

BAD SERINE 170 – REGULATION AND CELLULAR EFFECTS

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ABSTRACT

The balance between cell proliferation and cell death is imperative for homeostasis in multicellular organisms. This homeostasis had long been thought to be the result of two separate processes, but there is recent evidence indicating that the processes of proliferation and apoptosis are coupled. Here we demonstrate that Bad, a proapoptotic member of the Bcl-2 family of proteins that is thought to exert a death-promoting effect by heterodimerization with Bcl-xL, is able to interact directly with the cell cycle machinery. Immunoprecipitation experiments indicate that Bad interacts with both Cdk2 and its late G1 cyclin partner, Cyclin E. This finding is relevant as we also demonstrate that prior to the conversion of Bad into a death factor; Bad's phosphorylation state in healthy mammalian cells, specifically at Ser-170, is able to influence cell cycle progression. Here we show that transfection with Bad S170A, a mutant form of Bad which mimics the unphosphorylated form, results in a prolonged S phase during cell cycle. We also show that the kinase activity towards Bad Ser-170 increases in S phase of the cell cycle. Together this suggests that Bad Ser-170 is a phosphorylation site which is targeted during S phase and is able to interfere with the normal progression through S phase of the cell cycle. From this, it was of interest to elucidate the kinase responsible for phosphorylating Bad at Ser-170 as it may provide insight into signal transduction pathways that converge in terms of controlling both survival and cell proliferation, and ultimately cell expansion. Through a process of column purification, usage of chemical inhibitors, and gene knockdown, we show evidence that CaMKII- γ mediates the phosphorylation of Bad Ser 170, thus establishing a novel connection between CaMKII signaling and apoptosis in hematopoietic cells. We hypothesize that CaMKII- γ plays a major role in controlling Bad's ability to induce apoptosis and to affect cell cycle progression, by controlling Bad's phosphorylation state at Ser-170.

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LIST OF ABBREVIATION

α	anti
-/-	knockout
Ab	antibody
ADP	adenosine diphosphate
AIF	apoptosis inducing factor
APAF-1	apoptosis protease activating factor 1
Ask-1	apoptosis signal regulating kinase-1
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
ATR	ataxia telangiectasia related
Bad	Bcl-2 antagonist of cell death protein
Bcl-2	B cell lymphoma/ leukemia-2
BH	Bcl-2 homology
Bim	Bcl-2-interacting mediator of cell death
Bok	Bcl-2 associated ovarian killer
BrdU	bromodeoxyuridine
Caspase	cysteine aspartate specific protease
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
CAD	caspase activated DNAase
CARD	caspase recruitment domain
Cdk	cyclin dependent kinase
CED	cell death protein (<i>C. elegans</i>)
Chk1/ Chk2	checkpoint kinase I/ checkpoint kinase II
C-terminal	carboxy terminal
Da	dalton
DD	death domain
DED	death effector domain
DIABLO	direct IAP binding protein with Low pH
DISC	death-inducing signalling complex

DLC	dynein light chain
DNA	deoxyribonucleic acid
E. Coli	<i>Escherichia coli</i>
eIF	eukaryotic Initiation Factor
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FACS	fluorescence activated cell sorter
FADD	fas associated death domain
FasL	Fas ligand
GFP	green fluorescent protein
GMCSF	granulocyte macrophage colony stimulating factors
GSK-3	glycogen synthase kinase-3
h	hour
IAPs	inhibitor of apoptosis proteins
ICAD	inhibitor of caspase activated DNAase
IL3	interleukine-3
IP	immunoprecipitation
MAC	mitochondrial apoptosis-inducing channel
MAPK-AP	mitogen-activated protein kinase-activated protein
Mcl-1S	short Mcl-1
MEK	map kinase or erk kinase
MEKK1	MEK kinase 1
Mito	mitochondria
MMP	mitochondrial membrane permeabilization
MOI	multiplicity of infection
MOM	mitochondrial outer membrane
MPT	mitochondrial permeability transition
Ms	mouse
mTOR	mammalian target of Rapamycin
NF-AT	nuclear factor of activated T-cells

NK cells	natural killer cells
N-terminus	amino-terminus
P	phospho
P.I.	propidium iodide
PARP	poly ADP ribose polymerase
PBS	phosphate buffer saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDK-1	phosphoinositide dependent kinase -1
PDK-2	phosphoinositide dependent kinase -2
PEST	proline, glutamic acid, serine, threonin
PH	pleckstrin homology
PI	phosphatidylinositol
PI-3 kinase	phosphatidylinositol 3-kinase
PIKKs	PI-3 kinase related kinases
PIP	phosphatidylinositol phosphate
PKB	protein kinase B
PKC	protein kinase C
PSR	phosphatidylserine receptor
PtdIns	phosphoinositide
PtdIns _{3,4,5} P3	phosphatidylinositol 3,4,5-triphosphate
PtdIns _{4,5} P2	phosphatidylinositol 4,5-bisphosphate
PTEN	phosphatase and tensin homolog detected on chromosome ten
PTP	permeability transition pore
PUMA	p53 up-regulated mediator of apoptosis
Rb	rabbit
rpm	revolution per minute
S/ T	serine/ threonine
SDS/PAGE	sodium dodecyl sulphate/ polyacrylamide gel electrophoresis
Ser	serine

sFasL	soluble fas ligand
SH2	src homology-2
SiRNA	small interfering RNAs
SMAC	second mitochondria-derived activator of caspase
STAT	signal transducer and activator of transcription
t(14:18)	translocation (14:18)
tBid	truncated Bid
TCLs	total cell lysates
Thr	threonine
TNF	tumor-necrosis factor
TNF-R	tumor necrosis factor receptor
UV	ultra violet
VDAC	voltage dependent anion channel
WB	western blot

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Chapter 1

INTRODUCTION

1.1 APOPTOSIS

1.1.1 Apoptosis and the Bcl-2 Family

By the 1960's, several laboratories demonstrated that cell death was biologically controlled (programmed), requiring the cell's own proteins (1) and exhibited common morphological changes, including plasma membrane blebbing, cytoplasmic reorganization, chromatin condensation and DNA fragmentation (2). These morphological changes and their underlying molecular mechanism are now commonly referred to as apoptosis, a term coined in 1972 by Kerr, Wyllie, and Currie (3).

An early breakthrough in the study of apoptosis came with the observation that cell death is usually accompanied by rapid activation of endonucleases (4) and subsequently, electrophoresis of cleaved DNA "fragments" (5) were specifically associated with apoptosis (6), though only until recently was the endonuclease responsible (DFF/CAD) identified (7, 8). The observation that phosphatidylserine, normally found on the inner side of the cell's membrane, is exposed on the outer side of the cell membrane on dying cells (9) provided not only another apoptosis marker, but also led to our current understanding of how dead cells are recognized prior to their engulfment.

The first evidence of a genetic program dedicated to physiological cell death came from developmental studies in *C. elegans* (10, 11) and the generation of the first 'ced' (cell death abnormal) mutants in 1983 (12). Nobel-prize winning studies of Horvitz and colleagues (11) had determined that the demise of the 131 somatic cells fated to die during worm development required two genes, *CED-3* and *CED-4*, whereas another, *CED-9*, ensured the survival of all others (13, 14). However, the 'modern' era of cell death research and the explosion of interest in the field resulted from the identification of the first component of the mammalian cell death machinery, the gene *Bcl2* (15), and the convergence of two disparate fields brought about by studies showing that Bcl-2 was able to prevent programmed cell death in *C. elegans* (16) and that CED-9 was Bcl-2's

structural and functional counterpart (17). These landmark discoveries demonstrated that the programmed cell death observed in mammalian cells and in the nematode was the same highly conserved process (16).

Though Bcl-2 was the first component of the apoptotic system to be recognized, it had been originally cloned not because it was an apoptotic gene, but because it was found to be translocated in follicular lymphoma (B-cell CLL/lymphoma, (18)). Investigators first thought that Bcl-2 may be like other oncogenes involved in translocations, such as Abl and c-Myc which promote cell proliferation; however Bcl-2 was not shown to stimulate cell division, but rather prevented cell death when growth factor was removed (15). Several studies ensued providing early evidence that one function of p53 (19), the gene most commonly mutated in human cancers (20), is to cause apoptosis which can be blocked by Bcl-2 (21). Early experiments using transgenic mice over-expressing Bcl-2 also provided the first evidence connecting inhibition of cell death with autoimmune disease (22). These studies, among many others, gave birth to the concept, now widely embraced (23-26), that impaired apoptosis is a crucial step in tumorigenesis and dysregulated immune responses.

1.1.2 Bcl-2 Family Members

Over the past decade it has become evident that Bcl-2 belongs to an extended family of at least twenty five Bcl-2-related proteins in mammalian cells (27-30). The Bcl-2 family of proteins function to control the “life/death switch” by integrating diverse inter and intracellular cues to determine whether or not the signal should reach the mitochondrion. Members of the Bcl-2 family are characterized by the presence of distinct conserved sequence motifs known as Bcl-2 homology (BH) domains designated BH1, BH2, BH3 and BH4 (Fig. 1.1) . The Bcl-2 family can be divided into two broad classes: those that inhibit apoptosis and those that promote apoptosis.

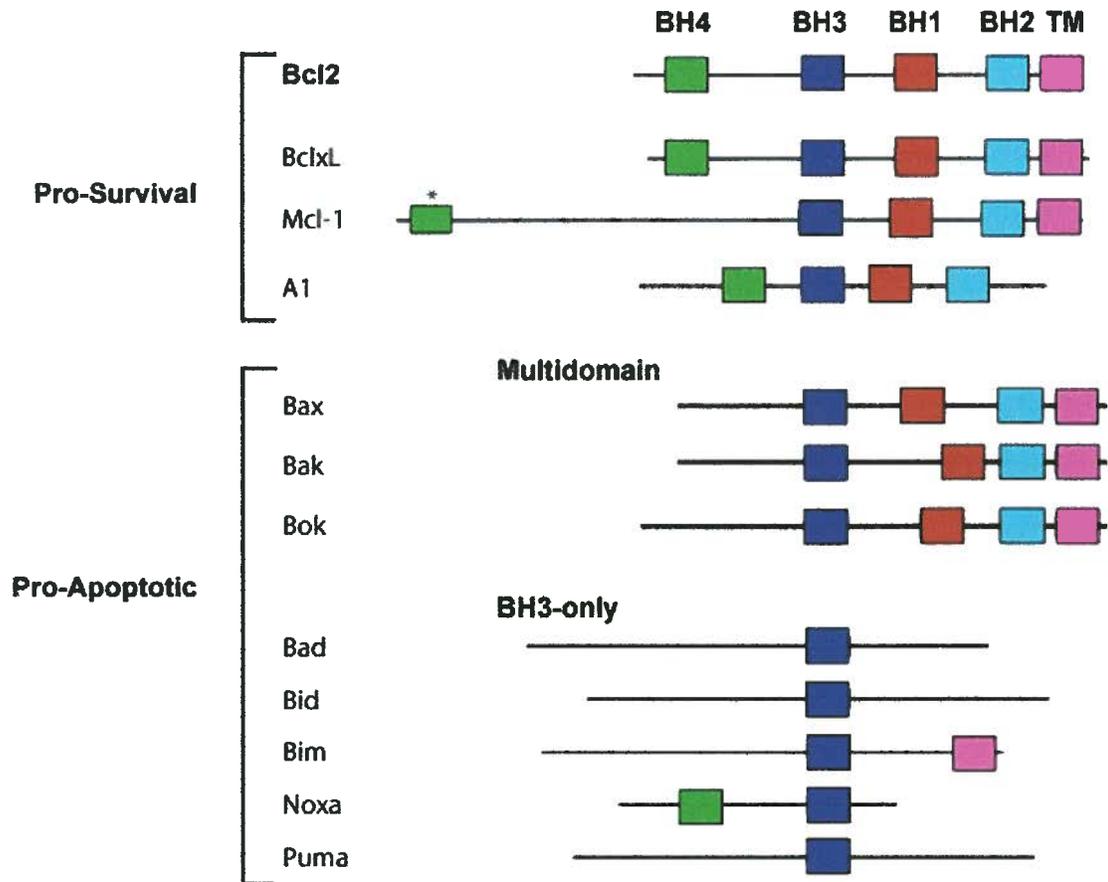


Figure. 1.1 Pro-Survival and Pro-Apoptotic Bcl-2 family members. Bcl-2 family members share regions of homology termed BH domains (BH1, BH2, BH3, and BH4). Several family members also contain a domain (TM) that mediates insertion into the outer membrane of the mitochondrion and/or endoplasmic reticulum. Pro-Survival family members contain BH1-4 domains. Pro-Apoptotic family members are subdivided into multi BH domain (Multidomain) or BH3-only domain proteins. Adapted from (31).

* The existence of a BH4 domain within Mcl-1 still remains controversial.

The pro-survival class has been divided into two subclasses based on the presence of one or more BH domains; Bcl-2 and its closest relatives, Bcl-xL and Bcl-w, and the more divergent group consisting of Al and Mcl-1.

The pro-apoptotic class, mainly identified as Bcl-2-binding proteins, promote rather than antagonize apoptosis and fall into two distinct groups: Bax subclass and BH3-only subclass. The Bax subclass members (Bax, Bak, Bok) have sequences that are similar to those in Bcl-2, especially in the BH1, BH2 and BH3 regions. The BH3-only subclass, exemplified by Bik, Bim and Bad, are largely unrelated in sequence to either Bcl-2 or each other (32, 33), apart from the short BH3 motif (hence their name). These disparate 'BH3-only proteins' cannot induce apoptosis in the absence of Bax and Bak (34, 35) and appear to function upstream, sensing death signals and intracellular damage; whereas the Bax-like proteins act further downstream, probably in mitochondrial disruption (see below).

1.1.3 Pro-Survival Bcl-2 Subfamily

The abundance and type of Bcl-2 pro-survival members is thought to dictate whether the cell will live when stimulated by physiological, pathogenic or cytotoxic stimuli. Bcl-2 and its closest homologues, Bcl-xL and Bcl-w, possess four BH regions and a hydrophobic carboxy-terminal domain. The C-terminal hydrophobic sequence targets or anchors these proteins to the cytoplasmic face of intracellular membranes including: the outer mitochondrial membrane, the endoplasmic reticulum (ER) and the nuclear envelope. The crucial interactions between pro-survival and pro-death Bcl-2 family members appears to occur on these membranes and most members either normally reside on these surfaces, or rapidly assemble there after an apoptotic signal (36). The three-dimensional structure of Bcl-xL (37), Bcl-2 (38) and Bcl-w (37) are remarkably similar and comprise a globular bundle of five amphipathic α -helices that surround two central hydrophobic α -helices. The resulting hydrophobic groove, formed by residues from BH1-3, can bind the BH3 (~24 residue α -helix) of an interacting BH3-only relative

(39); and this interaction interferes with the pro-survival protein's ability to prevent Bax or Bak from perturbing the integrity of intracellular membranes.

1.1.4 Apoptotic Bcl-2 subfamily

It is widely agreed upon that BAX and BAK are critical components of the cellular apoptotic machinery (40-47). In response to cytotoxic signals, Bax translocates from its cytoplasmic location to intracellular membranes, and both Bax and Bak change conformation and form membrane-associated homo-oligomers. The three-dimensional structure of monomeric Bax (48) closely resembles that of its pro-survival relatives. Bax has a BH1/2/3 hydrophobic groove which is occluded by its hydrophobic carboxy-terminal helix. In response to stress signals, Bax changes conformation and flips out its hydrophobic tail. Presumably the changes result in exposure of domains normally masked in the inactivated proteins (48), targeting the protein to the mitochondrial membrane (49) and enabling homo-oligomerization, which is the active form of the proteins. Unlike Bax, Bak is an integral mitochondrial membrane protein, though it too changes conformation during apoptosis (45, 47, 50-52). As the Bak BH3 domain is critical for Bak-mediated apoptosis (53), it has been suggested that this motif not only allows restraint of Bak by its pro-survival counterparts but also for its homo-oligomerization, perhaps by allowing a BH3-exposed conformer ('primed' Bak) to dimerize with an 'unprimed' receptor-like Bak conformer (53). Knockout studies suggest that both Bax and Bak are functionally similar as the loss of either gene has little effect in most cells and tissues. The absence of both proteins, however, blocks apoptosis in many cell types (47, 54) and impairs developmentally programmed attrition in several tissues, and results in perinatal death (55).

Bax and Bak oligomers are considered pro-apoptotic since they are thought to provoke or contribute to the permeabilization of the outer mitochondrial membrane, resulting in the release of apoptogenic proteins (28). The mechanism, however, remains controversial (56-58). One model, which is based on the structural resemblance of Bcl-2 family members and diphtheria toxin (37), is that Bax and Bak form channels. Consistent

with this hypothesis is the fact that Bax oligomers can form pores in liposomes (59) that allow passage of cytochrome c (60, 61), and it has also been shown that mitochondria from apoptotic cells contain a novel channel (62). Alternatively, Bax and Bak have been shown to influence the shape of the mitochondria possibly by affecting the fission/fusion apparatus (63) and it has also been suggested that Bax and Bak interact with the existing permeability transition pore to possibly create a larger channel (57, 58); however, several studies have found no evidence for such interactions (50, 56, 64).

1.1.5 BH3-only Subclass

BH3-only proteins, which include Bim, Bad, Bid, Bik, Bmf, Puma, Noxa and Hrk, act as sensors for distinct apoptotic pathways and are able to trigger apoptosis in response to developmental cues or intracellular damage (32). The primary structure of BH3-only proteins share no domains homologous with each other and with other members of the Bcl-2 family apart from the BH3 domain. Many studies have demonstrated that the association with pro-survival proteins is crucial for their pro-apoptotic effects and these interactions require a BH3 domain (65-73); however, how BH3-only proteins trigger apoptosis is still controversial. Two models, *direct* and *indirect*, have been put forward that attempt to account for the various experimental results. Both models conclude that some BH3-only proteins are more effective killers than others but they base this on different observations and reasoning. Another layer of complexity has been added by distinguishing proteins or their states as being cytoplasmic or membrane bound. Most notably, Leber et al., have proposed the *Embedding Together* model which proposes that both pro- and anti-apoptotic Bcl-2 family proteins undergo similar protein-protein interactions that are governed by membrane dependent conformational changes. These conformational changes are thought to culminate into either an aborted or executed permeabilization of the membrane depending on the final oligomeric state of pro-apoptotic Bax and/or Bak (36).

1.1.6 Direct Activation Model

The *direct activation* model suggests that certain BH3-only proteins, termed ‘activators’ namely Bim and the truncated form of Bid (tBid), can bind to Bax and Bak directly and promote their activation (42, 45, 74-77). Further studies have shown that if Bak (or Bax) is present, Bid is able to very rapidly trigger cytochrome c release and apoptosis (47, 78). Moreover, it has been suggested that tBid might act by inducing Bax and Bak to oligomerize and nucleate channel formation in the mitochondrial membrane (79). This hypothesis stems from Bid’s resemblance to the pore-forming subunit of some bacterial toxins (80). Bim and tBid can be bound and neutralized by Bcl-2 pro-survival proteins and the role of other BH3-only proteins, termed “sensitizers” in this model, is to bind to these pro-survival proteins and stop them from sequestering Bim/tBid. The “sensitizers” (eg. Bad, Bik, Noxa, Bmf) (42) exert their proapoptotic functions indirectly by competing for the BH3 domain-binding cleft in anti-apoptotic proteins, displacing or preventing the binding of activators (42, 45, 75, 81). Thus in this model “sensitizers” are considered inhibitors of the inhibitors of apoptosis and are different from direct “activators” (eg. Bid and Bim) (Figure 1.2).

Direct Activation Model		Indirect Activation Model	
Sensitizer	Activator	Selective	Promiscuous
Bad	Bim tBid	Bad	Bim tBid
Bcl-2		Bcl-2	Mcl-1
BclxL		BclxL	A1
Mcl-1			
A1	Bax Bak		Bax Bak

Figure 1.2 Direct and Indirect Activation Models. Adapted from (82).

The *direct activation* model was devised based on experiments in a cell-free system using synthetic BH3 peptides derived from representative BH3-only molecules. These BH3 peptides were applied to isolated mitochondria, and their ability to induce mitochondrial outer membrane permeabilization (MOMP) were investigated (45, 75). It was found that the BH3-peptides derived from Bim and Bid could induce cytochrome *c* release in the presence of Bax (45); while BH3 peptides, including those of Bad, Bik and Noxa, cannot directly activate Bax and Bak nor induce MOMP by themselves (45). Thus, although direct binding of the proteins were not observed in these particular experiments, the detectable collaboration implies direct activation, presumably by binding. However ensuing studies have indicated that both Bid and Puma BH3-peptides are able to bind to the N-terminus of Bax (41, 83); these results, however, should be interpreted carefully as it is uncertain whether whole BH3-only proteins could reproduce this binding. Furthermore, systems used in many of these experiments are in some points removed from the physiological conditions and the induction of mitochondrial outer membrane permeabilization (MOMP) on isolated mitochondria by Bid BH3 peptides, for example, requires a 1,000-fold higher concentration of BH3 molecule than that required in the induction of MOMP by the full-length activated Bid protein (45). It is also difficult to reconcile the direct binding model with recent data using gene-deficient mice. Activated T-cells die rapidly in culture; however this cell death is reduced in Bim-deficient T cells, slightly reduced in Puma-deficient T cells and almost completely prevented in Bim/Puma double-deficient T cells (84). These findings demonstrate that Bid is not sufficient for T-cell apoptosis (otherwise there would still be cell death in Bim/Puma-deficient T cells). It further shows that Puma is able to induce apoptosis similarly to Bim, as the difference in apoptosis between Bim-deficient and Bim/Puma-deficient T cells is clearly due to Puma (77). Therefore, the model needs to be modified to the extent that Puma can act like a direct activator, although the experiments with BH3-peptides did not suggest that (41, 83).

1.1.7 Indirect Activation Model

The *indirect activation* model (also termed the displacement model) on the other hand suggests that all the BH3-only proteins, through their BH3 domain (85), engage only their pro-survival relatives and that the pro-survival proteins function mainly by inhibiting Bax/Bak activation (53, 77, 86). In this model Bim, tBid and PUMA, for example, are considered to be strong apoptotic inducers because they can engage all the pro-survival proteins (41, 42, 53, 77, 87, 88) while other BH3-only proteins exhibit marked selectivity and as result show weaker apoptotic properties. This model is based on the observation that BH3-peptides derived from the various BH3-only proteins have vastly different affinities for Bcl-2-like proteins (42, 75, 82). Studies have revealed, for instance, Bad and Bmf bind only Bcl-2, Bcl-xL and Bcl-w, whereas Noxa binds only Mcl1 and A1 (82). Experiments stemming from these findings have suggested that the complementary binding profiles of Bad and Noxa represent an apoptosis requirement. More precisely, both classes of Bcl-2 pro-survival proteins, one comprising Bcl-2, Bcl-xL and Bcl-w and the other Mcl-1 and A-1, are required to be neutralized in order to induce a strong apoptotic effect (Figure 1.3). This explains in part how Bad and Noxa, known to be weak killers, potentially induce apoptosis when combined (82).

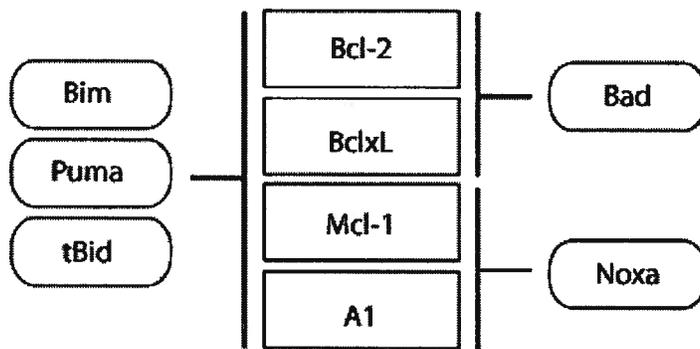


Figure 1.3 Pro-apoptotic Bcl-2 family proteins neutralize specific pro-survival proteins. Adapted from (82)

This model suggests that induction of apoptosis requires the activation of a combination of BH3-only proteins that cover and inactivate all Bcl-2 pro-survival proteins in the cell. Therefore, based on a system where all Bcl-2 pro-survival proteins are expressed, activation of Bim or Puma would be sufficient to induce apoptosis whereas Bad on its own would require an additional stimulus that could be provided by Noxa. This model also suggests that Mcl-1 is probably unable to block Bad (as it does not bind with high affinity), and Bcl-2 cannot inhibit Noxa or Bid efficiently. Functional studies supporting this model have been reported recently (53): first, Bak was found by co-immunoprecipitation experiments to be bound to Bcl-xL and Mcl-1 but not other Bcl-2-like proteins and, secondly, neutralization of both Bcl-xL and Mcl-1 was required to activate Bak and to induce apoptosis (77). Worth noting is that these experiments used cells expressing rather high levels of BH3-only proteins, and it is still unclear why neutralization of either Mcl-1 or Bcl-xL on its own does not release sufficient Bak to homo-dimerize and induce apoptosis. Additionally, co-expression of Noxa and Bad is able to induce apoptosis in cells lacking both Bim and tBid (77); which at the same time challenges the *direct activation* model as tBid and Bim are described as direct activators of Bax and Bak and are required for the induction of apoptosis. Further support for the *indirect activation* model comes from studies (86, 89, 90) using BH3 mimetic drug, ABT-737 (91). Despite its high affinity for Bcl-2, Bcl-x(L), and Bcl-w, many cell types proved refractory to ABT-737 and this resistance is due to ABT-737's inability to target pro-survival relative, Mcl-1 (92). Downregulation of Mcl-1 resulted in an increase in apoptosis when treated with ABT-737, where on the other hand, increased Mcl-1 expression conferred resistance (93). Interestingly cells overexpressing Bcl-2 remained highly sensitive to ABT-737 (92). Hence, ABT-737 may prove efficacious in tumors with low Mcl-1 levels, or when combined with agents that inactivate Mcl-1.

The *indirect activation* model isn't without its caveats. For instance, this particular model has difficulty explaining how cells tolerate relatively high levels of Bax and/or Bak expression without these proteins being constitutively bound to anti-apoptotic members (34, 94-97), as several studies have indicated the isolation of endogenous Bax and/or Bak does not generally stoichiometrically co-purify with anti-apoptotic proteins

(94). This suggests that further changes in the effector molecules are required for their interaction or permeabilization capability. Moreover, findings presented in this document, combined with other published reports, show that Bcl-2 family proteins bind to non-Bcl-2 family members. Some of these interactions have also been shown to be disrupted by only a specific BH3-only protein. For example, Puma has been shown to disrupt cytosolic p53 and Bcl-xL complex (98).

1.1.8 Embedding Together Model

Several lines of evidence suggest that the interaction between BH3-only proteins and anti-apoptotic members is dependent on a lipid environment. These observations have led to an emerging model termed *Embedding Together* (36) that combines attributes of both the indirect- and direct- activation models, in addition to providing a cellular context by also taking into account how the presence or absence of lipid membranes affect the binding of Bcl-2 members. Most notably, this model proposes that tBid changes conformation when bound to membranes and as result increases its affinity for Bax. The interaction between membrane bound tBid with peripherally-membrane-bound Bax triggers insertion of Bax into the membrane. Once integrated into the membrane, Bax can recruit additional Bax proteins and oligomerize to permeabilize the outer mitochondrial membrane. The model does not require that any one pro-apoptotic protein performs all of the functions ascribed to tBid, but rather suggests that most Bcl-2 family members will perform only a subset of them. Bak is proposed to have a similar function to Bax except that its insertion in the membrane will be controlled by different modulators (e.g. tBid, Bim) with different affinities. Since Bak's inactive conformation is constitutively membrane bound it is suggested that it will have a similar functional role as compared to the peripheral membrane form of Bax. Bcl-xL pro-survival role is thought to arise from its ability to inhibit both tBid and Bax. Although it may bind to both the membrane bound and cytoplasmic forms of tBid, the affinity of Bcl-xL for membrane bound tBid is higher. Binding of Bcl-xL to tBid triggers insertion of Bcl-xL into membranes and once inserted recruits other Bcl-xL proteins to insert into membranes

and/or tBid dissociates. Bcl-xL/tBid complexes are thought to be neutral complexes that neither prevent nor promote apoptosis. However, because the affinity of Bcl-xL for tBid is higher than that between Bax and tBid, interactions between tBid and Bcl-xL inhibits Bax recruitment to the membrane. Membrane bound Bcl-xL further prevents Bax inserting into the membrane by preventing the conformation change in Bax that occurs at the membrane. This model suggests that other pro-survival Bcl-2 family members function in a similar fashion by binding different pro-apoptotic proteins (e.g. tBid/Bax) with different affinities.

1.2 BCL-2 FAMILY REGULATION

1.2.1 Regulatory Mechanisms

The expression and activity of pro-survival and pro-apoptotic Bcl-2 family proteins influence whether or not cells undergo apoptosis, and thus can have a direct affect on tissue homeostasis. Overexpression of Bcl-2, for instance, provokes an abnormal accumulation of non-cycling cells (22, 99, 100). On the other hand, the tissue degeneration brought about by inadequate levels of these proteins can be prevented by a reduction in their BH3-only antagonist (101, 102). The levels and activity of pro-survival proteins are regulated by diverse mechanisms, including: transcriptional control, post-translational modification, and turnover. The levels of Bcl-xL, A1, Mcl-1, and Bcl-2 for example, are closely coupled to the supply of cytokines, which affects both their production and stability. Bcl-2 levels may also be controlled, in part, by micro-RNAs (103) and its activity is modulated in complex ways by phosphorylation (104). The Mcl-1 protein is particularly labile and is rapidly lost by proteasomal degradation early in response to several cytotoxic signals (105, 106). Although Bax levels have been reported to change during apoptosis (107, 108), in most, if not all cells the multi-domain proteins are present as inactive forms and the activity of the proteins appears to be regulated mainly at the post-translational level. This may explain why the levels of Bax and Bak do not seem to have great significance and the loss of single alleles of either, or even three of their (combined) four alleles, has little physiological impact (55). In contrast, levels of

BH3-only proteins can have profound effects on tissue homeostasis, exemplified by the loss of a single *Bim* allele which prevented renal failure provoked by loss of *Bcl2* gene and restored lymphocyte numbers (109).

