Regulation of Bcl2 Phosphorylation and Potential Significance for Leukemic Cell Chemoresistance

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Although considered tightly linked, the linkage effectors for proliferation and antiapoptotic signaling pathways are not clear. Phosphorylation of Bcl2 at serine 70 is required for suppression of apoptosis in interleukin 3 (IL-3)-dependent myeloid cells deprived of IL-3 or treated with antileukemic drugs and can result from agonist activation of mitochondrial protein kinase Cα (PKCα). However, we have recently found that high concentrations of staurosporine up to 1 μM can only partially inhibit IL-3-stimulated Bcl2 phosphorylation but completely block PKCα-mediated Bcl2 phosphorylation in vitro, indicating the existence of a non-PKC, staurosporine-resistant Bcl2 kinase (SRK). Although the RAF-1-MEK-1-mitogen-activated protein kinase (MAPK) cascade is required for factor-dependent mitogenic signaling, a direct role in antiapoptosis signaling is not clear. In particular, the role of phosphorylation in the regulation of death substrates is not yet clear. Our findings indicate a potential role for the MEK/MAPK pathway in addition to PKC in antiapoptosis signaling, involving Bcl2 phosphorylation that features a role for extracellular signal-regulated kinase (ERK)1 and 2 as SRKs. These findings indicate a novel role for ERK1 and 2 as molecular links between proliferative and survival signaling and may, at least in part, explain the apparent paradox by which Bcl2 may suppress staurosporine-induced apoptosis. Although the effect of phosphorylation on Bcl2 function is not clear, effector molecules that regulate Bcl2 phosphorylation may have clinical significance in patients with acute myelogenous leukemia (AML) who express detectable levels of Bcl2. Preliminary findings suggest that expression of PKCα, ERK2, and Bax in leukemic blast cells from patients with AML, although individually not prognostic, appears to have potential clinical value in predicting chemoresistance and survival outcomes. [J Natl Cancer Inst Monogr 2000; 28:30–7]

Hematopoietic growth factors, such as interleukin 3 (IL-3), mediate cell growth by stimulating proliferation and by suppressing the process of programmed cell death (1–4). A great deal is known about the molecular components and mechanisms that regulate IL-3 and other cytokine superfamilly receptor-mediated signal transduction pathways that result from receptor dimerization and activation of nonreceptor protein tyrosine kinases like JAK2, with coupling to the activation of cytoplasmic signal transducers and activators of transcription (3,5,6) or the activation of the Src-homology collagen, growth factor receptor-bound protein 2, son of sevenless–coupled RAS/RAF-MEK-1/mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK) pathway (3,7–9). However, relatively little is understood about the postreceptor signaling mechanism(s) by which growth factors, such as IL-3, might also couple to and regulate apoptosis.

Bcl2 and related family members are key regulators of programmed cell death or apoptosis, a natural process required for normal development, and they play a role in malignant transformation and autoimmune diseases (10–14). Bcl2 was discovered in the oncogene hunt as the oncogene fusion product of the immunoglobulin H (IgH) promoter and full-length Bcl2 characterized by the t14:18 breakpoint translocation found in approximately 80% of patients with indolent non-Hodgkin’s lymphoma (13–15). The survival function of Bcl2 as a potent suppressor of apoptosis was initially demonstrated when Bcl2 was shown to facilitate prolonged survival following exogenous expression in IL-3-dependent hematopoietic cells that were deprived of IL-3 (16) and later through studies with transgenic and knockout mice (17–20). Thus, in the absence of IL-3, cells default to a suicidal apoptotic pathway involving intracellular proteolysis, which, in turn, can be inhibited by Bcl2 (16). Subsequently, Bcl2’s ability to promote prolonged, but not indefinite, cell survival under various types of apoptotic stress (e.g., treatment with chemotherapy drugs, irradiation, exposure to toxins, or viral infection) was also discovered (16–18,21,22).

Work in our laboratory has uncovered a novel regulatory role for IL-3 in post-translational regulation of induced Bcl2 phosphorylation (23,24). The mechanism(s) by which IL-3 and other survival agonists may induce Bcl2 phosphorylation and the potential regulatory role for this post-translational modification on Bcl2’s function will be the focus of this study.

