[Frontiers in Bioscience 11, 1508-1521, May 1, 2006]

Posttranslational modifications of Bcl2 family members-a potential therapeutic target for human malignancy

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1. ABSTRACT

Apoptosis is a process that can occur normally, such as during tissue remodeling, embryogenesis or abnormally during certain pathologies, such as cancer (1-4). The identification of the Bcl2 (5) as well as IAP family members (6) has suggested that excessive inhibition of apoptosis may constitute a common feature of all known human cancers-the ability to influence their onset, progression and outcome. Bcl2 family proteins are frequently regulated by phosphorylation that affects their activity and conformation. The structural analysis of antiapoptotic members of Bcl2 family has contributed to a better understanding of the functional domains including the discovery of an unstructured "loop region" (LR) near the N-terminus exposed to the cytoplasm. The antiapoptotic members of Bcl2 family such as Bcl2/Bcl-xL/Mcl-1 are phosphorylated on specific serine/threonine residues within this unstructured loop in response to diverse stimuli including treatment with chemotherapeutic taxanes, survival factor addition or chemopreventive agents. In most instances, such phosphorylation has been associated with the loss of their biological function (7-71). The chemoresistant tumors overexpress Bcl2/Bcl-xL/Mcl-1(72). To this end, the apoptosis yielding effect due to phosphorylation of antiapoptotic Bcl2 family members is quite interesting. Phosphorylation-dephosphorylation pathway of these antiapoptotic proteins should be an ideal molecular target for therapy of subpopulation of cancer in which these death repressors are essential prognostic markers. Thus, further gaining the knowledge on the mechanism of inactivation of Bcl2/Bcl-xL/Mcl-1 by phosphorylation might be of paramount importance to therapy for human malignancies in which overexpression of these antiapoptotic proteins plays an essential role.

2. INTRODUCTION

2.1. Apoptotic signaling-an ideal target for cancer therapeutics

The knowledge of the molecular and genetic mechanisms regulating the development of cancer is emerging. At the same time, the range of options with which to combat this disease is on rise. Currently, the most promising approaches available to the patients to fight against cancer include immunotherapy, gene therapy, antisense or chemotherapy. While immunotherapy utilizes the body's immune system either directly or indirectly to boost body's cancer fighting mechanisms, gene therapy can be directed towards replacing some of the defective genes, believed to perturb normal cellular growth. On the other hand, chemotherapy is a traditional approach for the treatment of cancer and remains the subject of extensive research. The ongoing challenge in the development of chemotherapy or any therapy is the effectiveness of the therapeutic agent to kill tumor cells selectively without unnecessary damage of healthy tissue. Nonetheless, the advances in biotechnology have benefited us in the search for better drugs with fewer side effects.

The vast strides made in comprehending the abnormalities in signaling mechanisms of cancerous cells have opened therapies that can be targeted to disrupt the undesired pathways. By disrupting the signals within a cancer cell, that direct it to ignore the normal cellular process, the cancer cell can be destroyed or knocked into dormancy. In this respect, the modulation of cell death pathway in cancer cells is an enticing approach to target therapy of human cancer (73, 74).

2.1.1. Apoptosis at a glance

The programmed cell death, or apoptosis is a highly regulated process that is essential for the development and tissue homeostasis within all multicellular organisms. Diverse stimuli such as growth factor withdrawal, deregulation of the cell cycle, DNA damage or death receptor-ligand interaction might be responsible for initiating the apoptotic cascade (12, 13, 75-77). These pro-apoptotic signals induce several early events that converge by activating a common biochemical pathway, which then leads to the execution of apoptosis. Mitochondrion appears to be a core component of the cell death machinery (78-80). Several cell death signals have been reported to release cytochrome c from mitochondria into the cytosol (78-80). Released cytochrome c then binds to Apaf-1 (apoptosis protease activating factor-1) to form an apoptosome complex with procaspase-9 in an energy dependent manner (81-83). Subsequently activated caspase-9 triggers downstream caspases such as caspase-3 and caspase-7 (83). These downstream caspases, through the cleavage of several death substrates, are believed to cause execution of cell death. The pro-apoptotic members of Bcl2 family (84, 85) were previously shown to be inducers of mitochondrial damage in specific death signaling pathways. However, reports are available suggesting that apoptosis can also be triggered independent of cytochrome c release from mitochondria (86-89).

An astounding rate of progress has taken place in the field of apoptosis. The multiple apoptotic pathways emanate from mitochondria. Due to the exposure to an apoptotic trigger, the pro-apoptotic proteins such as Bax, Bak or Bid transduce signal to mitochondria by translocating themselves into the mitochondrial membrane. This signal can be neutralized by the anti-apoptotic proteins such as Bcl2 (5), Bcl-xL (90) or MCL1 (29) residing on the mitochondrial membrane. If this signal is further transduced for the absence or inability of anti-apoptotic proteins, mitochondrial damage is reflected by loss of membrane potential. Eventually, apoptogenic proteins such as cytochrome c, SMAC/Diablo, AIF and EndoG are released. Although Cytochrome c activates downstream caspase-9 by making complex with Apaf-1, SMAC /Diablo relieves IAP family of proteins from caspase-9. IAP family of proteins can bind to caspase-9 and can keep this caspase-9 in an inactivated state (6, 81). In some instances, concurrent with cytochrome c release, SMAC is also released from mitochondria to release caspase-9 from IAP family proteins such as survivin. Once released from IAPs, caspase-9 can freely form holomeric complex with Apaf-1 and cytochrome c to execute apoptosis. However, AIF or Endo G released from mitochondria can directly cause chromatin condensation bypassing caspases (87, 88). Despite a significant gain of knowledge acquired regarding mitochondria mediated apoptosis, many questions remain unanswered. The biochemical mechanism by which the apoptogenic proteins are released from mitochondria is still a mystery.

