Serine-70 Is One of the Critical Sites for Drug-induced Bcl2 Phosphorylation in Cancer Cells

Subrata Haldar, Aruna Basu, and Carlo M. Croce

Abstract

Taxoloids and other microtubule-damaging drugs are known to induce Bcl2 phosphorylation at the G2-M phase of the cell cycle, with concomitant apoptosis in malignant cells derived from a variety of human malignancies, including leukemia, lymphoma, and breast and prostate cancer. We have investigated the ability of another antineoplastic drug, dolastatin 10, in inducing Bcl2 phosphorylation and apoptosis. We also investigated the effects of a phosphatase inhibitor okadaic acid in the regulation of Bcl2 phosphorylation, cell cycle arrest, and programmed cell death. Moreover, site-directed mutagenesis studies were performed to determine the specific serine residues responsible for drug-induced Bcl2 phosphorylation. Our results indicate that these antimicrotubule agents or okadaic acid can induce posttranslational modification (phosphorylation) of Bcl2 protein at multiple serine residues. Interestingly, mutation of a serine residue at position 70 to alanine can significantly decrease drug-induced posttranslational modification (phosphorylation) of Bcl2 protein. Apparently, Ser70 seems to be a critical site for drug-induced posttranslational modification (phosphorylation) of the Bcl2 protein.

Introduction

All eukaryotic cells are capable of initiating an intrinsic cell death program often called programmed cell death or apoptosis. Apoptosis is necessary for regulated embryonic development and tissue homeostasis (1). During the past few years, genetic studies in both vertebrate and invertebrate systems have characterized a score of apoptosis regulatory elements. Dysregulation of genes involved in apoptosis may lead to a variety of diseases, such as cancer, autoimmune diseases, and neurodegenerative disorders (2). Interestingly, the BCL2 gene, originally isolated because of its juxtaposition to the immunoglobulin heavy chain locus in follicular lymphoma (3, 4), can delay the onset of programmed cell death triggered by many apoptotic stimuli, including glucocorticoids, growth factor withdrawal, and chemotherapeutic agents (5). BCL2 appears to be homologous to the nematode ced-9 gene, which has a death-inhibitory property (6). Low-stringency hybridization and the yeast two-hybrid system have allowed the discovery of other members of BCL2 family. Among them, some are antiapoptotic, and others are proapoptotic (7–10). One interesting characteristic of the members of this family is their propensity to form homo- and heterodimers. The stoichiometry of dimer formation between antiapoptotic and proapoptotic genes seems to determine the cellular fate (10).

BCL2 encodes a protein of 239 amino acids with a hydrophobic COOH terminus (11). Recently, the three-dimensional structure of Bcl-xL, an antiapoptotic member of the BCL2 family, has revealed its similarity with channel-forming membrane proteins, such as diphtheria toxin (12). Bcl-xL was found to form an ion channel on a synthetic lipid membrane (13). Despite several hypotheses that have been proposed with regard to the biochemical mechanism of Bcl2 action, the exact mechanism still remains enigmatic. We and others have recently shown that phosphorylation of the BCL2 product, Bcl2, can be induced by several microtubule-targeting drugs in a panel of cancer cell lines derived from leukemia, lymphoma, and breast and prostate cancer (14–18). Phosphorylation of Bcl2 is cell cycle dependent, occurring at G2-M (18), and results in concomitant apoptosis (18, 19). Here, we have studied the effect of a new antineoplastic microtubule-targeting drug, dolastatin 10, on the posttranslational modification (phosphorylation) of Bcl2 protein and apoptosis, alone or in combination with other anticancer drugs, such as Taxol or vincristine. To understand the functional significance of drug-induced Bcl2 phosphorylation, we substituted nine serine residues of Bcl2 protein with alanine by site-directed mutagenesis using the overlapping PCR method. Our investigation has determined that serine residues at position 70 is an important phosphorylation site for drug-induced apoptosis.

