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Loss of the Bcl-2 Phosphorylation Loop Domain Increases Resistance of Human Leukemia Cells (U937) to Paclitaxel-Mediated Mitochondrial Dysfunction and Apoptosis

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The impact of ectopic expression of an N-terminal phosphorylation loop deletant Bcl-2 protein (Bcl-2Δ_{32-80}) on the response of U937 monoblastic leukemia cells to paclitaxel was examined. In contrast to recent findings in HL-60 cells (Fang *et al., Cancer Res.* 58, 3202, 1998), U937 cells overexpressing Bcl-2Δ_{32-80} were significantly more resistant than those overexpressing full-length protein to caspase-3 and -9 activation, PARP degradation, and apoptosis induced by paclitaxel (500 nM; 18 h). Bcl-2Δ_{32-80} was also more effective than its full-length counterpart in opposing paclitaxel-mediated mitochondrial dysfunction, e.g., loss of mitochondrial membrane potential ($\Delta\psi_m$) and cytochrome *c* release into the cytoplasm. Enhanced resistance of U937/Bcl-2Δ_{32-80} cells to paclitaxel was observed primarily in the G_2M population. Together, these findings demonstrate that deletion of the Bcl-2 phosphorylation loop domain increases resistance of U937 leukemia cells to paclitaxel-mediated mitochondrial damage and apoptosis and suggest that factors other than, or in addition to, phosphorylation contribute to Bcl-2-related cytoprotectivity against paclitaxel in this model system.

Key Words: **apoptosis; Bcl-2 phosphorylation; paclitaxel; leukemia.**

Paclitaxel (Taxol) represents a prototypical member of the taxane family of antineoplastic agents. It acts by binding to microtubules and stabilizing α/β tubulin heterodimers, resulting in arrest of cells in the G_2M phase of the cell cycle [1]. Disruption of microtubule dynamics by paclitaxel and related agents leads, through mechanisms yet to be fully elucidated, to mitochondrial damage, including loss of the mitochondrial membrane potential ($\Delta\psi_m$) and release of cytochrome *c* into the cytosol [2]. These events ultimately trigger the organized cellular program of self-destruction referred to as apoptosis [3]. The lethal actions of paclitaxel may also involve perturbations in cell cycle regulation and signal transduction cascades, including inappropriate activation of the cyclin-dependent kinase $p34^{cdc2}$ [4] or induction of stress-related signaling pathways (e.g., JNK; Jun N-terminal kinase) [5].

The balance between cell death and survival is determined by interactions between members of the Bcl-2 family (reviewed in [6]), proteins that act at multiple levels to regulate mitochondrial integrity and the activity of apoptotic caspases responsible for cellular disassembly [7]. Treatment of cells with paclitaxel is associated with Bcl-2 phosphorylation [8], which in at least some model systems reduces Bcl-2 heterodimerization with the pro-apoptotic family member Bax [9]. In addition, paclitaxel-mediated Bcl-2 phosphorylation has been shown to proceed through a Raf-1-related pathway inasmuch as depletion of Raf-1 opposes both paclitaxel-associated Bcl-2 modifications as well as lethality [10]. However, it is more difficult to reconcile the notion that Bcl-2 phosphorylation is responsible for paclitaxel lethality with reports that (1) phosphorylation can enhance the cytoprotective effects of this protein [11], and (2) Bcl-2 phosphorylation may be more closely associated with G_2M arrest than with apoptosis [12].

