COMMITTEE CERTIFICATION OF APPROVED VERSION

The committee for Bryce P. Portier certifies that this is the approved version of the following dissertation:

CHARACTERIZATION OF A NOVEL PRO-APOPTOTIC ROLE FOR NUCLEAR ASSOCIATED BCL-2

Committee:

Giulio Taglialatela Ph.D., Supervisor

Shawn Newlands M.D., Ph.D.

Jay Foreman M.D., Ph.D.

Jeffery Ceci Ph.D.

Gracie Vargas Ph.D.

Dean, Graduate School

CHARACTERIZATION OF A NOVEL PRO-APOPTOTIC ROLE FOR NUCLEAR ASSOCIATED BCL-2

By

Bryce P. Portier, B.S.

Dissertation
Presented to the Faculty of The University of Texas Graduate School of
Biomedical Sciences at Galveston
in Partial Fulfillment of the Requirements
for the Degree of

Doctor of Philosophy

Approved by the Supervisory Committee

Giulio Taglialatela Ph.D.
Shawn Newlands M.D., Ph.D.
Jay Foreman M.D., Ph.D.
Jeffery Ceci Ph.D.
Gracie Vargas Ph.D.

September, 2006
Galveston, Texas

Key words: Bcl-2, Nuclear, Apoptosis, FKBP38, BH4

To my friends and family
This work was made possible by your continuous support and patience

Definition from Webster's Dictionary

Life:
1 a : the quality that distinguishes a vital and functional being from a dead body **b :** a principle or force that is considered to underlie the distinctive quality of animate beings **c :** an organismic state characterized by capacity for metabolism, growth, reaction to stimuli, and reproduction

Death:
1 a : a permanent cessation of all vital functions **:** the end of life **b :** an instance of dying

*******The work presented in this manuscript neither deals directly with life or death, but more accurately involves the study of transition from life to death under certain circumstances.

Relevant Quotation

"To examine the causes of life, we must first have recourse to death."
Mary Shelley, *Frankenstein*

ACKNOWLEDGEMENTS

I wish to express my greatest debt and sincere gratitude to my mentor Dr. Giulio Taglialatela, without whose continuous encouragement and guidance, the achievement of this work would have been impossible.

I would like to thank the members of my dissertation committee, Dr. Shawn Newlands, Dr. Jay Foreman, Dr. Jeffery Ceci, and Dr. Gracie Vargas for their helpful suggestions, valuable comments, and willingness to see this project through even through the difficulties of relocation.

Appreciation is also extended to Dr. Golda Leonard, my program director, for her bravery, continuous guidance, and listening ear throughout the past four years.

A huge thank-you goes to my family and extended family who provided me with support that carried me throughout my education.

Furthermore, I would like to thank the National Institute for Neurological Disease and Stroke (NINDS) for providing funding for this project through a Ruth L. Kirschstein pre-doctoral training grant.

Last, but certainly not least, I would like to thank Jill 'Nicole' for being a wonderful companion and great teammate. I truly couldn't have made it so far without her love, help, and support.

CHARACTERIZATION OF A NOVEL PRO-APOPTOTIC ROLE FOR NUCLEAR ASSOCIATED BCL-2

Publication No._____

Bryce P. Portier
The University of Texas Graduate School of Biomedical Sciences at Galveston, 2006

Supervisor: Giulio Taglialatela, Ph.D.

Both stroke and cancer are major public health problems that inflict immeasurable suffering to victims and their families. The treatment options currently in place for both stroke victims and cancer patients are limited. The underlying unifying characteristic of both conditions is that they are the end result of abrogation of apoptosis regulation. In the case of stroke, an initial increase in Bcl-2 expression traditionally follows ischemia/re-perfusion. A major goal of stroke therapy is to rescue the peri-ischemic region of cells that have up-regulated Bcl-2 but have not yet committed to the execution phase of apoptosis. A strong potential target to save neurons and supporting glia is to modulate Bcl-2 expression or localization in order to promote an anti-apoptotic function. Likewise, in the case of cancer, 80% of all tumors over-express Bcl-2. While malignant cells utilize mitochondrial localized Bcl-2 to gain protection from apoptosis, possession of Bcl-2 makes these cells a target for treatments that could alter Bcl-2's localization and potentially promote apoptosis by altering Bcl-2's sub-cellular localization/function. Thus, my Ph.D. project has focused on understanding the role of nuclear localized Bcl-2, Bcl-2's default sub-cellular localization, and on the mechanism of binding between Bcl-2 and its mitochondrial chaperone protein FKBP38. My work has characterized the function of nuclear-associated Bcl-2 as a pro-apoptotic protein and Bcl-2's BH4 domain as a critical domain for binding the chaperone protein FKBP38. Based on these findings we attempted a strategy for interfering with Bcl-2/FKBP38 binding via addition of a BH4 domain peptide both *in vivo* and *in vitro*. In both cases, addition of a BH4 domain peptide disrupted Bcl-2/FKBP38 binding, increased the level of nuclear associated Bcl-2, and induced apoptosis selectively in Bcl-2 bearing cells. The rationale behind this project was to determine molecular events underscoring Bcl-2's localization and function in order to identify novel therapeutic targets for treatment of conditions that would benefit from interventions that modify the execution of apoptosis. The long term goal spawning from my Ph.D. thesis project is to further our understanding of apoptotic control mechanisms and in turn develop future therapies to control diseases due to abrogation of apoptotic regulation.

TABLE OF CONTENTS

PAGE

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

AIF	Apoptosis Inducing Factor
ANT	Adenine Nucleotide
APAF-1	Apoptotic Protease-Activating Factor-1
APC	Allophycocyanin
ARC	Apoptosis Repressor with CARD
ATP	Adenine tri-phosphate
B-104	Cell line- rat neuroblastoma
BAR	Bi-functional Apoptosis Regulator
Bcl-2	Protein first isolated from B-Cell Lymphoma
BH	Bcl-2 Homology Domain
CAD	Caspase Activatable DNAse
CaM	Calmodulin
CaN	Calcineurin
CARD	Caspase Recruitment Domain
CNS	Central Nervous System
CsA	Cyclosporine A
CYT-C	Cytochrome-C
DD	Death Domain
DED	Death Effecter Domain
DEDD	Death Effecter Domain containing DNA binding domain
DFC	Dense Fibrillar Component
DIC	Differential Interference Contrast
DISC	Death Inducing Signaling Complex
ECL	Enzymatic Chemiluminescence
ELISA	Enzyme-Linked Immunosorbant Assay
ER	Endoplasmic Reticulum
FADD	Fas Associated Death Domain
FC	Fibrillar Center
FKBP	FK-506 Binding Protein
FLICE	Fas-associated death domain-like interleukin-1 β-converting enzyme
FLIP	FADD-like ICE- inhibitory protein
GC	Granular Component
HA	Hemagglutinin protein tag
HEK-293	Cell line- human embryonic kidney cells
HeLa	Cell line- human cervical epithelial cells
HL-60	Cell line- human acute myeloid leukemia
HRP	Horse Radish Peroxidase
IAP	Inhibitor of Apoptosis
ICAD	Inhibitor of Caspase Activatable DNAse
LDH	Lactate Dehyrdrogenase
NAD	Nicotinamide Adenine Dinucleotide

NF-AT	Nuclear Factor of Activated T cells
NFκB	Nuclear Factor Kappa B
NGF	Nerve Growth Factor
NGS	Normal Goat Serum
NUP	Nucleoporin
PC12	Cell line- Pheochromocytoma
PCD	Programmed Cell Death
PP2B	Calcineurin
PPIase	Peptidyl-Prolyl-cis-trans-Isomerase
PS	Phosphotidyl Serine
PT	Permeability Transition
RAIDD	Receptor Interacting Protein with Death Domain
REH	Cell line- acute lymphocytic leukemia
ROS	Reactive Oxygen Species
siRNA	Small interfering Ribonucleic acid
TM	Trans-Membrane
TNF	Tumor necrosis factor
TNF-R	Tumor Necrosis Factor-Receptor
TPR	Tetratricopeptide Repeat
TRADD	TNF-R1-Associated Death Domain
TRAF	TNF Receptor Associated Factor
TUNEL	Terminal Deoxynucleotidyl Transferase Nick End Labeling
UBP	Upstream Basal Protein
VDAC	Voltage Dependant Anion Channel
YFP	Yellow Fluorescent Protein

CHAPTER 1: BACKGROUND AND SIGNIFICANCE

I. PROGRAMMED CELL DEATH (APOPTOSIS)

I.I Apoptosis: Overview

Programmed cell death (PCD), also referred to as apoptosis, is an ATP-dependent process in which cells, based on either internal or external stimuli, induce a self mediated suicide process (Vogt, 1842; Glucksmann, 1951; Kerr et al., 1972). Proper initiation and execution of this process plays an important role in both development and normal tissue homeostasis (Milligan and Schwartz, 1997; Rudin and Thompson, 1997; Mirkes, 2002). Following development, it is often necessary to remove the superfluous cells that were produced. Since this cell removal process needs to be carried out efficiently and without damage to surrounding tissue, a program of organized cell elimination, i.e. apoptosis, is in place for the regulated removal of unwanted cells. Therefore, this program of events exists as a counter-balance to cell division and is in place to maintain homeostasis and insure proper function of tissues. The process of apoptosis includes many checks and balances to insure elimination of cells specifically targeted for removal. Following receiving a death induction signal, a molecular cascade is activated in which protein interactions and protein distribution is affected in order to promote or prevent the original death stimulus from executing apoptosis [reviewed in (Hengartner, 2000)]. One of the best characterized family of proteins involved in regulation of apoptosis is the Bcl-2 family. This family influences both pro- and anti-apoptotic functions and will be reviewed in detail in a subsequent section.

I.II Apoptosis: Early Discoveries

It is widely cited that PCD was first described by Glucksmann (1951). However, there are several published accounts of naturally occurring cell death that pre-date Glucksmann's report. One of the earliest reports occurred in 1842, not long after

establishment of the cell theory 1838, by Schleiden and Schwann, when Carl Vogt reported an observation of physiological cell death in the notochord of metamorphic toads (Vogt, 1842). Another significant characterization of PCD came following observations of cell death in rabbit ovarian follicles, a process which was given the title 'chromatolytic' cell death (Flemming, 1885), a title that would later be changed to apoptosis. The first report of PCD in humans was in 1890, when Thomas Watson Councilman described vacuolated acidophilic bodies in liver tissue from yellow fever patients (Councilman, 1890). The term 'apoptosis' was first suggested by professor James Cormack, and it means "to fall away from" (apo = from, ptosis = a fall) (Kerr et al., 1972). However the pathologist, Kerr, and his colleagues, significantly advanced the study of PCD by being the first to molecularly describe the PCD phenomenon and popularize the notion of apoptosis (Kerr et al., 1972). They succeeded not only in generating a collective term to label and pool together previous reports of physiological cell death but their major contribution to the field was the molecular characterization of apoptosis as an inherent two staged process. The first stage involved the formation of apoptotic bodies, while the second stage involved elimination of the apoptotic bodies via phagocytosis or degradation.

I.III. Apoptosis: Biological Relevance and Association with Disease

Apoptosis occurs in the setting of many physiological and pathological processes, such as development (Milligan and Schwartz, 1997), differentiation (Linette and Korsmeyer, 1994), tumorigenesis (Cappello et al., 2002; Malaguarnera, 2004), and infections (Rodenburg et al., 2000). Furthermore, disruption of pathways that regulate apoptosis can give rise to various diseases, including cancer and neurodegenerative disorders (Bold et al., 1997; Lyons and Clarke, 1997; Desjardins and Ledoux, 1998; Mizuno et al., 1998; Reed, 1999). What follows is a closer examination of apoptosis in development, tissue homeostasis, and pathological conditions follows.

Classical examples of apoptosis in development include studies from both the nervous and immune system. In the nervous system, neurons are created and connections are allowed to form. Following the initial proliferation, neurons that are not mapped or properly networked are starved of trophic support and eliminated by apoptosis (Oppenheim, 1989; Oppenheim et al., 1992; Meier et al., 2000; Yuan and Yankner, 2000). In the immune system, apoptosis plays a critical role in multiple pathways including giving rise to tolerance by removing auto-reactive T- and B-cells (Don et al., 1977; Shi et al., 1989), attenuating the immune response once an infection has been eliminated (Sanderson and Glauert, 1977; Sanderson et al., 1977), allowing resolution of inflammation, and elimination of host cells that become infected via action of cytotoxic T-cells (Bishop and Whiting, 1983; Krammer, 2000). In addition to the CNS and immune system, apoptosis is also necessary for deletion of interdigital areas, retinal development, and palate fusion (Farbman, 1968; Hammar and Mottet, 1971; Penfold and Provis, 1986), respectively. Apoptosis is also responsible for the removal of vestigial structures such as the pronephric kidneys (Hammerman, 1998). In the reproductive system, apoptosis allows adaptation of primordial organs for different functions as is the case with removal of the wolffian or mullerian ducts allowing for proper differentiation of the sex organs (Capel, 2000). Apoptosis also deletes cells from the center of the solid mass formed from the developing gastrointestinal tract, a process that results in a hollow and functional gastrointestinal system (Meier et al., 2000).

As in development, apoptosis also plays a critical role in tissue homeostasis. In tissue homeostasis, apoptosis serves as a mechanism to critically balance the process of proliferation and therefore is essential for maintaining proper cell number and function. Without apoptosis, cellular hyperplasia would result and if allowed to continue unchecked, this process would eventually give raise to neoplastic tissue. Loss of proper apoptotic regulation, typically through modulation of Bcl-2 family proteins or p53, have been directly linked to tumor formation (Lowe et al., 1993; Minn et al., 1996a). In addition, loss of regulation or the ability to execute apoptosis can result in tumors gaining resistance to chemotherapeutic treatments. On the other hand, if apoptosis is allowed to

proceed unchecked the result is tissue wasting, atrophy, and ultimately loss of organ function. The degree of tissue apoptosis required for proper homeostatic maintenance is almost directly related to the rate of cell proliferation. High turn-over tissues such as heamatopoitic, epithelial, and intestinal linings, all display high levels of apoptosis (Wyllie, 1987; Billig et al., 1995), while tissues with low levels of turn-over, including the kidneys, heart, and neuronal tissue display relatively low levels of apoptosis (Benedetti et al., 1988).

Equally important, apoptosis also plays a critical role in pathologic conditions. Some of the classical disease processes affiliated with apoptosis are summarized below. In autoimmune reactions, alterations in apoptosis can lead to targeted removal of self tissue (Greer et al., 2001). This form of apoptosis is most evident in graft vs. host rejection where the recipient's immune response is activated against an implanted organ that is determined to be foreign (French and Tschopp, 2000). In conditions of cerebral ischemia/ reperfusion, apoptosis is a leading cause of both vascular and neurological complications (Johnston et al., 2001). In diseases of the CNS such as Alzheimer's, Huntington's, Parkinson's, and demylinating neuropathies apoptosis plays a central part in removal of neurons (Mazarakis et al., 1997). Both viral pathogens and parasites have also evolved to take advantage of apoptosis in order to elude host defenses or promote replication. HIV is a classic example of a viral pathogen that can activate FAS ligand and directly initiate activation of apoptotic cascades (Kaplan and Sieg, 1998). Trypanosoma cruzi utilizes induction of apoptosis in T-cell populations in order to inactivate macrophage phagocytation, a step that is necessary for effective elimination of this parasite (Freire-de-Lima et al., 2000). There is also a tenuous tie between apoptosis and aging which is based on mitochondrial/reactive oxygen species (ROS) generation. This theory relies on a cycle of events that feeds forward and includes initial mitochondrial damage, which leads to increased production of ROS, increased ROS leads to increased mitochondrial damage, and the end result of the continual cycle is cellular apoptosis (Wei et al., 2001; Le Bras et al., 2005; Schuessel et al., 2006).

I.IV. Apoptosis: Morphology & Biochemistry

One of the first morphologically characterized forms of cell death was necrosis. Necrosis represents a form of non-controlled cell death that typically occurs in response to cell injury (Virchow, 1871). Morphologically, early stages of cell necrosis are characterized by an overall increase in cellular size, disorganization of cytosolic content, karyorrhexis (chromatin fragmentation) (**Fig. 1.1A**), and organelle swelling. At later stages, there is karyolysis (chromatin lysis) and release of proteases, nucleases, and lysosomal content due to rupture of the cell membrane (**Fig. 1.1B**) (Sauter et al., 2000; Proskuryakov et al., 2003). In contrast to the morphologic cell death phenomena observed in necrosis, morphological features of apoptotic cell death include cellular shrinkage, nuclear chromatin condensation and margination (**Fig. 1.1A**), nuclear pore clustering (**Fig. 1.1D-E**), and plasma membrane blebbing (**Fig. 1.1C**) (Kerr et al., 1972; Mondello and Scovassi, 2004).

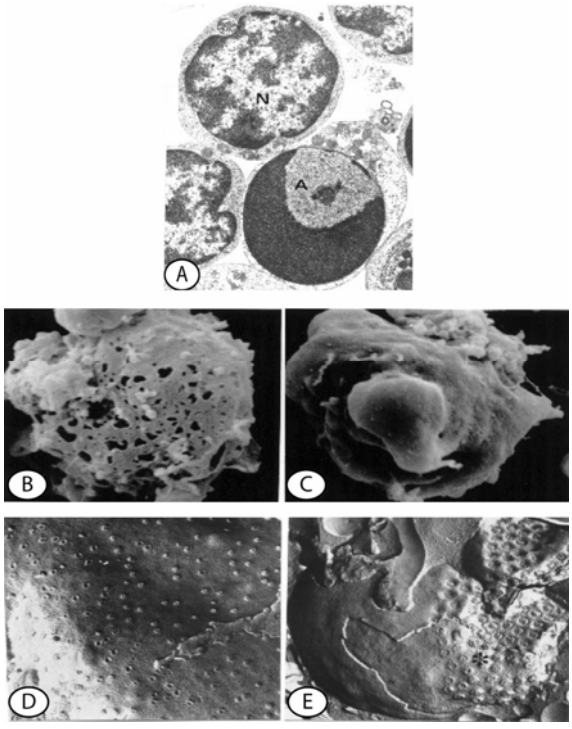


Figure 1.1 Morphology changes observed in necrosis versus apoptosis. **A**, Transmission electron microscopy image of normal (N) and apoptotic (A) cell demonstrating alterations in chromatin rearrangement. **B**, Scanning electron microscopy image of a necrotic cell with numerous lesions on the cell surface. **C**, Scanning electron microscopy of an apoptotic cell demonstrating surface blebbing. **D**, Freeze fracture image of a normal cells nuclear envelope. The regular distribution of nuclear pores is visible. **E**, Freeze fracture of an apoptotic cells, nuclear envelope demonstrating characteristic clustering of nuclear pores (*). Adapted from Vitale et al., Apoptosis vs. Necrosis, Purdue cytometry CD-ROM series Vol. 4.

In addition to these morphological changes, initiation of apoptosis results in formation of 180-200bp DNA strand breaks (Yamada et al., 1981; Wyllie et al., 1984). In apoptosis, unlike necrosis, the cytoplasmic organelles remain intact and exhibit only minor swelling. One of the earliest morphological manifestations of apoptosis is convolution of membranes which is often referred to as membrane blebbing. Membrane blebbing causes a loss in phospholipid symmetry found on the cells plasma membrane. This loss of symmetry results in exposure of phosphatidylserine (PS), phosphatidylethanolamine, and phosphatidylcholine on the outer leaflet of the plasmid membrane (Vance, 2003; Vance and Vance, 2004; Hanshaw and Smith, 2005). Exposure of phospholipids is a major factor in the recognition of apoptotic cells by phagocytes, involving membrane-bound receptors, such as the phosphatidyl serine receptor, vitronectin receptor, and several scavenger receptors (Fadok et al., 2001). At late stages of apoptosis, apoptotic cells shed apoptotic bodies which are membrane bound vesicles that contain both organelles and condensed chromatin. These apoptotic bodies are removed rapidly by either their cellular neighbors or by macrophages, in a process that ensures that neighboring cells are not damaged by release of the intracellular contents of the apoptotic cell. This process of organized cellular removal without release of cellular content helps prevent the invocation of an inflammatory response (Gregory and Devitt, 2004) (**Table 1.1**).

Characteristics	Apoptosis	Necrosis
Stimuli	Physiological	Phatolgoical (injury)
Occurrence	Single Cells	Groups of cells
Organelles	Late stage swelling	Very early swelling
Enzyme release	Absent	Present
Nucleus	Convulution and breakdown	Disappearance
Chromatin	Compaction into dense mass	Clumping not sharply defined
DNA Breakdown	Internucleosomal	Randomized
Cell	Form apoptotic bodies	Swelling and late disintegration
Phagocytosis	Present	Absent
Inflamation	Absent	Present

Table 1.1 Fundamental differences between apoptosis and necrosis. Adapted from Hengartner, M., Nature. Vol 407, 2000 page 771.

Detection of apoptosis in cell populations is predominantly based on morphological or biochemical changes specific to apoptotic cell death. The first identified assay for apoptosis detection utilized visualization of the 180-200bp DNA fragments using gel electrophoresis (Yamada et al., 1981). This form of apoptosis detection has been adapted for use via enzyme-linked immunosorbant assays (ELISA), and by Terminal Deoxynucleotidyl Transferase Nick End Labeling (TUNEL). Another common morphologic change utilized for apoptosis screening is detection of PS on the outer plasma membrane. Specific recognition of PS by Annexin-V, a calcium dependent phospholipid binding protein, allows rapid identification of cells following initiation of apoptosis (Hanshaw and Smith, 2005). Furthermore, apoptosis is also detected by staining cell populations with compounds such as cresyl violet, Hoechst, and silver (Northington et al., 2001) and analyzing changes in cell or nuclear morphology. Finally, apoptosis can also be detected by caspase assays in which, activation of effector caspases is quantified and used as a marker for active apoptosis (a detailed description of caspases is provided in section I.V.II.).

While several methods are available to detect apoptosis, rapid and uniform analysis of large populations of cells is difficult due to reliance on manual detection, antibody/stain efficiency, and false positive detection (Darzynkiewicz et al., 2001). One technique that overcomes these short falls is flow cytometry based detection of apoptosis via morphological changes. Changes in nuclear morphology as measured by physical light scatter parameters (forward vs. side scatter) give a close estimation of nuclear size and granularity, which are two parameters that decrease and increase respectively following initiation of apoptosis. Recently we developed a technique to measure apoptosis in populations of isolated nuclei via physical light scatter properties using flow cytometry and have shown that this technique accurately discriminates between healthy and apoptotic populations of nuclei isolated from both cells grown in culture and tissue (Portier et al., 2006).

Although specific morphological and biochemical changes can be used as guidelines for monitoring a cell in the process of dying, it should be recognized that the morphological changes described above for apoptosis and necrosis represent the two extremes of a continuum. Apoptosis can be initiated by various external or internal signals and is executed through several interrelated signaling pathways (**Fig. 1.2**). With a plethora of stimuli and proteins able to modulate the cell death process, at the level of individual cells the process may not be one of apoptosis or necrosis. Rather individual cells can show signs of both. In fact, despite the notion that apoptosis and necrosis are described as two distinct forms of cell death, recent evidence supports an idea of a cell death continuum, in which cells induced to die can show features of both forms of cell death (apoptosis and necrosis). Fuguali and colleagues demonstrated that the apoptotic/necrotic cell features displayed during cell death depend strongly on both the amount of caspase activation and availability of ATP to carry out the energy dependent cellular processes required for classical apoptosis. This continuum of cell death has recently been labeled with the chimeric title of "aponecrotic cell death" (Formigli et al., 2000).

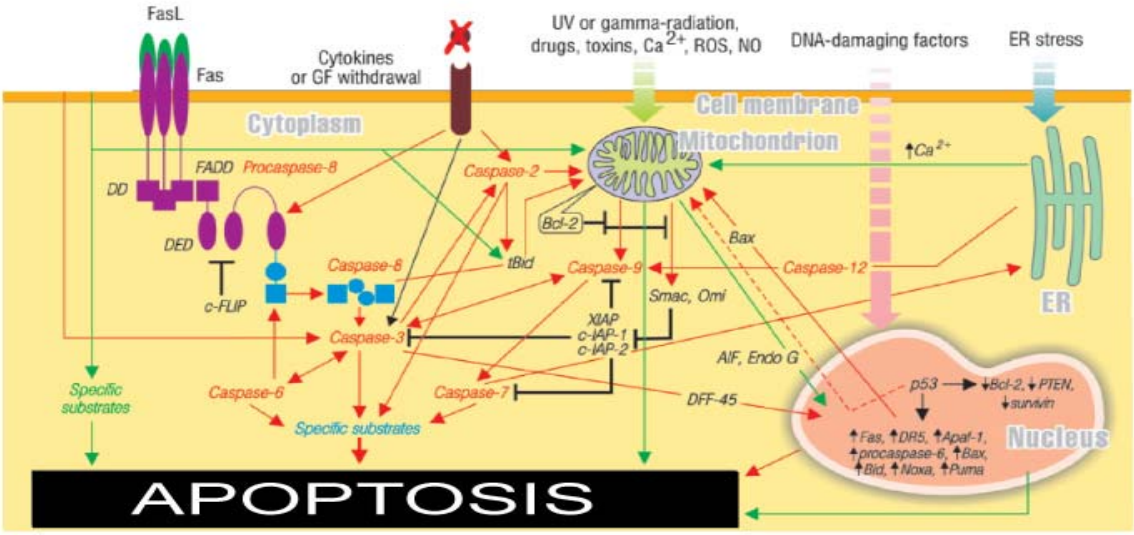


Figure 1.2 Multiple mechanisms and pathways to apoptosis. Red lines highlight the important role of caspases in apoptosis execution. Adapted from Philchenkov, A., J. Cell. Mol. Med. Vol 8, No 4, 2004 page 434.

I.V. Apoptosis: Cell death pathways

There are several well characterized initiation points for apoptosis. These include activation of cell surface death receptors, leak of mitochondrial proteins, and disregulation of endoplasmic reticulum calcium stores (Schultz and Harrington, 2003) (**Fig 1.2**). While the majority of research effort has been focused on investigating death receptor signaling as well as mitochondria mediated apoptosis, very little is known regarding the precise mode of action of the non-traditional pathways contributing to apoptosis which include mechanisms that are non-receptor and non-mitochondrial driven. Furthermore, an additional level of complexity is present in that there is a high degree of cross-talk between the different apoptotic cascades. Nevertheless, parallel mechanisms of induction, execution, and regulation of differentially triggered pathways exist and new parallels are sure to be uncovered. Our best current models for apoptosis follow one of two pathways. These two pathways include the death receptor mediated and the mitochondrial mediated pathways. Both are discussed in detail below along with the non-traditional and newly emerging ER and nuclear pathways.

I.V.I. Death Receptors

The interaction of surface receptors with their ligands is a fine-tuned mechanism that allows a rapid and selectively means to regulate cellular life and death. Several surface receptors are capable of transmitting cytotoxic signals into the cytoplasm, although many of these receptors display a wide range of apoptosis unrelated functions including cell activation, differentiation, and proliferation. In addition, for any specific signal it is highly cell type specific as to whether the signal will result in cell death or proliferation. One example of such a signal is the well characterized tumor necrosis factor

(TNF) which exerts a co-stimulatory effect on naive lymphocytes yet induces death signals in activated lymphocytes. The TNF-receptors (TNF-R) belong to a family of receptors, which possess roles in differentiation, survival, and cell death (Schulze-Osthoff, 1998).

Death receptors are members of the TNF receptor superfamily and are activated in response to external ligands (Idriss and Naismith, 2000). The best characterized members of this family include CD95, FAS, Apo 1, and TRAIL receptors (MacFarlane, 2003). TNF receptors contain highly conserved carboxy-terminal, cytoplasmically localized death domains (DD) which serve as a protein-protein binding domain and are utilized to both recruit and activate caspases (Sartorius et al., 2001). A standard activation of apoptosis via a TNF family receptor is outlined below. First a ligand, such as CD95L or FASL, binds to its cognate receptor. Following ligand binding three receptors are brought into close proximity and form a trimer, subsequently a death inducing signaling complex (DISC) is formed within the cytoplasmic portion of the receptor. The DISC is made up of the three receptors DD's which are necessary for interaction with death effector domain (DED) adaptor molecules such as the Fas-Associated Death Domain (FADD) (Chinnaiyan et al., 1995). Once the DD's bind the DED's and formation of the DISC is complete, then pro-caspase-8 is recruited and activated by cleavage to the activated form, caspase-8. Activation of caspase-8 can then go on to activate effector caspases such as caspase-3, which results in execution of apoptosis. Another pathway of CD95 may involve activation of procaspase-2 through RIP-interacting protein (RAIDD) which contains a DD and a caspase recruitment domain (CARD) (Duan and Dixit, 1997), that is also present in caspase-2 (Schulze-Osthoff et al., 1998). By RAIDD binding to pro-caspase-2, this allows auto activation and generation of active caspase-2, which can then go on to activate caspase-3 and promote apoptosis (**Fig. 1.3**).

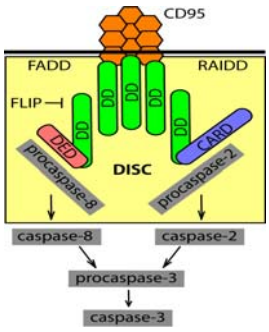


Figure 1.3 CD95-mediated signaling pathway. Activation of CD95 receptor by ligand-induced crosslinking, initiates recruitment of various adaptor molecules through homophilic death domain (DD) interactions. This includes FADD, which directly binds procaspase-

8 to the ligated receptor to form the death-inducing signaling complex (DISC-shaded yellow box). This, in turn, promotes trans-catalytic activation of caspase-8/2, which then processes and activates the effector caspase-3. Adapted from Macfarlane 2003. Toxicol. Lett. 2003 Apr 4;139(2-3):89-97. page.91

To add an additional level of complexity to TNF receptors mediated apoptosis, there are also adaptor proteins that are pro-caspase-negative regulators which contain DED. One well characterized apoptosis inhibiting adaptor proteins is FADD-like ICE-inhibitory protein (FLIP), which contains 2 DEDs and was initially discovered from herpes virus (Thome et al., 1997). There are two forms of FLIP, $FLIP_L$ and $FLIP_S$, both display significant anti-apoptotic activity by incorporation into the DISC. FLIP can bind FADD and prevent the release of caspase-8, an event that can block the initiation of apoptosis. The result of FLIP action is additionally responsible for inducing TNF-Receptor activating factor (TRAF) binding to inhibitors of apoptosis (IAP's) (Nachmias et al., 2004). When incorporated into the DISC, FLIP is also able to block autoproteolytic activation of procaspase-8 due to replacement of the caspase-8 active site cysteine by a tyrosine, this substitution renders the caspase proteolytically inactive (Irmler et al., 1997).

I.V.II. Caspases

Caspases are a family of cysteine proteases that cleave numerous protein substrates at aspartic acid residues, as reflected in their name, cysteinyl aspartate-specific proteases. Caspases can be activated by transactivation, proteolysis by other proteases, or by autoactivation. At the present time, there are 14 members of the caspase family, referred to as Caspase 1-14 (Wang et al., 2005). Caspases are highly conserved and were first identified in C. elegans following extensive genetic analysis which revealed *ced-3*, *ced-4*, c*ed-9*, and *egl-1* gene products as regulators of programmed death in 31 of the 1090 cells that undergo apoptosis in this organism during the course of morphogenesis (Stergiou and Hengartner, 2004). The ced genes are homologs to mammalian caspases which are expressed in cells as inactive pro-enzymes. These pro-enzymes become

proteolytically active following cleavage of their N-terminal pro-domain which allows the enzyme to form a heterotetramer of two small and two large subunits. Mammalian caspases can be broadly grouped into two categories based on initiator or effector caspase function. The upstream initiator caspases include caspases-2, -8, -9, and -10. Effector caspases-3, -6, and -7 cleave specific substrates that aid execution of apoptosis (Shiozaki and Shi, 2004). Caspases have a large subunit that contains a conserved active site, and a smaller subunit that determines substrate specificity. The two subunits bind to form a central 6 strand β-sheet core, flanked on either side by α-helices (Philchenkov, 2004). Caspase proteolytic signaling cascades are highly interconnected and, due to overlapping substrate specificity, they are also partially redundant.