Materials and Methods

Cells and Culture Conditions. Human lymphoid cells 697 and RS11846 [pre-B-cell line harboring a t(1;19) chromosomal translocation and a high-grade lymphoma carrying t(14;18) translocation, respectively] were maintained in RPMI 1640 supplemented with 10% FBS and 50 μg/ml gentamicin. Hormone-independent human prostate carcinoma cells (DU 145 or PC-3) were cultured in MEM with 10% FBS and 50 μg/ml gentamicin. In the case of hormone-dependent prostate cancer cells (LNCaP), MEM was substituted with RPMI 1640. Human mammary carcinoma cells (MCF-7) were grown in Iscove's modified Dulbecco's medium supplemented with 10% FBS (HyClone) and gentamicin.

Reagents. The antineoplastic drug dolastatin 10 was kindly provided by Dr. Peter O'Dwyer (Developmental Therapeutics Division, Kimmel Cancer Center, Philadelphia, PA). Antibody against PARP was a kind gift from Prof. Nathan Berger (Case Western Reserve University, Cleveland, OH). Monoclonal antibody against Bcl2 was purchased from Genosys (The Woodlands, TX). Taxol was available from Sigma Chemical Co. (St. Louis, MO). Vincristine was provided by Dr. Robert L. Comis (Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA). All other reagents used were of ultrapure grade.

Treatment of Cells with Spindle-damagingAgents or Phosphatase Inhibitor. Cells were treated with specified concentrations of dolastatin 10 (1–100 nM), Taxol (1–5 μM), OA (0.5 μM), or nocodazole (0.1 μM) for different time periods in a humidified 5% CO2 incubator. Following treatment, total cellular proteins were extracted as described previously (14, 15, 18).

Assessment of Bcl2 Phosphorylation. Equal amounts of proteins extracted from cells with or without treatment of drugs were subjected to 12% SDS-PAGE, followed by electrophoretic transfer on nitrocellulose membrane. Transferred proteins on nitrocellulose membranes were further processed for

The abbreviations used were: FBS, fetal bovine serum; PARP, poly(ADP) ribose polymerase; OA, okadaic acid; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.
immunoblotting using monoclonal antibody against Bcl2. The phosphorylated forms of Bcl2 protein were detected as slower migrating forms, as described previously (14, 15, 18).

**Determination of Apoptosis.** The extent of apoptosis was assessed by determining PARP degradation and TUNEL assay. For PARP degradation, the total proteins were extracted from the cells following scheduled exposure of specified drugs. For assessment of Bcl2 phosphorylation or analysis of PARP, the protein content of the total cellular extract was determined by bicinchoninic acid protein assay reagent (Pierce Chemical Co., Rockford, IL). Equivalent amounts of protein from control or drug-treated samples were fractionated by 5—15% SDS-PAGE, followed by immunoblotting with monoclonal PARP antibody. For TUNEL assay, an in situ cell death detection kit (Boehringer Mannheim) was used throughout the studies. Control or drug-treated cells were fixed with a 4% paraformaldehyde solution, followed by labeling with a TUNEL reaction mixture comprising terminal deoxynucleotidyl transferase and FITC-conjugated nucleotides. The samples were analyzed under a confocal microscope at the Kimmel Cancer Institute’s facility.

**Cell Sorting.** Control or drug-treated cells were sorted into two major subpopulations by using a fluorescence-activated cell sorter (EPIC cell sorter) at the Kimmel Cancer Institute’s core facility. Precisely, cells were stained with Hoechst 33342 (Sigma) at a concentration of 15 µg/ml for 1 h at 37°C. To increase the resolution of DNA distribution, 3,3'-dipentyloxacarbocyanine acid protein assay reagent (Pierce Chemical Co., Rockford, IL). Equivalent amounts of protein from control or drug-treated samples were fractionated by 5—15% SDS-PAGE, followed by immunoblotting with monoclonal PARP antibody. For TUNEL assay, an in situ cell death detection kit (Boehringer Mannheim) was used throughout the studies. Control or drug-treated cells were fixed with a 4% paraformaldehyde solution, followed by labeling with a TUNEL reaction mixture comprising terminal deoxynucleotidyl transferase and FITC-conjugated nucleotides. The samples were analyzed under a confocal microscope at the Kimmel Cancer Institute’s facility.