One strategy that has been employed to define the functional role of Bcl-2 phosphorylation in cell death/survival decisions has been to express mutant proteins lacking the N-terminal phosphorylation loop domain

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(e.g., Bcl-2Δ_{32-80}) [13]. In an IL-3-dependent hematopoietic cell line, loss of the phosphorylation loop rendered cells less rather than more sensitive to growth factor deprivation compared to cells expressing full-length protein [13]. Very recently, Fang and coworkers reported that expression of this mutant protein in human promeylocytic leukemia cells (HL-60) resulted in loss of protection against mitochondrial damage and apoptosis induced by paclitaxel, but not by other classes of antineoplastic agents [14]. This observation suggests a functional role for Bcl-2 phosphorylation in protection of human leukemia cells from paclitaxel-mediated lethality. In view of potential implications for attempts to understand the role of post-translational modifications of Bcl-2 in paclitaxel cytotoxicity, it would be important to determine the extent to which this finding might be generalized. To this end, we have recently generated a human myelomonocytic leukemia cell line (U937) stably overexpressing a Bcl-2 phosphorylation loop deletant protein (U937/Bcl-2Δ_{32-80}). In marked contrast to results previously described in HL-60 cells, we have found that the Bcl-2Δ_{32-80} mutant protein is significantly more potent than its full-length counterpart in protecting U937 leukemia cells from paclitaxel-mediated mitochondrial damage and apoptosis. This result indicates that the functional role of Bcl-2 phosphorylation in regulating paclitaxel-mediated cytotoxicity may be highly cell type-specific.

METHODS

Cells. U937 cells were obtained from ATCC (Rockville, MD), and maintained as previously reported [15]. They were transfected by electroporation as described elsewhere in detail [16] with plasmids encoding either full-length Bcl-2 (pcDNA 3.1) or an HA-tagged Bcl-2 lacking residues 32-80 encompassing the phosphorylation loop domain (pSFFV; provided by Dr. Craig Thompson, University of Chicago) [13] along with a G418 selection marker. Single cells were obtained by limiting dilution and expanded under selection pressure in medium containing 400 μg/ml G418. For all studies, cells containing empty vectors were used as controls.

Drugs. Paclitaxel was purchased from Sigma (St. Louis, MO), stored frozen at −20°C, and formulated in sterile DMSO (Sigma) prior to use. The final concentration of DMSO in all experiments was $<0.01\%$.

Experimental format. Logarithmically cells (2×10^5 cells/ml) were plastic 25-cm^2 T-flasks and exposed to the designated concentration of paclitaxel for various intervals (generally 18 h), after which cells were pelleted and subjected to analysis as outlined below.

Assessment of apoptosis. The percentage of apoptotic cells was determined by evaluating Wright-Giemsa-stained preparations by light microscopy and scoring 8–10 randomly selected fields containing $\geq$1000 cells for the classic morphologic features of apoptosis as previously described [15]. In some cases, results were confirmed by monitoring the sub-diploid population by flow cytometry or DNA fragmentation by agarose gel electrophoresis [17].

Western analysis. Cell lysates were prepared as previously described in detail [15] and 20 μg protein/condition separated by SDS–PAGE and electroblotted to nitrocellulose. After incubation with fresh blocking solution, blots were exposed to Bcl-2 primary antibody (1:1000 Santa Cruz Biotechnology, Santa Cruz, CA). This antibody recognizes an epitope on the COOH-terminal domain, and is therefore able to detect the Bcl-2 N-terminal deletant. For other studies, antibodies to PARP (Biomol, Plymouth Meeting, PA; 1:1000), caspase-3, (Santa Cruz; 1:2000), caspase-9 (Pharmingen, San Diego, CA; 1:500), and cytochrome *c* (Pharmingen, 1:1000) were employed. Blots were then incubated with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories, Hercules, CA) for 1 h at 22°C. Blots were again washed 3×5 min in PBS-T and then developed by enhanced chemiluminescence (Pierce, Rockford, IL).

In vivo labeling. [^{32}P]-labeling of U937/Bcl-2 and U937/Bcl-2Δ cells was carried out as previously outlined in detail [16]. Immunoprecipitates were obtained using anti-Bcl-2 [Santa Cruz] and anti-HA antibodies (anti-Rat monoclonal antibody; Boehringer-Mannheim GmbH, Mannheim, Germany) respectively, and separated (300 μg/ml per condition) by SDS–PAGE as described above, after which incorporation of [^{32}P] into Bcl-2 and Bcl-2Δ monitored by autoradiography.