1.2.2 BH3-only Regulation

Recent studies have shed light on the mechanisms by which BH3-only proteins are controlled. Depending on the stimulus, such as death signals or survival factors, they will be activated or deactivated, respectively. To avoid unwanted cell death, BH3-only proteins are restrained by a wide range of mechanisms at the mRNA as well as at the protein level, including post-translational modifications and sequestration in cellular structures (33, 92, 110). The multiplicity of mammalian BH3-only proteins and their complex regulation is thought to allow more precise control over apoptosis (33, 92). In healthy cells, BH3-only proteins remain either in an inactive state or are expressed at low levels, and modification or the induction of expression is usually required for their apoptotic action (32, 33). In some cell types BH3-only proteins are expressed at sufficiently high levels to induce apoptosis (40, 111, 112); however, they remain inactive and in many cases are sequestered away from the mitochondria, where they can interact with multidomain members and exert their pro-apoptotic effects. For instance, Bad, Bim and Bmf require post-translational activating-modifications (33) to translocate to the mitochondria and initiate apoptosis.

On the other hand, both Puma and Noxa, which are transcriptionally induced by p53 (68-70) in response to DNA damage, do not appear to be under post-translational control and are thought to translocate to the mitochondria unconditionally (33). Bim is an example of a BH3-only protein which is subject to several types of post-translational regulation. Bim's two predominant alleles, which are regulated downstream of the Akt signal pathway, are under transcriptional control of FOXO3a and Forkhead transcription factors (113). The BimEL and BimL proteins are sequestered in the cytoplasm by their association with dynein light chain (DLC) 1, a component of the microtubular dynein motor complex (114). Phosphorylation by c-Jun N-terminal kinase (JNK) enhances

Bim's pro-apoptotic activity (115) by allowing it to detach from the cytoskeleton. Beyond localization control, Bim protein levels are regulated by phosphorylation by Erk, which triggers its degradation by the proteasome (116-118). Regulation of Bid reveals yet another unique set of cellular controls. The engagement of cell surface death receptors activates caspase-8 (119, 120) which in turn cleaves the amino-terminal region of Bid. This gives rise to two fragments (p7/p15) that remain non-covalently bound to each other. The complex is then myristoylated on p15 (121). This process is thought to expose Bid's buried BH3 domain (79, 80) and target tBid to the mitochondria. Furthermore, Casein Kinases I and II can phosphorylate Bid and inhibit caspase-8-mediated cleavage of Bid (121). Apart from the post-translational regulations mentioned, Bid is transcriptionally induced by p53 (122), indicating the contribution of Bid to apoptotic pathways other than death-receptor-mediated pathways.

1.2.3 Bad Regulation

Bad, one of the highly regulated BH3-only proteins, is found constitutively expressed at varying levels in all mammalian cells (123, 124). In healthy cells, Bad is generally maintained in a hyperphosphorylated state by several kinase pathways and is sequestered in the cytosol by binding with 14-3-3 scaffold proteins (125-129). Death signals that result in dephosphorylation of Bad convert the Bad protein into a survival antagonist, whereby Bad selectively binds to and neutralizes anti-apoptotic molecules, particularly Bcl-xL (65, 96, 130); thereby, permitting the oligomerization and activation of pro-apoptotic molecules, Bax and Bak (123). Bad's pro-apoptotic function has been shown to be inhibited by phosphorylation (96, 119). Through its multiple phosphorylation sites (see figure 1.1), Bad is able to reflect the availability of survival factors on the cell surface and hence act as a convergence point for numerous pathways. The function of the various phosphorylation sites, independent of their position, is to render Bad unable to induce apoptosis (112, 131-133). A total of six phosphorylation sites have been identified on BAD; Ser¹¹² (134), Ser¹³⁶ (134), Ser¹²⁸ (77), Ser¹⁵⁵ (135, 136), Ser¹⁷⁰ (137) and Thr²⁰¹ (138).

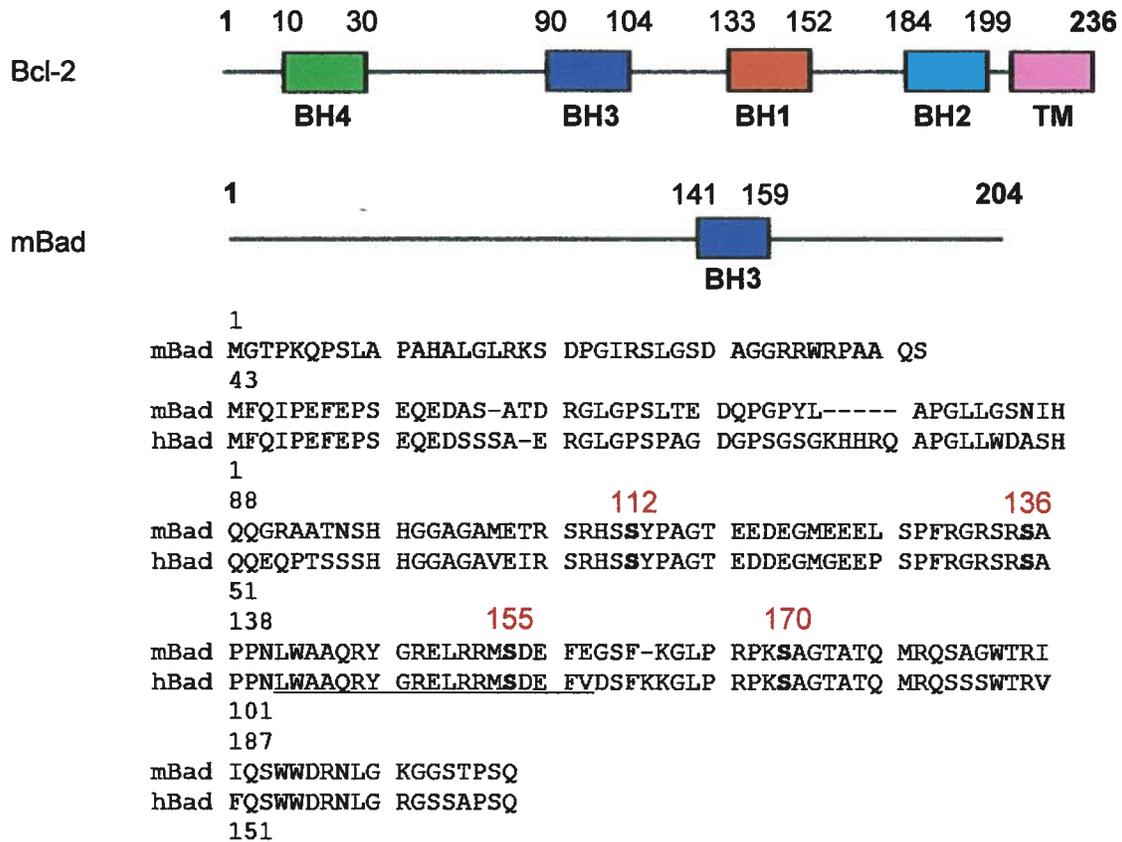


Figure 1.4 Schematic diagram of BH and transmembrane (TM) domains within Bcl-2 and Bad. The sequence of murine and human Bad are aligned to show conserved phosphorylation sites (red S112, S136, S155, S170) and the underlined BH3 domain.

The kinases involved in phosphorylation of Bad are numerous: PKC (139), Rsk (140), PKA (141), PKB (133), PI3K (142), p70S6K (143), CK2 (144), Rac1 (145), Jnk (138), Pak5 (146), Cdk1 (147), and Raf1 (148); many of these act on more than one of the phosphorylation sites. There are also several phosphatases, PP1 (149), PP2A (150) and PP2B (151), that have been shown to control the phosphorylation status of Bad. Moreover, phosphatases have been shown to compete with 14-3-3 for Bad binding upon

apoptosis induction, therefore suggesting that phosphatases do not act indiscriminately and are specific positive regulators of Bad-mediated apoptosis (53).

Though phosphorylating Bad at its various sites may have a common overall purpose, the mechanisms by which phosphorylation neutralizes Bad's apoptotic function are different. Among the 4 main sites of phosphorylation, Ser112 and Ser136 were shown to promote association of Bad with cytosolic 14-3-3 proteins (134), thus sequestering Bad away from Bcl-xL and the mitochondria. Neutralizing Bad's apoptotic function is also executed by phosphorylation at Ser155 which lies within the BH3 domain, therefore disrupting the interaction of Bad with Bcl-xL (126, 135, 152). Phosphorylation of Bad at Ser170 also abrogates Bad's apoptotic function; however, the precise mechanism is still unclear (137). Phosphorylation of yet another site on Bad at Ser128 was reported to promote apoptosis (153). Similarly, Thr201 of murine Bad was reported to be a target of phosphorylation, but the significance of this is unclear since the sequence of murine and human Bad are not conserved at that site (138).

1.3 CASPASES

1.3.1 Caspase Activation

Apoptosis is executed by caspases, a family of intracellular cysteine proteases. More than a dozen different caspases are synthesized in mammalian cells and are responsible for many of the morphogenic features of apoptotic cell death (154). For example, polynucleosomal DNA fragmentation is mediated by cleavage of ICAD (inhibitor of caspase-activated DNase), the inhibitor of the endonuclease CAD (caspase-activated DNase) that cleaves DNA into the characteristic oligomeric fragments (155). Likewise, proteolysis of several cytoskeletal proteins such as actin or fodrin leads to loss of overall cell shape, whereas degradation of lamin results in nuclear shrinking (154).

Caspases are present in healthy cells as catalytically dormant pro-enzymes (zymogens), which are activated in response to developmentally programmed cues and stress stimuli that trigger apoptosis. There are two types of caspases which are differentiated by their activation mechanism. One form of activation, exemplified by

caspases 1, 2, 4, 5, 8 – 12, have long N-terminal pro-domains that undergo homotypic interaction with specific adaptor proteins, such as FADD/MORT1 or Apaf-1. Though these zymogens have little proteolytic activity, it is thought that the close interactions between these molecules is sufficient to bring about autocatalytic processing resulting in the generation of polypeptides (~20 kDa and ~10 kDa) that assemble into the fully active caspases (156). The second type of caspases, for example caspases 3, 6, and 7, have short pro-domains and are activated predominantly through proteolysis by already active caspases (or by granzymes: aspartate-specific serine proteases). Thus, it is thought that the adaptor protein-regulated (long pro-domain) caspases ignite the death effector machinery and cause a self-amplifying (short pro-domain) caspase cascade that ultimately performs most of the proteolysis of vital substrate (157).

Caspase activity in the cell can be controlled at two levels. First, caspase activity is induced by upstream apoptosis signaling pathways that are highly regulated via the Bcl-2 family (discussed below). Second, certain caspases can be antagonized by the inhibitor of apoptosis proteins (IAPs). These IAPs bind to the active site of caspases, acting as competitive inhibitors. The anti-apoptotic effect of IAPs is neutralized upon release of certain mitochondrial proteins, such as Smac/DIABLO and HTRA2, which can sequester the IAPs (158).

1.3.2 Apoptotic Pathways Leading to Caspase Activation

Two principal apoptotic pathways, termed *extrinsic* and *intrinsic*, activate specific caspases involved in apoptosis. The evolutionarily conserved *intrinsic* pathway, also known as the ‘mitochondrial’ pathway, is primarily regulated by the Bcl-2 family and is triggered by developmental cues and diverse intracellular stresses. The *intrinsic* pathway leads to the perturbation of the mitochondrial membrane and the release of apoptogenic factors such as cytochrome *c*, apoptosis-inducing factor (AIF), Smac (second mitochondria-derived activator of caspase)/DIABLO (direct inhibitor of apoptosis protein (IAP)-binding protein with low PI), Omi/HtrA2 or endonuclease G from the mitochondrial intermembrane space (159-161). Cytochrome *c*, released from damaged

mitochondria, binds Apaf-1/caspase-9 to form what is known as the 'apoptosome' complex resulting in caspase-9 activation which then activates the effector caspase-3. Other proteins released from the mitochondria, Smac/DIABLO and Omi/HtrA2, promote caspase activation by neutralizing the inhibitory effects of IAPs (161). The triggering of caspase-3 activation leads to a caspase activation cascade that leads to complete cellular destruction as hundreds of cellular proteins are degraded (Fig. 1.5)

The *extrinsic* pathway begins outside a cell, when conditions in the extracellular environment determine that a cell must die. The *extrinsic* pathway, also known as the 'death receptor' pathway, involves at least five transmembrane receptors belonging to the TNF (tumor necrosis factor)/NGF (neuronal growth factor)-receptor superfamily or by perforin and granzyme B released from activated, cytotoxic lymphocytes. Cytotoxic lymphocytes express FasL and release granules containing granzyme B and perforin. Binding of Fas ligand to the death receptor CD95 (Fas) results in clustering of receptors and initiates the *extrinsic* pathway. The cytoplasmic portion of Fas contains a "death domain", which plays a crucial role in transmitting the death signal from the cell's surface to intracellular pathways (162). Unlike the intracellular regions of other transmembrane receptors involved in signal transduction, the death domain does not (162) possess enzymatic activity, but mediates signaling through protein-protein interactions. Stimulation of Fas by FasL results in receptor aggregation (152) and recruitment of the adaptor molecule Fas-associated death domain-containing protein (FADD) (163) through interaction between its own death domain and the clustered receptor death domains. FADD also contains a death-effector-domain (DED) that binds to an analogous domain repeated in tandem within the zymogen form of caspase-8 (164). Thus, activation of Fas results in receptor aggregation and formation of the so-called death-inducing-signaling-complex (DISC) (165), containing trimerized Fas, FADD and procaspase-8. Procaspase-8 oligomerization results in its autocatalysis and subsequent activation which results in the cleavage and activation of pro-effector caspases (caspase-3, 6, and 7) leading to apoptosis (149). In the case of cytotoxic lymphocytes, which express FasL and release granules containing granzyme B, granzyme B can enter target cells and directly activate procaspases such as caspase-3.

The two pathways are largely independent, as overexpressed Bcl-2 does not protect lymphocytes from apoptosis induced by death receptor ligands (166, 167). However in certain cell types (e.g. hepatocytes), the *extrinsic* pathway engages the *intrinsic* pathway via a cleaved form of BH3-only protein Bid (tBid) (168).

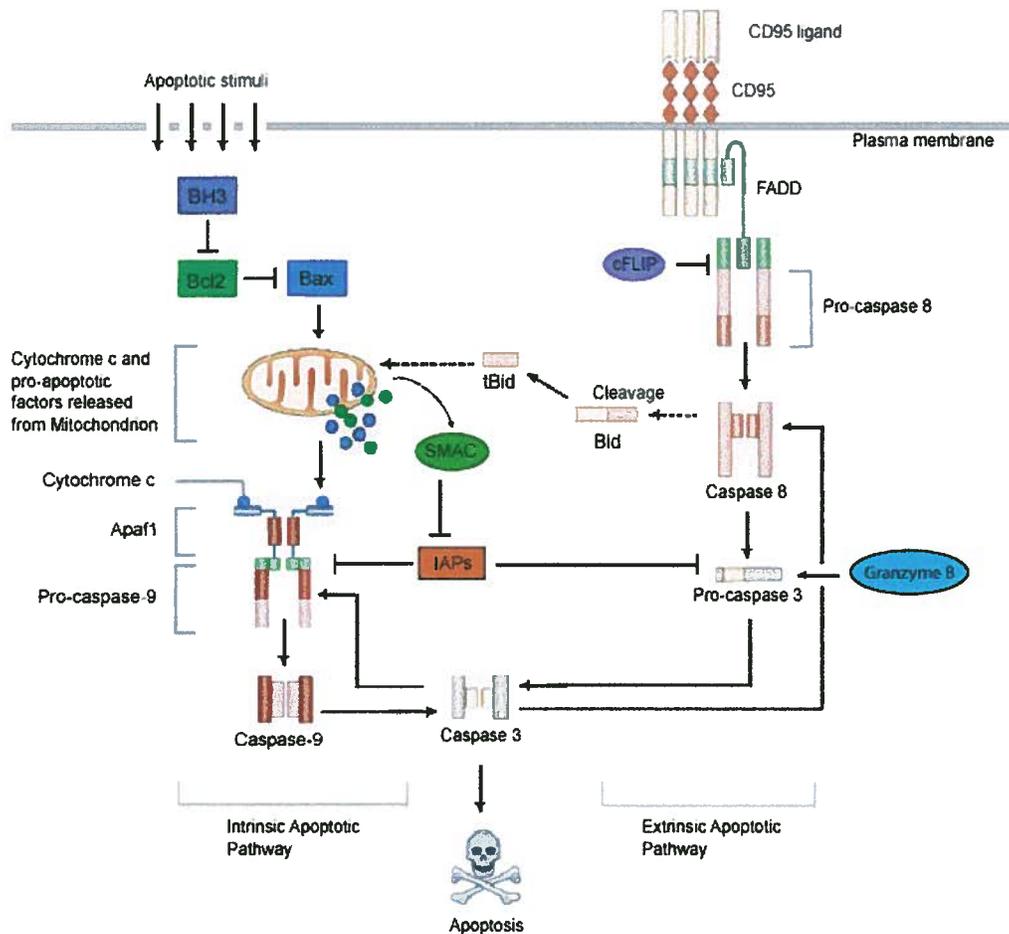


Figure 1.5 Apoptotic pathways overview

Intrinsic Apoptotic Pathway - Cellular stress induces pro-apoptotic Bcl-2 family members to translocate from the cytosol to the mitochondria, where they induce the release of cytochrome c. Cytochrome c promotes the oligomerization of Apaf-1, which recruits and promotes the activation of procaspase 9. This, in turn, activates procaspase 3, leading to apoptosis. Second mitochondrial activator of caspases (Smac/DIABLO) and Omi/HtrA released from the mitochondria upon induction of apoptosis relieves the inhibition on caspases by binding to the IAPs, thereby disrupting the association of IAPs with processed caspase 9, allowing caspase 9 to activate caspase 3, leading to apoptosis.

Extrinsic Apoptotic Pathway - Cellular stress induces pro-apoptotic Bcl-2 family members to translocate from the cytosol to the mitochondria, where they induce the release of cytochrome c. Cytochrome c catalyzes the oligomerization of Apaf-1, which recruits and promotes the activation of procaspase 9. This, in turn, activates procaspase 3, leading to apoptosis. In the case that cells are targeted by cytotoxic lymphocytes, Granzyme B is able to directly activate pro-caspase 3. Signaling from the Fas receptor to mitochondria involves cleavage of the BH3-only protein Bid by caspase-8. Bid subsequently induces cytochrome c release and downstream apoptotic events. Modified from (169).

1.4 Ca²⁺/CALMODULIN – DEPENDENT PROTEIN KINASE II

1.4.1 CaMKII Expression

Since we show evidence that Ca²⁺/Calmodulin (CaM)-dependent protein kinase II (CaMKII) phosphorylates the BH3-only protein, Bad, a short description of CaMKII's regulation and role in apoptosis is described below.

Ca²⁺/Calmodulin (CaM)-dependent protein kinases (CaMKs) - CaMKI, CaMKII, eEF2K (previously called CaMKIII), and CaMKIV - are Ser/Thr protein kinases that act as effectors by translating Ca²⁺ signaling into the appropriate cellular responses (170). These protein kinases, which are all linked to Ca²⁺ via the ubiquitous intracellular Ca²⁺ receptor CaM, have common as well as unique features with respect to their structure, regulation and activation.

CaMKII is found in most tissues, is responsive to numerous signal transduction pathways and has broad substrate specificity. Substrates phosphorylated by CaMKII are implicated in many aspects of cellular function, including the regulation of metabolism (glycogen synthetase and pyruvate kinase), membrane current (Ca²⁺, Cl⁻, and K⁺ channels), neurotransmitter synthesis (tyrosine hydroxylase and tryptophan hydroxylase) and release (synapsin I), transcription (C/EBP β and CRE-binding protein), cytoskeletal organization (microtubule-associated protein 2), intracellular calcium homeostasis (IP3 receptor and Ca²⁺/ATPase), long-term potentiation and neuronal memory (AMPA receptor) and as will be presented in this document, apoptosis (Bad).

1.4.2 CaMKII Isoforms

CaMKII comprises a family of isoforms derived from four closely related genes (α , β , γ , and δ), all of which, are highly conserved among mammalian species. For example, the coding region of rat CaMKII α mRNA is 93% identical to the human CaMKII α mRNA and at the protein level, the human and rat α isoforms are identical in amino acid composition. Four different isoforms of CaMKII (α , β , γ and δ) are encoded by four distinct genes: α (171-174), β (175-178), γ (179-181), and δ (182-184); however,

all the isoforms from all four genes have a conserved core structure and share 89%–93% sequence homology in their catalytic and regulatory domains (180). The molecular weight of these kinase isoforms range from 54 kDa (α subunit) to 58 - 72 kDa (β , γ , δ subunits).

CaMKII isoforms are expressed at varying levels in different tissues and differ in their cellular and subcellular localization. In the brain, for example, the α subunit is the predominant form in forebrain, whereas the β subunit is the dominant form in the cerebellum (185). The γ and δ isoforms are broadly distributed but at much lower levels than the α and β isoforms (186, 187). CaMKII isoforms also show distinct cellular localization; for example, as shown by immunohistochemistry, the distribution of the α and β subunits can differ even within the same neuronal cell (188).

1.4.3 CaMKII Holoenzymes

Interestingly, CaMKII isoforms expressed and localized in similar cellular locations are thought to assemble into large holoenzymes. Using measurements of the radii and sedimentation coefficients, holoenzymes are thought to be composed of 8-12 subunits (189-191). Co-expression and electron microscopy studies indicate that there is little or no selectivity in the assembly (192, 193) and since cells often express multiple isoforms, the possibility exists for the formation of heteromultimers. The formation of heteromultimers and their composition represents an additional means of modulating CaMKII activity, as the localization, sensitivity of activation and autophosphorylation (194) are altered depending on the number and type of isoforms involved in the heteromultimer (189, 195, 196). High resolution images of forebrain and cerebellar holoenzymes (193) depicts a central ring with a hole in the center. The ring appears to be composed of 8–10 smaller particles that appeared to be tethered by spokes that radiated from the central core (193). The N-terminal region of the kinase (catalytic/regulatory domain) is thought to be part of the small particles on the perimeter since calcium tracking experiments showed that Ca^{2+} /CaM appeared to gather at these particles. It was also noted that the rat forebrain kinase, which is predominantly α -CaMKII, appeared to

have 10 particles, whereas the rat cerebellar kinase, which is predominantly β -CaMKII, appeared to have only 8; indicating that the size of the holoenzyme also influences CaMKII's function and activity.

1.4.4 CaMKII Activation

The multimeric CaMKII is phosphorylated by an intra-holoenzyme autophosphorylation reaction that is directed at either the autoregulatory domain or the CaM-binding domain, producing diverse effects in its autoregulation and sensitivity to Ca^{2+} /CaM. Prior to CaMKII being activated, CaMKII is maintained in an inhibited basal state by an autoregulatory domain that acts as a pseudosubstrate preventing substrates from binding. The Ca^{2+} /CaM binding domain overlaps with the autoregulatory domain. The isoforms share autophosphorylation sites within the autoregulatory regions and based on the numbering of the α isoform, the sites are Thr286 (Thr287 for β , γ , δ) in the core regulatory domain and Thr305/Thr306 (Thr306/Thr307 for β , γ , δ) in the CaM binding domain (197, 198). By far the best explored and understood is the Ca^{2+} /CaM-stimulated autophosphorylation of Thr286. This phosphorylation involves a kinase cascade of sorts, with each subunit of the holoenzyme acting as both a kinase and a kinase of a CaMK. The resulting autophosphorylation of Thr286 produces a state of CaMKII, known as Ca^{2+} /CaM-independent activity, where it becomes autonomous of its normal stimulus, Ca^{2+} /CaM, without affecting its maximal Ca^{2+} /CaM-stimulated activity. This is a consequence of phosphorylation of Thr286 disabling the autoinhibitory domain. CaMKII, when autophosphorylated at Thr286 also undergoes a 1000-fold increase in its affinity for Ca^{2+} /CaM, known as CaM trapping; however, autophosphorylation within the CaM-binding domain following CaM dissociation of activated autophosphorylated enzyme restricts or prevents CaM from rebinding (CaM capping). The mechanisms and consequences of autophosphorylation are central to CaMKII's intricate autoregulation, potentially underlying its ability to become differentially activated in response to the length and frequency of calcium spikes. This ability to detect spike frequency allows CaMKII to act as a 'molecular switch' in learning and memory, as a readout of synaptic activity. All of these characteristics are possible due to the functional properties of

CaMKII, and its unique multimeric structure, autoregulation/activation and autophosphorylation.

It is worth noting that even though CaMKII isoforms share some homology with CaMKI, CaMKIII, and CaMKIV, CaMKII differs in terms of monomeric structure and mode of regulation (199, 200). Though each of these kinases, including CaMKII, require Ca^{2+} /CaM for activation and are activated by an upstream kinase that itself is a CaM kinase, CaMKI and CaMKIV also require phosphorylation of their activation loop for maximal enzymatic activity (200).

1.4.5 CaMKII and Apoptosis

Only recently has there been interest in CaMKII's role in apoptosis; however, a majority of these studies have been limited to neuronal and myocyte cells (201-206). Moreover, the few studies regarding the specific role of CaMKII in apoptosis have been inconsistent (207-211). For instance, CaMKII expression which was reportedly involved in IAP-2 regulation, prevented apoptosis in response to LPS and TNF α (212). However an earlier study suggested that TNF α -induced apoptosis in U937 cells was a result of Ca^{2+} -independent CaMKII activation and blocking CaMKII activation reduced the levels of apoptosis (213). The reasons for the variable reports regarding CaMKII function may be related to the differing expression levels of the 4 isoforms (and several reported splice variants) in different cell types. Since many of the studies mentioned use of a CaMKII inhibitor, which is not isoform specific, it's not surprising that the results will vary depending on the expression and the current activation level of each of the isoforms. For instance, many reports have indicated that increased activation of CaMKII results in increased survival in neuronal systems. Conversely, a majority of the myocyte studies, arising from cardiomyopathy and heart attack models, have indicated that CaMKII activation results in increased apoptosis and increased atrophy (201-204).

1.5 A LINK BETWEEN APOPTOSIS AND CELL CYCLE

1.5.1 Cell Cycle Summary

Here we describe a brief overview of the cell cycle with emphasis on the G1 to S phase transition control since several findings presented in this thesis relate to cell cycle regulation. The cell cycle is a term that refers to a series of events that take place in a eukaryotic cell leading to its replication. These events can be divided into two periods: i) interphase, during which the cell grows, accumulating nutrients needed for mitosis and duplicating its DNA and ii) the mitotic (M) phase, during which two distinct cells form. M phase is itself composed of two tightly coupled processes: a) mitosis, in which the cell's chromosomes are divided between the two daughter cells, and b) cytokinesis, in which the cell's cytoplasm divides forming distinct cells.

G1 phase, S phase, and G2 phase are known collectively as interphase. Activation of each phase is dependent on the proper progression and completion of the previous one. Cells that have temporarily or reversibly stopped dividing are said to have entered a state of quiescence called G₀ phase.

The complex macromolecular events of the eukaryotic cell cycle are regulated by a small number of heterodimeric protein kinases called cyclin dependent kinases (Cdks). The catalytic subunit of Cdks is only active as a protein kinase when bound to a regulatory cyclin protein. Whereas the levels of Cdks do not change significantly during the cell cycle, cyclin abundance is modulated during the cell cycle through programmed synthesis and degradation.

Because proper regulation of cell cycle phase transition is critical for an organism's survival, these protein kinases are exquisitely regulated at different mechanistic levels and in response to a large variety of intrinsic and extrinsic signals (214, 215). The most prominent Cdks that function at the G1/S phase transition are Cdk 2, 4, and 6 (214-216). Cdk4 and its close relative, Cdk6, are controlled by D-type cyclins (D1, D2, and D3) and act primarily by phosphorylating Retinoblastoma (Rb) and related proteins (214, 217, 218). G1 progression and S phase initiation, depends on the sustained expression of D-type cyclins, which, in turn, depends on continuous mitogenic

stimulation. This suggests that D-type cyclins may act as a link between mitogen signaling and the cell-cycle machinery. In support of this, D-type cyclin levels have been shown to be regulated at the translational level through the phosphatidylinositol 3-kinase (PI 3-kinase) pathway (218-224) via the initiation factor eIF-4E (225).

Following Cdk4/6 activation, Cyclin E binds and activates Cdk2 resulting in the phosphorylation of nucleophosmin, which is necessary for centrosome duplication. Phosphorylation of Nuclear Protein Ataxia-Telangiectasia (NPAT) then facilitates histone synthesis and establishment of pre-replication complexes (pre-RC) at origins of DNA replication (226). This entire process is thought to be necessary for proper S phase entry. Cyclin E expression appears to be more periodic than that of D-type cyclins. Cyclin E mRNA and protein begin to accumulate in late G1, peak at the G1/S transition and are downregulated during S phase (215). Cyclin E transcription is activated when pRb is hyperphosphorylated and no longer exerts repression via E2F/DP, the transcription factor complex that targets pRb-mediated repression (227). In addition to expression control, cyclin levels are controlled via degradation through the ubiquitin/proteasome pathway (228). The importance and the fidelity of cyclin regulation is highlighted in studies where overexpression of D-type cyclins or cyclin E during early G1 leads to untimely Cdk activation and premature S phase entry (218, 226).

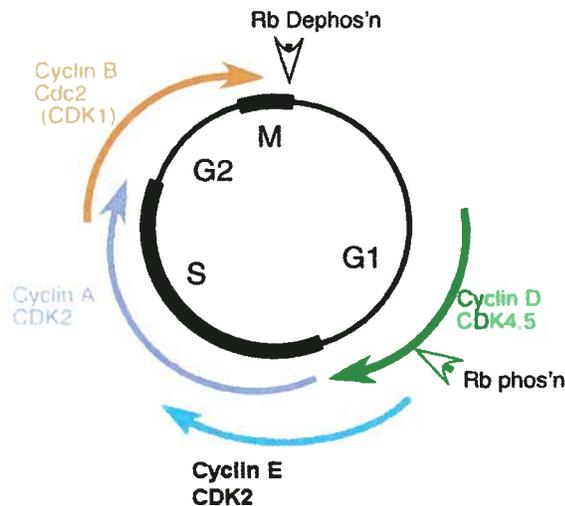


Figure 1.6 CDK/Cyclin Partners and the Regulators Cell Cycle Progression

In addition to Cyclin levels, Cdk activation is also regulated by two classes of Cdk inhibitors (CKIs). The two classes are the Cip/Kip family and the INK4 family. The Cip/Kip family is composed of three members: p21^{WAF1/CIP1}, p27^{KIP1}, and p57^{KIP2}. The INK4 family is composed of four members: p15, p16, p18 and p19 (229, 230). This classification is based on their structure as well as their Cdk affinity. The Cip/Kip CKIs have Cdk-inhibitory domains that are thought to function by anchoring the inhibitory polypeptide to the cyclin and occupying the substrate-binding site (231). Cip/Kip inhibitors are considered broad spectrum CKIs in that they can bind to and inhibit both cyclin-D–Cdk4/6 kinases, as well as cyclin-E/A–Cdk2, although it has been reported that the efficiency of Cdk4/6 inhibition may vary for the different Cip/Kip inhibitors (232).