**Construction of Bcl2 Mutants.** We started the purification of several serine residues in Bcl2 protein with well-known homologous alanine residues by site-directed mutagenesis, using overlap extension involving PCR (20). The sets of overlapping primers that were designed from the human Bc12 cDNA clone sequence (11) are shown in Table 1. In addition to the overlapping primers, two external primers (5' and 3' ends of Bcl2 cDNA) were also synthesized with HindIII and XbaI, respectively (Table 1).

In separate PCR, two fragments of the target gene sequence were amplified using wild-type Bcl2 cDNA (PB4) as template (1). Each PCR used one primer that hybridizes either the 5' or 3' end of the target gene sequence and one internal primer that hybridizes at the site of the mutation and contains the mismatched base(s). By using two internal primers that overlap, the two fragments generated in the first PCR were fused by denaturing and annealing in a subsequent primer extension reaction. PCR amplification was done using PCR core kit (Boehringer Mannheim) for 35 cycles. Each cycle consisted of denaturation at 95°C for 1 min, annealing at 60°C for 30 s, and extension at 72°C for 2 min. The PCR products were purified by a Qiagen spin column and digested with HindIII and XbaI restriction enzymes, followed by cloning into pcDNA3 (Invitrogen) at the HindIII and XbaI sites. Mutations in the clones were verified by automated DNA sequencing. Wild-type Bcl2 was also constructed in pcDNA3 vector by cloning the PCR amplification product generated by 5' and 3' end primers and PB4 template.

**Transfections of Wild-type and Mutant Bcl2 cDNAs.** Bcl2-negative DU 145 prostate cancer cells were transiently transfected with 10 µg of the respective DNA (purified by Qiagen column) using the calcium phosphate coprecipitation method (21). Total DNA amount was maintained at the 25 µg level using vector DNA as the filler plasmid. For rapid screening of the mutants, a Bcl2 phosphorylation-inducing agent, such as Taxol, was added 24 h posttransfection. Following 48 h of transfection, cells were harvested, followed by lysis (18), protein extraction, and immunoblotting with Bcl2 monoclonal antibody.

For the stable expression, DU 145 cells were transfected with wild-type and S70A mutant clones by electroporation (22). Following transfection, the cells were allowed to grow for two generations in nonselective medium and then transferred to selective medium containing the neomycin analogue genetin (Life Technologies, Inc.).