Reduction in mitochondrial membrane potential ($\Delta\psi_m$). Reductions in mitochondrial membrane potential following drug treatment were monitored by flow cytometric analysis of cells exposed to the cationic fluorophor $DiOC_6$ as we have previously described in detail [17]. Mitochondrial damage was detected by an increase in the percentage of cells displaying "low" uptake of $DiOC_6$ employing a Becton–Dickinson FacScan and user-set gating parameters.

Cytosolic cytochrome c release. Following drug treatment, cytosolic S-100 fractions were obtained as we have previously outlined in detail [16], and subjected to Western analysis using polyclonal cytochrome *c* antibodies (1:1000; Pharminigen, San Diego, CA).

Cell cycle analysis. Following drug treatment, cells were pelleted, fixed, and exposed to propidium iodide as previously described [17], after which cell cycle analysis was performed by flow cytometry using a commercially available software package (Modfit; Verity Winlist; Topsham, ME).

Statistical analysis. The significance of differences between experimental conditions was determined using the Student's *t* test for unpaired observations.

RESULTS

Following transfection of U937 cells with a plasmid encoding a Bcl-2 loop-deletant protein (Bcl-2Δ), several clonal lines were isolated and expanded. On Western analysis, lysates from these cells (clones G6, B11, and E6) revealed a 19 kDa protein, in contrast to the 26 kDa protein detected in lysates obtained from cells expressing full-length Bcl-2 (clones B3 and D4; Fig. 1A). In accordance with previous reports [13, 14], a reduction in expression of endogenous full-length Bcl-2 was observed in the former lines. In separate studies, levels of Bax and Bcl-x_L were equivalent in these cells (data not shown). Following a 18-h exposure to 500 nM paclitaxel, cells ectopically expressing full-length Bcl-2 displayed a significant reduction in the percentage of apoptotic cells compared to empty-vector controls (Fig. 1B). However, a significantly greater reduction in the extent of apoptosis was observed in each of the cell lines expressing the Bcl-2 loop deletants ($P \leq 0.05$ vs values for cells containing full-length Bcl-2 in each case). A corresponding attenuation in paclitaxel-

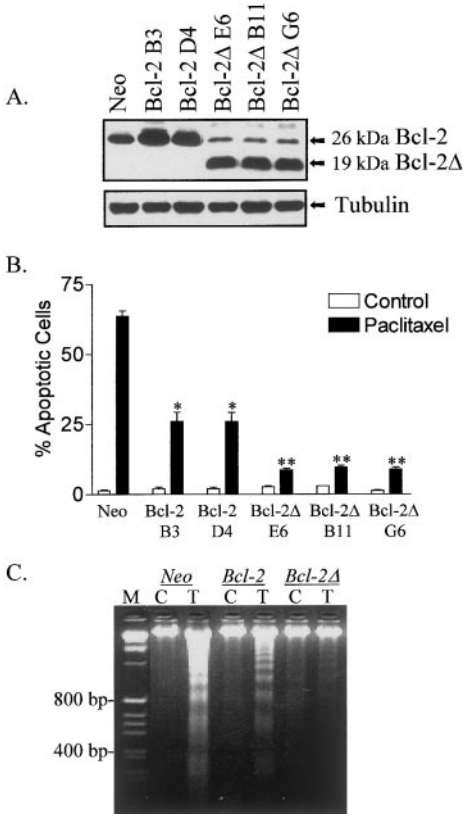

FIG. 1. Western analysis of lysates (25 μg protein/line) from cells transfected with an empty vector (Neo), the cDNA encoding full length Bcl-2 (clones B3 and D4) or the loop deletant Bcl-2Δ_{32-80} (clones ΔG6, ΔB11, and ΔE6) (A). Cells from each of these clonal populations were exposed to 500 nM paclitaxel for 18 h, after which the percentage of cells exhibiting the typical morphological features of apoptosis was determined as described in the text (B). Values represent the means for three separate determinations ± SD. *Significantly less than values for cells expressing empty vector (Neo); **significantly less than values for cells expressing full length Bcl-2; $P \leq 0.02$ in each case (B). Internucleosomal DNA fragmentation in U937 cells ectopically expressing empty vector (Neo), full-length Bcl-2 (clone B3) or Bcl-2Δ_{32-80} (clone ΔE6) following an 18-h exposure to 500 nM paclitaxel (C).

