Bcl-xL Is Phosphorylated in Malignant Cells following Microtubule Disruption

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ABSTRACT

The oncogenic protein Bcl-2 functions as a potent inhibitor of programmed cell death. This survival activity has been shown in some settings to be influenced by the Bcl-2 phosphorylation state. It has been demonstrated that treatment with microtubule-targeted agents results in phosphorylation of both Raf-1 kinase and Bcl-2. The Bcl-2-related family member Bcl-xL also exhibits a death suppressive activity, but its potential for phosphorylation following exposure to drugs that interact with microtubules has not been evaluated. Several tumor cell lines with low or undetectable levels of Bcl-2 protein expression were found to express Bcl-xL. A more slowly migrating Bcl-xL band was observed on immunoblots after cells were treated with microtubule-targeted agents. The appearance of this band was responsive to dose and was absent when the cell lysates were treated with a protein phosphatase. Using a Bcl-xL-specific monoclonal antibody, the phosphorylated form of Bcl-xL was immunoprecipitated from cells treated with paclitaxel and metabolically labeled with [32P]labeled inorganic orthophosphate. Herein, we report that Bcl-xL is phosphorylated in malignant cells after incubation with agents that target tubulin, including paclitaxel, vinristine, vinblastine, colchicine, and nocodazole. Moreover, paclitaxel-resistant ovarian carcinoma cell lines that have mutations in tubulin failed to exhibit phosphorylation of Bcl-xL after paclitaxel exposure, but they did demonstrate Bcl-xL phosphorylation in the presence of other tubulin-targeting agents. As observed for Bcl-2, phosphorylation of Bcl-xL was accompanied by phosphorylation of Raf-1. Interestingly, phosphorylation of these three proteins failed to occur or was much less pronounced when cells grown at high density were challenged with drug. Also, reduced Raf-1 expression, observed after treatment of cells with geldanamycin prior to and during incubation with the microtubule-active drugs, correlated with diminished Bcl-xL phosphorylation. Taken together, these results suggest that Bcl-xL, like Bcl-2, is phosphorylated by agents that disrupt microtubule architecture. By analogy with Bcl-2, this phosphorylation may play a critical role in modulating Bcl-xL function and may be an important determinant of microtubule-directed chemotherapeutic efficacy in human tumors.

INTRODUCTION

The discovery of the proto-oncogene Bcl-2 has paved the way for the identification of an expanding family of Bcl-2-related proteins (1, 2). These gene products exhibit various degrees of homology and conservation of structural and functional domains (1, 2) and are considered to be regulators of the effector stage of apoptotic signaling (1–3). Like Bcl-2, several of the proteins function as programmed cell death antagonists (Bcl-xL, Bcl-w, Mcl-1, and so forth), whereas others act in opposition as cell death agonists (Bax, Bak, Bcl-xL, Bad, and so forth; Ref. 2). The relative cellular abundance of these proteins has been shown to influence the predilection of a cell toward survival versus death, and homo- or hetero-dimers formed between these proteins appear to balance opposing actions (1, 4, 5). Members of the family possess several defined regions (BH1, BH2, and BH3) 2 that mediate interactions between the proteins (1, 2). In addition, antiapoptotic homologues, including Bcl-2 and Bcl-xL, have a conserved amino-terminal domain (BH4) that is necessary for the interaction of these proteins with death regulatory factors that are structurally unrelated to Bcl-2. The latter group includes Raf-1 kinase, which can be recruited to a mitochondrial location via an association with Bcl-2 (6). Bcl-2 phosphorylation has been observed to accompany Raf-1 phosphorylation following exposure of cells to microtubule-targeted drugs (7–10). The antiapoptotic function of Bcl-2 has been shown to be modulated by posttranslational phosphorylation (10–13). Deletion of the so-called “variable region” of Bcl-2, which lies between BH4 and BH3 and contains the major serine/threonine phosphorylation sites, confers a gain of Bcl-2 function accompanied by cell proliferation (14). Serine phosphorylation of Bad, a pro-apoptotic family member, has been shown to negatively affect its capacity for hetero-dimerization with other Bcl-2 family members and to also alter its intracellular location (15). The potential for posttranslational modification of Bcl-xL and a concomitant alteration in its function exists but has heretofore not been examined. The present study establishes that in several malignant cell lines that display low or negligible Bcl-2 protein expression, Bcl-xL is expressed and undergoes posttranslational modification in response to agents that alter microtubule architecture. This posttranslational modification was observed in conjunction with Raf-1 phosphorylation in all cell lines studied and paralleled the phosphorylation response of Bcl-2 seen in MCF7 cells (10). Our finding that the modified forms of Raf-1, Bcl-2, and Bcl-xL were sensitive to λ protein phosphatase confirmed that the modification observed involves phosphorylation. Likewise, the specific immunoprecipitation of phosphorylated Bcl-xL after paclitaxel treatment and metabolic labeling with [32P]P supports this conclusion. Interestingly, following paclitaxel treatment, our observation of the lack of phosphorylation of Raf-1 and Bcl-xL in paclitaxel-resistant cells (16) that were otherwise responsive to a variety of microtubule-destabilizing drugs further bolsters the relationship among loss of microtubule integrity, cytotoxicity, and modification of Raf-1 and Bcl-2 family members. Our demonstration of the existence of a phosphorylated form of Bcl-xL in cells treated with these cytotoxic agents raises the possibility that the antiapoptotic function of Bcl-xL can be potentially abrogated by its phosphorylation in a manner similar to that of its analogous family member Bcl-2.