The INK4 inhibitors share a common structural motif and mechanism of inhibition and are narrow-spectrum CKIs as they only bind to and inhibit Cdk4 and Cdk6. INK4 inhibitors consist of repeating structural units known as ankyrin repeats (231). Ankyrin repeats form a concave structure, which in the case of INK4 inhibitors bind across the back (non-catalytic) side of the target Cdk forcing a conformation that cannot support catalysis (231, 233). In addition, INK4-bound Cdks cannot bind to cyclin and thus are isolated as Cdk–INK4 heterodimers (233).

In addition to CKI's inhibition of Cdk activity, phosphorylation of Cdks represents another form of direct regulation. For instance, Cdk2 is inhibited if phosphorylated on tyrosine 15 (234). Conversely, Cdc25 phosphatase family members act at discrete times during the cell cycle to remove the inhibitory phosphates (235, 236). Experiments involving the ectopic expression of CDC25A show an accelerated G1/S phase transition. This suggests Cdc25 phosphatase activity may function to accumulate and maintain cyclin-E–Cdk2 complexes in a pre-active state (237). Recent studies have also shown that full activation of Cdk2, for example, requires phosphorylation of threonine 160 by Cdk-activating-kinases (CAK) or more precisely by homologs of the wee-1 kinase (238-242). All of which illustrates a complex mechanism aimed at controlling Cdk activity and ultimately cell cycle progression.

1.5.2 Apoptotic and Proliferative Signalling Pathways are Interconnected

The balance between cell proliferation and cell death is imperative for homeostasis in multicellular organisms. This homeostasis had long been thought to be the result of two separate processes, but there is evidence indicating that the processes of proliferation and apoptosis are coupled. For instance, evidence exists suggesting the involvement of cdks in the process of apoptosis. Studies have shown that cdks are activated in apoptosis arising in factor-deprived neurons, and during induction of apoptosis in lymphocytes by Granzyme B, tumor necrosis factor (TNF) or human immunodeficiency virus Tat protein (243-247). Conversely, it has been shown that several apoptosis regulatory proteins themselves can impinge on the cell cycle machinery (248-252). The concept of both survival and replication being coupled is further supported by observations that oncogenes sensitize cells to a wide range of mechanistically different triggers of apoptosis including DNA damage, nutrient deprivation, interferon, protein synthesis inhibitors, hypoxia, TNF, and CD95 (253-259), many of which exert no obvious direct effect on cell proliferation. Thus it appears as if the activation of cell proliferation primes the cellular apoptotic program and unless counteracted by appropriate survival signals, automatically removes the cell. Conversely, the process of undergoing apoptosis requires the engaging of the cell cycle machinery.

1.5.3 Bcl-2 Family and Cell Cycle

The Bcl-2 family of proteins, as mentioned previously, are recognized as critical regulators of apoptosis. However, accumulating evidence suggests Bcl-2 family members may also play an important role in cell cycle progression. The connection between cell cycle and cell death is supported by numerous observations describing cycling cells as more susceptible to cell death as compared to quiescent cells. The morphological appearance of apoptotic cells and its similarities to cells undergoing mitosis has also been noted. From this, suggestions have been made that the mechanisms that regulate mitosis may play a role in apoptosis (260). Conversely, cells undergoing programmed cell death

often exhibit activation of cell cycle events, such as cdk activation and abortive cell cycle progression (261, 262).

Bcl-2's pro-survival function was first observed to affect cell cycle progression in IL-3-dependent FDC-P1 cells over-expressing Bcl-2. It was observed that growth factor withdrawal resulted in the reduction of cell size as compared to unstarved cells and cells were mostly arrested in G₀/G₁ (15). From this, it was thought Bcl-2 arrested cells in G₀ to maintain viability when deprived of growth factor. A similar observation of Bcl-2's effect on proliferation was that bone marrow-derived IL-3-dependent BAF3 cells expressing Bcl-2 promoted survival upon IL-3 removal and arrested cells in G₀/G₁ (15). It was also noted that these cells did not undergo cell cycle re-entry upon IL-3 re-stimulation. From these early experiments, a series of papers ensued, including several studies examining the effect of Bcl-2 not only on G₀/G₁ arrest but also on cell cycle progression. Many of the early studies regarding the physiological effects of Bcl-2's cell cycle inhibitory role were demonstrated by Stan Korsmeyer's laboratory, by studying and comparing T-cells from models derived using *bcl2*-deficient, *bcl2*-heterozygous, wild-type, and *bcl2* transgenic mice (250). Studying cell cycle entry in response to T-cell activation, it was shown that increasing expression of Bcl-2 correlated with a higher G₀/G₁ fraction and lower S-phase fraction (252, 263, 264). Several experiments involving activation of quiescent T and B cells and serum stimulation experiments using arrested NIH3T3 cells showed that Bcl-2 expression delayed the onset of S phase, indicating inhibition of G₀ to S progression (264). Another early study which used HL60 cells (promyelocytic leukemia cell line) demonstrated that when cells overexpressing Bcl-2 were treated with DMSO, a differentiative stimulus that did not involve cell death, cells decreased RNA content more quickly than control cells. This suggested that Bcl-2 expression facilitated exit to G₀ (265) and this effect was separate from its anti-apoptotic function. Parallel studies using Bcl-2 homologs (Bcl-xL, BCL-w, and E1B19K) also showed retarded progression to S phase; demonstrating Bcl-2's ability to affect cell cycle progression is found in other antiapoptotic molecules within the Bcl-2 family, and is not cell type restricted (15, 266). Based on the above studies, Bcl-2 was thought to be generally growth inhibitory, however, growth rate measurements in conventional and

continuous chemostat cultures later revealed that in cycling cells, Bcl-2 does not significantly affect growth rates under normal growth conditions, but prolongs G1 in suboptimal conditions (250, 251, 263, 267-269). Furthermore, cells sorted for the same size, regardless of Bcl-2 or Bcl-xL expression level, entered cell cycle with similar kinetics, indicating that the main function of Bcl-2 and Bcl-xL is to drive cells into G₀ (270).

The notion that Bcl-2 may somehow be connected directly or indirectly to the cell cycle machinery prompted several more studies investigating the molecular mechanism by which Bcl-2 is able to inhibit cell cycle progression. One study, using murine IL-3–dependent NSF/N1.H7 cells, aimed at studying the effect of Bcl-2’s phosphorylation state on cell cycle progression, observed that Bcl-2 mono-(Ser70) or multi-site (Thr69, Ser70, and Ser87) phosphorylation in its flexible loop domain was shown to regulate intracellular reactive oxygen species levels; and subsequently inhibit cell cycle progression by delaying the G₁/S transition (271). Another study, using Bcl-2 transgenic mice, showed that Bcl-2 is able to delay cell cycle entry by delaying the accumulation of E2F1, a critical inducer of cell cycle entry (272). Various groups showed that negative cell cycle regulators p27 and p130, which binds E2F4 during G₀ to inhibit E2F-1 expression, were elevated significantly more than usual in Bcl-2 cells during arrest (249, 250, 272, 273). Further studies showed that activation of cyclinE/cdk2 and cyclinD/cdk4, which is required for normal G1 to S progression, was reduced in Bcl-2 and Bcl-xL cells; and this decrease in cdk activity was due to high p27 in the cyclin/cdk complexes (272, 274). Further evidence supporting p27 as an important mediator of Bcl-2 and Bcl-xL’s cell cycle effect was shown by the inability of Bcl-2 and Bcl-xL overexpression to delay proliferation in cells from *p27*^{-/-} transgenic mice (272, 274). It was found that the protein levels associated with the induction of G1 entry, including c-Fos, c-Jun, Myc, cyclin D expression, were not affected by Bcl-2 and Bcl-xL (250, 274), indicating the early signaling events initiating G₀ to G1 transition are intact. However, the critical activation of cdk2/4, which is required for transition into S phase, is delayed.

1.5.4 Bad and Cell Cycle

Interestingly Bcl-xL and Bcl-2 antagonist, BH3-only protein Bad, has been implicated in several studies with cell cycle regulation. Bad has been shown to be phosphorylated at S128 by Cdk1, a G2/M regulator in neuronal cells and Bad expression alters Cyclin D expression in breast cancer cells (275). However, neither of these findings have been supported in other cell types. Moreover, strong evidence involving Bad and cell cycle regulation arise from the finding that Bcl-xL can prolong the G₀ phase and induce cell cycle arrest, both of which can be blocked through Bad expression (270). Furthermore, it was demonstrated that a BH3 mutant form of Bad (L151A), which is unable to bind Bcl-xL and Bcl-2, had no effect on the ability of Bcl-xL to delay the onset of S phase. This demonstrated that Bad's ability to modulate Bcl-xL's cell cycle function is dependent on its interaction with Bcl-xL. Therefore, these experiments demonstrated that a molecule known to inhibit the anti-apoptotic function of Bcl-xL also inhibited its cell cycle activities. This further supports the suggestion that the cell cycle effects observed are a result of Bcl-xL expression. Furthermore, it was shown that overexpression of Bad not only prevented cell cycle arrest, but also showed increased activation of Cdk2 specifically during G1-S transition (248, 270). The precise timing of Cdk2 activation, caused by Bad expression, is worth noting as Cdk2 activation has been associated with Bax-induced cell death (276, 277). Interestingly, experiments using *Bclx*^{-/-} and *Bcl2*^{-/-} cells demonstrated that the cell cycle activity of Bad is not simply the result of inactivating Bcl-xL as cells lacking Bcl-xL arrest normally. Furthermore, it has been shown that *Bad*^{-/-} cells arrest normally and as such supports the hypothesis that neither Bad nor Bcl-xL is required for G₀/G1 arrest, though Bad/Bcl-xL heterodimerization can overcome the G₀/G1 checkpoint. Thus, the increase in Cdk2 activation and the ability of Bad/Bcl-xL heterodimers to push cells into S phase, demonstrates that the function of Bad may not only be to inactivate Bcl-xL or Bcl-2, but that Bad may be actively involved in cell cycle control. This is further supported by studies showing that phosphorylation of Bad at Ser-170 can promote cell cycle progression, an effect that appears to be separate from its pro-survival function (137).

1.6 BCL-2 FAMILY PROTEINS AS RATIONAL DRUG TARGETS

1.6.1 Cancer Cells are Primed for Death

The process whereby a normal cell becomes cancerous requires a minimum of two alterations: deregulation of proliferation and suppression of apoptosis. Deregulation of proliferation, as seen in many cancers, is a consequence of overproduction and/or increased activation of specific proteins that promote cell duplication. Cells, however, have a fail-safe mechanism that is able to detect extreme proliferative signals and activate the apoptotic machinery. For this reason even though oncogenes can stimulate cell proliferation and tissue growth, in many cases a net loss of cells results over time (278-280). Furthermore, studies have shown that cells with deregulated proliferation mechanism die more readily than normal cells in conditions where nutrients are scarce (281). For instance, up-regulation of Myc induces proliferation, but at the same time engages the apoptotic machine and as a result the cell becomes more vulnerable to death signals. Therefore in order to become cancerous, cells must undergo further cellular alterations that permit uncontrolled proliferation without promoting apoptosis. One common death-defying strategy is to enhance the activities of apoptosis-inhibiting proteins; therefore, blocking death signals from reaching the mitochondria (282). It is for this reason that transformed cells where oncogenes increase the rate of proliferation, in many cases, have also been shown to have up-regulated expression of pro-survival proteins such Bcl-2 or Bcl-xL (253, 258). It is noteworthy that while cancer cells inactivate elements of the apoptotic pathway, they never disable the entire signaling cascade. For instance, cells that have been transformed by Bcl-2:Myc synergy, have turned on the apoptotic machine, however the cell's demise is prevented by Bcl-2's ability to neutralize Bax-like proteins (15, 23, 253). As a result, even though the apoptotic machine is 'running,' Bcl-2 has disengaged it from being able to perturb the mitochondrion and trigger apoptosis. Nevertheless, the aggressive proliferative effect of Myc is thought to prime the cell for apoptosis and as a result, the prospect of re-engaging the apoptotic machinery to the mitochondrion makes Bcl-2 and its pro-survival relatives (Bcl-xL and Bcl-w) rational therapeutic targets. Hence, directly inhibiting Bcl-2 is likely

to restore a functional apoptotic system and thus engage the over-activated death machinery in cancerous cells while also increasing the sensitivity towards chemotherapy.

1.6.2 Targeting Bcl-2

Several proof-of-concept studies have been performed based on earlier work by Strasser *et al.* which showed that mice overexpressing both Bcl-2 and Myc develop an immature lymphoblastic leukemia, as seen by excessive amounts of white blood cells and enlarged spleen (283). Breakthrough studies by Letai *et al.* using transgenic mice that conditionally express Bcl-2 and Myc showed that reducing Bcl-2 results in a dramatic decrease in the number of white blood cells and the enlarged spleen returned to normal size within 2 weeks (284). Furthermore, mice that had Bcl-2 levels reduced lived on average 145 days as compared to mice with high levels of Bcl-2 which lived 82 days (284). This demonstrates that the elimination of Bcl-2 expression clearly results in remission of the leukemia and prolonged survival of the mice. Furthermore, normal cells have been shown to tolerate reduced Bcl-2 levels, and mice that have lowered expression of Bcl-2 are completely healthy (102). This further strengthens the rationale of Bcl-2 as a therapeutic target since the inhibition of the target protein must have little effect on cells other than those that are cancerous. These results combined with our increased understanding of the functional significance of specific Bcl-2 family members has fueled the pharmacological pursuit for therapeutic peptides and peptidomimetics that inhibit specific pro-survival proteins such as Bcl-2.

1.6.3 Bad - A drug template

Bad has been used as a template for peptidomimetic design since Bad has been shown to have high affinity for Bcl-2 and functional studies have demonstrated Bad's ability to antagonize Bcl-2's pro-survival effect (101, 285, 286). In addition, Bad^{-/-} mice have been shown to develop lymphomas with increased age, and in response to sublethal radiation (287, 288). Therefore, the lack of Bad clearly plays an important role in the

development of cancer and as such the introduction of a molecule with Bad-like-binding properties may be able to re-sensitize transformed cells to apoptosis signaling pathways. Further support comes from knock-in mice expressing Bad with Ser mutated to Ala at 112, 136 and 155 sites (Bad 3SA). Bad 3SA knock-in cells showed increased growth factor responsiveness and lowered threshold for mitochondrial disruption, as compared to Bad knock-in and wild type cells (287). These studies among the many others describing Bad's pro-apoptotic regulation, via phosphorylation, will undoubtedly lead to a more accurate template from which to design an effective therapeutic peptide. As such, our work investigating the cellular and molecular effects of Bad's phosphorylation state at Ser-170 will contribute to the overall understanding of Bad's regulation and possibly form the basis of a more potent and specific inducer of apoptosis.

1.6.4 Targeting the apoptotic and proliferative signaling pathways

The potential role of Bad in cell cycle regulation, as presented in this thesis, also represents a new direction in the study of this protein as well as a new potential for therapeutic development. The data presented (see Chapter 3) indicating Bad directly interacts with the proliferative pathway raises the possibility that Bad, when not phosphorylated, may harbor the potential of modulating both the apoptotic and proliferative pathways that are hyperactive in cancer cells, and redirect them towards the activation or amplification of the cell death machinery. Other data presented in Chapters 4 and 5, show that cells expressing Bad preferentially undergo apoptosis when treated with CaMKII inhibitor (KN93). This finding is relevant as we also show that CaMKII- γ phosphorylates Bad at Ser-170, a site known to modulate Bad's apoptotic ability. Thus targeting CaMKII activity may be another means of engaging the apoptotic machinery.

Moreover, well recognized genes involved in cancers, such as p53, which is a key regulator of the cell cycle and apoptosis (289, 290), have been the pharmacological target of many potential and current anti-cancer drugs (291-294). This highlights the importance of trying to understand the mechanism of Bad's regulation of cell cycle progression. Similar potential has attracted increased attention to cyclins and their

respective kinase partners, cdks, since both have been recently shown to play a role in apoptosis in addition to their well established role in cell cycle regulation (185, 277, 295-297). At least theoretically it should be possible to selectively harness their pro-apoptotic potential and direct it towards selective activation of programmed cell death in cancer cells. Thus, understanding the roles of these proteins will certainly pave a way towards more scientific advancement in the field of cancer biology and will provide solid foundations for the discovery of novel drug targets that would kill, or at least control cancer progression. Furthermore, the sophisticated dual role of the Bcl-2 family members in the intricate regulation of apoptosis and the cell cycle makes them ideal therapeutic targets in the numerous diseases characterized by deregulated cell cycle and apoptotic pathways.

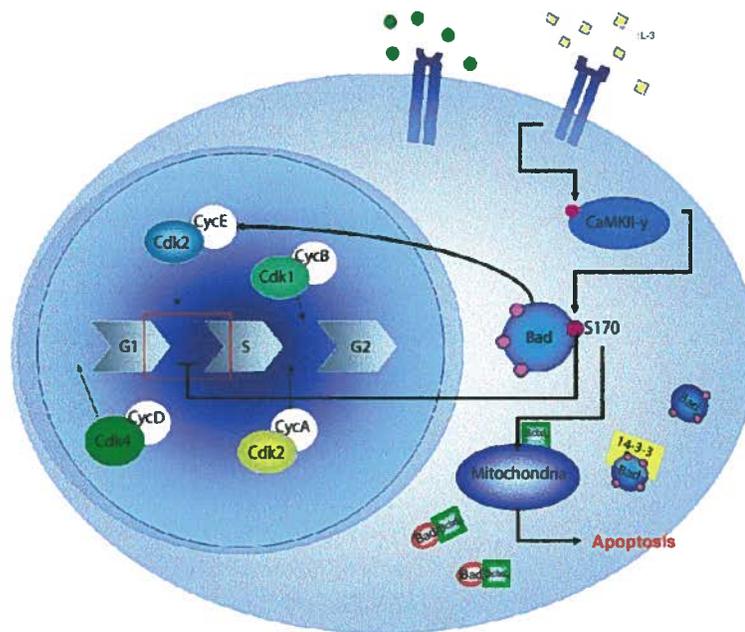


Figure 1.7 Bad Ser 170 – regulation and cellular effects

IL-3 stimulation was shown to increase the activity of levels of CaMKII and subsequently increase the level of phosphorylation of its activating phosphorylation site (Thr287). CaMKII-γ was shown to be capable of phosphorylating Bad at Ser 170. Bad's apoptotic ability was shown to be reduced when phosphorylated at Ser 170. Dephosphorylation of Bad at Ser 170 was shown to increase the time required for cells to transit from G1 to S phase of the cell cycle. Bad was also shown to interact with key G1/S cell cycle regulators, Cdk2 and Cyclin E.

1.7 AIMS OF STUDY

The studies in this thesis are aimed at furthering our understanding of Bad's regulation specifically with respect to its phosphorylation at the Ser 170 site. We were interested in knowing whether the dephosphorylation of Ser 170 site could contribute to the potency of the already apoptotic mutant protein, Bad Ser112,136,155Ala (Bad 3SA). Conversely, we were also interested in determining if phosphorylation of Ser 170 could reduce the apoptotic effect of Bad 3SA. Beyond studying the effects of Ser 170 on Bad's ability to induce apoptosis, we also describe a novel cell cycle effect that appears to be controlled at the level of phosphorylation at Ser 170. The increased replication time that occurs when cells overexpress Bad mutant form, mimicking dephosphorylation at Ser 170 (Bad S170A), was an intriguing observation. From this, we were interested in tracking the DNA content of cells expressing Bad S170A to see whether there was a specific stage in the cell cycle that was hindered or whether the entire cell cycle was slowed. From these studies, we show that cells expressing Bad S170A have marked increase in the percentage of cells in S phase which indicates a slowing of the cell cycle specifically at S phase. Based on this finding, we were interested in studying how Bad may be effecting cell cycle progression. Similar studies ongoing in our lab at the time, involving Mcl-1 and its association with Cdk1 (298), prompted us to investigate the potential association of Bad with cell cycle regulatory proteins. Endogenous Bad and flag-tagged Bad were immunoprecipitated and these IPs were probed for the presence of a number of cell cycle regulators. Association of Bad with both cyclin E and Cdk2 was detected. Interestingly, both cyclin E and Cdk2 are late G1 and S phase regulators, and as such we were interested in determining whether this novel interaction was responsible for the cell cycle effects described above. Unfortunately, we were unable to determine what regulates the interaction between Bad and Cdk2/cyclin E. We do show that the interaction occurs primarily in the cytosol and the overexpression of Bcl-xL appears to negatively affect the level of interaction of Bad and Cdk2/CyclinE. However, we did not observe a significant change in Bad's ability to interact with Bcl-xL or Cdk2 when Bad is mutated at Ser 170 to mimic phosphorylation (Bad S170D) or dephosphorylation (Bad S170A).

The second major experimental effort was aimed at identifying the kinase responsible for phosphorylating Bad at Ser-170. From these studies, we identified

CaMKII- γ as the kinase that phosphorylates Bad at Ser170. Parallel studies, investigating pathways involved in regulating Bad's phosphorylation at Ser 170 revealed that both cytokine stimulation and cell cycle stage affect the level of kinase activity against the Ser 170 site. Follow up studies involving the inhibition of CaMKII using small molecule inhibitor, KN93, demonstrated that FDC-P1 cells underwent apoptosis in conditions where CaMKII was inhibited. Both the apoptosis and kinase activity studies support the our newly formed hypothesis: CaMKII- γ is regulated by both proliferative and survival signaling pathways, and increased CaMKII- γ activity promotes survival, at least in part, by phosphorylating Bad at Ser 170.

Chapter 2

MATERIALS AND METHODS

2.1 CELL LINES AND TISSUE CULTURE

MC/9 and FDC-P1 cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2mM L-glutamine, 1mM sodium pyruvate, 50 nM β -mercaptoethanol. For MC/9 and FDC-P1 cells, the above medium was supplemented with 2.5% WEHI-3-conditioned medium as a source of mouse IL-3. MC/9 cells over-expressing Bcl-xL and Bcl-2 (MC/9-Bcl-xL), or Bcl-2 (MC/9-Bcl-2), and FDC-P1 cells over-expressing Bcl-xL (FDC-P1/Bcl-xL) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2mM L-glutamine, 1mM sodium pyruvate, 50 nM β -mercaptoethanol, and 2.5% WEHI-3-conditioned medium. The MC/9-Bcl-2 cell lines were maintained in the presence of (200 μ g/ml) hygromycin. The MC/9-Bcl-xL, and the FDC-P1/Bcl-xL were maintained in the presence of (0.5 mg/ml) G418. MC/9-Bcl-xL and FDC-P1/Bcl-xL expressing flag-tagged Bad WT and flag-tagged Bad mutants (S170A, S170D, 3SA, 4SA, 3SA170D) were maintained in the presence of (2.5mg/ml) puromycin in addition to (0.5 mg/ml) G418. 3T6 cells were grown in DMEM medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 1 mM sodium pyruvate. Cells were maintained at 37°C and 5% CO₂ in a humidified incubator. The MC/9-Bcl-2, MC/9-Bcl-xL and FDC-P1/Bcl-xL were constructed using retroviral infection. MC/9-Bcl-xL, and FDCP-1/Bcl-xL cells persisting after 7 days of continuous selection were used for transfection of BAD-pMXpuro constructs.

Bone marrow-derived mast cells (BMMCs) were derived by aspirating from the femurs and tibias of 6-8 weeks old female CD1 mice as described (299). Non-adherent cells BM cells were plated at 1×10^6 cells/ml in IMDM containing 30ng/ml of WEHI-3. Cell cultures were maintained between 2×10^5 and 8×10^5 cells per ml.

2.2 REAGENTS

Recombinant IL-3, was purchased from R&D Systems (Minneapolis, MN). The inhibitors LY-294002, wortmannin and rapamycin were from Calbiochem (EMD). Propidium iodide (P.I.) was from Sigma-Aldrich (Saint Louis, MO). The BP and LR clonase enzymes, Zeocin and Blastocidin were from *Invitrogen*. The Effectene and Lipofectamine 200 transfection reagents were from GIBCO BRL and Oligofectine transfection reagent, from *Invitrogen* (Burlington, ON) was used for siRNA knockdown experiments.

2.3 ANTIBODIES

Table 2.1: Antibodies used

Antibody	Type	Dilution	Procedure	Source
α -Bad	Rb	1:1000	W, IP	BD Biosciences, 610392
α -Bad	Ms	1:1000	W	Santa Cruz Biotechnology, SC-943
α -Flag	Ms	1:2500	W, IP	Sigma Chemical, F-3165
α -Bcl2	Rb	1:1000	W, IP	Santa Cruz Biotechnology SC-23960
α -BclxL	Rb	1:1000	W, IP	BD Biosciences, 556361
α -Cyclin A	Rb	1:500	W, IP	Santa Cruz Biotechnology SC-31084
α -Cyclin D	Rb	1:500	W, IP	Santa Cruz Biotechnology SC-450
α -Cyclin E	Rb	1:500	W, IP	Santa Cruz Biotechnology SC-25303
α -Cdk2	Rb	1:2000	W, IP	Santa Cruz Biotechnology SC-70829

Antibody	Type	Dilution	Procedure	Source
α-Cdk4	Rb	1:2000	W, IP	Santa Cruz Biotechnology SC-70832
α-Cdk6	Rb	1:2000	W, IP	Santa Cruz Biotechnology SC-53638
α-Chk1	Rb	1:2000	W, IP	Santa Cruz Biotechnology, SC-7898
α-CaMKII alpha	Ms	1:500	W, IP	Santa Cruz Biotechnology, SC-70492
α-CaMKII beta	Ms	1:500	W, IP	Santa Cruz Biotechnology, SC-1540
α-CaMKII gamma	Rb	1:500	W, IP	Santa Cruz Biotechnology, SC-1541
α-Gsk3	Rb	1:2500	W	Santa Cruz Biotechnology, SC-81463
α-Histone H1	Ms	1:2500	W	Santa Cruz Biotechnology, SC-8030
α-P Thr 286 CaMKII	P	1:500	W	Milipore, AB3827
α-Vinculin	Ms	1:5000	W	Santa Cruz Biotechnology, SC-73614

α =anti, P=phospho, IP=immunoprecipitation, W=western blot, Ab=antibody, Rb=rabbit,
Ms=mouse,

2.4 PLASMIDS

Plasmids CTV83 expressing human Bcl-xL and CTV87 expressing human Bcl-2 were a gift from Dr. Rob Kay. pMXpuro plasmids expressing Bad and Bad mutants Bad (S170A, S170D, 3SA) were constructed by Shaynoor Dramsi. The Bad 4SA construct was prepared commercially by Seqwright Inc.

PCR based site directed mutagenesis was performed using pMXpuro plasmid expressing mutated Bad 4SA to create Bad 3SA170D. Specifically the cytosine at position 977 was replaced with an adenine which resulted in the alanine (GCC) becoming aspartic acid (GAC). Primer used:

5'-GGACTTCCTCGCCCAAAGGACGCAGGCACTGCAACACAG-3'.

2.4.1 Bacterial Transformation

Constructs generated were propagated by transforming the DH5 α competent cells. Briefly, one microliter each of BP or LR reaction mix was added to 50 μ l of DH5 α competent cells. The reactions were incubated on ice for 30 minutes followed by heat shock at 42°C for 30 seconds. The cells were then put on ice for 1-2 minutes followed by addition of 450 μ l of LB medium and incubation at 37°C for 1 hour. The cells were then spread on LB plates containing 50 μ g/ml kanamycin and 100 μ g/ml ampicillin. The kanamycin or ampicillin resistant colonies were selected and the plasmids were propagated and purified using plasmid midi-prep kit (Qiagen).

2.5 PROTEIN ANALYSIS

2.5.1 Cell Treatments

For analysis of proteins by Western blotting, cytokine-dependent cells were starved of cytokine by overnight incubation leaving 10% of the original IL-3 containing medium, or alternatively, cells were washed three times with PBS and incubated in cytokine free medium for the indicated time. For experiments involving cytokine stimulation, cells were stimulated with 10 μ g/ml synthetic IL-3 in conditions previously

shown to induce maximal serine phosphorylation (Duronio *et al.*, 1992; Welham *et al.*, 1994; Ettinger *et al.*, 1997). In experiments involving KN93 (CaMKII inhibitor) or its inactive analog, KN92, cells were treated in the presence of cytokine for 24 hours. Final concentrations used were 10 µg/ml and 50 µg/ml KN93 or KN92.

2.5.2 Preparation of Total Cell Lysate (TCLs)

Total cell lysates were obtained by lysing cells in ice-cold solubilization buffer [50 mM Tris/HCl, pH 7.7, 1% Triton X-100, 10% (v/v) glycerol, 100 mM NaCl, 2.5 mM EDTA, 10 mM NaF, 40 µg/ml phenylmethylsulfonyl fluoride, 1 mM pepstatin, 0.5 mg/ml leupeptin, 10 mg/ml soybean trypsin inhibitor, 0.2 mM Na₃VO₄, 1 mM Na₃MoO₄ and 1 mg/ml microcystin-L for 5 min followed by centrifugation at 13000 rpm for 10 min. The supernatants containing the total cell proteins were boiled for 3 minutes in SDS sample buffer containing 1% β-mercaptoethanol and subsequently used for Western blotting.

2.5.3 Preparation of Nuclear and Cytoplasmic Extracts

For extraction of cytosolic and nuclear proteins, cells were lysed in buffer A containing 10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, 0.34 M sucrose, 10% glycerol plus 1 mM DTT, 0.1% Triton X-100, 0.2 mM Na₃VO₄, 1 mM Na₃MoO₄, and the protease inhibitor cocktail for 5 minutes followed by centrifugation at 4000 rpm (2000 g) for 5 minutes to pellet primarily the nuclei. The supernatants were further centrifuged at 13000 rpm (15000 g) for 10 minutes and subsequently stored as cytosolic fractions and pellets were used for extraction of nuclear proteins. The pellets were washed twice with buffer A and the nuclei were resuspended in buffer B containing 0.2 mM EGTA (pH 8), 3 mM EDTA (pH 8), plus 1 mM DTT, RNaseA, DNAaseA, 0.2 mM Na₃VO₄, 1mM Na₃MoO₄, and the protease inhibitor cocktail for 30 minutes followed by sonication using a Sonic Dismembrator 550 (Fisher Scientific, Nepean, ON, Canada) at setting 3 for 10 seconds. The extracted proteins were centrifuged at 4,000 rpm (2,000 g) for 5 minutes. The supernatants contained the nuclear proteins. The nuclear and cytosolic extracts were then boiled in SDS sample buffer containing 1% β-Mercaptoethanol for 5 minutes.