mediated internucleosomal DNA fragmentation was noted in Bcl-2ΔE6-expressing cells (Fig. 1C); similar results were obtained in other phosphorylation loop deletants lines (data not shown). Thus, these results stand in marked contrast to those of Fang *et al.* [14], who reported that ectopic expression of a Bcl-2 loop deletant protein failed to protect HL-60 leukemic cells from paclitaxel-mediated apoptosis. Consistent with these findings, full length Bcl-2 partially blocked the loss of mitochondrial membrane potential ($\Delta\psi_m$) in paclitaxel-treated cells (compared to empty-vector neo controls), whereas Bcl-2Δ_{32-80} was even more effective in this regard (Fig. 2A). Moreover, while ectopic expression of full-length Bcl-2 attenuated paclitaxel-mediated release of cytochrome *c* into the S-100 fraction relative to controls, this reduction was more marked in cells expressing Bcl-2Δ_{32-80} (Fig. 2B) Thus, loss of the phosphorylation loop domain increased the ability of Bcl-2 to protect cells from paclitaxel-mediated mitochondrial damage. In addition, cells ectopically expressing full-length Bcl-2 exhibited a diminution in activation of caspase-9 (manifested by cleavage of the full length 48 kDa protein to a 10 kDa cleavage product (Fig. 2C), as well as attenuated activation of the major caspase-9 target, caspase-3 (reflected by the appearance of 19 and 17 kDa cleavage products) (Fig. 2D). Ectopic expression of Bcl-2 was also associated with a reduction in degradation of PARP (manifested by the generation of an 85 kDa cleavage product) compared to vector controls (Fig. 2E). However diminution of paclitaxel-mediated caspase-3 and -9 activation and