### Table 1 Overlapping primers used for site-directed mutagenesis

<table>
<thead>
<tr>
<th>Site(s) of mutations</th>
<th>Overlapping primers</th>
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<tbody>
<tr>
<td>Ser24 → Ala24</td>
<td>1520</td>
</tr>
<tr>
<td>5’-ATA AGG TCG* CCC AGG GG-3’</td>
<td></td>
</tr>
<tr>
<td>3’-TAT TCG ACC GCG TCT CC-5’</td>
<td></td>
</tr>
<tr>
<td>Ser50-51 → Ala50-51</td>
<td>1591</td>
</tr>
<tr>
<td>5’-GTA TTC TCG* CCC AGC CC-3’</td>
<td></td>
</tr>
<tr>
<td>3’-CTG AGC AGG GCC GCG TCC-5’</td>
<td></td>
</tr>
<tr>
<td>Ser62 → Ala62</td>
<td>1634</td>
</tr>
<tr>
<td>5’-CAO CGG CAG* CCC GCC ACC-3’</td>
<td></td>
</tr>
<tr>
<td>3’-GTC GTC GTC GCG CCC TGC-5’</td>
<td></td>
</tr>
<tr>
<td>Ser70 → Ala70</td>
<td>1655</td>
</tr>
<tr>
<td>5’-GCT CGG AGA GCC CGC CCT GCA CAC C-3’</td>
<td></td>
</tr>
<tr>
<td>3’-CCA CGG AGT CGC GGG GGC CTT GGC-5’</td>
<td></td>
</tr>
<tr>
<td>Ser105 → Ala105</td>
<td>1760</td>
</tr>
<tr>
<td>5’-CGA CGA CCT CGC CCC CCG CTA-3’</td>
<td></td>
</tr>
<tr>
<td>3’-GCT GCT GAA GAG GCC GGC GAT-5’</td>
<td></td>
</tr>
<tr>
<td>Ser116-117 → Ala116-117</td>
<td>1794</td>
</tr>
<tr>
<td>5’-GCC CGC GAT GGC GCC CCA GCA GCA-3’</td>
<td></td>
</tr>
<tr>
<td>3’-GGC GCT CTA CGG GGT CGG CGA GAT-5’</td>
<td></td>
</tr>
<tr>
<td>Ser161 → Ala161</td>
<td>1817</td>
</tr>
<tr>
<td>5’-TCT GTG GTG GCG* GTC AAC CG-3’</td>
<td></td>
</tr>
<tr>
<td>3’-ACA CAC CTC CCG CAG TGG GC-5’</td>
<td></td>
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<tr>
<td>5’ end primer</td>
<td>1423</td>
</tr>
<tr>
<td>5’-GAG AAG CTT GTG CCG TGG GCC CCC-3’</td>
<td></td>
</tr>
<tr>
<td>3’ end primer</td>
<td>1437</td>
</tr>
<tr>
<td>5’-GCT TCT AGA ACA GCC TCC AAC TGC TT-3’</td>
<td></td>
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</tbody>
</table>

* The mutated nucleotides incorporated into the Bc12 gene. Numbers indicate the nucleotide position.
PHOSPHORYLATION SITE OF BCL2

Dolastatin (nM)

A. 0 1 10 100

Bcl2

B. 697 cells

C. RS11846 cells

D. LNCaP cells

E. PC-3 cells

MCF-7 cells

Fig. 1. Dolastatin 10 induces Bcl2 phosphorylation at a 1 nm concentration. A, 697 cells derived from human pre-B leukemic cells harboring a t(1;19) chromosomal translocation; B, RS11846 cells derived from a human B-cell lymphoma carrying t(14;18) and t(8;22) chromosomal translocations; C, LNCaP cells derived from a human metastatic prostate adenocarcinoma; D, PC-3 cells derived from a human grade IV prostate adenocarcinoma; E, MCF-7 cells derived from an estrogen receptor-positive human breast adenocarcinoma. All these cells were treated with 1–100 nm dolastatin 10 for 24 h in their respective culture media. Following treatment, total cellular proteins were extracted as described previously (18). Equal amounts of protein (100 μg) were fractionated by SDS-PAGE, followed by immunoblotting using a monoclonal antibody against Bcl2. Arrows, modified (phosphorylated) forms of Bcl2 protein.

Fig. 2. Kinetics of dolastatin 10-induced Bcl2 phosphorylation. 697 cells were challenged with dolastatin (0.1 μM) for several time periods between 0–48 h in a humidified 5% CO2 incubator, as described previously (18). Phosphorylated forms of Bcl2 protein were detected by Western blotting, as described in the legend to Fig. 1. Note the gradual emergence of two phosphorylated forms of Bcl2 protein. Arrows, phosphorylated Bcl2 protein.