Bcl2 Functions as a Docking Protein with Potential Pore-Forming Properties

The Bcl2 family, which now numbers some 16 members, is made up of both suppressors (Bcl2, BCL-XL, and MCL-1) and inducers (Bax, Bad, Bak, and Bid) of apoptosis [reviewed in (10–12)]. Briefly, Bcl2 has four conserved Bcl2 homology (BH) functional domains and seven α-helical regions providing structure. The BH1, BH2, and BH3 domains are also contained in some pro-apoptotic death effector members, and mutational studies have shown these domains to be necessary for Bcl2-Bax heterodimerization and any potential Bcl2 or Bax pore-forming properties (25–29). The heterodimerization of Bcl2 and Bax recently has been formally demonstrated in vivo (30) and is currently considered important, at least in part, for Bcl2’s ability to block Bax’s potent death effector properties (25,30). On the basis of the recent crystallographic and solution structure of Bcl2-Xc (31) and a BH3-only Bax-derived peptide, the BH3 domains in Bcl2, Bax, and other BH3-only members, such as Bid

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and Bim, are also modeled as death agonists (27,32,33). One popular model features their death-inducing effects occurring as a result of their potential membrane pore-forming properties that are potentially exerted as a result of binding to Bcl2 or Bcl-XL and/or integrating into mitochondrial membranes (25,30,34). For example, Bax and the BH3-only Bid death effectors can bind to Bcl2 and become associated with the mitochondrial membranes. Apparently, when their death properties are revealed, they become integrally associated with mitochondrial membranes that potentially open megapore channel(s) (35). This presumably is accompanied by migration of Bax to the mitochondria in association with leakage of caspase activators to the cytosol and collapse of the mitochondrial membrane potential (28–30). In support of this model, the crystal structure of Bcl-XL and Bax predicts a similar structure with that of bacterial colicins and diphtheria pore-forming toxins that function by disrupting membrane function (31,34–36). Furthermore, purified Bax can apparently directly induce mitochondrial membrane leakage in intact mitochondria in vitro by a process that can be blocked by Bcl2 (36). Thus, Bcl2 may function, at least in part, by dock ing with and/or somehow “neutralizing” Bax’s pore-forming properties (25,34–36). Alternatively, other functional properties of Bcl2 may result from its potential role as a multidocking site for other death regulators or components of mitogenic signal transduction pathways, including protein kinases (e.g., RAF-1) and phosphatases (e.g., protein phosphatase 2B [PP2B]) (Fig. 1).

**Bcl2 is Functionally Phosphorylated on Serine 70 by IL-3 and Other Survival Agonists**

Bcl2 was initially identified as a potential phosphoprotein when expressed in SF9 cells in which it was shown to prolong cell survival following baculovirus infection (43). Later studies in our laboratory (23,24) discovered that IL-3 could induce a rapid and robust serine phosphorylation of Bcl2. Importantly, this modification correlated closely with cell survival in factor-dependent cells and suggested a functional role for phosphorylation. It is interesting that the potent protein kinase C (PKC) agonist and natural product bryostatin-1 (Bryo), which can also support survival of IL-3-dependent myeloid cells following IL-3 withdrawal, were found to induce Bcl2 phosphorylation, which initially suggested a functional role for PKC (23,24,44). Phosphorylation of Bcl2 was found to occur at the same serine site, whether induced by Bryo, IL-3, or the related erythropoietic hormone erythropoietin (23,24). Bcl2 mutational studies confirmed a functional role for phosphorylation at the evolutionarily conserved ser70 site, which is located in a putative regulatory region known as the flexible loop domain (FLD) (30).

Thus, only Bcl2 containing the serine 70 to alanine (S70A) mutation failed to undergo phosphorylation by either IL-3 or Bryo, and this mutant also displayed a severely reduced survival function when stably expressed in factor-dependent cells (23,24). However, cells expressing the S70A Bcl2 mutant did fare slightly better with respect to survival than vector-only transduced parental cells, indicating that the nonphosphorylatable S70A Bcl2 mutant does retain some function under these conditions (24). This argues that phosphorylation may not be the only regulatory mechanism for Bcl2. In light of its multidocking and putative pore-channel properties, this is not surprising. By contrast, conversion of serine 70 to glutamate (S70E), a charged amino acid that could potentially mimic a phosphorylation site, resulted in an increased survival function. Thus, cells expressing S70E Bcl2 were more viable following the stress of either IL-3 withdrawal or etoposide chemotherapy treatment than cells expressing similar amounts of exogenous wild-type (wt) Bcl2 (24). These data strongly argue that ser70 is a regulatory site for Bcl2 (24).

