rs in generating pro-apoptotic Ca\textsuperscript{2+} signals**

Ca\textsuperscript{2+} elevation mediates apoptosis induction by a wide range of initiating stimuli, summarized in Figure 1.2. Cytoplasmic Ca\textsuperscript{2+} elevation triggers many pathways to apoptosis, including activation of proteases and endonucleases, as summarized in earlier reviews [24-27]. Understanding that cytoplasmic Ca\textsuperscript{2+} elevation induces apoptosis evolved following the seminal findings of Kaiser & Edelman [28] indicating that Ca\textsuperscript{2+} elevation mediates the death of immature lymphocytes in response to glucocorticosteroid hormones. A critical role for Ca\textsuperscript{2+} elevation in glucocorticoid-induced apoptosis is now well established [29-33] and accompanied by evidence that the Ca\textsuperscript{2+} elevation is initiated by release of Ca\textsuperscript{2+} from the ER and sustained by increased extracellular Ca\textsuperscript{2+} entry [31,
Moreover, Ca\textsuperscript{2+} signals generated by ER Ca\textsuperscript{2+} release induce apoptosis in response to a broad range of initiating stimuli, including growth factor withdrawal, the Ca\textsuperscript{2+}-ATPase inhibitor thapsigargin, hydrogen peroxide, staurosporine, ceramide, tumor necrosis factor and genotoxic stress [5, 6, 34-43].

In many cases, pro-apoptotic Ca\textsuperscript{2+} elevation is generated by Ca\textsuperscript{2+} efflux from the ER via IP3Rs [27]. Studies of the role of Ca\textsuperscript{2+} signaling in lymphocyte cell death have led the way to this understanding. Antisense-mediated repression of IP3R expression inhibited glucocorticosteroid-induced apoptosis in lymphocytes [44, 45]. Also, antisense-mediated knockdown of IP3Rs in Jurkat T cells inhibited apoptosis following TCR activation [45]. Similarly, genetic elimination of all three IP3R subtypes in chicken DT40 B lymphocytes inhibited apoptosis induction following B cell receptor activation (BCR) [46]. IP3R-mediated Ca\textsuperscript{2+} elevation is also implicated in mediating apoptosis during development and in response to neurotoxic damage. Elevated levels of IP3R mRNA and protein are detected in tissues known to have high rates of apoptosis, including developing postnatal cerebellar granule cells, dorsal root ganglia, embryonic hair follicles, and intestinal villi [47]. Moreover, elevated IP3R levels are associated with neurotoxic damage elicited by the glutamate agonist kainite and in chick dorsal root ganglia neurons undergoing apoptosis following nerve growth factor deprivation. Antisense-mediated IP3R repression inhibited apoptosis in each of these situations. Recently, supraphysiological concentrations of testosterone were found to induce sustained, IP3-mediated elevation of cytoplasmic Ca\textsuperscript{2+}, triggering apoptosis in neuronal cells [48].
IP3Rs also function to enhance as well as to initiate apoptotic signals (Figure 1.2). For example, cytochrome c released from mitochondria during the early stages of apoptosis binds to IP3Rs and potentiates IP3-mediated Ca\textsuperscript{2+} release, forming a positive feedback loop that enhances the mitochondrial phase of apoptosis [49]. Also, the IP3R sequence includes a consensus site for cleavage by the apoptosis-associated protease caspase-3 [50, 51]. The cleaved form of IP3R exhibits increased leakiness, thereby enhancing Ca\textsuperscript{2+} release from the ER during apoptosis.

The importance of IP3Rs in cell death induction is further illustrated by evidence that the N-terminal 1-225 amino acids of the Type 1 IP3R function as a suppressor domain to attenuate IP3-induced Ca\textsuperscript{2+} release and thereby reduce sensitivity to apoptosis [52]. Because of the capacity of IP3-receptor-mediated Ca\textsuperscript{2+} signals to induce apoptosis, cells have evolved a number of ways to modulate IP3R activity. For example, ERp44, a luminal chaperone, directly interacts with the third luminal loop of IP3Rs to sense pH, redox status, and Ca\textsuperscript{2+} concentration within the ER lumen and modulate IP3R activity accordingly, thereby protecting against apoptosis [53] (Figure 1.2).

Consistent with the central importance of mitochondria in apoptosis [54], considerable attention has been given to apoptosis induction by redistribution of Ca\textsuperscript{2+} from ER to mitochondria. The close proximity of the ER to mitochondria facilitates transfer of Ca\textsuperscript{2+} from one organelle to the other [16, 24, 55, 56] (Figure 1.2). Thus, mitochondria play an important role in Ca\textsuperscript{2+} homeostasis and signaling by buffering cytoplasmic Ca\textsuperscript{2+} elevation and modulating Ca\textsuperscript{2+} signals. Also, Ca\textsuperscript{2+} uptake in mitochondria activates oxidative
metabolism, generating high concentrations of ATP and favoring cell survival [56]. But IP3-mediated Ca\(^{2+}\) release can also sufficiently elevate mitochondrial Ca\(^{2+}\) concentration to induce apoptosis (reviewed in [26, 27, 57, 58]. Opening of IP3Rs on the ER can expose the mitochondrial Ca\(^{2+}\) uniporter to twenty-fold higher Ca\(^{2+}\) concentrations than elsewhere in the cytoplasm. Thus, mitochondrial Ca\(^{2+}\) signals evoked by IP3-mediated Ca\(^{2+}\) elevation trigger mitochondrial permeability transition and, in turn, cytochrome c release [56, 58, 59]. Importantly, the permeability transition pore reseals after the Ca\(^{2+}\) signal decays, providing an efficient mechanism to establish caspase activation, while mitochondrial metabolism is maintained to provide ATP necessary for the apoptotic process.

ER-mitochondria Ca\(^{2+}\) crosstalk mediates apoptosis in response to a variety of initiating factors (Figure 1.2). One of the earliest recognized examples is the redistribution of Ca\(^{2+}\) from ER to mitochondria induced by interleukin-3 (IL-3) withdrawal from IL-3-dependent cells [5]. Additional examples include mitochondrial Ca\(^{2+}\) waves initiated by IP3-mediated Ca\(^{2+}\) signals in cardiac myotubes exposed to ceramide and staurosporine [60], the increased mitochondrial Ca\(^{2+}\) uptake implicated in staurosporine-induced neural cell apoptosis [38], and the mitochondrial Ca\(^{2+}\) elevation induced by staurosporine and ATP in epithelial cells [61]. Recent findings by Mendes et al [62] indicate that one particular subtype of IP3R, type 3, preferentially transmits Ca\(^{2+}\) to mitochondria, thereby mediating apoptosis. Another mechanism involves cleavage of the integral ER membrane protein Bap31 by caspase-8 following engagement of cell death receptors (e.g., Fas) [42]. The p20 caspase cleavage fragment of Bap31 induces Ca\(^{2+}\) release from the ER, concomitant with increased Ca\(^{2+}\) uptake by mitochondria. As a consequence, the
Dynamin-related protein Drp1 is recruited to mitochondria, mediating scission of the outer mitochondrial membrane and cytochrome c release.

**Role of IP3Rs in generating pro-survival Ca\(^{2+}\) signals**

Ca\(^{2+}\) as a second messenger contributes to many vital cellular processes including fertilization, cell division, secretion, and neurotransmission [8]. Ca\(^{2+}\) signals positively regulate mitochondrial bioenergetics, thereby directly contributing to cell survival [63]. Ca\(^{2+}\) signals induced by TCR activation, BCR activation and ATP favor cell survival (Figure 1.2). As discussed above, both survival (positive selection) and death (negative selection) are mediated by Ca\(^{2+}\) signals in immature T cells [21] and distinctive patterns of cytoplasmic Ca\(^{2+}\) elevation appear to mediate these processes [22, 23] (Figure 1.1). Ca\(^{2+}\) oscillations optimally maintain activation of the protein phosphatase calcineurin, which in turn dephosphorylates and thereby activates the transcription factor NFAT. Thus, Ca\(^{2+}\) oscillations signal T cell proliferation and survival through activation of NFAT, which increases transcription of the pro-survival cytokine interleukin-2 (IL-2). TCR-mediated pro-survival Ca\(^{2+}\) signals also up-regulate expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL, via activation of the Ca\(^{2+}\)-sensitive transcription factors NFAT and CREB [64-68]. The anti-apoptotic protein, Bcl-xL, in turn enhances responses of IP3Rs to low concentrations of IP3, thereby enhancing Ca\(^{2+}\) oscillations triggered by BCR activation and so favoring cell survival [69].
IP3R and Bcl-2

*IP3R structure and function*

IP3Rs are intracellular ligand-gated ion channels that allow Ca\(^{2+}\) release from intracellular stores such as ER. IP3 is synthesized upon G-protein coupled receptor and receptor tyrosine kinase activation [70]. Upon receptor ligand binding, phospholipase C enzymes are activated and the phosphatidylinositol-4, 5-bisphosphate (PIP2) is hydrolyzed to generate IP3 and 1, 2-diacylglycerol (DAG). IP3 diffuses through the cytosol and binds to the IP3R, which is mainly located on the ER membrane. The IP3R channel opens and releases Ca\(^{2+}\) into the cytosol from ER. Three subtypes of IP3R have been identified with different expression levels throughout the body. The functional differences among these three types of IP3R are still not clear [27, 71, 72]. In this thesis, if not indicated, IP3R means the Type I IP3R which is used in most of experiments. The IP3R has four subunits, each of around 2700 amino acids (Figure 1.3). The C-terminal region of each subunit has six transmembrane segments, which interact to form the channel region. Each subunit has a large cytoplasmic domain containing a variety of regulatory sites. The IP3 binding site is close to the N terminus, and there is a long regulatory region between the IP3 binding region and the channel [12, 70]. Receptors can form homotetramers or heterotetramers. IP3R binds a number of proteins, thereby placing the IP3R at the key point in cellular signaling.

The primary channels for Ca\(^{2+}\) release from ER are IP3Rs and ryanodine receptors. Also some other proteins, including SERCA and GRP78/94, contribute to Ca\(^{2+}\) homeostasis in the ER [9]. It has become apparent that apoptosis can be positively or negatively affected
by changes of \( \text{Ca}^{2+} \) concentration in ER [8, 73]. Cellular \( \text{Ca}^{2+} \) overload or alterations of intracellular \( \text{Ca}^{2+} \) stores may lead to cytotoxic stress and trigger cell death. The amplitude, frequency and spatial properties of \( \text{Ca}^{2+} \) elevations regulate diverse cellular processes. In T cell activation, IP3R mediated \( \text{Ca}^{2+} \) release plays important roles in cell proliferation and apoptosis.

IP3R activity can be regulated by many kinases and phosphatases. IP3R-1 channel conductivity is regulated through phosphorylation at a lot of sites by protein kinase A (PKA), protein kinase C (PKC), protein kinase G (PKG), Fyn, and calcium/calmodulin-dependent protein kinase II, among others [70]. Meanwhile some phosphatases, calcineurin (PP2B) and protein phosphatase 1α (PP1α) can dephosphorylate IP3R [74-76]. The kinases and phosphatases regulate \( \text{Ca}^{2+} \) release from the ER by balancing the IP3R phosphorylation and dephosphorylation status. Calcineurin is a highly conserved, \( \text{Ca}^{2+}/\text{calmodulin-dependent serine/threonine phosphatase. Calcineurin anchored to the IP3R regulates the phosphorylation status of the receptor, resulting in a Ca}^{2+}\)-sensitive regulation of IP3-mediated \( \text{Ca}^{2+} \) flux [77, 78]. Both IP3R and Bcl-2 can interact with calcineurin. Direct interactions between Bcl-2 and calcineurin have been reported in BHK-21 cells transfected with both proteins [79, 80]. Recent immunoprecipitation experiments showed that in cortical and hippocampal slices, calcineurin interacted with Bcl-2 as well as IP3R1 and shuttled between these two substrates [81]. PP1α also can interact with IP3R and dephosphorylate IP3R [75, 76]. It was reported that Bcl-2 interacts with PP1α and targets it to Bad [82]. Fyn has also been reported in the regulation of IP3R mediated \( \text{Ca}^{2+} \) release. Fyn is a tyrosine kinase which is important in T cell activation.
During T cell activation Fyn interacts with and phosphorylates IP3R1 in T lymphocytes. Fyn-mediated tyrosine phosphorylation increases IP3R open probability by reducing Ca^{2+}-dependent inhibition of the channel [83, 84]. T cells from Fyn knock-out mice showed reduced Ca^{2+} release and reduced IP3R1 tyrosine phosphorylation in response to TCR ligation [84]. Since Bcl-2 can inhibit TCR induced Ca^{2+} release in WEHI7.2 cells in our work, it is worthwhile to investigate the effect of Bcl-2 on Fyn-IP3R interaction and phosphorylation.

The Bcl-2 family

Bcl-2 is an anti-apoptotic protein which has 239 amino acids and the founding member of a large family of both antiapoptotic and proapoptotic proteins [1, 2]. It is an integral membrane protein with a wide tissue distribution. Bcl-2 acts to inhibit apoptosis under a wide variety of circumstances. It was first identified through genetic analysis of B cell lymphomas two decades ago and is a major target of novel therapeutic approaches for cancer and other diseases [85-87]. In the most common type of human lymphoma, the region of chromosome 18 encoding Bcl-2 translocates to chromosome 14, downstream of the antibody heavy chain enhancer. Thus, in these t (14; 18) positive cells, Bcl-2 expression is driven by the IgH enhancer [1, 88] (Figure 1.4). Bcl-2 is elevated in many cancers, including breast cancer, colon cancer, prostate cancer, small cell lung cancer, chronic lymphocytic leukemia and low-grade lymphomas. The t (14:18) translocation is the mechanism only in lymphomas and a variety of mechanisms contribute to Bcl-2 dysregulation and overexpression in other types of cancer [86, 89]. The Bcl-2 protein exerts its oncogenic effects at least in part by inhibiting apoptosis. Impaired apoptosis is a
crucial step in tumorigenesis, neoplastic progression, metastasis and chemotherapy resistance.

The Bcl-2 family is generally divided into two categories (Figure 1.5): pro-survival Bcl-2 proteins including Bcl-2, Bcl-xL, A1 and Mcl1; and proapoptotic Bcl-2 proteins including Bax/Bak, Bim, Bad, Bik and Puma [1, 88]. The Bcl-2 family members in these two categories can interact each other, for example, Bcl-2/Bcl-xL can interact with Bim, thereby inhibiting Bim activation induced apoptosis. The balance between pro-survival and proapoptotic Bcl-2 family proteins is a major factor in determining whether or not cells undergo apoptosis in response to cell stress. Disorders of the apoptotic machinery can result in either undesirable cell accumulation as in cancer, or a loss of cells as seen in neurodegenerative, autoimmune and cardiovascular diseases [90]. Modulation of apoptosis has become a novel therapeutic concept. As one of the major apoptosis regulators, Bcl-2 has attracted considerable interest on the part of those involved in developing innovative therapies for cancer [87, 91]. These efforts have mainly targeted the inhibitory interaction of Bcl-2 with proapoptotic members of the Bcl-2 protein family, with the goal of disrupting this interaction and thereby abrogating the antiapoptotic action of Bcl-2. Except inhibiting apoptosis by interacting with proapoptotic Bcl-2 relatives, Bcl-2 also interacts with other apoptosis regulators.

Structure of Bcl-2 proteins

Based on sequence alignment Bcl-2 family members share considerable similarity in regions known as Bcl-2 homology domains (BH domains) [92, 93], shown in Figure 1.5. The prototypical antiapoptotic family members Bcl-2, Bcl-xL, and Bcl-w have four BH
domains (BH1-BH4), but Bax and Bak only have BH1, BH2 and BH3 domains. A third group that includes for example Bim, Bad and Bik, only have a BH3 domain and thus are referred to as BH3-only proteins.

The first X-ray and NMR structure of a Bcl-2 family protein to be determined was that of Bcl-xL [94]. The three dimensional structure of Bcl-2 was subsequently determined by NMR spectroscopy of Bcl-2/Bcl-xL chimeras in which part of the putative unstructured loop of Bcl-2, located between the BH4 and BH3 domains, was replaced with a shortened loop from Bcl-xL (Figure 1.6) [95]. The N-terminus of Bcl-xL contains an amphipathic α-helical BH4 domain and is located on the protein surface. This domain forms extensive hydrophobic interactions with the α2, α5 and α6 helices located in the region of the BH1, 2 and 3 domains [94]. The C-terminus of Bcl-2 contains a hydrophobic transmembrane region that inserts into intracellular membranes, including mitochondria and endoplasmic reticulum (ER), with the bulk of the Bcl-2 protein oriented on the cytoplasmic face of these organelles. The BH1 and BH2 domains of Bcl-2 are critical for heterodimerization with Bax and important for Bcl-2’s pro-survival function [92]. The BH1, BH2 and BH3 domains form a hydrophobic cleft on both the antiapoptotic Bcl-2/Bcl-xL proteins and proapoptotic proteins, Bax and Bak. This binding pocket on the antiapoptotic proteins Bcl-2 and Bcl-xL can be occupied by the α-helix of interacting BH3-only proteins, such as Bim and Bad. Through this mechanism Bcl-2/Bcl-xL sequester the BH3-only proteins, thereby preventing them from activating full-length proapoptotic proteins including Bax and Bak, although an alternative theory is that the prosurvival function of Bcl-2/Bcl-xL is neutralized by the BH3-only proteins.
The antiapoptotic function of Bcl-2

One of the characteristics of Bcl-2 family proteins is their ability to form heterodimers or homodimers, which contributes to the neutralizing competition between antiapoptotic and proapoptotic members. The detailed mechanisms accounting for Bcl-2’s antiapoptotic action are not fully understood and consequently there are many hypotheses. As already noted above, considerable attention has focused on the direct interactions between various Bcl-2 family members. It is generally held that through this process, the antiapoptotic proteins Bcl-2 and Bcl-xL prevent Bax and Bak from forming pores in the outer mitochondrial membrane that release factors such as cytochrome c [96, 97]. These factors, in turn, activate caspases, proteases that function to dismantle the cell during apoptosis. Also, Bcl-2 and Bcl-xL appear to sequester proapoptotic BH3-only proteins such as Bim and Bad, preventing them from conveying a death signal. Thus, the major current working models of apoptosis control are based on Bcl-2 family members’ mutual regulation [98-100]. Many Bcl-2 inhibitors intended for therapeutic application target these interactions, as discussed in Chapter 4.

Although the role of Bcl-2 family members in regulating mitochondrial membrane permeability have been generally emphasized in the apoptosis literature, the roles of Bcl-2 family members on the endoplasmic reticulum have received increasing attention in recent years, mainly focused on the role of Bcl-2 family members in regulating $\text{Ca}^{2+}$ release from ER. This $\text{Ca}^{2+}$ release elevates cytoplasmic $\text{Ca}^{2+}$, producing $\text{Ca}^{2+}$ signals that govern many cellular processes, including proliferation, development, cell cycle and
apoptosis. The Ca\(^{2+}\) ion is a versatile second messenger that regulates life and death signals \([8, 101]\). It induces apoptosis either by direct effects on mitochondria or indirectly by activating or inducing other proapoptotic proteins, including Bim, Bad, proteases and endonucleases \([24, 25, 102]\). Bcl-2 represses apoptosis by inhibiting \(\text{Ca}^{2+}\) release from ER. Two proposed mechanisms for how Bcl-2 regulates ER calcium, brought out in the past decade, are the inhibition of IP3R channel opening and the reduction of ER luminal calcium concentration (reviewed by \([73, 103]\). Recent studies indicate that Bcl-2 and Bcl-xL interact with the IP3R calcium channel on ER membrane and regulate its activity, thereby inhibiting proapoptotic sustained \(\text{Ca}^{2+}\) elevation without interfering with prosurvival \(\text{Ca}^{2+}\) oscillations \([23, 69, 104-106]\). Much of this work has been performed in T cells, in which T cell receptor activation (e.g., by antibody to the CD3 component of the T cell receptor complex) can trigger apoptosis, mediated in part through IP3-mediated \(\text{Ca}^{2+}\) elevation. Intriguingly, a Bcl-2 mutant (G145A) which does not form heterodimers with proapoptotic members of the Bcl-2 family, such as Bax, still protects T cells from anti-CD3-induced apoptosis \([107]\), suggesting that Bcl-2 inhibits this form of apoptosis by a mechanism other than that involving interactions between Bcl-2 family members. One candidate mechanism is the regulation of IP3-mediated \(\text{Ca}^{2+}\) elevation, based on our evidence that Bcl-2 inhibits anti-CD3-induced apoptosis by inhibiting IP3R-mediated \(\text{Ca}^{2+}\) release from the ER in T cells \([23, 104]\). Thus, Bcl-2 can inhibit apoptosis independent of, or in addition to, its association with Bax or other proapoptotic Bcl-2 family members in those situations where IP3-mediated \(\text{Ca}^{2+}\) elevation contributes to apoptosis induction.
Although most recent reports emphasize the interaction of Bcl-2 with other family members, Bcl-2 has been reported to also interact with a number of other proteins with the potential of regulating apoptosis, including protein phosphatase 1α, calcineurin, NF-κB, c-myc, FKBP38, Raf-1, NALP1, and Nur77/TR3 [82, 108-113]. Thus, a network of protein-protein interactions may contribute to Bcl-2’s antiapoptotic function in cells.

**Regulation of Ca^{2+} signals by Bcl-2 family members**

Anti-apoptotic Bcl-2 family members positively regulate pro-survival Ca^{2+} signals and negatively regulate pro-apoptotic Ca^{2+} signals, whereas pro-apoptotic Bcl-2 family members enhance pro-apoptotic Ca^{2+} signals (Figure 1.7). These counter-regulatory mechanisms are summarized here.

*Enhancement of pro-apoptotic Ca^{2+} signals by Bax, Bak and BH3-only proteins*

Whereas anti-apoptotic Bcl-2 family members inhibit ER Ca^{2+} release, pro-apoptotic family members enhance ER Ca^{2+} release and increase Ca^{2+} uptake by mitochondria (Figure 1.7). The pro-apoptotic family members Bax and Bak enhance ER-mitochondrial Ca^{2+} crosstalk. Nutt et al [114] discovered that Bax or Bak, when overexpressed in prostate cancer cells, localize to the ER membrane and induce Ca^{2+} redistribution from the ER to mitochondria, triggering cytochrome c release and the downstream biochemical events associated with apoptosis. Significantly, Bcl-2 inhibited both the Ca^{2+} redistribution and apoptosis. These investigators demonstrated the involvement of Bax-mediated ER-mitochondrial Ca^{2+} redistribution during apoptosis induction by staurosporine and doxorubicin [115]. Moreover, staurosporine-induced Ca^{2+} redistribution was prevented in Bax-deficient cancer cells, and restored by enforced Bax
expression. Also, ER stress induces a conformational change in Bak, resulting in oligomerization of Bak on the ER, as well as on mitochondria [116]. Thus, Bak acts at the ER to progressively deplete ER Ca^{2+} and to activate ER-associated caspase-12. Finally, recent findings by Carvalho et al [117] indicate that Bax, when microinjected into astrocytes, induces Ca^{2+} release from both mitochondria and the ER, evoking Ca^{2+} waves and wave propagation between cells.

The BH3-only members of the Bcl-2 family also play an active role in mediating ER-mitochondrial Ca^{2+} crosstalk (Figure 1.7). Genotoxic stress (e.g., radiation, doxorubicin) and p53 overexpression increase expression of the BH3-only protein Bik [43]. Bik is located on the ER and in response to stress releases Ca^{2+} from the ER, mediated through a Bax/Bak dependent process. The Ca^{2+} released by Bik also activates the dynamin-related GTPase DRP1, which is involved in p53-induced mitochondrial fission and release of cytochrome c to the cytosol. Therefore, endogenous Bik regulates a Ca^{2+}-mediated, Bax/Bak-dependent pathway that contributes to mitochondrial cytochrome c release and apoptosis. Whereas the BH3-only protein Bik promotes apoptosis by enhancing Ca^{2+} release from the ER, the activated BH3-only Bcl-2 family member Bid promotes apoptosis by increasing mitochondrial Ca^{2+} uptake [118]. The mechanism involves selective permeabilization of the outer mitochondrial membrane by Bid, thus increasing Ca^{2+} uptake sites on the inner mitochondrial membrane to locally high Ca^{2+} concentrations.

Although this discussion has emphasized the role of BH3-only proteins in transmitting Ca^{2+} signals from ER to mitochondria, Ca^{2+} signals also regulate the expression and activity of BH3-only proteins. For example, TCR activation mediates negative selection
in immature thymocytes by up-regulating expression of Bim [119] and L-glutamate-induced Ca\textsuperscript{2+} elevation in hippocampal neurons activates Bad via calcineurin-mediated dephosphorylation [120].

**Enhancement of pro-survival Ca\textsuperscript{2+} signals by Bcl-2 and Bcl-xL**

Bcl-2 not only supports cell survival by inhibiting pro-apoptotic Ca\textsuperscript{2+} signals, as described below, but also supports cell survival by enhancing Ca\textsuperscript{2+} signals that positively regulate cell proliferation and survival. In T cells, Bcl-2 enhances pro-survival Ca\textsuperscript{2+} oscillations induced by weak TCR activation [23]. Moreover, Bcl-xL increases the proportion of T cells that mount a Ca\textsuperscript{2+} oscillatory response to weak TCR activation, favoring NFAT activation and cell survival (Zhong, F. and Distelhorst, C., unpublished observations). The potential breadth of involvement of Bcl-2 in positive regulation of Ca\textsuperscript{2+} signals is illustrated by the findings of Jiao et al [121], indicating that Bcl-2 enhances Ca\textsuperscript{2+} signals that mediate neurite outgrowth and survival. In chicken DT40 B cells, Bcl-xL (and Bcl-2) enhanced Ca\textsuperscript{2+} oscillations induced by BCR activation, contributing to cell survival through enhanced mitochondrial bioenergetics [69]. The mechanism appears to involve an increased responsiveness of IP3Rs to low concentrations of IP3. Consistent with these findings, an earlier study demonstrated that ATP induces higher frequency Ca\textsuperscript{2+} oscillations in MCF-7 cells over-expressing Bcl-2 than in control cells [122].