2.2. Phosphorylation of Bcl2 family members and functional outcome

The Bcl2 family comprises of two counteracting groups of proteins: the pro-apoptotic and anti-apoptotic (1, 2). Among the anti-apoptotic members, Bcl2, Bcl-xL or Mcl-1 is phosphorylated by microtubule disarraying agents such as Taxol, nocodazole or 2-Methoxyestradiol (7-71). In most cases, phosphorylation of the Bcl2 family members leads to the loss of their biological function. The emerging concept yielded from these studies is that phosphorylation induced inactivation of Bcl2 protein on Ser 70 residue inside the unstructured "loop region" (LR) during mitosis might work as a checkpoint to permit apoptosis (13, 15, 18, 19). The LR of both Bcl2 and its close homologue Bcl-xL can negatively regulate their functions as evident by enhanced anti-death activity of LR deficient or phosphorylation-defective mutants (15, 18, 19, 41, 43). The screening of a library of phage-displayed peptides identifies human Bcl2 as a Taxol (paclitaxel) binding protein (91). By chemical approach, it was found that paclitaxel's core skeleton and its C-13 side chain significantly contribute to its interaction with LR of Bcl2 (92). By sequence comparison of various Bcl2 family proteins derived from different species, four structural domains have been characterized and termed as BH-1, BH-2, BH-3 and BH-4, where BH stands for Bcl2 homology domain (1, 2). Of interest is the absence of the BH4 domain in many of the proapoptotic Bcl2 family members including Bax, Bak, Bik and Bad. Another important point to note is the exclusive presence of variable loop region (LR) in antiapoptotic members of Bcl2 family. As described previously, the deletion of the loop region or mutation of specific phosphorylation sites in this LR augments antiapoptotic

activity of Bcl2 (15, 18, 19, 43). Of note, the endogenous phosphorylation of Bcl2 without treatment of any trigger can be detected in M phase of normally cycling cells (19). Another interesting observation demonstrates the ability of phosphorylated Bcl2 to regulate Ca^{2+} homeostasis and apoptosis (93). Phosphorylated Bcl2 binds less BH3 only BIM or multidomain BAX proapoptotic protein as observed earlier (11, 94-96). Precisely, phosphorylation of Bcl2 at mitosis would increase calcium in endoplasmic reticulum (ER) and could account for the increased G2/M susceptibility to apoptosis (93).

In addition to the Taxanes, kinase inhibitors (68), phosphatase inhibitors (7), proteasome inhibitors (23, 24), arsenic trioxide (25), radiation (26), chemopreventive agents (48, 94-95) also can induce Bcl2 phosphorylation with simultaneous apoptotic cell death. Interestingly, amongst these novel triggers of Bcl2 phosphorylation, proteasome inhibitors can cleave Bcl2 to its proapoptotic fragment as originally observed earlier (97). Besides, growth factor signaling molecule such as Insulin-like growth factor binding protein-3 (IGFBP-3) can mediate apoptosis induced by TNF-alpha through the inactivation of the cell survival protein Bcl2 via serine phosphorylation in prostate cancer cells PC-3 (69). Interestingly, studies have discovered a novel signaling between insulin receptor and Bcl2 phosphorylation (42). Insulin receptor substrate proteins can enhance antiapoptotic activity of Bcl2 by suppressing insulin triggered Bcl2 phosphorylation in cells derived from B-lymphocytes (42). Insulin receptor substrate protein, IRS-1 binds to the LR of Bcl2 to suppress its phosphorylation. Ueno *et al* (42) suggest that the apoptotic resistance property of Bcl2/Bcl-xL might be conferred due to sequestering of the loop region by IRS-1. The sequestering of the loop region that contains phosphorylation sites of Bcl2/Bcl-xL might hinder access of the Bcl2/Bcl-xL specific kinase to phosphorylate them.

Tubulin-binding anticancer drug triggered Bcl2 phosphorylation directs cells to apoptotic cascade, the outcome of cytokine dependent Bcl2 phosphorylation remains controversial. Previously, IL3 induced Bcl2 phosphorylation was demonstrated to exert antiapoptotic activity by creating point mutation on Ser 70 residue of Bcl2 protein (98). Subsequent report by Yamamoto *et al* (19) contradicts that by showing the ability of Ser 70 Ala mutant of Bcl2 protein to protect apoptosis in IL-3 dependent cell line, FL 5.12. However, the mechanism of IL3 induced Bcl2 phosphorylation might be different from that of tubulin-binding drugs.

Although studies with the inhibitor of Jun kinase (JNK) and phosphorylation defective mutant of Bcl2 reveal the role of JNK mediated Bcl2 phosphorylation in apoptosis of cancer cells (19,22,44,48,55,56, 60,67,94,95), it is true that multiple kinases including Cdc2 (33), Raf-1 (14,71), Protein kinases (16), mTOR kinase (38) can also phosphorylate Bcl2 in response to multiple stimuli. In addition, v-cyclin/cdk6 phosphorylates Bcl2 in U2OS and Cos-7 cells during G1/S phase of cell cycle (99). In line with this observation, Bassik *et al* (93) noted some phosphorylated Bcl2 in aphidicolin arrested G1/S cells. However, Ask1/JNK1 pathway appears to be responsible for the robust phosphorylation of Bcl2 during cell cycle progression as a normal physiologic process that inactivates Bcl2 at G_2/M (19). In this context, report by Miyoshi *et al* (48) is worth mentioning. Benzylisothiocyanate (BITC) induced apoptosis was accompanied by G2-M arrest and Bcl2 phosphorylation. The authors demonstrated here that the p38 MAP K pathway could be operative in cell cycle arrest induced by BITC, whereas JNK pathway plays a major role in apoptosis but not in the cell cycle regulation.

To the other end, growth factor suppression of apoptosis strongly relates with the phosphorylation of multiple proapoptotic proteins including Bcl2 family members BAD (101-108) or Bim (109,110). Growth factors induce the phosphorylation of BAD at three sites, Ser-112, Ser-136 and Ser-155, which inactivates the proapoptotic activity of BAD (101-108). Like Bcl2, several kinases that have been implicated in survival signaling have been proposed to trigger BAD phosphorylation including Akt, Rsk, PAK, $p70^{S6K}$, protein kinase A (101-108). In order to assess the contribution of BAD phosphorylation in cell survival, Datta *et al* (101) generated mice with point mutation in the BAD gene that abolish BAD phosphorylation at specific sites. BAD phosphorylation was shown to protect cells from the deleterious effects of apoptotic stimuli. BAD phosphorylation attenuates death pathway signaling by raising the threshold at which mitochondria releases cytochrome c to induce cell death. Similar to BAD, Harada *et al* (109) demonstrates the phosphorylation of proapoptotic BH3-only protein BIM by survival factor induced exrtracellular signal-regulated kinase. Phosphorylation of BIM inhibits its association with BAX and proapoptotic activity. When the proapoptotic protein BAD is phosphorylated on two different serine residues in the presence of a survival factor IL-3, phospho BAD is sequestered by 14-3-3 protein resulting in its inability to exert proapoptotic function. Apparently, phosphorylation of Bcl2 family proteins (Bcl2, Bcl-xL, BAD or Bim) interferes with their binding abilities to the respective partners, thus resulting in functional inactivation.