2.5.4 Co-Immunoprecipitations (IPs) and Western Blotting

Typically, 4-6 mg of protein extracts was used for immunoprecipitations. Extracted proteins were incubated overnight at 4°C with the antibodies listed in section 2.3 at the indicated concentrations. Immuno-complexes were captured with 40µl of protein G-Sepharose beads slurry at 4°C for 1 h. Beads were washed 3 times with cold lysis buffer and boiled in SDS sample buffer containing 2% β-Mercaptoethanol for 5 minutes.

TCLs and IPs were separated by SDS/PAGE followed by Western blot analysis. Transfers were made by semi-dry blotting on to nitrocellulose membranes. The membranes were blocked for 1 h in 5% (w/v) low-fat dry milk in Tris-buffered saline with 0.05% Tween 20 followed by overnight incubation at 4°C with the antibodies listed in section 2.3 at the indicated concentrations. Anti-rabbit, anti-mouse or anti-goat antibodies conjugated to horseradish peroxidase were used to detect the immunocomplexes by enhanced chemiluminescence (Amersham International, Oakville, ON, Canada)

2.6 ASSAYS

2.6.1 Apoptosis assays

Apoptosis was assayed by annexin V and propidium iodide (PI) staining. Samples were read using flow cytometry (BD FACSCanto) and analyzed with FCS Express v.2 (DeNovo software). The cells were cultured until reaching a density of no more than 5×10^5 cells per ml, followed by washing 3 times with complete RPMI 1640 medium without WEHI-3. Cells were harvested after various times of IL-3 starvation, washed with PBS twice and centrifuged at 200 x g for 4 min. The pellet (5×10^5 cells) was resuspended in 500 µL of binding buffer (PBS containing 0.1% glucose, 100 µg/ml RNase A). The staining solution [Annexin V-fluorescein labeling reagent: PI = 1:2 (v/v)] was incubated for 15 min at room temperature in the dark. Percentage of cells undergoing apoptosis was determined from both Annexin V positive cells together with cells that were both Annexin V and PI positive. Analysis of cells at various time points verified

that at early times, Annexin V positive cells were observed and at later times, these cells became double positive.

2.6.2 Cell Cycle Assay

Cells were synchronized in late G1 phase of the cell cycle by treating cells with Hydroxyurea (100 mM) for 18 h. Cells were released from G1 by washing cells three times with PBS and re-culturing in normal growth media plus WEHI-3. Cells were harvested at various time points after release and fixed in ice cold 70% ethanol for 15 minutes. The cells were then pelleted and incubated in PBS containing 0.1% glucose, 100 µg/ml RNase A and 50 µg/ml propidium iodide for 30 min in the dark. The samples were analyzed by flow cytometry. By analyzing cell size and DNA content, cells were categorized as being in G1, S and G2 phases of the cell cycle.

2.6.3 Cell Sorting Assay

Cells growing in normal growth media with WEHI-3 were used for cell sorting experiments. Cells were washed with PBS just prior to being sorted using the BD FACS Vantage SE Turbo sort cell sorter. Cells were sorted based on cell size and DNA content and are here referred to as cells in either: G1, S or G2 phases of the cell cycle. Sorted cells were lysed instantly as described in section 2.5.3.

2.7 RETROVIRAL INFECTION

The retroviral infection was done to over-express human Bcl-xL into cytokine dependent FDC-P1 and MC/9 cell lines. Briefly, Plat-E (300) packaging cells were plated in 6 well plates. Once cells were 50% confluent, cells were transfected with Effectene (Qiagen) according to the manufacturer's protocol. Approximately 1 µg of either CTV83-Bcl-xL (gift from Dr. Rob Kay) or CTV87-Bcl-2 retroviral vectors were used to transfect the cells using the Effectene transfection kit (Qiagen). Twenty-four hours following transfection, the medium throughout was replaced with fresh media and 72 hours later the viral supernatant was collected and centrifuged at high speed to remove all the cell

debris. The viral supernatant was subsequently filtered through a 0.45 μm filter and the supernatant was then used to infect FDC-P1 and MC/9 cells. The cell media was replaced with the viral supernatant and 48 hours after infection the cells were selected with either G418 (0.5 mg/ml) or Hygromycin (200 $\mu\text{g}/\text{ml}$) for cells infected with CTV83-Bcl-xL retrovirus or CTV-Bcl-2 retrovirus, respectively. MC/9-Bcl-xL, and FDC-P1/Bcl-xL cells persisting after 7 days of continuous selection were used for transfection of BAD-pMXpuro constructs. BAD-pMXpuro constructs include: Bad Ser170Ala (170A), Bad-Ser170Asp (170D), Bad- Ser112, 136, 155Ala (3SA), Bad-Ser112, 136, 155, 170Ala (4SA) and Bad-Ser112, 136, 155Ala, 170Asp (3SA170D). Stable FDC-P1/Bcl-xL and MC/9-Bcl-X_L transfectants expressing various FLAG-tagged Bad constructs in the pMX-puro vector were selected in complete medium containing 2.5 $\mu\text{g}/\text{mL}$ puromycin, and stable clones were isolated by serial dilution in 96 well plates.

2.8 COLUMN CHROMATOGRAPHY

Cells were pelleted at 200 x g for 4 minutes and lysed with ice-cold solubilization buffer. Lysates were spun down at 13,000 rpm (15,000 g) for 10 minutes at 4°C to remove the nuclei and insoluble material. The supernatant (500 μg of protein) was then loaded into Mono-Q HR 5/5 column from Pharmacia. Mono-Q-buffer-A contained 50 mM of Tris-HCl pH 8.8, 2.5 mM of EDTA pH 8.0, 1 mM of sodium vanadate and 0.01% Triton X-100. Mono-Q-buffer-B is the same as Buffer A except that it also contains a final concentration of 1 M NaCl and a linear gradient of 0 to 1 M NaCl was used to elute bound proteins. Samples of 0.5 ml were collected at flow rate of 0.25 ml/min.

For Superdex S200 column fractionation, the cell extracts (500 μg of protein) were loaded and the column washed with buffer containing 50 mM Tris 7.7 pH, 0.05% Triton X-100. Fractions of 0.5 ml were collected with a flow rate of 0.25 ml/min.

2.9 KINASE ASSAY

A peptide encoding the Ser170 site of Bad was synthetically prepared at the Biomedical Research Centre, UBC. The synthetic peptide contained the following sequence: RRGGPRPKSAGVA (PRPKSAG corresponds to residues 166-172 of murine Bad or 130-136 of human Bad). The substrate for CaMKII kinase activity was the peptide PLSTRLSVSS from Santa Cruz Biotechnology. For Chk1 kinase activity, the substrate was ChkTide (Sigma). For Cdk2 kinase activity, the substrate was purified histone H1 (Gibco-BRL). Kinase assays were performed by adding 5 µg of peptide, 10 µL of the kinase (fraction collected from column or IP'd kinase) and 10 µl of the γ -[^{32}P]-ATP prepared in assay dilution buffer which contained 25 mM of β -glycerophosphate, 20 mM MOPS, pH 7.2, 5 mM EGTA, 2 mM EDTA, 20mM MgCl₂, 250 µM DTT and 5 µM of β -methyl aspartic acid. The final reaction volume was 30 µl and reaction was incubated at 30° C for 15 min. Subsequently, 15 µl of the assay was spotted on Whatman p81 chromatography filter paper. The spotted filter papers were washed in 1% O-Phosphoric Acid for 40 minutes with multiple changes to remove unbound ^{32}P . The activity of the each sample was obtained by scintillation counting of the filter papers. In some experiments, KN-93, a selective inhibitor of CaMKII was incubated with the kinase at 10 µM final concentration, for 20 minutes at 37°C, prior to assay. In assays of commercial CaMKII, the CaM Kinase II Assay Kit (Upstate, 17-135) was used and Ca²⁺ and calmodulin were added for full activity of the enzyme. However, for immunoprecipitated CaMKII from cells, the presence or absence of Ca²⁺ was found to have no significant effect on the kinase activity.

3.0 siRNA

Gene Silencing by siRNA – 3T6 cells were plated at a density of 2×10^4 cells/cm² in 6-well plates. Twenty four hours after plating, cells were transfected using Oligofectamine (Invitrogen) as recommended by the manufacturer. Cells were harvested 48 h later. Three different CaMKII- β and γ siRNA variants were used together to knockdown expression.

The CaMKII- γ siRNA sequences are as follows:

5'-ACCAAGAAGUUGUCCGCCCGAGAU-3'

5'-AUCUCGGGCGGACCACUUCUUGGUA-3'

5'-UGAGAACUUGCUGCUGGCGAGUAAA-3'

The three CaMKII- β siRNA sequences are as follows:

5'-CCAGUGGACGGGAUUAAGGAAUCUU-3'

5'-AAGAUUCCUUAUCCCGUCCACUGG-3'

5'-CCAAAGCCCGGAAGCAGGAAAUCAU-3'

Chapter 3

Bad: Cross Talk between Cell Cycle and Apoptosis

3.1 INTRODUCTION

All mammalian cells have an intricate network of signaling molecules that are able to sense and interpret internal and external signals. Specific combinations of growth factors act as biological commands that direct cells to proliferate, grow, and differentiate while the absence of growth factors is a signal to either enter a state of quiescence, or to undergo apoptosis. Hemopoietic cells serve as an excellent model system to study apoptosis since their survival is dependent upon specific cytokines, which can stimulate cell growth as well as maintain cell viability by inhibiting apoptosis (301-304). The death of cytokine-deprived hemopoietic cells proceeds via the mitochondrial pathway, involving regulation of various Bcl-2 family proteins (113, 123, 305).

Bad is a pro-apoptotic member of the Bcl-2 family of proteins that is thought to exert a death-promoting effect by heterodimerization with Bcl-xL and disrupting its anti-apoptotic activity. Based on this feature, Bad has been labeled as a death effector; however, there have been several recent studies demonstrating its importance in healthy cells and implicating Bad in cell cycle regulation (270, 275).

Our laboratory reported that Bad is phosphorylated at a novel site, Ser 170 (137). The functional relevance of this phosphorylation was assessed by way of site directed mutagenesis. These studies revealed that transfection with the Bad S170A mutant, which mimics constitutive dephosphorylation at this site, enhanced the ability of this protein to induce apoptosis; whereas, Bad S170D, a mutant which mimics constitutive phosphorylation at S170, results in a protein that is virtually unable to promote apoptosis and causes at least a doubling in cell number over several days. Thus, it was hypothesized that Ser 170 represents a regulatory site able to inhibit Bad's apoptotic function and promote cell cycle progression.

In this chapter, results are presented that have further analyzed the function of the Ser170 residue in controlling Bad's apoptotic function. Here, we also present data supporting a novel cell cycle effect associated with the phosphorylation state of Bad Ser 170 along with a direct interaction between Bad and proteins involved in cell cycle regulation. All immunoblots shown in the following sections, are representative of a minimum of three replicates.

3.2 RESULTS

3.2.1 Phosphorylation at Ser170 Regulates Bad's Apoptotic Function

The BH3-only proteins, including Bad, can have a profound effect on apoptosis regulation by virtue of their interactions with other Bcl-2 family proteins. Bad is phosphorylated at several Ser residues, which control its interaction with Bcl-xL and/or 14-3-3 proteins. To further characterize the functional role of phosphorylation at the Ser-170 site on Bad, which was previously reported in cytokine dependent MC/9 mast cells (137), Flag-tagged Bad (WT) and the corresponding mutants at the Ser170 site (S170A and S170D) were expressed via retroviral transfer in another cytokine-dependent hemopoietic progenitor cell line, FDC-P1, in which Bcl-xL was also over-expressed. FDC-P1 cells were used for apoptosis studies since they were more sensitive to IL-3 withdrawal than MC/9 cells, in terms of increased levels of apoptosis within 24 hours of starvation. In our hands, as well in other laboratories (Dr. Schrader and Dr. McNagny personal communication), MC/9 cells became IL-3 independent and for this reason they were not as useful for apoptosis studies. Thus, while studies monitoring the proliferation and cell cycle status used MC/9 cells, we first wanted to confirm and extend the effect of changes to Bad at Ser170 in another cytokine-dependent cell line. Immunoblot analysis of stably infected cells demonstrated expression of the Flag-tagged mutant forms of Bad proteins (Bad S170A and Bad S170D), as well as wild type Bad, and showed that levels of endogenous Bad were unaffected as compared to control cells expressing only Bcl-xL (Fig. 3.1).

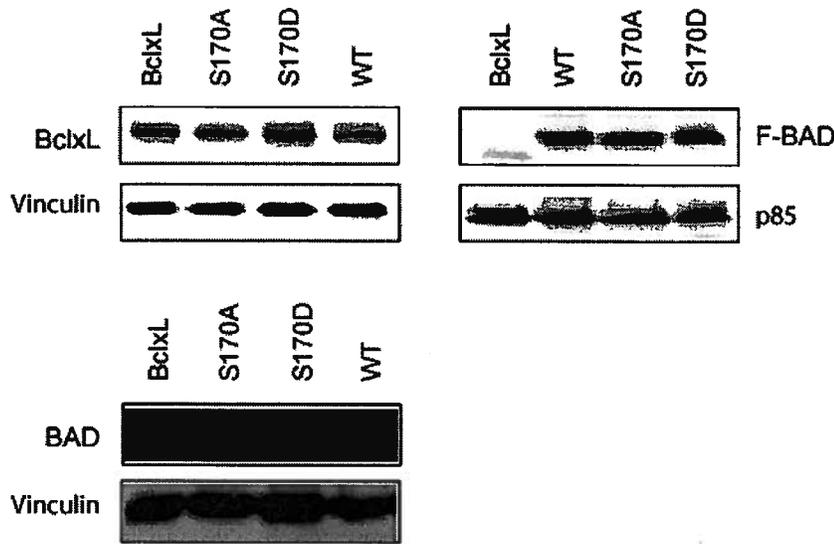


Figure 3.1. Stable infection of Bad S170 mutants

FDC-P1 cells expressing Bcl-xL (BclxL) were retrovirally infected with Flag-tagged Bad-Ser170Ala (S170A) or Bad-Ser170Asp (S170D) or Bad wild type (WT). Cell extracts were separated by SDS-PAGE and immunoblot analysis was performed using anti-Bad (BAD), anti-Flag (F-BAD) and anti-Bcl-xL (BCLxL) antibodies. F-BAD represents the amount of flag-tagged Bad; and BAD represents endogenous levels of Bad protein.

The IL-3 dependent FDC-P1 cells and the transfected variants were measured for signs of apoptosis after 24 hrs of cytokine starvation. Apoptosis was measured using flow cytometry analysis for binding of Annexin-V and incorporation of propidium iodide (P.I.). Annexin-V staining is regarded as an indicator of early stage apoptosis, while P.I. staining occurs at later stages of apoptosis. As shown in Figure 3.2, FDC-P1 cells expressing Bcl-xL were much more resistant to apoptosis following cytokine withdrawal when compared to parental cells. Expression of wild type Bad blocked the pro-survival effect of Bcl-xL as expected, and thus these cells regained cytokine-dependent survival. Supportive of previously published findings, cells expressing Bad-S170D underwent significantly less apoptosis following cytokine starvation than the same cells expressing Bad-S170A (t-test, $p < 0.05$; $n = 3$). When measuring Annexin V staining, cells expressing S170D were not significantly (t-test, $p > 0.05$) different from cells without the added Bad protein, suggesting that mutation of the S170 site to a phospho-mimetic residue is able to

prolong the onset of apoptosis. Conversely, cells expressing Bad-S170A showed similar levels of apoptosis as compared to cells expressing wild type Bad (Fig. 3.2).

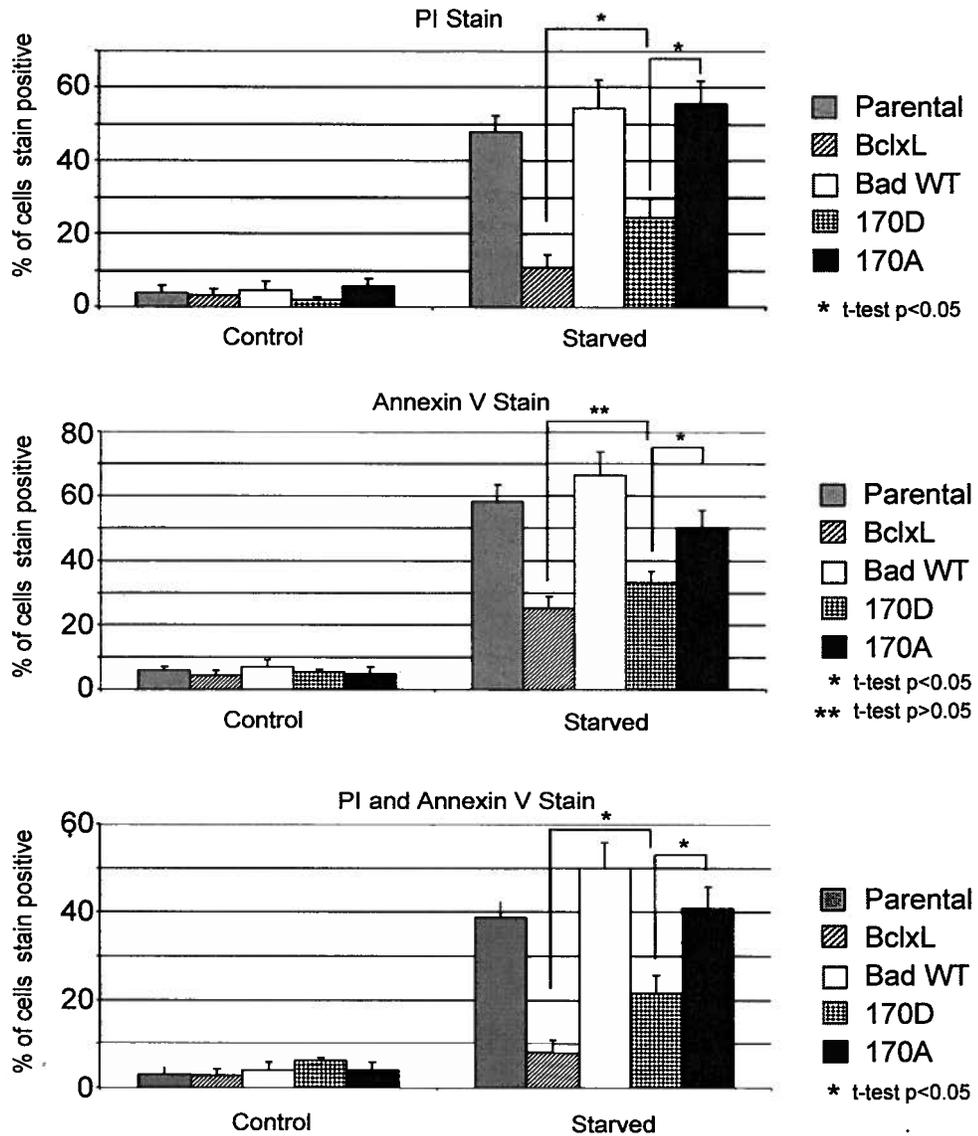


Figure 3.2 S170 modulates Bad's apoptotic function

FDC-P1 cells expressing Bcl-xL (BclxL) and Bad-Ser170Ala (S170A) or Bad-Ser170Asp (S170D) or Bad wild type (WT) were grown in normal conditions (Control) or where starved of cytokine for 24 hours (Starved). Using FACS, apoptosis was calculated as the percentage of cells showing either Annexin V or P.I. positive staining or double positive staining.

3.2.2 Phosphorylation at Ser170 mutes Bad's pro-apoptotic ability

Based on previous work in our lab showing that phosphorylation of Bad at Ser 170 could overcome the apoptotic effect of dephosphorylation of Ser 112; we were interested in testing whether phosphorylation at the Ser 170 site could overcome or diminish the apoptotic effect of Bad mutant 112A/136A/155A (3SA). Using FDC-P1-Bcl-xL cells (Fig 3.3A), apoptotic analysis studies comprising of Annexin V and P.I. staining revealed that cells expressing 112A/136A/155A/170D (3SA170D) had significantly lower levels of apoptosis during cytokine starvation, as compared to cells expressing Bad-3SA or Bad mutant 112A/136A/155A/170A (4SA) (Fig. 3.3B). From these results, it appears that phosphorylation at Ser 170 is not only able to reduce the apoptotic effect of Bad, but can at least partially overcome the potent pro-apoptotic effect of dephosphorylation at the three other key residues of Bad. Furthermore, the dephosphorylation of this site is able to transform Bad into a more potent death effector.

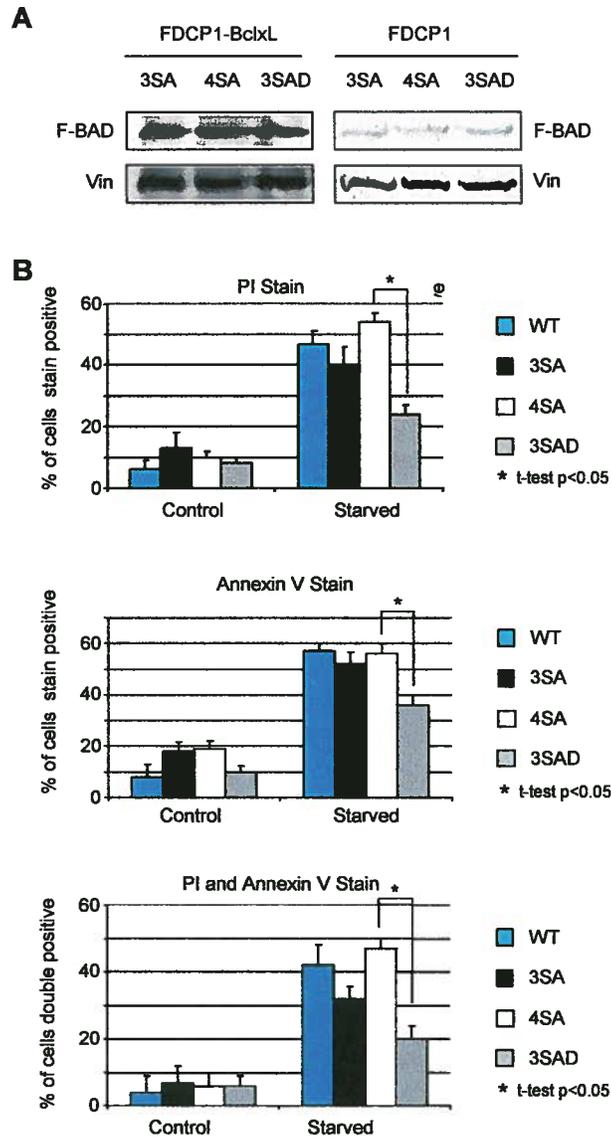


Figure 3.3 S170 modulates Bad's apoptotic function

- (A) FDC-P1 parental cells and FDC-P1 cells expressing Bcl-xL, were retrovirally infected to express Flag-tag-Bad-S112A/S136A/S155A (3SA), Bad-S112A/S136A/S155A/S170A (4SA) or S112A/S136A/S155A/S170D (3SAD). Lysates of cells expressing Bad mutants 3SA, 4SA and 3SAD were separated by SDS page and immunoblotted using anti-Flag antibody (F-Bad). Anti-Vinculin antibody (Vin) was used to ensure equal loading.
- (B) Using FACS, apoptosis was calculated as the percentage of cells showing either Annexin V or P.I. positive staining or double positive staining. Apoptosis was assayed using FDC-P1-BclxL cells expressing Flag-tagged Bad (WT), Bad-S112A/S136A/S155A (3SA) or Bad-S112A/S136A/S155A/S170A (4SA) and Bad-S112A/S136A/S155A/S170D (3SAD) under normal growth conditions (control) or after 24 hour cytokine starvation (Starved).

Further experiments examining the effect of phosphorylation of Ser 170 were performed using parental FDC-P1 cells (not overexpressing Bcl-xL). From this we were able to examine whether the observed increase in survival of cells expressing 3SA170D versus 3SA and 4SA required the co-expression of pro-survival protein, Bcl-xL. FDC-P1 cells were retrovirally infected with Bad constructs: 3SA, 4SA and 3SA170D. Cells were placed under selection for 48 h at which point cells were washed in order to remove dead non-transfected cells and debris. Following the initial selection, apoptosis was measured for 96 hours following infection under normal growth conditions. The high level of apoptosis observed when cells express Bad mutant 3SA or 4SA is consistent with our earlier findings as well as those of others (287). As shown in Figure 3.4, we did not observe a significant increase (t-test, $p > 0.05$) in levels of apoptosis in cell expressing Bad mutant 4SA as compared to 3SA; nevertheless, expression of either 3SA or 4SA showed a consistently high level of apoptosis. Interestingly, cells expressing Bad mutant 3SA170D showed a marked and significant decrease (t-test, $p < 0.05$) in the level of apoptosis as compared to 3SA and 4SA at 72 and 96 hours. In fact, FDC-P1 cells expressing 3SA170D continued to grow for weeks under constant selection without co-expression of Bcl-xL.

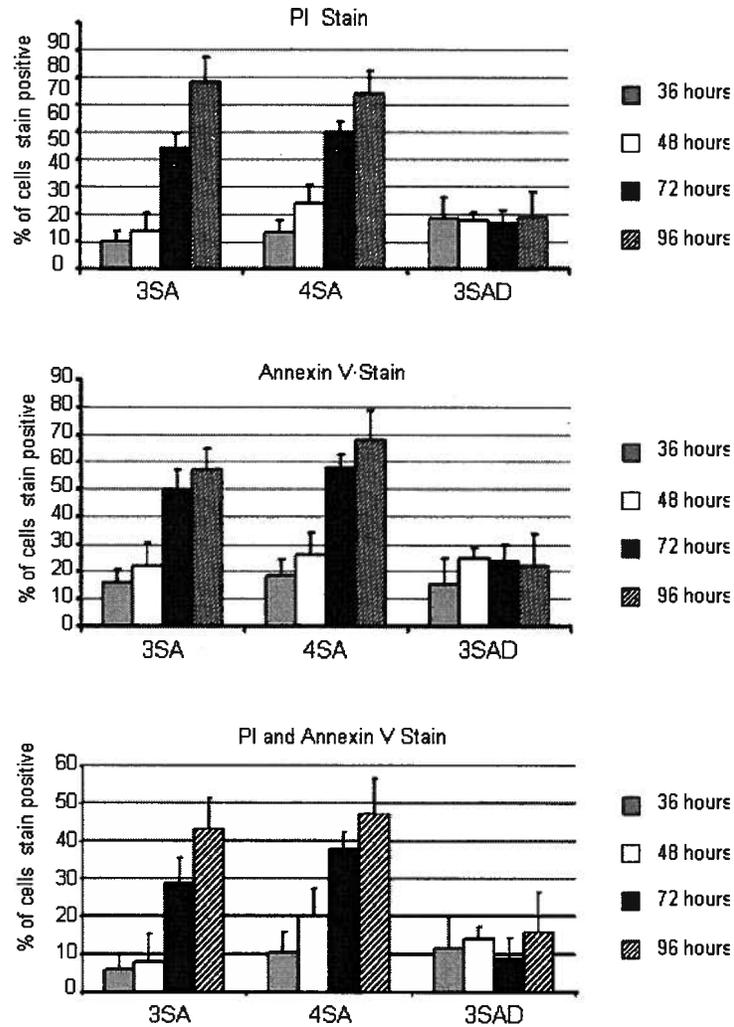


Figure 3.4 Mutation mimicking phosphorylation at Ser170 inhibits Bad's apoptotic function without overexpression of Bcl-xL

Using FACS, apoptosis was calculated as the percentage of cells showing either Annexin V or P.I. positive staining or double positive staining. Apoptosis was assayed using FDC-P1 cells transfected with Flag-tagged Bad-S112A/S136A/S155A (3SA), Bad-S112A/S136A/S155A/S170A (4SA) or Bad-S112A/S136A/S155A/S170D (3SAD) at 36, 48, 72 and 96 hours post transfection.

3.2.3 Phosphorylation of Bad Ser170 Regulates Novel Cell Cycle Effect

As mentioned, previous studies performed by Dramsi et al. (137) showed that a S170A mutation enhanced the pro-apoptotic activity of Bad, while the S170D Bad mutant showed very little apoptotic activity. Beyond the apoptotic effect, there was also the unexpected finding that the Bad S170D not only rendered Bad less apoptotic, but it also promoted cell proliferation.

We followed up the earlier studies, using MC/9 cells, to confirm that Bad's ability to affect cell cycle progression could be observed by comparing MC/9-BclxL-S170A and MC/9-BclxL-S170D cells (Fig. 3.5A). Cells expressing Bad S170A were found to require fewer passages to maintain appropriate cell numbers. In other words, these cells appeared to have a slower doubling time. From this we were interested in knowing if the increase in doubling time was due to a higher percentage of cells undergoing apoptosis or whether the cells have a slower rate of cell cycle. Using FACS analysis, double positive (P.I. and Annexin V) staining was assessed to determine apoptosis levels of MC/9-Bcl-xL cells expressing Bad S170A, Bad S170D and Bad WT (Fig. 3.5B). We observed nearly equal and very low level of apoptosis when cells were maintained in normal growth conditions (t-test $p > 0.05$; comparing BclxL to other cell types). These results were consistent with previous results (137) produced in our lab, using these MC/9 cells, and suggest that phosphorylation of Bad at S170 may be affecting cell cycle progression.

We examined the growth rate of MC/9 cells expressing Bad Ser170 mutants, by seeding cultures with the same number of cells and recorded cell numbers over 5 days (Fig. 3.5C). By the third day, there were a significantly lower (t- test, $p < 0.05$) number of cells expressing Bad-S170A as compared to cells expressing Bad-S170D and BclxL alone. However, the cells expressing Bad S170A had the longest doubling time, but in these analysis, the rate was not significantly different from cells expressing Bad WT (t-test, $p > 0.05$). Since apoptosis levels were nearly identical for all cells regardless of Bad mutant expressed, we were increasingly confident that the difference in cell numbers was due largely to the effects of mutations of Bad S170 affecting the cell cycle rate.