**Inhibition of pro-apoptotic Ca\textsuperscript{2+} signals by Bcl-2**

The regulation of ER Ca\textsuperscript{2+} release by Bcl-2 was recognized over a decade ago, but not until recent years has the mechanism begun to yield to investigation. Bcl-2 inhibits ER Ca\textsuperscript{2+} release and cytoplasmic Ca\textsuperscript{2+} elevation involved in apoptosis induction by hydrogen
peroxide, staurosporine, ceramide, tumor necrosis factor and genotoxic stress [35-43] (Figure 1.7). Also, Bcl-2 inhibits the cytoplasmic Ca\textsuperscript{2+} elevation and apoptosis induced by strong activation of TCR's in immature thymocytes [23]. Consistent with evidence that Bcl-2 represses pro-apoptotic Ca\textsuperscript{2+} signals, a small molecule inhibitor of Bcl-2, HA14-1, induced Ca\textsuperscript{2+} elevation in epithelial cancer cells, thereby triggering cytochrome c release and apoptosis [123]. Furthermore, the protective effect of transforming growth factor-\(\beta\) (TGF-\(\beta\)) against glutamate-mediated Ca\textsuperscript{2+} elevation and neuronal cell death correlates with induction of Bcl-2 expression by TGF-\(\beta\) [124].

A major action of Bcl-2 is to inhibit Ca\textsuperscript{2+} crosstalk between ER and mitochondria. In seminal work Baffy et al [5] discovered that Bcl-2 inhibits the redistribution of Ca\textsuperscript{2+} from ER to mitochondria associated with IL3 withdrawal from an IL3-dependent hematopoietic cell line. A study published shortly afterward provided evidence that Bcl-2 inhibits mitochondrial Ca\textsuperscript{2+} waves that precede the onset of apoptosis following serum withdrawal from fibroblasts [34]. Also, both Bcl-2 and Bcl-xL inhibited the mitochondrial Ca\textsuperscript{2+} uptake that mediates staurosporine-induced apoptosis in neural cells [38] and Bcl-xL inhibited mitochondrial Ca\textsuperscript{2+} waves initiated by IP3-mediated Ca\textsuperscript{2+} signals in cardiac myotubes exposed to ceramide and staurosporine [60]. Mcl-1, an anti-apoptotic family member located on mitochondria, inhibited mitochondrial Ca\textsuperscript{2+} elevation induced by staurosporine and ATP in epithelial cells [61].

Bcl-2 not only inhibits the mitochondrial Ca\textsuperscript{2+} elevation that triggers apoptosis, but also has the capacity to enhance mitochondrial Ca\textsuperscript{2+} buffering capacity. Thus, Bcl-2 potentiates the maximal Ca\textsuperscript{2+} uptake capacity of neuronal cell mitochondria [125]. This
appears to be the mechanism accounting for the protective effect of estrogen-induced Bcl-2 expression against glutamate-mediated excitotoxic neuronal cell injury [126]. Collectively, these findings indicate that the capacity of anti-apoptotic members of the Bcl-2 family to regulate ER-mitochondrial Ca\(^{2+}\) crosstalk contributes to their anti-apoptotic action.

**Mechanism of ER Ca\(^{2+}\) release inhibition by Bcl-2**

Because of the importance of IP3-mediated Ca\(^{2+}\) release in apoptosis, Bcl-2 appears to have evolved more than one mechanism by which it controls ER Ca\(^{2+}\) release. Two proposed mechanisms, both supported by considerable experimental evidence, are inhibition of IP3R channel opening and reduction of ER luminal Ca\(^{2+}\) concentration. Although these mechanisms are discussed separately, both serve the same ultimate purpose, to inhibit pro-apoptotic Ca\(^{2+}\) signals emanating from the ER, and thus are not mutually exclusive.

*Regulation of IP3R Ca\(^{2+}\) channel opening*

The recent discovery that Bcl-2 interacts with IP3Rs in T cells and inhibits IP3-mediated Ca\(^{2+}\) release from the ER following T cell receptor activation provides novel insight into the molecular mechanism by which Bcl-2 suppresses pro-apoptotic Ca\(^{2+}\) signals (Figure 1.8) [104]. Interaction of Bcl-2 with IP3Rs dampens IP3-induced cytoplasmic Ca\(^{2+}\) elevation induced by strong T cell receptor engagement, thereby inhibiting apoptosis [23]. In vitro, Bcl-2 inhibits the opening of IP3R channels incorporated into artificial lipid membranes [104]. The interaction of Bcl-2 with the IP3R appears to be direct and has been documented in vivo by FRET [127].
In recent studies we mapped the site of Bcl-2 interaction to the regulatory and coupling domain of the IP3R [127]. This domain, located between the IP3 binding domain and the transmembrane channel domain, is believed to regulate conformational changes of the IP3R [12]. It contains many regulatory sites including for Ca\(^{2+}\) and ATP binding, for phosphorylation and for binding of regulatory proteins. Although White et al [69] recently reported an interaction of Bcl-xL with the extreme C-terminal region of the IP3R, they did not test whether the regulatory and coupling domain could also interact. Our findings, employing stringent binding and washing conditions, indicate that both Bcl-2 and Bcl-xL interact most strongly in the regulatory and coupling domain. Moreover, a synthetic peptide, corresponding to the Bcl-2 interaction site in the regulatory and coupling domain of the IP3R, interrupts the Bcl-2-IP3R interaction and reverses the inhibitory effect of Bcl-2 on both IP3-mediated Ca\(^{2+}\) elevation and apoptosis [127], providing substantial evidence that the interaction of Bcl-2 with the IP3R is responsible for the inhibitory effect of Bcl-2 on IP3-mediated Ca\(^{2+}\) elevation and apoptosis.

The functional importance of the Bcl-2-IP3R interaction is further exemplified by evidence that this interaction in the sarcolemma of myotubes reduces mitochondrial Ca\(^{2+}\) uptake and apoptosis in dystrophic muscle cells [128]. Also, Bcl-2 over-expression in MCF-7 breast cancer cells inhibits IP3-mediated Ca\(^{2+}\) elevation induced by ATP-mediated activation of purinergic receptors [129].

Although a direct interaction of Bcl-2 with the IP3R appears sufficient to regulate IP3R channel activity and Ca\(^{2+}\) release, Bcl-2 may also regulate IP3R phosphorylation. IP3R
phosphorylation has been reported to enhance IP3R channel opening and IP3R dephosphorylation to reduce channel opening [77, 130, 131], but IP3R phosphorylation occurs on multiple sites, is regulated by a number of different kinases and phosphatases, and has varied effects on IP3-mediated Ca^{2+} release [132-134]. Therefore, potential effects of Bcl-2 on IP3R phosphorylation may be difficult to unravel. Nevertheless, overall IP3R phosphorylation appears reduced in T cells over-expressing Bcl-2 (Chen, R. and Distelhorst, C.W., unpublished data). Interestingly, Bcl-2 interacts with calcineurin (protein phosphatase 2B) [108] and Billingsley and coworkers found that Bcl-2 regulates IP3R phosphorylation in neural cells by docking calcineurin to intracellular membranes [81]. The calcineurin-Bcl-2 interaction in neural cells is increased after exposure to excitotoxic agents, hypoxia or low glucose and these interactions shuttle calcineurin to IP3Rs, providing a neuroprotective effect [79-81]. To complicate matters, Bcl-2 itself is a phosphoprotein and is dephosphorylated by calcineurin, a process that enhances Bcl-2's anti-apoptotic action, including its effect on pro-apoptotic Ca^{2+} signals [135, 136]. Furthermore, Bcl-2 interacts not only with calcineurin but also with protein phosphatase 1, and Bcl-2 appears to disrupt the complex between protein phosphatase 1 and protein kinase A on the IP3R [129]. Thus, additional work is required to sort out the respective roles of IP3R phosphorylation and Bcl-2 phosphorylation in regulating IP3-mediated Ca^{2+} signals and apoptosis.

Finally, it has been reported that Bcl-2 and Bcl-xL inhibit ER Ca^{2+} release by reducing IP3R expression levels [137]. Although this finding has not been confirmed by others [69, 104, 105, 136], whether or not Bcl-2 (or Bcl-xL) expression are coordinately regulated
with IP3R expression as a means to regulate Ca\(^{2+}\) homeostasis and signaling deserves further investigation.

**Bcl-2-mediated decrease in luminal Ca\(^{2+}\)**

The other proposed explanation of Bcl-2’s inhibitory effect on ER Ca\(^{2+}\) release is that Bcl-2 decreases the Ca\(^{2+}\) concentration within the ER lumen [138-140]. There are at least three proposed mechanisms as to how Bcl-2 decreases luminal Ca\(^{2+}\). One possible mechanism involves a Bcl-2-imposed increase in Ca\(^{2+}\) leakage through the ER membrane via IP3Rs [105]. It appears that interaction of Bcl-2 with IP3Rs in cells lacking Bax and Bak leads to IP3R hyperphosphorylation, enhancing ER Ca\(^{2+}\) leak and decreasing steady-state Ca\(^{2+}\) stores [105]. This in part appears to explain the decrease in luminal Ca\(^{2+}\) concentration detected in cells deficient in both Bax and Bak [141], suggesting that luminal Ca\(^{2+}\) concentration may be modulated by the ratio of anti-apoptotic Bcl-2 family members and pro-apoptotic family members. Another proposed mechanism is that Bcl-2 over-expression decreases the Ca\(^{2+}\) sequestering capacity of the ER by reducing levels of SERCA pumps on the ER membrane [140, 142, 143]. It is possible that this effect of Bcl-2 may be specific for certain cell types or certain SERCA pump subtypes, as a reduction in SERCA protein or activity by Bcl-2 has not been detected by others [6, 104, 136]. A third hypothesis is that Bcl-2 forms an ion transmitting pore in the ER membrane, a concept that is intriguing in view of the resemblance of Bcl-2 and Bcl-xL structures to that of pore-forming bacterial toxins, coupled with evidence that Bcl-2 and Bcl-xL form ion-transmitting channels in artificial lipid membranes in vitro (reviewed in [3]). But elegant experiments by Chami et al [144] discount this hypothesis by showing that
putative pore forming domains of Bcl-2 and Bcl-xL are not required for Bcl-2/Bcl-xL-imposed effects on Ca\(^{2+}\) homeostasis and signaling.

Although the theory that Bcl-2 decreases luminal Ca\(^{2+}\) has received strong experimental support, this concept remains somewhat controversial (reviewed in [145]), as a number of reports did not detect an effect of Bcl-2 on luminal Ca\(^{2+}\) or even found that Bcl-2 functions to preserve luminal Ca\(^{2+}\) concentration [5, 146-152]. Whether the variation in findings from different laboratories is due to different methods of Ca\(^{2+}\) measurement or reflects biological variation among different cell types remains to be determined.

Summary

There is now a wealth of evidence indicating that Bcl-2 family members regulate apoptosis on two fronts, the mitochondria and the ER. Mitochondria release cytochrome c to trigger apoptosis and the ER releases Ca\(^{2+}\) to trigger apoptosis. The Ca\(^{2+}\) released from the ER induces apoptosis through direct effects on mitochondria or indirectly by activating signaling pathways that up-regulate pro-apoptotic proteins such as Bim, activate pro-apoptotic proteins such as Bad, or activate proteases and endonucleases that execute cell destruction. Pro-apoptotic Bcl-2 family members mediate and anti-apoptotic family members inhibit cytochrome c release from mitochondria. Similarly, pro-apoptotic family members enhance ER Ca\(^{2+}\) release and mitochondrial Ca\(^{2+}\) uptake, whereas anti-apoptotic family members inhibit ER Ca\(^{2+}\) release. Understanding the regulation of pro-apoptotic Ca\(^{2+}\) signals by Bcl-2 family members has evolved slowly over the past decade, but will likely proceed much faster in upcoming years, building on the molecular
mechanism defined by recent recognition of the physical interaction of Bel-2 and Bcl-xL with IP3R channels. From this understanding should evolve novel therapeutics for cancer, where Bel-2 is often elevated, and other diseases, based on reversing Bel-2's anti-apoptotic effect.
STATEMENT OF PURPOSE

Bcl-2 is one of the most important oncogenes in many types of cancers. So far no biochemical activity has been ascribed to the oncoprotein that fully explains its function in the cell. Bcl-2 is thought to regulate apoptosis at the level of the mitochondria, but it also localizes to the ER and exerts antiapoptotic function. In addition to the interaction with other proapoptotic Bcl-2 family members, Bcl-2 also interacts with many other proteins including IP3R. Previous data suggested that Bcl-2 regulates IP3-mediated ER calcium release. We proposed that that Bcl-2 inhibits IP3-mediated proapoptotic Ca\(^{2+}\) signals by interacting with IP3Rs on the ER membrane (Figure 1.8). The purpose of this thesis was to examine four major questions: 1) Does Bcl-2 interact with IP3R? 2) Where are the binding sites of this interaction on Bcl-2 or IP3R? 3) Does this interaction contribute to Bcl-2’s inhibitory effect on Ca\(^{2+}\) and apoptosis? 4) Can we target to this interaction to regulate Ca\(^{2+}\) and apoptosis?
FIGURES

Figure 1.1 Distinct Ca\textsuperscript{2+} signals regulate cell survival and cell death.

TCR activation induces either cell survival or cell death, depending upon the avidity of TCR engagement by antigens. Strong TCR activation induces a sustained Ca\textsuperscript{2+} elevation (A), whereas weak TCR activation induces Ca\textsuperscript{2+} oscillations. (B), recent findings suggest that these distinctly different patterns of Ca\textsuperscript{2+} signaling encode cell death and cell survival, respectively.
Figure 1.2 Ca\(^{2+}\) signals regulate both cell survival and cell death. Ca\(^{2+}\) released from the ER via IP3Rs, in conjunction with Ca\(^{2+}\) entry through plasma membrane channels, can induce cell death or cell survival, depending on the pattern of Ca\(^{2+}\) elevation produced. Sustained Ca\(^{2+}\) elevation in response to a wide range of inducing agents induces apoptosis by triggering cytochrome c release from mitochondria and by activating calpains and caspases. Both caspases and cytochrome c participate in positive feedback loops that enhance Ca\(^{2+}\) release from the ER, thereby augmenting Ca\(^{2+}\) elevation and apoptosis. Ca\(^{2+}\) oscillations induced by ATP and by weak TCR or BCR activation favor cell survival by enhancing mitochondrial bioenergetics or by activating Ca\(^{2+}\)-sensitive transcription factors such as NFAT. Because of the importance of Ca\(^{2+}\) in mediating cell fate, cells have evolved multiple mechanisms to regulate IP3-mediated Ca\(^{2+}\) release from the ER, including for example Erp44, which directly interacts with the third luminal loop of IP3Rs to sense the pH, redox status and Ca\(^{2+}\) concentration within the ER lumen and modulate IP3R activity accordingly.
Figure 1.3 Structure and regions of type I IP3R.

A. IP3R has five domains. B. IP3R is mainly localized on ER membrane and mediates the calcium release from ER.
Figure 1.4 Chromosome translocation of Bcl-2 in B-cell lymphoma.

A region of the long arm of chromosome 18 encoding Bcl-2 translocates to chromosome 14 in a B-cell lymphoma. This places the Bcl-2 oncogene under the transcriptional regulation of an immunoglobulin heavy chain enhancer, leading to the over-expression of Bcl-2.
Figure 1.5 BH domains of Bcl-2 family members.

Bcl-2 family members share sequence similarity in Bcl-2 homology (BH) domains shown by different colored regions. Prosurvival Bcl-2 family members, Bcl-2, Bcl-xL and Bcl-w have all four BH domains, whereas proapoptotic Bax subfamily members do not have a BH4 domain and BH3-only subfamily members lack all but a BH3 domain. Known α-helical regions are indicated. TM, transmembrane domain.
Figure 1.6 Structure of Bcl-2

The structure of Bcl-2 is made by KiNG Viewer 2.12 (PDB accession No. 1G5M). The blue color ribbon is the N-terminus of Bcl-2. The pink color end is the C-terminus of Bcl-2.
Figure 1.7 Bcl-2 family members regulate pro-apoptotic and anti-apoptotic Ca\(^{2+}\) signals. Pro-apoptotic Bcl-2 family members enhance Ca\(^{2+}\) release from the ER and mitochondrial Ca\(^{2+}\) uptake, thereby promoting apoptosis. Anti-apoptotic family members inhibit Ca\(^{2+}\) release from the ER and thereby inhibit apoptosis. Pro-apoptotic Ca\(^{2+}\) signals also positively regulate the expression and activity of BH3-only proteins Bim and Bad, respectively, whereas pro-survival Ca\(^{2+}\) signals up-regulate expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL.
Figure 1.8 Bcl-2 interacts with IP3Rs in T cells and inhibits IP3-mediated Ca\(^{2+}\) release from the ER following T cell receptor activation.

Anti-CD3 antibody activates T cell receptor, thereby activates phospholipase c and generates IP3. IP3, a ligand of IP3R, stimulates the IP3R channel activity and release Ca\(^{2+}\) from ER store. Bcl-2 regulates Ca\(^{2+}\) signals by interacting with IP3R. The elevation of Ca\(^{2+}\) in cytoplasm results in many cell processes including cell division and apoptosis.
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ABSTRACT

The Bcl-2 protein inhibits apoptosis through the interaction with pro-apoptotic members of the Bcl-2 family. Here we report a novel mechanism through which Bcl-2 inhibits apoptosis by interacting with and regulating the opening of the inositol 1,4,5-trisphosphate (IP3) receptor (IP3R) calcium channel. In vivo interaction of Bcl-2 with the IP3R was demonstrated by FRET and the Bcl-2 interacting site on the IP3R was mapped to the regulatory and coupling domain. A small synthetic peptide corresponding to this site inhibits Bcl-2-IP3R interaction without blocking the interaction of Bcl-2 with BH3-only proteins. This peptide reversed Bcl-2's inhibitory effect on channel opening in vitro and on IP3-mediated calcium elevation and apoptosis in vivo. These findings establish a novel mechanism of apoptosis regulation by Bcl-2 and suggest the Bcl-2-IP3R interaction as a novel target for therapeutics based on overcoming Bcl-2's anti-apoptotic action.
INTRODUCTION

Bcl-2 family proteins are central regulators of cell death and cell survival [1, 2]. It is well recognized that Bcl-2 family members regulate mitochondrial membrane permeability [3, 153]. Hence, proapoptotic members, including Bax and Bak, trigger cytochrome c release from mitochondria, while anti-apoptotic members, including Bcl-2 and Bcl-xL, inhibit cytochrome c release [154]. The regulation of cytochrome c release by Bcl-2 family members is of undoubted importance because of the critical role of cytochrome c in mediating caspase activation during apoptosis [54, 155]. The mechanism of Bcl-2's antiapoptotic action is incompletely understood, although it involves, at least in part, binding BH3-only proteins (e.g. Bim, Bid) that activate Bax/Bak [88, 156].

Another mechanism of Bcl-2’s action involves inhibition of Ca\(^{2+}\) release from the endoplasmic reticulum [4, 27, 103]. The concept that Bcl-2 represses apoptosis by inhibiting Ca\(^{2+}\) release from the ER arose over a decade ago [5, 6] and is supported by reports that Bcl-2 inhibits ER Ca\(^{2+}\) release and cytoplasmic Ca\(^{2+}\) elevation involved in apoptosis induction by hydrogen peroxide, staurosporine, ceramide, tumor necrosis factor and genotoxic stress [35-43]. Ca\(^{2+}\) is a major intracellular messenger that mediates a wide range of cellular processes including fertilization, development, secretion, cell division and cell death [101]. As a second messenger, sustained Ca\(^{2+}\) elevation activates many pathways to apoptosis, including Ca\(^{2+}\) sensitive proteases and endonucleases [25]. Also, Ca\(^{2+}\) uptake by mitochondria triggers mitochondrial permeability transition and cytochrome c release.
In many cases, the Ca^{2+} elevation that mediates apoptosis is generated by Ca^{2+} efflux via inositol 1,4,5-trisphosphate receptor calcium channels on the ER membrane [27, 102]. IP3-mediated elevation of cytoplasmic Ca^{2+} via the IP3R can trigger apoptosis in response to a number of initiating stimuli, including T-cell receptor and B-cell receptor activation, glucocorticosteroids, neurotoxic damage and oxidative stress [44-47]. Three subtypes of IP3R (type 1, 2, 3) have been identified which differ in terms of their functional properties, expression patterns and subcellular localizations in different cell types [12, 14, 71, 157-159]. The IP3R is a large transmembrane protein with four subunits [160]. Each subunit has a cytoplasmic region containing an IP3-binding and an inhibitory and coupling domain close to the N-terminus and a long internal regulatory and coupling domain located between the IP3 binding domain and the channel domain, while the C-terminal tail of the IP3R consists of a so-called gatekeeper domain [161, 162]. The regulatory and coupling domain transfers the ligand binding signal from the N-terminal IP3 binding domain to the C-terminal channel domain. This domain also functions to keep the inactivated IP3R channel closed [51, 52, 162] and regulates the activity of the IP3R by binding Ca^{2+}, ATP, kinases, phosphatases and other accessory proteins [70, 163].

We recently discovered that Bcl-2 inhibits Ca^{2+} release from the ER following TCR activation in immature T cells, thereby inhibiting TCR-induced apoptosis [23, 104]. This novel Bcl-2 action appeared to be mediated through an interaction of Bcl-2 with the IP3R that inhibits IP3-mediated Ca^{2+} release from the ER. The present study was undertaken to determine whether Bcl-2 interacts with the IP3R in vivo and whether this interaction is responsible for the inhibitory effect of Bcl-2 on IP3-mediated Ca^{2+} elevation and
apoptosis following TCR activation. As reported here, we documented the *in vivo* interaction between Bcl-2 and IP3R type 1 by Fluorescence Resonance Energy Transfer (FRET) and mapped the site of Bcl-2 interaction on the IP3R regulatory and coupling domain. Accordingly, we designed an inhibitory peptide that abrogates the Bcl-2-IP3R interaction, thereby reversing Bcl-2's inhibitory effect on TCR-induced Ca\(^{2+}\) elevation and apoptosis. These findings establish Bcl-2-IP3R interaction and regulation of IP3-mediated Ca\(^{2+}\) signals as an important facet of Bcl-2’s anti-apoptotic mechanism and suggest this interaction as a potential therapeutic target mechanistically different from current approaches that target the interaction of Bcl-2 with proapoptotic family members.
MATERIALS AND METHODS

Reagents
Fura-2 AM and Hoechst 33342 were from Invitrogen. Hamster anti-mouse CD3ε chain mAb and mouse anti-hamster IgG1 were from BD Biosciences. Mouse anti-human CD3ε IgG was purchased from eBioscience. D-myo-IP3 ester was synthesized by SJ Conway, University of St Andrews [164].

Plasmids
IP3R fragments were fused with GST at their N-terminus and cloned into pGEX-6p2 vectors as described [165, 166]. Other plasmids and their source are: pECFP-Bcl-2, J Yuan; CFP-YFP cameleon, R Tsien; Bcl-2 mutants, T Parslow; ER-CFP (SRbeta-mCerulean), F Geng and D Andrews.

Cell culture and transfection
COS-7 cells and WEHI7.2 cells (wild type WEHI7.2, Bcl-2 overexpressing WEHI7.2 (Bcl-2(+) WEHI7.2) and WEHI7.2 cells transfected with empty vector (Bcl-2 (-) WEHI7.2)) were cultured as described [104]. Jurkat cells were cultured in RPMI1640 supplemented with 10% fetal bovine serum. Transfection in COS-7 cells was performed with FuGENE 6 from Roche. Transfection and cloning of Bcl-2 in WEHI7.2 cells were reported previously [104].

Förster resonance energy transfer (FRET).
Plasmids encoding combinations of CFP- and YFP-tagged proteins were transiently transfected into COS-7 cells or HEK293 cells. For acceptor photobleaching FRET, COS-7 cells were fixed in 4% paraformaldehyde for 10 min after 24 hr transfection. Each
FRET pair sample was measured by acceptor photobleaching and modified three-cube FRET method.

For acceptor photobleaching FRET, the samples were exposed 30 min to fluorescent light through YFP filter cube (excitation 500/20; beam splitter JP4 PC). Before and after acceptor YFP fluorophore photobleaching, the YFP or CFP channel images were taken by the camera on a conventional fluorescence microscope (Leica, DMLFSA, CFP excitation D436/10; CFP emission D470/30, YFP emission D535/30; all filters were obtained from Chroma). The microscope was equipped with a filter wheel on the emission side controlled by a Sutter lambda10-2 and a Sutter DG5 light source on the excitation side. A 63× water corrected 1.2NA objective was used for FRET sample observation. FRET efficiency was calculated according to the increase in CFP emission after acceptor YFP photobleaching: FRET efficiency = (1 - CFP before/CFP after) × 100%. Multiple regions of interest (ROI) (>60 ROI for each pair of samples) were randomly selected from the CFP and YFP colocalization regions in three individual experiments and analyzed by Volocity software (Improvision). The CFP fluorescence intensity was encoded by 256 gray values. The changes of CFP intensity were calculated by subtracting grey values of before bleaching from after bleaching pixel by pixel and shown with the grey value images. The intensity of the grey color represents the increase in CFP amplitude. The grey scale value outside of the cell of interest was typically under 20. The grey scale value obtained where FRET occurred was typically over 40. In our experimental settings we calculated that the YFP signal was effectively photobleached by ~70% after 30 min of light exposure. A number of negative control pairs, including ER-
CFP+YFP-IP3R, CFP+YFP-IP3R, YFP+CFP-Bcl-2, CFP+YFP, were used to eliminate any potential FRET artifacts.