Fig. 3. The mitotic spindle-damaging agent nocodazole can induce Bcl2 phosphorylation. 697 cells were exposed to the mitotic spindle-damaging agent nocodazole at a concentration of 0.1–5 μM for a 24-h period, and Bcl2 phosphorylation was assessed as described in legend to Fig. 1.
these mutants to undergo phosphorylation by transiently transfecting them into DU 145 prostate cancer cells, which do not express endogenous Bcl2 (15). As shown in Fig. 7, mutation of Ser70 to alanine (S70A) abolishes the ability of Taxol to induce Bcl2 phosphorylation on one site. As mentioned earlier, microtubule-targeting drugs can induce Bcl2 phosphorylation predominantly at two sites (Fig. 1). Similarly, in wild-type BCL2-transfected DU 145 cells, Bcl2 gets phosphorylated on two different sites, as is evident from two slower migrating Bcl2-specific protein bands (Fig. 7, Lane 1). Interestingly, in S70A mutant-transfected DU 145 cells, only one slower migrating (phosphorylated) Bcl2-specific protein was observed following Taxol treatment (Fig. 7, Lane 2). All other serine-to-alanine mutants failed to abolish both of the phosphorylated forms of Bcl2. This observation also nullifies the probable conformational change for homologous substitutions of serine to alanine. It is quite unlikely that only a S70A mutation will induce some conformational changes in Bcl2 protein. Subsequent stable transfection of S70A mutants in same cells indicated identical results (Fig. 8). DU 145 cells (lacking Bcl2 expression), when stably transfected with wild-type Bcl2, microtubule-damaging drugs (Taxol, vincristine, vinblastine, and dolastatin) or OA can trigger its phosphorylation at two different sites (Fig. 8A). Fig. 8B clearly documents the ability of S70A mutant to partially block drug- or OA-induced Bcl2 phosphorylation. In all, site-directed mutagenesis studies unravel Ser70 to be one of the critical sites necessary for Bcl2 phosphorylation. Investigations to determine the other site of phosphorylation are in progress.

Discussion

The studies reported here indicate that a new investigational drug dolastatin can induce posttranslational modification (phosphorylation) of Bcl2 protein with simultaneous apoptosis in a broad range of cancer cells. This is not surprising to us because its mechanism of action is mediated by inhibition of microtubule assembly. Unlike Taxol, which binds to tubulin polymers (26, 27),

A. PARP analysis

![Dolastatin-induced PARP degradation](image)

B. TUNEL assay

![Control vs 10 nM Dolastatin](image)
dolastatin binds to tubulin monomers (28) and is able to induce phosphorylation of Bcl2 at a very low concentration (at 1 nM). This observation is consistent with the studies on the anticancer activity of dolastatin 10, a unique peptide derived from the marine mollusk Dolabella auricularia (23). Dolastatin appears to be cytotoxic at very low concentrations in leukemia/lymphoma cells (29, 30). A few reports are also available on its cytotoxic effects against several malignancies, including melanoma and ovarian cancer (31, 32). The ability of spindle-damaging antineoplastic drugs to trigger Bcl2 phosphorylation, leading to tumor cell death, can be exploited in cancer treatment. It has been previously shown that Bcl2 can render cancer cells more resistant to treatment (2, 5). Thus, apparent inactivation of Bcl2 by phosphorylation can be exploited to stimulate apoptosis in Bcl2-positive cancer cells.

By site-directed mutagenesis studies reported in this article, we here demonstrate Ser70 is the critical site for drug-induced phosphorylation. At present, studies to identify the other phosphorylation site are in progress. However, the identification of Ser70 as one of the phosphorylation sites places our studies in an interesting stage. Ser70 is included in a 60-amino acid loop region connecting α1 and α2 helices, as revealed by three-dimensional structure of Bcl2 family members (12). Interestingly, Chang et al. (19) reported the complete abolition of Bcl2 phosphorylation when this loop region was deleted. One can speculate that either this loop region is the substrate for phosphorylation or it contains binding site for putative kinase(s) that can act on another place on Bcl2 molecule. Alternatively, of multiple phosphorylation sites, the major or primary one, along with the kinase-binding domain for another site, lies in this loop. Our results are in conformity with this alternative hypothesis. It seems that a cascade of kinases might be involved in the regulation of Bcl2 phosphorylation.