MATERIALS AND METHODS

Materials. Paclitaxel was obtained as a 6 mg/ml stock solution from Bristol-Myers Squibb, (Princeton, NJ). Vinristine and colchicine were obtained from Sigma Chemical Co. and dissolved in either DMSO (vincristine) or water (colchicine) as stock solutions. Vinblastine was obtained from Fujiwara USA as a 1 mg/ml stock. Nocodazole and sodium orthovanadate were Aldrich products and were dissolved in DMSO (nocodazole) or water (sodium orthovanadate). Geldanamycin was obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute and was dissolved in DMSO. Sodium fluoride was purchased from Sigma and dissolved in water. Antibody to Bcl-xL, 13.6, was a rabbit anti-Bcl-xL polyclonal antibody derived from a recent bleed following restimulation of the animal that generated the previously described 13.4 (17). Rabbit polyclonal antibodies, anti-Raf-1 antibody (Santa Cruz), and antiactin (Sigma), and murine monoclonal anti-Bcl-2 antibody (DAKO Corp.) were used for immunoblotting. Secondary horseradish...
ish peroxidase-conjugated antirabbit immunoglobulin, from donkey, and horseradish peroxidase conjugated antimouse immunoglobulin, from sheep, were each obtained from Amersham Pharmacia Biotech, as were the enhanced chemiluminescence immunoblotting detection reagents. Lambda protein phosphatase (400,000 u/ml) was obtained from New England Biolabs (Beverly, MA). For determination of the protein concentration of lysates, Bio-Rad protein assay dye reagent was used. All other reagents were of an analytical grade and purchased from Sigma.

**Cell Culture.** MCF7 and HS 578T are human breast cancer lines. HT29 and COLO205 are human colon cancer lines. SKOV3 is a human ovarian carcinoma cell line, and A549 is a human non-small cell lung carcinoma cell line. A2780(1A9) is a single-cell clone of the human ovarian carcinoma cell line A2780. I A9PTX18 and I A9PTX22 are paclitaxel-resistant sublines, each isolated initially as a single clone in a one-step selection by exposing A2780(1A9) cells to 5 ng/ml paclitaxel and 5 μg/ml verapamil (16). The paclitaxel-resistant cell lines were cultured in medium containing 15 ng/ml paclitaxel and 5 μg/ml verapamil and were removed from drug for 5–7 days before use in an experiment. All cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum. For most experiments, cells were sparsely plated so that following 2 days of growth they were 40–60% confluent. For low and high cell density growth experiments, MCF7, A2780(1A9), or SKOV3 cells were plated in 6-well dishes so that after 2 days of growth they were either 40–60% confluent or overgrown so that cell borders were difficult to distinguish; the latter situation was equivalent to 48 h postconfluence. Cells were then cultured either with or without 150 ng/ml paclitaxel or vincristine for 24 h prior to harvesting and cell lysis.