Later studies allowed us to conclude that phosphorylation is necessary for Bcl2’s full and potent survival phenotype, at least in factor-dependent myeloid cells. Presumably, this extends to other growth factor-sensitive cells expressing Bcl2 because nerve growth factor (NGF) also induces Bcl2 phosphorylation in PC12 pheochromocytoma cells in association with survival (45). Of interest, dephosphorylation of Bcl2, even in the presence of NGF, is closely linked to apoptosis in these cells.

The serine 70 site of Bcl2 is evolutionarily conserved and is located within the predicted unstructured FLD of Bcl2 (31,46).
The FLD is a stretch of approximately 50 amino acids (aa 30–80) that resides between the putative α1 and α2 helical structures that separate the amino terminal BH4 and BH3 domains of Bcl2 (Fig. 1) (26,27,46). The potential loop domain is conserved between Bcl2 and Bcl-XL, suggesting functional significance (46). It is interesting that deletion of this loop region from either Bcl-XL or Bcl2 results in a molecule with enhanced survival function under specific circumstances, such as when expressed in WEHI-231 cells that undergo apoptosis following exposure to IgM (46,47). It has, therefore, been proposed that the FLD may represent a negative regulatory region (46). However, one report (48) that uses the identical Bcl2 loop deletion mutant has found that this domain is required for its survival function, at least when cells are treated with certain chemotherapeutic agents, including paclitaxel (Taxol). One explanation for this apparent paradox is that deletion of the large loop domain may functionally represent a “phosphorylation equivalent” mutation. Thus, if the FLD region were a negative regulatory region, phosphorylation might somehow “inactivate” its negative effect on survival. This possibility would be consistent with most reported findings in IL-3-dependent cells because, in the absence of IL-3 or a survival agonist, Bcl2 phosphorylation is not easily detected and the negative regulatory properties of the FLD may then dominate (24). Also consistent with this notion, forced overexpression of a nonphosphorylatable Bcl2 mutant (S70A) was unable to prolong cell survival following IL-3 deprivation or treatment with etoposide chemotherapy compared with wt or S70E Bcl2 (24).

**Bcl2 IS A SUBSTRATE FOR AT LEAST TWO Bcl2 KINASES: PKCα AND A STAUROSPORINE-RESISTANT Bcl2 KINASE**

We have previously reported that PKCα is a physiologic Bcl2 kinase (44). However, the existence of another, non-PKC Bcl2 kinase(s) was also indicated, as overexpression of exogenous Bcl2 is reported to protect cells from apoptosis that would normally be induced by high concentrations of the potent PKC inhibitor staurosporine (49). Thus, a staurosporine-resistant Bcl2 kinase(s) (SRK) was sought. Involvement of an MAP kinase (i.e., ERK1 or ERK2) was considered likely according to reports that association of the MAP kinase phosphatase-1 (MKP-1) was associated with Bcl2 dephosphorylation and apoptosis in NGF-dependent PC12 cells treated with angiotensin 2 (45). Because IL-3 can rapidly activate the RAF-MEK-1-MAPK pathway (50), we tested a role for an MAPK in Bcl2 phosphorylation. Preliminary studies that used various protein kinase inhibitors indicated that PD98059, a specific MEK-1 inhibitor, could, like staurosporine, only partially block IL-3-induced Bcl2 phosphorylation. However, the combination of PD98059 and staurosporine could completely shut down IL-3-induced Bcl2 phosphorylation (51). Thus, ERK1 (p44) and ERK2 (p42) were identified as potential candidate Bcl2 kinases. It is interesting that a distinct population of cytosolic ERKs was found to be located in the heavy membrane mitochondrial subcellular fraction, indicating potential as direct Bcl2 kinases. When individually tested, ERK1 and ERK2 were found to be potent, direct Bcl2 kinases (Fig. 1) (51). Collectively then, although these findings identify ERK1 and ERK2 as physiologic Bcl2 kinases, they cannot exclude the formal possibility that other SRKs may also exist (Fig. 1) (51).