As would be expected with such potent regulators of cell death, both pro-caspase activation and caspase activity in cells is tightly controlled. In place to control caspase activity are multiple direct caspase inhibitors which include seven members of the mammalian inhibitors of apoptosis (IAP) family of proteins: FLIP, bi-functional apoptosis regulator (BAR) and the apoptosis repressor with CARD (ARC). Of special note, while an endogenous system is in place to regulate caspase activity, expression of these caspase inhibitors is tissue specific. Therefore, individual caspase inhibitors contribute to cell death machinery in both a cell type-specific as well as a signaling cascade-specific manner.

I.V.III. Mitochondria, ER, and Nucleus

The mitochondria are uniquely situated in regards to control of apoptosis. The mitochondria serve as a convergence point for both internal and extracellular signals that promote apoptosis. Since the discovery of release of cytochrome-c (Liu et al., 1996), much work on apoptosis has focused on the actions of mitochondrial proteins, which include both the mitochondrial localized Bcl-2 family of proteins and intrinsic mitochondrial proteins such as cytochrome-C (Cyt-C), apoptosis-inducing factor (AIF), and smac/diablo. While the mitochondria's role in apoptosis has been the focus for the

majority of apoptosis research, an area left largely unexplored is the activities that regulate apoptosis at organelles such as the ER and nucleus. The following section will highlight some of the specific roles of both the traditional mitochondrial and the non-traditional ER/nuclear mechanisms for modulating apoptosis.

Mitochondria

Permeabilization of mitochondrial membranes is a rate-limiting event in apoptosis due to the release of multiple apoptotic activator proteins. These intra-membrane proteins include Cyt-C, procaspases (-2, -3, and -9), heat shock proteins hsp10 and hsp60, Smac/Diablo, and AIF (Lorenzo and Susin, 2004). Upstream control over release of the intra-mitochondrial proteins is achieved via interaction of proteins at the mitochondrial surface. One of the best characterized class of mitochondrial membrane bound regulators of apoptosis is the Bcl-2 family of proteins. The predominant model of Bcl-2 family regulation of apoptosis at the mitochondria has been described in relation to the level of membrane associated Bax to membrane associated Bcl-2. Bax has been shown to form homo-dimers at the mitochondrial membrane and accelerate cell death. On the other hand, when Bcl-2 is overexpressed, it hetero-dimerizes with Bax and suppresses cell death. The stoichiometric balance between mitochondrial associated Bcl-2 and Bax appears to dictate the fate of individual cells. This relationship has been collectively termed the rheostat model and simply states that if Bcl-2 outnumbers Bax, cells survive, while if Bax outnumbers Bcl-2, cells die (Korsmeyer et al., 1993; Raisova et al., 2001). This model highlights the importance of the ratio of Bax to Bcl-2 in determining a cells susceptibility to apoptosis. A likely mechanism to account for pro- vs. anti-apoptotic outcomes following alteration in Bcl-2 family members is due to selective opening of the mitochondrial permeability transition (PT) pore (Vander Heiden et al., 2000). The adenine nucleotide translocase (ANT) and the voltage dependent anion channel (VDAC) make up the basic units of the PT pore (Crompton et al., 1999) and the complex is located at contact sites between the mitochondrial inner and outer-membrane. PT pore opening results in matrix swelling and outer-membrane rupture, which in turn causes Cyt-C and

AIF release (Zamzami et al., 1995). Therefore the regulation of PT pore opening via mitochondrial associated Bcl-2 proteins serves as a critical regulatory step, upstream of effector caspase activation. This characteristic places the Bcl-2 family of proteins at a critical decision point for the cell, one in which apoptosis can be accelerated or blocked, making the Bcl-2 family critical regulators of apoptosis.

During execution of apoptosis, the release of mitochondrial intramembrane proteins promotes the formation of a caspase-activating complex also known as the apoptosome (Kim et al., 2005). The apoptosome is formed in the presence of ATP by Cyt-C binding to Apaf-1 in the cytosol. Multimerization of Apaf-1, Cyt-C, and dATP induces a conformational change in Apaf-1, thereby exposing its CARD, thus mediating recruitment of procaspase-9 and allowing its subsequent autolytic activation (Srinivasula et al., 1998; Kuida, 2000). Interestingly, another CARD-protein, apoptosis repressor with CARD (ARC), has been identified in skeletal muscle and heart which interacts with procaspase-8 and procaspase-2 but not procaspase-9 (Koseki et al., 1998). This indicates cross talk between CD95/Fas and mitochondrial death inducing signaling pathways (**Fig. 1.4**). Released mitochondrial proteins Smac/Diablo induce apoptosis by binding inhibitors of apoptosis (IAPs) which are bound to active caspase-9 and caspase-3. Therefore Smac/Diablo mediated blocking of IAP's action results in increased action of caspases. Another protein liberated from the mitochondria is AIF. When released into the cytosol, AIF translocates to the nucleus and mediates caspase-independent DNA fragmentation (Susin et al., 1999; Daugas et al., 2000). Another protein capable of causing DNA fragmentation is caspase activated DNase (CAD). CAD is constitutively inhibited by the inhibitor of CAD (ICAD), which is cleaved by active caspase-3 which dissolves the complex and allows CAD to execute its DNase activity (Enari et al., 1998). This is in agreement with the findings that in some cells apoptosis is changed to a more necrotic phenotype by addition of caspase inhibitors (McCarthy et al., 1997).

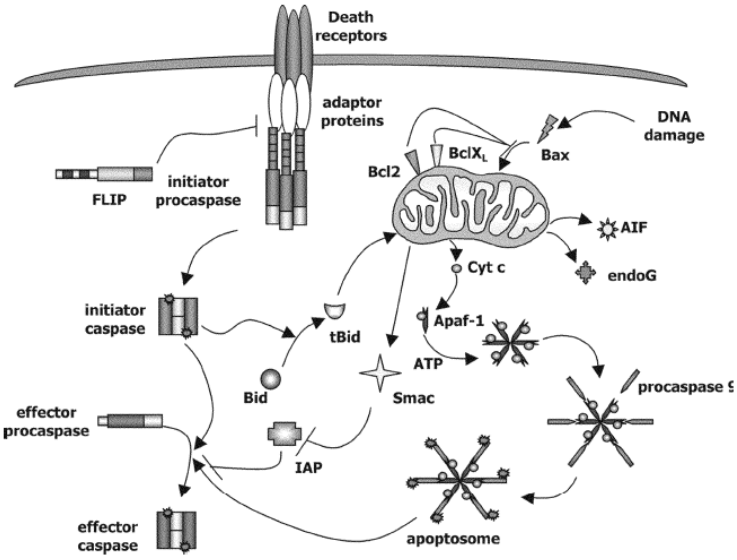


Figure 1.4 Extrinsic and intrinsic apoptosis pathways. The death-receptor pathway (extrinsic) is induced by ligand binding to TNFR superfamily members. The second pathway (intrinsic) is triggered by the mitochondria in response to extra- or intra-cellular injury such as DNA damage as shown above. Adapted from Delhalle et al. 2003 An introduction to the molecular mechanisms of apoptosis.

In addition to mitochondrial associated and intra-mitochondrial protein mediated apoptosis, it has also been shown that cells lacking a mitochondrial genome (ρ0 cells) can undergo apoptotic cell death (Jacobson et al., 1993). However, mitochondria are known to be a key component of the apoptotic machinery as described above, therefore, the presence of apoptosis in ρ0 cells point to the fact that not all apoptotic actions are coordinated via the mitochondria or mitochondria associated proteins. Additional evidence for non-mitochondrial based apoptosis has been generated from studies on the ER and nucleus and will be explored in further detail in the subsequent section.

Endoplasmic Reticulum (ER)

The contribution of the endoplasmic reticulum (ER) to apoptosis has only recently begun to be investigated for its active role in regulating apoptosis. The predominant initiator caspase in the ER mediated apoptotic pathway is caspase-12, which resides in

the cytosolic ER membrane and belongs to the caspase-1 subfamily. Activation of caspase-12 is triggered by ER stress and leads to inhibition of protein transport, inhibition of N-glycosylation, and disruption of calcium homeostasis. Experiments in caspase-12 knock out mice indicate that activation of caspase-12 is independent from death receptor signaling and mitochondrial targeted apoptotic signals but essential for ER-stress induced apoptosis (Nakagawa, 2000).

In addition to caspase-12, recent reports have described both pro- and anti-apoptotic roles for the ER in regulating apoptosis via calcium release (He et al., 1997; Kuo et al., 1998; Pinton et al., 2000; Vanden Abeele et al., 2002). Following the discovery that several Bcl-2 family members reside at the ER (Krajewski et al., 1993), it has been postulated that the one major role of ER regulation of apoptosis is via calcium homeostasis managed by the Bcl-2 family of proteins. While it was reported over a decade ago that Bcl-2 could regulate calcium levels and affect calcium distribution (Baffy et al., 1993), recent reports show that ER associated Bcl-2 family members might regulate apoptosis at the ER via a non-calcium related mechanism. These new reports center around the observation that several ER localized Bcl-2 family members are associated with non-calcium regulating proteins (Ng et al., 1997; Breckenridge et al., 2002; Breckenridge et al., 2003). In contrast to the early reports of an anti-apoptotic role for ER associated Bcl-2 proteins, the most recent reports on ER mediated regulation of apoptosis have proposed a role for the ER upstream of the mitochondria and have implicated ER associated Bcl-2 family proteins as initiators of apoptosis via activation of BH3 only members such as Bid. One model proposes that activation of Bid leads to its translocation to the mitochondria where it can directly promote apoptosis (Thomenius et al., 2003). Despite these observations, there is active debate as to the exact role and function of Bcl-2 family members at the ER.

Nucleus

While the mitochondria has received the most attention in regards to regulation of apoptosis, followed by new interest in the ER, the role of the nucleus in the regulation of apoptosis has been largely uncharacterized. Indirect evidence pointing to a nuclear

mediated regulation of apoptosis include nuclear envelope localization of Bcl-2 (Krajewski et al., 1993), apoptotic morphological changes induced in isolated nuclear preparations (Jones et al., 1989), and initiation of apoptosis in cells lacking mitochondria (ρ0) cells (Jacobson et al., 1993). With these observations, it would seem that the nucleus plays an active role in either the induction or execution stage of apoptosis. In the following section, specific nuclear sub-regions will be examined in detail with highlighted reports on their role in regulation of apoptosis.

Nucleolus

The nucleolus represents a specialized domain within the nucleus (**Fig 1.5**) and is truly a plurifunctional organelle, essential not only for ribosomal DNA transcription and ribosomal RNA processing (Sheer and Hock, 1999), it is also the site where cytoplasmic proteins are imported and preribosomes are formed (Schwarzacher and Wachtler, 1993). In addition, this organelle is a site for accumulation of DED proteins and other apoptosis related proteins during apoptosis, marking this organelle as a potential key regulator in nuclear changes seen during apoptosis. While the nucleolus is generally regarded as a nuclear sub-domain that is very resistant to apoptosis (Columbaro et al., 1998; Martelli et al., 1999), recent evidence has shown a potential role for the nucleolus in apoptosis induction. The cytosolic protein Death Effector Domain containing DNA binding domain (DEDD), has been shown to translocate to the nucleus following initiation of CD95-induced apoptosis (Stegh et al., 1998). This translocation event leads to accumulation of DEDD in the nucleolus where DEDD was shown to co-localize with upstream binding factor (UBF), a basal factor required for RNA polymerase I transcription. Therefore it is postulated that one crucial step in execution of apoptosis, mediated by CD95, is inhibition of cellular transcription machinery mediated by nucleolar localized DEDD.

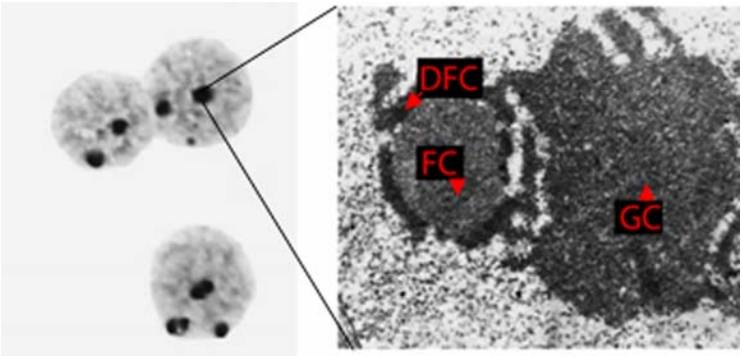


Figure 1.5: Nucleoli morphology. Isolated monocyte nuclei silver stained to reveal nucleoli (left). Electron micrograph of cell nucleolus demonstrating the Fibrillar Center (FC), Dense Fibrillar Component (DFC), and Granular Component (GC) (right). Adapted from Schwarzacher, Cytogenetics and Cell Genetics 91:243–252 (2000)

Nuclear Envelope

One of the earliest nuclear changes following initiation of apoptosis is degradation of the nuclear lamina (Oberhammer et al., 1994). In addition to nuclear lamina changes, other peripheral nuclear domains are altered due to initiation of apoptosis. Caspases cleave nuclear membrane proteins and nuclear pore complex proteins including: lamin B receptor, lamin-associated polypeptide-2α, nucleoporin (Nup) 153 and Nup 270, as well as RanGAP1 (Duband-Goulet et al., 1998; Buendia et al., 1999; Faleiro and Lazebnik, 2000; Gotzmann et al., 2000). Cleavage of lamin B receptor, lamin-associated polypeptide-2α, and Nup 153 proteins, which are on the inner surface of the nuclear envelope and bind chromatin, results in detachment of the nuclear envelope from chromatin. Furthermore, following cleavage of these proteins, nuclear pore complexes are found to cluster to one plane of the nuclear membrane (**Fig. 1.2E**) (Falcieri et al., 1994a; Reipert S, 1996). Activation of caspase-9, affects nuclear transport and increases the diffusion limit of nuclear pores. This increase then allows caspase-3, which is usually cytoplasmic, to gain access to nuclear targets (Falcieri et al., 1994b). Cleavage of RanGAP1, a component of the nuclear transport machinery (Cole and Hammell, 1998; Moroianu, 1999) and Nup 270, a nuclear pore complex protein (Stoffler et al., 1999; Ryan and Wente, 2000) are likely involved in increased nuclear pore permeability. While

these two proteins have shown to be directly cleaved by caspases, it is highly likely that several other nuclear pore components are caspase substrates and their degradation is responsible for the increase in nuclear permeability.

II. THE BCL-2 PROTEIN FAMILY

II.I. Bcl-2 protein family

The Bcl-2 family proteins are probably the best characterized effectors and modulators of cell apoptosis (Bruckheimer et al., 1998; Chao and Korsmeyer, 1998; Konopleva et al., 1999; Motyl, 1999). Bcl-2 is the founding member of this family and was isolated at the chromosomal breakpoint t(14;18) in B cell lymphoma (Tsujimoto et al., 1985; Boise et al., 1993). The Bcl-2 family of proteins consists of more than 20 members and can broadly be divided in two categories depending on their ability to inhibit or promote apoptosis (**Fig 1.6**). The anti-apoptotic Bcl-2 proteins, which include Bcl-2 and Bcl-X_L, contain four conserved domains termed Bcl-2 homology domains BH1-BH4. Additionally, this group also contains a C-terminus hydrophobic tail that anchors these family members to membranes at the mitochondria, ER, and nucleus (Krajewski et al., 1993; Akao et al., 1994; Strasser et al., 2000). The pro-apoptotic Bcl-2 protein category is much larger and includes members such as Bax, Bak, Bik, Bid, and Bim (Adams and Cory, 1998; Antonsson and Martinou, 2000). The pro-apoptotic Bcl-2 proteins can be further divided into two classes based on the presence or absence of one or more of the Bcl-2 Homology (BH) functional domains. The multi-domain, pro-apoptotic Bcl-2 proteins (e.g. Bax, Mtd, Bok, Bak, and Bcl-2-rambo) can directly promote the initiation of apoptosis, while the BH3-only, pro-apoptotic Bcl-2 proteins (e.g. Bik, Bad, Bim, Blk, Puma, and Bcl-G) cannot directly promote apoptosis but rather act by associating with and inhibiting the action of anti-apoptotic Bcl-2 proteins (Reimertz et al., 2003; Wilson-Annan et al., 2003).

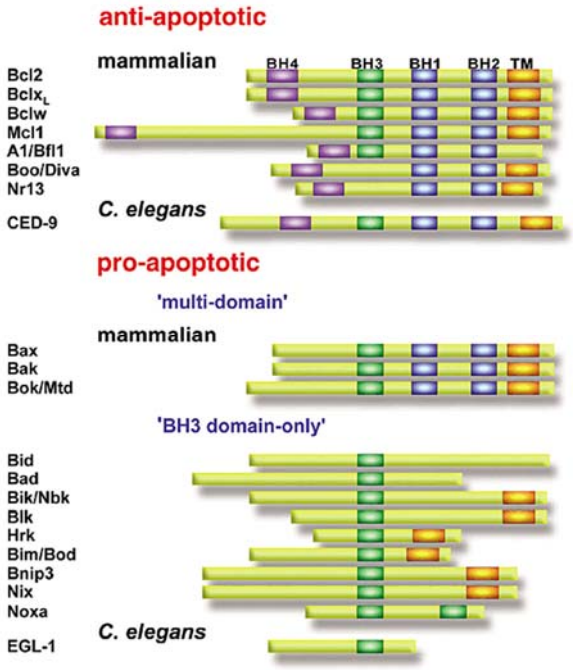


Figure 1.6: Summary of anti- and pro-apoptotic Bcl-2 family members. Bcl-2 homology domains are highlighted. Adapted from Ranger, A. et al., Nature Genetics 28: 113-118 (2001), p.114.

II.II. Bcl-2 Domains

Each of the identified domains found in Bcl-2 family members have been characterized with one or more specific functions. The alpha helices of BH1, BH2, and BH3 together form a hydrophobic pocket that serves as a binding site for the BH3 domain of other Bcl-2 family proteins, thereby granting a molecular basis for protein-protein interactions among the different members of the Bcl-2 family (Korsmeyer et al., 1993; Sattler et al., 1997; Chao and Korsmeyer, 1998; Borner, 2003). Both BH1 and BH2 domains of Bcl-2 are necessary to bind to and thereby inhibit the pro-apoptotic activity of Bax (Yin et al., 1994; Chittenden et al., 1995). The transmembrane domain (TM) is made up of a stretch of hydrophobic amino acids and allows post-translation insertion into membranes of the mitochondria, ER, and nucleus (Krajewski et al., 1993; Akao et al., 1994; Strasser et al., 2000). A slightly less conserved domain of the Bcl-2 family is the

loop domain. The loop domain of Bcl-2 is composed of amino acids 35 to 89. These amino acids make up a flexible domain that notably is capable of altering BH4 domain orientation and is a site for phosphorylation at amino acids Threonine-56, Serine-70, and Serine-87 (Chang et al., 1997; Petros et al., 2001; Kang et al., 2005).The BH4 domain, located closest to the N-terminus of the Bcl-2 protein, serves as a docking region for heterologous proteins to interact and bind Bcl-2 (Denis et al., 2003). Some heterologous interactions with Bcl-2's BH4 domain include binding to calcineurin (Shibasaki et al., 1997), *ced*-4 (Huang et al., 1998), and NFκB (Hour et al., 2000). Interestingly, all anti-apoptotic members of the Bcl-2 family contain the BH4 domain while the pro-apoptotic Bcl-2 proteins do not. Thus, the BH4 domain seems to be crucial to the anti-apoptotic activity of Bcl-2 proteins (Borner, 1996; Hunter et al., 1996; Reed, 1997). This hypothesis was confirmed by the seminal demonstration that endogenous caspase-3 can cleave Bcl-2 at position Asp34 during apoptosis, thus removing the BH4 domain, resulting in loss of Bcl-2 anti-apoptotic properties (Cheng et al., 1997). Furthermore, the remaining cleaved Bcl-2 (now comprising only BH1-3 and TM domains) acquired pro-apoptotic properties that resembled those of Bax. However, the mechanism(s) underscoring the switch of BH4-deprived Bcl-2 from an anti- to a pro-apoptotic protein was not examined. In order to gain a better understanding of the mechanisms controlling Bcl-2's regulation of apoptosis this dissertation specifically examines Bcl-2's BH4 domains role in regulating Bcl-2's apoptotic function.

II.III. Bcl-2 family localization and function

In the context of apoptotic regulation, Bcl-2 is classically described as a mitochondrial localized, anti-apoptotic protein (**Fig. 1.2**). While some Bcl-2 family members insert into the mitochondrial outer membrane, others have been found in the cytosol or associated with other subcellular compartments (Hockenbery et al., 1990; Krajewski et al., 1993; de Jong et al., 1994; Hoetelmans et al., 2000; Germain and Shore, 2003). For example, pro-apoptotic members Bad and Bax are found in the cytosol but relocate to the mitochondria in response to apoptotic stimuli (Yamaguchi and Wang,

2001). While at the mitochondria, Bad and Bax can bind the anti-apoptotic proteins Bcl-2 and Bcl- X_L and thereby prevent them from exerting their anti-apoptotic function. On the other hand, recent evidence showed that Bax and Bad can also be targeted to the ER where they can modulate apoptosis through altering calcium release from the ER (Demaurex and Distelhorst, 2003; Thomenius and Distelhorst, 2003). Also, increases in cytosolic calcium-activate calcineurin, an event that results in dephosphorylation of Bad. De-phosphorylated Bad has been shown to inhibit anti-apoptotic Bcl-2 family members through direct binding (Sheikh and Huang, 2004). In 2003, a major revision of our models for Bcl-2 family member localization and function came following isolation and characterization of FKBP38, which is an FK506-binding protein that was shown to bind Bcl-2 and Bcl-X_L and to chaperone these two proteins to the mitochondria (Shirane and Nakayama, 2003). This FKBP38 mediated placement of Bcl-2 and Bcl- X_L at the mitochondria promotes proper anti-apoptotic function, thus further indicating the physiological importance of Bcl-2 protein mobility and proper localization within the cell (FKBP38 is discussed in detail in a subsequent section).

The Bcl-2 family of proteins have multiple mechanisms to promote or inhibit apoptosis. One mechanism is the ability to form homo- and hetero-dimers which results in conformational changes in the complexed proteins. Based on the rheostat model (Korsmeyer et al., 1993), these conformational changes and overall expression levels influence the pro-/anti-apoptotic outcome of a cell. However this model is not all encompassing as illustrated by the discovery that heterodimerization is not required for pro-survival signaling (Cheng et al., 1996; Kelekar et al., 1997; Festjens et al., 2004). In addition to dimerization, Bcl-2 family proteins Bax, Bcl-2, and Bcl- X_L have been shown to form channels in lipid bilayers, albeit experiments have been largely limited to artificial liposomal based *in vitro* protocols (Minn et al., 1996b; Antonsson et al., 1997). Emphasis regarding the pore forming capability of the Bcl-2 family is given by the close spatial proximity of BH1, BH2, and BH3 domains which create an elongated hydrophobic cleft that structurally bears significant similarities to the pore forming

domains of several bacterial toxins (colchicins A, E1 and diphteria toxin) (Antonsson et al., 1997; Schlesinger et al., 1997; Schendel and Reed, 2000).

An additional function of the Bcl-2 family is regulation of PT pore formation via interaction with the voltage dependant anion channel (VDAC), which resides in the outer mitochondrial membrane. Both Bcl-2 and Bcl- X_L have been shown to interact with VDAC and thereby maintain exchange of complex anions between the cytosol and inter-membrane space of the mitochondria (Vander Heiden et al., 2000).

There are also post-translational mechanisms that influence Bcl-2 family protein function such as modification by phosphorylation. Multiple family members show functional changes in response to phosphorylation including the prototypical multi-domain family member Bcl-2 whose apoptotic function is modifiable based on phosphorylation at Ser-70 and Ser-82 (Breitschopf et al., 2000). In addition, the BH3-only members of the Bcl-2 family function is tightly regulated by phosphorylation. This was demonstrated by de-phosphorylation of Bad (Ser-112 and Ser-136), which results in Bad translocation to the mitochondria where it can bind and inactivate mitochondrial-localized Bcl-X_L as well as Bcl-2, an event that frees Bax to induce apoptosis (Condorelli et al., 2001). Finally, the significance of Bcl-2 protein localization in regards to pro- vs. anti-apoptotic function is also emerging as a primary mechanism for control of Bcl-2 family member function. Several reports have demonstrated that transiently expressed Bcl-2 acts in a pro-apoptotic fashion (Uhlmann et al., 1998; Wang et al., 2001), whereas stably expressed Bcl-2 acts in an anti-apoptotic fashion (Kroemer, 1997; Reed, 1997). The mechanism of Bcl-2's duality of function was unexplored until it was recently illustrated that the subcellular localization of Bcl-2 is altered in transient vs. stable expression (Massaad et al., 2004). This observation indicates that Bcl-2, and potentially other family members, posses multiple functions and sub-cellular localization is a critical aspect regulating Bcl-2 family member function.

II.IV. Bcl-2 and Calcium

Research on the physiologic and pathologic regulation of calcium, like research on Bcl-2, spans many fields and sub-specialties. While our understanding of calcium movement and function has grown, relatively little is known about Bcl-2's regulation of calcium movement or compartmentalization. The first link between Bcl-2 and calcium came from research showing Bcl-2 could regulate calcium levels and effect calcium distribution (Baffy et al., 1993). Additionally it was discovered that the amino acid sequence and structure of multi-domain Bcl-2 family proteins resembled pore-forming domains of bacterial toxins (Minn et al., 1997), thus opening the possibility that Bcl-2 family could directly regulate ion movement via direct pore formation. Several groups characterized Bcl-2 family member ion channels with little success in linking ion movement through a Bcl-2 pore to changes in compartment calcium concentration or apoptosis (Minn et al., 1997; Schlesinger et al., 1997; Schendel and Reed, 2000). Subsequent to investigations of Bcl-2 pores, research shifted following the discovery and characterization of a calcium dependent endonuclease (McConkey, 1996). Experiments that followed used isolated nuclei to demonstrate that the calcium dependent endonuclease activation only required micromolar calcium changes in the presence of ATP and NAD, at physiological concentrations (Jones et al., 1989). While interesting, this discovery failed to tie calcium changes and calcium mediated apoptosis control to the Bcl-2 family proteins. In 2003, the first paper to link Bcl-2 interaction with FKBP38, a component of the calmodulin/calcineurin pathway, was published (Shirane and Nakayama, 2003) and has set the field in a new direction, one in which the characterization of Bcl-2 role in modulating calmodulin/calcineurin interactions and calcium changes remains to be determined.

III. THE FK-506 BINDING PROTEIN FAMILY

III.I. Immunophilins

The FKBP family of proteins were discovered in an attempt to isolate a binding partner for the immunosuppressant macrolides FK506 and rampymycin. Technically, the use of affinity gels and radiolabeled FK506 assays (Harding et al., 1989; Siekierka et al., 1989b) yielded the isolation of a 12-kDa protein that was termed FK506 binding protein-1a, later renamed FKBP12 based on molecular weight. Additional screening led to the discovery of additional FKBP proteins and this family of proteins is highly conserved from bacteria to higher eukaryotes. Interestingly, shortly after discovery of FKBP12, sequencing revealed that this protein had previously been partially characterized and belonged to a class of proteins with peptidyl-prolyl-cis-trans-isomerase (PPIase) activity (Fischer et al., 1989; Harding et al., 1989). PPIase proteins are also referred to as rotamases and they help convert proteins from the cis/trans isomerization about a peptidylprolyl bond (Siekierka et al., 1989a; Standaert et al., 1990; Schiene and Fischer, 2000) Since the majority of proteins are synthesized at the ribosome in the trans-configuration, this class of enzymes has a distinct role in accelerating the often rate limiting step of cis/trans isomerization during protein folding.

While initially it was believed that the PPIase activity of FK506 binding proteins might account for this protein's immunosuppressive capabilities, further experiments which inhibited PPIase activity with no effect on immunosuppression revealed that the PPIase activity and the immunosuppressive capability of FK506 bound FKBP's were unrelated (Bierer et al., 1990; Liu et al., 1991; Dumont et al., 1992; Huai et al., 2002; Blankenship et al., 2003; Feske et al., 2003). Further investigation into the functional role of this newly identified FKBP proteins revealed that FK506-bound FKBP proteins promote immunosuppression via inhibiting calcineurin (CaN also known as PP2B) (**Fig 1.7**). Calcineurin is a calcium/calmodulin-dependent serine/threonine protein phosphatase involved in many physiological roles including dephosphorylation of NFAT and Bad as well as T-lymphocyte growth and differentiation (Mattila et al., 1990; Lin et

al., 1991; Liu et al., 1991). One atypical member of the FKBP family, FKBP38, has been shown to be intimately involved in modulating apoptosis by binding to and chaperoning Bcl-2 and Bcl-X_L to the mitochondria where they act as anti-apoptotic proteins (Shirane and Nakayama, 2003). The following section will review the limited reports on FKBP38 structure and function.

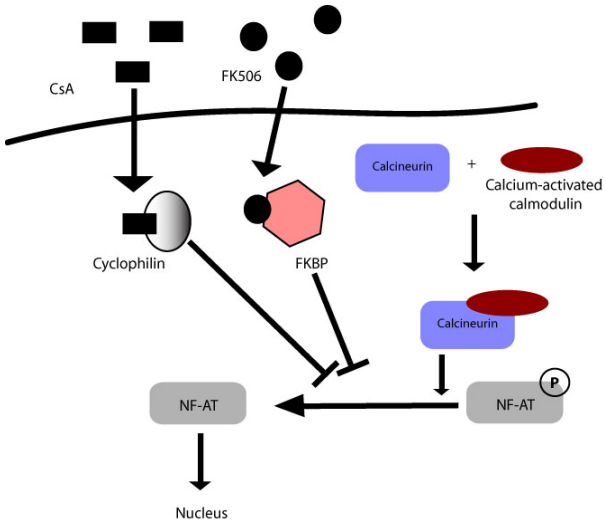


Figure 1.7 Mechanism of immunosuppression performed by FKBP's and cyclophilins. Addition of ligand cyclosporine A (CsA), or FK-506 binds to its respective immunophilin and blocks calcineurin/calcium activated calmodulin from de-phosphorylating NF-AT.