Bcl-xL (90), a close homologue of Bcl2, is an important regulator of apoptosis and is overexpressed in human cancer (72). Phosphorylation of Bcl-xL can also be induced by microtubule-damaging drugs such as Taxol or 2-ME (10, 34, 35). By site-directed mutagenesis studies, we have mapped phosphorylation sites for Taxol or 2-ME induced Bcl2/Bcl-xL phosphorylation in prostate cancer cells (Figure 1A & Refs. 9, 10, 13). While Bcl2 is phosphorylated on multiple serine/threonine (Ser 70, Ser 87, Thr 69) residues, Bcl-xL on the other hand gets phosphorylated on single serine residue at position 62 (10).

The substitution of phosphorylation site Ser to Glu enhances the negative charge of a protein and mimics the phosphorylated state of the protein. In the literature, it is quite common practice to test the functional effect of these phosphomimetic mutants (111-113). On this basis, we substituted serine 70 residues in Bcl2 protein with Glutamic acid residue. S70E mutant Bcl2 runs with slower mobility on SDS-PAGE (Figure 1C, lane 3), thus confirming the

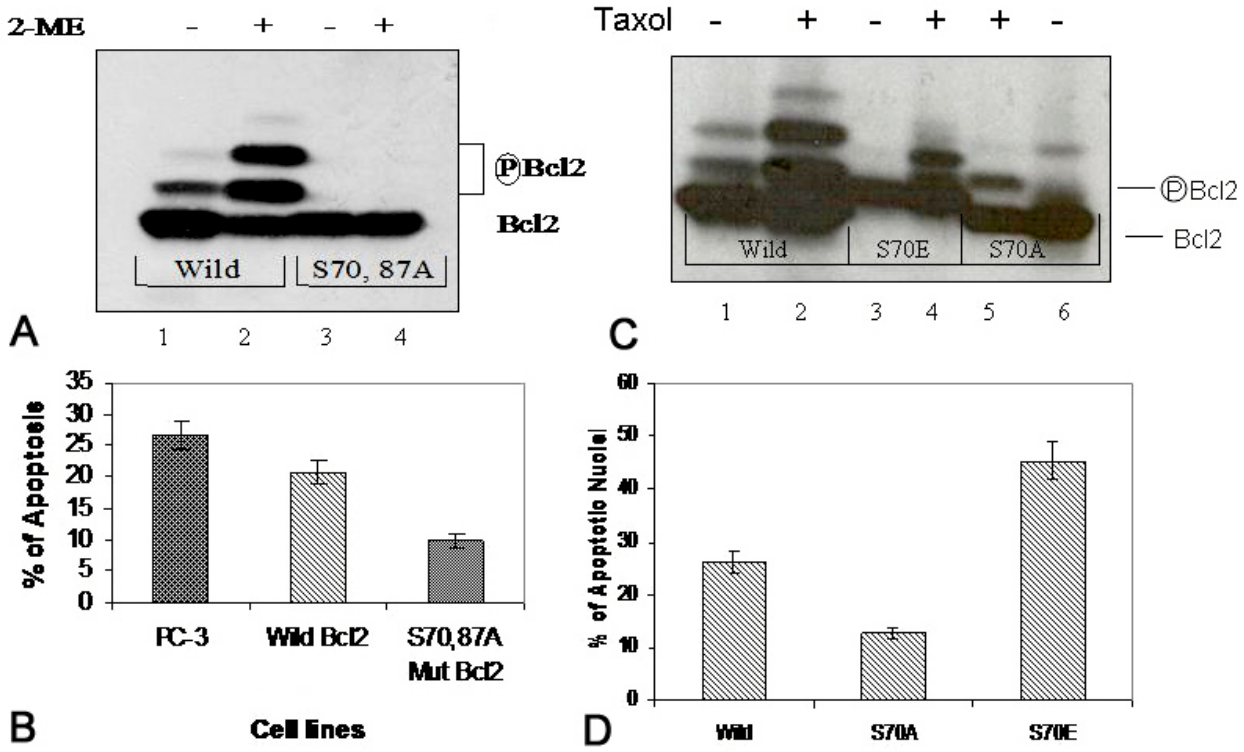

Figure 1. Taxol or 2-ME induced Bcl2 phosphorylation at Ser-70 residue regulates its antiapoptotic function. A & B. Substitution of Serine 70 & 87 residues with Alanine attenuates 2-ME induced Bcl2 phosphorylation and renders gain of antiapoptotic function. PC-3 cells genetically engineered to overexpress wild type and S70, 87A mutant Bcl2 were exposed to 5 μM 2-ME for 16 hrs. Cell free extracts were subjected to Western blot (A) using mouse monoclonal antibody against human Bcl2. Panel B, PC-3 parental cells as well as equally expressing wild type and S70, 87 A mutant transfected clones were treated with 5 μM 2-ME for 24 hrs. Cells were fixed with 4% paraformaldehyde followed by staining with DAPI. Each value represents mean ± S.D. of three independent experiments. C & D. Phosphomimetic mutant (S70E) of Bcl2 exhibits greater sensitivity to microtubule disarraying agents than phosphorylation defective mutant (S70A). PC-3 cells were transiently transfected with wild type, S70E as well as S70A mutant Bcl2 cDNA. 24 h post transfection, cells were treated with 100 nM Taxol for another 24 h. Total cellular extract was either subjected to Western blot (C) with Bcl2 antibody or determination of apoptotic index (D).

expression of phosphomimetic form of Bcl2. In addition, functional studies with S70E mutant and S70A mutant clearly indicate that prostate cancer cells transfected with phosphomimetic mutant (S70E) are more sensitive to Taxol than those with phosphorylation defective mutant (Figure 1D).

In line with our site directed mutagenesis studies, phosphorylation site-specific antibody against Bcl2/Bcl-xL developed in the laboratory also recognizes slower mobility (phosphoforms) of Bcl2/Bcl-xL (Ref. 8; Figure 2). Phosphorylation site specific antibody against Bcl-xL does not recognize Bcl-xL in the control lysate (lanes 1,3,5,7 & 9 of Figure 2B). However, this antibody can predominantly detect Bcl-xL in all Taxol treated lysate (lanes 2, 6, 8, 10 of Figure 2B) except Ser62Ala phosphorylation defective mutant (lane 4, Figure 2B). This observation using phospho Bcl-xL specific antibody strengthens the identity of the site of phosphorylation by mutagenesis studies.