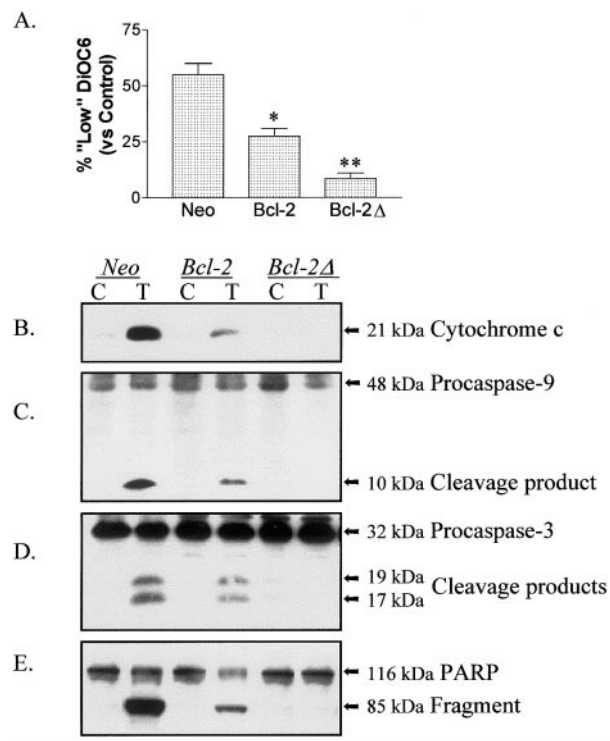

FIG. 2. Following a 18-h exposure to paclitaxel, U937 cells containing an empty vector or expressing either full-length Bcl-2 (clone 3B) or Bcl-2 lacking the phosphorylation loop domain (clone ΔE6) were monitored for loss of mitochondrial membrane potential ($\Delta\Psi_m$), reflected by reduced uptake of $DiOC_6$ (A). Values are expressed as the mean percentage of cells exhibiting "low" $DiOC_6$ (relative to controls) ± SD for three separate experiments. *Significantly less than values for empty-vector Neo controls; **significantly less than values for cells ectopically expressing full-length Bcl-2; $P \leq 0.05$ in each case. Alternatively, Western analysis was employed to assess release of cytochrome *c* into the cytosolic S-100 fraction as described in the text. In these and in subsequent studies, each lane was loaded with 25 μg protein (B). Cells were exposed to paclitaxel as above and activation of full-length (48 kDa) caspase-9 to yield the 10-kDa cleavage product, or full length (32 kDa) caspase-3 to yield 19 and 17 kDa cleavage products, was monitored by Western analysis as described in the text (C and D). Degradation of PARP to its 85-kDa cleavage product after paclitaxel exposure was also examined by Western analysis (E).

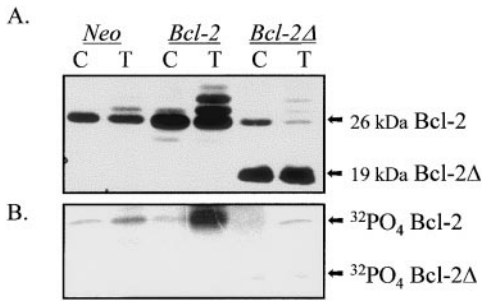

FIG. 3. Western analysis of lysates from cells expressing empty vector (Neo), full length Bcl-2 (clone B3), or Bcl-2Δ_{32-80} (clone ΔE6) following exposure to 500 nM paclitaxel for 18 h. Following separation on SDS–PAGE (25 μg protein/lane) and transfer to nitrocellulose, blots were probed with anti-Bcl-2 antibodies as outlined under Materials and Methods (A). Alternatively, cells were labeled with [^{32}P], exposed to paclitaxel, and Bcl-2 or Bcl-2Δ_{32-50} immunoprecipitates (300 μg protein/condition) were separated by SDS–PAGE and monitored for incorporation of labeled PO_4 by autoradiography as described in the text (B).

PARP degradation was even more striking in the Bcl-2ΔE6-expressing line (Figs. 2C–2E). Thus, in marked contrast to results obtained in HL-60 cells [14], the Bcl-2 loop-deletant protein was more, rather than less potent than the full-length protein in protecting U937 cells from paclitaxel-mediated mitochondrial dysfunction and activation of the apoptotic protease cascade.

Exposure of cells to paclitaxel results in altered mobility of the Bcl-2 protein, putatively representing a consequence of phosphorylation [8, 10]. Consistent with these findings, treatment of empty vector control cells with paclitaxel was associated with the appearance of a single slow mobility band on Western gels (Fig. 3A). When lysates from Bcl-2-overexpressing cells exposed to paclitaxel were examined, several slow mobility species were noted, presumably reflecting multiple phosphorylation states. Examination of loop-deletants revealed, in addition to the aforementioned reduction in endogenous Bcl-2, the absence of significant alterations in mobility of the Bcl-2Δ_{32-80} protein. It should be noted that although quite faint, several slow mobility species were observed in the 26 kDa region, suggesting that paclitaxel treatment of Bcl-2Δ_{32-80}-transfectants is associated with phosphorylation of endogenous Bcl-2. *In vivo* labeling of cells with [^{32}P] followed by immunoprecipitation of Bcl-2 and Bcl-2Δ_{32-80} demonstrated that whereas striking phosphorylation of the abundant full-length 26 kDa Bcl-2 protein occurred in paclitaxel-treated U937/Bcl-2 cells, minimal labeling of the 19-kDa Bcl-2Δ_{32-80} protein was detected under these conditions (Fig. 3B). It should be noted that paclitaxel treatment resulted in faint labeling of the residual 26-kDa Bcl-2 protein in the Bcl-2Δ_{32-80}-expressing cell line.