III.II. FKBP38

Background FKBP38

FKBP38 was originally cloned and characterized in 1995 using a reduced stringency PCR technique which yielded cDNA for a novel FKBP homolog that encoded a 38-kDa protein (Lam et al., 1995). The N-terminal FKBP like domain of FKBP38 was 33% identical to the founding FKBP member FKBP12. In addition to an FKBP domain, FKBP38 additionally contained 3 tetratricopeptide repeat domains (TPR), a putative calmodulin (CaM) binding domain, a lucine zipper repeat, and a putative transmembrane

domain. Based on the presence of the TPR's and the lucine zipper it was initially hypothesized that FKBP would form homomultimers or bind to other lucine-zipper or coil-coil motif containing proteins. One characteristic that differentiates FKBP38 from other FKBP's is that it has a Tryptophan to Leucine substitution at the critically conserved residue 59 of FKBP12. The Tryptophan was found to form the base of FKBP12's hydrophobic FK506 binding pocket (Michnick et al., 1991; Moore et al., 1991; Van Duyne et al., 1991). Additionally FKBP38 has a Phenylalanine to Valine substitution at amino acid 36 of FKBP12. In FKBP12, replacement of Phenylalanine 36 by a Tryptophan residue results in significantly lowered PPIase activity (Wiederrecht et al., 1992). Based on these two critical amino acid substitutions and the inability of to detect FK506 binding or PPIase activity from recombinant protein expressed in *E. coli* or from baculovirus-infected insect cells, it was concluded that FKBP38 was 'atypical' (Lam et al., 1995). Unlike other FKBP family members, FKBP38 does not appear to bind FK506 or have PPIase activity. However, consistent with other FKBP proteins the presence of FKBP38 mRNA was found to be highest in the brain.

FKBP38 Function

From the limited studies performed on FKBP38, three main characteristics have been examined and include FKBP38 mediated inhibition of calcineurin , FKBP38 mediated PPIase activity, and FKBP38's chaperone function. These three functions are reviewed below.

Calcineurin Inhibition

The initial characterization of FKBP38 by Lam et al. 1995, did not examine FKBP38's role in CaN inhibition in either the presence or the absence of FK506. Subsequent investigations including examination of structural arrangements due to amino acid substitutions (Maestre-Martinez et al., 2006) and NMR-based binding studies comparing FKBP38 to FKBP12 (Kang et al., 2005), showed that FKBP38 lacked CaN binding and CaN inhibition activity. Contrary to other reports, one group has reported

that FKBP38 directly binds CaN in the absence of FK506 and inhibits CaN function as measured both by *in vitro* and *in vivo* assays (Shirane and Nakayama, 2003). The *in vivo* FKBP38 mediated CaN inhibition was shown to block calcium mediated CaN dephosphorylation of NFAT (**Fig. 1.7**). This observation led to the hypothesis that FKBP38 was an inherent calcineurin inhibitor. This feature, while not supported by other published investigations, led this group to conclude that FKBP38, unlike the other members of the FKBP family, has the unique ability to mediate CaN inhibition without the need for FK506-binding.

PPIase Activity

To directly test PPIase activity of FKBP38, a standard *in vitro* assay which utilizes a tetrapeptide substrate (succinyl-AFPF-4-nitroanilide) in combination with recombinant FKBP38 is typically employed. One study using this method found recombinant FKBP38 exhibits no PPIase activity (Edlich et al., 2005). While Edlich's 2005 published findings were consistent with the the orginal findings of Lam et al. 1995, further experimentation by this same group revealed a significant increase in the peptitidyl-prolyl cis/trans isomerization of the tetrapeptide substrate following addition of calcium and CaM (Edlich et al., 2006). Interestingly, Edlich's 2006 publiation revealed that FKBP38's the cis/trans isomerization rate could be decrased when experiments were performed in presence of FK506 or Bcl-2. The concentration of calcium required for 50% FKBP38 PPIase activity was approximately 290nM, making activation of FKBP38 and its sensor protein CaM possible following bursts by second messenger calcium (only example of this) and not by non-activated cytosolic levels of calcium (Feske et al., 2001).

Chaperone/IP

Using a yeast two hybrid screen FKBP38 was identified as a potential Bcl-2 binding partner (Shirane and Nakayama, 2003). Due to the previously characterized lack of FK506 binding and PPIase activity (Lam et al., 1995), Shirane and Nakayama 2003, hypothesized that FKBP38 binding might be involved in apoptotic regulation via mechanisms other than through CaN or PPIase activity. Confirmation of the yeast two

hybrid results was generated by performing immunoprecipitation which showed that FKBP38 bound Bcl-2, Bcl-X_L, and CaN. This interaction between FKBP38 and its partners did not require exogenous addition of calcium, CaM, or FK506. Further investigation from the same authors revealed that Bcl-2 and FKBP38 co-localized at the mitochondria following co-expression and that expression of mutant FKBP38 constructs, which altered the localization of FKBP38, correspondingly altered the localization of Bcl-2. Finally, siRNA-mediated depletion of FKBP38 resulted in decreased mitochondrial localized Bcl-2 and increased apoptosis. From these observations it was determined that FKBP38 was both a Bcl-2 chaperone and that FKBP38 mediated transport of Bcl-2 to the mitochondia, away from the nuclus, was required for proper anti-apoptotic Bcl-2 function.

FKBP38 and Bcl-2 Binding

While Shirane and Nakayama in 2003 identified Bcl-2 as a FKBP38 binding partner, they did not characterize the molecular mechanism of protein interaction between the two proteins including which domains are necessary for proper interaction. The only publication that has attempted to address this mechanism used recombinant expression of both Bcl-2 and FKBP38, which demonstrated co-purification of both proteins in *E. coli*, yielding further evidence that these two proteins were capable of binding (Kang et al., 2005). In this report Kang and colleagues also propose a potential mechanism for Bcl-2/FKBP38 binding based on FKBP38 expression in the presence of loop domain deletion mutants of Bcl-2. This recombinant co-expression in *E. coli* revealed that loss of either the entire loop domain (AA35-89) or a large portion of the loop domain (AA55-89) prevented Bcl-2/FKBP38 binding. In addition, it was shown that other loop domain deletions (AA35-64, 65-89, and 35-79) decreased Bcl-2/FKBP38 binding but did not abolish binding. These results were further validated by yeast-two hybrid screen (Kang et al., 2005). Due to the fact that Bcl-2 is phosphorylated in the loop domain at amino acids Thr-56, Ser-70, and Ser-87, this group investigated whether or not the loss in Bcl-2/FKBP38 binding in the loop domain deletion mutants could be attributed to alterations

in the phosphorylation of Bcl-2. There results suggested that increased Bcl-2 phosphorylation resulted in a subsequent decrease in FKBP38 binding and therefore this group concluded that the mechanism of Bcl-2/FKBP38 binding was influenced by Bcl-2 phosphorylation. Therefore by deduction, since the loop domain contains three phosphorylation sites, Kang and colleagues implicated the loop domain of Bcl-2 as the critical domain responsible for regulating binding between Bcl-2 and FKBP38.

IV. PC12 CELLS

Pheochromocytoma (PC12) cells were derived from a spontaneous rat adrenal medullary tumor (Greene and Tischler, 1976). Undifferentiated PC12 cells undergo continual logarithmic multiplication and grow as an adherent cell line in culture (**Fig 1.8**). PC12 cells resemble cells of embryonic rat adrenal medulla by synthesizing, storing, and secreting both dopamine and acetylcholine following depolarization (Shafer and Atchison, 1991). PC12 cells also release catecholamines in response to nicotinic and muscarinic cholinergic agonists (Greene and Rein, 1977; Rabe et al., 1987). Differentiation of PC12 cells occurs following growth in media supplemented with nerve growth factor (NGF). PC12 cells exposed to NGF become differentiated and display a neuronal like phenotype. Differentiated PC12 cells also have altered surface and metabolic properties similar to that of sympathetic neurons (Meakin and Shooter, 1992). Therefore, this cell line is commonly employed as a 'neuron-like' model cell system. Finally, one important feature of PC12 cells is that they lack expression of endogenous Bcl-2 (**Fig. 4.1**), yet they still possess other Bcl-2 family members. The lack of endogenous Bcl-2 and possession of other Bcl-2 family members including Bcl-X_L, Bax, and Bad, make PC12 cells an ideal model system for studying the localization and apoptotic function of exogenously introduced Bcl-2. Presence of other Bcl-2 family members allows proper Bcl-2 dimerization and therefore allows experimental results to be examined in a cellular environment that is relevant to other disease related environments such as cerebral vasculature prior to ischemic insult. Also, the lack of

conflicting endogenous Bcl-2 activity eliminates the potential experimental bias that would occur using experimental models such as *in vitro* transient transfection into a Bcl-2 expressing cell line or using a transient ischemia/stroke *in vivo* tissue model.

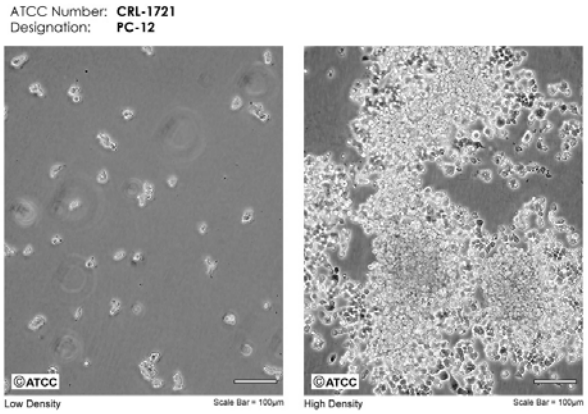


Figure 1.8 Undifferentiated PC12 cells in culture. Low plate density (left), high plate density (right). Adapted from American Type Culture Collection http://www.atcc.org/common/images/Cells/CRL-1721_mg1.jpg

V. OVERALL SIGNIFICANCE

It is well documented that multiple mechanisms regulate the initiation and execution of apoptosis, a process that is required for both physiological and pathological elimination of cells and tissue. The importance of regulating apoptosis becomes apparent when examined in the context of disease. Both transient ischemic attack and stroke result in cell loss that is modulated by the extent of apoptosis following initial insult. On the other hand, prevention of apoptosis by disregulated expression of apoptotic regulating proteins, such as Bcl-2, can result in neoplastic transformation, tumorigenesis, and acquired resistance to chemotherapeutic drugs. Thus, an intimate understanding of intracellular events that regulate the apoptotic process is a crucial step for the comprehension of molecular mechanisms that lead to pathological disregulation of apoptosis. This project characterizes the role of nuclear compartment-associated Bcl-2 in

the modulation of the apoptotic machinery. Understanding the role of proteins that regulate apoptosis will help establish new molecular targets for future therapies and therefore possibly reduce the severity of neuron loss following stroke or be used to specifically eliminate tumors which abnormally express high levels of Bcl-2.

CHAPTER 2: MATERIALS AND METHODS

CELL CULTURE

Rat pheochromocytoma cells (PC12), human embryonic kidney 293 cells (HEK 293), and human epithelial cells (HeLa) were obtained from the American Type Culture Collection (Manassas, Virgina). Rat neuroblastoma cells (B104) were a kind gift of Dr. H.M. Fishman (University of Texas Medical Branch – Galveston, Texas). Human Acute Lymphocytic Leukemia cells (REH), and human Acute Myeloid Leukemia cells (HL-60) were a kind gift of Dr. P. Ruvelo (MD Anderson- Houston, Texas). Cells were maintained in a humidified atmosphere with 5% CO2 in RPMI 1640 supplemented with 5% fetal bovine serum, 5% horse serum, 1% penicillin/streptomycin (Cellgro – Herndon, VA) [PC12 – REH – HL-60] or DMEM supplemented with 10% Fetal Bovine serum and 1% Penicillin/Streptomycin [HEK293 – HeLa], or DMEM-F12 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin [B104]. For maintenance of cell viability, 5ml of medium was replaced every other day and cells were split once per week.

DNA CONSTRUCTS

Expression vectors pMKitNeo (vector) and pMKitNeo-Bcl-2α were a kind gift of Dr. S. May (University of Florida – Gainesville, Florida). pHA-FKBP38 was a kind gift of Dr. K. I. Nakayama (Kyushu University – Fukuoka, Japan). Mito-Bcl-2 and Mito-Bcl-2-GFP were both kind gifts of Dr. C. W. Distelhorst (Case Western Reserve University – Cleveland, Ohio). Bcl-2ΔBH4 was generated by introducing a start codon at amino acid

position 25 of parental pMKitNeo-Bcl-2α, flanked by a XhoI site. The obtained fragment was then digested with XhoI/EcoRI and re-cloned into pMKit-Neo. pEYFP-N1 (YFP) vector was purchased from Clontech (Clontech – Mountain View, California). pBcl-2/YFP was generated by PCR amplification of amino acids 1-661 of parental pMKitNeo-Bcl-2α, substituting the stop codon of Bcl-2 with an alanine flanked by an XhoI site. The obtained fragment was then digested with XhoI/EcoRI and re-cloned into pEYFP-N1 in frame with its EYFP coding region.

TRANSFECTIONS

DNA transfection

All DNA transfections were performed using Lipofectamine and Plus reagent (Invitrogen – Carlsbad, California). Cells at 40 to 50% confluence received a total of 1.2 pmoles/ml of DNA coupled to Lipofectamin/plus at a ratio of 1:3 and diluted in serum-free medium, OptiMEM (Invitrogen – Carlsbad, California). To enhance transfection, it is essential to culture cells in serum-free medium for at least 3 hrs after addition of liposomes. After 3 hrs, transfection media was removed and normal culture media was added. Success of transient gene transfection and expression of the relevant protein was assayed 1-2 days later by Western blot. Stable transfectants were generated using the same transfection technique, followed by the addition of Geneticin antibiotic to the culture medium.

Peptide TAT-BH4 protein transfection

TAT-BH4 peptide and TAT-BH4scr peptide transfections were performed by diluting peptides to 2uM or 10uM in normal growth media without antibiotics and applying directly to cells. Cells were incubated at 37°C in normal growth media plus peptide until time of assay.

REPORTER ASSAYS

Lactate Dehydrogenase (LDH) Release

LDH activity was assayed in the cell culture medium using a colorimetric LDH detection kit (Roche, Indianapolis, Indiana) according to the manufacturer's instructions and applying appropriate controls.

GelCode Stain

Following immunoprecipitation and polyacrylamide gel electrophoresis, gels were stained using GelCode (Pierce Biotechnologies – Rockford, Illinois) following the manufacture's instructions. Briefly, gels were washed 3 times with deionized water for 15 min. Following washes, gels were incubated with approximately 50ml of GelCode (for 8.3x7.3cm gel) for 1 hr. Following staining, gels were rinsed 3 times with deionized water and then incubated overnight in water at room temperature. Following overnight wash, stained gels were placed on a light box and digital images were taken using UVP 12bit monochrome digital Chemi HR camera (UVP, Upland, California, USA).

WESTERN BLOT ANALYSIS

Protein Extraction and Subcellular Fractionation

For gene expression verification, total protein was collected from cells by lysis in SDS lysis buffer containing 5mM EDTA, 50mM Tris, 2% SDS, 1mM DTT, 1mM PMSF and 1% protease cocktail inhibitors (Sigma – St. Louis, Missouri). Following lysis, cells were sonicated for 15 sec to shear chromosomal DNA, and then centrifuged at 20,000 x g for 5 min. The supernatant was transferred to a clean tube and stored at -20^{o}C until further use. For protein localization experiments, nuclear and non-nuclear fractions were prepared as follows: Non-Nuclear Proteins- To collect non-nuclear proteins, PC12 cells were lysed for 15 min in 300µL ice-cold hypotonic lysis buffer (10mM Tris, pH 7.4, 0.1M sucrose, 10mM NaCl, 1mM dithiothreitol, 1mM phenylmethylsulfonyl

fluoride, 1mg/mL aprotinin, 1mg/mL leupeptin, 250mg/mL benzamide) and centrifuged at 800 x g for 5 min at 4°C. The resulting nuclear pellet was further purified as described under nuclear proteins section below. The supernate was transferred to a new 1.5mL tube and centrifuged at 1000 x g for 10 min at 4°C. The supernate from this second centrifugation (non-nuclear protein) was transferred to a new 1.5mL tube and protein concentration in each sample was measured using a BCA protein assay kit according to the manufacturer's instructions (Pierce – Rockford, Illinois). Nuclear Proteins- Following isolation of the nuclear pellet (as described above), nuclei were lysed in 50µL SDS lysis buffer (5mM EDTA, 50mM Tris and 2% SDS) and disrupted by sonication for 15 sec. Protein concentration was determined in each sample using the standard BCA protein assay (Pierce – Rockford, Illinois).

Electrophoresis and Electroblotting

Protein samples containing 40 µg of proteins were diluted in 4X SDS loading buffer (each 20 ml of buffer contains: 0.608g Tris, 0.8g SDS, 800µl β-mercaptoethanol, 0.005g Bromophenol Blue and 8ml Glycerol), boiled for 5 min and then subjected to SDS-PAGE using 12% gels. Samples were run until complete separation of molecular weight markers bands (Biorad – Hercules, California) in Tris-Glycine buffer (20mM Tris, 150mM glycine, 0.1% SDS, pH 8.3). Sample separation was followed by electrophoretic transfer to a methanol activated PVDF-membrane (Biorad – Hercules, California) in Tris-Glycine buffer (20mM Tris, 150 mM glycine, 20 Methanol, pH 8.0). Proteins were blotted onto the membrane by electrophoretic transfer at 400 mA for 4 h at 4^{o}C.

Immunodetection

Following transfer, membranes were incubated with 1:1000 (v:v) Anti-Bcl-2 polyclonal antibody (Pharmingen – San Diego, CA), or 1:10,000 Anti-FKBP38 monoclonal antibody (kind gift of Dr. K.I. Nakayama), or 1:50,000 anti β-actin

monoclonal antibody (Sigma – St. Louis, Missouri) or 1:2000 PanLamin (kind gift of Dr. A. Fields) or 1:2000 IκBα (Santa Cruz Biotechnology – Santa Cruz, California). This initial incubation was then followed by a horseradish peroxidase (HRP) conjugated secondary antibody (Biorad – Hercules, California) against rabbit IgG (for polyclonal primaries) or mouse IgG (for monoclonal primaries). Immunoreactive bands were detected by a chemiluminescent Western blot detection kit (Amersham Biosciences – Piscataway, New Jersey) according to the manufacturer's instructions.

Western Blot Analysis

Following incubation with the chemiluminescent agent (ECL), membranes were captured a using AlphaImager light cabinet (Alpha Innotech Corporation, San Leandro, California, USA). Images of immunoblots were analyzed and densitometry was measured using image analysis software from AlphaEase and Adobe Photoshop (AlphaEase Image Analysis Software – Alpha Innotech – San Leandro, California; Adobe Photoshop – Adobe Systems – San Jose, California)

FLOW CYTOMETRY BASED ASSAYS AND TECHNIQUES

Nuclei isolation from PC12 cells

PC12 cells were detached from the culture flask by tapping and collected cells were centrifuged at 800xg for 5 min. Media was removed and the cell pellet was washed 2x with cold PBS. The washed pellet was re-suspended in 1.25mL hypotonic lysis buffer (10mM Tris, pH 7.4, 0.1M sucrose, 10mM NaCl, 1mM dithiothreitol, 1mM phenylmethylsulfonyl fluoride, 1mg/mL aprotinin, 1mg/mL leupeptin, 250mg/mL benzamide), vortexed for 15 sec and set on ice for 15 min. Following lysis, 2.25mL of 1.8M sucrose was added and samples were mixed by vortexing. Next, samples were poured on top of a 1.25mL, 1.8M sucrose cushion and centrifuged at 15,500xg for 25 min at 4°C in a swinging bucket rotor (SW50.1). After centrifugation, the supernate was

removed, pellets (containing the nuclei) were re-suspended in 300µL PBS and centrifuged at 1000xg for 5 min at 4°C. Pelleted nuclei were then used for further assays.

Nuclei isolation from tissue

100mg of fresh or frozen tissue was weighed out and resuspended in 500µL cold PBS. Tissue was disrupted using a dounce cell homogenizer (20 strokes) and then centrifuged at 800xg for 5 min at 4°C. The pellet was then used for nuclei isolation as described above with the exception that 1.1M sucrose cushion (instead of 1.8M) was used for rat brain tissue.

Tissue preparation

According to protocols approved by the UTMB Institutional Animal Use and Care (IACUC) committee, three month old male Sprague Dawley rats (Harlan Sprague Dawley – Indianapolis, Indiana) were sacrificed by carbon dioxide asphyxiation and the hippocampus, cerebellum, and cortex rapidly dissected out. One half of each tissue was immediately processed for nuclei isolation and half were rapidly frozen in liquid nitrogen and stored at -80°C until nuclei isolation was performed.

Brain slice preparation

Immediately after sacrifice, rat brains were dissected out and sliced coronally at 225µm, using a Mcllwain tissue chopper (Wilmington, Deleware).

Apoptosis induction in PC12 cells

Cells were grown to 70% confluence under normal culture conditions as described above. To induce apoptosis, cells were treated with H_2O_2 as described previously (Konopleva et al., 2002). Briefly, cells were treated with 100µM H_2O_2 in

Opti-mem (Invitrogen – Carlsbad, California). After 20 min, Opti-mem/ H_2O_2 medium was replaced with normal growth medium. Apoptosis was detected by flow cytometry 12 hrs post treatment.

Apoptosis induction in PC12 nuclei

PC12 nuclei were isolated as described above. Induction of apoptosis using isolated nuclei was performed as described previously (Vanderbilt et al., 1982). Briefly, isolated nuclei were resuspended in buffer (0.35M sucrose, 5mM $MgCl_2$, 25mM Kcl, 10mM Tris pH 7.4) containing 1mM $CaCl_2$ and incubated at room temperature for 2hr. Following incubation, nuclei were suspended in PISS and then analyzed by flow cytometry.

Apoptosis induction in rat brain slices

This method was performed as described previously (Mattila et al., 1990). Briefly, coronal rat brain slices were placed in ice-cold buffer A continuously oxygenated with 95%/5% O_2/CO_2 (buffer A: pH 7.4, 10mM HEPES, 125mM NaCl, 5mM Kcl, 25mM $NaHCO_3$, $MgSO_4$ 15mM and 10mM glucose). Following division into random groups, brain slices were maintained in buffer A at 37°C for 30 min followed by buffer B for 30 min (buffer B: 10mM Hepes, pH 7.4, containing 125mM NaCl, 5mM Kcl, 25mM $NaHCO_3$, 5mM $MgSO_4$, 1.5mM $CaCl_2$ and 10mM glucose). Slices were totally immersed in the buffer (10mL) in glass vials. Brain slices for control group were continuously bubbled with 95%/5% O_2/CO_2. For the hypoxia/aglycia group, samples were made hypoxic by replacing 95%/5% O_2/CO_2 with 100% N_2 and aglycic using buffer C (buffer B lacking glucose) for 3hr. At the end of each treatment, populations of nuclei were isolated from brain slices for flow cytometry analysis or cytosolic fractions were collected for cell death ELISA.

Flow cytometry based Annexin-V assay

Cells were plated between 40-50% confluency into 12-well plates. Following 24 hrs of recovery, cells were treated with 10uM BH4 peptide or BH4scr peptide at set time points (3, 6, 24, and 48 hr) prior to assay. Immediately before assay, cells were washed 3 times with PBS (pH 7.4) then resuspended in 500µL Annexin Binding Buffer (10mM HEPES pH 7.4, 140mM NaCl, and 2.5mM $CaCl_2$). 100µl of this suspension was removed to a new 1.5mL eppendorff tube. 6µl of Annexin-V-APC (BD Pharmingen – San Diego, California, USA) was added to each tube and gently mixed. Tubes were incubated at room temperature for 15 min. Following incubation, 400µl of Annexin Binding Buffer (containing 1ng/mL propidium iodide) was added to each tube. Samples were gently mixed, placed on ice, and analyzed by flow cytometry within 60 min. Flow cytometry was performed on a Becton-Dickenson Canto with excitations of 488nm and 633nm. Emission wavelengths detected included >530nm (YFP), 660nm (APC), and 670nm (Propidium iodide). Data analysis was performed using WinMDI 2.8 software program (written by Joseph Trotter – Scripps Research Institute – La Jolla, California) and FCS express V3 (De Novo Software – Los Angeles, California).

Flow cytometry based physical light scatter apoptosis assay

Isolated populations of nuclei were re-suspended in 500µL Propidium Iodide Stain Solution (PISS: 1mg/mL sodium citrate, 1% Triton-X100, 50ng/mL propidium iodide, containing 50µg/mL RNAse (added immediately before application to nuclei), and then incubated at 4°C on a rotating shaker for 2-4 hrs. Following incubation, nuclei were pelleted at 1000xg for 5 min at 4°C and re-suspended in 500µL cold PBS. Next, populations of nuclei were analyzed on a Becton-Dickinson FACS-Scan using an argon laser (excitation = 488 nm). FSC: voltage E00, AmpGain 7.43, and Mode Lin. SSC: voltage 435, AmpGain 1.0, and Mode Lin. Propidium Iodide fluorescence was detected using a 620nm band pass filter. Data analysis was performed using WinMDI 2.8 software

program (written by Joseph Trotter – Scripps Research Institute – La Jolla, California) and FCS express V3 (De Novo Software – Los Angeles, California).

IMMUNOPRECIPITATION

General IP Protocol

PC12 cells grown in 75cm^2 flasks were washed 3 times with PBS and solubilized in 400µl of ice-cold immunoprecipitation buffer (1% Nonidet P-40 in 20mM Tris-HC1, pH 8.0, 137mM NaCl, 2mM EDTA, 10% glycerol, 1mM sodium orthovanadate, including the protease inhibitors phenylmethylsulfonyl fluoride (1mM), aprotinin (10 pg/ml), and leupeptin (10 pg/ml)) by gentle rocking for 1 hr at 4°C. Insoluble material was removed by centrifugation at 10,000g for 10 min. All immunoprecipitations were performed at 4°C.

Hemagglutinin (HA) pulldown

Performed according to manufacture's instructions (Profound HA immunoprecipiatation kit (Pierce Biotechnologies – Rockford, Illinois). Briefly, protein extracts (800µg) were incubated with agarose-coupled HA antibody rotating overnight at 4°C. Immunoprecipitated proteins were eluted from the beads using elution buffer, gel loading buffer was added, and samples were heated at 100°C for 5 min.

Bcl-2 mediated agarose A/G pulldown

Protein extracts (800µg) were incubated with primary antibody Bcl-2 N19 (8µl) with agarose A/G beads (50µl). Samples were incubated overnight rotating at 4°C and subsequently each immunoprecipitation was collected by centrifugation at 10,000g for 1 min, washed 3 times with ice-cold immunoprecipitation buffer and once with PBS (pH 7.4). For SDS-polyacrylamide gel electrophoresis of the immunoprecipitated proteins,

agarose A/G beads were heated at 100°C for 5 min in SDS-polyacrylamide gel electrophoresis buffer (each 100mL of buffer contained 3g Tris, 8g SDS, 2.5g DTT, 0.05g bromophenol blue, 40% (v/v) glycerol).

BH4 Peptide Immunoprecipitation

PC12 cells were transiently transfected with HA-FKBP38 construct for 24 hrs as described above. Cells were lysed in non-denaturing lysis buffer and total cell proteins were collected. Immunoprecipitation was performed using a HA antibody pulldown (Profound HA immunoprecipiatation kit – Pierce Biotechnologies – Rockford, Illinois), 800ug of total protein, and 2ug/ul of BH4 or BH4 scramble peptide. Following an overnight incubation rotating at 4°C, proteins were eluted and utilized for polyacrylamide gel electrophoresis.

CONFOCAL MICROSCOPY

Cells were grown on glass cover slips in 12-well plates. Following transient transfection as described above, cells were fixed with 4% paraformaldehyde for 30 min, washed in PBS for 5 min, permiabilized with 1% Triton-X in PBS for 30 min, washed in PBS for 5 min, incubated in blocking 5% normal goat serum (NGS) for 1 hr, incubated with primary antibody in 2% NGS for 1 hr, washed in 5% NGS in PBS three times for 10 min each, incubated in secondary antibody in 2% NGS for 1 hr, washed in 5% NGS three times for 10 min each, and mounted for imaging in Vecta-shield hard mount medium (Vector Laboratories – Burlingame, California) containing Hoechst. Images were obtained using a Zeiss LSM510 META laser scanning confocal microscope with laser excitations at 633nm, 351nm, 488nm, and long pass filters between 385 and 545nm (Hoechst) and >530nm (YFP). All imaging analysis was performed using Meta-morph imaging software V.6.0 (Universal Imaging Corporation – Downingtown, Pennsylvania).

PEPTIDE SYNTHESIS

Peptides were generated by solid-phase peptide synthesis using standard Fmoc chemistry and purified by HPLC. Identity was confirmed by amino acid analysis and mass spectrometry. TAT-BH4 (C-G-YARKARRQARR-DNREIVMKYIHYKLSQRG) and TAT BH4scramble (C-G-YARKARRQARR-VKRDYRSMIKGEHQLYIN) peptides were synthesized by GenScript Corp. (Piscataway, New Jersey) and HPLC-purified to >90% purity. Non-TAT labeled BH4 (DNREIVMKYIHYKLSQRG) and BH4scramble (VKRDYRSMIKGEHQLYIN) peptides were synthesized by the University of Texas Medical Branch protein synthesis core and were HPLC-purified to >95% purity.

Cell Death Enzyme-Linked Immune-Sorbant Assay (ELISA)

Apoptotic cell death in coronal rat brain slices was evaluated by ELISA (Cell Death Detection ELISAPLUS – Boehringer Mannheim/Roche – Indianapolis, Indiana) according to the manufacturer's instructions. The enrichment of mono- and oligonucleosomes released into the cytoplasm is calculated as the ratio of the absorbance of the sample cells to the absorbance of control cells.

Small-Interfering RNA

PC12 cells (1.0×10^5) were seeded into six-well plates and were grown to 50% confluency. The fkbp8 (FKBP38 small-interfering RNA (siRNA)) (ON TARGETplus SMARTpool siRNA – Dharmacon, Lafayette, Colorado) or control siRNA (ON TARGETplus SMARTpool siRNA) was combined with DharmaFECT transfection reagent #1, and the cells were transfected according to the recommended protocol with siRNA (100nM final concentration). After 48 hrs of transfection, cell lysates were prepared for Western blot analysis.

STATISTICAL ANALYSIS

For statistical evaluation, experiments were performed in triplicate (intra-assay variability). Final results were statistically evaluated on the average calculated out of the three independent replicate experiments. Statistical differences between groups were assessed using a Student's "t" test. An α level below 5% ($p<0.05$) between groups was considered statistically significant. All statistics were performed using the Microsoft Excel software (XP version).

The following chapter was previously published under the title “Rapid assay for quantitative measurement of apoptosis in cultured cells and brain tissue.” In Journal of Neuroscience Methods (155(1) 134-142, 2006). It was jointly authored by Bryce P. Portier, Ferrari DM, and Giulio Taglialatela.

CHAPTER 3: RAPID ASSAY FOR QUANTITATIVE MEASUREMENT OF APOPTOSIS IN CULTURED CELLS AND BRAIN TISSUE.

INTRODUCTION

Analysis of apoptosis in brain tissue following ischemia, hypoxia, or oxidative stress has technical limitations. The use of counting cells displaying apoptotic morphology is time intensive, vulnerable to sampling errors, and suffers from low numbers of total recorded events (Kerr et al., 1995; Saraste, 1999; Yasuhara et al., 2003). Other cell death assays such as agarose gel analysis of DNA fragmentation, TUNEL, or ELISA are time intensive, limited to a single endpoint measure, and can be technically difficult to perform or reproduce (Gold et al., 1993; Grasl-Kraupp et al., 1995; Stahelin et al., 1998). To overcome these limitations, we set out to develop a technique using flow cytometry to measure apoptosis based on the physical properties of light scatter produced from isolated nuclei. This dye/marker free approach would bypass many of the inherent encumbrances and reproducibility problems found in other apoptosis assays. Our hypothesis is that flow cytometry performed on isolated nuclei will allow rapid quantitation of apoptosis in nuclei isolated from both cells grown in culture as well as brain tissue. Here we investigated the ability to detect apoptosis in isolated nuclei populations using the functional application of a physical light scatter based flow cytometry assay.

METHODS

For a complete description of general methods and techniques, please refer to Chapter 2 Material and Methods.