For live cell FRET, after transfection HEK293 cells were incubated in extracellular solution containing 172 mM NaCl, 2.4 mM KCl, 10 mM HEPES, 10 mM glucose, 4mM CaCl$_2$, 4mM MgCl$_2$ (pH 7.3). FRET measurements were performed by using a modified version of the three-cube FRET method [167]. Briefly, the average FRET signal (FRET ratio) was calculated using the following equation: $FR = (FRET_{n} \times RD1 \times CFP_{n})/(RA1 \times (YFP_{n} \times RD2 \times CFP_{n}))$, where the constants RD1 and RA1, derived from the CFP and YFP constructs expressed alone in HEK293 cells, were determined by a multilinear regression (MLR) of the type $FRET_{fl} = \alpha \times CFP_{fl} + \beta \times YFP_{fl} + \gamma$[MLR] (where $fl$ indicates fluorescence). The constant RD1 has the relationships $RD1 = \alpha + FRET \times RA1 \times RD2$ and $RA1 = \beta / FRET$. Because the term $FRET \times RA1 \times RD2$ turns out to be very small in comparison to RD1, the constant RD1 was estimated by $\alpha$ to a good degree of approximation. The extensive details of the calculation and measurements are given by Erikson et al, 2001, (please see their supplemental appendix). The experiments were performed with the same Leica microscope (DMLFSA) and filters as in the above photobleaching experiment; because of the Sugger DG5 light source we did not need a filter wheel on the excitation side. For calculation of the FRET ratio over the whole image, images were saved as TIFF files and processed with Igor software (WaveMetrics, Inc.). The FRET equation was applied to every pixel of the image for all three images. FR within these images was encoded by 256 gray values. For FR calculation, the background regions which were outside of the cell of interest were subtracted from the
whole image. The same negative controls as in acceptor photobleaching experiment were used.

**Planar Lipid bilayer analysis of IP3R channel activity**

Planar bilayers were formed across a prepainted (150-µm in diameter) hole in the wall of a Delrin cup separating two chambers 1 ml each, using a 7:3 lipid mixture of phosphatidylethanolamine and phosphatidylcholine (50 mg/ml decane, Avanti Polar Lipids, Alabaster, AL). Single channel recordings of IP3R type 1 activities were performed by vesicle fusion of native rat cerebella IP3R type 1 microsomes into planar lipid bilayers. The bilayer separated two pools (cis and trans). The microsomes were added to the cis-side of the bilayer. IP3 (2 µM)-induced single IP3R channel opening events were observed on top of this baseline leak. The standard solution contained 20 mM HEPES-Tris, pH 7.4, 1 mM EGTA, [Ca\(^{2+}\)\text{FREE}] = 250 nM, and 220 mM CsCH\(_3\)SO\(_3\) in the cis-chamber (20 mM, trans). Free Ca\(^{2+}\) concentration was calculated using MaxChelator software and verified with a Ca\(^{2+}\)-specific microelectrode. The liquid junctional potential between cis and trans recording solutions was compensated before formation of bilayer. Open probability was determined by using the half-threshold crossing criteria from 3-min records at 0 mV. Unitary currents were recorded using a conventional patch clamp amplifier (Axopatch 200B, Axon Instruments, Union City, CA). A custom current-voltage conversion amplifier was used to optimize single channel recording. The current signal was digitized at 20 kHz with a 32-bit AD/DA converter (Digidata 1322A, Axon Instruments) and filtered at 1 kHz with a low pass eight pole Bessel filter (model 900, Frequency Devices, Haverhill, MA), stored on a computer hard drive and analyzed offline using pClamp Version 9.2.1.4 (Axon Instruments). For single channel analysis
currents were filtered digitally at 500 Hz, and for presentation of current traces data were filtered at 300 Hz. Statistical analysis, data processing and figure presentation were performed using Origin software (Microcal Software Inc., Northampton, M.A, USA).

Subcellular localization of Bcl-2 and IP3R

To confirm the distribution of Bcl-2 and IP3R, plasmids encoding CFP-Bcl-2 and YFP-IP3R were transfected into COS-7 cells. The ER marker (pDsRed2-ER, Clontech) and mitochondria marker (pDsRed2-Mito, Clontech; MitoTracker Deep Red 633, Molecular probes) were used. All confocal images were acquired using a Zeiss LSM 510 inverted laser-scanning confocal microscope. A 63× numerical aperture of a 1.4 oil immersion planapochromat objective was used for all experiments. To eliminate spectral crosstalk, lambda stacks of images were acquired and the linear unmixing function was performed.

Unidirectional \(45\text{Ca}^{2+}\) flux measurements

Murine embryonic fibroblasts were cultured in DMEM/Ham F12 supplemented with 10% fetal calf serum and 3.8 mM L-glutamine. The cells were seeded in 12-well plates (Costar, Cambridge, MA) at a density of \(2\times10^4\) cm\(^{-2}\). Experiments were carried out on confluent cell monolayers (~\(1\times10^6\) cells/well), five days after plating, essentially as described previously [168]. Cells were permeabilized by incubating them for 10 min with a solution containing 120 mM KCl, 30 mM imidazole-HCl, pH 6.8, 2 mM MgCl\(_2\), 1 mM ATP, 1 mM EGTA, and 20 µg/ml saponin at 26°C. The non-mitochondrial Ca\(^{2+}\) stores were loaded for 45 min at 26°C in 120 mM KCl, 30 mM imidazole-HCl, pH 6.8, 5 mM MgCl\(_2\), 5 mM ATP, 0.44 mM EGTA, 10 mM NaN\(_3\), and 150 nM free \(45\text{Ca}^{2+}\) (28 µCi/ml). After store loading the cells were washed twice with 1 ml of efflux medium containing 120 mM
The efflux was performed at 26°C and the efflux medium was replaced every 2 min. The additions of IP3 (0.6 μM) and of the peptides (pep2 or ctrlpep; 30 μM) or the vehicle (DMSO) are as indicated in the figures. For each experiment, peptides or vehicle were added 4 min before IP3. At the end of the experiment, the $^{45}\text{Ca}^{2+}$ remaining in the stores was assessed by incubation with 1 ml of a 2% sodium dodecyl sulfate solution for 30 min. Ca$^{2+}$ release is plotted as the fractional loss (i.e., the amount of Ca$^{2+}$ released in 2 min divided by the total store Ca$^{2+}$ content at that time). The latter value was calculated by summing in retrograde order the amount of tracer remaining in the cells at the end of the efflux and the amounts of tracer collected during the successive time intervals.

**Protein expression and GST pull-down**

GST-IP3R fragments were expressed and purified as described [165, 166]. WEHI7.2 cells were lysed with RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 1 mM NaF, 1 mM Na$_3$PO$_4$, and protease inhibitor cocktail (Roche)). Supernatant (250 μl), which contained 300 μg total protein, was pre-cleared with 25μl sepharose beads (Amersham Biosciences) for 1 hr, then pelleted to remove the beads. 10-20 μg of each GST-IP3R fragment was attached to a fresh batch of 25 μl sepharose beads and incubated with the pre-cleared cell supernatant for 1 hr at 4°C. The amounts of each GST-IP3R fragment used in pull-down assays were adjusted to similar levels. To reduce non-specific binding, 1% BSA was added to the pull-down reaction, and NaCl concentration was adjusted to 300 mM during the binding step. Beads were centrifuged, washed thoroughly 7-8 times with RIPA buffer, eluted with glutathione elution buffer and prepared for western blotting.
In certain experiments, three different buffer and wash conditions of differing stringencies were compared. The buffer/wash condition described above is termed buffer 2 and the same buffer/wash, except for replacement of the 1% NP40 with 1% CHAPS, is referred to as buffer 3. The Buffer 1 buffer/wash condition, which was employed previously in the work of White et al., 2005 [69], consists of PBS, 2% glycerol, 0.05% Triton, protease inhibitor cocktail. This buffer was used, without adding BSA or additional NaCl, for preparation of cell lysates, bead binding and three time washes.

Full length Bcl-2 with His tag was cloned into pPROEX-1 vector and expressed in \textit{E.coli} M15 cells. Ni-NTA spin (QIAGEN) was used to purify the His-Bcl-2 protein using the method described previously [169]. Eluted Bcl-2 protein was further dialyzed to remove traces of CHAPS and imidazole, then characterized by SDS-PAGE, followed by silver staining and western blotting.

\textit{Immunoprecipitation and Western blotting}

Coimmunoprecipitation methods were described previously [104]. The following antibodies were used: anti-human Bcl-2 (BD Biosciences), anti-mouse Bcl-2 (Santa Cruz Biotechnology), anti-IP3R type 1 (Calbiochem), anti-Bim (Sigma), anti-Bax (BD Pharmingen), anti-GST (Amersham Biosciences), and anti-actin (Sigma). In the peptide inhibition of immunoprecipitation experiments, $10^8$ cells were lysed with 500 μl lysis buffer. The supernatant of cell lysates was incubated with the peptides at 4°C for 30 min before the immunoprecipitation.
RNA interference

Jurkat cells were electroporated in a BioRad Gene Pulser with either a Non-Targeting smartpool or a human Bcl-2 smartpool from Dharmacon at a final concentration of 1 mM. Samples were then taken for western blotting at 0, 24, 48 and 72 hr after transfection and 3×10^5 cells were plated onto poly-L-lysine-coated coverslips at each time point to measure Ca^{2+}. Cells were split every 24 hr to ensure optimal growth conditions.

Peptide synthesis and delivery

The purity of synthesized peptides was >95%, verified by mass spectrometry and HPLC. Each peptide was delivered into cells by Chariot™ (Active Motif) reagent according to manufacturer instructions. Peptides were mixed with Chariot reagent, incubated for 30 min at 25ºC, and then added to suspensions of 10^6 cells in OPTI-MEM (Invitrogen) with the peptide concentration at 60 μM. After 30 min incubation, another 1.6 ml culture medium was added into the dish, diluting the peptide final concentration to ~20 μM. Cells were incubated for another 2-3 hr in 5% CO_2 at 37ºC before Ca^{2+} measurements.

Ca^{2+} imaging and fluorometric measurements

Anti-mouse CD3, anti-human CD3 antibodies (20 μg/ml) or IP3 ester (25 μM) were gently added to buffer overlaying poly-L-Lysine-coated coverslips on which cells were adhered and loaded with Fura-2 AM. Methods of Ca^{2+} imaging and Ca^{2+} measurements by fluorometry were described in detail previously [23, 104].

Anti-CD3 induced apoptosis

After 24 hr treatment with 5 μg/ml anti-human CD3 for Jurkat cells and 20 μg/ml hamster anti-mouse CD3 plus anti-hamster IgG for WEHI7.2 cells, cells were stained with 10
µg/ml Hoechst 33342 for 10 min and typical apoptotic nuclear morphology was detected by epifluorescence microscopy with a 40× objective as previously described [23].

Statistical analysis

Data were summarized as the mean ± SEM and comparisons were made using the two-tailed t-test for repeated measures. Differences between means were accepted as statistically significant at the 95% level (p<0.05).
RESULTS

_Bcl-2 interacts with IP3R in cells_

FRET between Bcl-2 and IP3Rs was measured by acceptor photobleaching in fixed cells, which measures dequenching of donor (CFP) emission by acceptor (YFP) photobleaching [170, 171], and by three-cube FRET (two-color ratio imaging) in live cells (Erickson et al., 2001). In the first method, YFP-IP3R type 1 was used as acceptor and CFP-Bcl-2 was used as donor (Figure 2.1). Fluorophores were tagged to the cytoplasmic N-terminus of both proteins (Figure 2.1B). When transiently expressed in COS-7 cells, using relatively low concentrations of expression vectors, CFP-Bcl-2 and YFP-IP3R were co-localized on the ER with a small fraction of CFP-Bcl-2 localized without YFP-IP3R to mitochondria (Figure 2.8). Regions where CFP and YFP co-localized were selected for FRET efficiency calculation; thus the calculated FRET efficiencies only represent the efficiencies in the regions where YFP fusions and CFP fusions co-localize, reducing the contribution from CFP on mitochondria. A cameleon Ca^{2+} sensor served as positive control [172]. CFP+YFP-IP3R, YFP+CFP-Bcl-2, CFP+YFP were negative controls, along with CFP targeted to the cytoplasmic face of the ER (ER-CFP) + YFP-IP3R. ER-CFP localizes to the ER (Figure 2.8), serving as a negative control to exclude the possibility that FRET arising from the CFP-Bcl-2 and YFP-IP3R pair is merely due to the expression of fluorescently tagged proteins in the same location. After 30 min photobleaching with the YFP excitation fluorescence light, the CFP channel fluorescence intensity increased in both CFP-Bcl-2+YFP-IP3R and CFP-YFP cameleon cells, but not in negative controls (Figure 2.1A). The CFP donor dequenching effect was clearly shown by subtracting pre-bleaching images from post-bleaching images through pixel-by-pixel
calculation (Figure 2.1A, CFP post-bleach minus CFP pre-bleach). The CFP signal increased an average of 28 ± 1% in YFP-IP3R+CFP-Bcl-2 cells and 37 ± 0.6% in CFP-YFP cameleon cells following YFP bleaching (Figure 2.1C). The CFP fluorescence did not increase appreciably in negative control pairs.

To measure FRET in live cells, the same pairs of fluorescent proteins were transiently expressed in HEK293 cells (Figure 2.9). In live HEK293 cells, FRET occurs in CFP-YFP cameleon cells and CFP-Bcl-2+YFP-IP3R cells, but not in control cells (Figure 2.9). These results, although less quantitative than those obtained with the acceptor photobleaching method [173], indicate that FRET occurs, providing evidence that the Bcl-2-IP3R interaction exists not only in fixed cells, but also in living cells.

To summarize, taking into consideration technical limitations of FRET (see Discussion), measurements of FRET in both fixed and live cells indicate that Bcl-2 interacts with IP3Rs in intact cells, verifying the interaction predicted by co-immunoprecipitation of these proteins from cell extracts.

*Endogenous Bcl-2 interacts with IP3R and inhibits TCR-mediated Ca\(^{2+}\) elevation*

Jurkat cells were used to test if endogenous Bcl-2 interacts with IP3Rs and regulates TCR-mediated Ca\(^{2+}\) elevation. The level of endogenous Bcl-2 in Jurkat cells is much less than that of exogenously expressed Bcl-2 in WEHI7.2 cells (Bcl-2(+) WEHI7.2 cells)
Nevertheless, Bcl-2 in Jurkat cells also co-immunoprecipitated with IP3Rs (Figure 2.2B) and siRNA-mediated knock down of this Bcl-2 (Figure 2.2C) enhanced anti-CD3-induced Ca^{2+} elevation (Figure 2.2D, E), indicating the regulatory action of Jurkat cell Bcl-2 on IP3-mediated Ca^{2+} elevation. Also, siRNA-mediated knock down of Bcl-2 in Jurkat cells did not alter thapsigargin-induced Ca^{2+} elevation (Figure 2.2F, G), suggesting that reducing the level of endogenous Bcl-2 did not affect ER Ca^{2+} concentration, a finding consistent with earlier indirect and direct measurements of ER Ca^{2+} in Bcl-2 overexpressing WEHI7.2 cells (see Discussion).

To summarize, evidence that endogenous Bcl-2 in Jurkat T cells interacts with IP3Rs and regulates IP3-mediated Ca^{2+} elevation alleviates concern that the previously reported inhibition of TCR-induced Ca^{2+} elevation by Bcl-2 might be secondary to either clonal selection or high Bcl-2 levels associated with exogenous expression. Moreover, these findings enable investigation of both exogenously and endogenously expressed Bcl-2 throughout the experiments that follow.

**Bcl-2 interacts with the regulatory and coupling domain of the IP3R**

The three IP3R subtypes differ in terms of functional properties, expression patterns and subcellular localization in different cell types [12, 14, 71, 157-159]. Each IP3R monomer has a cytoplasmic region containing an IP3-binding and an inhibitory and coupling domain close to the N-terminus and an internal regulatory and coupling domain between
the IP3 binding and channel domains, while the C-terminal IP3R tail includes a gatekeeper domain (Figure 2.3A) [161, 162].

To identify where Bcl-2 interacts on the IP3R, we used a series of GST-IP3R fusion constructs corresponding to 5 fragments covering the first N-terminal 2216 amino acids and 1 fragment corresponding to the last 160 amino acids of mouse IP3R type 1 (Figure 2.3A) [165]. These fragments are located in the cytoplasm and coincide with “natural” domains generated by limited proteolysis [174]. The transmembrane domain was not included. After purification, fragments migrated as single prominent bands corresponding to their expected molecular weights (Figure 2.3B, C), although some minor degradation products were detected.

The first set of GST pull-down experiments utilized extracts of Bcl-2(+) WEHI7.2 cells and Jurkat cells as two independent sources of Bcl-2. Cell extracts were incubated with GST-IP3R fragments prebound to glutathione sepharose resin, with equal loading of the GST-IP3R fragments (upper panels, Figure 2.3B, C). After pull-down, Bcl-2 bound to IP3R domains was detected by immunoblotting (lower panels, Figure 2.3B, C), indicating that Bcl-2 from each cellular source mainly interacted with IP3R domain 3. These experiments were repeated a total of five times with the same result. It is important to note that following incubation of cell extracts with GST-IP3R fragments on the glutathione sepharose resin, the resin was washed extensively (see Methods).
An interaction of Bcl-X<sub>L</sub> with domain 6 of IP3R 1 was previously reported [69], but only three IP3R fragments were tested: an N-terminal fragment (amino acids 1-600); a C-terminal fragment (amino acids 2512-2750); and a larger fragment, Δ1-600, encompassing both the regulatory and coupling domain and the C-terminus. Thus, on the basis of these mapping studies a potential interaction of Bcl-X<sub>L</sub>/Bcl-2 within the regulatory and coupling domain was not formally tested. Also, the GST pull-down experiments employed lower stringency conditions than in the present work. We therefore compared the interaction of Bcl-2 with domains 3 and 6 under three different buffer conditions, including the same buffer/wash conditions employed in our experiments described above (buffer 2), the buffer/wash condition employed by White et al., 2005 (buffer 1) [69], and a buffer employing CHAPS detergent (buffer 3) rather than the non-ionic detergents employed in the other two buffers (Figure 2.3D). The later control was included in view of earlier reports that non-ionic detergents affect the Bcl-2 family member interactions (Hsu and Youle, 1997). With the lower stringency condition (buffer 1, 0.05% Triton, 3 times washing), domain 6 may interact with Bcl-2; however, under all three buffer conditions the interaction of domain 3 with Bcl-2 was much more prominent than the interaction of domain 6. Thus, it is possible that Bcl-2 interacts with both of these IP3R regions, but because of the prominence of the interaction with domain 3, we choose to further analyze smaller fragments of domain 3, subdomains 3a (a.a.1347-1496) and 3b (a.a.1499-1649), to further narrow down the Bcl-2 binding region. These fragments correspond to smaller segments of the regulatory and coupling domain, as previously published [166] (Figure 2.3A). We found that Bcl-2 interacted with the 80 amino acid subdomain 3a1 (a.a.1347-1426) most strongly (Figure 2.3E). Therefore, this
subdomain was targeted to develop an inhibitory peptide and experiments employing this peptide further confirm the contribution of subdomain 3a1 to the interaction between Bcl-2 and IP3R (below).

To address whether Bcl-2 associates with the IP3R directly or not, GST pull-down experiments were performed using purified His-tagged full length Bcl-2 (Figure 2.3F). This protein retains its ability to bind Bim and Bax (Figure 2.10), as reported previously for GST-Bcl-2 [136]. Our findings, confirmed in three separate experiments, indicate that the purified His-tagged Bcl-2 also interacts with the GST-IP3R domain 3 fragment (Figure 2.3G). Thus, Bcl-2 directly interacts with the IP3R in vitro.

In preliminary experiments we found that deleting the BH4 domain from Bcl-2 prevented interaction of Bcl-2 with the IP3R (not shown). We therefore screened a series of dicodon mutations in the BH4 domain and found that mutating amino acids 6 and 7 abrogated the Bcl-2-IP3R interaction (Figure 2.11 A, B) and abrogated Bcl-2's inhibition of anti-CD3-induced calcium elevation (Figure 2.11C). These data indicate that the Bcl-2-IP3R interaction is necessary for Bcl-2’s inhibitory effect on calcium, a conclusion that is further substantiated by the following evidence using an inhibitory peptide that prevents the Bcl-2-IP3R interaction.
An IP3R peptide inhibits Bcl-2-IP3R interaction

Since the IP3R binding region was narrowed down to the 80 amino acid subdomain 3a1 (a.a.1347-1426), we designed two 20 amino acid peptides (peptide 1, a.a.1365-1384; peptide 2, a.a.1389-1408) based on the degree of homology in this region among different IP3R isoforms (Figure 2.4A). Also, preference was given to regions that have beta-turn and/or alpha-helical structures, rather than random-coil or non-structured sequences, expecting that one or both of the peptides could inhibit the Bcl-2-IP3R interaction. Furthermore, the C-terminal region of domain 3a1 was not selected because of its lower accessibility and unpredictable secondary structure (Invitrogen PeptideSelect™ Design Tool). The scrambled sequence of peptide 2 was also synthesized and used as a control (Figure 2.4A). The effect of the peptides on Bcl-2-IP3R interaction was initially tested in GST pull-down experiments using Bcl-2(+) WEHI7.2 extracts as a source of Bcl-2 (Figure 2.4B). Peptides (200 μM and 1 mM) were incubated with Bcl-2(+) WEHI7.2 cell extracts and GST-IP3R domain 3 attached to glutathione sepharose. The amount of bound Bcl-2 was significantly decreased by 200 μM or 1 mM peptide 2, but not by control peptide. At 1 mM, peptide 1 partially inhibited the interaction, but not as effectively as peptide 2. The inhibitory effect of peptide 2 on Bcl-2-IP3R interaction was also detected in co-immunoprecipitation assays employing both Bcl-2(+) WEHI7.2 cells (Figure 2.4C, D) and Jurkat cells (Figure 2.4E). Peptide 2 (400 μM) consistently inhibited Bcl-2-IP3R interaction, whereas control peptide and peptide 1 did not inhibit the interaction (Figure 2.4C, D, E). These results were confirmed in multiple experiments. As a control for specificity, peptide 2 disrupted Bcl-2-IP3R interaction but not interaction of Bcl-2 with the BH3-only protein Bim (Figure 2.4F, G).
Peptide 2 reverses Bcl-2’s inhibitory effect on IP3R channel opening in vitro
The effects of peptide 2 and purified Bcl-2 on IP3R channel activity were analyzed in planar lipid bilayers under steady-state conditions. Single IP3R type 1 channel activity was visualized as a series of discrete positive current fluctuations in the presence of 2 μM IP3 and 250 nM Ca^{2+} in the cis compartment (cytoplasmic side of channel) (Figure 2.5A). A significant reduction in single channel activity was observed after adding 0.1 μM purified Bcl-2 to the cytoplasmic face of the IP3R channel (Figure 2.5A, B) and addition of 10 μM peptide 2 reversed Bcl-2’s inhibitory effect on channel activity (Figure 2.5A). Average open probabilities under various conditions were calculated from multiple repeated experiments (Figure 2.5C). Addition of Bcl-2 reduced IP3R open probability by 11.5-fold to 0.018. Peptide 2 reversed Bcl-2’s inhibitory effect, increasing the open probability back to 0.16, whereas control peptide did not reverse Bcl-2’s inhibitory effect on channel activity (Figure 2.5C). These results demonstrate that peptide 2 reverses Bcl-2’s inhibitory effect on IP3R channel opening in a purified in vitro system, indicating that peptide 2 acts directly on the Bcl-2-IP3R interaction to reverse Bcl-2's inhibitory effect on channel opening.

Peptide 2 reverses Bcl-2’s inhibitory effect on TCR-induced Ca^{2+} elevation
Next we investigated whether peptide 2 also reverses Bcl-2’s inhibitory effect on Ca^{2+} release through the IP3R in Bcl-2(+) and (-) WEHI7.2 clones described previously [23, 104], newly-derived WEHI7.2 mixed populations exogenously expressing Bcl-2, and Jurkat cells. Optimal conditions for peptide delivery and anti-CD3 concentration were determined in preliminary experiments. Peptide delivery in the lipid bilayer is 100%,
whereas the penetration of peptide 2 into cells is much less, accounting for the lesser effect in cells. Therefore, a higher concentration of peptide (60 μM) was delivered into cells using the Chariot™ delivery reagent. Cytoplasmic Ca^{2+} concentration was continuously measured at a single-cell level by digital imaging during anti-CD3 treatment. Representative Ca^{2+} traces from Bcl-2(+) WEHI7.2 cells are shown in Figure 2.6A and the average Ca^{2+} elevation induced by anti-CD3 in seven experiments is summarized in Figure 2.6B. Observed variations in lag phase are at least partially attributable to the fact that lymphocytes are only loosely adherent to coverslips and thus rapid buffer exchange is not possible during antibody additions. Peptide 2 enhanced anti-CD3 induced Ca^{2+} elevation in Bcl-2(+) WEHI7.2 cells by 40%. Control peptide and peptide 1 did not enhance anti-CD3-induced Ca^{2+} elevation. In Bcl-2(-) cells, peptide 2 did not have a significant effect on Ca^{2+} (Figure 2.6B). Similarly, peptide 2 significantly enhanced anti-CD3-induced Ca^{2+} elevation in Jurkat cells, whereas control peptide and peptide 1 did not (Figure 2.6C, D). Peptide 2 had no effect on Ca^{2+} elevation when added to the cells in the absence of the Chariot™ reagent. Also, peptide 2 did not enhance anti-CD3-induced Ca^{2+} elevation in Jurkat cells where the endogenous Bcl-2 level had been knocked down by siRNA, further confirming that the effect of peptide 2 on Ca^{2+} is dependent upon its ability to disrupt the Bcl-2-IP3R interaction (Figure 2.12). Additionally, peptide 2 did not affect intracellular Ca^{2+} levels in the absence of anti-CD3 stimulation (data not shown). The low Bcl-2 level in Jurkat cells and the barely detectable endogenous Bcl-2 in Bcl-2(-) WEHI7.2 cells are consistent with the higher amplitude and longer duration of the Ca^{2+} response in these cells in contrast to Bcl-2(+) WEHI7.2 cells (compare Figure 2.6A, B to Figure 2.6C, D). Thus peptide 2 modulated anti-CD3 induced Ca^{2+} elevation in both Bcl-
2(+) WEHI7.2 cells and Jurkat cells, consistent with the ability of peptide 2 to disrupt Bcl-2-IP3R interaction.