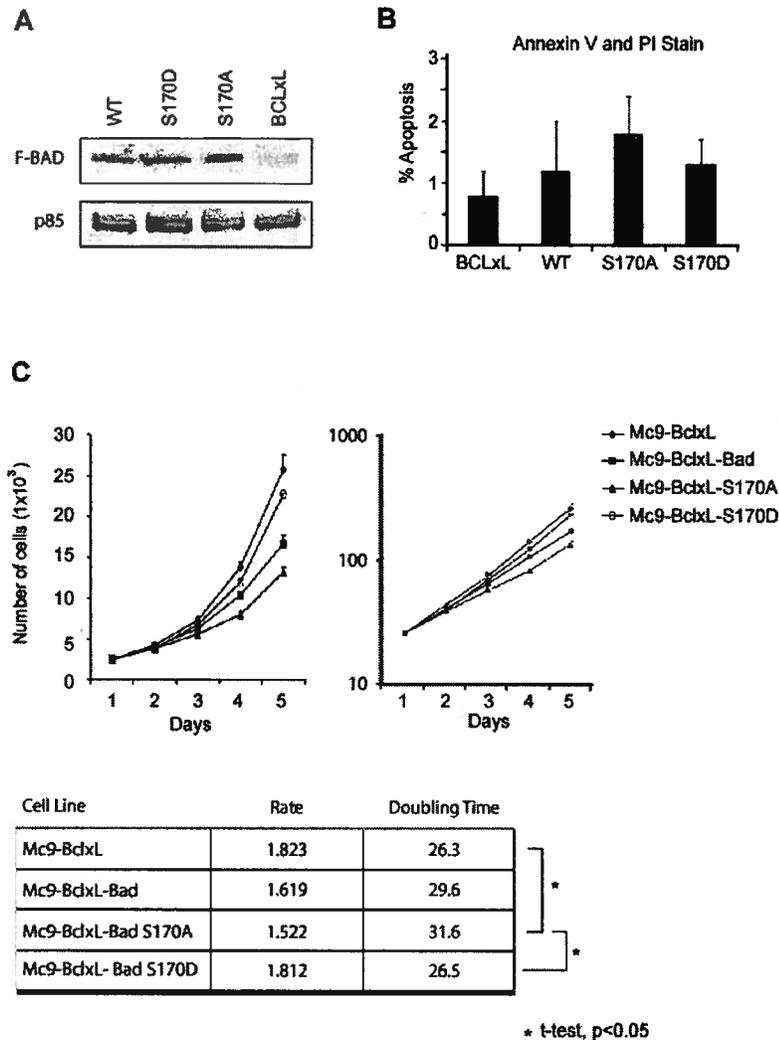


Figure 3.5 Cells Expressing Bad S170A Have a Longer Doubling Time

- (A) Flag-tagged Bad and the corresponding mutants at the Ser170 site (S170A and S170D) were expressed via retroviral transfer in MC/9 cells in which Bcl-xL was also overexpressed (BCLxL). Immunoblot analysis of stably infected cells demonstrated expression of the Flag-tagged Bad (WT) and mutant forms of Bad proteins.
- (B) MC/9-BclxL cells expressing Bad-wt (WT), Bad-S170A (S170A) or Bad-S170D (S170D) were stained with PI and Annexin-V. Using FACS, apoptosis was calculated as the percentage of cells showing Annexin and PI positive staining. Experiment repeated three times ($n = 3$).
- (C) MC/9-Bcl-xL cells expressing Bad WT, Bad S170A, or Bad S170D were seeded at a concentration 25,000 cells/mL and cell numbers were counted every 24 hours for 5 days. The Rate is defined as the multiple of times a cell doubles every twenty four hours. The Doubling Time is define the amount of time require for cells to double in number. Experiment repeated three times ($n = 3$).

3.2.4 Dephosphorylation at Bad Ser 170 Stalls Cell Cycle at S Phase

Careful examination at Bad's ability to affect cell cycle was accomplished by measuring the number of cells in G1, S and G2 stages of the cell cycle (Fig. 3.6A). Through this cell cycle analysis we observed that cells expressing Bad S170A mutant showed a consistent increase in the number of cells in S phase as compared to cells expressing Bad wild type and Bad S170D (Fig. 3.6A). The increase in the number of cells in S phase is indicative of an increase in the time required to transit through S phase; and as such we hypothesized that expression of Bad S170A prolongs the time during which cells are in S phase. In order to examine transit time through S phase, cells were first synchronized in G1 stage of the cell cycle using Hydroxyurea. Cells were released from cell cycle block and analyzed via FACS at various time points after release (Fig. 3.6B). The percentage of cells in either G1, S or G2 stage of the cell cycle was determined. From this we observed that expression of either Bad S170A or S170D had little to no affect on the the time require to enter S phase; however, we did note a digression in the time required to transit S phase. A majority of cells expressing Bad S170A are in S phase at 8 hours after release where cells expressing Bad S170D, Bad wt, or BclxL alone were shown to have entered G2 phase by that time. None of the mutants caused any difference in the exit of cells from G1, while the cells expressing BadS170A took longer to appear in G2. These results reaffirm our hypothesis that expression of Bad S170A slows progression through S phase of the cell cycle, suggesting that phosphorylation of Bad at Ser 170 may control both Bad's ability to induce apoptosis and Bad's novel cell cycle effect.

The results in Fig. 3.6 were obtained using mixed populations of infected cell lines. Thus, to further confirm Bad mutant S170A's effect on cell cycle, we were interested in studying the effect of expression levels of respective Bad Ser170 mutants on cell cycle progression. To do so, we created several clonal cell lines expressing varying levels of either Bad wt, Bad S170A or Bad S170D (Fig 3.7A). This enabled a more precise analysis as cells with known expression levels of respective forms of Bad.

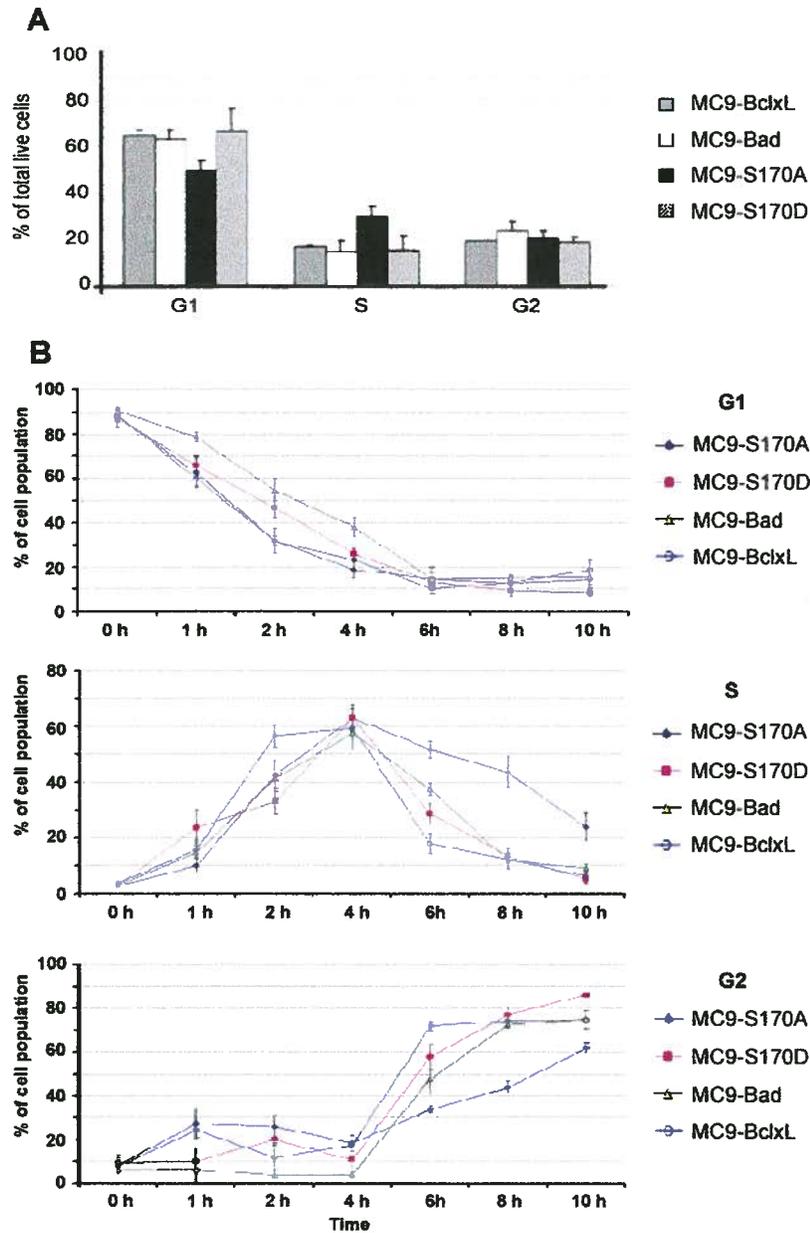


Figure 3.6 Bad S170A Expression Stalls Cells in S Phase

- (A) MC/9-Bcl-xL and MC/9-Bcl-xL Flag-Bad WT, S170A and S170D were fixed and stained with PI and analyzed using FACS. Cell cycle analysis was performed by measuring DNA content and cell size. Cells were classified as being in G1, S or G2 phase.
- (B) MC/9-Bcl-xL and MC/9-Bcl-xL Flag-Bad WT, S170A and S170D were synchronized at G1 and released. Cells were fixed and stained with PI and analyzed using FACS. The percentage of cells in G1, S or G2 was determined over a 0 to 10 hour time course after release.

Cells expressing higher or lower levels of Bad mutant (Bad S170A, Bad S170D) and wild type proteins were directly compared (Fig. 3.7B). This experiment supported initial findings and showed once again that cells expressing Bad S170A appeared to take longer to transit through S phase as compared to cells expressing Bad S170D (Fig 3.6). Interestingly, the cell line showing high level of expression of Bad S170D showed a decrease in the percentage of cells in S phase. Conversely, increase expression of Bad S170A resulted in an even greater increase in the number of cells in S phase. Therefore the effects of Bad S170A and S170D expression appears to be dose dependent, which further validates our initial findings indicating that phosphorylation of Ser170, or lack of, is responsible for the observed cell cycle effect.

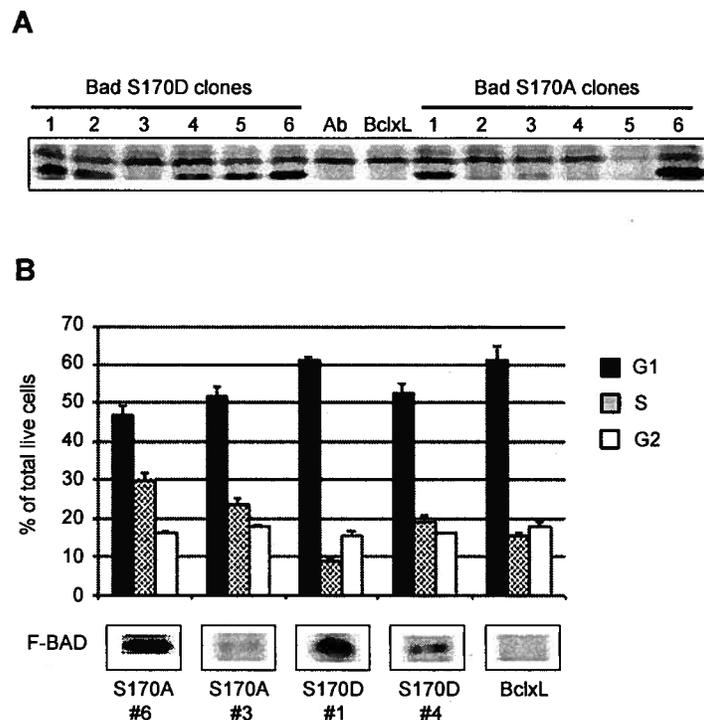


Figure 3.7 Increased levels of Bad S170A or Bad S170D results in increased cell cycle effect.

- (A) Lysates from clonal populations of MC/9-BclxL cells (BclxL) expressing Bad S170A and Bad S170D, were immunoprecipitated using anti-Flag antibody and western blotted using anti-Flag antibody.
- (B) Cell cycle analysis was performed using clonal populations of MC/9-BclxL cells (BclxL) expressing Bad S170A and Bad S170D. Cell lysates of respective clonal populations were separated by SDS-PAGE and immunoblotted using anti-Flag antibody.

3.2.5 Bad Interacts with Cell Cycle Machinery

Based on the above findings demonstrating that Bad, depending on its phosphorylation state at Ser 170, can influence the time required for cells to transition through S phase; we were interested in examining the molecular mechanism by which Bad is able to affect cell cycle progression. We first examined the possibility that Bad interacts with cell cycle regulatory proteins. This was assessed by means of immunoprecipitation experiments. Endogenous Bad was immunoprecipitated and bound proteins were separated using SDS PAGE. Immuno-detection was performed using a series of antibodies against a wide range of cell cycle regulatory proteins (for example Cdk1, Cdk2, Cdk4, Cdk6, Cyclin B, Cyclin D, p27, and p21); however we were only able to consistently detect Cyclin-Dependent Kinase 2 (Cdk2) within the immuno-complex. This Bad/Cdk2 interaction was observed using both MC/9 cells and primary mast cells (mouse) (Fig. 3.8A) while immunoprecipitating endogenous Bad.

Based on the above results, we were interested in examining if the observed Bad/Cdk2 interaction is part of a greater complex of proteins. More specifically, we were interested in knowing if Cdk2's G1/S cyclin partners, Cyclin A and E, also formed part of this interaction. Performing similar experiments as described above, we observed a consistent detection of Cyclin E; however, we were unable to detect any Cyclin A within the complex (Fig. 3.8A). To further strengthen the specificity argument of these interactions, cells expressing Flag-tagged Bad were used. When Flag-Bad was immunoprecipitated using anti-Flag antibody, association with both cyclin E and Cdk2 were also observed (Fig. 3.8B), showing that this was not a result of non-specific co-immunoprecipitation.

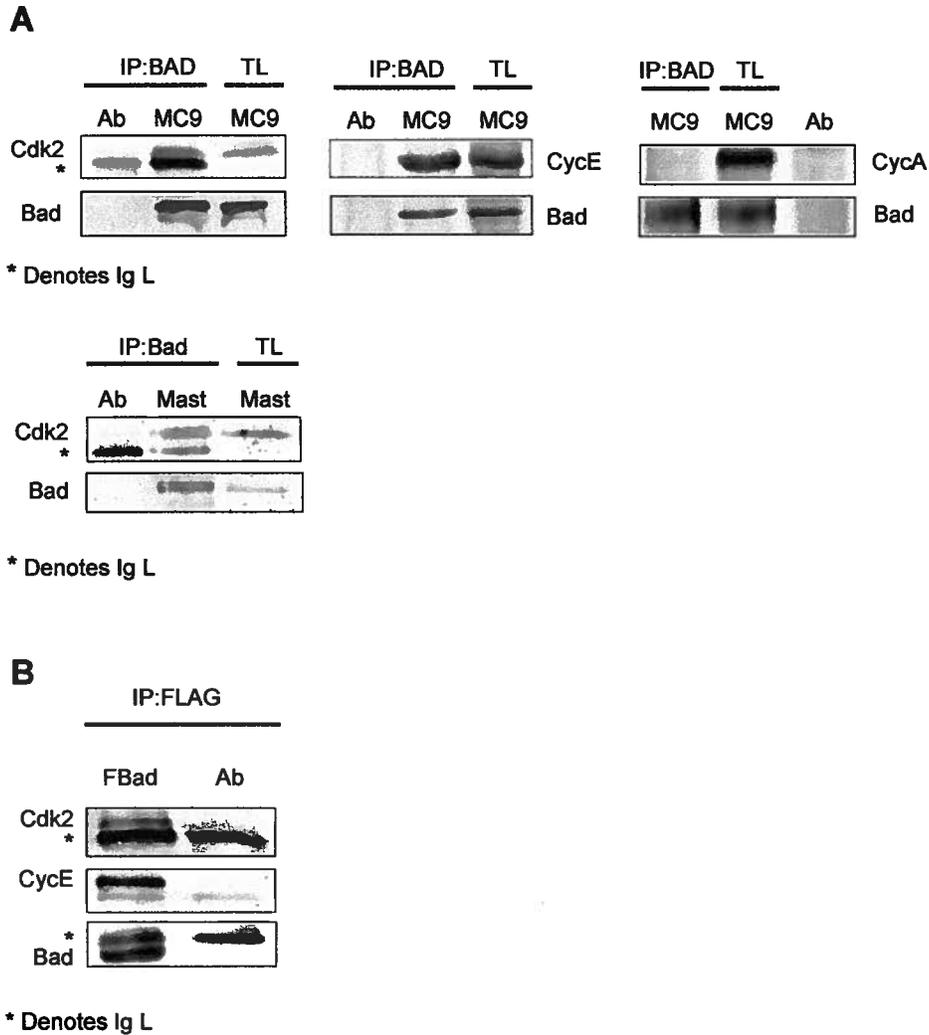


Figure 3.8 Bad Co-Immunoprecipitates with Cdk2 and CyclinE

- (A) MC/9 (MC9) and bone-marrow derived mast (Mast) cell extracts were immunoprecipitated (IP) using anti-Bad antibody and the immuno-complex was separated by SDS-PAGE and immunoblotted with anti- Bad (Bad), Cdk2 (CDK2), Cyclin E (CycE) and Cyclin A (CycA) antibodies.
- (B) MC/9-BclxL-Flag-tagged Bad (FBad) cell extracts were immunoprecipitated (IP) using anti-Flag antibody and separated by SDS-PAGE and immunoblotted with anti-Bad (BAD), Cdk2 (CDK2) and Cyclin E (CycE) antibodies.

We also performed immunoprecipitation experiments using Cdk2 and Cyclin E antibodies and immuno-blotted using Bad antibody (Fig. 3.9A,B). In both cases, Bad was detected within the immunoprecipitated complex. Together, these results suggest that Bad, Cdk2, and Cyclin E form a complex or are part of a larger complex of proteins.

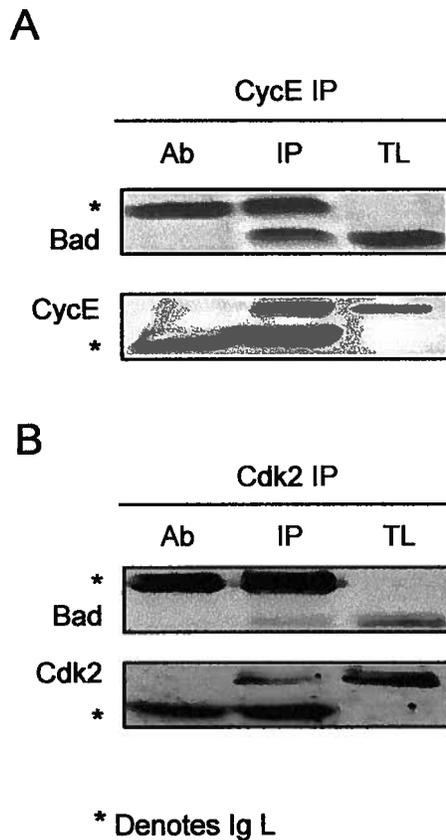


Figure 3.9 Cdk2 and CyclinE Co-Immunoprecipitate with Bad

- (A) MC/9 cell extracts were immunoprecipitated (IP) using anti-CyclinE antibody (CycE IP) and the immuno-complex was separated by SDS-PAGE and immunoblotted with anti- Bad (BAD) and Cyclin E (CycE) antibodies.
- (B) MC/9 cell extracts were immunoprecipitated (IP) using anti-Cdk2 antibody and separated by SDS-PAGE and immunoblotted with anti-Bad (Bad), Cdk2 (CDK2) antibodies.

3.2.6 Bad's Interaction with Cdk2 and Cyclin E occurs in the cytosol

Since both Cyclin E and its catalytic subunit, Cdk2, translocate to the nucleus where they regulate the phosphorylation of many proteins including retinoblastoma, it was of interest to determine if the observed Bad/Cdk2 and Bad/CyclinE interactions occur specifically in the nucleus. Figure 3.10A is meant to demonstrate the efficacy of the nuclear preparations. This was performed by immunoblot analysis of both the cytosolic and nuclear fractions using GSK-3 and Histone H1 antibodies. GSK-3 is known to localize to the cytosol and Histone H1 is found within the nuclear fraction. The detection of both these proteins enabled us to demonstrate that our nuclear preparations had minimal contamination of cytosolic proteins (Fig. 3.10A). Nuclear and cytosolic lysate fractions were immunoprecipitated using anti-Bad antibody and immuno-detection of bound proteins was performed using anti-Cdk2 and Cyclin E antibodies. We did not detect any interaction between Bad and Cdk2 or Cyclin E when using nuclear fractions; though, we did observe Bad/Cdk2/CyclinE interactions when using cytosolic fractions (Fig. 3.10B,C). Since there is little evidence in the literature that Bad is present in the nucleus, in addition to the great majority of Cdk2 and Cyclin E expression being in cytosolic fraction, it was not surprising that no association of the proteins in the nucleus was detected.

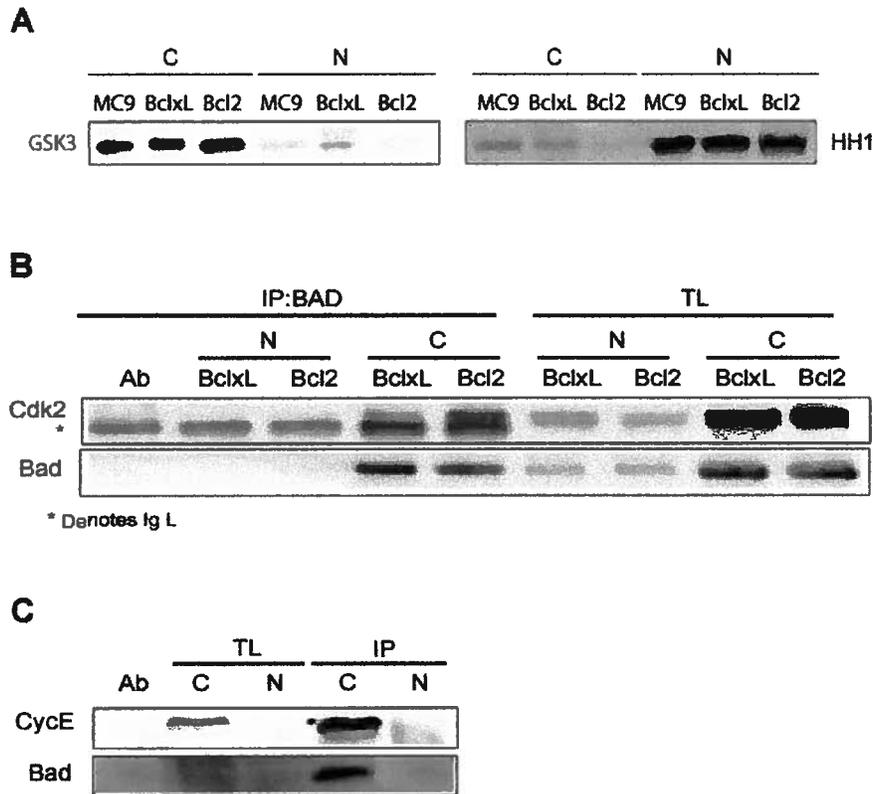


Figure 3.10 Bad interacts with Cdk2/CyclinE in the cytosol

- (A) Cytosolic and nuclear fractions of MC/9 (MC9); MC/9- BclxL (BclxL); and MC/9-Bcl2 (Bcl2) cell lysates were separated by SDS-PAGE and immunoblotted using anti-Gsk3 (GSK3) and Histone H1 (HH1) antibodies.
- (B) Nuclear (N) and cytosolic (C) fractions of MC/9 cells expressing either BclxL (BclxL) or Bcl-2 (Bcl2) were used to perform immunoprecipitation experiments using anti-Bad antibody (IP:BAD), followed by separation by SDS-PAGE and immunoblotting with anti- Bad (BAD)and Cdk2 (CDK2),
- (C) Nuclear (N) and cytosolic (C) fractions of MC/9 cell extracts were used to perform immunoprecipitation experiments using anti-Bad antibody (IP), followed by separation by SDS-PAGE and immunoblotting with anti-Cyclin E (CycE) and Bad (Bad) antibodies.

It has been recently reported that Bad/Bcl-xL complex affects cell cycle progression (248); therefore, we investigated the effects of overexpressing Bad's pro-survival binding partners, Bcl-xL and Bcl-2, on the formation of the Bad/Cdk2 and Bad/Cyclin E complexes. Using parental MC/9 and MC/9 cells overexpressing either Bcl-xL or Bcl-2 (Fig. 3.11A), immunoprecipitation experiments using anti-Bad antibody showed that overexpression of BclxL, as compared to the overexpression of Bcl-2 and endogenous levels of Bcl-xL, negatively affected the amount of Cdk2 associated with Bad (Fig 3.11B); however, we did not observe a significant change in the amount of associated Cyclin E (Fig 3.11C).

Together these findings indicate Bad, a pro-death Bcl-2 member, is able to hinder cell cycle progression, specifically at the G1-S transition; and Bad is able to interact directly with established G1/S cell cycle regulators Cdk2 and Cyclin E in the cytosol; and furthermore, expression of Bad's survival antagonist, Bcl-xL, appears to effect Bad's ability to associate with Cdk2.

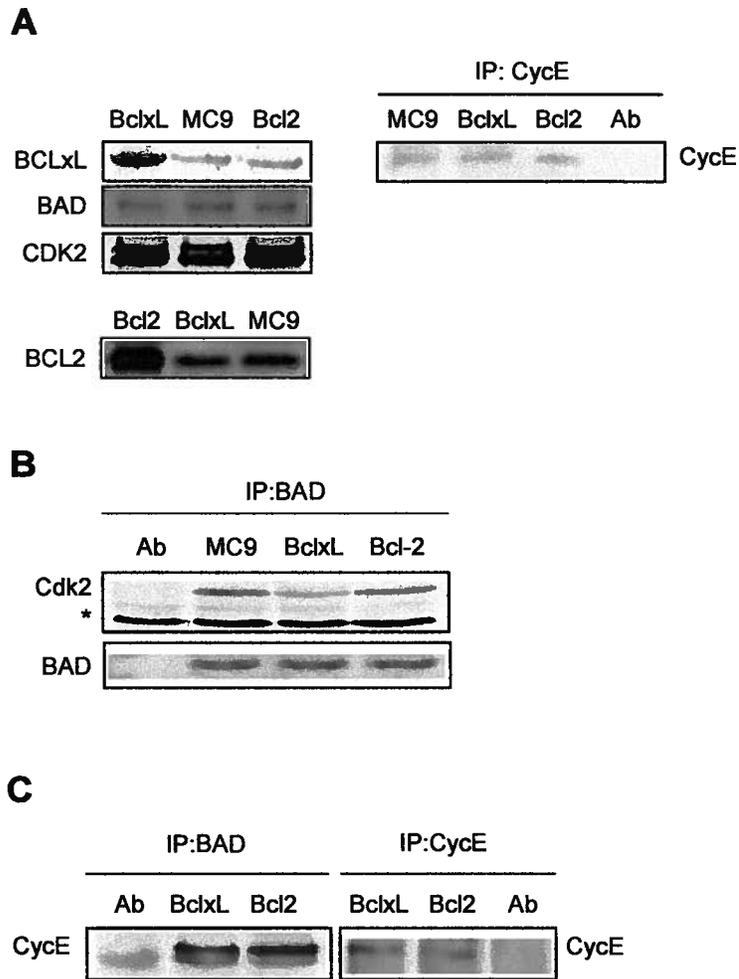


Figure 3.11 Bcl-xL expression interferes with Bad/Cdk2 interaction

- (A) MC/9 (MC9); MC/9- BclxL (BclxL); and MC/9-Bcl2 (Bcl2) cells were assessed for Bcl-xL (BCLxL), Bcl-2 (BCL2), Bad (BAD), and Cdk2 (CDK2) expression. Cyclin E expression levels were assessed via immunoprecipitation of Cyclin E using anti-Cyclin E antibody (CycE).
- (B) MC/9 (MC9); MC/9- BclxL (BclxL); and MC/9-Bcl2 (Bcl2) cell lysates were immunoprecipitated using anti-Bad antibody. Proteins were separated by SDS-PAGE and immunoblotted using anti-Cdk2 (CDK2), Bad (BAD) and
- (C) anti-Cyclin E (CycE) antibody.

3.2.7 Bad's Interactions and Cell Cycle Stage

Since overexpression of Bad S170A and Bad S170D showed a cell cycle effect at S phase transition, we were interested in determining whether Bad's interaction with Cdk2 and Cyclin E was altered at various stages of the cell cycle. Using a G1 cell cycle inhibitor, hydroxyurea, which arrests cells by blocking DNA synthesis, we were able to synchronize cells (Fig 3.12A). Over a specified time course we analyzed the expression of Bad, Cdk2, and Cyclin E (Fig. 3.12B,C) and over the same specified time course we determined if the observed Bad/Cdk2/CyclinE interactions change throughout the cell cycle. These experiments revealed that Cyclin E expression was highest at 0 h and decreased over the observed time points (0, 2, 4, 6, 8 h) after hydroxyurea release, whereas Cdk2 levels remained relatively constant. Bad IP experiments over a similar time course showed that Bad/Cdk2 interaction remained unchanged after release (Fig 3.12B), while the amount of interaction between Bad and Cyclin E changed over the course of the cell cycle. More precisely, we observed a greater amount of Cyclin E complexed with Bad at G1/S and early S phase of the cell cycle (Fig 3.12C). At this point we cannot be sure whether the observed changes in Bad/Cyclin E interactions are simply the result of increased expression of Cyclin E at late G1 and early S phase (Fig 3.12C) or this interaction is truly indicative of a molecular mechanism, involving Bad and Cyclin E, that acts to regulate cell cycle progression. However, since the level of Cdk2 associated with Bad appears to remain constant, the increase co-immunoprecipitation of Cyclin E may result from its association with Cdk2.