Finally, cell cycle analysis revealed that exposure of empty vector control cells and cells expressing full-length Bcl-2 or Bcl-2Δ_{32-80} to paclitaxel (500 nM; 18 hr) produced equivalent reductions in the G_0/G_1 and S-phase populations (Fig. 4). However, paclitaxel-mediated increases in G_2 M arrest (relative to untreated controls) were greatest in U937/Bcl-2Δ_{32-80} cells, intermediate in U937/Bcl-2 cells, and lowest in the empty vector-containing line. Conversely, increases in the sub-diploid (apoptotic) cell fractions exhibited a reciprocal pattern (e.g., empty vector controls > U937Bcl-2 > U937/Bcl-2Δ_{32-80}). This suggests that ectopic expression of Bcl-2Δ_{32-80} primarily acts to block apoptosis in cells arrested in the G_2M phase of the cell cycle.

DISCUSSION

Anti-apoptotic proteins such as Bcl-x_L (and Bcl-2) consist of two central hydrophobic α-helices surrounded by 5 amphipathic α-helices and a large unstructured N-terminal loop [18] encompassing the phosphorylation sites [13]. The results described herein indicate that human leukemia cells ectopically expressing a Bcl-2 protein lacking the N-terminal phosphorylation domain (residues 32–80) are if anything, more resistant to paclitaxel-mediated mitochondrial damage and apoptosis than cells expressing the full length protein. This finding is in accord with those of Chang *et al.*, who reported that murine FL5.12 hematopoietic cells expressing Bcl-2Δ_{32-80} were less susceptible to IL-3-deprivation-induced apoptosis than those expressing full length Bcl-2 [13]. They are also consistent with results described by Uhlmann *et al.*, who found that N-terminal deletions in the Bcl-2 protein (Bcl-2Δ_{51-85}) increased its ability to protect BRK and Rat1 cells from p53- and c-Myc-mediated cell death [19]. Together, such findings suggest that regions exist within the Bcl-2 protein that negatively

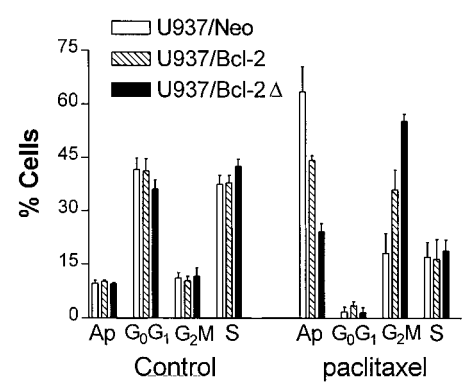

FIG. 4. Following treatment of U937/pCEP4, U937/Bcl-2 (clone B3), and U937/Bcl-2Δ_{32-80} (clone E6) cells with paclitaxel (500 nM; 18 h), they were stained with propidium iodide and subjected to cell cycle analysis as described in the text. Values, corresponding to the percentage of cells in the sub-G_1 (apoptotic; Ap), G_0/G_1, S, and G_2M fractions, represent the means ± SD for three separate experiments.

regulate its cytoprotective function. Whether this phenomenon specifically involves phosphorylation or a conformational change conferred by loss of the N-terminal region of the protein remains to be determined. Our results do, however, differ markedly from those of Fang *et al.,* who reported that human promyelocytic leukemic cells (HL-60) ectopically expressing a Bcl-2 protein lacking the phosphorylation loop domain were fully sensitive to paclitaxel-mediated cell death, but not that induced by VP-16 or ara-C [14]. While the basis for this difference is unclear, such divergent results suggest that the role of the Bcl-2 phosphorylation loop in resistance to apoptosis depends not only upon the apoptotic stimulus, but also the cell type under investigation.