RESULTS

Purity of Nuclei Post Isolation

Nuclei isolation and flow cytometry conditions were optimized using the neuron like model cell line PC12 (Greene and Tischler, 1976). A phase contrast image (**Fig. 3.1A**) demonstrates architecture of the PC12 nucleus after sucrose gradient isolation. The phase contrast image is representative of three separate isolation procedures and illustrates the purity (lack of cytosolic contaminates) and intact architecture of nuclei isolated using the sucrose gradient procedure detailed in the methods section. Western blot analysis (**Fig. 3.1B**) shows the amount of cross contamination between nuclear and non-nuclear separated fractions. In this blot the presence of ER protein Calnexin, mitochondrial protein COX IV, and nuclear protein Pan Lamin (A- and B-type lamins) were analyzed in both the nuclear and non-nuclear fractions. Densitometry revealed 10% of expressed Calnexin localized within the nuclear fraction. Since it is known that the ER is continuous with the nuclear envelope (Voeltz et al., 2002), finding a small portion of ER protein localized within the nuclear fraction was expected, especially since our preparation does not disturb the outer nuclear membrane. Based on this, we set a standard of purity for nuclear fractions at ≤10% ER protein within the nuclear fraction. The mitochondrial protein COX IV was solely detected in the cytosolic fraction which demonstrates no mitochondrial contamination within the nuclear fraction. Additionally the nuclear marker Pan Lamin was found solely in the nuclear fraction demonstrating that nuclei were not lost in the initial steps of isolation (i.e. while obtaining the non-nuclear proteins).

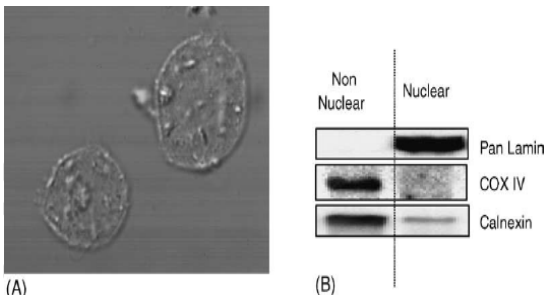


Figure. 3.1. Assessment of isolated nuclei purity. A, Representative phase contrast image of nuclei isolated from PC12 cells using the sucrose gradient protocol described in methods. B, Western blot analysis detecting Pan Lamin (nuclear marker), COX IV (mitochondrial marker) and Calnexin (ER

marker) in nuclear and non-nuclear protein fractions prepared from PC12 cells

Apoptosis detection by Physical Light Scatter Properties of Isolated Nuclei

To demonstrate the technique of using flow cytometry to detect apoptosis in isolated populations of nuclei by physical light scatter, we compared matched physical light scatter and propidium iodide staining following growth under normal culture conditions and following 12hr treatment with 100µM H_2O_2 (**Fig. 3.2A**). This dose of H_2O_2 induces a moderate degree of apoptosis in PC12 cells and was chosen based on previously published evidence (Konopleva et al., 2002) and a dose response (**Fig. 3.2B**), that shows an increase in LDH release from our PC12 cells. The light scatter profile revealed that isolated nuclei separate into three distinct populations (**Fig. 3.2A, Top**). Analysis of the corresponding propidium iodide staining (**Fig. 3.2A, Bottom**) revealed that these three populations from top to bottom, represent nuclei with G2, G1, and sub-G1 DNA content respectively. Nuclei located between the G2 and G1 populations represent nuclei in various stages of the S phase of cell division. As expected, there was a marked increase in the sub-G1 DNA (arrows) in the population of nuclei isolated from PC12 cells exposed to H_2O_2.

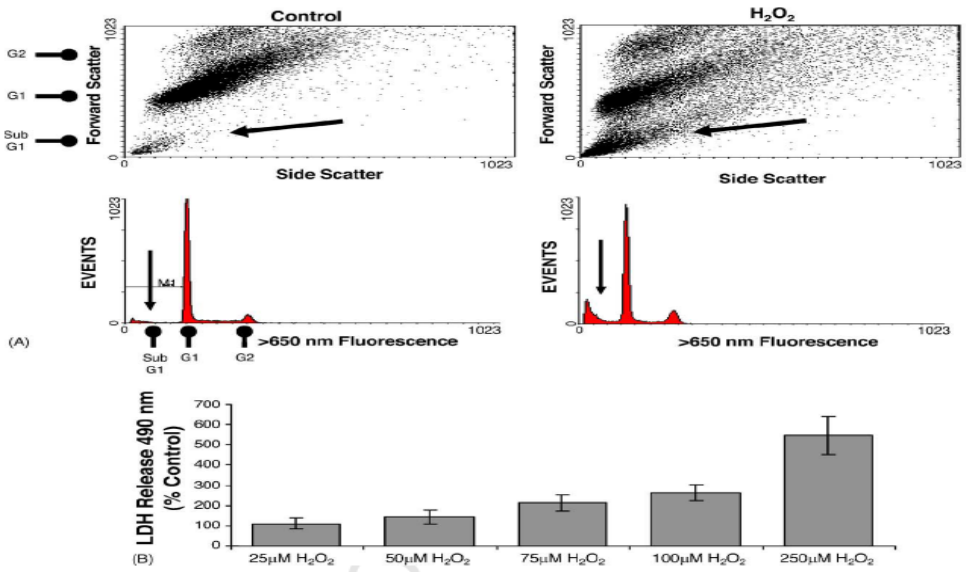


Figure 3.2. Detection of apoptotic nuclei by physical light scatter and propidium iodide DNA staining using flow cytometry. **A**, Physical light scatter and DNA histogram of nuclei isolated from PC12 cells grown under normal culture conditions or 12hr post H_2O_2 treatment. Physical light scatter generated a dot plot with 3 distinct populations. These 3 populations correspond (from top to bottom) to nuclei containing G2 DNA content (nuclei undergoing cell division), G1 DNA content (normal nuclei), and sub-G1 DNA content (apoptotic nuclei –solid arrows). The physical light scatter and corresponding propidium iodide DNA histogram for control cells show only a trace amount of sub-G1 (apoptotic) nuclei. Physical light scatter and corresponding propidium iodide DNA histogram revealed an increase in the sub-G1 population in cells treated with H_2O_2 as compared to control. **B**, PC12 cell death elicited by increasing concentrations of H_2O_2. Cell death is represented as percent of LDH release relative to non-treated control PC12 cells.

To further test the ability of flow cytometry to detect nuclei containing sub-G1 DNA on the basis of their physical light scattering, a population of nuclei isolated from control PC12 cells were treated with 1mM calcium, which results in massive DNA fragmentation due to activation of calcium-dependent endonucleases as described previously (Vanderbilt et al., 1982; Jones et al., 1989). The population of nuclei treated with calcium (**Fig. 3.3**) were solely detected as shrunken nuclei by physical light scatter and the propidium iodide stained nuclei histogram revealed that all nuclei contained sub-G1 DNA.

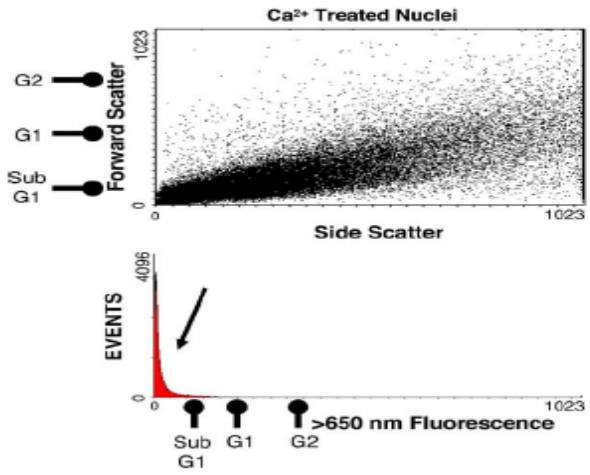


Figure 3.3. Physical light scatter plot and propidium iodide DNA histogram of isolated nuclei exposed to 1mM calcium 2 hrs prior to flow cytometry analysis. Physical light

scatter shows all nuclei detected belong to the small apoptotic nuclei population. The DNA histogram also reveals that all nuclei, forced to undergo apoptosis via calcium treatment, now are detectable solely as sub-G1 apoptotic nuclei.

Nuclei Isolation in Fresh and Frozen Brain Tissue

When translating protocols from cultured cells to brain tissue it was unknown whether freezing tissue would affect nuclei isolation or artificially increase the amount of detectable apoptosis. To address this concern, nuclei were isolated from matched fresh and frozen samples from cortex, hippocampus, and cerebellum and subsequently analyzed for physical light scatter and propidium iodide staining. Nuclei propidium iodide histograms and corresponding physical light scatter profiles are shown for fresh (**Fig. 3.4A**) and frozen tissue (**Fig. 3.4B**). No significant difference was detected in the amount of sub-G1, apoptotic nuclei detected by either propidium iodide staining or by physical light scatter between fresh and frozen tissue. The amount of sub-G1 DNA detected by propidium iodide staining in fresh and frozen specimens from each brain region was as follows: cerebellum (fresh: 12.96%±.26 frozen: 12.56%±.24), cortex (fresh: 6.36%±.17 frozen: 6.15%±.32), and hippocampus (fresh: 17.78%±.32 frozen: 18.31%±.25). Similarly, the amount of sub-G1 DNA detected by physical light scatter in fresh and frozen tissue was as follows: cerebellum (fresh: 12.74%±.29 frozen: 12.02%±.30), cortex (fresh: 6.0%±.49 frozen: 5.9%±.41), and hippocampus (fresh: 17.92%±.21 frozen: 18.22%±.33). In addition, physical light scatter plots (**Fig. 3.4A-B**) revealed that populations of nuclei prepared from brain tissues yielded fewer nuclei in S and G2 phase of the cell cycle as compared to populations of nuclei isolated from cultured cells (**Fig. 2A**). This was not unexpected considering, unlike PC12 cells (rapidly dividing cell line), the majority of neural cells are post mitotic.

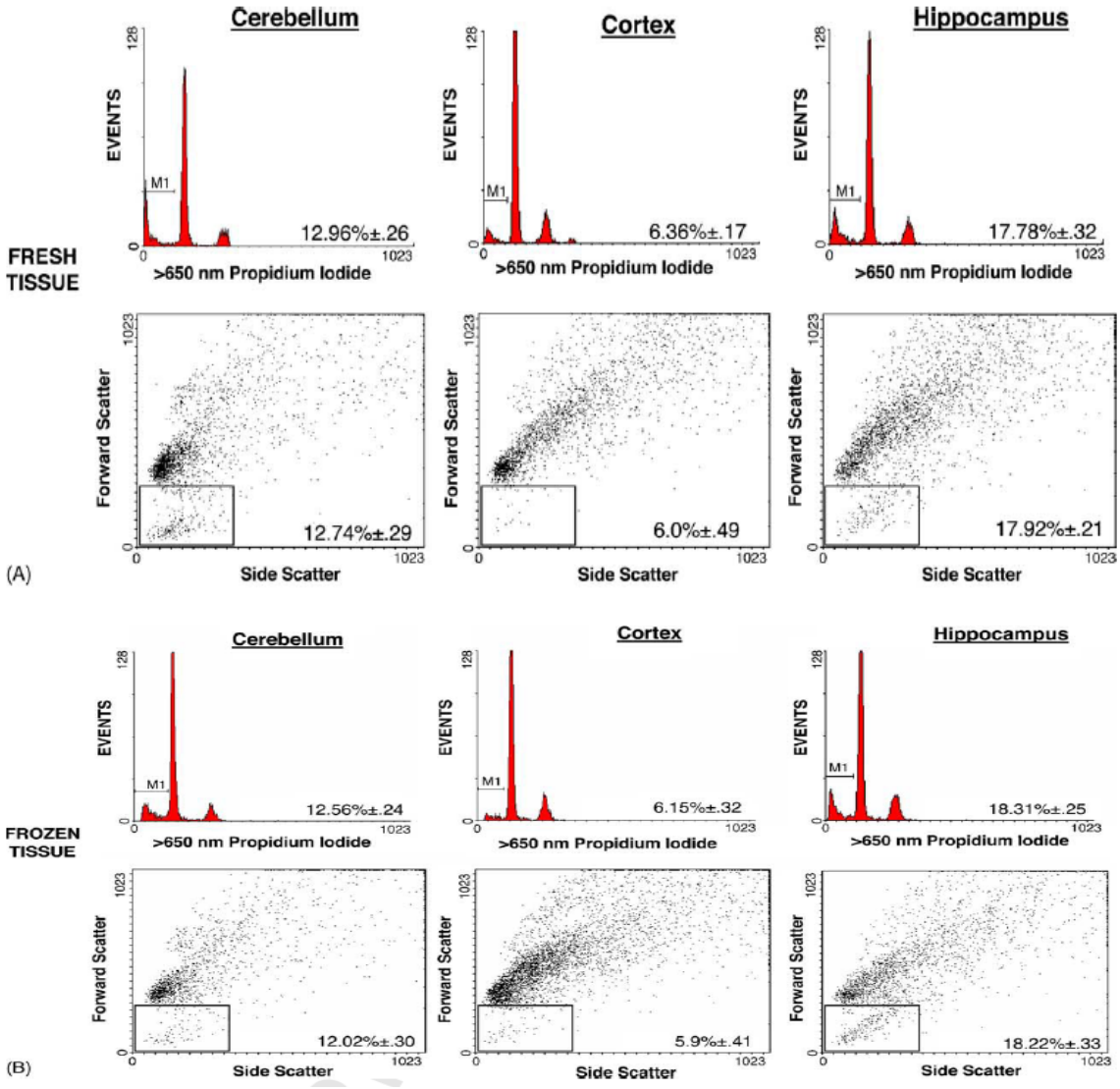


Figure 3.4. Comparison of sub-G1 DNA content and physical light scatter in nuclei isolated from fresh vs. frozen brain tissue. Rat brains were dissected and cerebellum, hippocampus, and cortex were isolated. Half of each brain region was stored at -80°C for one week prior to isolating nuclei. The other half of each brain region was used immediately after dissection for nuclei isolation followed by flow cytometry. The brain preparation, nuclei isolation, and flow cytometry was repeated 3 times. The average sub-G1 DNA content (histogram) and small apoptotic nuclei (boxed area within the physical light scatter dot plot) ± the standard deviation from fresh tissue is represented in **A** and frozen tissue in **B**. The propidium iodide DNA histograms along with the physical light scatter dot plots show no significant change in sub-G1 DNA content from nuclei isolated from fresh or frozen tissue.

Detection of Apoptosis in Brain Slices Post Aglycia/Hypoxia Treatment

To demonstrate the utility of detecting neuronal apoptosis by physical light scatter, we prepared coronal brain slices from Sprague Dawley rats and exposed them to apoptosis inducing conditions of aglycia/hypoxia as described previously (Mattila et al., 1990). Following treatment conditions (3hr hypoxia/aglycia and 3hr recovery) or standard conditions (control) both groups were assayed for induction of apoptosis using a classical apoptosis detection method, fragmented DNA ELISA. The ELISA (**Fig. 3.5A**) reveals a significant increase in the presence of fragmented, apoptotic DNA following aglycia/hypoxia treatment (+71%; $p<0.001$ compared to controls). Flow cytometry measuring physical light scatter of nuclei isolated from the same brain slices revealed a 143% increase (11% control vs. 26.73% treatment) in apoptotic nuclei from brain slices exposed to hypoxia/aglycia (**Fig. 3.5B**). Consistent with this measurement, analysis of propidium iodide stained DNA also revealed a 130% increase (11.36% control vs. 26.08% treatment) in sub-G1 DNA containing nuclei as compared to controls. The discrepancy between the extent of apoptosis measured by the ELISA (+71%) as compared to physical light scatter (+143%) can be explained by the fact that the two methods measure different parameters of apoptosis (amount of cytosolic light molecular weight DNA vs. number of apoptotic nuclei containing Sub-G1 DNA). The amount of fragmented DNA released in the cytosolic fraction may not necessarily be proportional to the absolute number of apoptotic nuclei as each apoptotic nucleus may release different amounts of fragmented DNA, depending on the stage of the apoptotic process. Using physical light scatter, every nucleus undergoing apoptosis is counted regardless of the amount of cytosolic DNA released. Therefore, it is not surprising that the percent increase of the number of apoptotic nuclei may exceed the percent increase of cytosolic fragmented DNA determined in the same tissue sample.

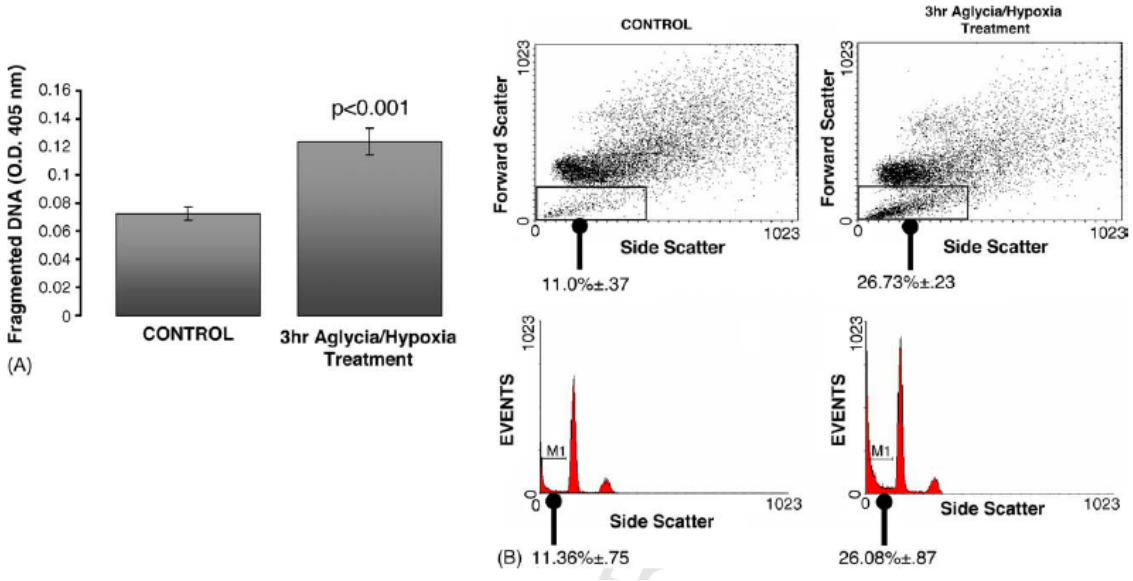


Figure 3.5. Demonstration of apoptosis detection by flow cytometry using physical light scatter of isolated nuclei from coronal rat brain slices. Coronal rat brain slices were placed under standard conditions for 6hr (control) or hypoxic/aglycic conditions for 3hr followed by 3hr recovery (treatment). Following treatment, brain slices were analyzed by fragmented DNA ELISA. The ELISA in **A**, showed a significant increase in fragmented DNA in the samples receiving treatment ($p<0.001$, 71% increase in cytosolic fragmented DNA release compared to control). In panel **B**, the same coronal rat brain slices were subjected to nuclei isolation and examined for apoptosis via physical light scatter and propidium iodide DNA histogram. The nuclei contained within the box represent shrunken apoptotic nuclei. The percent shown below each graph is the average amount of shrunken apoptotic nuclei within the box ± standard deviation as compared to the total population from three separate nuclear isolations (11.0%±.37 control vs. 26.73±.23 treated which represents a 143% increase in apoptotic nuclei). Nuclei were additionally propidium iodide stained and analyzed for sub-G1 DNA content. The number below the DNA histogram represents the average percent of sub-G1 DNA containing nuclei from three separate nuclear isolations ± standard deviation (11.36%±.75 control vs. 26.08±.87 treated which represents a 130% increase in apoptotic nuclei).

DISCUSSION

The primary goal of this study was to develop a reproducible, rapid assay that could be used to measure apoptosis in neuronal tissue based solely on nuclear

morphology. Our results show that flow cytometry analysis can identify nuclei containing sub-G1 levels of DNA based on their size/morphology as detected by light scattering properties. A similar correlation between nuclear morphology/size detected by light scattering and DNA content was observed in nuclei isolated from both fresh and frozen brain tissue. In addition, we detected an increase in the number of small, sub-G1 DNA containing nuclei isolated from brain slices exposed to aglycia/hypoxia, a treatment known to induce apoptosis in ex-vivo preparations of brain slices (Mattila et al., 1990). We therefore conclude that our method of measuring apoptosis based on physical light scattering from populations of isolated nuclei reliably detects and allows quantification of apoptosis in fresh or frozen brain tissue as well as cultured cells.

Current techniques for measuring apoptosis in neuronal tissue have technical problems that can bias collection or quantification of neuronal cell death. The method described here for apoptosis detection resolves many problems inherent in other assays such as reliance on stains/dyes and manual counting. This method offers a major advantage over the TUNEL assay and silver staining in which non-specific labeling generates false positives (Gold et al., 1993; Grasl-Kraupp et al., 1995; Martin et al., 1998). Furthermore, the nuclei isolation/flow cytometry technique described here provides a reliable method to measure apoptosis in multiple regions of the brain. The nuclei isolation process is rapid, taking only two hours to complete and the nuclei isolated using this procedure are intact. These isolated nuclei can be analyzed by flow cytometry for apoptosis detection or could be separated (apoptotic vs. non-apoptotic) by flow separation and subsequently used for further analysis such as run-on assay, microarray, reverse-transcript PCR or western blot post nuclei sorting. This latter possibility is significant as current knowledge on molecular nuclear mechanisms involved in apoptosis is often acquired on whole cell populations including apoptotic and non-apoptotic cells. Thus, appreciating nuclear events selectively occurring in apoptotic cells as compared to non-apoptotic cells can pose a challenge when using classical techniques. Having the opportunity to isolate populations of nuclei selectively from cells undergoing apoptosis out of a mixed cell population as described here will overcome this problem

and should clarify specific nuclear events occurring in apoptotic versus non-apoptotic cells.

One significant concern regarding using this technique on brain tissue samples is the presence of small, non-apoptotic, G1 DNA-containing nuclei from glia (Gittins and Harrison, 2004) that may thus be erroneously accounted among the apoptotic nuclei. This is of particular concern, especially in conditions know to increase glia infiltration such as chronic neuroinflamation and Alzheimer disease (Blasko et al., 2004). Depending on the experimental conditions, this problem can be addressed by the addition of two preliminary experiments. Experiment 1, stain nuclei from control and treated brain tissue with propidium iodide (as described in methods), measure both physical light scatter and propidium iodide fluorescence, and then quantify the number of small apoptotic nuclei and the corresponding amount of sub-G1 DNA between the two samples. An increase in both the number of small nuclei and sub-G1 DNA will indicate increased apoptosis, whereas an increase in small nuclei without a corresponding increase in sub-G1 DNA content would reflect glia infiltration. Alternatively, experiment 2, nuclei could be labeled with the neuronal specific nuclear marker NeuN as described previously (Herculano-Houzel and Lent, 2005). Following NeuN labeling, compare the forward vs. side scatter of NeuN-positive nuclei to that of NeuN-negative nuclei (presumably population of glia nuclei). Addition of one or both of these preliminary experiments will allow an accurate assessment of glia contamination and adjustments to nuclei isolation procedure or data collection methods could be applied accordingly.

CONCLUSION

Using the physical light scatter profile from isolated populations of nuclei for analysis of apoptosis in neuronal tissue is rapid, reliable, and adaptable to multiple research applications. This procedure represents a general approach that can be applied to rapidly quantify apoptosis in cells grown in culture as well as tissue from CNS areas that can be reliably dissected, regardless of the method of apoptosis initiation or specific

region of brain being investigated. Given the limitations that can be encountered under conditions leading to glia proliferation/infiltration as discussed above, there are three main advantages to using our technique 1) isolation of nuclei can be reproducibly performed using fresh or frozen brain tissue from different CNS regions, 2) nuclei can be used to quantify the amount of apoptosis within a specific brain region without addition of markers/dyes, and 3) populations of nuclei (apoptotic or non-apoptotic) can be sorted and then further analyzed by run-on assay, EMSA, or any assay in which initial isolation of intact nuclei is required.

.

The following chapter is in press under the title “Bcl-2 localized at the nuclear compartment induces apoptosis following transient transfection” J. Biol. Chem., 10.1074/jbc.M606181200. It was jointly authored by Bryce P. Portier and Giulio Taglialatela.

CHAPTER 4: BCL-2 LOCALIZED AT THE NUCLEAR COMPARTMENT INDUCES APOPTOSIS FOLLOWING TRANSIENT TRANSFECTION

INTRODUCTION

Bcl-2 is the best characterized member of a large family of proteins that regulate apoptosis. While it is established that Bcl-2 localized at the mitochondria functions as an anti-apoptotic protein (Korsmeyer et al., 1993), the function of Bcl-2 at the nucleus remains unclear. We have previously shown that in aged rats there is an oxidative stress-dependent up-regulation of nuclear compartment-associated Bcl-2 (Kaufmann et al., 2001; Kaufmann et al., 2003). In addition, this nuclear compartment-associated Bcl-2 failed to protect cells from apoptosis induced by oxidative stress (Kaufmann et al., 2002). More recently, we reported that Bcl-2 localized at the nuclear compartment decreased activity of several transcription factors, particularly nuclear factor kappa B (NFκB), likely through depression of nuclear trafficking (Massaad et al., 2004). Given the requirement of proper NFκB activity to prevent cell death both *in vitro* (Taglialatela et al., 1997) and *in vivo* (Taglialatela et al., 1998), these results suggested that nuclear Bcl-2 may induce rather than prevent apoptosis. The notion that Bcl-2 can act in a pro-apoptotic fashion, albeit counter-intuitive, has been previously reported. Both transient expression of Bcl-2 (Uhlmann et al., 1998; Wang et al., 2001) and caspase 3 cleavage of Bcl-2 (Cheng et al., 1997; Liang et al., 2002), an event that removes Bcl-2's BH4 domain and converts Bcl-2 into a Bax like protein, have been shown to induce apoptosis. Furthermore, interaction of Bcl-2 with the nuclear orphan receptor Nur-77, a protein that leaves the nucleus during apoptosis and binds Bcl-2 at the mitochondrion, also converts Bcl-2 into a pro-apoptotic protein (Lin et al., 2004). Based on these observations, we hypothesized that presence of Bcl-2 at the nucleus may induce rather than protect cells from apoptosis. Here, we investigated the putative apoptotic role of nuclear

compartment-associated Bcl-2. Additionally, we examine the role of Bcl-2's BH4 domain in mediating binding to FKBP38, Bcl-2's mitochondrial chaperone.

METHODS

For a complete description of general methods and techniques, please refer to Chapter 2 Material and Methods.

RESULTS

Apoptosis induction following transient expression of Bcl-2 or Bcl-2ΔBH4.

Rat PC12, human HeLa, mouse B104, and human HEK293 cells were transiently transfected with YFP, Bcl-2/YFP, or Bcl-2ΔBH4/YFP. Following transient transfection, cells were stained with Annexin-V and Propidium Iodide (PI) and analyzed by flow cytometry. Cells expressing the transfected constructs, as determined by YFP fluorescence, were gated (R1) and the degree of apoptosis specifically in the R1 population was determined by assessing the number of cells that were simultaneously Annexin-V-positive and PI-negative (**Fig. 4.1A**) (see Appendix, supplemental Fig. I for an example of R1 gating). Analyses performed on three independent experiments revealed that all cell lines assayed underwent a significant degree of apoptosis upon transient transfection of Bcl-2/YFP or Bcl-2ΔBH4/YFP, as compared to cells transfected with the YFP control vector. Western blot analysis detecting Bcl-2 in total protein extracts from non-transfected PC12, HeLa, B104, and HEK293 revealed different degrees of endogenous Bcl-2 expression in these cell lines, with the highest expression in HeLa cells and levels below the limit of detection in PC12 cells (**Fig. 4.1B**). Since induction of apoptosis by transient expression of exogenous Bcl-2 seemed not to be restricted to any specific cell line and was independent of endogenous Bcl-2 expression, we elected to focus our subsequent studies on PC12 cells.

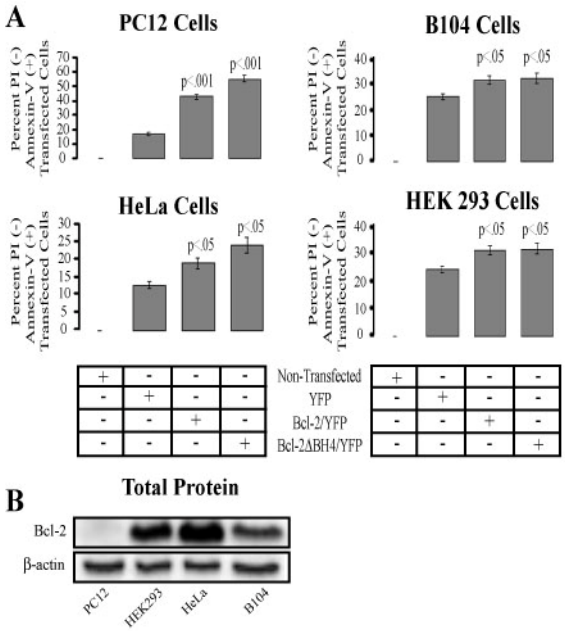


Figure 4.1. Examination of apoptosis induced following transient overexpression of Bcl-2 and Bcl-2ΔBH4. **A**, Annexin-V based quantification of apoptosis in YFP expressing populations from PC12, HEK293, HeLa, and B104 cells following transient transfection with YFP, Bcl-2/YFP, or Bcl-2ΔBH4/YFP. Each bar represents the mean of 3 separate experiments ± standard deviation and indicated statistical significances are vs. YFP-vector transfected cells (two-tailed Student's t-test). **B**, Western blot displaying the level of Bcl-2 and β-actin expression from total protein collected from non-transfected PC12, HEK293, HeLa, and B104 cell lines.

Next, we determined whether the fusion protein Bcl-2/YFP used in these experiments retained function similar to wild type Bcl-2. To test this, PC12 cells stably expressing either Bcl-2 or Bcl-2/YFP were exposed to serum withdrawal or Staurosporine treatment. Both of these apoptosis-inducing stimuli have been shown to be attenuated by stable, mitochondrial compartment-localized Bcl-2 in PC12 cells (Hunter et al., 1996; Maroto and Perez-Polo, 1997). Both stably expressed wild type Bcl-2 and Bcl-2/YFP provided significant protection from cell death following 24 hr serum withdrawal or 24 hr treatment with Staurosporine (see Appendix, supplemental Fig. IIA). In addition,

both stably expressed Bcl-2 and Bcl-2/YFP shared a non-nuclear subcellular localization (see Appendix, supplemental Fig. IIB).

Nuclear localization of Bcl-2 or Bcl-2ΔBH4 is associated with a significant increase in apoptosis.

The representative Western blot shown in **Fig. 4.2A** compares the sub-cellular distribution of exogenously-expressed Bcl-2 and Bcl-2ΔBH4 following either transient or stable transfection. While transiently-expressed Bcl-2 localized to both nuclear and non-nuclear fractions, transiently-expressed Bcl-2ΔBH4 solely localized within the nuclear fraction. Conversely, stably-expressed Bcl-2 was predominately found in the non-nuclear protein fraction, while expression of Bcl-2ΔBH4 in the non-nuclear fraction was not detected following long-term growth in selection medium. Purity of nuclear and non-nuclear fractions was confirmed by re-probing with antibodies specific for nuclear and non-nuclear proteins (IκBα for cytosolic, Pan Lamin for nuclear, and Calnexin for ER) (**Fig. 4.2A**). While satisfactory separation of nuclear from non-nuclear fractions was confirmed, the small degree of calnexin identified in the nuclear fraction was expected since the ER is contiguous with the outer nuclear envelope (Voeltz et al., 2002) and our method of nuclei isolation preserves the outer nuclear membrane (Portier et al., 2006).