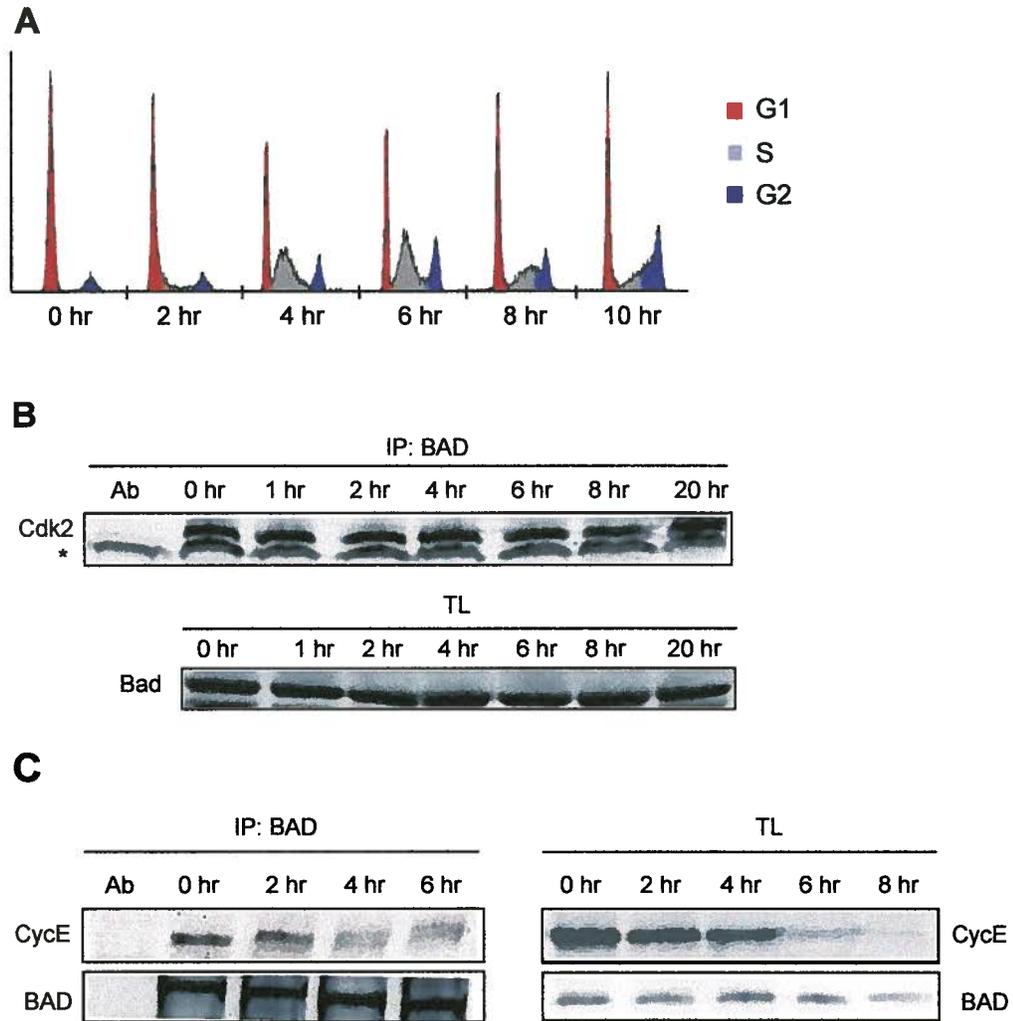


Figure 3.12 Bad associates with CyclinE during G1/S phase

- (A) MC/9 cells were synchronized at late G1/S. Times refer to hours after release. Cells were fixed and stained with PI and were analyzed using FACS.
- (B) Cell extracts, from corresponding time points, were immunoprecipitated using anti-Bad antibody (IP:BAD). Immunoprecipitated proteins and total lysates (TL) from corresponding extracts were separated by SDS PAGE and immunoblotted with anti- Cdk2, and Bad antibodies.
- (C) Time point extracts were immunoprecipitated using anti-Bad antibody (IP:BAD). Immunoprecipitated proteins and total lysates (TL) were separated by SDS PAGE and immunoblotted with anti- Cyclin E and Bad antibodies.

3.2.8 Co-Immunoprecipitated Cdk2 shows kinase activity against Histone H1 protein

Since we have shown that Bad co-interacts with Cdk2 and CyclinE, we were interested in determining whether the co-immunoprecipitated Cdk2 was active. Histone H1 peptide substrate, an established Cdk2 substrate (306, 307), was used as a Cdk2 substrate while performing kinase assays. Using a G1 chemical cell cycle inhibitor, hydroxyurea, we synchronized MC/9 cells at late G1 phase of the cell cycle. Cell lysates from designated time points after release were used to immunoprecipitate Bad. We evaluated whether Bad's co-immunoprecipitated complex showed kinase activity against Histone H1 and whether the activity changed depending on what stage of the cell cycle the immunoprecipitate was assessed. These experiments reveal that the anti-Bad immunoprecipitated complex has kinase activity against Histone H1 (Fig. 3.13). This result suggests that Cdk2 may be active, though it is difficult to say with certainty that the observed activity is not a consequence of another kinase that forms part of the co-immunoprecipitated complex that is also able to phosphorylate Histone H1. We did however observe a consistent and significant (t-test, comparing the 3 hour time point with other time points) increase in activity in immunoprecipitated fractions at the 3 hour time point after release compared to all other time points except for 0 hour. This finding corresponds with published data (307, 308) showing peak Cdk2 activity during G1/S phase transition and our data showing a majority of cells entering S phase within 4 hours of Hydroxyurea release (see Fig 3.6 and Fig 3.12). Thus, the increase in activity is likely due to the overall increase in Cdk2 activity rather than an increase in Cdk2/Bad interaction since we have shown that the level of Cdk2/Bad interaction is not affected by cell cycle stage (see Fig. 3.6). Moreover, we can safely suggest that the increased Cdk2 activity in the anti-Bad ip's may be directly correlated to the increased level of cyclin E associated with Cdk2/Bad complex..

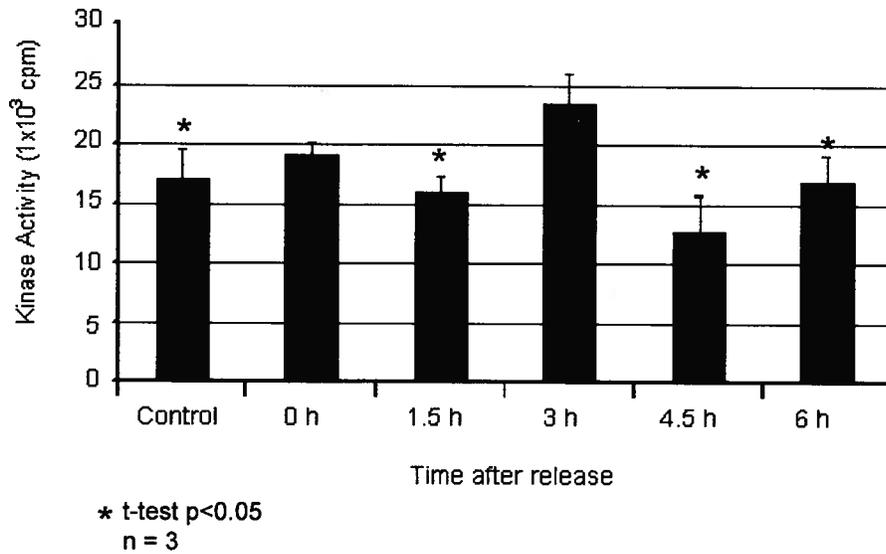


Figure 3.13 Bad's co-immunoprecipitated complex shows kinase activity against Histone H1 protein

MC/9 cells were synchronized using Hydroxyurea and then released. Lysates corresponding to various time points after release were used to immunoprecipitate Bad using Bad antibody. Control represents non-synchronized cells. Kinase assays were performed against Histone H1 peptide. The consistently observed peak in activity at 3 hours after release was statistically (*) different from all other time points measured except 0 hour. The results presented are representative of three replicate experiments (n=3).

3.3 DISCUSSION

Regulation of apoptosis and cell cycle control are both important to oncogenesis and vital to normal homeostasis. Disruption of cell cycle regulation can result in apoptosis and demise of the organism (309-311). Conversely, failure to execute apoptosis correctly can lead to a cancerous phenotype with abnormal cell cycle kinetics (312, 313). This homeostasis had long been thought as a result of two separate processes, but there is growing evidence indicating that the processes of proliferation and apoptosis are coupled.

The first portion of this chapter presents data supporting that Bad is highly regulated by numerous phosphorylation events, at multiple Serine residues (35, 75, 314, 315), for which Bad thus serves as a cell 'sensor.' We demonstrate that phosphorylation of Bad at Ser170, as shown indirectly by expression of a mutant form of Bad in which the Ser170 site is mutated to the phospho-mimetic residue aspartate, can reduce its apoptotic effect. This was shown in experiments comparing the effects of Bad mutants: 3SA versus 3SA170D and 170A versus 170D. The apoptotic ability of Bad, depending on the level of dephosphorylation, strongly supports Bad's role as an integral sensor of apoptotic and survival signals. Furthermore, we show that the neutralizing effect of phosphorylation of Ser 170 may act independently of Bcl-xL expression. Thus, Bad's ability to promote apoptosis may not be limited to a mechanism in which Bad antagonizes Bcl-xL. It is important to note that since cells studied are overexpressing proteins, one should view the observed cellular effects with caution. The various fold increase in Bad expression may result in cells becoming far more sensitive to cytokine withdrawal regardless of the phosphorylation state of Bad. The high expression of Bad may result in the inability of the cell to contain or control Bad's activity, which may result in Bad protein being located in cellular compartments or interacting with various proteins that in normal conditions it would not.

The Bcl-2 family, an established class of regulators of apoptosis, has more recently been associated with other physiological roles such as cell cycle regulation (250-252, 266, 272, 273, 277). For instance, Bcl-2 and Bcl-xL have been shown to delay cell

cycle re-entry from the resting G₀ state, and BAX can accelerate entry into S phase (249, 316). Bid^{-/-} knockout mice have revealed an intriguing role in both cell proliferation (317) and DNA damage response via ATM (318, 319); and more recently, our group has shown that Mcl-1, a Bcl-2 family member known to suppress cell growth when overexpressed, interacts with inactive Cdk1 in the nucleus (298), as well as playing a role in checkpoint response via Chk1 kinase (320).

Other related studies have implicated Bad, a member of the 'BH3-only' sub-group of the Bcl-2 family of proteins (32, 87, 314), in cell cycle regulation (137, 248, 321). A recent study has shown that Bad can cause continued cell cycle progression in serum starvation or contact inhibition conditions and this ability to overcome cell cycle arrest is directly affected by the heterodimerization of Bad and Bcl-xL (248). More specifically, it has been suggested that the ability of Bad/Bcl-xL heterodimers to push cells into S phase without causing significant apoptosis is an indication that the function of Bad may not merely be to inactivate Bcl-xL or Bcl2, but that Bad may be actively involved in cell cycle control. In 2001, Dramsi et al. (137) reported that Bad, when phosphorylated at Ser 170, loses much of its apoptotic potency and promotes cell cycle progression. This cell cycle phenomenon, along with parallel studies involving Mcl-1 and G2/M cell cycle regulators being performed in our lab, initiated many of the studies presented involving Bad and G1/S cell cycle regulatory proteins. In this chapter we have presented evidence that dephosphorylation of Ser 170 not only primes the cell for death, but also hinders cell cycle progression. Specifically, cells expressing Bad S170A, a mutation mimicking dephosphorylation, had a higher percentage of cells in the S phase of the cell cycle; indicating the cells were taking longer to duplicate their DNA as compared to cells expressing a mutant form mimicking phosphorylation (Bad S170D) and wild type Bad (Bad WT). This was further supported when examining individual mutant clones with varying levels of expression. If Bad's phosphorylation state at Ser 170 was affecting cell cycle progression, one would expect that increasing the expression level of either mutant should magnify the effects, and this is precisely what we observed. In addition, it is important to note that the observed cell cycle effect was unrelated to apoptosis, since these cells, regardless of which Bad Ser 170 mutant was expressed, underwent similar low levels of apoptosis when maintained in the appropriate growth conditions. These

observations together suggest that phosphorylation of Bad at Ser170 can control a unique function of this protein. Hence, Bad may be playing a role in the coupling of two processes involved in cell expansion: cell survival and cell proliferation.

Even though several studies have implicated Bcl-2 family members with cell cycle regulation, very few studies have revealed any direct connection between the cell cycle and apoptotic machines. Here we have presented evidence that Bad was able to associate with the cyclin dependent kinase, Cdk-2, and its G1/S cyclin partner, Cyclin E. This association was tested using antibodies against Bad, Flag-Bad, Cdk2 and Cyclin E. In all cases, independent of which antibody is used for immunoprecipitation, we were able to observe Bad's association with Cdk2 and Cyclin E.

The G1/S cell cycle checkpoint represents a critical period for cells to commit to growth arrest or proliferation. This stage also represents a period where the cells are responsive to cytokines; therefore, once cells are committed to enter S phase, additional stimulation by growth factors is surplus to the cell's proliferation requirements (214). That is, once the Rb is activated by phosphorylation by Cyclin D/Cdk-4, or -6 complexes in early G1 and in late G1 by Cyclin E/Cdk2 complexes; and Rb is able to dissociate from its repressor, E2F1, the cell has committed to entering the cell cycle process. Since Cdk2/Cyclin E phosphorylates Rb in the nucleus, we were interested in examining whether the observed association between Bad, Cdk2 and Cyclin E was altered depending on its cellular localization. From these studies we observed no interaction in the nucleus and all the observed interaction was shown to occur in the cytosol. To determine whether Bad's association with Cdk2 and Cyclin is altered depending on the stage of the cell cycle, we synchronized the cells and immunoprecipitated Bad from cell lysates from G1 through late S phase. Bad's association with Cdk2 did not alter throughout the cell cycle, however Bad showed increased association with Cyclin E during late G1 and early S phase. The association decreased substantially by mid S phase. This observation is complicated by the fact that Cyclin E showed its greatest levels of expression during the same period of the cell cycle; therefore it is difficult to say with certainty that the increase in association between Bad and Cyclin E is not a reflection of the increased Cyclin E expression as opposed to an insight into the mechanism by which

Bad may function to regulate cell cycle progression. We also tested whether the immunoprecipitated complex show kinase activity against Histone H1, a known Cdk2 kinase target. We showed that the immuno-complex has kinase activity against Histone H1 and this activity is highest when cells are in S phase. Together this, supports our finding that Bad interacts with Cdk2, however it has not yet been shown with certainty that Cdk2 is responsible for the observed kinase activity.

Further attempts to understand which factors may be affecting Bad's association with the above cell cycle regulators lead us to test whether expression of Bad's apoptosis antagonists, Bcl-xL and Bcl-2, had an effect. We did not observe an effect on Bad's association with cyclin E and Cdk2 when expressing Bcl-2; however we did observe a decrease in the levels of association between Bad and Cyclin E in cells expressing high levels of Bcl-xL. Since both Bcl-xL and Bcl-2 are known Bad heterodimer partners, the difference observed may be due to Bad's higher affinity for Bcl-xL as compared to Bcl-2. On the other hand, the observed difference may in fact be a hint into how Bad's association with Cdk2 and Cyclin E is controlled.

We performed further studies examining the effect of phosphorylation of Ser 170 and Bad's association with Cdk2, Cyclin E and Bcl-xL. In support of previously published findings, we did not observe a relationship in the levels of Bad and Bcl-xL association and the phosphorylation state of Bad Ser 170. When examining the levels of Cdk2 (Cyclin E exp. in progress) associated with Bad, we failed to observe any significant changes regardless of whether Bad was mutated to mimic phosphorylation or dephosphorylation at Ser 170. These findings indicate that even though phosphorylation of Bad at Ser 170 appears to have a specific effect on late G1 and early S phase, the interaction of Bad and Cdk2 or Bad and Cyclin E may be an indirect relationship controlled by means other than Bad's phosphorylation state at Ser-170. Thus, the molecular target of Bad when dephosphorylated at Ser 170 remains to be elucidated. The cell cycle mechanism of the Bcl-2 family may involve complex regulation of both inhibitors and activators of key cell cycle mediators. Bad may be playing an active role in cell cycle regulation or it is possible that Bad may be acting similarly to its proposed sensory function. Therefore, the association may be a result of a mechanism allowing the

apoptotic machinery to sense the proper operation of the cell cycle. Furthermore, studies demonstrating cell cycle activities of Bcl2, Bcl-xL, and Bad, together with their known mitochondrial membrane localization, also raises the possibility that the mitochondria and their energy producing function may have a role in the observed cell cycle effect.

Together, the results in this chapter strengthen the notion that Bad's apoptotic effect is modulated by its numerous phosphorylation sites and that phosphorylation of Bad at the Ser170 site plays an important role in the regulation of its pro-apoptotic function. Moreover, the neutralizing effect of the phospho-mimetic mutation at Ser 170 does not appear to require the expression of Bcl-xL. This observation is in agreement with previous findings demonstrating that the phosphorylation state of Ser 170 does not effect Bad's ability to heterodimerize Bcl-xL. Moreover, the observations that Bad, depending on the phosphorylation state of Ser170, can influence cell cycle progression, combined with the novel interaction with cell cycle regulators Cdk2 and Cyclin E, reveals a novel convergence between two major events controlling total cellular content in any complex organism: apoptosis and cell division.

Chapter 4

CAMKII- γ MEDIATES PHOSPHORYLATION OF BAD AT SER170

4.1 INTRODUCTION

One of the highly regulated BH3-only proteins is Bad, whose pro-apoptotic function was shown to be inhibited by phosphorylation at multiple sites (123, 134). As discussed earlier, Bad and its multiple phosphorylation sites (Ser-112, 128, 136, 155, and 170 in the murine protein) is able to converge signals from numerous pathways as well as sensitize cells to apoptotic signals. These apoptotic signals are manifested in the the blocking of upstream kinases that are able to phosphorylate Bad.

Bad is constitutively expressed at varying levels in all healthy mammalian cells and is generally maintained in a hyperphosphorylated state by several kinase pathways including PKA (135, 136), p90rsk (322) and PKB/AKT (131, 133). The latter, which is the most widely reported, is thought to play an important role in the well known survival effects of the PI3-kinase pathway. However, we and others have found that this pathway may have no role in Bad phosphorylation in some cell types (112, 323). Nevertheless, death signals that result in dephosphorylation of Bad convert the Bad protein into a survival antagonist. As mentioned earlier, Bad selectively binds and neutralizes anti-apoptotic molecules, particularly Bcl-xL (65, 96, 130); thereby permitting activation of the multi-domain pro-apoptotic molecules, Bax and Bak.

Studies analyzing phosphorylation of Bad, performed in our laboratory, discovered a site near the carboxy terminus, Ser170, as an additional site of phosphorylation involved in regulation of Bad (137). As presented in chapter 3, the degree of phosphorylation of Ser170 site appears to affect not only Bad's ability to promote apoptosis, but also cell cycle progression. In this chapter we describe the studies and results that lead to the identification of the kinase that regulates phosphorylation of Ser170 on murine Bad (corresponding to Ser134 of human Bad), and thus demonstrate a direct role of this kinase in cell survival and proliferation.

4.2 RESULTS

4.2.1 Identification of the Bad Ser170 kinase

We sought the identity of the kinase that phosphorylates Bad at Ser 170 by assaying kinase activity against a peptide based on the sequence corresponding to the Ser 170 in murine Bad (PRPKSAG). As will be shown below, kinase activity that phosphorylates this site could be readily assayed using extracts from MC/9 cells, which were the cell type first used to identify this phosphorylation, as well as from other cell types, including FDC-P1. We first tested a series of known kinase inhibitors: LY-294002, wortmannin, U0126, rapamycin, SB203580 and Ro-31-8220 to obtain clues regarding the identity of the kinase; however, little or no effect was observed at concentrations known to selectively block various protein kinases (data not shown). Work done in our lab had previously shown, through the use of standard *in vitro* kinase assays, that the following immunoprecipitation of kinases: PKB/akt, erk1/2, Cdk-2, Cdk-4, Cdk-6, and GSK-3 showed no significant activity against the peptide compared to their activity against standard substrates (data not shown).

The search for alternative potential kinases that may phosphorylate the S170 site of Bad was carried out using ScanProsite (<http://expasy.org/tools/scanprosite/>) against the SwissProt database. The short sequence PRPKS was found to be surprisingly rare in the database, with only 37 hits found (3 of which were Bad – human, mouse and rat). A motif scan under high stringency was performed on all 37 proteins to analyze potential serine/threonine phosphorylation sites. Residue S846 within the protein MSulf-1, which corresponded to the serine of the PRPKS sequence, was the only protein found to have a predicted kinase that phosphorylated the site. In this case, Calmodulin-dependent Kinase II was predicted to be a possible kinase that phosphorylated this site. It was also reported recently that the checkpoint kinase, Chk1, was able to phosphorylate the Ser170 site in Bad (324). As will be described below, these two kinases were further investigated to determine their potential role in phosphorylating the Ser170 site of Bad.

4.2.2 MonoQ Column Chromatography

The separation of Bad-S170 kinase activity by column chromatography was performed. Cell lysates of both MC/9 and FDC-P1 cells were each separated by MonoQ and Superdex S200 fractionation. MonoQ fractionation of either MC/9 or FDC-P1 resulted in a single peak of kinase activity that co-migrated when fractions were assayed using the peptide corresponding to Bad-S170, or the peptide: PLSTRLSVSS, a reported CaMKII substrate (Fig. 4.1A). The peak activity eluted between 0.4 and 0.5 M NaCl. When the same column fractions were assayed using a peptide that is commonly used to detect Chk1 activity, there were two major peaks of activity that eluted at earlier points in the salt gradient (Fig. 4.1B). This result suggested that Chk1 was unlikely to be the kinase responsible for S170 phosphorylation.

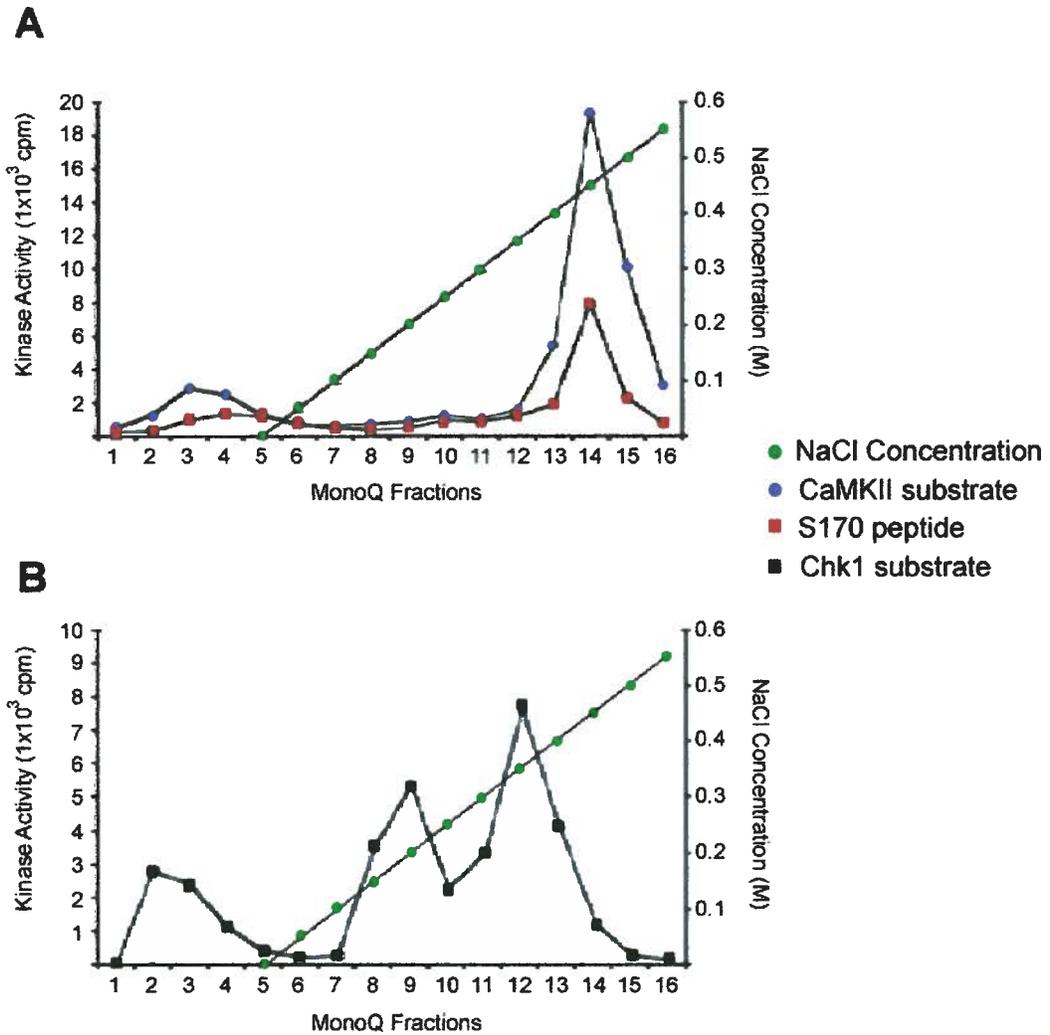


Figure 4.1 Mono-Q column fractionation and kinase Assay

- (A) Mono-Q fractionation of MC/9 cell extracts assayed for kinase activity. The first 5 fractions represent column flow-through, at which point the 0-1.0 M NaCl gradient was begun. In (A), red squares represent activity against Bad-S170 peptide and blue circles represent activity against a CaMKII substrate, PLSTRLSVSS. The green circles represent the NaCl concentration.
- (B) Data represents activity against the Chk1 substrate in identical fractions to those shown in panel (A).

4.2.3 Superdex S200 Column Chromatography

Superdex S200 fractionation of MC/9 lysate also showed a single major peak of kinase activity that was capable of phosphorylating the Bad-S170 peptide. This peak was centered at an approximate molecular weight of 400-500 kDa and overlapped with a peak of activity measured against the CaMKII substrate (Fig. 4.2A). As shown in Fig. 4.2B, the same fractions assayed against a peptide used to detect Chk1 activity showed an overlapping profile, but the major peaks of activity were clearly distinct and the Chk1 activity migrated with a much smaller molecular weight. It is important to note that the migration of the CaMKII activity with an apparent size of greater than 440 kDa is expected for the multimeric forms of this kinase family.

In order to further purify the fraction containing the Bad S170 kinase, we performed a double column purification; where the peak Superdex S200 fraction was run on the MonoQ column. Unfortunately, despite numerous attempts, we found that most of the kinase activity was lost following any combination of these two column purification steps and thus further purification by conventional chromatography was not pursued.

To verify that CaMKII and Chk1 protein are present in peak fractions predicted from the kinase assays, Superdex S200 column fractions were precipitated with cold acetone, then run on SDS-PAGE and immunoblotted to detect CaMKII or Chk1. As shown in Figure 2C, the antibody to CaMKII detected bands corresponding to the isoforms of the enzyme, which are known to migrate at approximately 60-65 kDa. These bands appeared in Superdex S200 fractions 9-12, which correspond to the peak of kinase activity that could phosphorylate the Ser170-containing peptide and the CaMKII substrate. Immunoblotting for the Chk1 enzyme was performed to determine where it migrated on the same Superdex S200 column. The band at approximately 50 kDa corresponding to Chk1 was detected in fractions 17-19, which corresponded to the peak of activity against the ChkTide substrate. Together, our data appears to rule out Chk1 as the relevant kinase that is responsible for phosphorylation of Bad at Ser170, and subsequent studies focused on the activity of CaMKII.

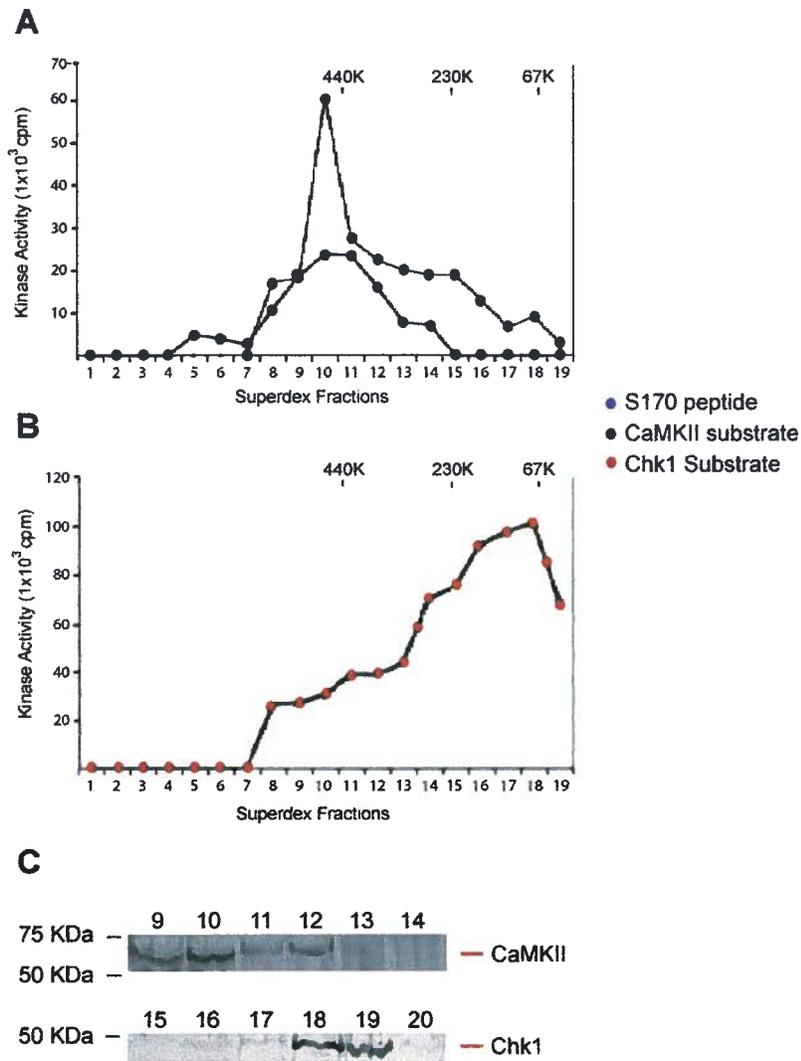


Figure 4.2 Superdex S200 fractionation and kinase Assay

- (A) Superdex S200 fractionation of MC/9 cell extracts. The blue dots represent kinase activity against Bad-S170 peptide and black dots represent activity against the CaMKII substrate.
- (B) The activity against the Chk1 substrate was monitored in the same fractions as in panel (A). Migration of three molecular weight standards is indicated in panel (A) and (B).
- (C) Column fractions numbers correspond to those in Fig. 2 (A) and (B). Peak fractions of Ser170 and ACT III kinase activity (9-11) correspond to fractions in which CaMKII isoforms of 60-65 kDa are detected. Fractions 18 and 19, which show a peak of activity against the Chk1 substrate, have a band below 55kDa, which is the size expected for Chk1.