Further studies with the inhibitor of Jun kinase (JNK) and phosphorylation null mutant of Bcl2/Bcl-xL reveal the significant role of JNK mediated Bcl2/Bcl-xL phosphorylation in apoptosis of different cancer cells (10,19,22). Precisely, studies by others and us suggest that the phosphorylation of Bcl2/Bcl-xL by stress response kinase signaling might oppose the antiapoptotic function of Bcl2/Bcl-xL to permit leukemic, prostate or breast cancer cells to die by apoptosis. The mechanism by which tubulin-binding drugs can bypass the death suppressor effect of Bcl2/Bcl-xL is not known. However, in the pursuit of dissecting the molecular events associated with Bcl-xL phosphorylation and microtubule damaging anticancer drugs, we have observed: i) phospho Bcl-xL is dephosphorylated due to longer exposure of Taxol and proteasome inhibitor can stabilize phosphoforms of Bcl-xL**.** ii) phospho Bcl-xL has shorter half-life than native Bcl-xL.

As evident in Figure 3A, the extent of Bcl-xL phosphorylation reaches a peak at 16-24 hrs (lanes 2 & 3) whereas a longer period of exposure of Taxol such as 48 hrs leads to the decline of phosphoforms of Bcl-xL (lane 4*).*We have already shown that the dephosphorylation of phospho Bcl2 can be blocked when proteasomes are

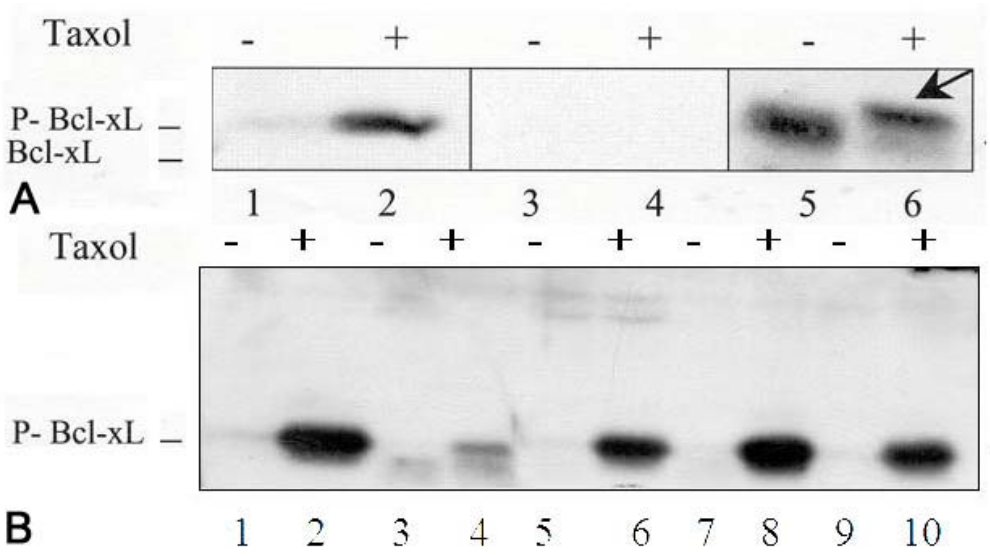

Figure 2. Characterization of Phospho Bcl-xL specific antibody. A. Phospho Bcl-xL (Ser 62) specific antiserum specifically recognizes phosphoform of Bcl-xL on Western blot (composite Figure). Lanes 1,3,5: Untreated PC3 cell lysate, Lanes 2,4,6: 100 nM Taxol treated PC3 cell lysate. Lanes 1 and 2: Phospho Bcl-xL-specific antiserum; Lanes 3 and 4: Preimmune serum; Lanes 5 and 6: Bcl-xL monoclonal antibody. Arrows indicate phospho Bcl-xL. In order to develop phospho Bcl-xL specific antibody the 15 mer Bcl-xL peptide, "P S W H L A D S* P A V N G A T" (S* indicates phosphorylated serine-62 residue) was synthesized. HPLC purified peptide was coupled to KLH. Phosphopeptide antibody was developed in rabbit and characterized as described for phospho Bcl-2 specific antibody (8). B. Phospho Bcl-xL antibody can detect Bcl-xL in all Taxol- treated lysate except Ser62Ala mutant. Lanes 1& 2: Wild-type; Lanes 3&4: Ser62 Ala mutant; Lanes 5& 6: Ser56 Ala mutant; Lanes 7& 8: Thr47 Ala mutant; Lanes 9 & 10: Thr115 Ala mutant. Control and Taxol treated lysate from DU145 cells stably transfected with wild, Ser62Ala, Ser56Ala, Thr47Ala, Thr115Ala mutant Bcl-xL were subjected to immunoblot analysis with phospho (Ser62) Bcl-xL specific antibody.