The present findings have implications for attempts to define the role of Bcl-2 phosphorylation in paclitaxel-related cell death. There have been a number of conflicting reports on this issue, as well as on the impact of Bcl-2 phosphorylation on cell death in general. For example, induction of Bcl-2 phosphorylation (e.g., by PKC down-regulation) has been associated with cell death in Jurkat cells [20], and in various cell types exposed to paclitaxel and related agents that interfere with microtubule dynamics [9, 10, 21]. Moreover, paclitaxel-mediated Bcl-2 phosphorylation has been shown to be dependent upon Raf-1 [10, 21], and disruption of this pathway reportedly opposes paclitaxel-induced cell death [10]. Taken in conjunction with evidence that deletion of the N-terminal Bcl-2 phosphorylation loop promotes cell survival [13, 19], such findings support the notion of a functional role for Bcl-2 phosphorylation in paclitaxel-associated lethality. In contrast, several groups have demonstrated that Bcl-2 phosphorylation correlates with G_2M arrest but not with apoptosis [12, 22]. Moreover, Ito [23] and Ruvolo [24] have reported that Bcl-2 phosphorylation is necessary for protection of cells from growth factor deprivation- and cytotoxic drug-related apoptosis respectively. The observation that loss of the phosphorylation loop opposes Bcl-2-mediated protection from paclitaxel-induced cell death in HL-60 cells [14] is most consistent with the latter findings. However, the ability of phosphorylation loop-deletants to protect U937 leukemia cells from paclitaxel-mediated lethality indicates that in at least some cell types, Bcl-2 phosphorylation is not required for the cytoprotective activity of this protein. It should be noted that in the studies by Ito and Ruvolo [23, 24], site-directed mutagenesis was employed to prevent Bcl-2 phosphorylation at serine residues, and it is possible that conformational changes in the Bcl-2 protein accompanying loss of the phosphorylation loop may have functional consequences distinct from those resulting from amino acid substitutions. Thus, while the present findings do not provide direct evidence of a role for Bcl-2 phosphorylation in mediating paclitaxel lethality, they do suggest that in U937 leukemia cells, protection from paclitaxel-induced apoptosis involves factors other than, or in addition to, this process.

The mechanism by which Bcl-2 and related anti-apoptotic proteins oppose cell death is uncertain, but may involve multiple actions, including binding to and inactivation of Bax [25], induction of cell cycle perturbations [26], blockade of the mitochondrial permeability transition and/or cytochrome *c* release [27], and binding to *apaf-1* (apoptosis-activating factor-1), thereby preventing cleavage/activation of *apaf-3* (procaspase-9) and other downstream proteases, notably caspase-3 [28]. Modulation of Bcl-2 cytoprotectivity by phosphorylation could therefore act at one or more of these levels. For example, in one study, Bcl-2 phosphorylation induced by paclitaxel was associated with decreased Bax/Bcl-2 heterodimerization [9]; moreover, we have previously observed increased free Bax levels in paclitaxel-treated U937 cells [15]. However, the cytoprotective capacity of Bcl-2 loop deletants does not appear to depend upon direct interactions with Bax [13]. It is also noteworthy that in the study by Fang *et al.,* in which cells expressing the loop-deletant Bcl-2 protein were shown to be fully susceptible to paclitaxel toxicity, a reduction in Bcl-$2\Delta_{32-80}$ (relative to the full length protein) coimmunoprecipitating with *apaf-1* was observed [14]. If such a phenomenon is cell line-specific, it could potentially account for the discordant effects of loss of the N-terminal phosphorylation loop on paclitaxel sensitivity in HL-60 versus U937 leukemia cells. Alternatively, differential responses to paclitaxel might stem from the greater abundance of Bcl-$2\Delta_{32-80}$ relative to full-length Bcl-2 in the U937 line employed in the present study.

In summary, the present results demonstrate that a Bcl-2 protein lacking the phosphorylation loop domain, in contrast to results previously reported in the HL-60 promyelocytic leukemia cell line, is more rather than less effective than the full-length protein in protecting U937 leukemia cells from paclitaxel-mediated mitochondrial damage, caspase activation, and cell death. While these findings do not resolve the question of whether Bcl-2 phosphorylation plays a causal role in paclitaxel lethality, they do indicate that factors other than, or in addition to, phosphorylation of this protein contribute to protection against paclitaxel-induced apoptosis, at least in some cell types. Further insights into the nature of factors negatively regulating Bcl-2 function could provide a basis for developing novel strategies capable of enhancing the activity of paclitaxel and possibly other cytotoxic drugs.

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