4.2.4 CaMKII inhibitor studies

The column fractionation experiments and immunoblot analysis provided good evidence that CaMKII co-migrated with the peak fractions from either the MonoQ or Superdex S200 columns. In order to test whether CaMKII was responsible for the observed activity against both the Bad S170 and commercially available CaMKII substrate (AutoCamTide II peptide), we performed a series of experiments using a CaMKII inhibitor, KN-93 (10 μ M final concentration). KN93 is widely used and has been described as a selective inhibitor of CaMKII kinase activity (325). Our first set of experiments involved verifying KN93's ability to inhibit CaMKII activity (Fig. 4.3A). Using purified CaMKII protein, we assessed the activity against AutoCamTide II peptide (ACTII) and tested whether the activity was specific. We observed very little phosphorylation of Histone H1 and H2B; and the high levels of phosphorylation against ACTII were reduced by nearly 70% in the presence of KN93. These confirmatory experiments led to further inhibitor studies where we examined whether the fractions showing peak activity against both the CaMKII substrate and Bad S170-containing peptide could be inhibited using KN93. Based on these studies we observed that peak fractions from either the MonoQ (Fig. 4.3B) or Superdex S200



Figure 4.3 Column activity and KN93

- (A) The kinase activity of purified CaMKII was assayed against commercially available CaMKII substrate, Histone H1, Histone H2B; in the presence and absence of CaMKII inhibitor, KN93 (10 μ g/ml). This figure is a representative of 3 separate experiments.
- (B) The kinase activity was determined for MonoQ column peak fraction (corresponding to fraction 14 in Fig. 4.1A) with and without KN93.

(Fig. 4.4A) are greatly inhibited by KN93. Inhibition by KN93 reduced activity of peak fractions from either column by nearly 80%. Furthermore, the inactive analog (KN92) had no inhibitory effect (Fig. 4.4B).

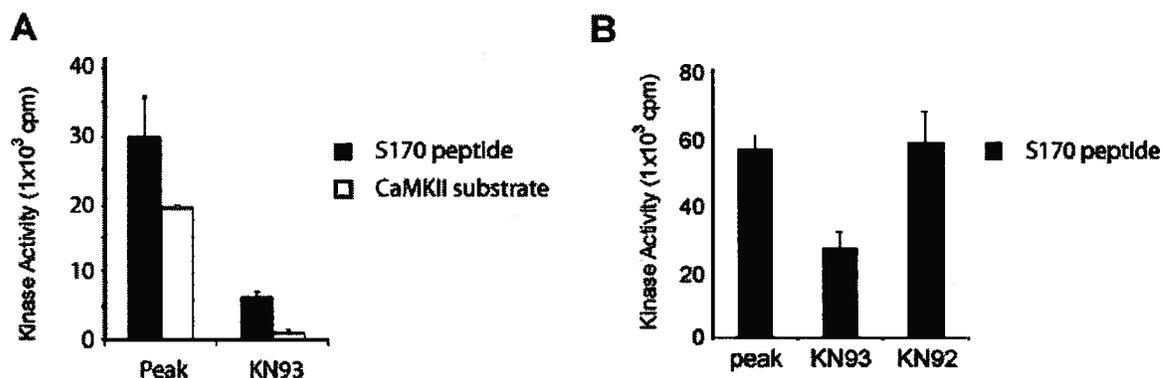


Figure 4.4 Superdex column activity and KN93

- (A) Superdex S200 column peak fraction (corresponding to fraction 10 in Fig. 2A) was assayed for kinase activity against either the Bad S170-containing peptide, or the AutoCamTide II peptide (ACT III); in the presence or absence of the CaMKII inhibitor, KN-93 (10 μ g/ml). Results are averages of triplicate determinations \pm standard deviation from a single experiment. Similar results were obtained in at least 3 independent experiments.
- (B) Superdex S200 column peak fraction (corresponding to fraction 10 in Fig. 4.2A) was assayed for kinase activity against Bad S170-containing peptide; in the presence or absence of the CaMKII inhibitor, KN-93 (10 μ g/ml) and its inactive analog KN92. Similar results were obtained in at least 3 independent experiments.

Further data supporting CaMKII as a Bad Ser170 kinase, was derived from immunoprecipitation experiments. Using MC/9 cell lysates, CaMKII was immunoprecipitated, using anti-CaMKII antibody, and subsequently tested to determine whether the immunoprecipitate showed kinase activity against Bad Ser 170 peptide. As shown in figure 4.5, immunoprecipitated CaMKII showed activity against both a commercially available substrate and the Bad Ser170 peptide. Moreover, KN93 inhibited the observed activity, hence eliminating the possibility that unknown proteins forming a complex with CaMKII may be responsible for the observed activity.

Therefore, fractionation of cellular proteins by size or by charge results in single fractions, shown to contain CaMKII protein, which is able to phosphorylate both CaMKII substrate peptide and the S170 peptide; and the activity against both substrates are greatly reduced by the selective CaMKII inhibitor KN93. This marked decrease in activity against the S170 peptide in the presence of CaMKII inhibitor, suggests that CaMKII is able and is responsible for the majority of the observed phosphorylation of Bad at Ser 170.

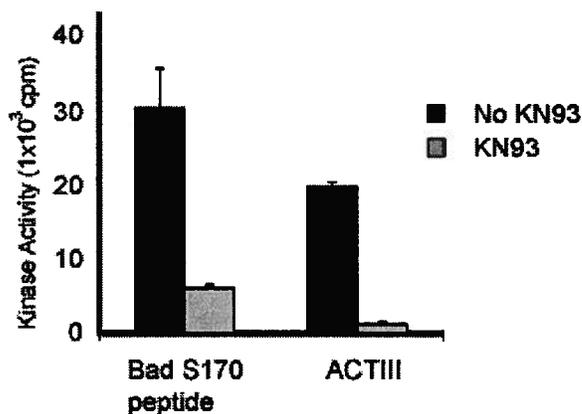


Figure 4.5 Kinase activity of immunoprecipitated CaMKII

CaMKII was immunoprecipitated using anti-CaMKII antibody and kinase activity of the immuno-complex was assayed against both the ACTIII and S170 containing peptide; with and without KN93 (10 µg/ml). Similar results were obtained in at least 3 independent experiments.

4.2.5 Kinase activity using purified CaMKII isoforms

The column purification experiments along with the CaMKII inhibitor studies provided strong evidence that CaMKII is able to phosphorylate Bad S170-containing peptide. Therefore, we were interested in determining which of the four major isoforms of CaMKII (α , β , γ , and δ) were present in the peak fractions of both the MonoQ and Superdex S200 column purification. The peaks from the column fractionation containing kinase activity were separated using SDS PAGE and we probed immunoblots for the presence of CaMKII isoforms using antibodies against CaMKII- α , β , γ , and δ . Unfortunately, the antibodies available to CaMKII isoforms, particularly anti-CaMKII- γ , and $-\delta$, were not found to be useful in immunoblots to distinguish between them. As we were unable to detect which protein isoforms were expressed in cell types of interest, we did however obtain commercial sources of purified isoforms of CaMKII- α , β , γ , and δ which allowed us to test for their ability to phosphorylate the peptide corresponding to Bad S170 or the CaMKII substrate peptide. As shown in Figure 4.6, all of these kinases phosphorylated the CaMKII substrate, with some differences in their relative level of activity that might be explained by specific activities of the enzyme preparations. However, when assayed using the peptide corresponding to Bad S170, there was a striking difference in the activities, with only the gamma isoform of CaMKII having robust activity, while the others had little to no activity. As expected, the KN-93 inhibitor was able to block the activity of each of the isoforms, in assays against both substrates, although in our hands it may have been slightly less effective against CaMKII- α .

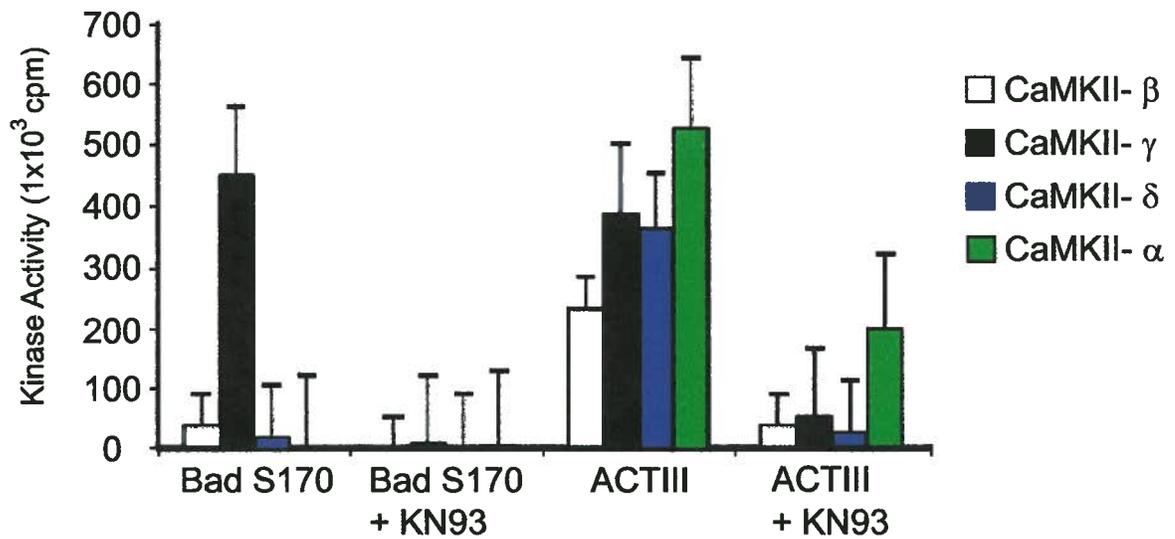


Figure 4.6 Activity of purified CaMKII isoforms

Recombinant CaMKII- α , β , γ , and δ were used in kinase assays against either the Bad S170-containing peptide (Bad S170) or AutoCamTide III (ACTIII), in the presence or absence of KN-93 (10 $\mu\text{g/ml}$).

4.2.6 siRNA knockdown experiment of CaMKII

To confirm that CaMKII- γ was the cellular kinase responsible for the Bad S170 kinase activity, we used 3T6 cells treated with siRNA to knock down expression of CaMKII- γ . 3T6 cells were used since siRNA knockdown, based on past experience, cannot be successfully done in either MC/9 or FDC-P1 cells. To ensure 3T6 cells had similar kinase activity profiles, as compared to MC/9 and FDC-P1, we performed Superdex S200 fractionation of 3T6 lysates (Fig 4.7A). The fractions were tested for activity against Bad Ser-170 peptide. The profile was nearly identical to that seen when using MC/9 and FDC-P1 cells (see Fig. 4.2). Fraction #10 showed the greatest amount of kinase activity against the Bad S170 peptide and this activity was greatly reduced when treated with CaMKII inhibitor KN93 (Fig 4.7B).

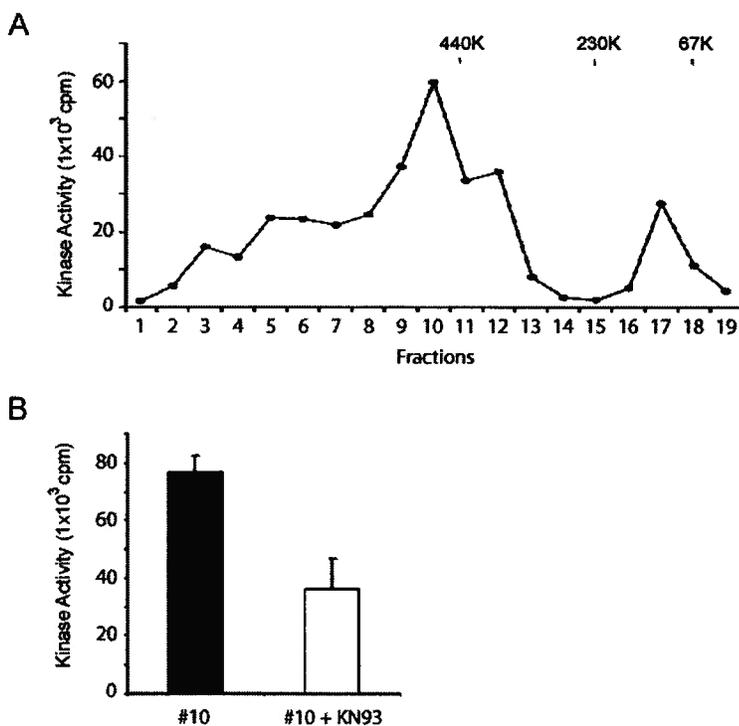


Figure 4.7 Superdex S200 fraction and kinase assay of 3T6 lysates

- (A) Superdex S200 fractionation of 3T6 cell extracts. The black dots represent kinase activity of respective fractions against Bad-S170 peptide.
- (B) Kinase activity of superdex S200 peak fraction (#10) with and without CaMKII inhibitor (KN93)

We chose siRNA specific for CaMKII- β as a negative control, since we had already demonstrated that the purified form of this isoform was unable to phosphorylate Bad S170 peptide. In support of this result we did not observe any decrease in kinase activity against S170 peptide when cells were treated with siRNA, specific for CaMKII- β . However, when cells were treated with siRNA specific for CaMKII- γ , the activity of cell lysates against both substrates was significantly reduced (Fig.4.8). Knockdown of CaMKII- γ had a greater effect on the kinase activity against Bad Ser170 peptide than against the CaMKII substrate peptide. The appearance of a differential inhibition was somewhat expected as the activity of the remaining isoforms is thought to contribute to the total CaMKII activity observed. We were eager to follow up these studies with western blot analysis in order to verify that CaMKII- γ protein levels had in fact been knocked down; but at the time these experiments were performed, there were no commercially available CaMKII- γ antibodies.

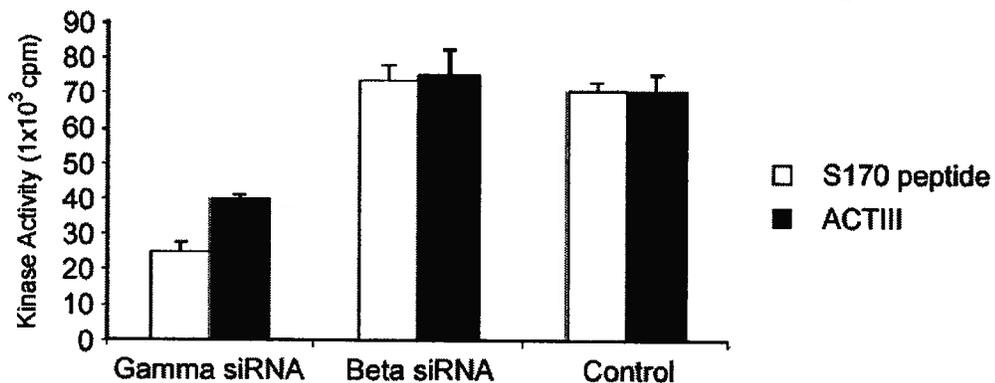


Figure 4.8 CaMKII- γ -directed siRNA decreases kinase activity against Bad S170

Represent studies performed three times. 3T6 cells were untreated (Control), or incubated with siRNA directed against CaMKII- β or - γ , as indicated. Cell lysates were used in a kinase assay using Bad S170-containing peptide (S170) or AutoCamTide III (ACT III) as indicated. Results are averages of triplicate determinations +/- standard deviation from a single experiment. Similar results were obtained in at least 3 independent experiments.

4.3 DISCUSSION

Much work has been conducted to understand the mechanism by which Bcl-2 family members control mitochondrial mediated cell death. The BH3-only subfamily of proteins have been described as “sensitizers” since they influence the apoptotic response but are unable to disrupt the mitochondrial membrane as can be done by the “effector” type Bcl-2 family proteins, Bax and Bak. Bad is one of the BH3-only proteins, and it has a unique role as a sensor of multiple upstream kinases that target its phosphorylation at multiple sites. Phosphorylation of Bad at Ser112 and Ser136 can allow association with cytosolic 14-3-3 proteins, and phosphorylation at Ser155, which is in the BH3 domain, serves to disrupt associations with Bcl-2 and Bcl-xL. In earlier sections, we show evidence that phosphorylation of Bad at Ser170 plays a role in regulation of Bad’s pro-apoptotic activity and a novel cell cycle regulatory function. Even though the Ser170 site was first discovered in 2000, no studies to date had characterized the kinase responsible for phosphorylation of Bad at Ser170, which became the focus of the studies in this chapter.

When the Ser170 phosphorylation site was first described, it was shown that its phosphorylation was not dependent upon either PI3K/PKB or MEK/erk dependent pathways (137, 326). Other relevant studies reported that a peptide based on this phosphorylation site could be phosphorylated by the checkpoint kinase, Chk1 (324). A more recent study describes Pim3 as being able to phosphorylate Bad at multiple serine residues including Ser170 (327), though the cellular effect of Bad being phosphorylated at Ser170 is not pursued.

Our initial studies established conditions under which we could readily detect kinase activity in specific cell extracts (MC/9 and FDC-P1). Using Bad Ser 170 containing peptide, we then proceeded to examine a series of rational kinase candidates by testing their respective inhibitors. We were unsuccessful in our initial attempts, though predictive software did eventually help us identify a promising lead candidate.

In a series of in vitro assays of kinase activity, we have presented convincing evidence that the primary activity that is responsible for phosphorylation of a peptide

encoding the S170 of Bad, corresponds to CaMKII. The use of a selective inhibitor of CaMKII, KN-93, reinforces the identity of the S170 kinase as CaMKII. The analog, KN92, which has no effect on CaMKII, also does not inhibit S170 activity. Furthermore, kinase activity of immunoprecipitated CaMKII also showed activity against the S170 site. In further characterization of the different CaMKII isoforms that might be responsible for phosphorylation of Bad-S170, we were surprised to find a striking difference in the ability of the four enzymes to phosphorylate this site. While each of the purified enzymes could phosphorylate the CaMKII substrate, and the activity was inhibitable by KN-93, only CaMKII- γ phosphorylated the peptide encoding S170.

Another intriguing aspect of the studies is the specificity of the CaMKII- γ enzyme. Comparison of the four isoforms of CaMKII shows that they are highly homologous, particularly in the kinase domain. The Ser170 site in Bad is a non-conventional site of phosphorylation that is unlike any other known kinase substrate. Therefore, it will be interesting to investigate the features of the CaMKII- γ enzyme that results in its unique ability to phosphorylate Bad at Ser170. Finally, the role of CaMKII- γ in phosphorylating Bad combined with the data indicating the effects of Bad Ser 170 phosphorylation on promoting cell survival and cell cycle survival may lead to further novel studies, most likely having a greater impact in the field of neuroscience as both Bad and CaMKII have already been established as key proteins in the function and survival of neuronal cells.

Chapter 5

Cellular Effects of CaMKII Activation and Bad Phosphorylation at Ser170

5.1 INTRODUCTION

CaMKII is a ubiquitous serine/threonine protein kinase that is activated by calcium and CaM and is known to phosphorylate diverse substrates involved in multiple cellular functions including metabolism, neurotransmitter release, membrane fusion, cell cycle control and apoptosis (328-331). As shown in chapter 4, we have provided evidence that CaMKII, and more specifically, CaMKII- γ , is able to directly phosphorylate Bad at Ser170, indicating CaMKII- γ can act as a signaling intermediate relaying survival signals by neutralizing Bad's apoptotic ability as well as affecting Bad's cell cycle effect.

Bad is a pro-apoptotic member of the Bcl-2 family of proteins that is thought to exert a death-promoting effect by heterodimerizing with Bcl-xL and disrupting its anti-apoptotic activity. Bad's apoptotic function is modulated by its phosphorylation at multiple serine sites including Ser-170. The Ser-170 site was first discovered as a site that was hyperphosphorylated when MC/9 cells were stimulated with IL-3. Moreover, phosphorylation at Ser-170 was found to inhibit Bad's apoptotic functions and thus provides a probable means by which IL-3 promotes survival. However, the kinase responsible for relaying the IL-3 survival signal and phosphorylating Bad at Ser170 had remained elusive until now. As such, this chapter aims to describe the effects of various stimulations on CaMKII activation and whether the inhibition of CaMKII effects cell survival.

5.2 RESULTS

5.2.1 Cytokine activation of CaMKII activity

The ability of cytokines to activate CaMKII activity has never been described and therefore we investigated whether the CaMKII activity in FDC-P1 cells was modulated

by cytokine deprivation and re-addition. We utilized the peak kinase activity from Superdex S200 fractionation which we had previously shown to contain CaMKII. When FDC-P1 cells were deprived of cytokine for 4 hours, which we have shown previously was able to almost completely suppress activity of other kinases such as PKB or erk1/2 (332), the kinase activity within the peak fraction was reduced by approximately 30% when assayed against Bad S170 peptide (5.1A). However, when the kinase activity was assayed against CaMKII substrate (5.1B), the reduction in activity was not as pronounced (15%). These results suggest that IL-3 signaling increases CaMKII activity against both Bad Ser170 and CaMKII substrate. The difference in activity levels may be a consequence of CaMKII isoforms being differentially activated by IL-3 stimulation. Since all the isoforms have been shown to phosphorylate the CaMKII substrate, the change in one or more isoforms activity levels would be difficult to capture in this type of experiment. Whereas we have shown that CaMKII- γ is likely the only isoform that phosphorylates Bad Ser170 in FDC-P1 cells and as such we can expect a more pronounced effect against the Bad S170 peptide if IL-3 stimulation activates CaMKII. Moreover, the fact that cytokine starvation did not lead to complete suppression of CaMKII activity is consistent with our previous results showing that when cells were starved of cytokine, there was always a basal level of Bad phosphorylation specifically at the Ser170 site, and the level of phosphorylation was increased by cytokine treatment of cells (peptide 2 in (326)).

Re-addition of IL-3 to the starved cells for 20 min resulted in an increase in activity back to the pre-starvation levels, when assayed against either the Bad S170 peptide or the CaMKII substrate peptide. The results shown in figure 5.1A and B are representative of several replicate experiments. The data is presented in this manner since the experiments were performed with different age radioisotope and as such the radioactivity levels cannot be compared directly.

Similar results were obtained when CaMKII activity was assayed following immunoprecipitation from cytokine-starved cells and from starved cells treated with IL-3 (Figure 5.1C). At the time these experiments were performed, we did not have antibodies selective for each of the isoforms, thus we were unable to determine which of the

CaMKII isoforms were affected by IL-3 stimulation. In more recent studies done by P. Hojabrpour, immunoprecipitation of CaMKII- γ using a specific antibody has demonstrated that stimulation of cytokine-starved FDC-P1 cells with IL-3 results in a robust stimulation of kinase activity.

The level of activity of the CaMKII group of enzymes can be closely correlated with the level of phosphorylation at a highly conserved autophosphorylation site, Thr286 (numbering from CaMKII-alpha), found in all isozymes of CaMK-II. This has been shown in studies with specific inhibitors, as well as specific substrates of the kinase (333, 334). Phosphorylation at this "autonomy site" enables CaMK-II to remain active after the interaction with Ca⁺²/CaM has ended (333-335). Consistent with our previous findings using Superdex S200 peak fractions, stimulation of cytokine-starved FDC-P1 cells with IL-3 resulted in an increase in phosphorylation of Thr286 on CaMKII (Figure 5.1D). Related studies just recently performed by P. Hojabrpour, have shown that a similar increase in autophosphorylation can be observed in the immunoprecipitated CaMKII- γ isoform. Together, these findings indicate that Bad Ser-170 phosphorylation and its associated anti-apoptotic effect are controlled by IL-3 signalling via CaMKII- γ activation.

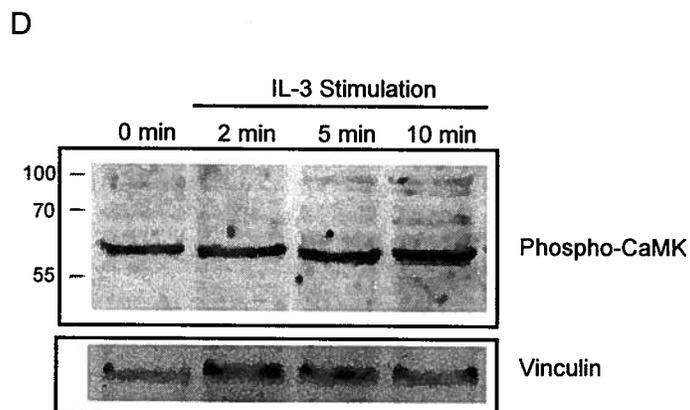
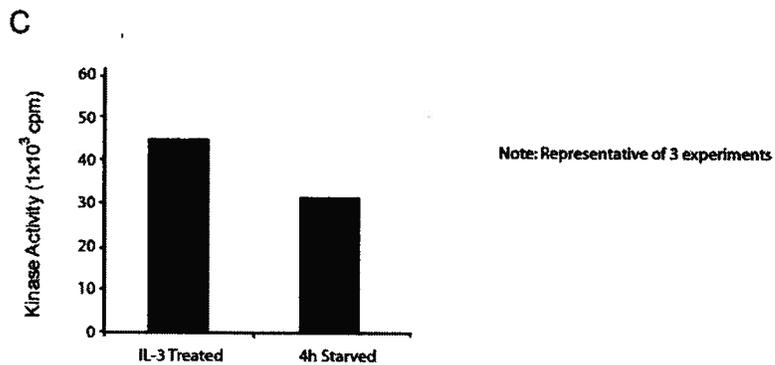
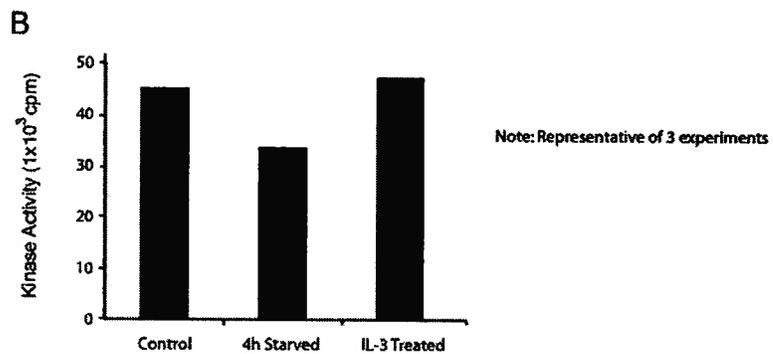
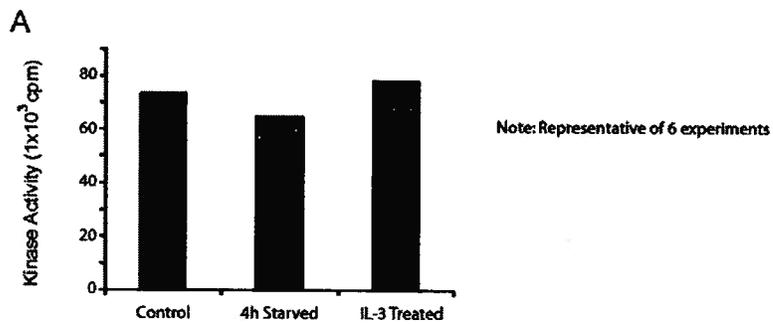


Figure 5.1 IL-3-stimulates kinase activity against Bad S170 peptide

- (A) FDC-P1 cells were harvested after growth under normal conditions (Control), or after starvation of cytokine for 4 hours (4h Starvation), or cells were starved for 4 hours and re-stimulated with IL-3 for 20 minutes (IL-3 treated). Cell extracts were separated by Superdex S200 and kinase activity against the peptide corresponding to Bad-S170 was assayed. This is a representative result showing average of duplicate samples from a single experiment that was repeated 6 times with similar results.
- (B) Same experimental setup as in A, except Superdex S200 fractions were assayed for kinase activity against the CaMKII substrate peptide. This is a representative result showing average of duplicate samples from a single experiment that was repeated 3 times with similar results.
- (C) FDC-P1 cells were starved of cytokine or re-stimulated with IL-3 as in A. Lysates were immunoprecipitated with anti-CaMKII antibody and the immunoprecipitates were used in a kinase assay with the Bad-S170 peptide as substrate. Results shown are from a single experiment, but similar results were obtained in at least 4 independent experiments.
- (D) IL-3 stimulates phosphorylation of CaMKII at Thr286. FDC-P1 cells were starved for 4 hours, followed by stimulation with IL-3 for the time indicated. Cell lysates were separated by electrophoresis and immunoblotted using an antibody directed against the phosphorylated T286 of CaMKII (phospho-CaMKII). Numbers at left indicate relative molecular weight (in kDa) of molecular weight standards. The same blot was blotted with anti-Vinculin antibody.

5.2.2 Inducing apoptosis via cytokine starvation does not affect Bad/Cdk2 interaction

Stemming from experiments analyzing CaMKII's kinase activity against Bad Ser-170 when cells are starved of cytokine (Fig 5.1), we chose to also test whether cytokine starvation affects Bad's ability to interact with Cdk2. As described previously, Bad's phosphorylation status is altered when starved of cytokine and Bad's Ser-170 site had been shown to become hyper-phosphorylated when stimulated with cytokine.

Admittedly, without understanding the mechanism or the cellular consequences of Bad/Cdk2 interaction, we thought to interrogate the effects of cytokine withdrawal on the formation of the Bad/Cdk2 complex. We show in Figure 5.2A that 6 hours of cytokine starvation has very little effect on Bad's ability to interact with Cdk2 as compared to unstarved cells. As to avoid the complications associated with comparing studies when cells are undergoing apoptosis, we were limited to a narrow window of starvation time points. It was shown that by 8 hours there was a sharp increase in the percentage of cells showing initial signs of apoptosis (Fig. 5.2B). The lack of any detectable change in Bad-Cdk2 association following cytokine starvation again gave us no conclusive results regarding this event and the phosphorylation of Ser170.