Our results along with others demonstrate that Bcl2 phosphorylation is triggered specifically by microtubule-targeting antineoplastic drugs, such as Taxol, vincristine, and vinblastine (14–19). Thus, Bcl2 phosphorylation can be due to mitotic arrest of cancer cells expressing Bcl2. Interestingly, very recent reports (12, 19) indicated the identification of a novel regulatory domain in Bcl2 and Bcl-XL. The three-dimensional structure of Bcl-XL revealed a 60-amino acid loop lacking defined structure. Despite this 60-amino acid loop not being conserved among all family members, structural modeling suggested that Bcl2 also contains this large unstructured region. Compared with the full-length protein, loop deletion mutants of Bcl2 and Bcl-XL displayed an enhanced ability to inhibit apoptosis. Full-length Bcl2 was unable to prevent
Fig. 8. Stable transfection of a Ser70 → Ala70 mutant in DU 145 cells. Wild-type (A) and S70A mutant (B) Bcl2 were stably transfected in DU 145 cells by electroporation. Bcl2-positive clones were treated with 2 μM Taxol (Lanes T), vincristine (Lanes V), vinblastine (Lanes Vc), and OA (Lanes OA) for a 24-h period. For dolastatin (Lanes DT), the concentration used was 100 nM for the same time period. Phosphorylation was assessed by the method described in legend to Fig. 7. Arrows, slower mobility (phosphorylated) forms of Bcl2 protein.

anti-IgM-induced cell death of the immature B-cell line WEHI-231. On the contrary, a Bcl2 deletion mutant lacking this loop region protected these cells from death. Bcl2 phosphorylation was found to be dependent on the presence of the intact loop domain. These results suggest that the loop domain in Bcl-xL and Bcl2 can suppress the apoptotic function of these genes and may be a target for posttranslational modification. Interestingly, our studies reveal that Ser70 is one of the multiple phosphorylation sites (Figs. 7 and 8) and that it is located in this nonconserved loop. Another recent report (33) suggesting that Ser70 phosphorylation of Bcl2 protein is necessary for antiapoptosis function does not fit to the model proposed by two individual laboratories (19, 34). The enhanced antiapoptotic effect was observed in cells transfected with deletion mutant (amino acids 51–85) of Bcl2. Because, in both cases, deletion mutants comprising Ser70 abolition confer an enhanced survival advantage, it is extremely difficult to explain the results of May and colleagues (33). Moreover, a very recent report by Korsmeyer and colleagues (25) also does not conform to the notion of survival advantage by Bcl2 phosphorylation; rather, it demonstrates a death advantage (14–19).

Apoptosis can be subdivided into two general categories (35): one form showing rapid initiation of apoptotic events in cells arrested in the G1 phase of the cell cycle ("primed apoptosis"), and a secondary form occurring with a longer time course in cells that may require passage through the cell cycle before apoptotic death ("unprimed apoptosis"). Primed apoptosis, for example, is found in lymphocytes in response to treatment with glucocorticoids (36) or in hormone-depenuendent cells after the rapid withdrawal of hormone (37). The apoptotic response of human carcinoma cells to chemotherapy may be more likely to be unprimed, such as Taxol-induced cell death in human carcinoma cells (27, 38). Perhaps Bcl2 phosphorylation regulates primed and unprimed apoptosis by a different manner. Alternatively, it could exert different effects on mouse cells (33). From our studies, it is apparent that phosphorylation at multiple sites on Bcl2 protein might regulate Taxol-induced unprimed apoptosis in cancer cells by mitotic spindle damage, followed by growth arrest. The identification of other phosphorylation site(s) should enable us to dissect the functional significance of drug-induced Bcl2 phosphorylation.

Acknowledgments

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References


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