**Bcl2 PHOSPHORYLATION IS A DYNAMIC PROCESS INVOLVING PHYSIOLOGIC Bcl2 KINASE(S) AND A PHOSPHATASE**

Although Bcl2’s survival function can be regulated, at least in part, by phosphorylation at ser70, phosphorylation is not a static process (52). Rather, Bcl2 phosphorylation represents a balance between a Bcl2 kinase(s) and a phosphatase(s) (Fig. 1). Thus, the potential for perturbing Bcl2’s survival function through agonist-induced Bcl2 phosphorylation may also occur via phosphatase activation (52). Concerning this possibility, it is interesting to note that ceramide, a potent PP2A activator (53,54), can be rapidly generated intracellularly after treatment of cells with various types of cell death stimuli, including cytotoxic cytokines like tumor necrosis factor α (55), chemotherapeutic drugs (56,57), ischemia/reperfusion injury (58), FAS antigen activation (59), irradiation (60), and corticosteroids (61). Indeed, the production of ceramide is so common during apoptosis that it has been considered a universal feature of this process (62,63).

Whether ceramide is a trigger for cell death is not clear, but C2-ceramide can induce cell death when added directly to cells (55). We have discovered that C2-ceramide, but not the functionally inactive C2-dihydro-ceramide, can potently inhibit Bcl2 phosphorylation induced by either IL-3 or Bryo (64). Reversal of phosphorylation resulted from the rapid activation of a mitochondrial-associated, okadaic acid-sensitive PP2A-like activity that was directly associated with Bcl2. Of interest, however, cells expressing the functionally potent S70E Bcl2 mutant fail to undergo apoptosis after treatment with high concentrations of C2-ceramide that can potently activate PP2A and would readily induce apoptosis in cells expressing wt or S70A Bcl2 (64).

These findings indicate that inhibition of Bcl2 phosphorylation may be one mechanism by which C2-ceramide can induce apoptosis in IL-3-dependent myeloid cells that express Bcl2. In support of this possibility, it was demonstrated that, although NGF can induce Bcl2 phosphorylation and survival in PC12W pheochromocytoma cells, NGF-induced Bcl2 phosphorylation could be inhibited and cells induced to undergo apoptosis after addition of angiotensin-2 (45). Angiotensin-2 was found to potently activate the MAPK-phosphatase, MKP-1, that resulted in apparent inhibition of MAPK/ERK activity and was associated with loss of phosphorylation of Bcl2 (45). These findings are consistent with the notion that inhibition of Bcl2 phosphorylation is associated with apoptosis. However, because protein phosphatases and kinases seldom have a solitary substrate, it may be possible that the phosphorylation of other potential molecule(s) may affect the survival status of the cell. Furthermore, other potential Bcl2 kinases and phosphatases may also exist.

It has been reported that Bcl2 may bind and sequester the protein phosphatase PP2B in association with protection of Jurkat T cells from apoptosis induced by PP2B/calcinurin overexpression (39). Thus, although PP2B could be a potential Bcl2 phosphatase on this basis, we found that, at least in vitro, PP2B is a much weaker Bcl2 phosphatase than PP2A or PP1 (52). This finding suggests that PP2B’s role in Bcl2’s binding may not have a direct effect on phosphorylation and function; alternatively, Bcl2 may regulate PP2B’s role in FAS-ligand-induced apoptosis of T cells by actively soaking up PP2B (65). Alternatively, because PP2B is, like BAD, located primarily in the cytosol, one other consequence of PP2B binding to Bcl2 may be to sequester this enzyme and to prevent dephosphorylation of...
cytosolic substrates such as BAD, which can help trigger apoptosis under some circumstances (66–68).