To exclude the possibility that peptide 2 may interfere with the upstream TCR activation pathway, a cell-permeant IP3 ester (D-myo InsP3 hexakisbutyryloxymethyl ester) was used to bypass TCR activation and anti-CD3-induced IP3 generation. Peptide 2 enhanced IP3 ester-induced Ca\(^{2+}\) elevation in Jurkat cells (Figure 2.13 A&B), indicating that peptide 2 acts at the level of the IP3R rather than interfering with upstream components of the TCR signaling pathway.

The conclusion that peptide 2 acts directly on the Bcl-2-IP3R interaction is supported not only by experiments described above (Figure 2.5) in which peptide 2 reversed Bcl-2-mediated inhibition of IP3R channel opening in vitro, but also by unidirectional \(^{45}\)Ca\(^{2+}\) flux experiments (Figure 2.13 D, E) that allow for very accurate quantification of Ca\(^{2+}\) release through IP3Rs located on the ER. These experiments require firmly adherent cells and therefore murine embryonic fibroblasts (MEFs), which have a somewhat lower level of endogenous Bcl-2 than Jurkat cells (Figure 2.13 C), were employed. The rate of IP3-induced \(^{45}\)Ca\(^{2+}\) efflux was measured in the presence of thapsigargin, which completely prevents ER Ca\(^{2+}\) reuptake. Peptide 2, but not control peptide, significantly increased the rate of \(^{45}\)Ca\(^{2+}\) efflux (Figure 2.13 D, E), confirming that peptide 2 acts at the ER level to reverse Bcl-2's inhibition of IP3R activity.
Peptide 2 enhances TCR-induced apoptosis

TCR-induced apoptosis is mediated by Ca^{2+} release from the ER via the IP3R and, as noted above, Bcl-2 inhibits TCR-induced apoptosis by inhibiting IP3-mediated Ca^{2+} release [23]. Since peptide 2 reverses Bcl-2’s inhibitory effect on Ca^{2+} release, we next investigated whether peptide 2 also attenuates the inhibitory effect of Bcl-2 on anti-CD3-induced apoptosis. Peptides were delivered into Bcl-2(+) WEHI7.2 cells by Chariot™ reagent. After 3 hr, cells were treated with anti-CD3 antibody and the percentage of apoptotic cells was measured 24 hr later. In Bcl-2(-) WEHI7.2 cells, anti-CD3 induced ~18% apoptotic cells while the Bcl-2 overexpressing Bcl-2(+) WEHI7.2 cells display markedly inhibited anti-CD3-induced apoptosis (Figure 2.7B). Peptide 2 enhanced anti-CD3-induced apoptosis by 2-fold in Bcl-2(+) WEHI7.2 cells, whereas control peptide did not have a significant effect.

Peptide 2 also attenuated Bcl-2’s inhibition of anti-CD3-induced apoptosis in Jurkat cells. In preliminary studies (data not shown), we tested several anti-CD3 concentrations. Higher concentrations of anti-CD3 induced high levels of apoptosis that might obscure the effect of the peptide. We therefore used 5 μg/ml anti-CD3, sufficient to induce 20-30% apoptosis. Peptide 2 enhanced anti-CD3-induced apoptosis to 40-45%, whereas the control peptide, peptide 1, or peptide 2 in the absence of Chariot™ reagent did not have an effect on anti-CD3-induced apoptosis (Figure 2.7A, C). In contrast to the effect of peptide 2 on anti-CD3-induced apoptosis, peptide 2 did not enhance Fas-induced Ca^{2+}-independent apoptosis in Jurkat cells (Figure 2.12B).
In summary, consistent with the effects of peptide 2 on the Bcl-2-IP3R interaction and on anti-CD3-induced Ca\(^{2+}\) elevation, peptide 2 enhanced anti-CD3-induced apoptosis in both Jurkat cells and Bcl-2(+) WEHI7.2 cells. In considering these findings, it is important to keep in mind that Bcl-2 also inhibits apoptosis by binding to BH3-only proteins and that the effect of peptide 2 selectively interrupts Bcl-2-IP3R interaction, not the interaction of Bcl-2 with BH3-only proteins (Figure 2.4F). Thus, it is to be expected that peptide 2 would only partially reverse Bcl-2's anti-apoptotic activity.
DISCUSSION

Here we have used FRET measurements to document Bcl-2-IP3R interaction within intact cells and have mapped the site of Bcl-2 interaction on the IP3R, thereby developing an inhibitory peptide used to establish the importance of the Bcl-2-IP3R interaction in regulating Ca^{2+} signals. This peptide, referred to as peptide 2, corresponds to a 20 amino acid sequence in subdomain 3a1 of the IP3R, a sequence that displays a high degree of homology in all three IP3R isoforms. The GST-IP3R domains used to map the Bcl-2 binding region represent well-folded structural components retaining significant IP3-induced Ca^{2+} release activity in vitro as long as they remain assembled [162, 174]. Thus, the interaction between Bcl-2 and subdomain 3a1 identified in our experiments likely corresponds to the interaction occurring in the natural situation. That this IP3R subdomain is critical for the interaction of Bcl-2 with IP3R's is substantiated by the ability of peptide 2 to inhibit this interaction in GST pull-down and co-immunoprecipitation experiments employing both Bcl-2(+) WEHI7.2 cells and endogenous Bcl-2 expressing Jurkat cells. Moreover, this action of peptide 2 in Jurkat cells is consistent with evidence, also presented here, that endogenously expressed Bcl-2 interacts with endogenously expressed IP3Rs. These findings alleviate concern that the Bcl-2-IP3R interaction might be dependent upon over-expression of Bcl-2 at high levels.

Domain 3a1, where Bcl-2 interacts, is located in the regulatory and coupling domain of the IP3R, which transfers the ligand binding signal from the N-terminal IP3 binding domain to the C-terminal channel domain. This domain also functions to keep the inactivated IP3R channel closed [12, 51, 52, 162] and contains many target sites for
regulators of IP3R activity [70, 163]. Moreover, recent electron microscopic findings suggested that conformational changes within the IP3R type 1 regulatory and coupling domain switch IP3R states between a “windmill”-like and “square”-like structure which may represent the “open” and “closed” channel, respectively [161, 175, 176]. Thus, it is interesting to speculate that the binding of Bcl-2 in this region may regulate this conformational change, thereby regulating IP3R channel opening.

The development of peptide 2 as an inhibitor of Bcl-2-IP3R interaction provided a useful tool with which to investigate the fundamental mechanism by which Bcl-2 inhibits Ca^{2+} release from the ER, at a molecular level. To this end, we employed peptide 2 in a multiplicity of experimental strategies to remove any doubt that the interaction of Bcl-2 with IP3Rs contributes to Bcl-2’s inhibition of IP3-mediated ER Ca^{2+} release. First, peptide 2 reversed Bcl-2’s inhibition of IP3R channel opening in vitro. Second, peptide 2, when introduced into cells using Chariot reagent, reversed Bcl-2’s inhibition of TCR-mediated Ca^{2+} elevation both in Bcl-2(+) WEHI7.2 cells and in Jurkat cells. These findings indicate that Bcl-2 inhibits pro-apoptotic Ca^{2+} signals at least in part through interaction with the IP3R. Third, special care was taken to control for the outside possibility that peptide 2 might act in cells at some other level than the IP3R itself (e.g., the TCR signaling pathway upstream of the IP3R). For this purpose, we demonstrated that peptide 2 antagonized both unidirectional $^{45}$Ca^{2+} efflux from the ER in permeabilized cells and IP3 ester-induced Ca^{2+} elevation in intact cells. Thus, the present findings provide strong evidence that the Bcl-2-IP3R interaction is an important component of the process through which Bcl-2 inhibits IP3-mediated Ca^{2+} release from the ER.
One proposed component of this process upon which there is not complete agreement is the role of Bcl-2 in regulating ER Ca\(^{2+}\) concentration, a topic discussed in recent reviews [25, 73, 103, 145]. In our earlier work [104] and in the present study, as well as findings in other laboratories (see Introduction), a Bcl-2-imposed decrease in ER Ca\(^{2+}\) concentration was not detected. Nevertheless, the previously reported detection of a Bcl-2-imposed decrease in Ca\(^{2+}\) concentration by others and our present findings regarding the interaction of Bcl-2 with the IP3R are not necessarily mutually exclusive. In fact, recent reports propose a role for Bcl-2-IP3R interaction in regulating ER Ca\(^{2+}\) concentration [105] and indicate that the ability of Bcl-2 to decrease ER Ca\(^{2+}\) concentration is dependent upon both Bcl-2 phosphorylation state [136] and which of the three IP3R isoforms is expressed [177]. Overall, the major focus of the current work is not on reexamining potential effects of Bcl-2 on ER Ca\(^{2+}\) concentration, but on the Bcl-2-IP3R and designing a peptide inhibitor that reverses Bcl-2’s inhibitory effect on calcium release and apoptosis. These findings stand whether or not Bcl-2 decreases ER Ca\(^{2+}\) concentration.

That peptide 2, when introduced into intact cells, reverses Bcl-2’s inhibition of TCR- or cell permeable IP3 ester-induced Ca\(^{2+}\) elevation suggests that the Bcl-2-IP3R interaction previously detected by co-immunoprecipitation in cell extracts indeed occurs within intact cells. This conclusion is further substantiated by FRET measurements. Bcl-2 is known to localize to both the ER and mitochondria, but IP3R's are mainly localized to the
ER and Bcl-2's inhibitory effect on IP3-mediated Ca\(^{2+}\) elevation is observed in cells where Bcl-2 is selectively targeted to the ER [104]. The FRET measurements reported here document an interaction of Bcl-2 with IP3R's on the ER, although FRET techniques are challenging and have recognized pitfalls and limitations [173]. Therefore, in this work we employed two different, independent methods of FRET measurement, acceptor photobleaching in fixed cells and direct FRET measurements in living cells, as complementary methods to determine if Bcl-2 interacts with IP3R in cells. Also, because of the known limitations of both of these methods, a stringent set of controls was included. The most straightforward method to detect FRET is to photobleach the acceptor and to monitor the change in donor emission. For this method the actual FRET signal and the FRET efficiency could be overestimated depending on conditions (e.g. fixation) used. Thus, the quantification of FRET efficiencies in acceptor photobleaching experiments at present is valid only for establishing the presence or absence of FRET [170]. A number of negative controls reduced the possibility of false positive FRET. Although live cell FRET by ratiometric imaging is the simplest FRET method, potential artifacts due to the spectral bleed-through have to be considered. The corrective ratiometric method employed in our measurements of live cell FRET has been established to subtract the crosstalk between the fluorescence proteins (see appendix of Erickson et al., 2001). Though actual FRET values depend on the exact amount of acceptor proteins interacting with donor proteins, it is not necessary for the donor and acceptor concentrations to be equal in both of the methods employed here [173, 178]. Nevertheless, according to the experimental conditions, the CFP-Bcl-2 and YFP-IP3R concentrations do not vary dramatically. Our data indicate that donor and acceptor pairs
interact in sufficiently close proximity and orientation to allow FRET. With these precautions in mind, both FRET techniques provided positive evidence that Bcl-2 does indeed interact with IP3Rs within cells.

Overall, the findings presented here indicate that Bcl-2 operates on two fronts, blocking BH3-only protein activity and blocking proapoptotic Ca$^{2+}$ signals. Recently developed Bcl-2 antagonists target the Bcl-2-BH3-only protein interaction, whereas peptide 2 interferes selectively with the Bcl-2-IP3R interaction. Perhaps in the future small molecule inhibitors of the latter interaction could be developed that mimic the activity of peptide 2 and complement the therapeutic activity of small compounds targeting the interaction of Bcl-2 with BH3-only proteins.
FIGURES

Figure 2.1 FRET detection of Bcl-2-IP3R interaction.

FRET was detected by the increase of CFP fluorescence following YFP bleaching in COS-7 cells expressing CFP-Bcl-2 + YFP-IP3R and CFP-YFP cameleon, but not control combinations of fluorescently tagged proteins. (A) Representative images of CFP fluorescence intensity before and after YFP photobleaching (left and middle columns). Grey value images (right column) were obtained by pixel-by-pixel subtraction of CFP pre-bleach images from post-bleach images, with relative intensity differences represented by the grey scale. Bar, 5μm. (B) Diagram of C-terminal location of CFP and YFP on Bcl-2 and IP3R, respectively. (C) Multiple regions of interest (>60 for each pair of samples) were randomly selected from the CFP and YFP colocalization regions in three individual experiments. FRET efficiency was calculated according to the increase in CFP emission by Volocity software. Symbols represent mean ± SEM. *, p<0.01.
Figure 2.2 Endogenous Bcl-2 inhibits anti-CD3-induced Ca\(^{2+}\) elevation in Jurkat cells. 

(A) Bcl-2 levels in wild type WEHI7.2, Jurkat and Bcl-2(+) WEHI7.2 cells by immunoblotting. (B) Coimmunoprecipitation of Bcl-2 with IP3R in Jurkat extracts; immunoblot analysis using anti-Bcl-2 antibody. (C) Immunoblot of Bcl-2 in Jurkat extracts 24 and 48 hr after transfection with non-targeting control siRNA (siNT) or Bcl-2 siRNA (siBcl-2). (D) Digital imaging traces (average 160 cells per sample) monitoring Ca\(^{2+}\) elevation induced by 20 µg/ml anti-CD3 in presence of extracellular Ca\(^{2+}\). (E) Peak Ca\(^{2+}\) elevation induced by 20 µg/ml anti-CD3 in presence of extracellular Ca\(^{2+}\) (mean ± SEM, three experiments). (F) Peak Ca\(^{2+}\) elevation induced by 100 nM thapsigargin, measured fluorometrically in the absence of extracellular Ca\(^{2+}\) (mean ± SEM, three experiments). (G) Area under the cytosolic Ca\(^{2+}\) 340nM/380nM ratio curve in (F) (mean ± SEM, three experiments)
Figure 2.3 Bcl-2-IP3R interaction mapping.

(A) Diagram of type 1 IP3R domains. (B,C) GST-IP3R fragment pull-downs employing cytosolic extracts from Bcl-2(+) WEHI7.2 or Jurkat cells as Bcl-2 source. Upper panel, Coomassie blue stained gel showing input GST-IP3R fragments; bottom panel, Bcl-2 detected by immunoblotting. Findings representative of 5 experiments using Bcl-2(+) WEHI7.2 extract and 2 experiments using Jurkat extract indicate that Bcl-2 mainly interacts with domain 3. (D) Cell extracts from Bcl-2(+) WEHI7.2 cells were prepared in three different buffers which differ mainly by detergent type and concentration. The same buffers were used during GST-IP3R pull-down and wash steps, except that in buffers 2 and 3 1% BSA was added and the NaCl concentration was increased to 300 mM in the binding steps to increase binding specificity. Also, wash steps were repeated 3 times with buffer 1, but 7-8 times with buffers 2 and 3. Upper panel, Coomassie blue staining of GST-IP3R fragments, repeated twice with the same result. (E) GST pull-down, performed as in B, using GST-IP3R subdomain fragments 3a, 3b, 3a1 and 3a2. Upper panel, anti-GST immunoblot documenting input levels of IP3R fragments. Lower panel, anti-Bcl-2 immunoblot documenting that subdomain 3a1 is the major binding region of Bcl-2 on the IP3R. This experiment was repeated twice with the same result. (F) Silver-stain gel indicating the purity of His-tagged Bcl-2. (G) GST pull-down with purified His-tagged Bcl-2. Upper panel, input GST-IP3R fragments resolved by SDS-PAGE and stained with Coomassie blue. Bottom panel, immunoblot for Bcl-2 showing that purified 30 kDa His-Bcl-2 directly interacted with GST-IP3R domain 3, repeated three times with the same result.
Figure 2.4 An IP3R peptide inhibits Bcl-2-IP3R interaction but not Bim-Bcl-2 interaction.

(A) Sequences of peptides 1 and 2, corresponding to regions within IP3R subdomain 3a1, and control peptide representing a scrambled sequence of peptide 2. (B) Peptide 2 inhibits the interaction between Bcl-2 and GST-IP3R domain 3 in GST pull-down assays. Bcl-2(+) WEHI7.2 cell extracts were pre-incubated with 200 μM or 1 mM peptides for 20 min before adding the glutathione sepharose resin with GST-IP3R domain 3 fragment attached. (C, E) IP3R was immunoprecipitated from extracts of Bcl-2(+) WEHI7.2 cells (C) or Jurkat cells (E) in the presence of 400 μM peptides. Peptide 2, but not peptide 1 or control peptide, inhibited the Bcl-2-IP3R coimmunoprecipitation. (D) Summary of Bcl-2 immunoblot signal intensities in three GST pull-down experiments identical to panel C (mean ± SEM). (F) Bcl-2 was immunoprecipitated from Bcl-2(+) WEHI7.2 cell extracts in the presence of 400 μM peptides and coimmunoprecipitation of Bim was detected by anti-Bim immunoblotting. Neither control peptide nor peptide 2 interfered with the Bcl-2-Bim interaction. (G) Summary of Bim immunoblot signal intensities in three experiments identical to panel F (mean ± SEM).
Figure 2.5 Peptide 2 reverses Bcl-2’s inhibition of IP3R channel opening in vitro.

(A) IP3R type 1 single channel recordings at 0 mV in planar lipid bilayers with 0.2 mM Ca\(^{2+}\) and 2 μM IP3 in the cis (cytosolic) compartment (zero-current level marked). Current traces at the expanded time scale are shown in the bottom panel. Purified Bcl-2 (0.1 μM), added to the cis compartment, blocked channel activity. Subsequent addition of peptide 2 (10 μM) reversed Bcl-2’s inhibition of channel activity. (B) Shown is a single channel recording documenting that significant reduction of channel activity is detected within less than 3 min after Bcl-2 addition. (C) Summary of multiple experiments (mean ± SEM) measuring effects of Bcl-2 and peptides on IP3R channel open probability. n=number of individual channels examined. Symbols represent mean ± SEM. * p<0.05.
Figure 2.6 Peptide 2 reverses Bcl-2's inhibition of anti-CD3 induced Ca$^{2+}$ elevation.

(A) Representative Ca$^{2+}$ traces (each the mean of ~65 cells) recording anti-CD3 (arrow, time of addition) induced Ca$^{2+}$ elevation in Bcl-2(+) WEHI7.2 cells. Peptide (60 µM) uptake was facilitated by Chariot reagent. (B) Peak anti-CD3 induced Ca$^{2+}$ elevation in Bcl-2(-) and Bcl-2(+) WEHI7.2 cells treated with peptides as in A. (mean ± SEM, 5 experiments for Bcl-2(-) and 7 experiments for Bcl-2(+) cells, with mean 68 cells per sample per experiment). (C) Representative Ca$^{2+}$ traces in Jurkat cells (each the mean of ~85 cells), with treatment conditions the same as in A, with addition of a no Chariot control. (D) Peak anti-CD3-induced Ca$^{2+}$ elevation in Jurkat cells treated with peptides as in C (mean ± SEM, 7 experiments, mean 81 cells per sample per experiment).
Figure 2.7 Peptide 2 enhances anti-CD3-induced apoptosis in Bcl-2 positive cells.

(A) Typical apoptotic nuclear morphology (arrow, Hoechst 33342 stain) 24 hr after 5μg/ml anti-CD3 treatment of Jurkat cells. Bars, 10 μm. (B) Bcl-2(-) and Bcl-2(+) WEHI7.2 cells were pre-incubated ± 60 μM peptides plus Chariot reagent and then incubated with 20 μg/ml anti-CD3 for 24 hr. Symbols represent the percentage of cells (mean±SEM) with apoptotic nuclei in 3 experiments for Bcl-2(-) and 5 experiments for Bcl-2(+) cells (200 cells counted per coverslip). (C) Jurkat cells were pre-incubated ± 60 μM peptides ± Chariot reagent and then incubated with 5 μg/ml anti-CD3 for 24 hr. Symbols represent the percentage of cells (mean ± SEM) with apoptotic nuclei in 5 experiments (240 cells counted per coverslip).
Figure 2.8 Localization of CFP-Bcl-2 and YFP-IP3R in COS-7 cells.

pDsRed2-ER or pDsRed2-Mito was co-transfected with CFP-Bcl-2, YFP-IP3R or ER-CFP. Some samples were stained by the MitoTracker Deep Red 633 dye (10nM) for 10 min. First and second rows show that the CFP-Bcl-2 distribution pattern corresponds more to the DsRed2-ER pattern rather than the DsRed2-Mito pattern. The third row shows the CFP-Bcl-2 distribution in the same cell compared with DsRed2-ER and DsRed2-Mito. The fourth and fifth rows show that the YFP-IP3R localizes on ER rather than mitochondria. The sixth row shows that ER-CFP does localize on ER in mammalian COS-7 cells. Bars, 10 μm.
Figure 2.9 Live cell FRET measurements documenting Bcl-2-IP3R interaction.

The left and middle columns are fluorescence images of HEK293 cells expressing the various proteins identified on the left side of the figure. The right column is the calculated FRET image of living HEK293 cells, in which the FRET ratio per pixel was calculated using the equation: FR = (FRET_n - RD1 x CFP_n)/(RA1 x YFP_n), and encoded in grey values (Cameleon was used as a positive control, which made the strongest FRET). The *bar on the right* relates the calculated FR to gray scale values. These experiments were repeated twice with the same results.
Figure 2.10 Purified His-Bcl-2 from *E.coli* cells interacts with Bax and Bim in Bcl-2(-) WEHI7.2 cell lysates.

(A) His-Bcl-2 was preincubated with Bcl-2(-) WEHI7.2 cell lysates for 1 hr. Bim was coimmunoprecipitated with His-Bcl-2 using anti-Bcl-2 antibody and analyzed by western blotting using anti-Bim antibody. Control IgG did not coimmunoprecipitate Bim.

(B) Bax also coimmunoprecipitated with His-Bcl-2.
Figure 2.11 Mutating Bcl-2 in the BH4 domain abrogates Bcl-2's interaction with the IP3R and Bcl-2's inhibition of anti-CD3-induced Ca\(^{2+}\) elevation.

(A) Bcl-2(6-7) dicodon mutant (RS was mutated to GG) has similar expression level as wild type Bcl-2 in WEHI7.2 cells. (B) Bcl-2 was coimmunoprecipitated with IP3R with anti-Bcl-2 antibody and analyzed by western blotting using Bcl-2 antibody and IP3R antibody. Control IgG and Bcl-2(-) cells do not have Bcl-2-IP3R coimmunoprecipitation. More IP3R1 coimmunoprecipitated with wild type Bcl-2 than with the Bcl-2(6-7) mutant, while the anti-Bcl-2 antibody pulls down the same amount of wild type Bcl-2 as Bcl-2(6-7) mutant. (C) In Bcl-2 mix populations or Bcl-2 single clone cells (Bcl-2 sc), the anti-CD3 induced Ca\(^{2+}\) elevation was inhibited. The Bcl-2(6-7) mutant did not inhibit anti-CD3 induced Ca\(^{2+}\) elevation (mean ± SEM, p<0.004, three individual experiments).
Figure 2.12 Peptide 2 does not work in Bcl-2 knock down Jurkat cells and anti-Fas induced apoptosis

(A) Peptide 2 does not enhance anti-CD3 induced Ca2+ elevation in Bcl-2 knock down Jurkat cells. The level of endogenous Bcl-2 in Jurkat cells was knocked down by siRNA as shown in Figure 2.2C. Preincubation with peptides and measurement of anti-CD3-induced Ca2+ elevation was performed as described in Figure 2.6C&D. Symbols represent mean ± SEM of three separate experiments, in which peak Ca2+ elevations were normalized to the peak elevation induced by anti-CD3 in the absence of peptide treatment.