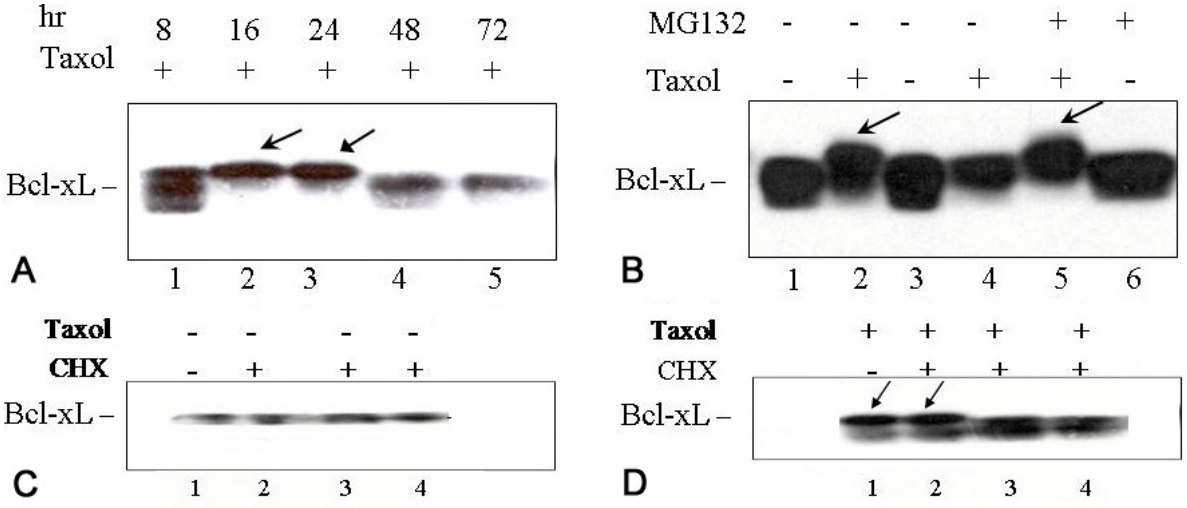

Figure 3. Phospho Bcl-xL is stabilized by proteasome inhibitor. A. Time-course studies of Bcl-xL phosphorylation in the presence of Taxol. DU145-2 cells (DU145 cells ectopically overexpressed with wild type Bcl-xL) were challenged with 1μM Taxol for different time-periods (8-72 hrs. Total protein extract was immunoblotted using monoclonal antibody against Bcl-xL. The slower mobility form indicated by arrows represents phosphorylated Bcl-xL. B. Effect of MG132 on dephosphorylation of Bcl-xL in PC-3 cells. Lane 1: 24 hr DMSO control; lane 2: 1μM Taxol for 24 hrs; lane 3: 48 hr DMSO control; lane 4: 1μM Taxol for 48 hrs lane 5: 48 hr Taxol (1μM) and last 24 hr MG132 (20 μM); lane 6: 24 hr MG132 alone. C. Phospho Bcl-xL disappears at a faster rate than native Bcl-xL in DU145-2 cells. Panel C, CHX treatment in the absence of Taxol; Panel D, CHX treatment in the presence of Taxol. Panels C & D, lane 1: 0 hr; lane 2: 2 hr CHX; lane 3: 4 hr CHX; lane 4: 6 hr CHX; Arrows indicate phosphoform of Bcl-xL

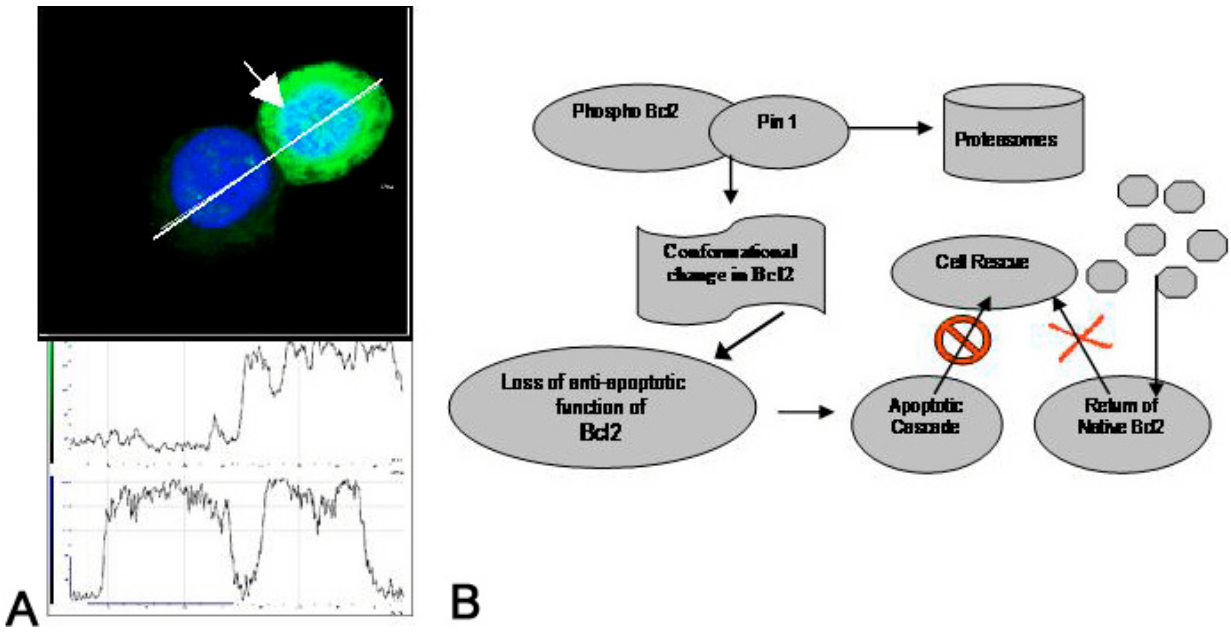

Figure 4. Confocal Microscopy with phospho Bcl2 specific antibody. A. 2-ME exposed W-34 cells (PC-3 cells genetically engineered to overexpress wild type Bcl-2) show merging of phospho Bcl2 inside the nucleus in a cell affected with 2-ME (indicated by arrow). Histogram of the above image further shows superimposition of FITC stain (Green) with the nuclear TO-PRO stain (Blue) in the affected cell. Y-axis represents FITC (upper histogram) and TOPRO (lower histogram) fluorescence intensity. X-axis in both histograms represents distance along the designated line on the image. B. Schematic presentation of interaction of phospho Bcl2 and Pin1. Phosphorylated Bcl2 can associate with nuclear peptidyl prolyl isomerase Pin1 due to nuclear envelope breakdown in mitotic arrested cells. Pin1 induced conformational change may hinder its antiapoptotic function. Proteasomal degradation of Pin1reverts phosphorylated Bcl2 to dephosphorylated state. But the cells reach to an irreversible executioner phase of apoptosis.

inactivated (8). Similar observation was made in the case of Bcl-xL dephosphorylation in DU145-2 cells. Phospho Bcl-xL gets dephosphorylated due to 48 hr Taxol exposure (lane 4, Figure 3B), but this dephosphorylation can be prevented if cell permeable proteasome inhibitor, MG132 is added after 24 hr Taxol treatment (lane 5, Figure 3B)