**Bcl2 Phosphorylation May Potentially Affect the Proteolytic Cleavage of Both Bcl2 and Bax**

It was reported that the N-terminal domain of Bcl2 could be proteolytically cleaved at a recognized caspase 3 proteolytic site at D34 (40). Furthermore, cleavage of Bcl2 renders a truncated form (Δ34N-Bcl2) that is nonfunctional in protecting cells from IL-3 deprivation. These data suggest that the cleaved N-terminal region of Bcl2, which contains the BH4 domain that is the docking site for such signaling proteins as RAF-1, PP2B, and p53, is potentially required for its potent antiapoptotic activity. Thus, IL-3 postreceptor signaling may somehow protect Bcl2 from inactivation by caspase cleavage (40). Our preliminary findings (24) support this notion. We found that steady-state expression of Bcl2 is maintained and cell survival prolonged after IL-3 deprivation in cells that express wt but not S70A-Bcl2 (Fig. 2). This finding suggests that the ser70 site phosphorylation of Bcl2, although not being required for Bax heterodimerization, may protect it from proteolytic cleavage. Precisely how phosphorylation may affect this process is not yet clear. It is interesting that phosphorylation can apparently protect pro-caspase 9 and presenilin-2 from proteolytic cleavage (69,70). Presenilin-2 is a transmembrane protein potentially involved in early onset of Alzheimer’s disease (70). Phosphorylation at a serine site residing c-terminal to an aspartate target site for caspase apparently retards the neuronal apoptotic process characteristic of this neurodegenerative disease (70).

Although Bcl2 is an integral mitochondrial membrane protein that heterodimerizes with Bax, the majority of Bax is not an integral membrane protein, at least during normal cell growth (71,72). Rather, Bax is primarily cytosolic and/or only peripherally associated (i.e., not membrane integrated) with the mitochondria membranes (such that it can fractionate with mitochondria unless extracted by a pH 11.5 alkali treatment to remove peripherally associated proteins) (71). Bax can be translocated during stress from the cytosol to the outer mitochondrial membrane, where it will apparently integrate into the membrane via its hydrophobic c-terminal transmembrane domain (71–73). However, how Bax is cleaved and/or translocated from the cytosol to become an integral membrane protein that may trigger or be involved in apoptosis is not yet clear. Bax is a 21-kd protein. It was found that p21 Bax can be cleaved at the N-terminus to yield a p18 Bax form that apparently is more efficient at membrane insertion, at least in part, the stability of the interaction between Bcl2 and Bax (25) and potentially retard Bax cleavage. Our findings also indicate that an intact Bcl2 ser70 phosphorylation site is required to maintain the tight association between Bax and Bcl2 observed during co-immunoprecipitation from detergent lysates of cells (Fig. 2). Thus, the nonphosphorylatable, severe loss of function of S70A Bcl2 displays a significantly decreased association with Bax. Although Bcl2 phosphorylation is not required for Bax: Bcl2 association, it may stabilize such an association. Indeed, ceramide-induced Bcl2 dephosphorylation also appears to correlate with a similar decrease in Bcl2:Bax association (Fig. 3). Importantly, cells expressing the S70E Bcl2 mutant, which mimic phosphorylation but cannot be dephosphorylated, are viable even at elevated concentrations of ceramide (i.e., 50 μM). By contrast, cells expressing wt Bcl2 are killed at ceramide levels (10 μM) in which serine 70 is dephosphorylated (64). Furthermore, under conditions of IL-3 deprivation, Bax undergoes rapid proteolytic cleavage from a p21 to a p18 Bax form (Deng X, Ruvolo P, Carr BK, May WS: unpublished data). Thus, Bax cleavage is pronounced and occurs in cells that express S70A Bcl2 after IL-3 withdrawal. This finding suggests...
that phosphorylation of Bcl2 at ser70 can modulate Bcl2 : Bax stability and potentially protect p21 Bax from proteolysis. How Bcl2 phosphorylation may protect Bax and/or Bcl2 itself from proteolysis and whether cleavage of these substrates may be involved in initiating and/or amplifying the process as compared with simply being degraded during apoptosis are not yet clear.

In addition to Bcl2 and Bax, pro-caspase 9, an initiator of the intrinsically activated caspase cascade (74,75), is also apparently regulated by phosphorylation and proteolytic cleavage (69). Thus, following AKT-induced pro-caspase 9 phosphorylation at ser196, the pro-caspase form remains intact and catalytically inactive (67). A flag-tagged ser196 ala pro-caspase 9 mutant was created that was found to be resistant to AKT-mediated phosphorylation and, importantly, unable to undergo caspase 3 cleavage and enzymatic activation. Thus, post-translational phosphorylation mechanisms may be commonly employed in the regulation of cleavage substrates in the apoptotic pathway. Our finding that phosphorylation of Bcl2 at ser70 is required for its full and potent survival function may potentially be explained by a role in regulating cleavage of itself and/or its heterodimeric, pro-apoptotic partner Bax (Fig. 2).