(B) Peptide 2 does not enhance anti-Fas induced apoptosis in Jurkat cells. 0.1ug/ml anti-Fas antibody was incubated with Jurkat cells for 24 hours after peptide treatment. Apoptosis was quantified according to nuclear morphological changes by Hoechst staining.
Figure 2.13 Peptide 2 works directly at the IP3R, rather than in upstream TCR-signaling pathways, to reverse Bcl-2’s inhibitory effect on IP3-mediated Ca\(^{2+}\) release from the ER. (A) Representative Ca\(^{2+}\) traces recording the Ca\(^{2+}\) elevation induced by 25 µM IP3 ester in Jurkat cells treated with or without control peptide and peptide 2. The arrow designates the addition of IP3 ester. The data represent the mean of 68 no peptide-treated cells, 73 control peptide-treated cells and 75 peptide 2-treated cells. (B) Summary of the peak Ca\(^{2+}\) elevation induced by 25 µM IP3 ester in control peptide- and peptide 2-treated cells. Symbols represent mean ± SEM in four separate experiments (288 peptide 2-treated cells and 303 control peptide-treated cells). (C) The endogenous IP3R expression level and Bcl-2 level in MEF cells are lower than in Bcl-2(+) WEHI7.2 cells. (D) Peptide 2 enhances IP3-induced Ca\(^{2+}\) release in permeabilized murine embryonic fibroblasts. The effect of control peptide, no peptide and peptide 2 on IP3-induced Ca\(^{2+}\) release. After loading the ER Ca\(^{2+}\) stores with \(^{45}\)Ca\(^{2+}\), the unidirectional efflux of \(^{45}\)Ca\(^{2+}\) was followed as a function of time. Ca\(^{2+}\) release was induced by the addition of 0.6 µM IP3. Cells were treated with 30 µM control peptide, no peptide (DMSO) or 30 µM peptide 2 from 4 min before until 2 min after IP3 addition. Data are plotted as fractional loss (the amount of \(^{45}\)Ca\(^{2+}\) leaving the stores in 2 min divided by the total store Ca\(^{2+}\) content at that time) as a function of time. The data are from experiment 070421 and each data point represents the mean ± SEM for three wells. (E) Bar graph demonstrating the difference between the fractional loss after the addition of IP3 and the fractional loss before the addition of IP3 as measured in the presence of 30 µM control peptide, no peptide or 30 µM peptide 2. Symbols represent the mean ± SEM for 5 individual experiments, each performed in triplicate.
CHAPTER 3: BCL-2 TAT-BH4 PEPTIDE INTERACTS WITH THE IP3R AND INHIBITS TCR-MEDIATED CALCIUM ELEVATION AND APOPTOSIS
ABSTRACT

Bcl-2 is the founding member of a large family of apoptosis regulating proteins. A major function of Bcl-2 is to interact with proapoptotic members of the Bcl-2 family to inhibit apoptosis. A separate function of Bcl-2 is to interact with the inositol 1,4,5-trisphosphate receptor (IP3R) Ca$^{2+}$ channel and inhibit IP3-mediated Ca$^{2+}$ signals that promote apoptosis. Here we report that the BH4 domain of Bcl-2 is necessary and sufficient for Bcl-2 interaction with the IP3R, based in part on coimmunoprecipitation and GST pull-down experiments. Also a synthetic peptide corresponding to the BH4 domain of Bcl-2 interacts with the same IP3R domain as full length Bcl-2. Fusion of this peptide with HIV TAT facilitates its uptake by cells. This fusion peptide TAT-BH4 possesses the same functional activity as full length Bcl-2, based on evidence that it inhibits both cytoplasmic Ca$^{2+}$ elevation and apoptosis induced by T cell receptor activation. Two experimental findings confirm that these actions of TAT-BH4 are mediated through interaction with the IP3R, rather than an unexpected effect on upstream signaling pathways. First, TAT-BH4 inhibits Ca$^{2+}$ elevation induced by a cell permeant IP3 ester. Second, a peptide that blocks Bcl-2-IP3R interaction reverses the inhibitory effect of TAT-BH4 on both Ca$^{2+}$ elevation and apoptosis following T cell receptor activation. The anti-apoptotic action of TAT-BH4 is already recognized and is being applied in therapeutic models of diseases associated with accelerated apoptosis. The findings reported here suggest a novel mechanism of TAT-BH4 action.
INTRODUCTION

Bcl-2 is a potent inhibitor of apoptosis that exerts its anti-apoptotic action mainly by binding pro-apoptotic members of the Bcl-2 family. Bcl-2 also inhibits apoptosis by regulating Ca^{2+} homeostasis and signaling (reviewed in [73, 103]. At least in part this regulation is mediated through an interaction of Bcl-2 with the inositol 1,4,5-trisphosphate receptor (IP3R), an IP3-gated Ca^{2+} channel on the endoplasmic reticulum [69, 104-106, 129, 177]. IP3R mediated Ca^{2+} signals regulate many processes, including cell differentiation, proliferation, transcriptional activation and apoptosis [8, 101]. Ca^{2+} release from ER through IP3R induces apoptosis in response to a broad range of stimuli, including T cell receptor activation. The role of Ca^{2+} elevation in apoptosis has been reviewed previously [25, 73, 103]. Recently, we determined that Bcl-2 interacts with the regulatory and coupling domain of the IP3R to inhibit IP3-mediated pro-apoptotic Ca^{2+} signals [127]. Based on the IP3R sequence where Bcl-2 binds we synthesized a peptide that disrupts the Bcl-2-IP3R interaction. This peptide reversed the inhibitory effect of Bcl-2 on IP3-mediated Ca^{2+} elevation and apoptosis, confirming that the interaction of Bcl-2 with the IP3R contributes to Bcl-2’s anti-apoptotic action.

Bcl-2 and other members of the Bcl-2 protein family share homology in regions known as Bcl-2 homology domains (BH domains). The anti-apoptotic proteins Bcl-2 and Bcl-xL have four BH domains (BH1-4) whereas the full length pro-apoptotic proteins Bax and Bak have only three BH domains (BH1-3). A third group, the BH3-only proteins have only a BH3 domain in common with other Bcl-2 family members. The three dimensional structure of Bcl-2 was determined by NMR spectroscopy of Bcl-2/Bcl-xL chimeras in
which part of the putative unstructured loop of Bcl-2 was replaced with a shortened loop from Bcl-xL [94, 95]. The BH1, BH2 and BH3 domains form a hydrophobic groove where proapoptotic proteins bind [179]. Thus, Bcl-2 inhibits apoptosis mainly by interacting with the BH3 domain of pro-apoptotic Bcl-2 family members, including the BH3-only proteins (e.g., Bim, Bad) [92, 180, 181]. The BH4 domain of Bcl-2 is located in an N-terminal α-helix. The role of the BH4 domain in Bcl-2’s anti-apoptotic activity is not as well understood as that of the other three BH domains. Whether or not the BH4 domain is required for binding pro-apoptotic proteins is uncertain. Interestingly, deletion of the BH4 domain has been reported to convert Bcl-2 into a pro-apoptotic protein without impairing Bcl-2’s homodimerization or heterodimerization [182]. This suggests that the BH4 domain may have an anti-apoptotic activity separate from the anti-apoptotic activity mediated by binding pro-apoptotic Bcl-2 family proteins.

The current study was undertaken to identify the region of Bcl-2 responsible for this interaction of Bcl-2 with the IP3R. We determined that the BH4 domain of Bcl-2 is critical for Bcl-2-IP3R interaction and that a BH4 peptide itself can interact with IP3R and inhibit IP3-mediated Ca^{2+} release from the ER. Also, the BH4 peptide inhibited Ca^{2+} elevation and apoptosis induced by anti-CD3-mediated T cell receptor activation as well as full length Bcl-2. These results indicate that Bcl-2 can inhibit apoptosis not only through its binding of pro-apoptotic Bcl-2 family members, but also through its interaction with and regulation of IP3R channel opening. Moreover, the current work indicates that these two distinct activities are mediated through different regions of the Bcl-2 protein. The BH4 domain is both necessary and sufficient to interact with the IP3R.
and regulate proapoptotic calcium signals independent of other regions of Bcl-2 involved in heterodimerization with proapoptotic relatives.
MATERIALS AND METHODS

Reagents and plasmids
Hamster anti-mouse CD3ε chain mAb and mouse anti-hamster IgG1 were obtained from BD Biosciences. Mouse anti-human CD3ε IgG was purchased from eBioscience. D-myo-IP3 ester was synthesized by Stuart J. Conway in University of St Andrews, St Andrews [164]. Fura-2 AM and Hoechst 33342 were obtained from Invitrogen.

IP3R fragments were fused with GST at their N-terminus and cloned into pGEX-6p2 vectors as described [165, 166]. ER-dsRed and Mitotracker deep red dye was purchased from Molecular Probes.

Cell culture and transfection
WEHI7.2 cells (wild type WEHI7.2, Bcl-2 overexpressing WEHI7.2 (Bcl-2(+)) WEHI7.2) and WEHI7.2 cells transfected with empty vector (Bcl-2 (-) WEHI7.2)) were incubated in DMEM supplemented with 10% bovine calf serum, 2 mM L-glutamine, and 100 μM of non-essential amino acids. Jurkat cells (clone E6-1) were cultured in RPMI1640 supplemented with 10% fetal bovine serum. Transfection and cloning of Bcl-2 in WEHI7.2 cells were reported previously [104].

Peptide synthesis and delivery
The purity of synthesized peptides was >95%, verified by mass spectrometry and HPLC. TAT-BH4 Bcl-2 peptide: NH2-GRKKRRQRRRGGRTGYDNREIVMKYIHLYKLSQRYEW-COOH, TAT-control peptide: NH2-RKRRQRRRGGGLKNDICLRVTPSVILVNE-COOH, TAT-peptide 2: NH2-RKRRQRRRGGNVETYEIKCNSSLPLDDIVRV-COOH. FITC was attached at
the N terminus of TAT-BH4 and control peptides. Peptides were incubated with 10^6 cells in OPTI-MEM (Invitrogen) for 60 min at 25°C before Ca^{2+} measurements.

**Confocal microscopy of FITC labeled TAT-BH4**

To detect the fTAT-BH4 peptide in cells, 5 µM fTAT-BH4 peptides were incubated with WEHI7.2 cells or COS-7 cells for 1-2 hrs. After incubation, the cells were washed with PBS three times and twice with HBSS buffer (Hanks' balanced salt solution with calcium and magnesium, 10 mM HEPES, and 1% fetal bovine serum). The ER marker (pDsRed2-ER, Clontech) and mitochondria marker (MitoTracker Deep Red 633, Molecular probes) were used according to the manufacture protocols. All confocal images were acquired using a Zeiss LSM 510 inverted laser-scanning confocal microscope. A 63× numerical aperture of a 1.4 oil immersion planapochromat objective was used for all experiments. To eliminate spectral crosstalk, lambda stacks of images were acquired and the linear unmixing function was performed.

**GST pull-down of FITC labeled peptides**

GST-IP3R fragments domain 3 and domain 6 were expressed and purified as described previously [165, 166]. 10-20 µg GST fusion fragments were attached to a fresh batch of 25 µl sepharose beads for 1 hr and washed four times with 1XPBS. 40 µM peptides were mixed with GST fusion fragments in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 1 mM NaF, 1 mM Na_3PO_4, and protease inhibitor cocktail (Roche Applied Science)). The amounts of each GST-IP3R fragment used in pull-down assays were adjusted to similar levels. After binding for 2 hrs, beads were centrifuged, washed 4-5 times with RIPA buffer, eluted with 100 µl
glutathione elution buffer. The fluorescence counts of the eluted solution were measured at 485nm/535nm by Victor3 1420 multilabel counter (PerkinElmer).

$Ca^{2+}$ imaging

Anti-mouse CD3, anti-human CD3 antibodies (20 μg/ml) or IP3 ester (40 μM) were gently added to buffer overlaying poly-L-Lysine-coated coverslips on which cells were adhered and loaded with 1μM Fura-2 AM in extracellular buffer. Methods of $Ca^{2+}$ imaging were described in detail previously [23].

Immunoprecipitation and Western blotting

Coimmunoprecipitation methods were as described previously [104]. The following antibodies were used: anti-human Bcl-2 (BD Biosciences, 15131A), anti-mouse Bcl-2 (Santa Cruz Biotechnology, sc7382), anti-IP3R type 1 (Calbiochem, 407144), anti-Fluorescein (Abcam, ab19492), anti-GST (Amersham Biosciences), and anti-actin (Sigma, A5441).

Anti-CD3 induced apoptosis

Jurkat cells and WEHI7.2 cells were plated into 96-well plates at $4 \times 10^5$/ml and $2 \times 10^5$/ml respectively. After 24 hr treatment with 5 μg/ml anti-human CD3 for Jurkat cells and 20 μg/ml hamster anti-mouse CD3 plus anti-hamster IgG for WEHI7.2 cells, the cells were stained with Hoechst 33342 (final concentration 10 μg/ml) for 10 min, and typical apoptotic nuclear morphology was detected by epifluorescence microscope with a 40× objective (Carl Zeiss MicroImaging, Inc.) as previously described [23].
Statistical analysis

Data were summarized as the mean ± SEM and comparisons were made using the two-tailed \( t \)-test for repeated measures. Differences between means were accepted as statistically significant at the 95% level (\( p<0.05 \)).
RESULTS

BH4 domain is necessary for Bcl-2 interaction with IP3R

In Chapter 2 we mapped the Bcl-2-IP3R binding site in the regulatory and coupling domain (domain 3) of the IP3R by using a series of GST-IP3R fragments encompassing the entire IP3R, with exception of the transmembrane channel region [127]. These fragments are located in the cytoplasm and coincide with “natural” domains generated by limited proteolysis [174]. Since in our previous work full length Bcl-2 interacted with domain 3 but not with domain 6, here we used GST-IP3R domain 3, GST-IP3R domain 6, and GST alone, in GST pull-downs with wild type full-length Bcl-2 and BH4 deletion Bcl-2 (ΔBH4Bcl-2). Lysates of WEHI7.2 cells overexpressing human Bcl-2 or ΔBH4Bcl-2 were incubated with GST-IP3R fragments in pull down experiments indicating that deletion of the BH4 domain from Bcl-2 abrogates its interaction with IP3R domain 3 (Figure 3.1A). Similarly, deletion of the BH4 domain prevented Bcl-2 co-immunoprecipitation with full length IP3R in cell extracts (Figure 3.1B). Thus, the BH4 domain of Bcl-2 is necessary for Bcl-2-IP3R interaction.

TAT-BH4 peptide interacts with IP3R

To determine if the BH4 domain of Bcl-2 interacts with the IP3R, a TAT-BH4 fusion peptide was synthesized and labeled with FITC. BH4 was fused to the HIV-1 TAT protein transduction domain in anticipation of subsequent experiments designed to introduce BH4 peptide into cells. The use of TAT peptides for protein, peptide, drug and gene delivery across cellular membranes has been demonstrated in many cell types [183-185]. The FITC labeled TAT-BH4 peptide (fTAT-BH4) was incubated with GST-IP3R
fragments and the amount of fTAT-BH4 bound by GST-IP3R fragments was quantified by fluorescence measurements. Compared with the FITC-TAT (fTAT) negative control, fTAT-BH4 interacted with GST-IP3R-d3, but not with GST-IP3R-d6 or either GST alone or glutathione beads alone (Figure 3.2A). Also fTAT-BH4 interaction with full length IP3R was detected by coimmunoprecipitation, whereas the negative control fTAT peptide did not coimmunoprecipitate with the IP3R (Figure 3.2B). These results indicate that the BH4 domain of Bcl-2 is sufficient to interact with the IP3R and interacts in the same IP3R region as full length Bcl-2.

**TAT-BH4 peptide inhibits anti-CD3 induced calcium elevation**

Since the preceding experiments indicated that the TAT-BH4 peptide binds to the same region of IP3R as full length Bcl-2, we tested if the peptide has the same inhibitory effect on IP3-induced Ca\(^{2+}\) elevation as we reported previously for full length Bcl-2. Two T cell lines, WEHI7.2 and Jurkat, commonly used by us and others to study T cell receptor (TCR)-mediated Ca\(^{2+}\) signaling [19, 127] were employed in these studies. WEHI7.2 cells have virtually no detectable endogenous Bcl-2, whereas Jurkat cells have readily detectable levels of endogenous Bcl-2 [127]. In these experiments, IP3-mediated Ca\(^{2+}\) elevation was induced by treating cells with antibody to the CD3 component of the TCR complex. Optimal conditions for peptide delivery and anti-CD3 concentration were determined in preliminary experiments. After incubating cells with 2 μM TAT-BH4 peptide, cytoplasmic Ca\(^{2+}\) concentration was measured by single-cell digital imaging as reported previously [23]. Anti-CD3 induced Ca\(^{2+}\) elevation was inhibited by TAT-BH4 peptide but not control peptide in both cells lines (Figure 3.3). Representative Ca\(^{2+}\) traces
are shown in panels A, B, D, and E. Anti-CD3-induced Ca\(^{2+}\) elevations (mean ± SEM) from 7 experiments in WEHI 7.2 cells and 4 experiments in Jurkat cells are summarized in Figure 3.3 C and F, demonstrating significant inhibition of anti-CD3-induced Ca\(^{2+}\) elevation by TAT-BH4 peptide compared to control peptide in both WEHI7.2 and Jurkat cells.

TAT-BH4 peptide inhibits cell permeable IP3 ester induced cytoplasmic calcium elevation

In view of the complexity of the TCR pathway activated by anti-CD3 antibody, a cell permeable IP3 ester (D-myo InsP3 hexakisbutyryloxymethyl ester) was added to WEHI7.2 cells to bypass steps in the TCR activation pathway upstream of the IP3R. After preincubation with 2 µM TAT-BH4 peptide or control peptide, cells were loaded with the calcium indicator Fura-2 AM and cytoplasmic Ca\(^{2+}\) concentration was measured at a single cell level by digital imaging. TAT-BH4 peptide also inhibited IP3 ester-induced Ca\(^{2+}\) elevation (Figure 3.4). Panels A and B are representative Ca\(^{2+}\) traces, while panels C and D summarize results of multiple experiments. The findings indicate that the TAT-BH4 peptide acts at the level of the IP3R to inhibit anti-CD3-induced Ca\(^{2+}\) elevation rather than at a step in the upstream TCR signaling pathway.

The effect of TAT-BH4 on calcium is reversed by peptide 2

In our previous work, we designed a peptide (pep2), which can displace Bcl-2 from the IP3R and abrogate Bcl-2’s inhibitory effect on IP3 dependent IP3R channel opening and IP3-induced Ca\(^{2+}\) release from the ER [127]. We synthesized TAT-pep2 to facilitate entry
of pep2 into cells instead of using Chariot reagent as we had done in our earlier studies. We first confirmed that TAT-pep2 reverses the Bcl-2 imposed inhibition of anti-CD3-induced Ca^{2+} elevation (Figure 3.5A). WEHI7.2 cells stably overexpressing Bcl-2 (labeled 'Bcl-2(+)) were compared with control cells transfected with empty vector (labeled 'Neo'). As reported in our previous studies, Bcl-2 significantly inhibited anti-CD3-induced Ca^{2+} elevation [23, 104, 127]. TAT-ctrl (TAT-control peptide) did not reverse the inhibitory effect of Bcl-2, whereas 10 µM TAT-pep2 did reverse Bcl-2-mediated inhibition of anti-CD3-induced Ca^{2+} elevation. Since TAT-BH4 peptide has an inhibitory effect on the IP3R similar to that previously reported with full length Bcl-2, we tested if TAT-pep2 can reverse the inhibitory effect by TAT-BH4 on anti-CD3-induced Ca^{2+} elevation in Neo WEHI7.2 cells (Figure 3.5B). The results indicate that TAT-pep2 does reverse BH4 peptide’s inhibitory effect on anti-CD3-induced Ca^{2+} elevation. These findings indicate that the TAT-BH4 peptide inhibits anti-CD3-induced Ca^{2+} elevation by binding to and inhibiting IP3R channels.

**TAT-BH4 peptide does alter luminal Ca^{2+}**

In the following experiments we controlled for potential effects of TAT-BH4 on ER Ca^{2+} content by analyzing both the thapsigargin-induced Ca^{2+} peak and area under the Ca^{2+} release curve (Figure 3.5 C, D). Since thapsigargin-induced Ca^{2+} elevation is an indirect measure of the mobilizable ER Ca^{2+} pool, this finding indicates that TAT-BH4 does not alter the ER luminal Ca^{2+} level. This finding is consistent with extensive evidence that Bcl-2 inhibits IP3-induced Ca^{2+} release from the ER following TCR activation by inhibiting IP3R channel opening.
**TAT-BH4 peptide does not disrupt ER structure**

It has been reported that transiently overexpressing high levels of full length Bcl-2 can disrupt ER structure and induce cell death [186]. Therefore, we investigated the effect of TAT-BH4 peptide on ER structure. WEHI7.2 cells and COS-7 cells were incubated with 5 µM fTAT-BH4 peptide for 1 hr and then examined by confocal microscopy. As shown in Figure 3.6A, fTAT-BH4 (green) was distributed around Hoechst dye-stained (blue) nuclei. Although confocal microscopy confirmed that fTAT-BH4 enters WEHI7.2 cells, due to their small size ER structure was difficult to evaluate in WEHI7.2 cells. COS-7 cells are larger and therefore better for this purpose. Therefore, COS-7 cells incubated with fTAT-BH4 peptide were also stained with ER-dsRed and Mitotracker markers (Figure 3.6B). Although the fTAT-BH4 appeared in a punctate distribution that appeared to coincide more with the ER (visualized by ER-targeted dsRed) than mitochondria (visualized by Mitotracker dye), the peptide did not exclusively localize to either organelle. The delivery efficiency was 50-60% based on the observed fluorescence signal. Cells with an aggregated or disrupted ER structural pattern were estimated at less than 10% of the total cells examined. Thus, fTAT-BH4 peptide did not significantly alter ER structure under the experimental conditions (5 µM 1 hr incubation) employed here (Figure 3.6C).
**TAT-BH4 peptide inhibits anti-CD3 induced apoptosis**

IP3-induced Ca\(^{2+}\) elevation mediates apoptosis following TCR activation. The elevation of Ca\(^{2+}\) stimulates cell death by activating proteases, decreasing mitochondria membrane potential and elevating expression of CD95L and the proapoptotic protein Bim [23, 119, 187]. We recently reported that anti-CD3-induced Ca\(^{2+}\) elevation triggers apoptosis in WEHI7.2 and Jurkat cells, and that this process is negatively regulated by the interaction of Bcl-2 with the IP3R [23, 127]. Therefore, to determine if TAT-BH4 has a similar action to Bcl-2 in this regard, we preincubated Jurkat cells with TAT-BH4 peptide for 1 hr and then treated the cells with 5 µg/µl anti-CD3 antibody. After 24 hrs, cells were stained with Hoechst dye and apoptosis was quantified according to the percentage of cells displaying apoptotic nuclear morphology. The results indicate that the TAT-BH4 peptide inhibited anti-CD3 induced apoptosis (Figure 3.7A). Co-incubation with TAT-pep2 reversed the inhibitory effect of TAT-BH4 on anti-CD3-induced apoptosis, whereas TAT-ctrl peptide did not (Figure 3.7B). Note that in Figure 3.7B anti-CD3 alone induce ~30% apoptosis, whereas the combination of anti-CD3 and TAT-pep2 induced ~45% apoptosis. This is consistent with our previous findings in Jurkat cells, in which endogenous Bcl-2 represses anti-CD3 induced apoptosis [127]. Treating Jurkat cells with TAT-BH4 further inhibited apoptosis, and TAT-pep2 reversed this apoptosis inhibition. The ability of TAT-pep2 to reverse the inhibitory effect of TAT-BH4 on anti-CD3 induced apoptosis further indicates that TAT-BH4's antiapoptotic function is mediated through its interaction with the IP3R.
We also investigated whether TAT-BH4 peptide might interfere with the interaction between Bcl-2 and Bim. This would be unexpected since it is well-known that BH3 only proteins interact with the hydrophobic groove formed by BH1, 2 and 3 domains of Bcl-2 and not with the BH4 domain. Indeed, we found that TAT-BH4 does not disrupt the co-immunoprecipitation of Bcl-2 and Bim, indicating that TAT-BH4 peptide does not affect the Bcl-2-Bim interaction (Figure 3.7C). This finding excludes the possibility that the TAT-BH4 peptide regulates calcium and apoptosis by regulating the interaction of Bcl-2 with other Bcl-2 family members. Thus, antiapoptotic function of TAT-BH4 is independent of the interaction of Bcl-2 with other pro-apoptotic Bcl-2 family proteins.
DISCUSSION

Here we report that the BH4 domain of Bcl-2 is both necessary and sufficient for the interaction of Bcl-2 with the IP3R. By fusing a TAT peptide to a BH4 peptide (TAT-BH4), we were able to deliver a peptide corresponding to the BH4 domain of Bcl-2 into cells. The results indicate that the BH4 peptide binds to the same region of IP3R as does full length Bcl-2 and inhibits TCR activation-induced Ca\(^{2+}\) elevation and apoptosis.

IP3-mediated Ca\(^{2+}\) elevation induces Bim upregulation and activates endonucleases, proteases (calpains and caspases) and the BH3-only protein Bad [26, 119, 120]. The important and broad roles of Ca\(^{2+}\) in apoptosis were also suggested by the positive feedback of cytochrome c on IP3R-mediated Ca\(^{2+}\) release [49]. Cytochrome c released from mitochondria during the early stages of apoptosis binds to IP3Rs and potentiates IP3-mediated Ca\(^{2+}\) release that enhances the mitochondrial phase of apoptosis. Moreover, the IP3R sequence includes a consensus site for cleavage by the apoptosis associated protease caspase-3 [50, 51, 188]. The cleaved form of IP3R exhibits increased leakiness, thereby enhancing Ca\(^{2+}\) release from the ER during apoptosis.