In order to determine whether phosphorylated form of Bcl-xL disappears faster than non-phospho form, Bcl-xL overexpressing DU145-2 cells were first treated with 1μM Taxol for 16 hrs. The levels of Phospho Bcl-xL were monitored in the presence or absence of 20 μg /ml cycloheximide for several time-periods (2-6 hrs). Similar approaches using cycloheximide were undertaken to determine the half-lives *(t ½)* of other proteins such as Bcl2, p53 and Survivin (8). In the presence of protein synthesis inhibitor, cycloheximide (CHX), phospho Bcl-xL disappears at 4 hr (Panel D, Figure 3). In contrary, the status of native Bcl-xL remains unchanged at this time point (Panel A, Figure 3). Of note, phosphoforms of Bcl2 also disappear faster than non phospho form (8). The mechanism of faster disappearance of phospho Bcl2/Bcl-xL might be attributed to the association with a nuclear cis-trans peptidyl prolyl isomerase, Pin1 (33, 114). Interestingly, the half lives ($t_{1/2}$) of both Pin1 and phospho Bcl2/ Bcl-xL are similar and phosphoforms of Bcl2/Bcl-xL are detected inside the nucleus (Refs.8, 66 & Figure 4A). Perhaps Pin1 can facilitate dephosphorylation of phospho Bcl2/Bcl-xL in a proteasome dependent manner (8). Apparently, the association of phospho Bcl2/Bcl-xL might induce a conformational change to modulate their antiapoptotic function (Figure 4B).

2.2.1. Bcl2 phosphorylation studies *in vivo*

The phosphorylation of Bcl2 at the G2-M phase of normally cycling cells (19) indicates that the phosphorylation of Bcl2 is a normal physiologic process rather than exclusively a response to microtubule damage. Taxanes treatment represents a convergence of G2-M arrest, microtubule polymerization followed by phosphorylation of Bcl2. The accumulating evidences in the field would convene that phosphorylation of Bcl2 is functionally linked to apoptosis. It has been shown that cells in which Bcl2 is phosphorylated are more sensitive to environmental stress or in other words phosphorylation of Bcl2 is necessary to decrease the threshold at which mitochondria release cytochrome c in response to apoptotic stimuli.

Despite the phosphorylation of Bcl2 family members provided a clue for apoptotic signaling, there lies a major caveat: all experiments have been performed in cell culture or *in vitro*. The physiological significance of Bcl2 phosphorylation in the context of multicellular organism is not clear. The most comprehensive way to test the *in vivo* function of a gene is to place it transgenically into the germ line of mice. This renders a prospective opportunity to examine the effects of this gene on multiple cellular

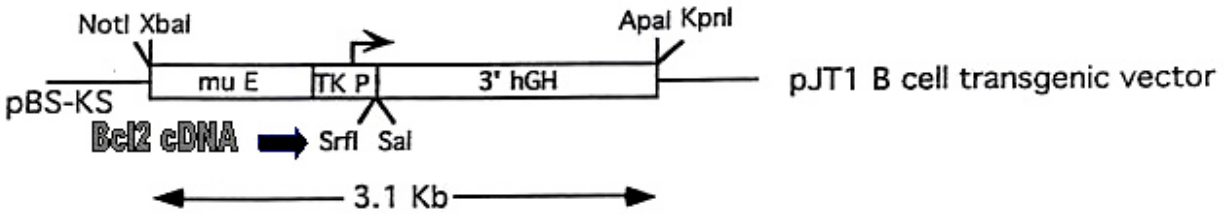

Figure 5. Preparation of the transgenic constructs. S70, 87A mutant Bcl2 cDNA cloned in pcDNA3 was digested with Hind III and XbaI. The purified insert was blunt ended and ligated to Srf1 digested, dephosphorylated expression vector pJT1. The positive clones were identified by Kpn1 and Xba1 digestion. The clones were verified by automated sequencing. The transcription unit was liberated from the positive clones by digestion with Kpn1 and Xba1 prior to microinjection in mice.

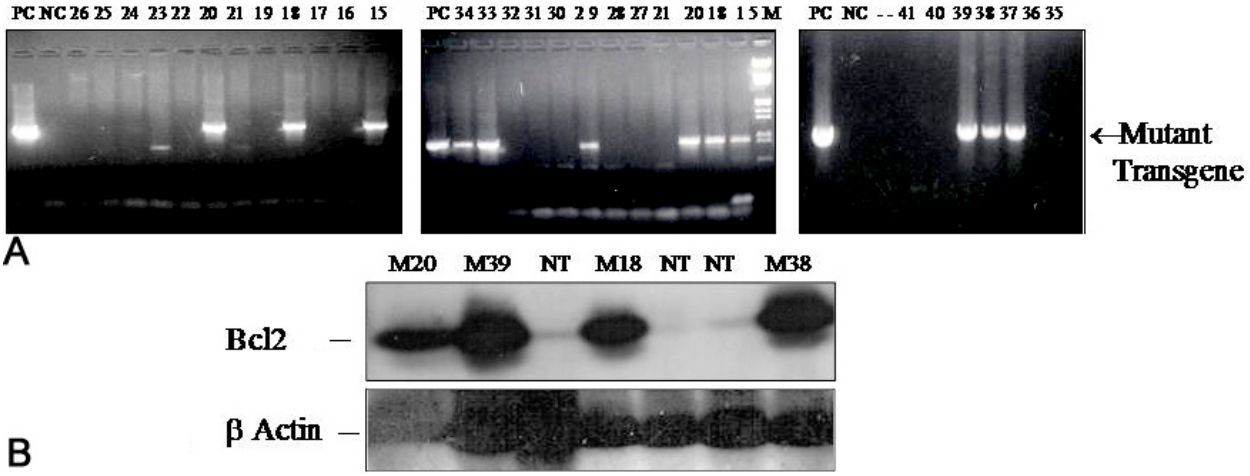

Figure 6. Expression of mutant Bcl2 in transgenic founders. A. PCR genotyping of F_0 founder tail DNA from Ser 70, 87 Ala mutant Bcl2 mouse. PC: Positive control PCR using 2.5 pmole Bcl2 cDNA as template. NC: Negative control PCR using water instead of template DNA. M: Molecular weight marker (EcoR1 and Hind III digested λ DNA). Numbers on each lane denote the identity of individual mouse. Tail biopsy was done in 5 weeks old pups. Genomic DNA was isolated from tails and PCR reaction was performed using 5' (5'GAG AAG CTT GTG CCG TTG GCC CCC 3') and 3' (5'GGT TCT AGA ACA GCC TGC AGC TTT 3') end primers of Bcl2 cDNA. This process results in the amplification of approximately 0.85 kilobase fragment representing human Bcl2 cDNA transgene. However, it does not amplify endogenous mouse Bcl2. S70, 87A mutant Bcl2 DNA sequences were verified in PCR reaction products. B. Expression of the protein product of the mutant Bcl2 transgene in spleen tissue of F_1 mice. Western blot of total proteins extracted from spleen of individual mouse were performed with Bcl2 antibody that specifically detects human Bcl2 and does negligibly cross react with endogenous mouse Bcl2. Numbers denote transgenic line. NT: Nontransgenic littermate.

lineages during the development of an organism. Cells expressing mutant form of Bcl2 might accumulate genetic alterations because of the lack of phosphorylation. Thus, we thought that the generation of S70, 87A mutant Bcl2 transgenic mice, in which two regulatory serine residues are changed to alanine should provide an important genetic tool.