Fig. 4.2B shows representative confocal photomicrographs of nuclei isolated from PC12 cells transiently transfected with either Bcl-2/YFP or Bcl-2ΔBH4/YFP. These isolated nuclei showed a peri-nuclear association of Bcl-2 and Bcl-2ΔBH4, indicating that nuclear Bcl-2 is retained through the process of nuclei isolation. To investigate the extent of apoptosis in cells bearing Bcl-2 and Bcl-2ΔBH4 at the nuclear compartment, we employed flow cytometry-based analysis of physical light scattering properties (forward vs. side scatter as described above) to determine apoptotic status of isolated nuclei (Portier et al., 2006). Using this new technique coupled with transfection of Bcl-2/YFP and Bcl-2ΔBH4/YFP fusion proteins, it was possible to compare apoptosis in both

populations of nuclei that contained or lacked Bcl-2 or Bcl-2ΔBH4. Analysis from three independent experiments using the physical light scatter technique on nuclei isolated from Bcl-2/YFP and Bcl-2ΔBH4/YFP-transiently-trasfected cells showed that there was a significant increase in apoptosis in nuclei bearing Bcl-2 (46%±1.73 as compared to 12%±3 in nuclei devoid of Bcl-2) (**Fig. 4.2C)**. Similarly, there was a significant increase in apoptotic nuclei bearing nuclear Bcl-2ΔBH4 (50.7%±2.7 as compared to 13.74%±1.46 in nuclei devoid of Bcl-2ΔBH4) (**Fig. 4.2D**). (Representative physical light scatter dot plots are shown in Appendix, supplemental Fig. IIIA&B)

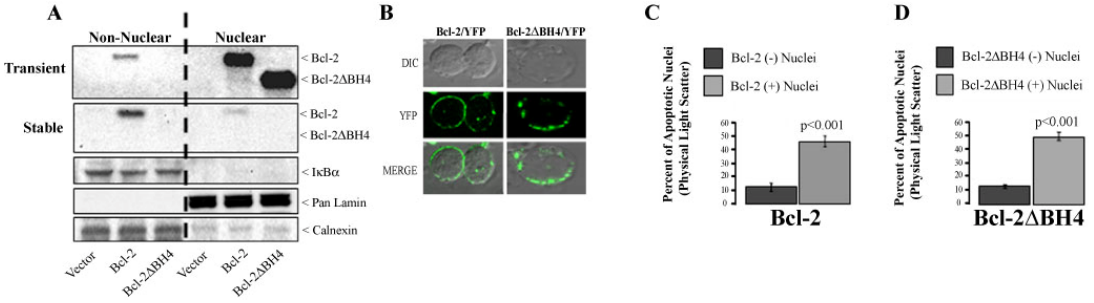


Figure 4.2. Nuclear localization of Bcl-2 and Bcl-2ΔBH4 following transient transfection induces cell death. **A,** Western blot analysis detecting Bcl-2 and Bcl-2ΔBH4 in non-nuclear and nuclear protein fractions prepared from PC12 cells transiently or stably transfected with vector, Bcl-2, or Bcl-2ΔBH4. Blots were re-probed with antibodies directed against organelle-specific markers to determine fractionation purity; antibodies used were IκBα (cytosolic), Pan Lamin (nuclear), and Calnexin (ER). **B**, Representative confocal photomicrographs of nuclei isolated from PC12 cells transiently transfected with Bcl-2/YFP or Bcl-2ΔBH4/YFP. **C**, Physical light scatter based quantification of apoptosis performed on isolated nuclei from PC12 cells following transient transfection with Bcl-2/YFP. Each bar represents the mean of 3 separate experiments ± standard deviation and indicated statistical significances are Bcl-2 (+) nuclei vs. Bcl-2 (-) nuclei (two-tailed Student's t-test). D, Physical light scatter based quantification of apoptosis performed on isolated nuclei from PC12 cells following transient transfection with Bcl-2ΔBH4/YFP. Each bar represents the mean of 3 separate experiments ± standard deviation and indicated statistical significances are Bcl-2ΔBH4 (+) nuclei vs. Bcl-2ΔBH4 (-) nuclei (two-tailed Student's t-test).

Cell death induced by transiently-transfected Bcl-2 is affected by modulation of FKBP38 expression levels.

Cell fractionation (nuclear vs. non-nuclear) was utilized in conjunction with Western blotting to determine changes in Bcl-2 and Bcl-2ΔBH4 localization following transient co-expression with FKBP38 (**Fig. 4.3A**). Co-transfection of Bcl-2 and FKBP38 reduced nuclear compartment-associated Bcl-2 and increased nuclear FKBP38. Co-transfection of Bcl-2ΔBH4 and FKBP38 increased nuclear compartment-associated FKBP38 but failed to decrease the level of nuclear Bcl-2ΔBH4. Flow cytometry-based counting of nuclei was also used to determine the percent of nuclei bearing Bcl-2 or Bcl-2ΔBH4 following transient transfection in the presence or absence of co-transfected FKBP38. As shown in **Fig. 4.3B,** and consistent with the Western blot data shown in Fig. 3*A*, addition of FKBP38 significantly reduced the percent of Bcl-2-positive nuclei from 26%±2.51 to 15%±2.05, but did not significantly alter the percent of Bcl-2ΔBH4-positive nuclei (34%±1.76 in the absence of FKBP38 vs. 36%±3.2 in the presence of FKBP38). Furthermore, physical light scatter analysis revealed a significant reduction ($p<0.01$) in apoptosis, 41.7%±1.74 for nuclei bearing Bcl-2 in the absence of co-expressed FKBP38 compared to 22.9%±3.26 following FKBP38 co-transfection (**Fig. 4.3C**). On the other hand, no significant reduction in apoptosis was observed for nuclei bearing Bcl-2ΔBH4 following FKBP38 co-expression, 49.3%±3.0 in the absence of co-expressed FKBP38 compared to 53.6%±3.4 following FKBP38 co-transfection (**Fig. 4.3D**). (Representative physical light scatter dot plots are shown in Appendix, supplemental Fig. IIIC&D)

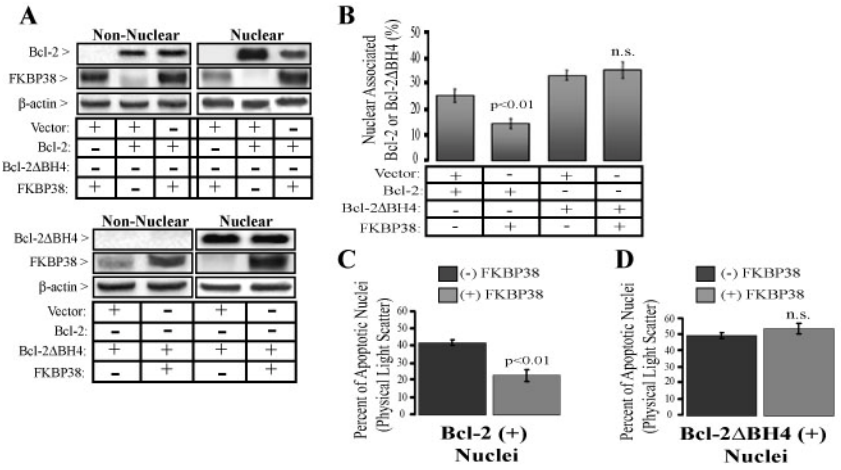


Figure 4.3. Expression of FKBP38 reduces nuclear associated Bcl-2 and reduces apoptosis in cells bearing nuclear associated Bcl-2. **A**, Western blot analysis performed on non-nuclear and nuclear protein fractions isolated from PC12 cells following transient transfection with Bcl-2 or Bcl-2ΔBH4 in the presence or absence of co-transfected FKBP38. Membranes were re-probed for β-actin to control for equal protein loading. **B**, Percent of Bcl-2 or Bcl-2ΔBH4-bearing nuclei following transient transfection of Bcl-2/YFP or Bcl2ΔBH4/YFP in the presence or absence of co-transfected FKBP38. Each bar represents the mean of 3 separate experiments ± standard deviation; indicated statistical significances are vs. FKBP38 (-) cell populations (two-tailed Student's t-test). **C**, Physical light scatter based quantification of apoptosis following transient transfection of Bcl-2 in the presence or absence of co-transfected FKBP38. Each bar represents the mean of 3 separate experiments ± standard deviation; indicated statistical significances are vs. FKBP38 (-) cell populations (two-tailed Student's t-test). **D**, Physical light scatter based quantification of apoptosis following transient transfection of Bcl-2ΔBH4 in the presence or absence of co-transfected FKBP38. Each bar represents the mean of 3 separate experiments ± standard deviation; indicated statistical significances are vs. FKBP38 (-) cell populations (two-tailed Student's t-test).

Fig. 4.4 shows the results from a flow cytometry-based Annexin-V assay in which cells were transiently transfected with Bcl-2ΔBH4 (nuclear localized Bcl-2) in the presence or absence of a co-transfected Bcl-2 mutant that is selectively targeted to the mitochondrion (Mito-Bcl-2) (subcellular localization of both Bcl-2 constructs has previously been characterized (Wang et al., 2001; Massaad et al., 2004)). Transiently-transfected Bcl-2ΔBH4 induced a significant increase ($p<0.01$) in apoptosis as compared

to cells transfected with Mito-Bcl-2 (**Fig. 4.4A**). Furthermore, co-transfection of Mito-Bcl-2 failed to prevent apoptosis induced by Bcl-2ΔBH4 (**Fig. 4.4B**). The results from this experiment suggest that the FKBP38-mediated rescue of cells described in **Fig. 4.3C** was likely due to the reduced presence of nuclear Bcl-2 rather than increased mitochondrial delivery of Bcl-2 by co-transfected FKBP38.

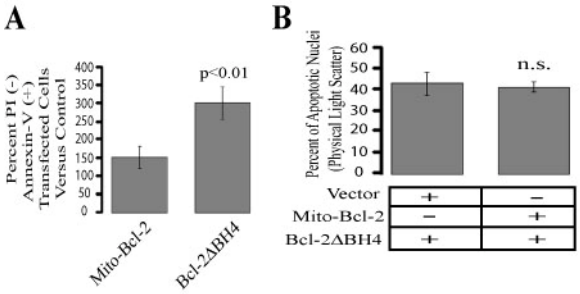


Figure 4.4. Targeted delivery of Bcl-2 to the mitochondria fails to prevent apoptosis induced by nuclear localized Bcl-2ΔBH4. **A**, Annexin-V based quantification of apoptosis following transient transfection with vector, mitochondrial-targeted Bcl-2 (mito-Bcl-2), or nuclear localized Bcl-2 (Bcl-2ΔBH4). Each bar represents the mean of 3 separate experiments ± standard deviation; indicated statistical significances are vs. mito-Bcl-2 expressing cell populations (two-tailed Student's t-test). **B**, Physical light scatter based quantification of apoptosis performed on isolated nuclei from PC12 cells following transient transfection with Bcl-2ΔBH4 in the presence and absence of co-transfected mitochondrial-targeted Bcl-2. Each bar represents the mean of 3 separate experiments ± standard deviation and indicated statistical significances are vs. Bcl-2ΔBH4 (+) nuclei populations in the absence of transiently transfected Mito-Bcl-2 (two-tailed Student's t-test).

To further characterize the apoptotic role of nuclear associated Bcl-2 we examined the changes in subcellular distribution of Bcl-2 and the extent of apoptosis following suppression of endogenous FKBP38 expression using siRNA. As shown in **Fig. 4.5A**, FKBP38 expression was almost completely abolished 72 hrs following the addition of FKBP38 siRNA. Sub-cellular fractionation and Western blots performed following 24 hr transient transfection with Bcl-2 in cells pre-treated for 72 hrs with siRNA for FKBP38 (**Fig. 4.5B**, left panel) revealed a dramatic decrease in non-nuclear

Bcl-2 paralleled by an appreciable increase of nuclear compartment-associated Bcl-2. Similar experiments performed using Bcl-2ΔBH4 instead of full-length Bcl-2 revealed no significant difference in levels of nuclear associated Bcl-2ΔBH4 between cells pretreated with the FKBP38 siRNA as compared to cell pretreated with a non-targeted siRNA (**Fig. 4.5B,** right panel). Apoptosis measured by Annexin-V assay performed in similarly treated cells revealed a significant increase ($p<0.05$) in apoptosis in cells transfected with Bcl-2 but not Bcl-2ΔBH4 following FKBP38 siRNA knockdown (**Fig. 4.5C**).

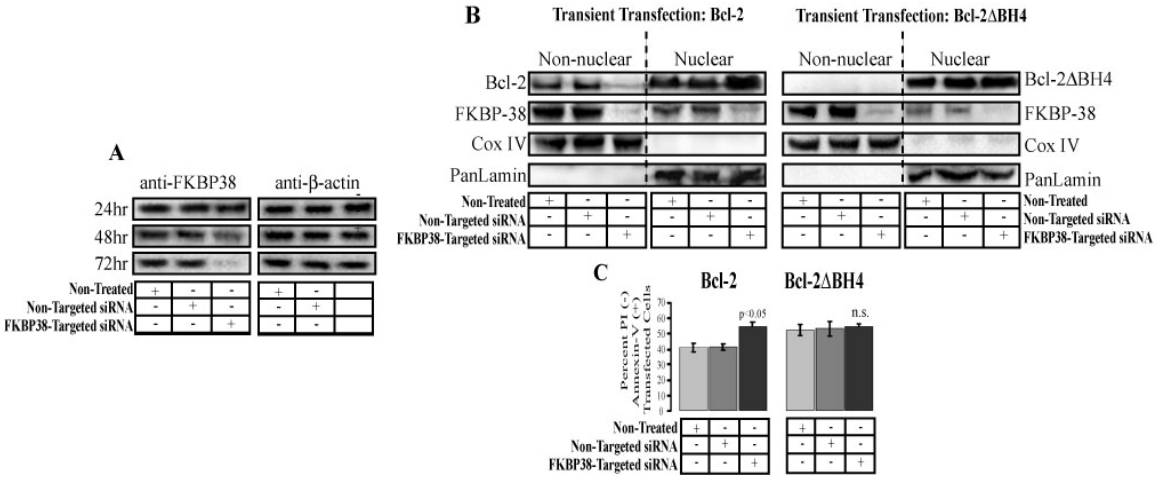


Figure 4.5 Targeted siRNA knockdown of FKBP38 expression increases the pro-apoptotic activity of transiently transfected Bcl-2. **A**, Western blot analysis of FKBP38 and β-actin expression level following 24 hr, 48 hr, and 72 hr treatment with control (non-treated), non-targeted siRNA, and FKBP38-targeted siRNA. **B**, Western blot performed following non-nuclear and nuclear protein fractionation in PC12 cells treated for 72 hrs with FKBP38-targeted siRNA, non-targeted siRNA, and control (non-treated) and transiently transfected for 24 hrs with Bcl-2 (LEFT) or Bcl-2ΔBH4 (RIGHT). **C**, Annexin-V based quantification of apoptosis on PC12 cells treated for 72 hrs with FKBP38-targeted siRNA, non-targeted siRNA, and control (non-treated) and transiently transfected for 24hr with Bcl-2 (LEFT) or Bcl-2ΔBH4 (RIGHT). Each bar represents the mean of 3 separate experiments ± standard deviation; indicated statistical significances are vs. non-targeted siRNA treated cell populations (two-tailed Student's t-test).

FKBP38 binds Bcl-2 utilizing Bcl-2's BH4 domain.

Since addition of FKBP38 failed to remove Bcl-2ΔBH4 from the nuclear compartment (**Fig. 4.3A&B**), we asked whether the BH4 domain of Bcl-2 was critical for binding to FKBP38. To address this question, we performed immunoprecipitations (IP) 48 hrs after transient co-expression of Bcl-2 and FKBP38 or Bcl-2ΔBH4 and FKBP38. Western blots performed on input (total non-denatured protein extract), IP (eluate from agarose beads with conjugated HA antibody), and beads (eluate from agarose beads without antibody) were probed for FKBP38 (IP), Bcl-2 (co-IP), and Bcl-2ΔBH4 (co-IP). While Bcl-2 co-immunoprecipitated with FKPB38, Bcl-2ΔBH4 failed to co-immunoprecipitate (**Fig. 4.6A**). This result was confirmed by performing a reciprocal co-IP, using an anti-Bcl-2 antibody for pull down (**Fig. 4.6B**).

Lack of co-IP between FKBP38 and Bcl-2ΔBH4 suggested that the BH4 domain of Bcl-2 was involved in FKBP38 binding but did not distinguish whether the BH4 domain directly bound FKBP38 or simply stabilized the binding. Therefore, to determine whether the association between Bcl-2 and FKBP38 was directly mediated by Bcl-2's BH4 domain, we synthesized a BH4 domain peptide and control BH4 domain scrambled amino acid sequence peptide (BH4scr) for use in FKBP38 immunoprecipitation assays. Incubation of the BH4 domain peptide with total cell extracts from PC12 cells that were previously transiently transfected with FKBP38 revealed that the BH4 domain peptide co-immunoprecipitated with FKBP38 (**Fig. 4.6C**). In addition, immunoprecipitation performed using the control BH4scr peptide failed to co-immunoprecipitate with FKBP38 (**Fig. 4.6D**). Due to the lack of an antibody that detects BH4scr peptide, we performed gel staining using GelCode stain reagent for detection of loaded (input) and bound (Immunoprecipitated) proteins. As shown in **Fig. 4.6C**, GelCode stain revealed the

BH4 peptide in input and IP lanes, whereas the BH4scr peptide was detected solely in the input but not in the IP fraction (**Fig. 4.6D**).

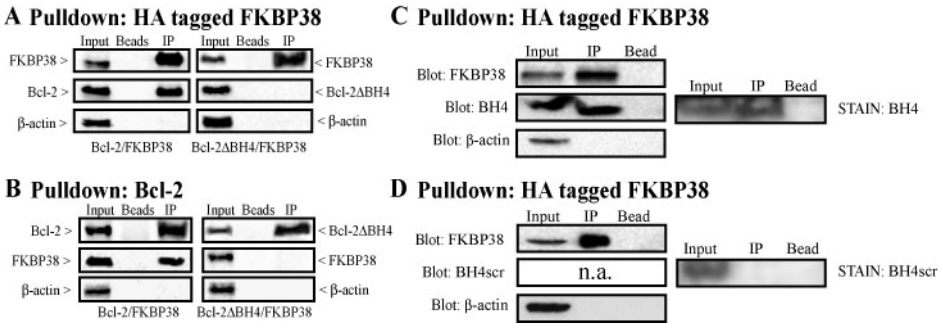


Figure 4.6. Bcl-2's BH4 domain is necessary and sufficient for binding FKBP38. **A&B**, Western blot analysis detecting Bcl-2, Bcl-2ΔBH4, and FKBP38 in immunoprecipitated fractions from total protein extracts prepared from PC12 cells transiently transfected with Bcl-2 or Bcl-2ΔBH4 in the presence of co-transfected HA-tagged FKBP38. Extracts were immunoprecipitated using anti-HA (**A**) or anti-Bcl-2 **(B)** antibodies. Loaded samples are: Input (total non-denatured protein extract), IP (eluate from HA-agarose beads or Bcl-2-protein A/G agarose beads), and beads (eluate from agarose beads/no antibody). **C**, Western blot analysis detecting BH4 peptide and FKBP38 in immunoprecipitated fractions from total protein extracts prepared from PC12 cells transiently transfected with HA-tagged FKBP38. Extracts were immunoprecipitated using anti-HA antibody and blots were probed with HA, Bcl-2 (epitope N-terminal Bcl-2 i.e. BH4 domain), and β-actin antibodies. In addition a parallel gel was ran and stained with GelCode to reveal immunoprecipitated proteins via chemical stain in place of immunodetection. **D**, Western blot analysis detecting BH4 scramble peptide (BH4scr) and FKBP38 in immunoprecipitated fractions from total protein extracts prepared from PC12 cells transiently transfected with HA-tagged FKBP38. Extracts were immunoprecipitated using anti-HA antibody and blots were probed with HA and β-actin antibodies. In addition a parallel gel was ran and stained with GelCode to reveal immunoprecipitated proteins via chemical stain in place of immunodetection.

DISCUSSION

We present here evidence of a pro-apoptotic role for nuclear compartment-associated Bcl-2. Previous studies have demonstrated that Bcl-2 localizes to multiple

organelles including the mitochondria, ER, and nuclear envelope (Krajewski et al., 1993). While most studies have focused on the anti-apoptotic role of mitochondrial localized Bcl-2, recent evidence has suggested that Bcl-2 can act in a pro-apoptotic fashion (Cheng et al., 1997; Uhlmann et al., 1998; Wang et al., 2001). Our results indicate that one putative sub-cellular localization where the pro-apoptotic action of Bcl-2 can be exerted, is at the nuclear compartment, specifically the peri-nuclear area. These studies have far reaching implications because they reveal a dual role for Bcl-2 as a cell protector or cell killer, dependent on sub-cellular localization thus opening the possibility for therapeutic intervention by directing Bcl-2's sub-cellular distribution to dictate cells fate.

We previously reported the ability of nuclear compartment-associated Bcl-2 to block transcription factor activation in multiple cell lines including PC12 (Massaad et al., 2004). Based on our earlier studies in which we demonstrated the necessity of transcription factor activation in PC12 cells for survival (Taglialatela et al., 1997), we hypothesized that blockade of transcription factor activation by nuclear Bcl-2 would induce apoptosis. Our present findings support this hypothesis and illustrate that the presence of nuclear Bcl-2 is incompatible with long term cell survival. While our results indicate that transient transfection of Bcl-2 induces apoptosis in several different cell types (**Fig. 4.1**), we chose to utilize PC12 cells as a cell model. Our reason for focusing on PC12 cells was based on this cell lines lack of endogenous Bcl-2 expression ((Maroto and Perez-Polo, 1997) and **Fig. 4.1A**) and the fact that this cell line does not depend on Bcl-2 for survival under normal culture conditions. Also, working with cells that lack endogenous Bcl-2 expression afforded us the capability to specifically examine the effects of exogenously introduced Bcl-2 into specific subcellular compartents without the competing effects of endogenous Bcl-2.

Our results show that transient expression of Bcl-2 leads to high levels of nuclear compartment-associated Bcl-2 while stable expression results in a predominant non-nuclear (mitochondria and ER) localization (**Fig. 4.2A**). On the other hand, transient expression of Bcl-2ΔBH4 results in Bcl-2ΔBH4 solely localized to the nuclear

compartment, while attempts to create a stable Bcl-2ΔBH4 expressing cell line were unsuccessful. Furthermore, Annexin-V assays revealed significant apoptosis induced by transient expression of both Bcl-2 and Bcl-2ΔBH4 as compared to vector-transfected control cells (**Fig. 4.1A**). We interpret these results as suggestive of two distinct phenomena: one, when localized at the nuclear compartment, Bcl-2 is incompatible with long-term cell survival due to induction of apoptosis and two, Bcl-2's BH4 domain plays a crucial role in Bcl-2's intra-cellular transport.

To address the question as to whether Bcl-2 has a pro-apoptotic role following nuclear association, we applied a flow cytometry-based physical light scatter analysis (Chapter 3) for characterization of apoptosis in isolated nuclei. This assay allows detection of nuclear compartment-associated proteins while simultaneously determining the apoptotic nature of the cell from which the nucleus was isolated. Analysis of nuclei isolated from PC12 cells transiently transfected with Bcl-2 or Bcl-2ΔBH4 (**Fig. 4.2B**), revealed a significant increase in the percent of apoptotic nuclei, specifically in those nuclei bearing Bcl-2 or Bcl-2ΔBH4 as compared to nuclei devoid of Bcl-2 or Bcl-2ΔBH4 (**Fig. 4.2C&D**). While these findings linked the presence of nuclear associated Bcl-2 and Bcl-2ΔBH4 to the induction of apoptosis, they did not provide conclusive evidence for a causal relationship between the two events. However, both Western blot and flow cytometry-based analysis of isolated nuclei showed that the presence of FKBP38 reduced the presence of nuclear Bcl-2 (**Fig. 4.3A&B**) while significantly reducing apoptosis (**Fig. 4.3C***)*. On the other hand, co-expressed FKBP38 failed to remove nuclear compartment-associated Bcl-2ΔBH4 (**Fig. 4.3A&B**) and failed to enhance the survival of cells expressing Bcl-2ΔBH4 (**Fig. 4.3D**). Collectively these results suggest that there is either a causal link between the presence of Bcl-2 at the nuclear compartment and initiation of apoptosis in PC12 cells or that the added FKBP38 increased mitochondrial localized Bcl-2 and the increased survival observed was due to protection exerted by the increased mitochondrial Bcl-2.

To directly test whether the decrease in apoptosis observed following addition of FKBP38 was due to increased mitochondrial Bcl-2, we performed co-transfections with Bcl-2ΔBH4 and a mitochondrial-targeted Bcl-2 (Mito-Bcl-2). Our result show that directly adding Bcl-2 to the mitochondria in the presence of nuclear trapped Bcl-2 (Bcl-2ΔBH4) failed to significantly protect cells from nuclear Bcl-2-mediated apoptosis (**Fig. 4.4B**). These data indicate that the increase in cell survival observed via co-expression of FKBP38 and Bcl-2 but not FKBP38 and Bcl-2ΔBH4 was predominantly mediated by reduced pro-apoptotic nuclear Bcl-2 rather than increased anti-apoptotic mitochondrial Bcl-2. A similar conclusion could be drawn from the data shown in **Fig. 4.1** where transient expression of Bcl-2 induces apoptosis in cell lines that express substantial levels of endogenous Bcl-2, thus suggesting that the presence of endogenous Bcl-2 (mostly mitochondrial) does not protect cells from apoptosis induced by transiently-expressed Bcl-2, which localizes substantially at the nucleus. Furthermore, suppression of endogenous FKBP38 expression by siRNA (**Fig. 4.5**) shifted the localization of Bcl-2 almost completely to the nuclear compartment and made transiently-transfected Bcl-2 as toxic as Bcl-2ΔBH4, which is solely nuclear. Thus these results provide evidence for a causal relationship between the toxicity of transiently-transfected Bcl-2 and its presence at the nuclear compartment. Collectively, these results also suggest that mitochondrial Bcl-2 is unable to block apoptosis triggered by nuclear Bcl-2. While this observation argues for a novel mechanism of initiation of apoptosis triggered by nuclear Bcl-2 that bypasses the mitochondrial checkpoint, further studies are needed to elucidate the exact molecular pathways involved.

In characterizing the pro-apoptotic nature of nuclear Bcl-2 we generated evidence that the BH4 domain of Bcl-2 was critically involved in regulating Bcl-2 movement, likely through facilitating interaction with the chaperone protein FKBP38. To determine if Bcl-2's BH4 domain was the critical domain responsible for Bcl-2 mediated binding to FKBP38, we performed co-immunoprecipitation experiments with FKBP38 and Bcl-2, Bcl-2ΔBH4, and a synthetic BH4 domain peptide (**Fig 4.6A-D**). While our results

confirmed previously published reports that Bcl-2 and FKBP38 co-immunoprecipitate (**Fig. 4.6A&B**), they also showed for the first time that removal of Bcl-2's BH4 domain results in loss of co-immunoprecipitation with FKBP38 (**Fig. 4.6A&B**). This finding in conjunction with the observation that FKBP38 fails to remove Bcl-2ΔBH4 from the nuclear compartment (**Fig. 4.3A&B**) strongly suggests that the BH4 domain of Bcl-2 is critically involved in regulating the binding between Bcl-2 and FKBP38 and the subsequent delivery of Bcl-2 to the mitochondrion. Indeed, we found that a BH4 domain peptide co-IPed with FKBP38, thus providing direct evidence that the BH4 sequence of Bcl-2 is sufficient to mediate binding to FKBP38. This is not surprising, given the extensive literature describing the BH4 domain as a "docking" domain that allows Bcl-2 to interact with several heterologous proteins (Wang et al., 1996; Shibasaki et al., 1997).

In light of these data it is interesting that others, using a prokaryotic overexpression approach, concluded that Bcl-2's loop domain, located next to the BH4 domain, was directly responsible for mediating FKBP38/Bcl-2 binding (Kang et al., 2005). While this report appears to contradict our current findings, both results could be explained by the fact that the loop domain is a flexible structure that positively displaces Bcl-2's BH4 domain for heterologous protein interactions (Chang et al., 1997; Minn et al., 1997; Petros et al., 2001). It is therefore possible that deletions within this domain induced conformational changes that blocked or hindered access of FKBP38 to Bcl-2's BH4 domain. This argument is strengthened by the fact that the same authors failed to identify an exact binding domain sequence within the loop domain and found that several deletions of non-congruent sections of the loop domain resulted in alteration in FKBP38 binding. Therefore the previous report that FKBP38 binds to the loop domain of Bcl-2 is not a direct contradiction with our current results that indicate that the BH4 domain of Bcl-2 is the critical domain required for Bcl-2/FKBP38 binding.

CONCLUSION

We have shown that nuclear compartment-associated Bcl-2 promotes rather than protects cells from apoptosis. The ability to regulate Bcl-2's movement and thus dictate an anti-apoptotic (mitochondria) or pro-apoptotic (nuclear) mechanism appears to depend upon proper interaction with the chaperone protein FKBP38, an interaction in which Bcl-2's BH4 domain plays a critical role. This observation offers a potentially significant target for future therapies aimed at eliminating Bcl-2-expressing cells or enhancing Bcl-2-mediated cell survival. The future development of possible BH4 domain mimetic molecules could be proposed to disrupt Bcl-2/FKBP38 interaction and thus selectively target Bcl-2 to the nucleus to induce apoptosis, specifically in high Bcl-2-expressing cells such as several types of human cancers (Kirkin et al., 2004). On the other hand, neurons and supporting glia expressing high levels of Bcl-2 following transient ischemic attack/stroke (Honkaniemi et al., 1996; Ferrer et al., 1998) could potentially be saved by promoting mitochondrial rather than nuclear association of Bcl-2. Feasibility of pharmacologically-relevant techniques that will allow such manipulation of Bcl-2's localization remains to be determined.

The following chapter is in preparation for journal submission following addition of further BH4 peptide treatments in AML stem cell populations (October 2006). Tentative title “Delivery of Bcl-2’s BH4 domain by protein transduction induces cell death selectively in cells expressing Bcl-2”. It was jointly authored by Bryce P. Portier, Wen-Ru Zhang, and Giulio Taglialatela.

CHAPTER 5: DELIVERY OF BCL-2'S BH4 DOMAIN BY PROTEIN TRANSDUCTION INDUCES CELL DEATH SELECTIVELY IN CELLS EXPRESSING BCL-2

INTRODUCTION

Apoptosis plays a fundamental role in physiological and pathological events such as tissue development and cancer (Hamburger, 1949; Glucksmann, 1951; Kerr et al., 1972; Arends and Wyllie, 1991; Hengartner, 2000). The Bcl-2 family of proteins function as central regulators of apoptosis (Bruckheimer et al., 1998; Chao and Korsmeyer, 1998; Konopleva et al., 1999; Motyl, 1999). The prototypical member of this protein family, Bcl-2, has been extensively characterized as a mitochondrial-localized anti-apoptotic protein (Korsmeyer et al., 1993). Bcl-2 localization at the mitochondria requires the carrier action of FKBP38, an atypical calcineurin inhibitor and chaperone protein without which Bcl-2 localizes primarily to its default localizations at the ER and nuclear envelope (Germain and Shore, 2003; Shirane and Nakayama, 2003). In the studies described in Chapter 4 we showed that nuclear compartment-associated Bcl-2, contrary to mitochondrial-localized Bcl-2, induces apoptosis (Portier and Taglialatela, 2006). In that same studies we also showed that Bcl-2's BH4 domain was critical in regulating the interaction between Bcl-2 and chaperone protein FKBP38. In addition, removal of Bcl-2's BH4 domain resulted in increased nuclear (pro-apoptotic) and decreased mitochondrial (anti-apoptotic) localized Bcl-2. Based on these observations, we hypothesized that targeted disruption of Bcl-2/FKBP38 binding would promote nuclear localization of Bcl-2 and thereby induce cell death selectively in Bcl-2 expressing cells. Here, we investigated the ability of a synthetic Bcl-2 BH4 domain peptide to disrupt Bcl-2/FKBP38 binding and thus induce apoptosis in Bcl-2-expressing cells, including high Bcl-2-expressing malignant cells from bone marrow and plasma of acute myeloid leukemia (AML) patients.