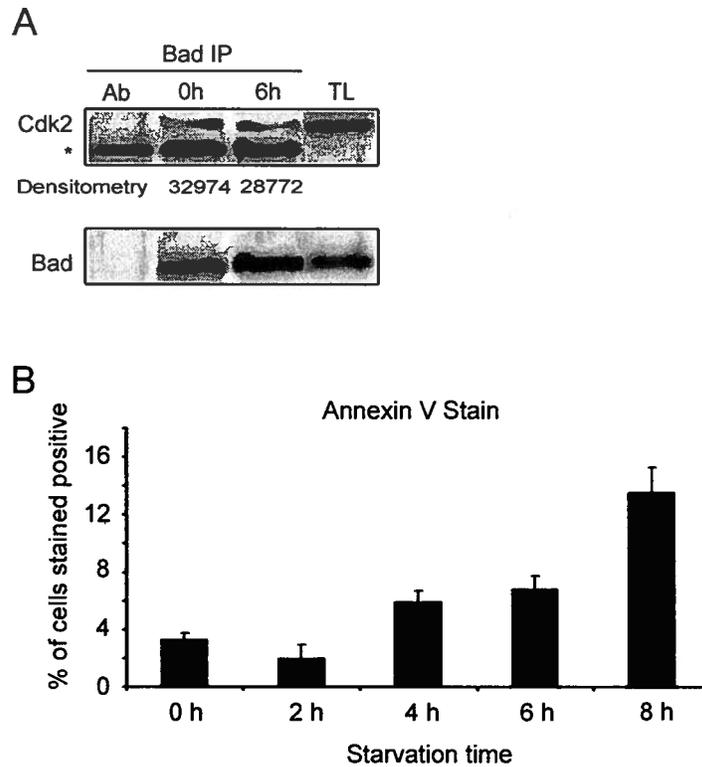


Figure 5.2 Inducing apoptosis via IL-3 starvation does not appear to effect Bad's ability to interact with Cdk2

- (A) MC/9 cells were starved of cytokine for 0 hours (0h) and 24 hours (24h). Cell extracts were immunoprecipitated using anti-Bad antibody (Bad IP) and the immuno-complex was separated by SDS-PAGE and immunoblotted with anti-Cdk2 (CDK2) and anti-Bad (Bad) antibodies. Densitometry was performed in order to accurately compare the amount of Cdk2 pulled down within the Bad's immunoprecipitated complex. Densitometry measurements of bands corresponding to co-immunoprecipitated Cdk2 at 0 hour and 6 hour after IL-3 stimulation; 32974 and 28772 respectively.
- (B) MC/9 cells were starved of cytokine for 0 (0h), 2 (2h), 4 (4h), 6 (6h), and 8 hours (8h). Cells were stained with Annexin V and the percentage of cells was determined using FACS.

5.2.3 Apoptosis mediated by CaMKII inhibitor is dependent upon Bad S170

The possible involvement of CaMKII activity as a regulator of cytokine-dependent cell survival, and the corresponding phosphorylation of Bad at Ser170, was tested by use of the inhibitor KN93. The pharmacological compound with selective inhibitory activity against CaMKII, the methoxybenzene sulfonyl derivative KN-93 (336-341), which prevents the activation of CaMK-II by antagonizing CaM binding, is membrane permeant and has been used in numerous functional studies on living cells (342, 343). As shown in Figure 5.3, when FDC-P1 cells, in the presence of complete growth medium with IL-3, were incubated with KN93 for 24 hours, at 10 and 50 μ M concentrations, there was a dramatic increase in the level of apoptosis as measured by the extent of annexin V and PI staining. However, when FDC-P1 cells expressing Bcl-xL were treated in the same way, cell death was reduced considerably. This finding confirms that the apoptosis caused by KN93 was due to its effects on the mitochondrial death pathway. As previously shown (Fig. 3.2) Bad expression in the cells expressing Bcl-xL caused them to revert to being dependent upon cytokine for their survival. When these cells expressing Bad were incubated with KN93 (and still in the presence of cytokine), they also regained sensitivity to the effects of the CaMKII inhibitor. This provides strong evidence in support of the suggestion that the effect of KN93 in causing apoptosis is due to its inhibition of CaMKII-mediated phosphorylation of Bad at Ser170. We next tested the effect of the inhibitor on cells in which Bad S170D was expressed, and on these cells there was much less effect of KN93, further supporting the hypothesis. Statistical analysis shows that the level of apoptosis caused by KN-93 is significantly different between Bad-S170D expressing cells, and Bad wt cells or parental cells ($p < 0.05$), but not compared to the cells expressing Bcl-xL alone. It should be noted that the inactive analog, KN92, did not induce apoptosis at the same concentrations. Together, these results suggest that at least part of the pro-survival effect of cytokines in blocking Bad's pro-apoptotic activity is due to phosphorylation of Bad by CaMKII at the Ser170 site.

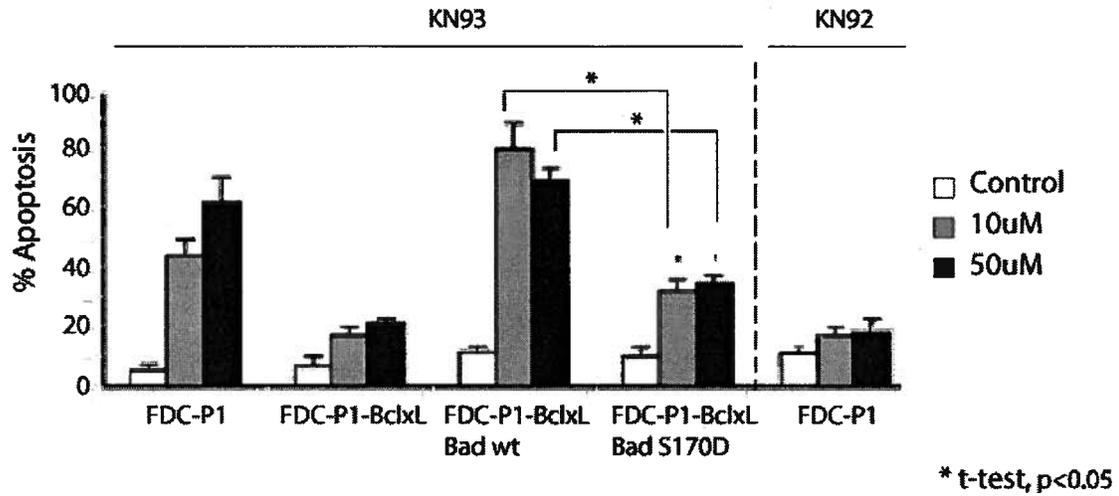


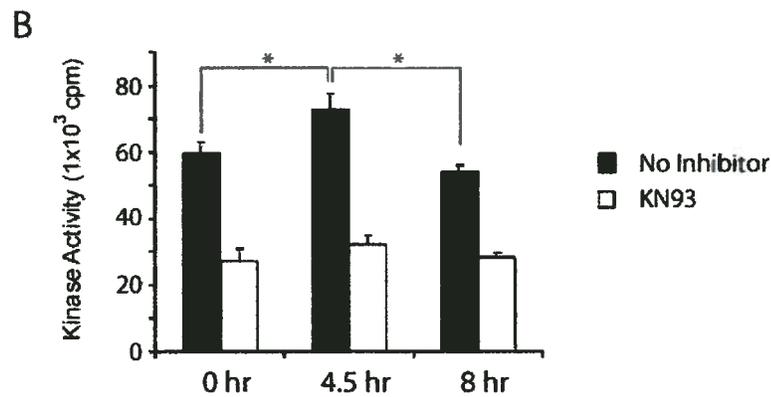
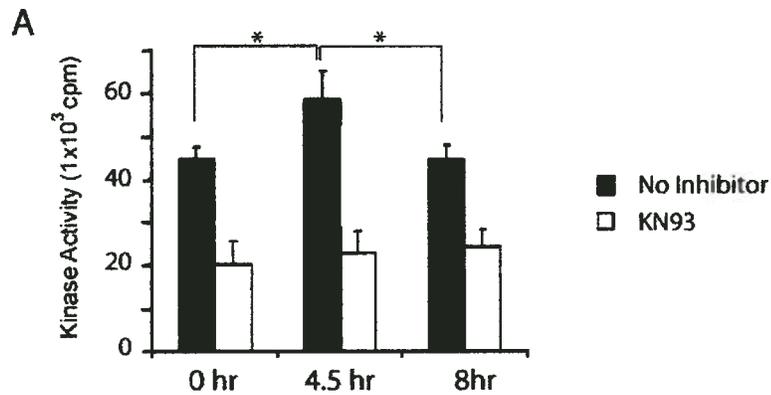
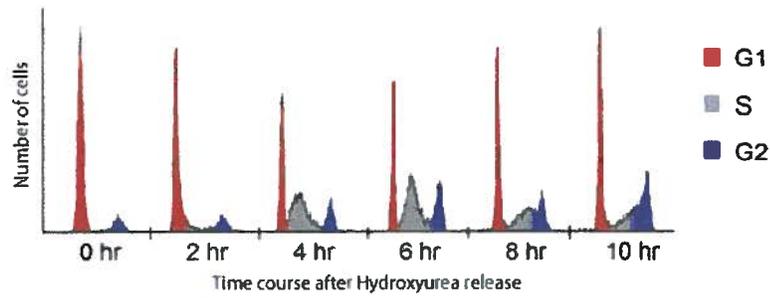
Figure 5.3 Induction of apoptosis by the CaMKII inhibitor, KN93

KN93 and KN92 was added to cells at 0 μ M (Control), 10 μ M (grey bars) or 50 μ M (black bars) overnight, after which cells were assayed for P.I and Annexin V stain. Results were compiled from 3 experiments and indicate average values +/- standard deviation. Statistical analysis shows that the level of apoptosis caused by KN-93 is significantly different between Bad-S170D expressing cells and Bad wt cells ($p < 0.05$), but not compared to the cells expressing Bcl-xL alone. Experiment repeated three times ($n=3$).

5.2.4 Kinase activity against Ser170 peptide increases in S phase of cell cycle

A few studies have suggested that CaMKII may play a cell cycle regulatory role at both the G2-M and G1-S transitions. For instance, CaMKII inhibitory drugs have been shown to arrest cells in G1, and S (336, 344). Other studies, for example, using peptides that inhibit CaMKII activity, block sea urchin eggs at the G2-M transition (345) and expression of a constitutively active CaMKII arrests certain mold and mammalian cells in G2 (346, 347). CaMKII activity and its inhibition is likely to have different cellular and physiological effects due to the differential expression profiles of CaMKII isoforms in each of the cell types. Nevertheless, studies have amounted sufficient data to support a connection between CaMKII activity and cell cycle progression. Together with our data indicating CaMKII phosphorylates Bad at Ser-170, a site shown to effect cell cycle progression, we were interested in examining CaMKII activity at different stages of cell cycle progression.

Using a G1 chemical cell cycle inhibitor, hydroxyurea, we were able to synchronize cells (same as Fig. 3.6A) and over a specified time course determine if the kinase activity against CaMKII substrate (Fig. 5.4A) and Bad Ser170 peptide (Fig. 5.4B) changed throughout the cell cycle. These experiments revealed a consistent, statistically significant, increase in CaMKII kinase activity when cells were synchronized in S phase (3-5 hours after hydroxyurea release). In all cases, when cell extracts were treated with CaMKII inhibitor, KN93, we observed a decrease in kinase activity.



* t-test, $p < 0.05$

Figure 5.4 Cells synchronized in S phase show relative increase in CaMKII kinase activity

- (A) Cell extracts, from corresponding time points after hydroxyurea release, were used to perform kinase assays against CaMKII substrate.
- (B) Cell extracts, from corresponding time points after hydroxyurea release, were used to perform kinase assays against Bad Ser170 peptide.

In addition to hydroxyurea synchronization, similar kinase assay experiments were performed using cells sorted based on cell cycle stage (Fig. 5.6). Again, we observed that cells in S phase show greater kinase activity against Bad Ser170 peptide than cells in G1 and G2 phases of the cell cycle. Interestingly, the increase in activity at S phase corresponds to the same stage we originally observed an effect of overexpressing Bad S170A mutant. Together, these results indicate that CaMKII activity is increased in S phase, as compared to G1 and G2 phases. Furthermore, together with the cell cycle experiments shown above with the Bad Ser170 mutants, we can conclude that CaMKII activity in phosphorylating Bad at Ser170 may serve a regulatory role in cells progressing through S phase of the cell cycle.

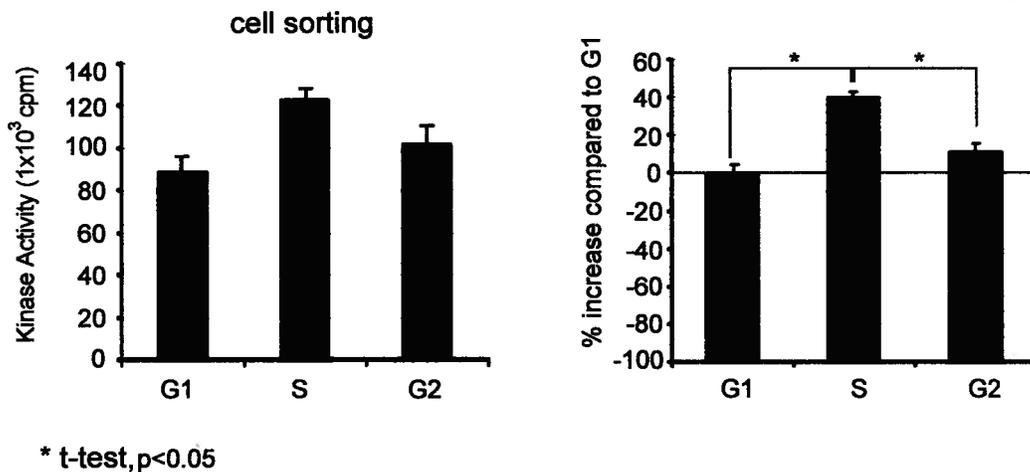


Figure 5.5 CaMKII kinase activity increase in S phase

Cells sorted on the basis of cell cycle stage: G1, S or G2 were used to perform kinase assays against Bad Ser-170 peptide. Results are representative of three separate experiments.

5.3 DISCUSSION:

The most convincing evidence we have obtained to show that CaMKII activity can mediate phosphorylation of Bad at S170 and provide a survival effect comes from incubation of cells with the inhibitor of CaMKII, KN93. In parental cells, or those expressing Bad, KN93 induces apoptosis. Expression of Bcl-xL largely overcomes the pro-apoptotic effect of KN-93. However, when cells were expressing Bad-S170D, the KN93 caused much less apoptosis. This result provides clear evidence to support the suggestion that CaMKII is responsible for phosphorylation of Bad at the Ser170 site and phosphorylation at that site blocks the pro-apoptotic activity of Bad. Thus we can firmly suggest that the effect of KN93 in inducing apoptosis is largely due to its effect on blocking phosphorylation of Bad at Ser170. Nevertheless, one should note that Bad mutant S170A expression in FDC-P1-BclxL was only shown to sensitize cells to apoptosis and requires IL-3 withdrawal for complete induction of apoptosis. However, these results are difficult to assess as the contribution of endogenous Bad is not controlled for. Moreover, we know that the Ser170 site is constitutively phosphorylated, even in starved cells there is some level of Ser170 phosphorylation (137), which is also supported by the CaMKII activity assays presented.

Our results showing KN93 treatment induces apoptosis are supported by a recent study using cells transfected with Bad and CaMKII. The spiral ganglion neuron (SGN) cells, overexpressing Bad, when grown in reduced serum level (1%) media would undergo apoptosis unless co-transfected with CaMKII (206). The authors suggested that depolarization of SGN cells increased CaMKII activity which resulted in the functional inactivation of Bad, implying that Bad inactivation is a means by which depolarization and CaMKII promote SGN survival (206, 348). These studies also relate to another recent finding that demonstrated KN-93 was a much more potent inducer of cell death than wortmannin, a PI3K inhibitor, in prostate cancer cells (349). Similarly, it has also been shown that overexpression of CaMKII- α gene results in resistance to apoptosis when treated with doxorubicin, thapsigargin and TRAIL. It was suggested that, based on these finding, CaMKII expression plays an important role in prostate cancer cell resistance to apoptosis (208, 349).

In order to elucidate the mechanisms controlling Bad's association with Cdk2, we performed a series of experiments examining the effects of DNA damage through the use of UV radiation. Observing the effects of increasing radiation, we were unable to detect a change in the levels of Bad/Cdk2/Cyclin E association. These results however were difficult to assess as there was a very narrow window of radiation where DNA damage could be caused without triggering high levels of apoptosis. These findings are similar to those observed when testing whether cytokine starvation affected the Bad/Cdk2/Cyclin E complex. We did not observe a significant change in the levels of association during the starvation time course; however, these experiments are difficult to assess as cells began to undergo apoptosis within 8 hours of starvation (data not shown). The early onset of apoptosis limited the time course to six hours and, as mentioned above, depending on the stage of the cell cycle cells may respond differently to cytokine withdrawal.

We did however show that the kinase activity phosphorylating the Ser170 site is greater in the S phase of the cell cycle (Fig. 5.4). This was initially done using cells blocked at G1/S by hydroxyurea incubation, then released from the block by washing cells. The kinase activity is consistently higher in the time that most cells are in S phase, and decreases as cells progress into G2. The same type of analysis was also done with normal cells (not blocked by use of drug) that were sorted into G1, S and G2 populations. Again, consistently higher Ser170 kinase activity can be detected when cells are in the S phase of the cell cycle. Thus, we have this additional evidence that the phosphorylation of Bad at the Ser170 site is cell cycle regulated, as is the kinase that phosphorylates that site.

The activity was also shown to be decreased following cytokine starvation of cells, and activated by treatment of starved cells with cytokine. Unlike some other downstream kinases regulated by cytokine, such as PKB or erk1/2, we did not see a complete reduction in CaMKII activity during the 4h of cytokine starvation. This result is consistent with the observation we made previously showing that the S170 site in Bad is always phosphorylated to some extent in cytokine-starved cells, and its phosphorylation increases, along with other sites in Bad, upon cytokine stimulation.

In summary, we discovered that in FDC-P1 cells CaMKII activity is increased in response to cytokine treatment, and inhibition of CaMKII correlates with enhanced apoptosis, which is dependent upon the ability of CaMKII to phosphorylate Bad at Ser170. It is important to specify the cell line used, since it has been shown that CaMKII's activity, the expression profile of CaMKII isoforms, and its cellular effects differ between cell types. Nevertheless, these findings provide insight into a novel mechanism regulating Bad and its cell survival and cell cycle functions.

Future studies will need to address the question of what protein interactions may be mediated by the domain of Bad encoding the Ser170 site, and how phosphorylation of that site affects those interactions. For example, we know that Bad-S170D, although much less pro-apoptotic, can still bind to Bcl-xL (137). However, we have not determined whether these interactions occur in the cytosol, or at the mitochondrial membrane. Another interesting question is whether Bad phosphorylated at Ser170 effects the binding to specific 14-3-3 isoforms. The interactions that Bad makes with these, or perhaps other, proteins will likely provide clues as to how the Ser170 site controls Bad's apoptotic and cell cycle effects. Furthermore, it would be of interest to determine if expression of constitutively active CaMKII, specifically CaMKII- γ , de-sensitizes FDC-P1 cells from IL-3-withdrawal-related apoptosis or whether the cell cycle is perturbed.

Chapter 6

CONCLUSION

Apoptosis is controlled by two major pathways, initiated either via cell surface receptors (extrinsic) or via disruption of mitochondria and release of contents into the cytosol (intrinsic) (162, 350). The disruption versus the maintained integrity of the mitochondria is a highly regulated cellular mechanism controlled in part by the Bcl-2 family of proteins. At the mitochondria, 'pro-survival' Bcl-2 family members maintain mitochondrial integrity, while pro-apoptotic proteins such as Bax and Bak, play a role in perturbing the mitochondrial wall. Disruption of the mitochondrial wall results in the release of mitochondrial contents including cytochrome c and numerous other proteins that play a role in initiating caspase activation. The activation of caspases results in the degradation of intracellular molecules which eventually leads to the death of the cell (162).

Bcl-2 was first characterized as an oncogene that was over-expressed in B-cell lymphomas (15). However, unlike the growth-promoting oncogenes that had been described at that time, the oncogenic function of Bcl-2 was shown to be due to its ability to prevent apoptosis (124). Once researchers demonstrated that Bcl-2's form and function were evolutionarily conserved, this gave way to a plethora of studies investigating both the mechanisms controlling and the importance of apoptosis in the development of diseases such as cancer and neurological degenerative disorders. With respect to cancer, many of the chemotherapeutic agents or radiation treatments that were found to be efficacious stem from their ability to cause apoptosis of cancer cells (28, 351-355). Further interest was fuelled by findings indicating p53 kills cells mainly by a Bcl-2-dependent mechanism, since Bcl-2 overexpression can block most forms of cell deaths induced by p53 (257, 356, 357). Both clinical observations and mouse model experiments suggest that inhibition of apoptosis (e.g. p53 mutation, Bcl-2 overexpression) (283, 358) greatly promote oncogenic transformation caused by mutations that promote cellular proliferation alone (e.g. c-Myc overexpression, Ras mutations). Thus, reversing the process of tumorigenesis by promoting cell death, such as by activating p53 function or by inhibiting Bcl-2 function, may allow novel ways to complement our current treatments

for malignancies. Thus, agents that directly mimic the BH3-only proteins would be predicted to induce cell death and since they are known to antagonize the pro-survival Bcl-2 family members, therefore, be of value therapeutically. In particular, since many of the oncogenic mutations, such as those to p53 results in defects in sensing cellular damage that would normally result in cell death by a Bcl-2-dependent mechanism, directly targeting Bcl-2 and its homologs may circumvent such mutations. This may also permit an alternative route to overcome tumor resistance to current treatments.

To date, the solution structures of Bcl-xL (37) and Bcl-2 (38) have been solved, and NMR structural analysis of Bcl-xL complexed with the BH3 domains of Bak (39) and Bad (359) shows that the BH1, BH2, and BH3 domains of Bcl-xL form a hydrophobic groove which envelops the α -helical BH3 domain of Bad or Bak. Given this level of understanding it has been possible to design BH3 peptidomimetics (86, 89, 90) such as ABT-737 (91) which is currently in clinical trials (360). ABT-737 (BH3 mimetic) development represents an overall shift towards rational, targeted approaches to inducing apoptosis.

As such, there has been a large effort directed towards understanding the precise role of Bcl-2 family members in controlling apoptosis. Stemming from such studies, Bad's function in the regulation of apoptosis has become well established (287, 361). Knock-in mice expressing Bad with Ser mutated to Ala at 112, 136 and 155 sites showed effects in growth factor responsive or apoptosis-inducing conditions, affecting the threshold for mitochondrial disruption (287). The complete knockout of Bad is viable, but those mice confirmed the role of Bad as a 'sensitizer' BH3 molecule, since there were signs of altered growth factor survival signaling (288). Studies have also shown that animals with non-functional forms of Bad or those with reduced Bad expression are more susceptible to developing cancer with increased age, and in response to sublethal radiation (287, 288). Thus, the lack of functional Bad can have a major effect in homeostasis and therefore, understanding how Bad is regulated may have major therapeutic implications.

It has been shown that phosphorylation of Bad at multiple serine residues controls whether it is able to bind with pro-survival proteins such as Bcl-xL (65, 96, 130), and consequently neutralize their pro-survival function. The interaction and consequences of Bcl-2 member interactions and functional control of Bad through phosphorylation lead to several studies investigating how the presence of survival cytokines and growth factors are able to promote the phosphorylation of Bad (112). Stemming from these studies, five main sites of phosphorylation have been characterized. Phosphorylation of Ser112 and Ser136 were shown to promote association of Bad with cytosolic 14-3-3 proteins, thus inhibiting Bad's ability to interact with Bcl-xL at the mitochondria (125). Phosphorylation at Ser155 appears to represent another mechanism controlling Bad/Bcl-xL interaction. Ser155 which lies within the BH3 domain, has been shown to disrupt the interaction of Bad with Bcl-xL (135, 136). More recently, phosphorylation of Bad at Ser128 was reported to promote apoptosis (77), but the significance of this is unclear since the sequence of murine and human Bad are not completely conserved at that site. Investigators in Duronio's lab reported a fifth site of phosphorylation on Bad at Ser170 (137). Interestingly, this site was shown to alter a novel growth inducing characteristic of Bad. Though the mechanism by which phosphorylation at Bad-Ser170 functions to control the activity of Bad is not yet clear, the thesis does present data indicating that CaMKII-gamma phosphorylates Bad-Ser170, therefore providing insight into the mechanism controlling phosphorylation state of this site. Furthermore, we show through the use of Bad mutants mimicking dephosphorylation at Ser 112, 136, and 155, that dephosphorylation of Ser170 is able to transform the Bad protein into a more potent inducer of cell death. Conversely, Bad mutant mimicking phosphorylation at Ser 170 was shown to be less able to promote apoptosis regardless of whether the other three sites (S112, S136, and S155) were dephosphorylated.

Moreover, we were intrigued by the fact that the Bad-Ser170Asp mutant can promote an increase in cell number (as well as blocking normal apoptotic activity of Bad); thus, we further investigated the potential connection between phosphorylation at Ser170 and cell cycle regulation. The work presented in chapter 4 shows that the expression of Bad S170A results in a slowing of cell cycle progression through S phase

and this effect appears to be unrelated to Bad's apoptotic functions. We speculate that this intersection of Bad's pro-apoptotic and cell cycle regulatory activity may represent a sensory mechanism by which Bad acts as signaling node where apoptotic and cell cycle signals converge. The work in this thesis has provided some evidence to further explore the potential role of the Ser170 site, which may regulate protein associations via phosphorylation of Ser170. Considering we have shown that CaMKII (specifically CaMKII- γ) kinase activity against Bad Ser170 increases not only in response to pro-survival IL-3 signalling but at specific stages of the cell cycle, this may represent a survival signal threshold prior to cells committing to undergoing DNA replication. Experiments examining the effects of CaMKII inhibition on cell cycle stage transition, specifically at the G1/S transition, may provide insight into a mechanism involving Bad and cell cycle regulation.

Bad's cell cycle effect is supported by work describing Bad affecting cell proliferation (321) and similar studies suggesting a role for Bcl-2 or Bcl-xL in cell cycle re-entry from G₀ into G1/S (248). The premise for several of the studies presented in this thesis originated from other work being pursued in our lab. Work by Jamil et al. showed that Mcl-1, which is known to suppress cell growth when overexpressed, interacts with inactive Cdk1 in the nucleus (298). This interaction, and other interactions, including with the checkpoint kinase, Chk1, has been recently shown to be involved in DNA damage response signaling (320). Based on some of these findings, we were interested in learning whether Bad was able to interact directly with proteins known to regulate cell cycle progression. Since we had data showing Bad's effect was specific to the S phase, we examined Bad's ability to interact with known S phase regulators. From these experiments we demonstrate that Bad is able to interact with Cdk2 and Cyclin E. It is likely very significant that Cdk2 together with Cyclin E are known to play a key role in the entry into S phase of the cell cycle and we show that the kinase activity phosphorylating the Ser170 site is greatest in the S phase of the cell cycle. Thus, we have this additional evidence that the phosphorylation of Bad at the Ser170 site is cell cycle regulated, as is the kinase that phosphorylates that site.

As detailed in Chapter 4 we have shown that the kinase that phosphorylates the Bad-Ser170 site, and which is responsible for the pro-survival effect of that phosphorylation, is calmodulin-dependent kinase II gamma (CaMKII- γ). Furthermore, we show that CaMKII acts as a cytokine-dependent survival kinase, as we demonstrate CaMKII activity levels increase when cells are stimulated with IL-3. In addition, more recent work done by lab colleagues have gone on to show that IL-3 stimulation specifically increases the activity level CaMKII- γ . Recent experiments done in our laboratory have also shown that CaMKII- γ immunoprecipitated from cell lysates, shows activity against Bad Ser170 peptide. Together, all of our data supports a role for CaMKII (more precisely CaMKII- γ) in mediating cytokine-dependent survival by virtue of its phosphorylation of Bad at Ser170.

CaMKII's effects on both apoptosis and cell cycle appear to be cell type specific. It is likely that the variability observed in different cell types is a consequence of differential expression patterns of CaMKII isoforms. It is known that CaMKII holoenzymes have altered functions depending on their size and isoform composition (196). Thus a careful characterization of the CaMKII isoforms expressed will likely be necessary before predictive cellular consequences of increased or decreased CaMKII activity can be made. From this, it will be interesting to determine the effects of overexpressing CaMKII- γ in response to IL-3 survival signaling and cell cycle progression.

Another aspect of our studies that will be important to pursue in future is the mechanism by which a cytokine such as IL-3 can activate CaMKII activity. As shown, we found that IL-3 stimulated phosphorylation of CaMKII activation site (Thr286) and Bad Ser170. Understanding which signaling pathways and kinases are responsible for initiating the phosphorylation and activation of CaMKII in haemopoietic cells will be of much interest. Furthermore, understanding how CaMKII- γ is regulated in haemopoietic cells and what are the cellular consequences of altering CaMKII- γ expression and activity, through knockdown siRNA or overexpression experiments and/or expressing constitutively active mutant forms of CaMKII- γ .

The potential role of Bad in cell cycle regulation combined with findings associated with CaMKII as the Ser170 kinase represents a new direction in the study of this protein. Though the cell cycle effect of Bad may not represent its main function there does appear to be a growing level of interest into the intricate connections between proteins that were formerly thought to be solely responsible for either cell cycle or apoptosis regulation.

Future work exploring the cellular consequences of Bad/CyclinE/Cdk2, as it relates to cell cycle progression and cell death would be of great interest. In several attempts, experiments were carried out to examine the effect of Bad phosphorylation at Ser170 on the association with Cdk2 and CyclinE; however we were unsuccessful in achieving a clear result. This was mostly due to the difficulty of working with CyclinE given its unstable characteristics along with inconsistent results associated with non-detection due to poorly functioning antibodies. However, given sufficient time to troubleshoot with additional reagents, these results may shed light on a novel mechanism which may involve the signaling convergence between what is thought of as two separate mechanisms: apoptosis and cell cycle. Moreover, understanding the cellular signals that affect these interactions may also provide insight into the mechanism by which Bad affects cell cycle. Stemming from studies in progress in our lab, examining the potential involvement of Bad in DNA surveillance and G1/S or S phase checkpoint may also result in a greater understanding of Bad's cell cycle related role.

Understanding how Bad Ser170 is able to play a key regulatory role in both apoptosis and cell cycle, the two most widely studied aspects of cancer progression, may hold therapeutic promise. Peptidomimetics based on the specific sequence surrounding Ser170 may be able to elicit a similar effect as what has been observed with full length Bad S170A. That is cells under stressful conditions, similar to IL-3 starvation, undergo apoptosis while normally growing cells are relatively unaffected. The other therapeutic avenue relates to CaMKII inhibition, which we have shown to cause apoptosis. Since we have shown evidence that specifically CaMKII- γ phosphorylates Bad, gamma isoform specific inhibitors may represent another therapeutic strategy.

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