2.2.2. Germ line transmission of Ser 70,87 Ala mutant Bcl2 cDNA in mice

For this purpose, we have cloned phosphorylation-defective mutant cDNAs in B-cell specific expression vector pJT1 (kindly provided by Dr. Tim Behrens, University of Minnesota), because Bcl2 was originally implicated with B cell lymphoma. The transcription unit of the mutant Bcl2 transgenic construct (Figure 5) was injected into the pro-nuclei of fertilized eggs of donor mice. The injected eggs were implanted to the oviduct of pseudopregnant foster mother.

The presence or absence of mutant Bcl2 transgene in the founder mice (C57BL6/SJL-F2) was determined by PCR genotyping (Figure 6A). Genotyping revealed that 9 transgenic founders were positive out of 38 total Ser 70, 87 Ala mutant F0 mice. These positive mice were mated with C57BL/6 mice for producing F1 progeny. The expression of the protein product of human Bcl2 transgene in F1 progeny was confirmed by Western blot (Figure 6B).

2.2.3. Histopathology of lymphatic tissues of Ser 70,87Ala Bcl2 transgenic mice

Initial examination of spleens from 15-30 weeks old mice exhibited enlargement of spleen in phosphorylation defective mutant as compared to the nontransgenic animal (Figure 7A). The above phenotype with approximately two-fold increase in weight of the spleen than nontransgenic littermate was evident in all mutant lines. Ten percent of the S70, 87A mutant Bcl2

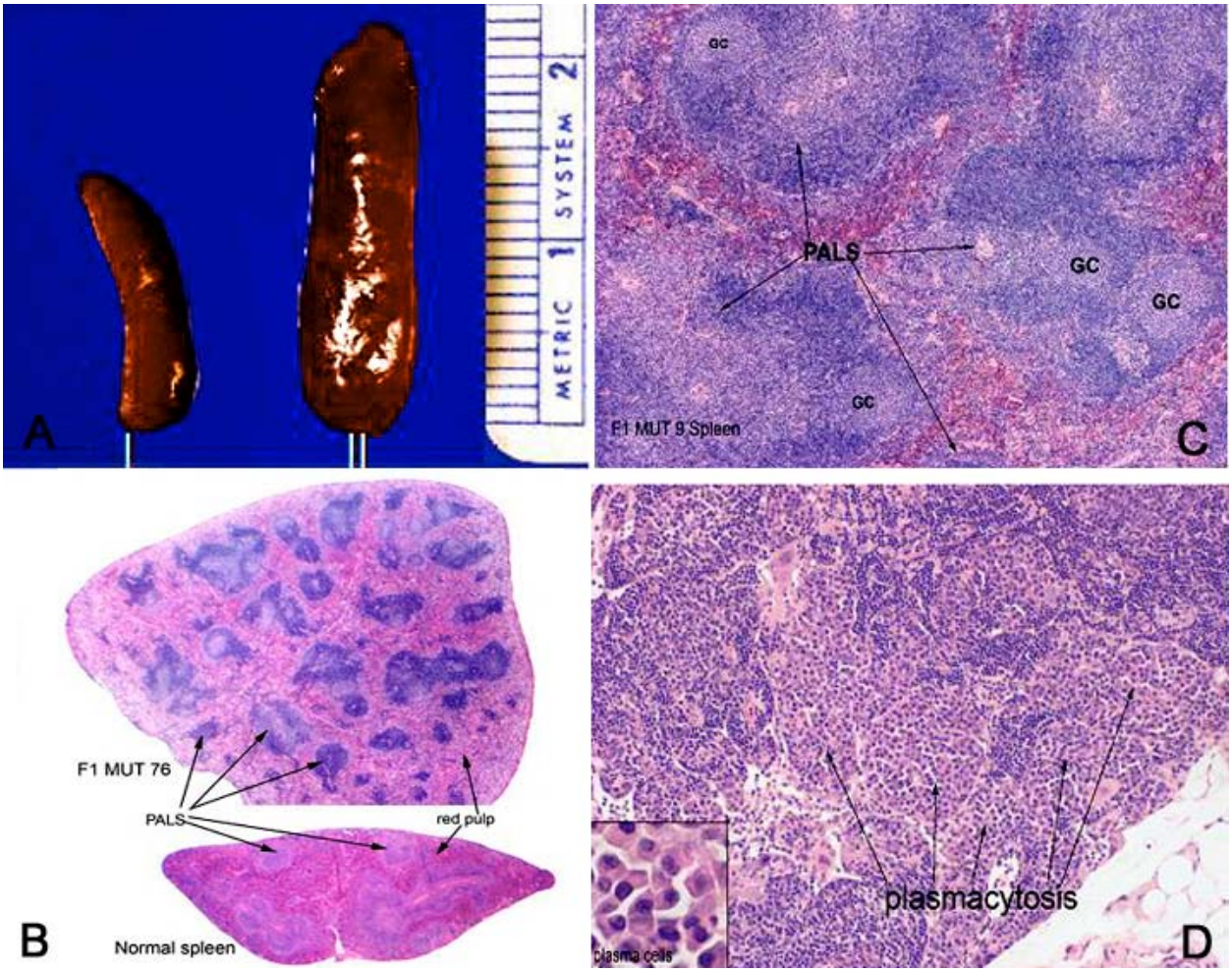

Figure 7. Light microscopic studies of lymphoid tissues from S70, 87A mutant Bcl2 transgenic mice. A. Phosphorylation defective mutant Bcl2 causes splenic enlargement. Photographs of spleens from 10 week old S70, 87A Mutant Bcl2 (II), and Nontransgenic mouse (I) are presented. B, C & D. Histopathological evaluation of Spleen and lymph node from different S70, 87A mutant Bcl-2 transgenic lines. Tissues were fixed in neutral buffered formalin followed by paraffin embedding, sectioning and staining with hematoxylin and eosin. B: Normal spleen and spleen of F1 Mut 76 mouse. Mutant expressing spleen exhibits follicular hyperplasia and plasmacytosis. C: Spleen of F1 MUT 9. The PALS are expanded and coalesced and the white pulp contains multiple follicles with active germinal center (GC). D: H&E stained section of lymph node of F1 MUT 11 mouse. The follicles are easily visible because of active germinal centers and the cortex has generally increased cellularity. The medullary cords are aggregates of plasma cells.

mice exhibited approximately 5-fold increase in spleen weight as compared to control mice. This phenotype is not due to lack of uniform genetic background, since it has been observed up to F4 generation resulting from crossing with C57BL/6 mice.