METHODS

For a complete description of general methods and techniques, please refer to Chapter 2 Material and Methods.

RESULTS

BH4 domain mediated alterations in Bcl-2/FKBP38 binding

Fig. 5.1 shows representative Western blots detecting FKBP38, Bcl-2, and TAT-BH4 peptide following co-immunoprecipitation (co-IP) experiments. Protein extracts from cells transiently transfected with HA-tagged FKBP38 were incubated with 5ng/ul of Bcl-2, in the presence or absence of 100ng/ul of TAT-BH4 or scrambled TAT-BH4 (TAT-BH4scr) peptide. After incubation, the extracts were immunoprecipitated with an anti-HA antibody and eluate was run on a Western blot. As displayed in **Fig. 5.1A**, following incubation with either Bcl-2 or TAT-BH4, both Bcl-2 and TAT-BH4 peptide could be co-immunoprecipitated with FKBP38. However, simultaneous incubation of Bcl-2 and TAT-BH4 with FKBP38 containing protein extract, reduced the amount of FKBP38 bound Bcl-2 and TAT-BH4 peptide, compared to single protein co-IP. A parallel experiment performed using TAT-BH4scr, showed no co-immunoprecipitation with FKBP38 and did not reduce the amount of Bcl-2 that co-immunoprecipitated with FKBP38 (**Fig. 5.2B**). Quantification of Western bolt data from three independent co-immunoprecipitations experiments showed addition of the TAT-BH4 peptide significantly reduced ($p<0.01$) the amount of Bcl-2 bound to FKBP38 while addition of the TAT-BH4scr peptide did not change Bcl-2/FKBP38 binding (**Fig. 5.1C,D**). Collectively, these results illustrate that the TAT-BH4 domain peptide binds FKBP38, Bcl-2 binds FKBP38, and that addition of a TAT-BH4 domain peptide competes with FKBP38/Bcl-2 binding, thereby reducing the level of Bcl-2 detected following FBKP38 immunoprecipitation. Due to the lack of an antibody recognizing an epitope on TAT-

BH4scr, gel staining was performed using GelCode in order to detect immunoprecipitated proteins. As seen in **Fig.5.1A&B**, both Bcl-2 and TAT-BH4 were detected following co-IP with FKBP38 while only Bcl-2 was detected following co-IP with FKBP38 in the presence of BH4scr blot.

	5x TAT	Bcl-2's BH4 Domain (AA 11-28)
TAT-BH4:	C-G-YARKARRQARR-	DNREIVMKYIHYKLSQRG
TAT-BH4 glycine:	C-G-YARKARRQARR-	DNREGGMKYIHYKLSQRG
TAT-BH4 scramble:	C-G-YARKARRQARR-	VKRDYRSMIKGEHQLYIN

Table 5.1. Amino acid sequence of synthesized TAT-fused-BH4 domain peptides.

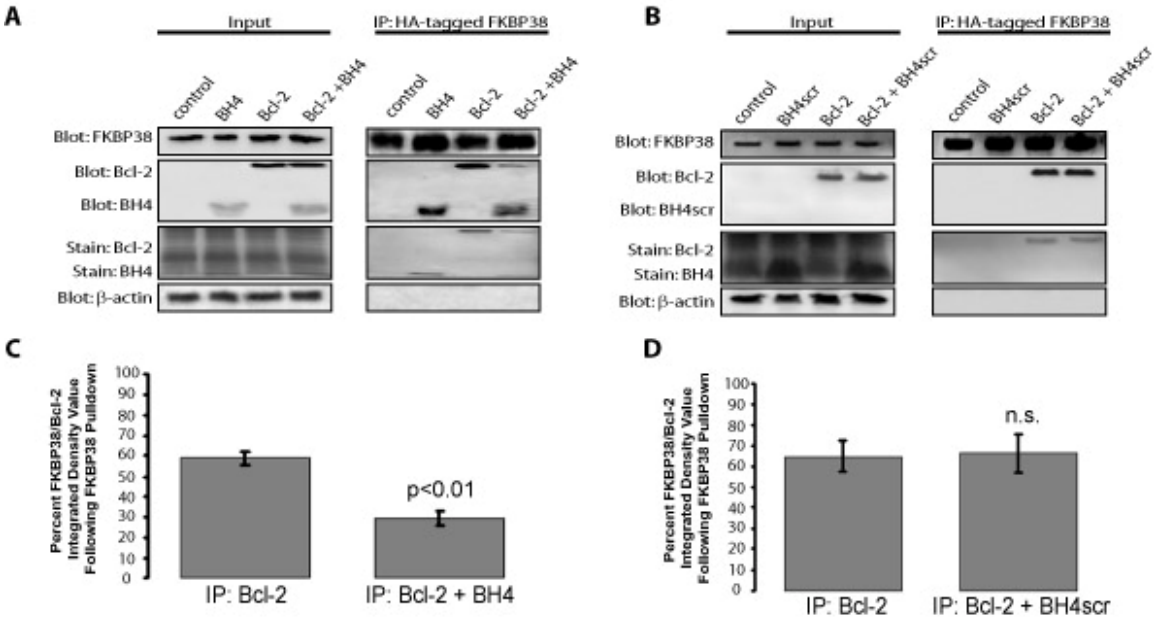


Figure 5.1. Disruption of Bcl-2/FKBP38 binding by BH4 domain peptide. **A**, Co-immunopreciptation performed using non-denatured protein extracts following 24hr

transient transfection with FKBP38. Exogenous BH4, Bcl-2, and BH4 + Bcl-2 were added and co-immunopreciptated with FKBP38. **B**, Co-immunopreciptation performed using non-denatured protein extracts following 24hr transient transfection with FKBP38. Exogenous BH4scr, Bcl-2, and BH4scr + Bcl-2 were added and FKBP38 co-immunopreciptation performed. Western blots of IP and co-IPed proteins along with input samples were probed for FKBP38, Bcl-2, BH4/BH4scr, and b-actin. In addition, gels were stained with GelCode to reveal proteins follwing IP in order to reveal protins in a non-epitope specific fashion. **C**, Quantification of the ratio of co-IPed Bcl-2 to IPed FKBP38 in the presence and absence of BH4 domain peptide. Each bar represents the mean of 3 separate experiments ± standard deviation and indicated statistical significances are vs. non-peptide added populations (two-tailed Student's t-test). **D**, Quantification of the ratio of co-IPed Bcl-2 to IPed FKBP38 in the presence and absence of BH4scr domain peptide. Each bar represents the mean of 3 separate experiments ± standard deviation and indicated statistical significances are vs. non-peptide added populations (two-tailed Student's t-test).

BH4 mediated cell death in Bcl-2 expressing cells

With *in vitro* evidence that the TAT-BH4 domain peptide binds FKBP38 and disrupts FKBP38/Bcl-2 binding, we next tested the ability of TAT-BH4 to gain entry into PC12 cells stably expressing either vector (SV) or Bcl-2 (SB). Following a 2 hr incubation with TAT-BH4, Western blot was performed on total protein extracts and revealed that TAT-BH4 was taken up by the cells at both 2μM and 10μM concentrations (**Fig. 5.2**). To ensure that the detection of the peptide was not an artifact due to non-specific adherence of the TAT-BH4 peptide to the cell surface, cells were trypsinized to remove surface bound TAT-BH4 and washed three times in PBS prior to total protein isolation and Western blotting.

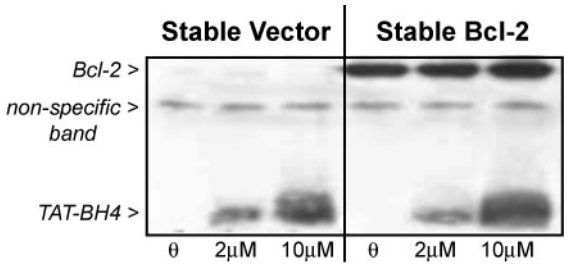


Figure 5.2. Intra-cellular delivery of TAT-BH4 domain peptide into PC12 cells. Western blot analysis performed on total protein extracts from PC12 cells stably expressing vector or Bcl-2 following 2hr incubation with either 2uM or 10uM TAT-BH4 peptide. Blot was probed with anti-Bcl-2 anti-body with epitope recognition of Bcl-2's BH4 domain.

With a functional mechanism of cell entry, we next investigated the sub-cellular localization of Bcl-2 and FKBP38 following addition of TAT-BH4 in SB and SV PC12 cells. Western blot analysis of non-nuclear and nuclear protein fractionations after 48 hr treatment with TAT-BH4 and TAT-BH4scr revealed that nuclear Bcl-2 was elevated following addition of TAT-BH4 but not TAT-BH4scr in SB cells (**Fig. 5.3**).

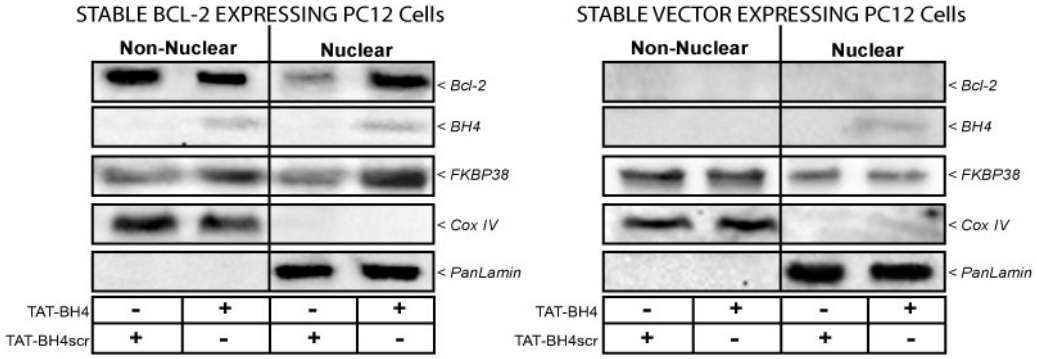


Figure 5.3. BH4 domain mediated re-localization of Bcl-2 to the nucleus. **A**, Western blot analysis detecting Bcl-2 and FKBP38 in non-nuclear and nuclear protein fractions prepared from stable Bcl-2 expressing PC12 cells treated with BH4scr or BH4 domain peptides. Blots were re-probed with antibodies directed against organelle-specific markers to determine fractionation purity; antibodies used were Cox IV (non-nuclear) and Pan Lamin (nuclear). **B**, Western blot analysis detecting Bcl-2 and FKBP38 in non-nuclear and nuclear protein fractions prepared from stable vector expressing PC12 cells treated with BH4scr or BH4 domain peptides. Blots were re-probed with antibodies directed against organelle-specific markers to determine fractionation purity; antibodies used were Cox IV (non-nuclear) and Pan Lamin (nuclear).

Additionally, the level of FKBP38 was elevated in both the nuclear and non-nuclear protein fractions following treatment with TAT-BH4 peptide but not TAT-BH4scr. Due to increased localization of Bcl-2 at the nucleus following addition of TAT-

BH4 and the recently recognized pro-apoptotic role for nuclear associated Bcl-2 (Portier and Taglialatela, 2006), we investigated the degree of cell death following BH4 peptide treatment both in cells that lack Bcl-2 (SV) as well as a Bcl-2 expressing cell line (SB). As seen in **Fig. 5.4A**, significant cell death was induced in SB cells following 48 hr TAT-BH4 peptide treatment at 2uM ($p<0.01$) and 10uM ($p<0.001$), whereas SB cells treated with TAT-BH4scr showed no significant increase in cell death. In addition, SV cells treated with TAT-BH4 or TAT-BH4scr peptide, showed no significant cell death at either 2uM or 10uM peptide concentration (**Fig. 5.4B**). In order to verify proper expression of Bcl-2 in SV and SB cell lines, an additional Western blot was performed using total cell extracts and revealed that SV lacked detectable Bcl-2 expression whereas SB expressed Bcl-2 (**Fig. 5.4C**).

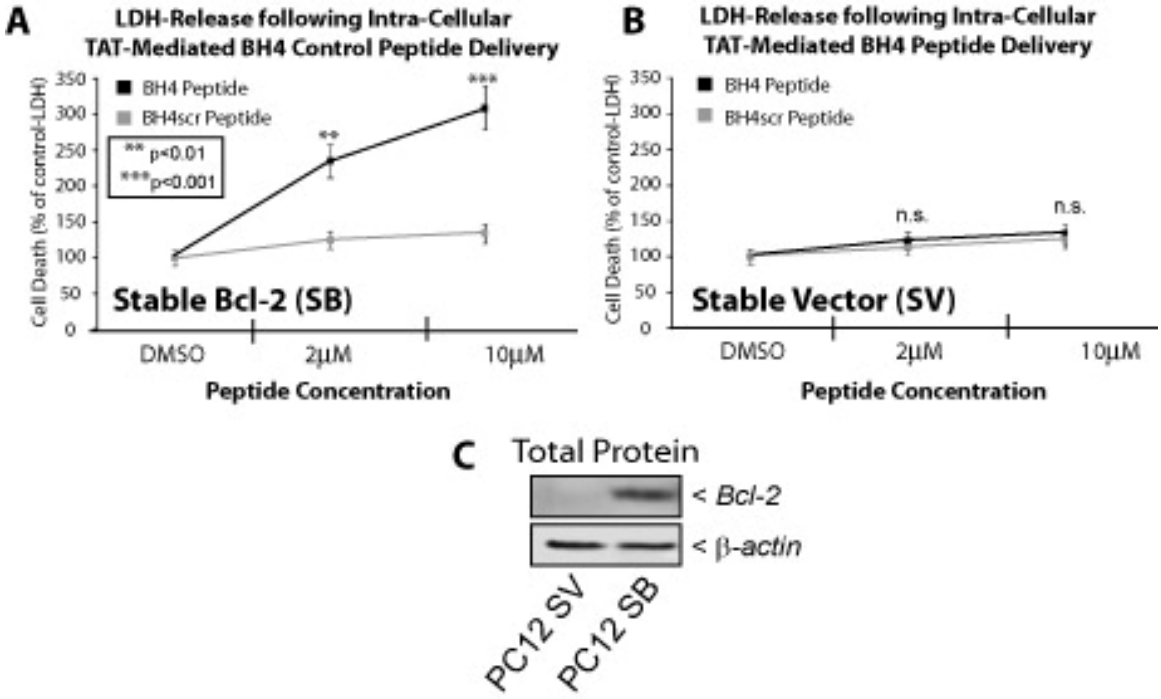


Figure 5.4. Addition of BH4 domain peptide induces cell death specifically in Bcl-2 expressing PC12 cells. A, Stable Bcl-2 expressing PC12 cells were treated with BH4 or BH4scr domain peptide at 2µM or 10µM concentrations. LDH release was measured 48 hrs post peptide administration. B, Stable vector expressing PC12 cells were treated with

BH4 or BH4scr domain peptide at 2μM or 10μM concentrations. LDH release was measured 48 hrs post peptide administration. C, Western blot detecting Bcl-2 expression in stable vector (SV) and stable Bcl-2 (SB) PC12 cells. Blot was re-probed with β-actin to control for protein loading.

BH4 mediated cell death in Bcl-2-expressing human leukemia cell lines and primary AML cancer cells.

REH and HL-60 are two human leukemia-derived cell lines that express significant levels of Bcl-2 (**Fig. 5.5A**). Treatment of these cells with TAT-BH4 peptide significantly induced cell death as compared to cells treated with the control TAT-BH4scr peptide. Cell death was measured by LDH release assay which revealed a progressive increase in cell death following TAT-BH4 peptide treatment (**Figs. 5.5A&B**, upper panel), whereas Annexin-V labeling showed that the number of cells undergoing apoptosis peaked 6 hrs after the addition of the TAT-BH4 peptide (**Figs. 5.5C&D**, lower panel).

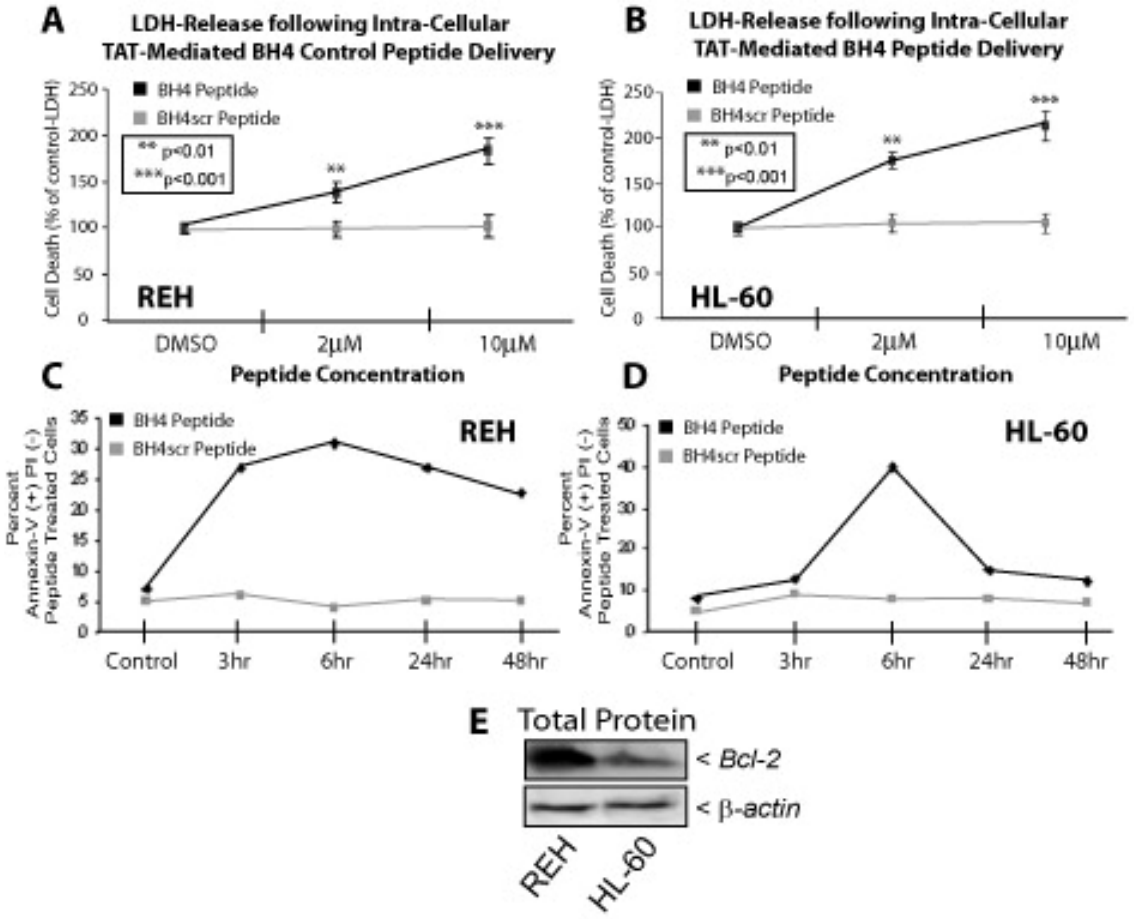


Figure 5.5. BH4 domain peptide induces cell death via induction of apoptosis in REH and HL-60 cells. **A**, REH cells were treated with BH4 or BH4scr domain peptide at 2μM or 10μM concentrations. LDH release was measured 48 hrs post peptide administration. **B**, HL-60 cells were treated with BH4 or BH4scr domain peptide at 2μM or 10μM concentrations. LDH release was measured 48 hrs post peptide administration. **C**, Time course measuring induction of apoptosis in REH cells following addition of either BH4 or BH4scr domain peptide at 10μM concentration. **D**, Time course measuring induction of apoptosis in HL-60 cells following addition of either BH4 or BH4scr domain peptide at 10μM concentration. **E**, Western blot detecting Bcl-2 expression in REH and HL-60 cells. Blot was re-probed with β-actin to control for protein loading.

Administration of BH4 peptide to isolated bone marrow and peripheral blood from AML patients revealed an increase in apoptosis in progenitor cancerous stem cells

(**Fig. 5.6A**) and circulating mature cancer cells (**Fig. 5.6B**) as compared to cells treated with the TAT-BH4scr peptide or naive control cells.

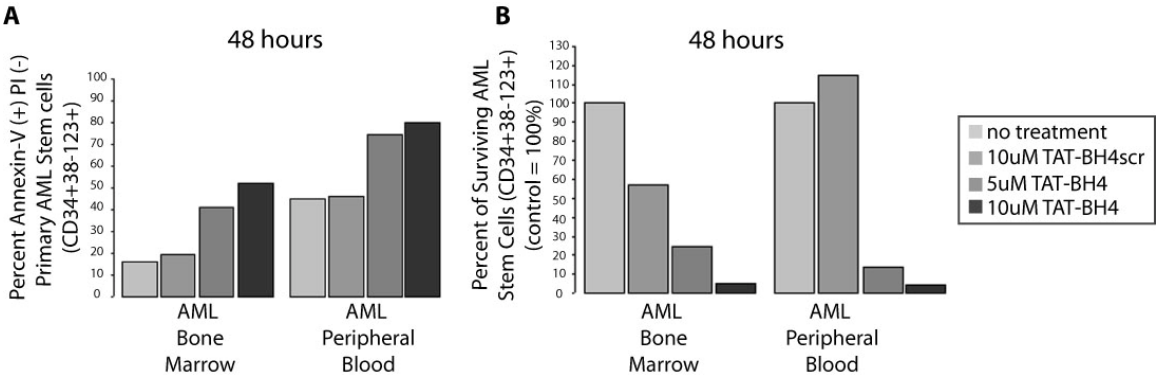


Figure 5.6. BH4 peptide mediated induction of apoptosis in AML stem cells from bone marrow and peripheral blood. **A**, Apoptosis induced in AML stem cells isolated from bone marrow or peripheral blood following 48hr incubation with 10μM BH4scr, 5μM BH4, or 10μM BH4 domain peptide. **B**, Survival of AML stem cells isolated from bone marrow or peripheral blood following 48hr incubation with 10μM BH4scr, 5μM BH4, or 10μM BH4 domain peptide.

DISCUSSION

In these experiments we provide evidence for a novel molecular strategy to induce apoptosis selectively in Bcl-2 expressing cells by promoting nuclear localization of Bcl-2. While the majority of Bcl-2 studies have focused on its anti-apoptotic role at the mitochondria, recent evidence has shown that Bcl-2 also acts in a pro-apoptotic fashion following transient expression (Uhlmann et al., 1998; Wang et al., 2001) or following caspase cleavage, an event that results in removal of Bcl-2's BH4 domain (Cheng et al., 1997). While pro-apoptotic function for Bcl-2 has been previously described, it was not until recently that nuclear localized Bcl-2 was shown to promote apoptosis therefore giving a cellular location for pro-apoptotic action of Bcl-2 (Chapter 4). Our data presented here outlines a strategy for targeted disruption of the binding between Bcl-2

and its mitochondrial chaperone FKBP38, an event that results in increased nuclear localization and subsequent nuclear Bcl-2-mediated cell death.

We previously reported that nuclear compartment-associated Bcl-2 induces apoptosis in multiple cell lines (Massaad et al., 2004). In the course of investigating nuclear Bcl-2's function, we observed that a Bcl-2 deletion mutant lacking the BH4 domain (Bcl-2ΔBH4), failed to bind FKBP38 and failed to localize to the mitochondria (**Figs. 4.6 and 4.3** respectively). Consistent with our observations, previous reports have shown that caspase-3 cleavage of endogenous Bcl-2 results in removal of Bcl-2's BH4 domain and converts Bcl-2 from a cell protector into a cell killer (Cheng et al., 1997; Subramanian and Chinnadurai, 2003), while suppression of FKBP38 expression via siRNA inhibits the anti-apoptotic effect of Bcl-2 (Shirane and Nakayama, 2003). This critical link between presence of Bcl-2's BH4 domain and proper mitochondrial localization for anti-apoptotic Bcl-2 function, along with our own observations of a pro-apoptotic activity of nuclear Bcl-2, prompted us to further investigate the potential use of a Bcl-2 BH4 domain peptide to disrupt Bcl-2 binding to FKBP38, thus modulating Bcl-2 sub-cellular localization and ultimately dictating the cell's fate by directing Bcl-2 to the nucleus to induce apoptosis.

Based on our previous work demonstrating that a synthetic BH4 domain peptide binds FKBP38 (**Fig. 4.6**), here we specifically tested the ability of such a BH4 peptide to disrupt normal Bcl-2/FKBP38 binding in such a way as to prevent mitochondrial trafficking and promote nuclear localization. As shown in **Fig. 5.1**, BH4 peptide and Bcl-2 both co-immunoprecipitate with FKBP38. However, co-immunoprecipitation of FKBP and Bcl-2 in the presence of the BH4 peptide resulted in a significant reduction in the amount of Bcl-2 pulled down by FKBP38. Parallel experiments performed using a control TAT-BH4scr peptide, showed that TAT-BH4scr failed to bind to FKBP38 and failed to disrupt Bcl-2 and FKBP38 binding which demonstrates the specificity of the BH4 amino acid sequence for binding to FKBP38. While this experiment demonstrated that TAT-BH4 peptide was capable of disrupting Bcl-2/FKPB38 binding, this assay was

performed *in vitro* and therefore the applicability of this strategy to intact cells was unknown. To accomplish intra-cellular delivery of BH4, we synthesized a BH4 peptide fused to a modified HIV TAT domain, which had previously been utilized for intra-cellular peptide delivery (Ho et al., 2001). Incubation of cells with TAT fused BH4 peptide results in intracellular delivery in a dose dependent fashion into Bcl-2 and non-Bcl-2 expressing PC12 cells (**Fig. 5.2**).

Following demonstration of a functional delivery mechanism for the BH4 peptide, and based on BH4's ability to disrupt Bcl-2/FKBP38 binding *in vitro,* we tested whether addition of BH4 peptide would increase nuclear localization of Bcl-2. Examination of Bcl-2 and FKBP38 sub-cellular localization following addition of TAT-BH4 into cells transfected with Bcl-2 demonstrated that intracellular delivery of TAT-BH4 in Bcl-2-transfected cells increases nuclear localization of Bcl-2 and elevates FKBP38's expression in both the nuclear and non-nuclear fractions (**Fig. 5.3**). On the other hand, the TAT-BH4 peptide did not affect FKBP38 sub-cellular localization or FKBP38 expression level in vector-transfected PC12 cells. Measurements of cell death following addition of BH4 peptide mediated increased nuclear Bcl-2 revealed a significant increase in cell death selectively in Bcl-2-expressing PC12 cells, while addition of TAT-BH4 to vector-expressing cells, that do not have Bcl-2, failed to induce a significant change in cell death (**Fig. 5.4**). It is important to emphasize that the PC12 cells used in these experiments do not express endogenous Bcl-2 (Massaad et al., 2004) and therefore that the PC12 cells stably-transfected with Bcl-2 differed from the cells stably transfected with vector only because of the presence of absence of Bcl-2. Thus, only in the presence of Bcl-2 was apoptosis induced by the addition of the BH4 peptide, indicating that the BH4 peptide per se was not toxic to cells; rather, the nuclear shift of Bcl-2 resulting from the BH4 peptide-mediated disruption of Bcl-2/FKBP38 binding triggered apoptosis. Accordingly, only cells expressing Bcl-2 were affected. This finding is highly significant due to the selective nature of cell death induced by the BH4 peptide and suggests a possible use for BH4 peptide in selective elimination of Bcl-2 expressing malignant cells such as that found in the majority of human neoplasms.

The meaning of the observed increase of cytosolic and nuclear FKBP38 expression after treatment with the BH4 peptide (**Fig. 5.3**) is not clear. One possibility is that the engagement of FKBP38 by the BH4 peptide results in a feedback signal promoting de novo synthesis of FKBP38. However, since the expression of FKBP38 was not up-regulated by the BH4 peptide in vector-transfected cells (**Fig. 5.3**) this possibility seems unlikely. Alternatively, either the loss of Bcl-2 binding onto FKBP38 or the presence of Bcl-2 at the nuclear compartment may deliver a feedback signal for increased FKBP38 synthesis. While second possibility would be consistent with the lack of any effect of the BH4 peptide on FKBP38 expression in vector-transfected cells, further studies are needed to elucidate the involved mechanisms.

While illustrating a novel strategy to induce nuclear Bcl-2-triggered apoptosis, these results were obtained in PC12 cells which do not express endogenous Bcl-2 and do not depend on Bcl-2 for survival (Massaad et al., 2004). Lack of endogenous Bcl-2 expression makes PC12 cells a useful tool to determine the effects of exogenously-expressed Bcl-2 as emphasized above. However, this feature also renders PC12 cells a distant model for cancer cells that express and often depend on endogenous Bcl-2 and that thus could be targeted by a potential BH4-based treatment. Therefore, to directly test the efficacy of the BH4 peptide in eliminating Bcl-2 expressing malignant cells, we investigated the effect of a synthetic TAT-BH4 peptide in REH and HL-60 leukemia cell lines as well as in stem-cells isolated from bone marrow and mature cancer cells from peripheral blood from AML patients. All of these investigated cells express high levels of Bcl-2 and in addition, Bcl-2 has been shown to play a substantial role in their malignant transformation, expansion, and resistance to apoptotic stimuli (Andreeff et al., 1999; Konopleva et al., 1999; Deng et al., 2001; Konopleva et al., 2002; Milella et al., 2002; Kurinna et al., 2006). Our results indicate that the BH4 peptide induces apoptosis in REH and HL-60 cells as well as in progenitor cancer cells in the bone marrow and mature cancer cells in peripheral blood from AML patients and confirm that the presence of endogenous Bcl-2 can mark cancer cells for killing by the BH4 peptide.

CONCLUSION

We have shown that Bcl-2's interaction with mitochondrial chaperone protein FKBP38 can be disrupted using a synthetic BH4 domain peptide. Disruption of Bcl-2/FKBP38 binding via addition of BH4 domain peptide shifts the distribution of Bcl-2 away from the mitochondria to its default location at the nuclear envelope. As previously demonstrated, nuclear localized Bcl-2 specifically induces apoptosis (Chapter 4). Therefore, addition of a BH4 domain peptide makes it possible to selectively target and eliminate cells that express Bcl-2. This strategy for mediating cell death has been demonstrated using PC12, REH, HL-60, and AML stem cells isolated both from human bone marrow and mature AML cancer cells from peripheral blood. These studies have far reaching implications because they offer a mechanism in which it is possible to utilize Bcl-2's dual role as a cell protector or cell killer, following directed sub-cellular localization of Bcl-2. These studies thus show the possibility for therapeutic intervention by manipulating Bcl-2's sub-cellular distribution in order to dictate a cell's apoptotic fate.