Bcl-2 has four BH domains, BH1-4, and the hydrophobic groove formed by the BH1, 2, 3 domains contributes to Bcl-2’s interaction with BH3-only Bcl-2 members. Although there are two prominent theories as to how exactly how this interaction regulates apoptosis and which is correct is still controversial, the binding of Bcl-2 with Bax/Bak, Bim, Bad, Bid through this hydrophobic groove contributes to Bcl-2’s anti-apoptotic function [89, 98, 189]. Although this heterodimerization activity of Bcl-2 has been a major focus of
apoptosis research, the roles of Bcl-2 BH4 domain are getting increasing attention recently. One of the major differences between pro-apoptotic Bcl-2 members and anti-apoptotic Bcl-2 members is the presence of a BH4 domain in the former but not in the later. Bcl-2 without the BH4 domain functions like a pro-apoptotic protein, even though it still binds Bax, Bak, Bad, and Bik [190]. Thus, the BH4 domain is critical for Bcl-2’s anti-apoptotic function, although its precise role has not been defined. It has also been reported that the BH4 domain is not only involved in inhibiting apoptosis, but also seems to be required for Bcl-2’s inhibitory effect on the cell cycle [191]. Also, in recent years the BH4 domain was reported to bind to a number of proteins involved in mediating or regulating apoptosis, including the voltage dependent anion channel (VDAC), the \( \text{Ca}^{2+} \)/calmodulin-dependent phosphatase calcineurin, Ras, Raf-1, NF-kappaB, paxillin and ASPP2 (Apoptosis Stimulating Protein of p53) [108, 111, 182, 192-195]. These interactions may contribute to some of Bcl-2’s anti-apoptotic function. For example, the TAT-BH4 peptide was reported to inhibit VDAC channel activity and mitochondrial calcium uptake, thereby inhibiting apoptosis [192]. However, knocking down VDAC was reported to have no effect on mitochondria permeability transition and cell death, leading to the prediction that VDAC is dispensable for mitochondria dependent cell death [196]. However, in those experiments one isoform of VDAC was incompletely knocked down, raising suspicion on the part of some investigators that VDAC may still be involved in mitochondria-mediated cell death pathways. Thus, whether or not the anti-apoptotic function of TAT-BH4 peptide also involves inhibition of the VDAC channel remains uncertain. Our results described here suggest another mechanism that involves interaction of the BH4 domain of Bcl-2 with IP3R’s to inhibit IP3-mediated \( \text{Ca}^{2+} \) elevation. Thus,
TAT-BH4 inhibits anti-CD3 induced apoptosis by inhibiting IP3R mediated Ca\(^{2+}\) elevation and apoptosis in the same manner as reported for full length Bcl-2. Moreover, TAT-BH4 fusion peptide inhibits apoptosis induction by etoposide, radiation, oxidative stress, and sepsis, indicating that the BH4 domain itself has an anti-apoptotic function independent of the other BH domains [134, 197-199].

The specificity of TAT-BH4 peptide was confirmed by IP3 ester experiments and peptide 2 reversing experiments. Cell permeable IP3 ester specifically and directly activates IP3R's and induces Ca\(^{2+}\) release from the ER. Therefore, the inhibitory effect of TAT-BH4 peptide on IP3 ester-induced calcium elevation observed here excludes the possibility that TAT-BH4 peptide acts on other components of the TCR signaling pathway. Also we previously found that peptide 2, which was derived from the IP3 receptor binding site for Bcl-2, specifically reverses Bcl-2’s inhibitory effect on IP3 receptor channel activity. In the present study, peptide2 similarly reversed TAT-BH4 peptide’s inhibitory effect on IP3-mediated Ca\(^{2+}\) elevation and apoptosis. These findings further substantiate that TAT-BH4’s inhibitory effect on IP3-mediated Ca\(^{2+}\) elevation is mediated through its inhibitory effect on the IP3R channel.

The TAT-BH4 peptide and our previously reported pep2 have opposite effects on IP3R mediated Ca\(^{2+}\) elevation and apoptosis. Both peptides target the Bcl-2-IP3R interaction. Peptide2 enhances IP3R activity, IP3 mediated Ca\(^{2+}\) elevation and apoptosis by blocking the interaction of Bcl-2 with the IP3R. On the other hand, TAT-BH4 peptide inhibits IP3R activity, IP3 mediated Ca\(^{2+}\) and apoptosis by mimicking Bcl-2. It has already been
reported that TAT-BH4 peptide is functional in some diseases models by inhibiting apoptosis. TAT-BH4 peptide improves functional recovery after spinal cord injury by inhibiting apoptosis in neurons [200]. It also protects against excitotoxic insults and ischemia reperfusion induced cardiac dysfunction [201]. Recently, IP3R mediated Ca\(^{2+}\) release has been associated with Alzheimer’s disease [202, 203]. Interestingly, TAT-BH4 peptide reduces beta-amyloid peptide’s toxicity on capillary endothelium [204]. TAT-BH4 peptide’s inhibitory effect on IP3R-mediated Ca\(^{2+}\) elevation will bring some thoughts on its application in Alzheimer’s disease. Taken together, our data explain one mechanism through which TAT-BH4 peptide inhibits apoptosis and suggest the possibility of applying TAT-BH4 peptide or small molecules that mimic its activity as a potential pharmacological agent in diseases that involve IP3R-mediated apoptosis.
FIGURES

Figure 3.1 The BH4 domain is required for Bcl-2-IP3R interaction.

(A) Equal amounts of GST alone, GST-IP3R domain 3 (d3) and domain 6 (d6) (upper panel, coomassie blue staining) were bound to glutathione sepharose resins and incubated with cell lysates containing similar levels of ΔBH4Bcl-2 or wild type Bcl-2 (wtBcl-2), respectively. As shown in the lower panel, wtBcl-2 interacted with domain 3, but ΔBH4Bcl-2 did not. (B) IP3R was immunoprecipitated from Bcl-2(+) WEHI7.2 cells (wtBcl-2) or stably ΔBH4Bcl-2 overexpressing WEHI7.2 cells using an anti-IP3R type 1 antibody or using rabbit IgG as a control. Immunoprecipitates were analyzed by western blotting using a Bcl-2 antibody that recognizes both wtBcl-2 and ΔBH4Bcl-2. The input levels of wtBcl-2 and ΔBH4Bcl-2 in the cell extracts before immunoprecipitation are shown in right panels.
Figure 3.2 BH4 peptide interacts with IP3R.

(A) FITC-TAT-BH4 peptide interacts with domain 3 of IP3R. GST fusion domain 3 of the IP3R (GST-d3), GST alone (GST) or GST fusion domain 6 of the IP3R were incubated with 10 µM FITC-TAT-BH4 peptide (fTAT-BH4). The pull-down peptide was monitored by fluorescent counts. GST-EB (GST elution buffer), beads without binding protein (beads+fTAT-BH4) and FITC-TAT peptide (fTAT) were used as controls. (B) Coimmunoprecipitation of fTAT-BH4 peptide with IP3R1 in WEHI7.2 cell extracts; immunoblot analysis with anti-IP3R1 antibody.
Figure 3.3 TAT-BH4 peptide inhibits anti-CD3 induced Ca\(^{2+}\) elevation

(A, B) Representative Ca\(^{2+}\) traces recording the Ca\(^{2+}\) elevation induced by 20 μg/ml anti-CD3 antibody in wild type WEHI7.2 (Bcl-2 (-)) cells in the presence of 2 μM TAT-control peptide (ctrlpep) or TAT-BH4 peptide. The antibody was added around 1-2 min after recording was started. (C) Histograms summarize the average peak anti-CD3 induced Ca\(^{2+}\) elevation in WEHI7.2 cells treated with different peptides. Symbols represent the mean±SEM of 7 individual experiments (>50 cells per sample per experiment). Statistical analysis was performed with the t-test. (D, E) Representative Ca\(^{2+}\) traces recording the Ca\(^{2+}\) elevation induced by 20 μg/ml anti-CD3 antibody in Jurkat cells in the presence of 2 μM TAT-control peptide (ctrlpep) or TAT-BH4 peptide. The antibody was added 1-2 min after recording was started. (F) Histograms summarize the average peak anti-CD3 induced Ca\(^{2+}\) elevation in Jurkat cells treated with different peptides. Symbols represent the mean ± SEM of 4 individual experiments (>50 cells per sample per experiment). Statistical analysis was performed with the t-test.
Figure 3.4 TAT-BH4 peptide inhibits IP3 ester induced Ca\(^{2+}\) elevation.

(A, B) Representative Ca\(^{2+}\) traces recording the Ca\(^{2+}\) elevation induced by 100 µM IP3 ester in wild type WEHI7.2 (Bcl-2 (-)) cells in the presence of 2 µM TAT-control peptide (ctrlpep) or TAT-BH4 peptide. The IP3 ester was added 1-2 min after the recording was started. (C) Summary of the peak Ca\(^{2+}\) elevation induced by 100 µM IP3 ester in TAT-control peptide- and TAT-BH4-treated cells. Symbols represent mean±SEM in three separate experiments (>50 cells per sample per experiment). (D) Summary of the area under the curve of Ca\(^{2+}\) elevation induced by 100 µM IP3 ester in TAT-control peptide- and TAT-BH4-treated cells. Symbols represent mean ± SEM in three separate experiments (>50 cells per sample per experiment).
Figure 3.5 TAT-BH4 peptide’s effect is reversed by peptide 2 and TAT-BH4 peptide does not decrease the thapsigargin induced Ca^{2+} elevation.

(A) Summary of the peak Ca^{2+} elevation induced by 20μg/ml anti-CD3 antibody in 10 μM TAT-peptide2-treated (TAT-pep2), TAT-control peptide (TAT-ctrl) or non-treated Bcl-2 (+) WEHI7.2 cells. Data are from three separate experiments (>50 cells per sample per experiment). Symbols represent mean±SEM of these experiments. (B) Summary of the peak Ca^{2+} elevation induced by 20 μg/ml anti-CD3 antibody in non-treated, 10 μM TAT-ctrl-treated, 2 μM TAT-BH4-treated, 2 μM TAT-BH4 + 10 μM TAT-peptide2-treated, 2 μM TAT-BH4 + 10 μM TAT-control peptide-treated wild type WEHI7.2 cells (Bcl-2(-)). Data are from four separate experiments (>50 cells per sample per experiment). Symbols represent mean±SEM of these experiments. (C) Peak Ca^{2+} elevation induced by 100 nM thapsigargin in wild type WEHI7.2 cells, measured by fluorescence microscopy in the absence of extracellular Ca^{2+} (mean ± SEM, three experiments). (D) Area under the cytosolic Ca^{2+} 340nM/380nM ratio curve in (C) (mean ± SEM, three experiments)
Figure 3.6 FITC-TAT-BH4 peptide is taken up by cells and does not alter ER structure. (A) WEHI7.2 cells incubated with 5 µM fTAT-BH4 peptide (green color) for 1 hr were observed by confocal microscopy. The cells were stained with Hoechst 33342 dye (blue color) to show nuclei. Fluorescence images were merged with phase contrast channel image in the last column. (B) 5 µM FITC-TAT-BH4 (fTAT-BH4) peptide (green color) was incubated with COS-7 cells for 1 hr; the latter had been transfected with ER-dsRed plasmid (red color) (Molecular Probes) or stained with MitoTracker Deep Red 633 (red color) (Molecular Probes). Bars, 5 µm. (C) CO3-7 cells remained untreated or treated with fTAT-BH4; 24 hrs later the cells were examined by confocal microscopy to examine ER structure. The percentage of cells displaying an abnormal ER structural pattern was recorded. Data are from two separate experiments (>150 cells were counted per sample per experiment). Symbols represent mean ± SEM.
Figure 3.7 TAT-BH4 peptide inhibits anti-CD3-induced apoptosis in WEHI7.2 and Jurkat cells without perturbing Bim-Bcl-2 interaction.

(A) Apoptotic WEHI7.2 cells were counted based on apoptotic nuclear morphology (Hoechst 33342 staining) 24 hr after 20 μg/ml anti-CD3 treatment. The cells were pre-incubated ± 2 μM TAT-BH4 peptide for 1 hr and then incubated with 20 μg/ml anti-CD3 for 24 hr. Symbols represent the percentage of cells (mean ± SEM) with apoptotic nuclei in four experiments (>200 cells counted per coverslip). (B) Jurkat cells were pre-incubated ± 2 μM TAT-BH4 peptide or 10 μM TAT-control peptide in the presence or absence of 10 μM TAT-peptide 2 and then incubated with 5 μg/ml anti-CD3 for 24 hr. Symbols represent the percentage of cells (mean ± SEM) with apoptotic nuclei in four experiments (>200 cells counted per coverslip). (C) Bcl-2 was immunoprecipitated from Bcl-2(+) WEHI7.2 cell extracts in the presence of 10 μM TAT-BH4 peptide or TAT-control peptide (ctrl) and Bim was detected by anti-Bim immunoblotting.
CHAPTER 4: BH4 DOMAIN OF BCL-2, A TARGET TO REGULATE IP3-MEDIATED CALCIUM AND APOPTOSIS
ABSTRACT

Bcl-2 is the founding member of a large family of apoptosis regulating proteins. Bcl-2 is a prime target for novel therapeutics because it is elevated in many forms of cancer and contributes to cancer progression and therapy resistance based on its ability to inhibit apoptosis. Bcl-2 interacts with proapoptotic members of the Bcl-2 family to inhibit apoptosis and small molecules that disrupt this interaction have already entered the cancer therapy arena. A separate function of Bcl-2 is to inhibit Ca\(^{2+}\) signals that promote apoptosis. This function is mediated through interaction of the Bcl-2 BH4 domain with the inositol 1, 4, 5-trisphosphate receptor (IP3R) Ca\(^{2+}\) channel. A novel peptide inhibitor of this interaction enhances proapoptotic Ca\(^{2+}\) signals. In preliminary experiments this peptide enhances ABT-737 induced apoptosis in chronic lymphocytic leukemia cells. These findings draw attention to the BH4 domain as a potential therapeutic target. This review summarizes what is currently known about the BH4 domain of Bcl-2, its interaction with the IP3R and other proteins, and the part it plays in Bcl-2's anti-apoptotic function. In addition, we speculate on how the BH4 domain of Bcl-2 can be targeted therapeutically not only for diseases associated with apoptosis resistance, but also for diseases associated with accelerated cell death.
INTRODUCTION

In Chapter 3 we found that the BH4 domain of Bcl-2 is critical for Bcl-2-IP3R interaction. TAT-BH4 peptide mimics the function of Bcl-2 on proapoptotic Ca\textsuperscript{2+} signals. Also the results in Chapter 2 indicate that peptide2 targets the BH4 domain of Bcl-2 to reverse Bcl-2’s inhibitory effect on Ca\textsuperscript{2+} and apoptosis. Therefore this Chapter reviews the recent evidence that the BH4 domain of Bcl-2 mediates an interaction between Bcl-2 and a number of factors involved in regulating cell growth and survival, including the IP3R\textsuperscript{[69, 104, 127]}. By interacting with this intracellular Ca\textsuperscript{2+} channel, Bcl-2 modulates Ca\textsuperscript{2+} signals that mediate apoptosis. It is proposed that interactions involving the BH4 domain of Bcl-2 may also prove to be a worthwhile target for therapeutic development.

The BH4 domain of Bcl-2/Bcl-xL is crucial for its antiapoptotic function

The BH4 domain is highly conserved among antiapoptotic proteins and across species. Thus, for example, there is considerable sequence homology between the BH4 domains of Bcl-2 and Bcl-xL and swapping the BH4 domains between these proteins does not diminish their antiapoptotic function \textsuperscript{[182]}. The BH4 region is the only conserved domain among Bcl-2 family members that is present only in those members with antiapoptotic activity, including Bcl-2, Bcl-xL and Bcl-w, but absent from proapoptotic family members. This fact alone suggests that the BH4 domain plays a critical role in determining Bcl-2’s antiapoptotic potential. This concept is confirmed by evidence that BH4 domain deletion or mutation eliminates the prosurvival activity of Bcl-2 \textsuperscript{[190, 205, 206]} without interfering with the ability of Bcl-2 to bind BH3-only proteins with an affinity similar to that of full length Bcl-2 \textsuperscript{[182]}. Therefore, the BH4 domain-mediated antiapoptotic function of Bcl-2 appears to be independent of Bcl-2’s ability to dimerize
with proapoptotic Bcl-2 family members. BH4-deleted Bcl-2 (ΔBH4Bcl-2) lacks protective function in multiple forms of apoptosis, including that induced by IL-3 deprivation, staurosporine, γ-irradiation and dexamethasone [182, 205]. Also, ΔBH4Bcl-2 may possibly function as a dominant negative inhibitor of Bcl-2 [205]. Others have suggested that ΔBH4Bcl-2 functions like Bax to promote rather than inhibit cell death [207].

**BH4 domain interacting proteins**

The BH4 domain represents a critical region within the Bcl-2 molecule for the prevention of apoptosis through its ability to interact with the following apoptosis regulating proteins. The regions in the BH4 domain where several proteins are known to interact are shown in Figure 4.1.

**Calcineurin**

Calcineurin is reported to interact with the BH4 domain of Bcl-2 in baby hamster kidney cells, Jurkat cells and SUDHL-4 B-cell lymphoma cells [108, 208]. Amino acids 1-20 at the N terminus of Bcl-2 (Figure 4.1), located in the BH4 domain, are sufficient and necessary to interact with calcineurin. Bcl-2 sequesters active calcineurin from NF-AT, thereby inhibiting calcineurin-dependent NF-AT signaling. Interestingly, the proapoptotic protein Bax interferes with the interaction between Bcl-2 and calcineurin. It is still not clear if the Bcl-2-calcineurin interaction disrupts activation induced cell death in T cells or contributes to lymphoproliferation.

Calcineurin is not only reported to be anchored to Bcl-2 but also to the IP3R. The interaction of calcineurin with the IP3R is thought to regulate the phosphorylation status
of the receptor, resulting in a Ca^{2+}-sensitive regulation of IP3-mediated Ca^{2+} flux [77]. The regulation of IP3R-mediated Ca^{2+} signaling by calcineurin has been most extensively characterized in the immune system [21, 74, 209]. Billingsley and coworkers reported that calcineurin interacts with both Bcl-2 and the IP3R in cortical and hippocampal slices and proposed that calcineurin shuttles between these two proteins [79, 80]. This triple protein complex contributes to cell survival in primary neuronal cells.

*VDAC*

The IP3R is not the only intracellular channel with which antiapoptotic Bcl-2 family members interact. The BH4 domain of Bcl-xL has been reported to interact with the voltage dependent anion channel (VDAC), which is located on mitochondria and responsible for regulating mitochondrial membrane potential [210-212]. The interaction of the BH4 domain with VDAC is required for Bcl-xL to inhibit etoposide-induced cytochrome c release and preserve VDAC channel activity and mitochondrial membrane potential [192]. Although the N-terminus of Bcl-xL (amino acids 2-19aa) coimmunoprecipitates with VDAC (Figure 4.1), △BH4 Bcl-xL still interacts with VDAC suggesting that other regions of Bcl-2 in addition to the BH4 domain may interact with the IP3R. BH4 peptide’s antiapoptotic function has been attributed to the inhibition of VDAC channel activity, thereby preventing apoptotic mitochondrial changes [192]. But knocking out VDAC in mice has no effect on mitochondrial permeability transition and cell death, indicating that VDAC is dispensable for mitochondria-dependent cell death [196]. This has led some to question the importance of the VDAC channel in mitochondria-mediated apoptosis, and hence to question whether the interaction between Bcl-xL and VDAC contributes significantly to Bcl-xL’s antiapoptotic function. However,
one isoform of VDAC was not completely eliminated in the VDAC knockout mouse, leaving open the possibility that VDAC may still be involved in mediating apoptosis.

**Raf-1**

Raf-1, a serine/threonine protein kinase in the MAPK/ERK signal transduction pathway, plays important roles in cell cycle, apoptosis, and differentiation [213, 214]. It has been reported to interact with the BH4 domain of Bcl-2 (residues 11-33) and in this manner to regulate apoptosis (Figure 4.1) [111, 215], although this interaction may not be stable in cellular extracts [216]. One theory is that Bcl-2 targets Raf-1 to mitochondria to block cell death by phosphorylating the BH3-only proapoptotic protein Bad. Ras also interacts with Raf-1 and targets it to the plasma membrane [217]. Thus, it is possible that competition between Ras and Bcl-2 for binding to limiting amounts of Raf-1 determines Raf-1’s localization, substrates and effects on cell survival or death [111].

**Ras**

The BH4 domain of Bcl-2 is also reported to interact with activated Ras and thereby block Ras-mediated apoptotic signaling [194]. Ras is a small GTPase oncogene involved in many cell processes, including proliferation, differentiation and apoptosis, mainly through its effects on signaling pathways regulated by MAP kinase, growth factors, and Fas [218-220]. Deletion of the BH4 domain from Bcl-2 abrogates the coimmunoprecipitation of Bcl-2 with Ras and eliminates the antiapoptotic effect of Bcl-2 in Fas-induced apoptosis.

**CED-4**

Both Bcl-2 and Bcl-xL interact with the non-mammalian proapoptotic protein CED-4, originally identified in *C. elegans*, and the BH4 domain of Bcl-2 appears required for this
interaction [182]. CED-4 enhances CED-3-induced cell death and full length Bcl-xL, but not ∆BH4 Bcl-xL, antagonizes the apoptotic activity of CED-4. Apaf-1 is a CED-4 mammalian homologue. However, although there were initially reports that Bcl-2 interacts with Apaf-1, these reports were not subsequently confirmed by other groups [221-223].

*Paxillin*

Paxillin is a focal adhesion-associated adaptor protein, serving as a docking protein to interact with focal adhesion and cytoskeleton or signal transduction proteins. It is required in embryonic development and plays critical roles in cell spreading and motility [224]. Cell adhesion determines tissue architecture during morphogenesis and has an impact on apoptosis. Recent work by Sorenson showed that the BH4 domain of Bcl-2 interacts with paxillin in embryonic kidney lysates and lysates from HEK293 and NIH3T3 cells [195]. Amino acids 17-31 in the BH4 domain of Bcl-2 are necessary for the Bcl-2 interaction with paxillin. Tyrosines 21 and 28 in the BH4 domain are especially critical for this interaction (Figure 4.1). A BH4 domain peptide is also sufficient to interact with paxillin and disrupt nephrogenesis. Although how Bcl-2 regulates apoptosis by interacting with paxillin is still not understood, it has been proposed that Bcl-2 protects cells from apoptosis caused by loss of adhesion. Bcl-2 may bypass integrin-mediated survival signals via interactions with paxillin/FAK, circumventing the need for adhesion. This provides integration of adhesive and survival signals which is critical for normal development without the threat of apoptosis [225].

*NF-κB*
Nuclear factor κB, a transcription factor, plays an important antiapoptotic function in mammalian cells [226, 227]. NF-κB activation is required for Bcl-2’s antiapoptotic function in ventricular myocytes [193]. Also, the presence of Bcl-2-NF-κB complexes has been confirmed in nuclear fractions of NIH3T3 cells and it is thought that this interaction contributes to Bcl-2’s roles in cell cycle control and apoptosis [109]. Full length Bcl-2 has been shown to enhance NF-κB’s DNA binding activity, but this activity is lost when the BH4 domain is deleted from Bcl-2. Also, both the level and activity of the NF-κB inhibitor IκBα were suppressed by Bcl-2 but not by the ΔBH4Bcl-2.

**IP3R**

Recently we found that Bcl-2 interacts with all three subtypes of IP3R, documented by multiple experimental approaches, including coimmunoprecipitation, Blue Native Gel Electrophoresis, GST pull-down (Figure 2.3) and Fluorescence Resonance Energy Transfer (Figure 2.1) [104, 127]. The interaction of Bcl-2 and Bcl-xL with the IP3R has been confirmed by a number of laboratories [69, 105, 106, 128, 129]. Although Bcl-2 is widely known to localize to mitochondria, it is also well documented on the ER where it interacts with the IP3R, an IP3 sensitive intracellular Ca\(^{2+}\) channel. The IP3R transmits Ca\(^{2+}\) from the ER lumen to the cytoplasm, elevating cytoplasmic Ca\(^{2+}\) concentration and thereby generating Ca\(^{2+}\) signals that mediate a wide range of cellular processes, including apoptosis. Through its interaction with IP3R’s, Bcl-2 inhibits IP3-dependent opening of IP3R channels reconstituted in planar lipid bilayers and also inhibits IP3-dependent Ca\(^{2+}\) elevation induced by T cell receptor (TCR) activation or by a cell permeant IP3 ester. We recently mapped the Bcl-2 interacting site to an eighty amino acid sequence within the regulatory and coupling domain of the IP3R, and based on a twenty amino acid sequence
within this region developed an inhibitory peptide, referred to as peptide 2, that disrupts the Bcl-2-IP3R interaction \[127\] (Figure 4.2). By using peptide 2 to abrogate the Bcl-2-IP3R interaction, we established that this interaction is indeed necessary for Bcl-2’s inhibitory effect on IP3-mediated Ca\(^{2+}\) elevation and apoptosis in lymphocytes following TCR activation. Thus, the regulatory effect of Bcl-2 on IP3-induced Ca\(^{2+}\) elevation contributes to Bcl-2’s antiapoptotic action by a mechanism different from the well-known inhibitory effect of Bcl-2 on proapoptotic members of the Bcl-2 protein family.

In Chapter 3, we found that the BH4 domain of Bcl-2 is necessary and sufficient to interact with the IP3R. Moreover, a peptide corresponding to the BH4 domain, when coupled with HIV-TAT to facilitate its entry into cells, inhibits IP3-dependent Ca\(^{2+}\) elevation and apoptosis in lymphocytes following TCR activation. These findings further indicate that Bcl-2’s inhibitory effect on IP3R-mediated Ca\(^{2+}\) elevation and apoptosis is independent of other BH domains or binding with other Bcl-2 family members.

In summary, the BH4 domain of Bcl-2 interacts with a number of different factors and thereby plays an important role in apoptosis inhibition. Although our laboratory has focused mainly on the interactions of Bcl-2 and Bcl-xL with the IP3R, the other interactions discussed above may contribute together with the Bcl-2-IP3R interaction to enable Bcl-2 to regulate a wide range of signaling pathways, perhaps including pathways not directly related to apoptosis.
**Bcl-2 inhibitors**

Many diseases can be attributed directly or indirectly to dysregulation of apoptosis. Targeting apoptosis has become an attractive therapeutic strategy in the treatment of these disorders, especially cancer which is often associated with resistance to apoptosis, often due to elevated levels of Bcl-2 or other antiapoptotic Bcl-2 family members. A number of proteins and small molecules designed to trigger cell death have entered the clinic for use against cancer. Due to Bcl-2’s antiapoptotic and oncogenic function in cancer cells, Bcl-2 draws a lot of attention as one of the major targets in apoptosis targeting therapies. Many small molecule Bcl-2 inhibitors are in the pipeline. Genasence (Genta), an antisense oligonucleotide targeted against Bcl-2, can reduce Bcl-2’s expression level [228, 229]. It has entered a Phase III clinical trial for chronic lymphocytic leukemia (CLL) and metastatic melanoma [133, 230]. ABT-737 and ABT-263 by Abbott Pharmaceuticals [231], Obatoclax by Gemin X [232], and AT-101 by Ascenta [233] are all BH3 only protein mimetics that bind to the hydrophobic groove of Bcl-2/Bcl-xL and displace proapoptotic proteins from Bcl-2's inhibitory grip. A small molecule Bcl-2 inhibitor by Infinity Pharmaceuticals also interrupts the interactions between Bcl-2 and its proapoptotic binding partners. Some other natural and chemical inhibitors of Bcl-2, including HA14-1 [234], BH3I-I [235], chelerythrine [236], and gossypol [237] appear mainly to regulate the Bcl-2 interaction with other Bcl-2 family members. As described before, the site targeted by these novel therapeutics is the hydrophobic groove formed by the BH1, BH2 and BH3 domains. Surprisingly, none of them target the BH4 domain of Bcl-2 to antagonize Bcl-2’s antiapoptotic function, even
though the BH4 domain contributes to the antiapoptotic function of Bcl-2 as summarized as above.