**MULTISITE BCL2 PHOSPHORYLATION**

In addition to our previous findings (23,24,44) and those of others (76–78) concerning a role for phosphorylation in regulating Bcl2’s survival function, serine phosphorylation of Bcl2 has also been reported to result from the treatment of cells with specific antimitotic chemotherapeutic agents, including paclitaxel, vincristine, vinblastine, and dolastatin 10 (79–84). Because cells undergo apoptosis after exposure to these toxins, it was proposed that phosphorylation could negatively regulate or inactivate Bcl2 (79). However, drug-induced Bcl2 phosphorylation is markedly different from that seen after the addition of growth factors or other survival agonists. First, although IL-3-induced Bcl2 phosphorylation is rapid and occurs within minutes, paclitaxel induces a slow phosphorylation (i.e., 2 hours) that occurs during mitosis only (79–84). Second, unlike IL-3- or NGF-induced Bcl2 phosphorylation (23,24,44,45), paclitaxel-induced phosphorylation is associated with a nonreversible or slowly reversible mobility shifted form of Bcl2 detected by western blot analysis following denaturing electrophoresis (77–84). Third, the Bcl2 kinases responsible for this drug-induced phosphorylation mechanism are reported to be protein kinase A (PKA) and c-Jun N-terminal kinase (JNK) (76,82,83). PKCα and ERK1 or ERK2 are apparently not involved. Furthermore, paclitaxel-induced Bcl2 phosphorylation apparently occurs at three sites, including thr69, ser70, and ser87 (81,83), and Rac-1-activated JNK was found to phosphorylate Bcl2 directly in vitro at multiple sites, including thr56, thr74, ser70, and ser87 (76). Thus, it is possible that mono-site (i.e., ser70) versus multiple-site Bcl2 phosphorylation may differentially affect Bcl2 function, perhaps by inducing different conformational changes in the molecule. However, although antimitotic drug treatment is associated with cell death, the cells expressing the nonshifted and unphosphorylated Bcl2 form are apparently the ones that actually undergo apoptosis (85). Thus, apoptosis likely occurs from the well-characterized mechanism by which such drugs deregulate the dynamic microtubule function (86), and Bcl2 phosphorylation may not be required or involved. To date, it has not been experimentally demonstrated that multisite phosphorylation of Bcl2 renders Bcl2 functionally inactive to suppress apoptosis. Such conclusions have been largely based on circumstantial data. An alternative explanation not yet tested is whether multisite Bcl2 phosphorylation might represent an unsuccessful attempt by the cell to activate and engage any survival mechanism(s) available, but, as a result of the irreversible microtubule damage sustained, cell death is inevitable. This alternate possibility predicts that cells expressing wt Bcl2 will display prolonged cell survival versus nonexpressing cells when treated with antimitotic drugs even if the cells eventually undergo apoptosis. Furthermore, expression of Bcl2 mutants containing a double mutation at both ser70 and ser87 sites to nonphosphorylatable amino acids would be predicted to inhibit apoptosis after paclitaxel treatment. Although there is no evidence available yet that tests the latter prediction, it has been reported that expression of wt Bcl2 can significantly prolong cell survival after exposure to paclitaxel (87). These findings then appear to indicate that treatment with antimitotic agents does not block Bcl2’s antiapoptotic function but rather that Bcl2 can protect against such drug-induced death. Further studies will be required to test the effect of multisite Bcl2 phosphorylation on survival function.

**EXPRESSION OF PKCα, BAX, AND ERK1 AND ERK2 IN CLINICAL AML SAMPLES MAY MODULATE BCL2’S PROGNOSTIC SIGNIFICANCE FOR PATIENT OUTCOMES**

On the basis of the above findings and our preliminary studies, we have tested whether expression of the Bcl2 kinase PKCα and Bcl2 and Bax may have clinical relevance. Earlier, we reported that increased expression of the Bcl2 protein in patient samples of AML cells displaying favorable or intermediate prognosis cytogenetics (FIPC) correlated with decreased rates of successful remission-induction treatment and event-free survival (88). Samples of leukemic blast cells from 165 patients with newly diagnosed AML were obtained from peripheral blood samples (approximately 85% blasts) and analyzed as individual and interactive variables (89). When assessed individually, the expression levels of PKCα or Bax, as compared with Bcl2 (89), were not prognostic of successful standard induction-remission or survival outcomes. However, when evaluated as interactive