CHAPTER 6: GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

GENERAL CONCLUSIONS

The discovery and characterization of the Bcl-2 family of proteins has provided insight into many functional mechanisms of apoptosis regulation. By far the best characterized protein in this family is the founding member, Bcl-2. Extensive research on Bcl-2 has revealed several functional roles for mitochondrial localized Bcl-2. These roles include heterdimerization with and subsequent inactivation of pro-apoptotic family members, formation of ion channels, control over permeability transition pore formation, and blockade of the release of pro-apoptotic intra-mitochondrial proteins. While mitochondrial localized Bcl-2 is well established as an anti-apoptotic regulator, Bcl-2's functional role at other sub-cellular locations such as the nuclear envelope and ER remain relatively un-characterized. Collectively, this evidence has shaped our general prospective of Bcl-2 as a mitochondrial localized anti-apoptotic protein. With this dogma of anti-apoptotic Bcl-2 function, it seems almost counter intuitive that several reports have identified cases of pro-apoptotic function of Bcl-2. For example, during the execution of apoptosis caspase-3 has been shown to cleave Bcl-2's BH4 domain and convert Bcl-2 into a pro-apoptotic protein (Cheng et al., 1997). Moreover, transient overexpression of Bcl-2 has also been linked to induction of apoptosis (Uhlmann et al., 1998; Wang et al., 2001). These results demonstrate that Bcl-2 can function in a pro-apoptotic manner; however, the mechanisms that control the conversion of Bcl-2 from a pro- to an anti-apoptotic protein remained largely uncharacterized.

Previous work from our own laboratory has focused on Bcl-2's role at the nuclear envelope. This interest in nuclear compartment-associated Bcl-2 came from our observation of oxidative stress-dependent up-regulation of nuclear compartment-associated Bcl-2 in aged rats (Kaufmann et al., 2001). Following this initial observation we demonstrated that nuclear compartment-associated Bcl-2 failed to protect cells from

apoptosis induced by oxidative stress (Kaufmann et al., 2003). Additionally, in an attempt to further characterize the role of nuclear associated Bcl-2, we investigated the role of nuclear localized Bcl-2 on regulation of transcription factor activity. These studies showed that localization of Bcl-2 at the nuclear compartment decreased activity of several transcription factors, particularly nuclear factor kappa B (NFκB) (Massaad et al., 2004), a transcription factor required to prevent cell death both *in vitro* (Taglialatela et al., 1997) and *in vivo* (Taglialatela et al., 1998). Our results suggested that nuclear Bcl-2 may induce apoptosis rather than prevent it, an event that could tie earlier observation of pro-apoptotic Bcl-2 function to a particular sub-cellular localization.

The body of work presented here builds on our laboratory's previous studies and specifically characterizes nuclear compartment associated Bcl-2's function in apoptosis regulation. This dissertation presents the functional characterization of nuclear Bcl-2's role in apoptosis regulation, molecularly characterizes the binding between Bcl-2 and mitochondrial chaperone protein FKBP38, and additionally tests the functionality of a BH4 domain-mediated strategy for directing Bcl-2 to the nucleus to induce apoptosis selectively in Bcl-2 expressing cells. To investigate the role of nuclear associated Bcl-2 in apoptosis regulation, I first generated an apoptosis assay that could simultaneously report the presence/absence of Bcl-2 at the nucleus paired with cells apoptotic status. This was accomplished utilizing flow cytometry based analysis of physical light scatter properties of isolated nuclei, a technique that allowed rapid quantitative measurement of apoptosis in nuclear Bcl-2-containing cells from both cell culture and brain tissue (Chapter 3) (Portier et al., 2006). Validation of this assay required standardized induction of apoptosis by H_2O_2 in cells culture or aglycia/hypoxia in tissue, followed by quantification of apoptosis using physical light scatter properties paired with a standard sub-G1 propidium iodide staining apoptosis assay. Measurement of physical light scatter properties of isolated nuclei offered several advantages over standard apoptosis assays. One advantage includes the ability to rapidly screen populations of nuclei for induction of apoptosis without the need for staining or antibody recognition. A second advantage of

this assay is reduction of false positive inclusion due to measuring fundamental morphology changes that occur specifically in apoptosis rather than biochemical changes that can occur in some cases of necrosis.

With a functional assay allowing co-analysis of nuclear associated Bcl-2 and apoptosis, I was able to demonstrate that Bcl-2 localized at the nuclear compartment induces apoptosis following transient transfection (Chapter 4). To further dissect the function of nuclear Bcl-2 I employed two strategies 1) reducing nuclear Bcl-2 via addition of mitochondrial chaperone FKPB38 and 2) increasing nuclear Bcl-2 via siRNA mediated knock-down of endogenous FKBP38. Addition of FKBP38 decreased nuclear association of Bcl-2 and resulted in a significant increase in cell survival. On the other hand, siRNA knock-down of FKBP38 resulted in increased nuclear associated Bcl-2 and a significant increase in apoptosis. The use of these two strategies directly linked apoptosis induction to the presence of nuclear Bcl-2 and therefore demonstrates that nuclear Bcl-2 is a pro-apoptotic regulator protein.

In order to translate Bcl-2's pro-apoptotic activity into a functional therapeutic strategy, I showed that protein transduction-mediated delivery of a synthetic peptide based on Bcl-2's BH4 aminoacid sequence induces cell death selectively in cells expressing Bcl-2 (Chapter 5). I also showed that *in vitro* administration of a BH4 domain peptide specifically disrupts Bcl-2/FKPB38 binding. In addition, TAT-mediated introduction of the BH4 peptide into cells revealed an increase in nuclear association of Bcl-2 and a selective induction of cell death in Bcl-2 expressing cells. Application of the BH4 peptide to AML and ALL cells, both of which are Bcl-2-expressing human leukemia cell lines, induced prompt apoptosis. Finally, treatment of bone marrow and peripheral blood samples from AML patients using a cell-permeable TAT-BH4 peptide revealed significant and selective elimination of Bcl-2 expressing progenitor cancer cells. These results demonstrate the therapeutic potentials of using the pro-apoptotic activity of nuclear localized Bcl-2 in order to specifically eliminate cells expressing high levels of Bcl-2.

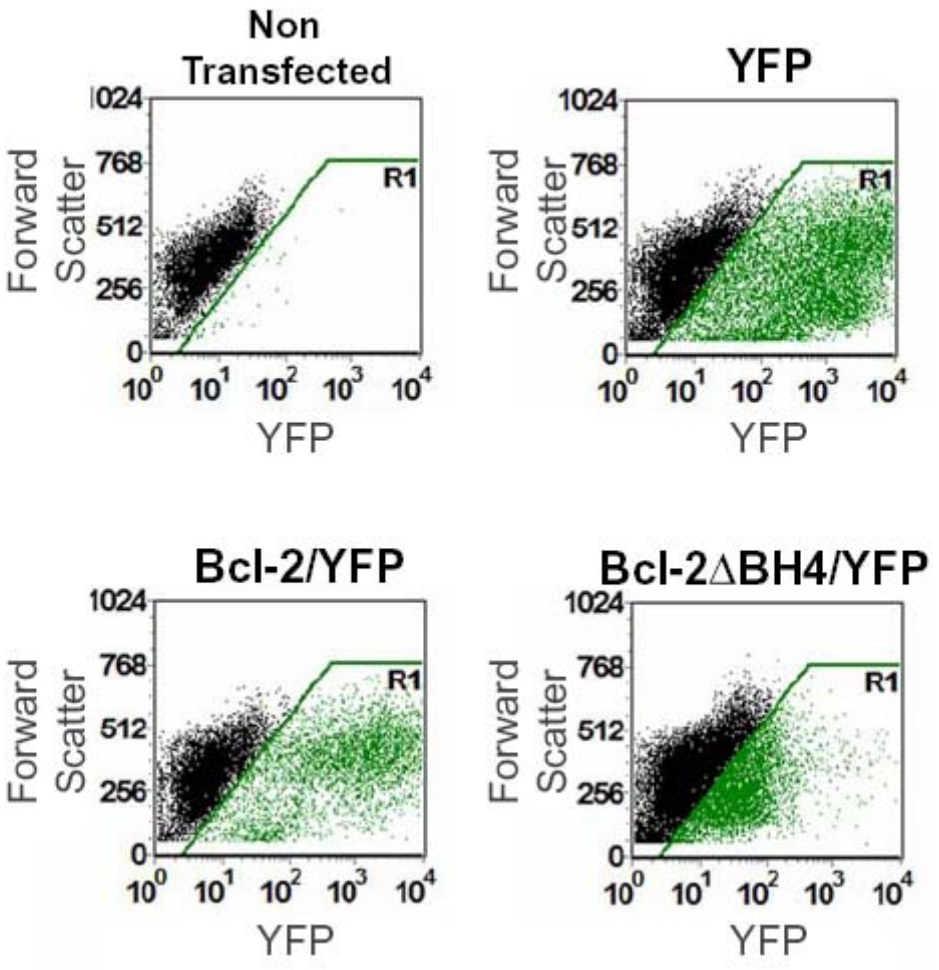
The goal of this dissertation was to determine nuclear Bcl-2's role as a pro- or anti-apoptotic regulator in order to further our understanding of basic apoptotic control mechanisms. This dissertation initially outlined a novel flow cytometry-based apoptosis assay that makes characterization of apoptosis tied to presence of nuclear localized proteins possible. Furthermore, I demonstrated that nuclear Bcl-2 functions as a pro-apoptotic protein. Lastly, I defined the critical binding requirements for Bcl-2/FKBP38 interaction. In conclusion this dissertation demonstrates a strategy for utilizing the pro-apoptotic action of Bcl-2 to selectively target and eliminate cells that express Bcl-2.

FUTURE DIRECTIONS

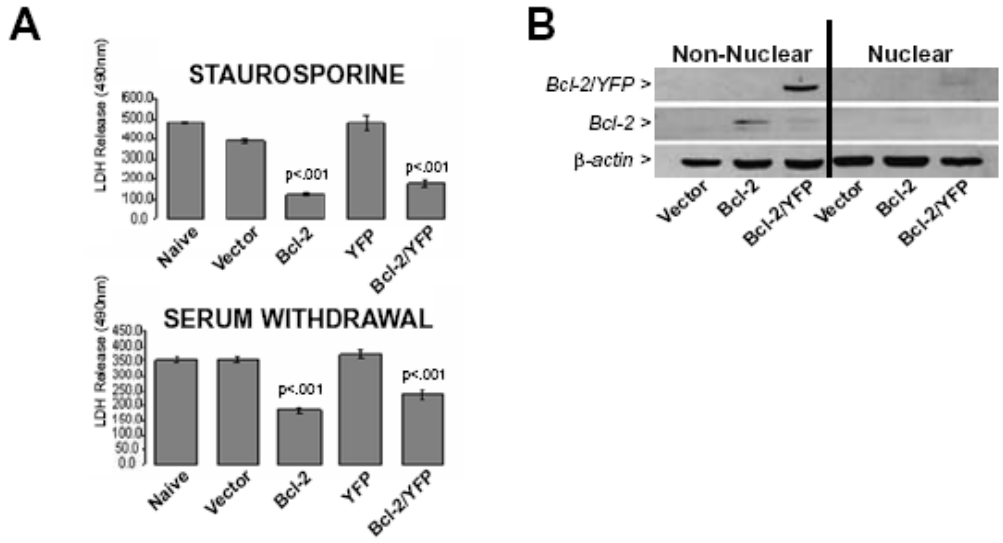
The next logical steps to be undertaken with this project follow three main lines of investigation. The first extension of this project would involve utilizing the molecular tools and techniques optimized in this project in order to determine the critical minimum BH4 domain amino acid sequence needed to bind FKBP38. This could be accomplished by synthesizing BH4 domain peptides with a reduced number of conserved amino acids and subsequently testing each peptide's ability to bind to FKBP38 via immunoprecipitation. A second extension of this project would require molecular protein modeling and physical design of a BH4 mimetic small molecule that would be therapeutically effective and clinically more attractive. A third extension of this project would include testing the mimetic drug/minimal BH4 peptide on Bcl-2 expressing AML-stem cells in order to determine its effectiveness as an *ex-vivo* therapy for AML. Furthermore, it would also be possible to test the mimetic/minimal BH4 peptides therapeutic potential in a nude mouse model of tumor induction. Based on the in vivo nude mouse model data further applications and potential clinical trials could be proposed.

APPENDIX

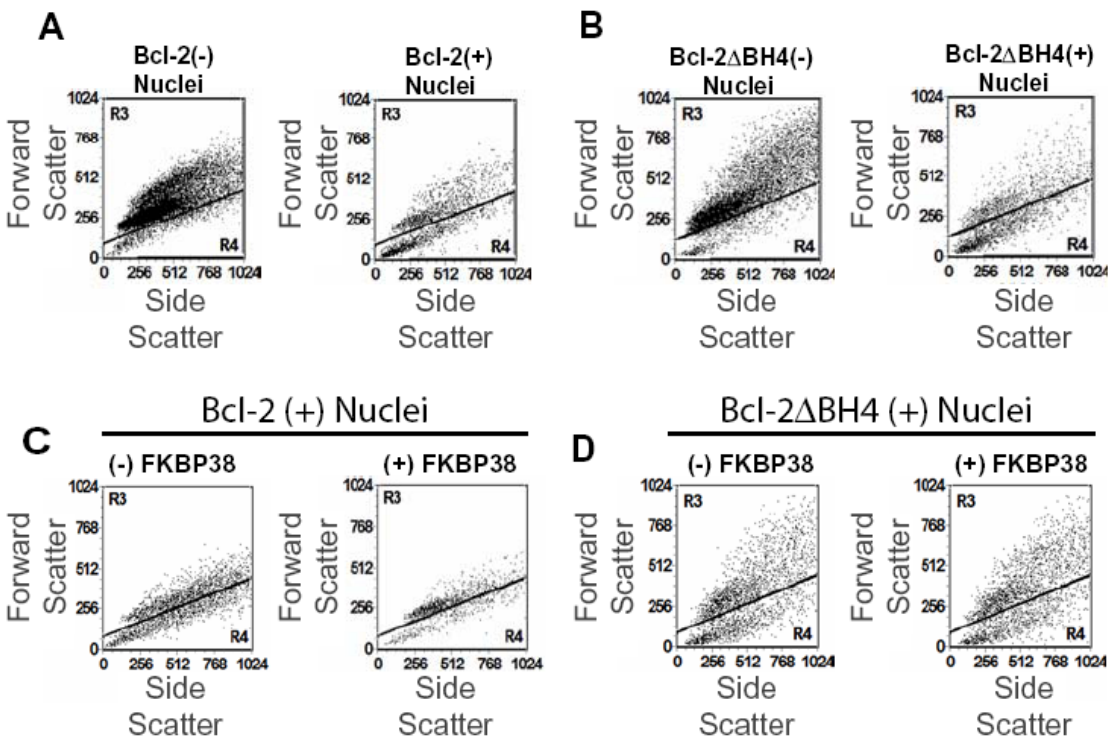
Supplemental Figures



Supplemental Fig. I. Flow cytometry assay detecting fluorescent protein expression in PC12 cells following transient transfection with YFP, Bcl-2/YFP, Bcl-2ΔBH4/YFP or control (non-transfected). R1 population: cells expressing YFP, Bcl-2/YFP, or Bcl-2ΔBH4/YFP.



Supplemental Fig. II. Test anti-apoptotic function of stable expressed Bcl-2/YFP in PC12 cells. **A**, LDH release detected in culture medium 24 hrs post Staurosporine treatment or serum withdrawal in PC12 cells stably transfected with vector, Bcl-2, YFP, or Bcl-2/YFP. Each bar represents the mean of 3 separate experiments ± standard deviation and indicated statistical significances are vs. pmKitneo-vector or YFP-vector transfected cells (two-tailed Student's t-test). **B**, Western blot analysis detecting Bcl-2 and Bcl-2/YFP in non-nuclear and nuclear protein fractions prepared from PC12 cells stably transfected with vector, Bcl-2, and Bcl-2/YFP.



Supplemental Fig. III. Representative dot plots from physical light scatter assays and Annexin-V assays. **A**, Representative physical light scatter dot blots following transient transfection of Bcl-2/YFP in PC12 cells. R3 represents healthy nuclei (G1,S,or G2 phase of cell cycle) while R4 represents apoptotic nuclei. **B**, Representative physical light scatter dot blots following transient transfection of Bcl-2ΔBH4/YFP in PC12 cells. R3 represents healthy nuclei (G1,S,or G2 phase of cell cycle) while R4 represents apoptotic nuclei. **C**, Representative physical light scatter dot blots following transient transfection of Bcl-2/YFP in the presence or absence of FKBP38 performed in PC12 cells. R3 represents healthy nuclei (G1,S,or G2 phase of cell cycle) while R4 represents apoptotic nuclei. **D**, Representative physical light scatter dot blots following transient transfection of Bcl-2ΔBH4/YFP in the presence or absence of FKBP38 performed in PC12 cells. R3 represents healthy nuclei (G1,S, or G2 phase of cell cycle) while R4 represents apoptotic nuclei.

BIBLIOGRAPHY

Adams JM, Cory S (1998) The Bcl-2 protein family: arbiters of cell survival. Science 281:1322-1326.

Akao Y, Otsuki Y, Kataoka S, Ito Y, Tsujimoto Y (1994) Multiple subcellular localization of bcl-2: detection in nuclear outer membrane, endoplasmic reticulum membrane, and mitochondrial membranes. Cancer Res 54:2468-2471.

Andreeff M, Jiang S, Zhang X, Konopleva M, Estrov Z, Snell VE, Xie Z, Okcu MF, Sanchez-Williams G, Dong J, Estey EH, Champlin RC, Kornblau SM, Reed JC, Zhao S (1999) Expression of Bcl-2-related genes in normal and AML progenitors: changes induced by chemotherapy and retinoic acid. Leukemia 13:1881-1892.

Antonsson B, Martinou JC (2000) The Bcl-2 protein family. Exp Cell Res 256:50-57.

Antonsson B, Conti F, Ciavatta A, Montessuit S, Lewis S, Martinou I, Bernasconi L, Bernard A, Mermod JJ, Mazzei G, Maundrell K, Gambale F, Sadoul R, Martinou JC (1997) Inhibition of Bax channel-forming activity by Bcl-2. Science 277:370-372.

Arends MJ, Wyllie AH (1991) Apoptosis: mechanisms and roles in pathology. Int Rev Exp Pathol 32:223-254.

Baffy G, Miyashita T, Williamson JR, Reed JC (1993) Apoptosis induced by withdrawal of interleukin-3 (IL-3) from an IL-3-dependent hematopoietic cell line is associated with repartitioning of intracellular calcium and is blocked by enforced Bcl-2 oncoprotein production. J Biol Chem 268:6511-6519.

Benedetti A, Jezequel AM, Orlandi F (1988) A quantitative evaluation of apoptotic bodies in rat liver. Liver 8:172-177.

Bierer BE, Mattila PS, Standaert RF, Herzenberg LA, Burakoff SJ, Crabtree G, Schreiber SL (1990) Two distinct signal transmission pathways in T lymphocytes are inhibited by complexes formed between an immunophilin and either FK506 or rapamycin. Proc Natl Acad Sci U S A 87:9231-9235.

Billig H, Furuta I, Rivier C, Tapanainen J, Parvinen M, Hsueh AJ (1995) Apoptosis in testis germ cells: developmental changes in gonadotropin dependence and localization to selective tubule stages. Endocrinology 136:5-12.

Bishop CJ, Whiting VA (1983) The role of natural killer cells in the intravascular death of intravenously injected murine tumour cells. Br J Cancer 48:441-444.

Blankenship JR, Steinbach WJ, Perfect JR, Heitman J (2003) Teaching old drugs new tricks: reincarnating immunosuppressants as antifungal drugs. Curr Opin Investig Drugs 4:192-199.

Blasko I, Stampfer-Kountchev M, Robatscher P, Veerhuis R, Eikelenboom P, Grubeck-Loebenstein B (2004) How chronic inflammation can affect the brain and support the development of Alzheimer's disease in old age: the role of microglia and astrocytes. Aging Cell 3:169-176.

Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, Mao X, Nunez G, Thompson CB (1993) bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell 74:597-608.

Bold RJ, Termuhlen PM, McConkey DJ (1997) Apoptosis, cancer and cancer therapy. Surg Oncol 6:133-142.

Borner C (1996) Diminished cell proliferation associated with the death-protective activity of Bcl-2. J Biol Chem 271:12695-12698.

Borner C (2003) The Bcl-2 protein family: sensors and checkpoints for life-or-death decisions. Mol Immunol 39:615-647.

Breckenridge DG, Nguyen M, Kuppig S, Reth M, Shore GC (2002) The procaspase-8 isoform, procaspase-8L, recruited to the BAP31 complex at the endoplasmic reticulum. Proc Natl Acad Sci U S A 99:4331-4336.

Breckenridge DG, Germain M, Mathai JP, Nguyen M, Shore GC (2003) Regulation of apoptosis by endoplasmic reticulum pathways. Oncogene 22:8608-8618.

Breitschopf K, Haendeler J, Malchow P, Zeiher AM, Dimmeler S (2000) Posttranslational modification of Bcl-2 facilitates its proteasome-dependent degradation: molecular characterization of the involved signaling pathway. Mol Cell Biol 20:1886-1896.

Bruckheimer EM, Cho SH, Sarkiss M, Herrmann J, McDonnell TJ (1998) The Bcl-2 gene family and apoptosis. Adv Biochem Eng Biotechnol 62:75-105.

Buendia B, Santa-Maria A, Courvalin JC (1999) Caspase-dependent proteolysis of integral and peripheral proteins of nuclear membranes and nuclear pore complex proteins during apoptosis. J Cell Sci 112 (Pt 11):1743-1753.

Capel B (2000) The battle of the sexes. Mech Dev 92:89-103.

Cappello F, Bellafiore M, Palma A, Bucchieri F (2002) Defective apoptosis and tumorigenesis: role of p53 mutation and Fas/FasL system dysregulation. Eur J Histochem 46:199-208.

Chang BS, Minn AJ, Muchmore SW, Fesik SW, Thompson CB (1997) Identification of a novel regulatory domain in Bcl-X(L) and Bcl-2. Embo J 16:968-977.

Chao DT, Korsmeyer SJ (1998) BCL-2 family: regulators of cell death. Annu Rev Immunol 16:395-419.

Cheng EH, Levine B, Boise LH, Thompson CB, Hardwick JM (1996) Bax-independent inhibition of apoptosis by Bcl-XL. Nature 379:554-556.

Cheng EH, Kirsch DG, Clem RJ, Ravi R, Kastan MB, Bedi A, Ueno K, Hardwick JM (1997) Conversion of Bcl-2 to a Bax-like death effector by caspases. Science 278:1966-1968.

Chinnaiyan AM, O'Rourke K, Tewari M, Dixit VM (1995) FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell 81:505-512.

Chittenden T, Flemington C, Houghton AB, Ebb RG, Gallo GJ, Elangovan B, Chinnadurai G, Lutz RJ (1995) A conserved domain in Bak, distinct from BH1 and BH2, mediates cell death and protein binding functions. EMBO J 14:5589-5596.

Cole CN, Hammell CM (1998) Nucleocytoplasmic transport: driving and directing transport. Curr Biol 8:R368-372.

Columbaro M, Gobbi P, Reno F, Luchetti F, Santi S, Valmori A, Falcieri E (1998) A multiple technical approach to the study of apoptotic cell micronuclei. Scanning 20:541-548.

Condorelli F, Salomoni P, Cotteret S, Cesi V, Srinivasula SM, Alnemri ES, Calabretta B (2001) Caspase cleavage enhances the apoptosis-inducing effects of BAD. Mol Cell Biol 21:3025-3036.

Councilman T (1890) Report on the etiology and prevention of yellow fever. Public Health Bulletin Washington DC: US Govt Print Off 2:151–159.

Crompton M, Virji S, Doyle V, Johnson N, Ward JM (1999) The mitochondrial permeability transition pore. Biochem Soc Symp 66:167-179.

Darzynkiewicz Z, Bedner E, Traganos F (2001) Difficulties and pitfalls in analysis of apoptosis. Methods Cell Biol 63:527-546.

Daugas E, Nochy D, Ravagnan L, Loeffler M, Susin SA, Zamzami N, Kroemer G (2000) Apoptosis-inducing factor (AIF): a ubiquitous mitochondrial oxidoreductase involved in apoptosis. FEBS Lett 476:118-123.

de Jong D, Prins FA, Mason DY, Reed JC, van Ommen GB, Kluin PM (1994) Subcellular localization of the bcl-2 protein in malignant and normal lymphoid cells. Cancer Res 54:256-260.

Demaurex N, Distelhorst C (2003) Cell biology. Apoptosis--the calcium connection. Science 300:65-67.

Deng X, Kornblau SM, Ruvolo PP, May WS, Jr. (2001) Regulation of Bcl2 phosphorylation and potential significance for leukemic cell chemoresistance. J Natl Cancer Inst Monogr 15:30-37.

Denis GV, Yu Q, Ma P, Deeds L, Faller DV, Chen CY (2003) Bcl-2, via its BH4 domain, blocks apoptotic signaling mediated by mitochondrial Ras. J Biol Chem 278:5775-5785.

Desjardins P, Ledoux S (1998) The role of apoptosis in neurodegenerative diseases. Metab Brain Dis 13:79-96.

Don MM, Ablett G, Bishop CJ, Bundesen PG, Donald KJ, Searle J, Kerr JF (1977) Death of cells by apoptosis following attachment of specifically allergized lymphocytes in vitro. Aust J Exp Biol Med Sci 55:407-417.

Duan H, Dixit VM (1997) RAIDD is a new 'death' adaptor molecule. Nature 385:86-89.

Duband-Goulet I, Courvalin JC, Buendia B (1998) LBR, a chromatin and lamin binding protein from the inner nuclear membrane, is proteolyzed at late stages of apoptosis. J Cell Sci 111 1441-1451.

Dumont FJ, Staruch MJ, Koprak SL, Siekierka JJ, Lin CS, Harrison R, Sewell T, Kindt VM, Beattie TR, Wyvratt M, et al. (1992) The immunosuppressive and toxic effects of FK-506 are mechanistically related: pharmacology of a novel antagonist of FK-506 and rapamycin. J Exp Med 176:751-760.

Edlich F, Weiwad M, Erdmann F, Fanghanel J, Jarczowski F, Rahfeld JU, Fischer G (2005) Bcl-2 regulator FKBP38 is activated by Ca2+/calmodulin. EMBO J 24:2688-2699.

Edlich F, Weiwad M, Wildemann D, Jarczowski F, Kilka S, Moutty MC, Jahreis G, Lucke C, Schmidt W, Striggow F, Fischer G (2006) The specific FKBP38 inhibitor N-(N',N'-dimethylcarboxamidomethyl)cycloheximide has potent neuroprotective and neurotrophic properties in brain ischemia. J Biol Chem 281:14961-14970.

Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S (1998) A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature 391:43-50.

Fadok VA, de Cathelineau A, Daleke DL, Henson PM, Bratton DL (2001) Loss of phospholipid asymmetry and surface exposure of phosphatidylserine is required for phagocytosis of apoptotic cells by macrophages and fibroblasts. J Biol Chem 276:1071-1077.

Falcieri E, Gobbi P, Cataldi A, Zamai L, Faenza I, Vitale M (1994a) Nuclear pores in the apoptotic cell. Histochem J 26:754-763.

Falcieri E, Zamai L, Santi S, Cinti C, Gobbi P, Bosco D, Cataldi A, Betts C, Vitale M (1994b) The behaviour of nuclear domains in the course of apoptosis. Histochemistry 102:221-231.

Faleiro L, Lazebnik Y (2000) Caspases disrupt the nuclear-cytoplasmic barrier. J Cell Biol 151:951-959.

Farbman AI (1968) Electron microscope study of palate fusion in mouse embryos. Dev Biol 18:93-116.

Ferrer I, Lopez E, Blanco R, Rivera R, Ballabriga J, Pozas E, Marti E (1998) Bcl-2, Bax, and Bcl-x expression in the CA1 area of the hippocampus following transient forebrain ischemia in the adult gerbil. Exp Brain Res 121:167-173.

Feske S, Okamura H, Hogan PG, Rao A (2003) Ca2+/calcineurin signalling in cells of the immune system. Biochem Biophys Res Commun 311:1117-1132.

Feske S, Giltnane J, Dolmetsch R, Staudt LM, Rao A (2001) Gene regulation mediated by calcium signals in T lymphocytes. Nat Immunol 2:316-324.

Festjens N, van Gurp M, van Loo G, Saelens X, Vandenabeele P (2004) Bcl-2 family members as sentinels of cellular integrity and role of mitochondrial intermembrane space proteins in apoptotic cell death. Acta Haematol 111:7-27.

Fischer G, Wittmann-Liebold B, Lang K, Kiefhaber T, Schmid FX (1989) Cyclophilin and peptidyl-prolyl cis-trans isomerase are probably identical proteins. Nature 337:476-478.

Flemming W (1885) Uber die Bildung von Richtungsfiguren in Säugetrhiereiern beim Untergang Graff scher Follikel. Arch Anat Physiol 221- 244.

Formigli L, Papucci L, Tani A, Schiavone N, Tempestini A, Orlandini GE, Capaccioli S, Orlandini SZ (2000) Aponecrosis: morphological and biochemical exploration of a syncretic process of cell death sharing apoptosis and necrosis. J Cell Physiol 182:41-49.

Freire-de-Lima CG, Nascimento DO, Soares MB, Bozza PT, Castro-Faria-Neto HC, de Mello FG, DosReis GA, Lopes MF (2000) Uptake of apoptotic cells drives the growth of a pathogenic trypanosome in macrophages. Nature 403:199-203.

French LE, Tschopp J (2000) Fas-mediated cell death in toxic epidermal necrolysis and graft-versus-host disease: potential for therapeutic inhibition. Schweiz Med Wochenschr 130:1656-1661.

Germain M, Shore GC (2003) Cellular distribution of Bcl-2 family proteins. Sci STKE 2003:10-11.

Gittins R, Harrison PJ (2004) Neuronal density, size and shape in the human anterior cingulate cortex: a comparison of Nissl and NeuN staining. Brain Res Bull 63:155-160.

Glucksmann A (1951) Cell death in normal vertebrate ontogeny. Biology Review 26:59-86.

Gold R, Schmied M, Rothe G, Zischler H, Breitschopf H, Wekerle H, Lassmann H (1993) Detection of DNA fragmentation in apoptosis: application of in situ nick translation to cell culture systems and tissue sections. J Histochem Cytochem 41:1023-1030.

Gotzmann J, Vlcek S, Foisner R (2000) Caspase-mediated cleavage of the chromosome-binding domain of lamina-associated polypeptide 2 alpha. J Cell Sci 113 3769-3780.

Grasl-Kraupp B, Ruttkay-Nedecky B, Koudelka H, Bukowska K, Bursch W, Schulte-Hermann R (1995) In situ detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. Hepatology 21:1465-1468.

Greene LA, Tischler AS (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc Natl Acad Sci U S A 73:2424-2428.

Greene LA, Rein G (1977) Synthesis, storage and release of acetylcholine by a noradrenergic pheochromocytoma cell line. Nature 268:349-351.

Greer JP, Kinney MC, Loughran TP, Jr. (2001) T cell and NK cell lymphoproliferative disorders. Hematology Am Soc Hematol Educ Program 15:259-281.

Gregory CD, Devitt A (2004) The macrophage and the apoptotic cell: an innate immune interaction viewed simplistically? Immunology 113:1-14.

Hamburger V, Levi-Montalcini, R. (1949) Proliferation, differentiation and experimental conditions. J Exp Zool 111:457-501.

Hammar SP, Mottet NK (1971) Tetrazolium salt and electron-microscopic studies of cellular degeneration and necrosis in the interdigital areas of the developing chick limb. J Cell Sci 8:229-251.

Hammerman MR (1998) Regulation of cell survival during renal development. Pediatr Nephrol 12:596-602.

Hanshaw RG, Smith BD (2005) New reagents for phosphatidylserine recognition and detection of apoptosis. Bioorg Med Chem 13:5035-5042.