The concept that the Bcl-2 BH4 domain may be a worthy therapeutic target arises in part from our studies of the Bcl-2-IP3R interaction, which is mediated through the BH4 domain. As discussed earlier, a 20 amino acid peptide (peptide 2), mimicking the Bcl-2 binding site sequence on the IP3R, abrogates the Bcl-2-IP3R interaction, thereby reversing Bcl-2’s inhibitory effect on IP3-mediated Ca\(^{2+}\) signaling and apoptosis. Peptide 2 is derived from the sequence of IP3R that binds to the BH4 domain of Bcl-2. Peptide 2 does not interfere with the interaction of Bcl-2 with Bim. Thus, peptide 2 targets the BH4 domain of Bcl-2 instead of the hydrophobic groove formed by BH1, 2 and 3 domains. Peptide 2’s proapoptotic effect makes it of potential value in cancer therapy.

**BH4 peptide has antiapoptotic function**

Interestingly, although the BH4 domain has not previously been targeted to reverse Bcl-2’s antiapoptotic action, the BH4 domain peptide is used to mimic Bcl-2 as an antiapoptotic reagent in many apoptosis models. Shimizu et al used a TAT-BH4 peptide to facilitate BH4 peptide entry into cells and found that the TAT-BH4 peptide prevents Ca\(^{2+}\)-induced loss of mitochondrial membrane potential and cytochrome c release [192]. Moreover, the TAT-BH4 peptide inhibited X-ray- and VP-16 (Etoposide)-induced apoptosis, but not tunicamycin-induced apoptosis in PC-12 or Hela cells. Injection of TAT-BH4 peptide into the peritoneum of C57BL6J mice conferred protection against X-ray-induced cell death in the small intestine [197]. Also, TAT-BH4 peptide suppressed
anti-Fas induced fulminant hepatitis [197] and improved ischemia-reperfusion-induced cardiac dysfunction, therefore attenuating ischemia-reperfusion injury in the rat heart [238]. Other groups found that BH4 peptide inhibits oxidative stress induced coronary endothelial cell apoptosis [199], human islet cell apoptosis, staurosporine and serum deprivation-induced apoptosis [239, 240], radiation-induced apoptosis in human T lymphocytes and B cells [198], beta-amyloid peptide-induced apoptosis, and sepsis-induced apoptosis [134]. In addition, BH4 peptide’s convenient delivery into cells and antiapoptotic effect endow it protective roles in spinal cord injury [200], islet transplantation [239], neurotoxicity and hippocampal damage in vivo [201].

Recently, IP3R mediated Ca\(^{2+}\) release has been implicated as contributing to the pathogenesis of Alzheimer’s disease [202]. Interestingly, TAT-BH4 peptide reduces the toxic effect of beta-amyloid peptide on capillary endothelium [204]. The mechanism of the protective action of TAT-BH4 in Alzheimer’s disease is not fully elucidated. We have found TAT-BH4 peptide, like full length Bcl-2, inhibits IP3R-mediated Ca\(^{2+}\) release from the ER and apoptosis in T cells (Figure 3.6 and 3.7). This raises the possibility that a similar mechanism might be relevant in Alzheimer’s disease.

In summary, there is considerable interest in development of antiapoptotic therapies for diseases associated with accelerated cell death, based on the BH4 domain of Bcl-2 or Bcl-xL, even though the role of the BH4 domain in Bcl-2/Bcl-xL function has not been fully elucidated.
In view of BH4 domain’s importance as a potential target to regulate Ca\(^{2+}\) signaling and apoptosis, the concept of blocking BH4 domain interactions with other proteins involved in regulating or mediating apoptosis (e.g., IP3R, calcineurin, paxillin) by small molecules or peptides, as illustrated by peptide 2, provides another opportunity to design Bcl-2 inhibitors. Not only does the BH4 domain's role in apoptosis regulation provide an opportunity to develop therapeutics designed to enhance apoptosis by abrogating Bcl-2’s antiapoptotic function, but the ability of the BH4 peptide by itself to inhibit apoptosis encourages efforts to identify or engineer molecules that mimic the BH4 domain and thus have antiapoptotic function similar to that of full length Bcl-2/Bcl-xL. These would likely prove to be useful in diseases associated with accelerated cell death. Based on what is known about the structure and location of the BH4 domain in Bcl-2 these goals should be feasible. The BH4 domain is an \(\alpha\)-helical region located at the N-terminus of Bcl-2. Based on conservation and surface accessibility, the Bcl-2 BH4 domain residues predicted to be most like involved in binding other proteins are D10 and R12 (on one face) and H20, Y21, Q25, R26, and Y28 (on the opposite face) (Figure 4.3). These residues are conserved in BH4 domains of seven of the closest mammalian Bcl-2 family member sequences and have highly accessible exposed side chains available for specific binding to other molecules. A positive and a negative residue are located on one face, and positively charged residues on the opposite face which may contribute to ionic or hydrogen bond interactions. The residues R12, I14, V15, Y18, I19 and L23 have been reported indispensable for the anti-apoptotic activity of Bcl-2 [182, 206].
Notably, the hydrophobic groove formed by the BH1-3 domains, responsible for interaction with BH3-only proteins and targeted therapeutically by BH3-mimetics, is separate from the BH4 domain in the three dimensional structure of Bcl-2. Thus, it will be interesting to explore targeting the predicted binding sites of the BH4 domain to develop therapeutics that act by a different mechanism to inhibit Bcl-2’s antiapoptotic activity.

Summary

The BH4 domain is present only in antiapoptotic members of the Bcl-2 protein family and thus is a major distinguishing feature that separates the antiapoptotic and proapoptotic family members at a molecular level. Although this is the case, exploring the BH4 domain for its therapeutic potential has lagged far behind the concentrated efforts focused on the interaction of antiapoptotic family members with proapoptotic family members mediated through the BH1, BH2 and BH3 domains. The BH4 domain is not only a promising target for small molecule therapeutics intended to reverse the antiapoptotic functions of Bcl-2 and Bcl-xL, but also a useful model for therapeutics intended to inhibit apoptosis based on considerable evidence that a BH4 peptide itself inhibits proapoptotic Ca\(^{2+}\) signals and apoptosis in a variety of settings. The BH4 domain-mediated interactions with IP3R and other apoptotic regulators is another dimension of Bcl-2’s function that is not sufficiently understood. The development of peptide inhibitors or small molecule peptide mimetics directed at the BH4 domain of Bcl-2 will be one way to further elucidate the function of the BH4 domain. This is illustrated by our recent development of peptide 2 based on detailed analysis of the interaction of Bcl-2 with the IP3R. The use of peptide 2 has already established the importance of the
Bcl-2-IP3R interaction in regulating Ca\(^{2+}\) signals and apoptosis, and even suggested potential therapeutic utility of targeting the BH4 domain for treatment of diseases associated with elevated Bcl-2. Perhaps in the future BH4 domain-based Bcl-2 targeting strategies combined with BH3 mimetic strategies will inhibit Bcl-2’s function more efficiently than either alone in cancer therapy. Alternatively, perhaps therapeutics based on the intrinsic antiapoptotic activity of the BH4 domain will be useful for treatment of diseases associated with accelerated cell death.

Here, we tested TAT-peptide2 in ABT-737-induced chronic lymphocytic leukemia (CLL) cell apoptosis. Since ABT-737 targets to the hydrophobic groove of Bcl-2, and peptide 2 targets to another location (BH4 domain) on Bcl-2, we propose that peptide 2 may have synergistic killing effect with ABT-737 in CLL cells.
MATERIALS AND METHODS

Cell Culture

WEHI7.2 cells (wild type WEHI7.2, Bcl-2 overexpressing WEHI7.2 (Bcl-2(+) WEHI7.2) and WEHI7.2 cells transfected with empty vector (Bcl-2 (-) WEHI7.2)) were incubated in DMEM supplemented with 10% bovine calf serum, 2 mM L-glutamine, and 100 μM of non-essential amino acids. Jurkat cells (clone E6-1) were cultured in RPMI1640 supplemented with 10% fetal bovine serum. Bcl-2 (+) WEHI7.2 cells and vector control cells were generated by transfecting the Bcl-2 cDNA in pCDNA3.1 vector into wild type WEHI7.2 cells using electroporation.

Chronic lymphocytic leukemia cells separation and culture

Lymphocytes were freshly separated from heparinized peripheral blood obtained from adult patients with chronic lymphocytic leukemia meeting standard diagnostic guidelines. We conformed to all guidelines and regulations in accordance with Internal Review Board protocols ICC2902/11-02-28 (Case Western Reserve University Cancer Center/University Hospitals of Cleveland Ireland Cancer Center). Cells were separated by centrifugation through Ficoll-Hypaque and cultured in RPMI medium (10% fetal bovine serum) at a density of 2.0×10⁶ cells/ml. CLL cells were treated in vitro with 2 μM ABT-737 in the presence or absence of 5 μM TAT-peptide 2 (pep2) or TAT-control peptide (ctrlpep). Cell death was detected by trypan blue staining after 24 hr treatment.
Caspase apoptosis assay

Caspase-Glo 3/7 assay (Promega) was used to measure the cell caspase activity. The cells in 96-well plates were equilibrated to room temperature before the measurement. 100 µl of Caspase-Glo 3/7 reagent was added into each well of a white-walled 96-well plate containing 100 µl of blank, negative control cells or treated cells in culture medium. Gently mix contents of wells using a plate shaker at 300-500rpm for 30 sec. Incubate at room temperature for 30 min to 3 hours, depending upon cell culture system. Then measure the luminescence of each sample on Victor 3 multilabel counter (Perkin Elmer)
RESULTS

*Peptide 2 enhances ABT-737 induced apoptosis in CLL cells*

We tested the effect of peptide 2 in CLL, a common human malignancy associated with elevated levels of Bcl-2. CLL cells are "addicted to Bcl-2" because they have elevated levels of both BH3-only proteins and Bcl-2; consequently, CLL cells undergo apoptosis if the ability of Bcl-2 to bind and inhibit BH3-only proteins is abrogated by treatment with the BH3-mimetic ABT737 [91]. In preliminary experiments, we found that the control inactive ABT-737 (IABT) has toxic effects similar to that of active ABT-737 when used at a high concentration (10 µM). Thus, IABT appears to have a nonspecific toxic effect to some degree, especially at high concentrations. Therefore we chose a lower concentration of ABT-737 (0.01-2 µM) to induce apoptosis. Although peptide 2 by itself was not toxic to primary CLL cells, 5 µM peptide 2 significantly enhanced ABT-737 induced cell death (Figure 4.4). We also confirmed these results by caspase assay. Peptide 2 enhanced the caspase activity by ~15% at 6 hrs in four CLL patient samples. It would appear that a combinatory inhibitory effect is achieved by simultaneously targeting two different sites on Bcl-2. Although this preliminary study involved CLL cells from only six patients, the results are promising enough to cast light on the potential importance of the BH4 domain as a therapeutic target.

We also tested the effect of peptide 2 in Jurkat and WEHI7.2 cells. At 6 hrs we did not detect significant cell death by trypan blue staining. But 15%-30% cells started to die by 18-24 hrs after ABT-737 treatment. In all the conditions, 10 µM TAT-peptide 2 enhanced ABT-737-induced cell death in B17 (a Bcl-2 overexpressing WEHI7.2 clone) or Jurkat.
cells. We are still optimizing the conditions of ABT-737 concentration and time point in cells lines.

**DISCUSSION AND FUTURE DIRECTIONS**

In our experiments, ABT-737 induces apoptosis in Bcl-2 overexpressing WEHI7.2 cells, Jurkat cells and primary CLL cells. Peptide 2 enhanced ABT-737-induced apoptosis by 15-30%. The sensitivity of the CLL cells to ABT-737 is different among patient samples. It is probably dependent on the different Bcl-2 levels in different samples. CLL is a heterogeneous disease which accounts for the variation in efficacy of ABT-737 and peptide 2 at inducing cell death in different patient samples. Peptide 2 and ABT-737 treatment appear more effective in Bcl-2 overexpressing WEHI7.2 cells than in Bcl-2 (-) WEHI7.2 cells. This is consistent with the hypothesis that effects of both peptide 2 and ABT-737 are dependent on Bcl-2 proteins. In Bcl-2 (-) WEHI7.2 cells there is a detectable level of Bcl-xL, which can bind ABT-737. This accounts for the ability of ABT-737 to induce apoptosis in those cells.

The detailed mechanisms involved in the enhancement of ABT-737 induced apoptosis by TAT-peptide 2 are not understood yet. ABT-737 induces apoptosis by abrogating the interaction of Bcl-2/Bcl-xL with BH3 only proteins, such as Bim and Bid. It is not clear if ABT-737 induces calcium signals in CLL cells. Peptide 2 ostensibly interacts with the BH4 domain of Bcl-2 to disrupt Bcl-2-IP3R interaction. We did find that peptide 2 inhibited Bcl-2-IP3R interaction (Figure 2.4 B, C and E). However, we have not tested if peptide 2 directly interacts with the BH4 domain of Bcl-2. Evidence presented in Chapter
2 (Figure 2.4F, G) indicates that peptide 2 does not regulate the hydrophobic groove-mediated Bcl-2-Bim interaction. Since peptide 2 targets to the BH4 domain of Bcl-2, it may interfere with other BH4 domain interacting proteins as described in the Introduction. It is not possible at this point to be sure whether the mechanism of action of peptide 2 in this context is the disruption of Bcl-2’s interaction with the IP3R or one of the other BH4 domain interacting partners described above. Based on this evidence, two major hypotheses are proposed to explain how peptide 2 enhances ABT-737-induced apoptosis.

1. Peptide 2 enhances proapoptotic calcium signals by reversing Bcl-2’s inhibitory effect on IP3R channel activity. If ABT-737 directly or indirectly induces proapoptotic calcium signals, peptide 2 will be able to enhance apoptosis by enhancing these calcium signals. Alternatively, ABT737 does not regulate any calcium signals, but it demolishes the Bcl-2’s inhibitory effect on proapoptotic BH3-only proteins. The apoptotic pathway mediated by BH3-only proteins could be downstream of peptide-induced proapoptotic calcium signals. Therefore peptide 2 alone would not be expected to induce apoptosis due to the blockage of the BH3-only protein-mediated apoptotic pathway by Bcl-2. But, one may speculate that in the presence of ABT-737, peptide 2-induced calcium signals would be able to trigger cell death by activating BH3 only proteins.

2. Peptide 2 enhances apoptosis by disrupting the interaction between the BH4 domain of Bcl-2 with other interacting proteins, such as calcineurin, VDAC, NF-kB, Raf-1 (see introduction). As we know, peptide 2 targets the BH4 domain of Bcl-2. We have not checked how specific the peptide 2 is. It is possible that peptide 2 not only displaces IP3R from BH4 domain but also interfere with the
binding of other BH4 domain interacting proteins. Calcineurin, VDAC, NF-kB have been reported to regulate cell apoptosis and proliferation. Our preliminary data has suggested that peptide 2 may not interfere with Bcl-2-VDAC and Bel-2-Calcineurin interactions during coimmunoprecipitation. We have not tested other BH4 domain binding proteins. This may also contribute to peptide’s synergistic proapoptotic effect with ABT-737.

We already have some preliminary data indicating that peptide 2 alone can induce significant calcium oscillations in WEHI7.2 cells. Next we are going to study if these calcium oscillations contribute to cell death and how peptide 2 induces these calcium oscillations. As summarized above, peptide 2 enhances anti-CD3-induced apoptosis and ABT-737-induced apoptosis. This drives us to test if peptide 2 affects other types of apoptosis. We have tested dexamethasone-induced apoptosis in WEHI7.2 cells, etoposide-induced apoptosis and anti-CD20-induced apoptosis. However, preliminary experiments failed to show an enhancing effect of peptide 2 on apoptosis induction by these agents. Interestingly, peptide 2 appears to enhance proteasome inhibitor MG132-induced cell death in T cells in the preliminary tests. Further study of peptide 2’s proapoptotic effect in other types of apoptosis will explore a broader therapeutic application for the BH4-IP3R targeting strategy.
FIGURES

Figure 4.1 The binding regions of various proteins on the N-terminus of Bcl-2 (BH4 domain).
Bcl-xL (1-24): MSQSNRELVDFLSYKLSQKGYSW
Bcl-2 (1-30): MAHAGRTGYDNREIVMKYIHYKLSPQRGEYW

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VDAC
Figure 4.2 Bcl-2 interacts with the regulatory and coupling domain of the IP3R.

The locations of various structural and functional domains in the IP3R [12, 70, 161] are shown here, in addition to the site within the regulatory and coupling domain of the IP3R where Bcl-2 interacts. The sequence of peptide 2, which inhibits the interaction of Bcl-2 with the IP3R, was derived from the sequence of the Bcl-2 binding site on the IP3R [127].
Figure 4.3 BH4 domain residues in the Bcl-2 structure.

The NMR structure of Bcl-2 bound to a sulfonamide inhibitor is shown as space-filling spheres. BH4 domain residues which are most conserved in the 7 closest homolog sequences are purple (conserved residues 6, 8-15, 17-21, 23, 25-28, and 30) while those that are not conserved are gray. The remainder of the Bcl-2 structure is cyan, with the bound sulfonamide inhibitor in yellow. (Figure drawn using MolScript [241] and Raster3D [242] using coordinates with PDB accession code 1ysw [231]).
Figure 4.4 Peptide 2 enhances ABT-737-induced apoptosis in CLL cells.

CLL cells were treated in vitro with either 2 µM ABT-737 (A) or 0.01 µM ABT-737 (B) in the presence or absence of 5 µM TAT-peptide 2 (pep2) or TAT-control peptide (ctrlpep). Cell death was detected by trypan blue staining after 24 hr treatment. Six individual experiments were analyzed by the two-tailed Student’s $t$ test, using a significance level of $p<0.05$. 
CHAPTER 5: PRELIMINARY RESULTS, FUTURE DIRECTIONS AND SUMMARY
INTRODUCTION

Bcl-2 mutants

As discussed in Chapters 2 and 3, we found that the BH4 domain of Bcl-2 is necessary for Bcl-2-IP3R interaction. In order to study which amino acids in the BH4 domain of Bcl-2 are critical for Bcl-2-IP3R interaction, individual clones of WEHI7.2 cells expressing a series of Bcl-2 mutants were prepared. Dr. Tristram Parslow provided 14 deletion scanning mutants of human Bcl-2, 12 dicodon BH4 domain Bcl-2 mutants and 6 single point mutants of the BH4 domain (I14G, V15G, Y18G, I19G, K22G, L23G). The 14 deletion scanning Bcl-2 mutants virtually spanned the entire Bcl-2 coding sequence [205]. Each contained an in-frame deletion of 14-60 native codons, which were replaced by a BglII restriction site encoding the dipeptide Arg-Ser. The mutants were designated as Δ1 to Δ13. The 6-20aa region of BH4 domain was mutated by oligonucleotide-directed mutagenesis. In each mutant, sequences encoding a pair of adjacent residues were replaced by glycine (or, where glycine was normally present, alanine) codons [206]. Previously, attempts were made to express most of the mutants in QT6 quail cells. However, the Δ4, 8, 9, G18-19, G24-25, 26-27 mutants had very low expression levels [206].

Parslow et al. have shown that Bcl-2 activity was only partially reduced after deletion of the hydrophobic tail (C-terminus) that anchors Bcl-2 on membrane [205]. The BH4 domain of Bcl-2 is not required for Bcl-2 dimerization in vivo, but its deletion mutant promoted rather than inhibited apoptotic death. Δ2 and Δ3 (30-91aa) still maintain the
majority of Bcl-2 anti-apoptotic function, but the antiapoptotic function of other deletion mutants was impaired. Ile-14, Val-15, Tyr-18, Ile-19 and Leu-23 amino acids in the BH4 domain are essential for Bcl-2’s anti-apoptotic activity, based on evidence that transient transfection of these five point mutants inhibited Bcl-2’s activity, whereas other residues (6-29aa) could be replaced by glycine or alanine without significant loss of activity.

We obtained the plasmid constructs of these Bcl-2 mutants and tested them in our WEHI7.2 cells. The purpose was to identify Bcl-2 mutants that do not interact with the IP3R. Therefore, we measured the effect of these Bcl-2 mutants on anti-CD3-induced calcium elevation and and apoptosis (see Results Figure 5.2 and 5.3).

**IP3R is regulated by phosphatases and kinases**

As introduced in Chapter 1, the IP3R is regulated by many phosphatases and kinases, including calcineurin, PP1α, Fyn, PKC, and PKA. Previously, Rui Chen in our lab found that IP3R phosphorylation was decreased in Bcl-2 overexpressing WEHI7.2 cells. Although we have shown that Bcl-2 regulates IP3R channel activity in lipid bilayer experiments, the IP3R employed in these experiments was derived from cerebellar microsomes and hence not pure; therefore, we cannot exclude the possibility that Bcl-2’s effect on IP3R involve other proteins in association with Bcl-2.

Bcl-2 directly interacts with IP3R shown by GST pull down experiments involving interaction of purified recombinant Bcl-2 with purified GST-IP3R domains (Figure 2.3). Therefore, we proposed three possible models of how Bcl-2 regulates IP3R channel activity, described in Figure 5.1. Model 1 suggests that Bcl-2 may directly regulate IP3R
by itself. Model 2 proposes that Bcl-2 may displace kinases from IP3R. In this regard, kinases Fyn and Lck are investigated in this thesis since both are involved in TCR signaling and interact with the IP3R. Model 3 shows that Bcl-2 docks one or more phosphatases (e.g., PP1, calcineurin) to the IP3R to inhibit IP3R phosphorylation. Importantly, these models are not mutually exclusive and can coexist at the same time.

Protein phosphatase 1 (PP1) is a major serine/threonine phosphatase that regulates many cellular functions. PP1 is one of the PP1 family members in mammalians. Its catalytic subunit (PP1c) can interact with over fifty different regulatory subunits [243]. Protein Kinase A, protein phosphatase PP1 and the IP3R form a macromolecular complex in the cerebellum [76]. PKA activates while PP1 inhibits the activity of IP3R1 channel activity in planar lipid bilayer experiments [76]. The IP3R activity can be regulated by the balance of the activities between PP1 and PKA. Recent findings suggest that PP1 is involved in Bel-2’s inhibitory effect on the IP3R [129].

Calcineurin (PP2B) is a Ca\(^{2+}\) calmodulin dependent protein phosphatase which has an important role in the control of intracellular Ca\(^{2+}\) signaling. The protein has one catalytic (CnA) subunit and one regulatory (CnB) subunit. The regulatory subunit is essential for the high specific activity of the phosphatase. CnA has two isoforms, \(\alpha\) and \(\beta\). A model of FKBP12, calcineurin and IP3R interaction has been claimed. FKBP12 binds to the IP3R at 1400-1401 amino acids (within domain 3a1) and docks calcineurin to this site [77, 78]. However, these findings were not confirmed by other groups [244, 245]. The phosphorylation of IP3R by calcineurin has been extensively reviewed [74]. Calcineurin-regulated Ca\(^{2+}\) signals play an important role in the T cell receptor activation pathway [209]. Bel-2 interacts with calcineurin as reviewed in Chapter 4 [108]. Recently,
Billingsley’s group reported that Bcl-2, calcineurin and IP3R form a complex. According to their findings, calcineurin-IP3R interaction is mediated by Bcl-2. The Bcl-2-calcineurin interaction increases in response to excitotoxic agents and hypoxia/aglycia. Their theory is that Bcl-2 may shuttle calcineurin to the IP3R, because the IP3R-calcineurin interaction also increases under these conditions [79, 80]. Therefore, in this chapter we checked if Bcl-2 overexpression increases the calcineurin-IP3R interaction. The increase of this interaction may dephosphorylate IP3R and inhibit IP3R channel activity.

Fyn and Lck are Src family tyrosine kinases which have a tyrosine kinase (SH1) domain located at the C-terminal end [246]. The center of the protein contains two modulatory Src Homology (SH) domains which mediate protein-protein interactions. Lck is more abundantly found at the plasma membrane, whereas Fyn largely colocalizes with centrosomal and mitotic structures. Lck specifically interacts with the CD4/CD8 complex through a dicysteine motif present in its unique N-terminal domain [247]. Fyn has two isoforms. The binding specificities of the SH2 and SH3 domains of Lck and Fyn are different. Fyn (T) is expressed in T cells by using exon 7B. Fyn (B) is expressed in brain and other tissues from exon 7A. Both Fyn and Lck play important roles in the T cell receptor signaling pathway. The Fyn (T) knockout mice cannot respond to stimulation of the TCR with mitogen or antigen [248]. Fyn binds to the CD3-TCR complex and is activated when the TCR is crosslinked [246]. Fyn has been shown to activate an IP3-gated calcium channel in vitro. In Fyn knockout mice, tyrosine phosphorylation of the IP3R is reduced during T cell activation [84]. T cell receptor activation triggers the IP3R-Fyn interaction which induces phosphorylation of IP3R at tyrosine 353 [83]. As discussed
before [23], Bcl-2 may play a role in T cell development by regulating T cell receptor activation induced Ca\(^{2+}\) signals. Therefore, we propose that Bcl-2 regulates the interaction between Fyn and IP3R by interacting with the IP3R.
METHODS

Generation of stable mixed populations of Bcl-2 mutants

10 million cells were collected by centrifugation and then resuspended in 800 µl cold OPTI-MEM medium (Invitrogen) containing 50 µg plasmid DNA. Cells were electroporated in a 0.4 cm cuvette at 1125 µF, 800V, then immediately placed into a T-75 flask containing 30 ml of complete growth medium. Two hours after electroporation, 0.6 ml (1mg/ml) G418 was added to the flask. 7 days later, transfected cells were collected by centrifugation, and resuspended in 5 ml PBS. Ficoll-hypaque (5 ml) was placed into a 15 ml conical tube and carefully overlayered with 5 ml of the cell suspension in PBS. Following centrifugation at 2000 rpm for 25 min, the live lymphocytes which will form a band at the interface between the two solutions were collected. Cells were washed twice with 20 ml PBS and diluted into growth medium with G418 for culture. When enough cells were available, cells were harvested and analyzed to determine the mutant Bcl-2 expression level.