Histopathological evaluation (Table 1) revealed that spleens from mutant transgenic mice remarkably varied from their normal littermate. Multifocal lymphoid hyperplasia of white pulp zones was observed in all lines. Mutant expressing spleen exhibits follicular hyperplasia and plasmacytosis. The PALS (periarteriolar lymphatic sheath) are expanded (Figure 7C), coalesced and the white pulp contains multiple follicles with active germinal centers (GC). In addition, microscopic examination of lymph node indicated reactive lymphoid hyperplasia characterized by follicular hyperplasia and plasmacytosis (Figure 7D). Precisely, lack of *in vivo* phosphorylation of Bcl2 in mouse model leads to hyperplastic splenic follicles perhaps by expansion of B cell compartment. Thus our investigation suggests that Bcl2 phosphorylation is not associated with survival signaling pathway in lymphoid system *in vivo*.

3. PERSPECTIVES

The focus of our research program is orientated towards investigating the role of Bcl2/Bcl-xL phosphorylation in the regulation of its antiapoptotic activity. Taxol-induced Bcl2/Bcl-xL phosphorylation occurs at Ser residues (s) that are followed by proline. The

Table 1. Summary of Microscopic Diagnoses of S70, 87A Mutant Mice

Animal ID	Size of Section(mm) [1]	Splenic Histology
F1MUT9	5x5	Expansion of PALS, multifocal; follicular hyperplasia, multifocal; plasmacytosis, multifocal
F1MUT10	3x5	Expansion of PALS, multifocal; follicular hyperplasia, multifocal; plasmacytosis, multifocal
F1MUT53	5x5	Follicular hyperplasia, multifocal
F1MUT57	4x6	Expansion of PALS, multifocal; melanosis
F1MUT59	3x5	Expansion of PALS, multifocal
F1MUT76	5x5	Follicular hyperplasia, multifocal; plasmacytosis, multifocal
F2MUT33	6x6	Follicular hyperplasia, multifocal; plasmacytosis, multifocal

[1] All sections from mutant mice are somewhat larger than typical sections of normal spleen, H & E stained sections of spleen tissues isolated from different S70, 87A mutant mice were analyzed by light microscopy. Mice from F1 and F2 generation were used for Histopathological studies. PALS: Periarteriolar lymphatic sheath.

posttranslational modifications induced in Bcl2/Bcl-xL during cell cycle arrest triggered by antimicrotubule drugs promote association with a mitotic protein Pin1, a member of peptidyl prolyl isomerase (PPIase) family This PPIase binds through its WW domain to proline containing phosphoserine and/or phosphothreonine motifs in target proteins (114-117). The phosphorylation site(s) of Bcl2/Bcl-xL comprise of the consensus motif ideal for being a substrate for PPIase (8). The region in Bcl-xL containing phosphorylation sites represents a proline-rich "loop region" (LR) that has been associated with autorepression of their antiapoptotic activity. The discovery of novel downstream interactions of phosphorylated Bcl2/Bcl-xL raises the potential conformation alteration of Bcl2/Bcl-xL by Pin1 association and thereby might modulate their function in M phase arrested cells. Immunofluorescence experiments performed in synchronized HeLa cells indicate that mitotic phosphorylated forms of Bcl-2 can be detected in nuclear structures in prophase cells (66). However, the elaborative studies should be undertaken in cancer cells where Pin1 gene will be post-transcriptionally silenced by siRNA approach (118-119). The results yielded from these studies in the presence and absence of Pin1 should give us a clear understanding on the significance of their association.

The endogenous phosphorylation of Bcl2 without treatment of any trigger can be detected if the M phase cells are elutriated (19). However, the role of endogenous phosphorylation of Bcl2 family members remains elusive. Our recent knowledge on the phosphorylation site has enabled to generate phosphomimetic mutant of Bcl2/Bcl-xL**.** Further investigation employing phosphomimetic as well as phosphorylation defective mutant mice are warranted.

To the other end, a clinical study revealed that 83% of breast tumors with high phospho Bcl2 expression were sensitive to treatment to paclitaxel or docetaxel in contrary to tumors expressing low phospho Bcl2 (96). In order to validate that the presence of phosphorylable Ser residue (s) of Bcl2/Bcl-xL might be of therapeutic advantage for tubulin-binding anticancer drugs *in vivo*. This can be accomplished by implanting wild type, phosphomimetic and phosphorylation null mutant Bcl2/Bcl-xL overexpressing cancer cells in athymic mice. The effect of Taxanes needs to be assessed by measuring tumor growth, monitoring phosphorylation of Bcl2/Bcl-xL and apoptosis. These future studies should unravel findings related to *in depth* mechanism of Bcl2/Bcl-xL phosphorylation. The knowledge gained on the mechanism of altered apoptotic signaling by anticancer tubulin binding drugs should uphold a great promise for potential therapeutic approaches.

4. ACKNOWLEDGEMENTS

The work was supported by grants (CA 77328 & CA109181) from National Cancer Institute. We gratefully acknowledge Dr. Tim Behrens, University of Minnesota for providing us with pJT1 vector. We wish to thank Drs. Yingli He and Rachael Mann of Transgenic mouse core facility, Case Western Reserve University for their assistance in microinjection and helpful discussions in preparing the transgenic lines. We also thank Dr. Meredith A Simon for reviewing hematoxylin and eosin (H & E) stained tissue sections from transgenic mice.

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Key Words: Apoptosis, Cell death, Bcl2, Bcl-xL, Phosphorylation; Cancer, Tumor, Neoplasia, Review

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