Harding MW, Galat A, Uehling DE, Schreiber SL (1989) A receptor for the immunosuppressant FK506 is a cis-trans peptidyl-prolyl isomerase. Nature 341:758-760.

He H, Lam M, McCormick TS, Distelhorst CW (1997) Maintenance of calcium homeostasis in the endoplasmic reticulum by Bcl-2. J Cell Biol 138:1219-1228.

Hengartner MO (2000) The biochemistry of apoptosis. Nature 407:770-776.

Herculano-Houzel S, Lent R (2005) Isotropic fractionator: a simple, rapid method for the quantification of total cell and neuron numbers in the brain. J Neurosci 25:2518-2521.

Ho A, Schwarze SR, Mermelstein SJ, Waksman G, Dowdy SF (2001) Synthetic protein transduction domains: enhanced transduction potential in vitro and in vivo. Cancer Res 61:474-477.

Hockenbery D, Nunez G, Milliman C, Schreiber RD, Korsmeyer SJ (1990) Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature 348:334-336.

Hoetelmans R, van Slooten HJ, Keijzer R, Erkeland S, van de Velde CJ, Dierendonck JH (2000) Bcl-2 and Bax proteins are present in interphase nuclei of mammalian cells. Cell Death Differ 7:384-392.

Honkaniemi J, Massa SM, Breckinridge M, Sharp FR (1996) Global ischemia induces apoptosis-associated genes in hippocampus. Brain Res Mol Brain Res 42:79-88.

Hour TC, Chen L, Lin JK (2000) Suppression of transcription factor NF-kappaB activity by Bcl-2 protein in NIH3T3 cells: implication of a novel NF-kappaB p50-Bcl-2 complex for the anti-apoptotic function of Bcl-2. Eur J Cell Biol 79:121-129.

Huai Q, Kim HY, Liu Y, Zhao Y, Mondragon A, Liu JO, Ke H (2002) Crystal structure of calcineurin-cyclophilin-cyclosporin shows common but distinct recognition of immunophilin-drug complexes. Proc Natl Acad Sci U S A 99:12037-12042.

Huang DC, Adams JM, Cory S (1998) The conserved N-terminal BH4 domain of Bcl-2 homologues is essential for inhibition of apoptosis and interaction with CED-4. EMBO J 17:1029-1039.

Hunter JJ, Bond BL, Parslow TG (1996) Functional dissection of the human Bc12 protein: sequence requirements for inhibition of apoptosis. Mol Cell Biol 16:877-883.

Idriss HT, Naismith JH (2000) TNF alpha and the TNF receptor superfamily: structure-function relationship(s). Microsc Res Tech 50:184-195.

Irmler M, Thome M, Hahne M, Schneider P, Hofmann K, Steiner V, Bodmer JL, Schroter M, Burns K, Mattmann C, Rimoldi D, French LE, Tschopp J (1997) Inhibition of death receptor signals by cellular FLIP. Nature 388:190-195.

Jacobson MD, Burne JF, King MP, Miyashita T, Reed JC, Raff MC (1993) Bcl-2 blocks apoptosis in cells lacking mitochondrial DNA. Nature 361:365-369.

Johnston MV, Trescher WH, Ishida A, Nakajima W (2001) Neurobiology of hypoxic-ischemic injury in the developing brain. Pediatr Res 49:735-741.

Jones DP, McConkey DJ, Nicotera P, Orrenius S (1989) Calcium-activated DNA fragmentation in rat liver nuclei. J Biol Chem 264:6398-6403.

Kang CB, Tai J, Chia J, Yoon HS (2005) The flexible loop of Bcl-2 is required for molecular interaction with immunosuppressant FK-506 binding protein 38 (FKBP38). FEBS Lett 579:1469-1476.

Kaplan D, Sieg S (1998) Role of the Fas/Fas ligand apoptotic pathway in human immunodeficiency virus type 1 disease. J Virol 72:6279-6282.

Kaufmann JA, Bickford PC, Taglialatela G (2001) Oxidative-stress-dependent up-regulation of Bcl-2 expression in the central nervous system of aged Fisher-344 rats. J Neurochem 76:1099-1108.

Kaufmann JA, Bickford PC, Taglialatela G (2002) Free radical-dependent changes in constitutive Nuclear factor kappa B in the aged hippocampus. Neuroreport 13:1917-1920.

Kaufmann JA, Perez M, Zhang W, Bickford PC, Holmes DB, Taglialatela G (2003) Free radical-dependent nuclear localization of Bcl-2 in the central nervous system of aged rats is not associated with Bcl-2-mediated protection from apoptosis. J Neurochem 87:981-994.

Kelekar A, Chang BS, Harlan JE, Fesik SW, Thompson CB (1997) Bad is a BH3 domain-containing protein that forms an inactivating dimer with Bcl-XL. Mol Cell Biol 17:7040-7046.

Kerr JF, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26:239-257.

Kerr JF, Gobe GC, Winterford CM, Harmon BV (1995) Anatomical methods in cell death. Methods Cell Biol 46:1-27.

Kim HE, Du F, Fang M, Wang X (2005) Formation of apoptosome is initiated by cytochrome c-induced dATP hydrolysis and subsequent nucleotide exchange on Apaf-1. Proc Natl Acad Sci U S A 102:17545-17550.

Kirkin V, Joos S, Zornig M (2004) The role of Bcl-2 family members in tumorigenesis. Biochim Biophys Acta 1644:229-249.

Konopleva M, Zhao S, Xie Z, Segall H, Younes A, Claxton DF, Estrov Z, Kornblau SM, Andreeff M (1999) Apoptosis. Molecules and mechanisms. Adv Exp Med Biol 457:217-236.

Konopleva M, Zhao S, Hu W, Jiang S, Snell V, Weidner D, Jackson CE, Zhang X, Champlin R, Estey E, Reed JC, Andreeff M (2002) The anti-apoptotic genes Bcl-X(L) and Bcl-2 are over-expressed and contribute to chemoresistance of non-proliferating leukaemic CD34+ cells. Br J Haematol 118:521-534.

Korsmeyer SJ, Shutter JR, Veis DJ, Merry DE, Oltvai ZN (1993) Bcl-2/Bax: a rheostat that regulates an anti-oxidant pathway and cell death. Semin Cancer Biol 4:327-332.

Koseki T, Inohara N, Chen S, Nunez G (1998) ARC, an inhibitor of apoptosis expressed in skeletal muscle and heart that interacts selectively with caspases. Proc Natl Acad Sci U S A 95:5156-5160.

Krajewski S, Tanaka S, Takayama S, Schibler MJ, Fenton W, Reed JC (1993) Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. Cancer Res 53:4701-4714.

Krammer PH (2000) CD95's deadly mission in the immune system. Nature 407:789-795.

Kroemer G (1997) The proto-oncogene Bcl-2 and its role in regulating apoptosis. Nat Med 3:614-620.

Kuida K (2000) Caspase-9. Int J Biochem Cell Biol 32:121-124.

Kuo TH, Kim HR, Zhu L, Yu Y, Lin HM, Tsang W (1998) Modulation of endoplasmic reticulum calcium pump by Bcl-2. Oncogene 17:1903-1910.

Kurinna S, Konopleva M, Palla SL, Chen W, Kornblau S, Contractor R, Deng X, May WS, Andreeff M, Ruvolo PP (2006) Bcl2 phosphorylation and active PKC alpha are associated with poor survival in AML. Leukemia 20:1316-1319.

Lam E, Martin M, Wiederrecht G (1995) Isolation of a cDNA encoding a novel human FK506-binding protein homolog containing leucine zipper and tetratricopeptide repeat motifs. Gene 160:297-302.

Le Bras M, Clement MV, Pervaiz S, Brenner C (2005) Reactive oxygen species and the mitochondrial signaling pathway of cell death. Histol Histopathol 20:205-219.

Liang Y, Nylander KD, Yan C, Schor NF (2002) Role of caspase 3-dependent Bcl-2 cleavage in potentiation of apoptosis by Bcl-2. Mol Pharmacol 61:142-149.

Lin B, Kolluri SK, Lin F, Liu W, Han YH, Cao X, Dawson MI, Reed JC, Zhang XK (2004) Conversion of Bcl-2 from protector to killer by interaction with nuclear orphan receptor Nur77/TR3. Cell 116:527-540.

Lin CS, Boltz RC, Siekierka JJ, Sigal NH (1991) FK-506 and cyclosporin A inhibit highly similar signal transduction pathways in human T lymphocytes. Cell Immunol 133:269-284.

Linette GP, Korsmeyer SJ (1994) Differentiation and cell death: lessons from the immune system. Curr Opin Cell Biol 6:809-815.

Liu J, Farmer JD, Jr., Lane WS, Friedman J, Weissman I, Schreiber SL (1991) Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell 66:807-815.

Liu X, Kim CN, Yang J, Jemmerson R, Wang X (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell 86:147-157.

Lorenzo HK, Susin SA (2004) Mitochondrial effectors in caspase-independent cell death. FEBS Lett 557:14-20.

Lowe SW, Schmitt EM, Smith SW, Osborne BA, Jacks T (1993) p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature 362:847-849.

Lyons SK, Clarke AR (1997) Apoptosis and carcinogenesis. Br Med Bull 53:554-569.

MacFarlane M (2003) TRAIL-induced signalling and apoptosis. Toxicol Lett 139:89-97.

Maestre-Martinez M, Edlich F, Jarczowski F, Weiwad M, Fischer G, Lucke C (2006) Solution structure of the FK506-binding domain of human FKBP38. J Biomol NMR 34:197-202.

Malaguarnera L (2004) Implications of apoptosis regulators in tumorigenesis. Cancer Metastasis Rev 23:367-387.

Maroto R, Perez-Polo JR (1997) BCL-2-related protein expression in apoptosis: oxidative stress versus serum deprivation in PC12 cells. J Neurochem 69:514-523.

Martelli AM, Bortul R, Fackelmayer FO, Tazzari PL, Bareggi R, Narducci P, Zweyer M (1999) Biochemical and morphological characterization of the nuclear matrix from apoptotic HL-60 cells. J Cell Biochem 72:35-46.

Martin LJ, Al-Abdulla NA, Brambrink AM, Kirsch JR, Sieber FE, Portera-Cailliau C (1998) Neurodegeneration in excitotoxicity, global cerebral ischemia, and target deprivation: A perspective on the contributions of apoptosis and necrosis. Brain Res Bull 46:281-309.

Massaad CA, Portier BP, Taglialatela G (2004) Inhibition of transcription factor activity by nuclear compartment-associated Bcl-2. J Biol Chem 279:54470-54478.

Mattila PS, Ullman KS, Fiering S, Emmel EA, McCutcheon M, Crabtree GR, Herzenberg LA (1990) The actions of cyclosporin A and FK506 suggest a novel step in the activation of T lymphocytes. EMBO J 9:4425-4433.

Mazarakis ND, Edwards AD, Mehmet H (1997) Apoptosis in neural development and disease. Arch Dis Child Fetal Neonatal Ed 77:F165-170.

McCarthy NJ, Whyte MK, Gilbert CS, Evan GI (1997) Inhibition of Ced-3/ICE-related proteases does not prevent cell death induced by oncogenes, DNA damage, or the Bcl-2 homologue Bak. J Cell Biol 136:215-227.

McConkey DJ (1996) Calcium-dependent, interleukin 1-converting enzyme inhibitor-insensitive degradation of lamin B1 and DNA fragmentation in isolated thymocyte nuclei. J Biol Chem 271:22398-22406.

Meakin SO, Shooter EM (1992) The nerve growth factor family of receptors. Trends Neurosci 15:323-331.

Meier P, Finch A, Evan G (2000) Apoptosis in development. Nature 407:796-801.

Michnick SW, Rosen MK, Wandless TJ, Karplus M, Schreiber SL (1991) Solution structure of FKBP, a rotamase enzyme and receptor for FK506 and rapamycin. Science 252:836-839.

Milella M, Estrov Z, Kornblau SM, Carter BZ, Konopleva M, Tari A, Schober WD, Harris D, Leysath CE, Lopez-Berestein G, Huang Z, Andreeff M (2002) Synergistic induction of apoptosis by simultaneous disruption of the Bcl-2 and MEK/MAPK pathways in acute myelogenous leukemia. Blood 99:3461-3464.

Milligan CE, Schwartz LM (1997) Programmed cell death during animal development. Br Med Bull 53:570-590.

Minn AJ, Boise LH, Thompson CB (1996a) Expression of Bcl-xL and loss of p53 can cooperate to overcome a cell cycle checkpoint induced by mitotic spindle damage. Genes Dev 10:2621-2631.

Minn AJ, Boise LH, Thompson CB (1996b) Bcl-x(S) anatagonizes the protective effects of Bcl-x(L). J Biol Chem 271:6306-6312.

Minn AJ, Velez P, Schendel SL, Liang H, Muchmore SW, Fesik SW, Fill M, Thompson CB (1997) Bcl-x(L) forms an ion channel in synthetic lipid membranes. Nature 385:353-357.

Mirkes PE (2002) 2001 Warkany lecture: to die or not to die, the role of apoptosis in normal and abnormal mammalian development. Teratology 65:228-239.

Mizuno Y, Mochizuki H, Sugita Y, Goto K (1998) Apoptosis in neurodegenerative disorders. Intern Med 37:192-193.

Mondello C, Scovassi AI (2004) Telomeres, telomerase, and apoptosis. Biochem Cell Biol 82:498-507.

Moore JM, Peattie DA, Fitzgibbon MJ, Thomson JA (1991) Solution structure of the major binding protein for the immunosuppressant FK506. Nature 351:248-250.

Moroianu J (1999) Nuclear import and export pathways. J Cell Biochem Suppl 32-33:76-83.

Motyl T (1999) Regulation of apoptosis: involvement of Bcl-2-related proteins. Reprod Nutr Dev 39:49-59.

Nachmias B, Ashhab Y, Ben-Yehuda D (2004) The inhibitor of apoptosis protein family (IAPs): an emerging therapeutic target in cancer. Semin Cancer Biol 14:231-243.

Ng FW, Nguyen M, Kwan T, Branton PE, Nicholson DW, Cromlish JA, Shore GC (1997) p28 Bap31, a Bcl-2/Bcl-XL- and procaspase-8-associated protein in the endoplasmic reticulum. J Cell Biol 139:327-338.

Northington FJ, Ferriero DM, Graham EM, Traystman RJ, Martin LJ (2001) Early Neurodegeneration after Hypoxia-Ischemia in Neonatal Rat Is Necrosis while Delayed Neuronal Death Is Apoptosis. Neurobiol Dis 8:207-219.

Oberhammer FA, Hochegger K, Froschl G, Tiefenbacher R, Pavelka M (1994) Chromatin condensation during apoptosis is accompanied by degradation of lamin A+B, without enhanced activation of cdc2 kinase. J Cell Biol 126:827-837.

Oppenheim RW (1989) The neurotrophic theory and naturally occurring motoneuron death. Trends Neurosci 12:252-255.

Oppenheim RW, Yin QW, Prevette D, Yan Q (1992) Brain-derived neurotrophic factor rescues developing avian motoneurons from cell death. Nature 360:755-757.

Penfold PL, Provis JM (1986) Cell death in the development of the human retina: phagocytosis of pyknotic and apoptotic bodies by retinal cells. Graefes Arch Clin Exp Ophthalmol 224:549-553.

Petros AM, Medek A, Nettesheim DG, Kim DH, Yoon HS, Swift K, Matayoshi ED, Oltersdorf T, Fesik SW (2001) Solution structure of the antiapoptotic protein bcl-2. Proc Natl Acad Sci U S A 98:3012-3017.

Philchenkov A (2004) Caspases: potential targets for regulating cell death. J Cell Mol Med 8:432-444.

Pinton P, Ferrari D, Magalhaes P, Schulze-Osthoff K, Di Virgilio F, Pozzan T, Rizzuto R (2000) Reduced loading of intracellular Ca(2+) stores and downregulation of capacitative Ca(2+) influx in Bcl-2-overexpressing cells. J Cell Biol 148:857-862.

Portier BP, Taglialatela G (2006) Bcl-2 localized at the nuclear compartment induces apoptosis following transient transfection. J Biol Chem (in press).

Portier BP, Ferrari DC, Taglialatela G (2006) Rapid assay for quantitative measurement of apoptosis in cultured cells and brain tissue. J Neurosci Methods.

Proskuryakov SY, Konoplyannikov AG, Gabai VL (2003) Necrosis: a specific form of programmed cell death? Exp Cell Res 283:1-16.

Rabe CS, Delorme E, Weight FF (1987) Muscarine-stimulated neurotransmitter release from PC12 cells. J Pharmacol Exp Ther 243:534-541.

Reed JC (1997) Double identity for proteins of the Bcl-2 family. Nature 387:773-776.

Reed JC (1999) Dysregulation of apoptosis in cancer. J Clin Oncol 17:2941-2953.

Reimertz C, Kogel D, Rami A, Chittenden T, Prehn JH (2003) Gene expression during ER stress-induced apoptosis in neurons: induction of the BH3-only protein Bbc3/PUMA and activation of the mitochondrial apoptosis pathway. J Cell Biol 162:587-597.

Reipert S RB, Hickman JA, Allen TD. (1996) Nuclear pore clustering is a consistent feature of apoptosis in vitro. Cell Death and Differentiation 3:131-139.

Rodenburg RJ, Raats JM, Pruijn GJ, van Venrooij WJ (2000) Cell death: a trigger of autoimmunity? Bioessays 22:627-636.

Rudin CM, Thompson CB (1997) Apoptosis and disease: regulation and clinical relevance of programmed cell death. Annu Rev Med 48:267-281.

Ryan KJ, Wente SR (2000) The nuclear pore complex: a protein machine bridging the nucleus and cytoplasm. Curr Opin Cell Biol 12:361-371.

Sanderson CJ, Glauert AM (1977) The mechanism of T cell mediated cytotoxicity. V. Morphological studies by electron microscopy. Proc R Soc Lond B Biol Sci 198:315-323.

Sanderson CJ, Hall PJ, Thomas JA (1977) The mechanism of T cell mediated cytotoxicity. IV. Studies on communicating junctions between cells in contact. Proc R Soc Lond B Biol Sci 196:73-84.

Saraste A (1999) Morphologic criteria and detection of apoptosis. Herz 24:189-195.

Sartorius U, Schmitz I, Krammer PH (2001) Molecular mechanisms of death-receptor-mediated apoptosis. Chembiochem 2:20-29.

Sattler M, Liang H, Nettesheim D, Meadows RP, Harlan JE, Eberstadt M, Yoon HS, Shuker SB, Chang BS, Minn AJ, Thompson CB, Fesik SW (1997) Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis. Science 275:983-986.

Sauter B, Albert ML, Francisco L, Larsson M, Somersan S, Bhardwaj N (2000) Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. J Exp Med 191:423-434.

Schendel SL, Reed JC (2000) Measuring pore formation by Bcl-2 family proteins. Methods Enzymol 322:274-282.

Schiene C, Fischer G (2000) Enzymes that catalyse the restructuring of proteins. Curr Opin Struct Biol 10:40-45.

Schlesinger PH, Gross A, Yin XM, Yamamoto K, Saito M, Waksman G, Korsmeyer SJ (1997) Comparison of the ion channel characteristics of proapoptotic BAX and antiapoptotic BCL-2. Proc Natl Acad Sci U S A 94:11357-11362.

Schuessel K, Frey C, Jourdan C, Keil U, Weber CC, Muller-Spahn F, Muller WE, Eckert A (2006) Aging sensitizes toward ROS formation and lipid peroxidation in PS1M146L transgenic mice. Free Radic Biol Med 40:850-862.

Schultz DR, Harrington WJ, Jr. (2003) Apoptosis: programmed cell death at a molecular level. Semin Arthritis Rheum 32:345-369.

Schulze-Osthoff K, Ferrari D, Los M, Wesselborg S, Peter ME (1998) Apoptosis signaling by death receptors. Eur J Biochem 254:439-459.

Schwarzacher HG, Wachtler F (1993) The nucleolus. Anat Embryol (Berl) 188:515-536.

Shafer TJ, Atchison WD (1991) Transmitter, ion channel and receptor properties of pheochromocytoma (PC12) cells: a model for neurotoxicological studies. Neurotoxicology 12:473-492.

Sheikh MS, Huang Y (2004) TRAIL death receptors, Bcl-2 protein family, and endoplasmic reticulum calcium pool. Vitam Horm 67:169-188.

Shi YF, Sahai BM, Green DR (1989) Cyclosporin A inhibits activation-induced cell death in T-cell hybridomas and thymocytes. Nature 339:625-626.

Shibasaki F, Kondo E, Akagi T, McKeon F (1997) Suppression of signalling through transcription factor NF-AT by interactions between calcineurin and Bcl-2. Nature 386:728-731.

Shiozaki EN, Shi Y (2004) Caspases, IAPs and Smac/DIABLO: mechanisms from structural biology. Trends Biochem Sci 29:486-494.

Shirane M, Nakayama KI (2003) Inherent calcineurin inhibitor FKBP38 targets Bcl-2 to mitochondria and inhibits apoptosis. Nat Cell Biol 5:28-37.

Siekierka JJ, Staruch MJ, Hung SH, Sigal NH (1989a) FK-506, a potent novel immunosuppressive agent, binds to a cytosolic protein which is distinct from the cyclosporin A-binding protein, cyclophilin. J Immunol 143:1580-1583.

Siekierka JJ, Hung SH, Poe M, Lin CS, Sigal NH (1989b) A cytosolic binding protein for the immunosuppressant FK506 has peptidyl-prolyl isomerase activity but is distinct from cyclophilin. Nature 341:755-757.

Srinivasula SM, Ahmad M, Fernandes-Alnemri T, Alnemri ES (1998) Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. Mol Cell 1:949-957.

Stahelin BJ, Marti U, Solioz M, Zimmermann H, Reichen J (1998) False positive staining in the TUNEL assay to detect apoptosis in liver and intestine is caused by endogenous nucleases and inhibited by diethyl pyrocarbonate. Mol Pathol 51:204-208.

Standaert RF, Galat A, Verdine GL, Schreiber SL (1990) Molecular cloning and overexpression of the human FK506-binding protein FKBP. Nature 346:671-674.

Stegh AH, Schickling O, Ehret A, Scaffidi C, Peterhansel C, Hofmann TG, Grummt I, Krammer PH, Peter ME (1998) DEDD, a novel death effector domain-containing protein, targeted to the nucleolus. EMBO J 17:5974-5986.

Stergiou L, Hengartner MO (2004) Death and more: DNA damage response pathways in the nematode C. elegans. Cell Death Differ 11:21-28.

Stoffler D, Fahrenkrog B, Aebi U (1999) The nuclear pore complex: from molecular architecture to functional dynamics. Curr Opin Cell Biol 11:391-401.

Strasser A, O'Connor L, Dixit VM (2000) Apoptosis signaling. Annu Rev Biochem 69:217-245.

Subramanian T, Chinnadurai G (2003) Pro-apoptotic activity of transiently expressed BCL-2 occurs independent of BAX and BAK. J Cell Biochem 89:1102-1114.

Susin SA, Lorenzo HK, Zamzami N, Marzo I, Brenner C, Larochette N, Prevost MC, Alzari PM, Kroemer G (1999) Mitochondrial release of caspase-2 and -9 during the apoptotic process. J Exp Med 189:381-394.

Taglialatela G, Robinson R, Perez-Polo JR (1997) Inhibition of nuclear factor kappa B (NFkappaB) activity induces nerve growth factor-resistant apoptosis in PC12 cells. J Neurosci Res 47:155-162.

Taglialatela G, Kaufmann JA, Trevino A, Perez-Polo JR (1998) Central nervous system DNA fragmentation induced by the inhibition of nuclear factor kappa B. Neuroreport 9:489-493.

Thome M, Schneider P, Hofmann K, Fickenscher H, Meinl E, Neipel F, Mattmann C, Burns K, Bodmer JL, Schroter M, Scaffidi C, Krammer PH, Peter ME, Tschopp J (1997) Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. Nature 386:517-521.

Thomenius MJ, Distelhorst CW (2003) Bcl-2 on the endoplasmic reticulum: protecting the mitochondria from a distance. J Cell Sci 116:4493-4499.

Thomenius MJ, Wang NS, Reineks EZ, Wang Z, Distelhorst CW (2003) Bcl-2 on the endoplasmic reticulum regulates Bax activity by binding to BH3-only proteins. J Biol Chem 278:6243-6250.

Tsujimoto Y, Gorham J, Cossman J, Jaffe E, Croce CM (1985) The t(14;18) chromosome translocations involved in B-cell neoplasms result from mistakes in VDJ joining. Science 229:1390-1393.

Uhlmann EJ, Subramanian T, Vater CA, Lutz R, Chinnadurai G (1998) A potent cell death activity associated with transient high level expression of BCL-2. J Biol Chem 273:17926-17932.

Van Duyne GD, Standaert RF, Karplus PA, Schreiber SL, Clardy J (1991) Atomic structure of FKBP-FK506, an immunophilin-immunosuppressant complex. Science 252:839-842.

Vance JE (2003) Molecular and cell biology of phosphatidylserine and phosphatidylethanolamine metabolism. Prog Nucleic Acid Res Mol Biol 75:69-111.

Vance JE, Vance DE (2004) Phospholipid biosynthesis in mammalian cells. Biochem Cell Biol 82:113-128.

Vanden Abeele F, Skryma R, Shuba Y, Van Coppenolle F, Slomianny C, Roudbaraki M, Mauroy B, Wuytack F, Prevarskaya N (2002) Bcl-2-dependent modulation of Ca(2+) homeostasis and store-operated channels in prostate cancer cells. Cancer Cell 1:169-179.

Vander Heiden MG, Chandel NS, Li XX, Schumacker PT, Colombini M, Thompson CB (2000) Outer mitochondrial membrane permeability can regulate coupled respiration and cell survival. Proc Natl Acad Sci U S A 97:4666-4671.

Vanderbilt JN, Bloom KS, Anderson JN (1982) Endogenous nuclease. Properties and effects on transcribed genes in chromatin. J Biol Chem 257:13009-13017.

Virchow R (1871) Die Cellularpathologie in ihrer Begründung auf physiologische

Gewebelehre. Auflage Hirschwald Berlin 4.

Voeltz GK, Rolls MM, Rapoport TA (2002) Structural organization of the endoplasmic reticulum. EMBO Rep 3:944-950.

Vogt C (1842) Untersuchungen bei der Entwicklungsgeschichte der Geburtshelferkroete (Alytes obstericans). Jent and Gassmann Solothurn.

Wang HG, Rapp UR, Reed JC (1996) Bcl-2 targets the protein kinase Raf-1 to mitochondria. Cell 87:629-638.

Wang NS, Unkila MT, Reineks EZ, Distelhorst CW (2001) Transient expression of wild-type or mitochondrially targeted Bcl-2 induces apoptosis, whereas transient expression of endoplasmic reticulum-targeted Bcl-2 is protective against Bax-induced cell death. J Biol Chem 276:44117-44128.

Wang ZB, Liu YQ, Cui YF (2005) Pathways to caspase activation. Cell Biol Int 29:489-496.

Wei YH, Ma YS, Lee HC, Lee CF, Lu CY (2001) Mitochondrial theory of aging matures--roles of mtDNA mutation and oxidative stress in human aging. Zhonghua Yi Xue Za Zhi (Taipei) 64:259-270.

Wiederrecht G, Martin MM, Sigal NH, Siekierka JJ (1992) Isolation of a human cDNA encoding a 25 kDa FK-506 and rapamycin binding protein. Biochem Biophys Res Commun 185:298-303.

Wilson-Annan J, O'Reilly LA, Crawford SA, Hausmann G, Beaumont JG, Parma LP, Chen L, Lackmann M, Lithgow T, Hinds MG, Day CL, Adams JM, Huang DC (2003) Proapoptotic BH3-only proteins trigger membrane integration of prosurvival Bcl-w and neutralize its activity. J Cell Biol 162:877-887.

Wyllie AH (1987) Apoptosis: cell death in tissue regulation. J Pathol 153:313-316.

Wyllie AH, Morris RG, Smith AL, Dunlop D (1984) Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. J Pathol 142:67-77.

Yamada T, Ohyama H, Kinjo Y, Watanabe M (1981) Evidence for the internucleosomal breakage of chromatin in rat thymocytes irradiated in vitro. Radiat Res 85:544-553.

Yamaguchi H, Wang HG (2001) The protein kinase PKB/Akt regulates cell survival and apoptosis by inhibiting Bax conformational change. Oncogene 20:7779-7786.

Yasuhara S, Zhu Y, Matsui T, Tipirneni N, Yasuhara Y, Kaneki M, Rosenzweig A, Martyn JA (2003) Comparison of comet assay, electron microscopy, and flow cytometry for detection of apoptosis. J Histochem Cytochem 51:873-885.

Yin XM, Oltvai ZN, Korsmeyer SJ (1994) BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. Nature 369:321-323.

Yuan J, Yankner BA (2000) Apoptosis in the nervous system. Nature 407:802-809.

Zamzami N, Marchetti P, Castedo M, Decaudin D, Macho A, Hirsch T, Susin SA, Petit PX, Mignotte B, Kroemer G (1995) Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. J Exp Med 182:367-377.

VITA

Bryce Patrick Portier was born on September 22nd, 1976 in Houston, Texas. He is the son of Glen Patrick Portier and Sheri Steffek. He obtained a Bachelor of Science degree in Genetics from Texas A&M University in 1999. From 1997 to 2000, he worked as a research assistant in the Horticultural Genetic Program at Texas A&M University. In August of 2000, he enrolled in the MD/PhD program at the University of Texas Medical Branch and in 2002 he began his doctoral research under the supervision of Dr. Giulio Taglialatela. Bryce is the recipient of the Blocker Scholar memorial award and a recipient of a Ruth L. Kirschstein F30 predoctoral fellowship from the National Institute for Neurological Disease and Stroke (NINDS).

EDUCATION

B.S. Genetics, December 1999, Texas A&M University, College Station, Texas

PUBLICATIONS

Portier BP and Taglialatela G (2007) Pro-apoptotic role of Bcl-2; function dictated by localization: Mini-review. (*In Prep)*

Portier BP and Taglialatela G (2007) Pitfalls of Mifepristone induced gene expression in PC12 cells. (*In Prep)*

Portier BP, Zhang, WR, and Taglialatela G (2007) Delivery of Bcl-2's BH4 domain by protein transduction induces cell death selectively in cells expressing Bcl-2. (*In Prep)*

Portier BP and Taglialatela G (**2006**) Bcl-2 localized at the nuclear compartment induces apoptosis following transient overexpression PC12 cells. J Biol Chem. (*In Press)*

Portier BP, Ferrari DM, Taglialatela G (**2006**) Rapid assay for quantitative measurement of apoptosis in cultured cells and brain tissue. J Neurosci Methods. 15;155(1):134-42.

Massaad CA, **Portier BP**, Taglialatela G (**2004**) Inhibition of transcription factor activity by nuclear compartment-associated Bcl-2. J Biol Chem. 24;279(52):54470-8.

Smith JS, Tachibana I, Pohl U, Lee HK, Thanarajasingam U, **Portier BP**, Ueki K, Ramaswamy S, Billings SJ, Mohrenweiser HW, Louis DN, Jenkins RB. (**2000**) A transcript map of the chromosome 19q-arm glioma tumor suppressor region. Genomics. 15;64(1):44-50.

Peters N, Smith JS, Tachibana I, Lee HK, Pohl U, **Portier BP**, Louis DN, Jenkins RB (**1999**) The human glia maturation factor-gamma gene: genomic structure and mutation analysis in gliomas with chromosome 19q loss. Neurogenetics. 2(3):163-6.