Immunoprecipitation, western blot, GST pull-down and calcium measurement

As described in previous Chapters, anti-IP3R type1 (Calbiochem, 407144), anti-calcineurin (BD Biosciences, 610259), anti-FKBPI2 (BD Biosciences, 610808), anti-PP1α (Upstate Biotechnology, 07-273), anti-Bcl-xL (H-5) (Santa Cruz Biotech.), anti-Fyn (Santa Cruz Biotech. sc-16) and FK506 (LC laboratories) were used. Methods for immunoprecipitation, western blotting, GST-pulldown and calcium measurement were as described in previous Chapters.
RESULTS

The expression of Bcl-2 mutants in WEHI7.2 cells

WEHI7.2 mix population cells expressing Bcl-2 mutants were characterized by western blotting. Most of the Bcl-2 deletion mutants did not express well in WEHI cells. Mutants Δ3 and Δ 13 did express and had higher expression levels than most other mutants in WEHI7.2 cells. The 12 dicodon mutants were also transfected into WEHI7.2 cells. G6-7, A8G9, G36A27, Y28 mutants had expression levels similar to that of wild type Bcl-2. G12-13, G16-17, G20-21, G28-29 had lower expression level compared to wild type Bcl-2. Other dicodon mutants did not express well in WEHI 7.2 cells. The only expressed single point mutants are K22 and Y28A (Figure 5.2). We also tried to express ΔBH4 Bcl-2 and ER targeted ΔBH4 Bcl-2 in pTag2B vector (Stratagene) with a Flag tag instead of in pSFFV vector. The expression levels were still lower than wild type Bcl-2. Alternatively we expressed these Bcl-2 mutants in 3T3 cells. Some of Bcl-2 mutants did not express well (data not shown).

The effects of mutating Bcl-2 on Bcl-2-IP3R interaction and calcium regulation

Next, we compared the IP3R binding ability of wild type Bcl-2 with selected Bcl-2 mutants by GST pull down assay (Figure 5.3A). Beads alone and GST alone were used as negative controls. The Bcl-2 mutants were pulled down by GST-domain 3 of the IP3R and detected by western blot. We found that ΔBH4 Bcl-2 does not interact with IP3R domain 3 as described in Chapter 3. Δ13, G26-27, G16-17 had lower affinity for domain
3 of IP3R than did wild type Bcl-2. The other Bcl-2 mutants interacted with IP3R comparable to wild type Bcl-2.

We measured anti-CD3 induced calcium elevation in the Bcl-2 mutant expression WEHI7.2 cells. We found that ∆13, ∆3, ∆8-9 still inhibited anti-CD3 induced calcium elevation as did wild type Bcl-2 (Figure 5.3 B&C). Although G16-17, G20-21 and ∆BH4 mutants did not inhibit calcium, their expression levels were much lower than wild type Bcl-2. Only G6-7, G26A27, Y28 expression levels similar to wild type Bcl-2 but did not inhibit anti-CD3 induced calcium elevation. As mentioned in Chapter 2 (Figure 2.11), we further tested the G6-7 dicodon mutant of Bcl-2. We found that the G6-7 mutant did not interact with the full length IP3R by coimmunoprecipitation (Figure 2.11). This contradicted the GST pull down data which showed that G6-7 still interacted with the domain 3 of the IP3R (Figure 5.3 A). We do not know the reason for this. One possibility is that the mutant may have a higher affinity with domain 3 under GST pull down conditions. It probably does not represent the binding situation with full length IP3R in cells. Due to the different expression level of Bcl-2 mutants and some contradictions between GST pull-down and coimmunoprecipitation results, it is difficult to evaluate the effect of these mutants on calcium regulation.

**Y28 Bcl-2 mutant**

Previously, we consistently found that the Y28 Bcl-2 mutant did not inhibit anti-CD3 induced calcium release. It intrigued us to figure out the reason behind this. We did GST pull downs with the Y28 Bcl-2 mutant and GST-domain 3 of the IP3R. The GST pull-
down suggested that the Y28 Bcl-2 mutant still interacted with domain 3 of the IP3R (Figure 5.4A). However, the coimmunoprecipitation of Y28 Bcl-2 mutant with the full length IP3R indicated that the Y28 Bcl-2 mutant did not interact with the IP3R (Figure 5.4B). Next we checked the Y28 Bcl-2 effect on calcium and cell survival. Interestingly, Y28 Bcl-2 did not inhibit anti-CD3 induced calcium elevation compared with wild type Bcl-2 (B17 clone) in WEHI7.2 cells (Figure 5.4C). Also Y28 Bcl-2 mutant’s antiapoptotic activity was not as effective as wild type Bcl-2 in dexamethasone-induced WEHI7.2 cell apoptosis (Figure 5.4D). These results suggested that the Y28 mutation of Bcl-2 abrogated the Bcl-2-IP3R interaction, thereby eliminating Bcl-2’s inhibitory effect on the IP3-mediated cytoplasmic calcium elevation that at least partially contributes to Bcl-2’s antiapoptotic function.

**IP3R interacts with calcineurin, PP1α, FKBP12**

Although we found that Bcl-2 directly interacts with the IP3R in vitro by GST pull down (Chapter 2), the question remains whether or not Bcl-2 directly regulates IP3R activity by itself. Our preliminary data suggests that Bcl-2 overexpression decreases the phosphorylation of IP3R. Therefore, Bcl-2 may also indirectly regulate the IP3R activity by inhibiting IP3R phosphorylation. We investigated here if Bcl-2 alters the interaction between several phosphatases/kinases and IP3R. FKBP12 has been reported to dock calcineurin to IP3R [77], so we also tested the FKBP12-IP3R interaction.

First, the expression levels of calcineurin, PP1α, FKBP12 were detected in Bcl-2(-) WEHI7.2 cells (Neo), Bcl-2(+) WEHI7.2 cells (B17) and ΔBH4 Bcl-2 WEHI7.2 cells
Overexpression of Bcl-2 or ΔBH4 Bcl-2 did not alter their protein levels. By GST pull-down, we found that the interaction between PP1α and IP3R was similar in Bcl-xL overexpressing WEHI, ΔBH4 Bcl-2 WEHI, Y28 Bcl-2 WEHI, wild type Bcl-2 WEHI (B17) and Neo control WEHI cells (Figure 5.5B). However, we did not detect an interaction between FKBP12 and IP3R, which is consistent with observations of other investigators [74].

Calcineurin is another phosphatase reported to regulate IP3R phosphorylation and interact with both Bcl-2 and IP3R. My GST pull-down and coimmunoprecipitation experiments confirmed that calcineurin does interact with the IP3R, in domain 3. We did not find any differences in the binding of calcineurin with IP3Rs in B17, Neo, Y28 and ΔBH4 WEHI7.2 cells (Figure 5.5C, D). These results suggested that overexpression of Bcl-2 did not dock or sequester PP1α and calcineurin to IP3R. The reduction of phosphorylation of IP3R may not be due to Bcl-2’s “docking effect” on calcineurin/PP1α.

**IP3R interacts with Fyn and Lck by GST pull down**

Besides phosphatases, we also studied the IP3R interacting kinases Fyn and Lck. Overexpressing Bcl-2 did not regulate Fyn protein expression levels (Figure 5.6A). By GST pull-down, we found that Fyn mainly interacted with IP3R domain 4 (Figure 5.6B). Domains 2 and 5 may have some weaker interaction with Fyn. However, Bcl-2 did not regulate the Fyn-IP3R interaction, according to GST pull-down results (Figure 5.6C). We also found that Lck interacts with IP3R domain 5 (Figure 5.6D). We have not studied if
Bcl-2 regulates Lck-IP3R interaction. Overall, these findings suggest that Bcl-2 may not reduce IP3R phosphorylation by blocking other IP3R interacting kinases.

**Peptide 2 does not interfere with IP3R-calcineurin interaction**

The binding site of Bcl-2 on the IP3R (domain 3a1) is also close to the calcineurin binding site on the IP3R. In order to test if peptide 2 also disrupts the IP3R-calcineurin interaction, we performed immunoprecipitation in the presence or absence of peptide2, control peptide and peptide1. Coimmunoprecipitation data shows that peptide2 does not interfere with the interaction between calcineurin and IP3R (Figure 5.7).

**Bcl-xL interacts with IP3R domain 3**

In addition to Bcl-2, we also mapped the binding region of Bcl-xL on the IP3R by GST pull-down. Bcl-xL interacts with the same region (domain 3) of IP3R as Bcl-2 does (Figure 5.8). However, peptide 2 appeared not to enhance anti-CD3 induced calcium elevation in Bcl-xL overexpressing WEHI7.2 cells. This may be due to the relatively large amount of Bcl-xL located in the cytoplasm compared to the amount on organelles. This cytoplasmic Bcl-xL may bind peptide 2 and reduce the interaction of peptide 2 with Bcl-xL located on organelles, where it would be expected to interact with the IP3R.
DISCUSSIONS

One of the possible reasons for the lack of success in expressing Bcl-2 mutants is the toxic effect of some Bcl-2 mutants. For example, deletion of BH4 domain of Bcl-2 has been reported to convert Bcl-2 from an antiapoptotic protein to a proapoptotic protein. Therefore, we cannot get stably overexpressed Bcl-2 mutant cell lines in which the mutant forms of Bcl-2 are at levels as high as wild type Bcl-2, or in some cases expressed at all. Transient overexpression may overcome this problem, but it is not feasible for the planned calcium and apoptosis measurements. Fortunately, we found that G6-7 (the 6th and 7th amino acids were replaced with glycine) and Y28 mutants have similar expression levels as wild type Bcl-2. These two mutants did not interact with IP3R by coimmunoprecipitation. Also they did not inhibit anti-CD3 induced calcium elevation. It will be interesting to study how these two Bcl-2 mutants regulate different types of apoptosis compared with wild type Bcl-2. If their antiapoptotic functions are distinct from wild type Bcl-2 in some types of apoptosis, it will suggest that these types of apoptosis are mediated by Bcl-2-IP3R interaction-regulated calcium signals.

We previously found that Bcl-2 can inhibit IP3R channel activity in planar lipid bilayer experiments (Chapter 2). His-tagged Bcl-2 directly interacts with the GST fusion of IP3R domain 3 in vitro. However, the possibility that Bcl-2 indirectly regulates IP3R channel activity via other proteins cannot be excluded. The purified microsomes which contain IP3R protein may also have other IP3R interacting proteins. Those proteins could be involved in the inhibitory effect of Bcl-2 on IP3R channel activity in lipid bilayer experiments. Particularly, Bcl-2 decreases IP3R phosphorylation in WEHI7.2 cells. Therefore, as we proposed in Figure 5.1, Bcl-2 may either dock one or more
phosphatases to IP3R or block kinases from associating with the IP3R. Here we studied calcineurin, PP1α and Fyn. The negative preliminary data suggesting that Bcl-2 does not alter the association of these proteins with the IP3R suggests that Bcl-2 may directly regulate IP3R channel activity (Figure 5.1, Model 1). The fast response of IP3R to Bcl-2 (within 2 min) in lipid bilayer experiments may also hint that Bcl-2’s effect is direct, instead of a slower phosphorylation or dephosphorylation process. The calcineurin binding region (domain 3) we found here is consistent with reports by other investigators [78]. Interestingly, there is a Ca^{2+} binding site localized in domain 3a1 where Bcl-2 interacts [127, 166]. Is Ca^{2+} involved in Bcl-2-IP3R interaction? If this is true, the Bcl-2-IP3R interaction could be controlled by a Ca^{2+} feedback loop. PP1α was reported to interact with the RXGX motif in the C-terminus of IP3R by yeast two-hybrid [76], though potential interactions with other IP3R regions were not checked. However, in our preliminary experiments, PP1α interacts with domain 3 of the IP3R. We did not detect significant binding with IP3R domain 6 (C-terminus). Further study is required to test other possible kinases or phosphatases, such as protein kinases A or C for a potential role in regulating IP3R function.

Notably, Peptide2 did not inhibit IP3R-calcineurin interaction based on coimmunoprecipitation experiments. Also, FK506, an inhibitor of calcineurin, did not reverse Bcl-2’s inhibitory effect on calcium in WEHi7.2 cells in our preliminary experiments. Furthermore, peptide2 did not enhance anti-CD3 induced calcium release in Bcl-2(-) WEHI7.2 cells. All of the above findings suggested that peptide2’s effects on calcium and apoptosis are not mediated via interfering with IP3R-calcineurin interaction. However, we have not tested if peptide 2 interferes with Bcl-2-calcineurin interaction.
This could be another potential mechanism that peptide 2 uses to regulate calcium and apoptosis, as discussed in Chapter 4.

**FUTURE DIRECTIONS**

Although the project in this thesis has established that Bcl-2 interacts with the IP3R and regulates IP3R-mediated proapoptotic Ca\(^{2+}\) signals, there are still several questions that need to be addressed in the future.

1. Do peptide 2 and TAT-BH4 peptide regulate other types of IP3R-mediated calcium signals and apoptosis?

   So far we have checked the effect of peptide 2 in etoposide, Fas, dexamethasone, and MG132 induced apoptosis. We did not detect a significant enhancement of cell death by peptide 2 in these types of apoptosis. But peptide 2 did enhance ABT737-induced apoptosis. These data imply that peptide 2 can only enhance apoptosis when Bcl-2’s classical antiapoptotic pathway (the inhibition of Bax/Bak/BH3 only proteins by Bcl-2, for example, by ABT-737) is unblocked. In anti-CD3 induced apoptosis, calcium plays the most critical roles. Therefore Bcl-2 mainly inhibits apoptosis by inhibiting IP3-mediated calcium signals. In some other types of apoptosis, Bcl-2 mainly blocks apoptosis by binding and inhibiting Bax/Bak. So peptide 2 has no proapoptotic effect in these types of apoptosis. Peptide 2 could be used to test if particular types of apoptosis are predominantly calcium mediated. How do the enhanced Ca\(^{2+}\) signals contribute to the increase of apoptosis induced by anti-CD3 or ABT737?
Peptide 2 is able to enhance anti-CD3 induced calcium elevation and induce calcium oscillations without other treatments. Whether peptide 2-induced calcium signals contribute to its proapoptotic function is still not clear. Two strategies can be developed to study the link between calcium and apoptosis. 1) Inhibit peptide 2 induced calcium signals. Since the effect of peptide 2 on calcium is dependent on Bcl-2-IP3R interaction, according to the data in Chapter 2, we can test if peptide 2 still enhances cell death in Bcl-2/IP3R function defect cells, such as using Bcl-2/IP3R knockout or negative cells, Y28A/6-7 dicodon Bcl-2 mutant, IP3R inhibitors etc. It will also be interesting to check if the proapoptotic effect of peptide 2 is dependent on Bax/Bak by using Bax/Bak double knockout cells. This will give an idea if calcium induces apoptosis via Bax/Bak pathway or not. 2) Direct test the downstream effectors’ activity of proapoptotic calcium signals. Calpain, NFAT, CREB and BH3 only proteins, such as Bim/Bid/Bad, are potential candidates. A measurement of mitochondria membrane potential is also another alternative way to show how calcium signals lead to apoptosis.

2. How does peptide 2 induce calcium oscillation by itself? Our preliminary results suggested that peptide 2 induces significant calcium oscillation in WEHI7.2, Jurkat, CEM and CLL cells. Is this peptide 2-induced oscillation dependent on Bcl-2 and IP3R? Does peptide 2 regulate other store operated calcium channel at plasma membrane, since its effect is very fast (a couple of minutes after delivery)?

3. How can peptide 2 or BH4 peptide be further optimized? The 20-aa peptide 2 is not efficient on stability, delivery and synthesis. Further narrowing down peptide 2/BH4 peptide or modification will improve the peptide’s function in cells. We
also need study the biochemical properties of peptide 2, including its binding with Bcl-2, the binding affinity, the localization and the stability in cells. How does peptide 2 bind to the BH4 domain of Bcl-2? Will this interaction change Bcl-2 conformation, for example displacing BH4 domain from the Bcl-2 c terminal as Nur77-derived peptide does? Will the peptide 2 regulate Bcl-2’s localization in cells, such as translocating Bcl-2 from ER to mitochondria? Although we have found that peptide does not interfere with Bcl-2-VDAC, Bcl-2-Calcineurin, Bcl-2-Bim interactions, will peptide 2 interfere with the interactions of other BH4 domain interacting proteins described in Chapter 4?

4. As summarized in Chapter 4, the interaction between IP3R and the BH4 domain of Bcl-2 is a potential target to regulate calcium and apoptosis. Based on the structural studies of the peptide 2-Bcl-2 interaction by NMR, some small molecules which mimic peptide 2’s binding structure can be tested. Another strategy is to directly screen small molecules which can disrupt the Bcl-2-IP3R interaction. At least two step screening should be employed, the screen based on the interaction assay and cytotoxicity assay. If possible, a further screen based on calcium regulation will greatly improve the specificity for Bcl-2-IP3R interaction. There are some commercial interaction assays for high throughput screening, such as HTRF, in yeast assay, Fluorescence polarization (Hybrigenics) and AlphaScreen (PerkinElmer).

5. The difference between Bcl-xL and Bcl-2 on IP3R-mediated proapoptotic calcium signals is another interesting direction to study. Does Bcl-xL bind to the same site of IP3R as Bcl-2 with the similar affinity? Does Bcl-xL also inhibit the IP3R
channel activity in lipid bilayer experiment? Why does peptide 2 not enhance IP3-mediated calcium signals in Bcl-xL overexpressed cells? Can we design a new peptide to abrogate both Bcl-2 and Bcl-xL interactions with IP3R?

6. The roles of IP3R-mediated calcium signals in Alzheimer disease have attracted more and more interest recently. Presenilin has been reported to enhance IP3R channel activity and calcium signals. Therefore, this disease model could be an interesting one in which to test peptide 2 and BH4 peptide, except anti-CD3 induced calcium and apoptosis. TAT-BH4 peptide can reduce Aβ peptide-induced apoptosis in Alzheimer disease. Does the inhibition effect of TAT-BH4 peptide on IP3R channel activity also play roles in this process?

7. Although the current data suggest that Bcl-2 may directly regulate IP3R channel activity, it does not exclude the possibility that Bcl-2 indirectly regulate IP3R phosphorylation status and activity. The findings in Chapter 5 have shown that Bcl-2 seems not to regulate calcineurin-IP3R or Fyn-IP3R interaction. We used GST-d3-IP3R to test the PP1α-IP3R interaction and did not find that Bcl-2 affects GST-d3-IP3R-PP1α interaction. However, some other researchers have reported that the C terminus of the IP3R is the binding region of PP1α. It is worth to further test this by using GST-d6-IP3R. There are also some other potential kinase and phosphatase candidates, such as Lck, PKA and SHP-2, to be examined in future studies.

8. Will Bcl-2, peptide 2 and BH4 peptide work on ryanodine receptor, since the domain 3a1 region of IP3R is very similar with the corresponding region of
ryanodine receptor? Do Bcl-2, Bcl-xL, peptide 2 and BH4 peptide have any specificity on different types of IP3R?

CONCLUSIONS
This project was initiated in order to better understand the mechanism by which Bcl-2 regulates IP3-mediated Ca^{2+} signals in T lymphocytes with the ultimate goal of targeting Bcl-2 in cancer therapy. Although there is considerable information about the mechanisms of action of both the proapoptotic (e.g., Bax, Bak) and antiapoptotic (e.g., Bcl-2, Bcl-xL) family members at the level of mitochondria, the roles of Bcl-2 on the regulation of ER calcium are just beginning to emerge. In this project, we determined that Bcl-2 directly interacts with the regulatory and coupling domain of IP3R in vitro by GST pull-down. The interaction between Bcl-2 and IP3R in cells has been confirmed by FRET. Peptide 2 and the BH4 peptide represent the binding regions of this interaction on the IP3R and Bcl-2, respectively. Peptide 2 disrupts the Bcl-2-IP3R interaction. The BH4 peptide can bind to the IP3R by itself. These two peptides oppositely regulate IP3-mediated proapoptotic Ca^{2+} signals. Peptide 2 reverses, but BH4 peptide mimics the Bcl-2’s inhibitory effect on anti-CD3 induced Ca^{2+} and apoptosis. The Bcl-2-IP3R interaction is required for Bcl-2’s regulation of IP3-mediated Ca^{2+} signals. In conclusion, this work has advanced our understanding of the mechanism of Bcl-2 action and identified a novel molecular target for the next generation of antineoplastic agents.
FIGURES

Figure 5.1 Models of Bcl-2 regulating IP3R activity.

Model 1: Bcl-2 directly interacts with IP3R and regulates IP3R. Model 2: Bcl-2 regulates IP3R by blocking Fyn or other kinases’ phosphorylation on IP3R. Model 3: Bcl-2 regulates IP3R by docking calcineurin or PP1a on IP3R.
Figure 5.2 Expression of Bcl-2 mutants in WEHI7.2 cells.

Bcl-2 deletion scanning mutants (Δ1-Δ13), BH4 domain dicodon mutants (G6-7 – G28-29), single point mutants (I14, V15, Y18, I19, K22, L23 and Y28) were stably expressed in WEHI7.2 cells. Flag tagged ΔBH4, ΔBH4-cb5 in pTag2B vector were also expressed to compare the protein level with Flag tagged wild type Bcl-2. The protein levels of Bcl-2 mutants were detected by western blot.
Figure 5.3 The effects of mutating Bcl-2 on Bcl-2-IP3R interaction and calcium.

(A) GST pull down of GST-d3-IP3R with Bcl-2 mutants. The amounts of cell extracts were adjusted based on the Bcl-2 mutants’ expression levels. (B) Bcl-2 deletion mutants (∆3 and ∆13) reduced the anti-CD3 induced calcium peak. Symbols represent Mean±SEM of three experiments. (C) Bcl-2 dicodon mutants and Y28’s effects on anti-CD3 induced calcium elevation.
A

**Input control**
IB: Bcl-2

**GST pull down**
IB: Bcl-2

B

**Bcl-2 deletion mutants**

Calcium (nM)

- Neo
- Bcl-2
- Δ3
- Δ3

C

**Bcl-2 dicodon mutants**

Calcium (nM)

- Neo
- Bcl-2
- Δ6-7
- Δ8-9
- Δ16-17
- Δ18-20
- Δ21-23
- Δ26-27
- ΔBH4
- Y28
Figure 5.4 Characterization of the properties of Y28 Bcl-2 mutants on IP3R interaction, calcium and apoptosis.

(A) GST pull-down of Y28 Bcl-2 with the GST-d3-IP3R by using B11 cell extracts and Y28 Bcl-2 WEHI7.2 cell extracts. (B) Coimmunoprecipitation of wild type Bcl-2 (B17) or Y28 Bcl-2 mutant with the endogenous full length IP3R. (C) Y28 Bcl-2 mutant does not regulate anti-CD3 induced calcium elevation by fluorescence microscopy based calcium imaging. *, p<0.05. (D) Wild type Bcl-2 cells (B17), Y28 Bcl-2 mutant cells (Y28) and Bcl-2 negative cells (WEHI7.2) were treated with 0.1 µM dexamethasone. The cell viability was monitored until 72 hours after treatment by trypan blue assay.
Figure 5.5 Interactions between IP3R and phosphatases.

(A) Calcineurin, PP1α and FKBP12 were detected by western blot with the corresponding antibodies in Neo, B17 and ΔBH4 WEHI7.2 cells. Actin was used as input control. (B) GST pull-down of PP1α and FKBP12 by using GST-d3-IP3R in different cells. (C) GST pull-down of calcineurin by using GST-d3-IP3R in different cells. (D) Coimmunoprecipitation of calcineurin with the full length endogenous IP3R in B17 and Neo cells.
Figure 5.6 Interactions between IP3R and kinases.

(A) Fyn protein level in B17 and Neo cells. (B) GST pull-down of Fyn with the GST-IP3R fragments in non-antiCD3-treated B17 cell extracts. Coomassie blue staining shows the GST-IP3R fragments input. (C) GST pull-down of Fyn with the GST-d4-IP3R in non-antiCD3 treated B17 and Neo cell extracts. (D) GST pull-down of Lck with the GST-IP3R fragments in non-antiCD3-treated wild type WEHI7.2 cell extracts.
Figure 5.7 Peptide 2 does not interfere with IP3R-calcineurin interaction. Calcineurin was immunoprecipitated with IP3R in the presence or absence of peptide 1 (pep1), peptide 2 (pep2) and control peptide (ctrl).
Figure 5.8 Bcl-xL interacts with IP3R domain 3.

GST-IP3R fragment pull-downs employing cytosolic extracts from Bcl-xL overexpressing WEHI7.2 cells. Upper panel, coomassie blue-stained gel showing input GST-IP3R fragments; Bottom panel, Bcl-xL detected by immunoblotting after pull-down.
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