![Fig. 3. C2-ceramide addition to cells induces Bcl2 dephosphorylation and is associated with a decrease in Bcl2 : Bax association. Ceramide has been demonstrated to promote the dephosphorylation of wild-type (wt) Bcl2 (64). Co-immunoprecipitation of wt Bcl2 : Bax. Bcl2 : Bax is reduced in a dose-dependent manner, following addition of C2-ceramide to cells before detergent lysis and processing as described in the legend to Fig. 2. Bcl2 : Bax ratio is calculated with the use of densitometry analysis and is expressed as a percentage of the bound Bcl2 : Bax ratio in untreated cells. Ceramide has been demonstrated to promote the dephosphorylation of wild-type (wt) Bcl2 (64). Co-immunoprecipitation of wt Bcl2 : Bax. Bcl2 : Bax is reduced in a dose-dependent manner, following addition of C2-ceramide to cells before detergent lysis and processing as described in the legend to Fig. 2. Bcl2 : Bax ratio is calculated with the use of densitometry analysis and is expressed as a percentage of the bound Bcl2 : Bax ratio in untreated cells.](image-url)
variables, we found that the ratio of either Bcl2 to Bax (B2/Bx) or PKCα · B2/Bx (PK · B2/Bx; i.e., ratios of expression levels of the protein relative to the median level of expression of the individual protein) was highly prognostic for 100 patients with AML who exhibited FIPC (Table 1) (89). Results indicate that the AML samples that displayed a lower ratio of either B2/Bx or PKCα · B2/Bx had a significantly higher initial remission-induction rate (88% versus 69%; \( P = .04 \)) and a prolonged survival (median 141 weeks versus 80.5 weeks, \( P = .007 \)) compared with patients whose blasts demonstrated higher ratios (89). Because a previous correlation was established for Bcl2 expression, a poor outcome but no correlation was observed in these preliminary studies to indicate that expression of individual levels of PKCα or Bax affected outcomes. The expectation was that, when forming the interactive variable terms (i.e., ratios), any prognostic value of Bcl2 alone would be lost (because of the expression of essentially random levels of PKCα or Bax). Surprisingly, however, forming the interactive terms gave greater prognostic discrimination, suggesting that, although the relationships were not immediately apparent on the basis of raw expression levels, a functional relationship among these variables exists. More recent preliminary studies have also suggested that expression of higher levels of ERK2 may also affect Bcl2’s poor prognostic effect on AML (Kornblau SM, RuvoI P, Deng X, May WS; unpublished data). A similar analysis of ERK1 as another Bcl2 kinase is now pending. No definitive conclusions should be drawn at this point from this retrospective analysis because the actual Bcl2 phosphorylation state and the apoptosis rate of individual AML leukemic blast cells were not measured. However, these results were found to be statistically significant and thus suggest that a functional relationship may exist between these variables. Further studies are now in progress to test the role for these variables in a prospective study. If a correlation can be established between cell survival and increased Bcl2 phosphorylation, mitochondrial localization of PKCα and/or ERK1 and ERK2, and increased cell survival following exposure to induction-remission chemotherapy in vitro, these data would support the hypothesis that phosphorylation of Bcl2 may have clinical relevance. In this case, developing novel antineoplastic strategies to block Bcl2 phosphorylation would be one novel strategy to block Bcl2 phosphorylation with a survival signaling pathway induced by growth factors like IL-3 that feature Bcl2 phosphorylation. This finding now directly links these two critical pathways (Fig. 1). Furthermore, by serving as an SRK, the ERKs can potentially cooperate with other survival signaling pathways, including PKC activation, to ensure Bcl2 survival function. Our findings also help to explain the apparent paradox in which Bcl2 may remain functionally phosphorylated and at the same time protect cells from apoptosis induced by high concentrations of staurosporine, the potent inhibitor of PKC. Finally, if these findings are shown to have clinical relevance, this would suggest that novel apoptosis-inducing antineoplastic strategies aimed at functionally inactivating Bcl2 may require targeting of multiple agonist-activated upstream pathways.

**References**


NOTE
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