**Microscopy.** MCF7 cells were plated at low or high cell density in 24-well dishes, as described above, and after 2 days of growth were cultured for an additional 24 h with or without 150 ng/ml paclitaxel. Cultures were fixed in ice cold methanol for 10 min and stained with DAPI reagent (Sigma) used at a dilution of 1:2500. Fluorescence microscopy was performed using a Zeiss Axioshot (Oberkochen, Germany) photomicroscope.

**Immunoblotting.** Cells were lysed at 4°C in TNE (50 mM Tris, pH 7.5, 2 mM EDTA, 1% Triton X-100), 0.1% Nonidet P-40, 5% sodium deoxycholate, 25 mM sodium fluoride, 2 μg/ml leupeptin, and 1% NP40). Lysates were incubated at 4°C for 15 min, and centrifuged at 14,000 rpm, and protein concentrations were determined. For a particular experiment, equal amounts of total protein (ranging from 75 to 125 μg) from different cell lysates were separated on 7.5% (Raf-1) or 16% (Bcl-2 and Bcl-x<sub>L</sub>) SDS-polyacrylamide gels alongside Rainbow molecular weight standards (Amersham Pharmacia Biotech), transferred to Immobilon-P membranes (Millipore Corp, Bedford, MA), and probed with antibodies. Raf-1 antibody was used at a dilution of 1:1000, Bcl-2 antibody at 1:300, and Bcl-x<sub>L</sub> 13.6 rabbit antisera at 1:2500. In most cases, blots were stripped with 0.2 M NaOH and reprobed with antiactin antibody used at a dilution of 1:400. Blocking steps, antibody incubations, and blot washes (the latter including 0.05% Tween 20) were carried out in either 100 mM Tris, pH 7.5, 154 mM NaCl with 5% nonfat dry milk or in TNE (50 mM Tris, pH 7.5, 100 mM NaCl, 2 mM EDTA) (8).

**Phosphatase Treatment.** Exponentially growing subconfluent cells were treated for 20–24 h with either paclitaxel or vincristine, 150 ng/ml, before lysis at 4°C in a buffer consisting of 10 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, with phenylmethylsulfonyl fluoride and aprotinin added to a final concentration of 1 mM and 2 mg/ml, respectively. Lysates were incubated at 4°C for 1 h and centrifuged at 14,000 rpm, and the protein concentrations were determined for each of the supernatants. Aliquots containing 75 μg of protein were adjusted with the 1X phosphatase reaction buffer (New England Biosciences), with MnCl<sub>2</sub> added to a final concentration of 2 mM. Either 400 or, in later experiments, 75 units of calf intestinal phosphatase were added to each reaction mixture and samples were incubated at 30°C for 3–4 h. Control samples containing phosphatase and the phosphatase inhibitors sodium fluoride and sodium orthovanadate or those without enzyme addition were incubated either at 30°C or 4°C for the same length of time prior to analysis by gel electrophoresis.

**Metabolic Labeling and Immunoprecipitation.** Prior to labeling, exponentially growing subconfluent SKOV3 cells were pretreated for 3 h with 150 ng/ml paclitaxel in complete culture medium followed by a 2 h incubation in phosphate-free RPMI containing 10% fetal bovine serum. Cells were then metabolically labeled for 16 h in the same medium containing 125 μCi/ml [32P]<sub>PI</sub> (specific activity, 370 MBq/ml; Amersham Pharmacia Bio-tech). Control cells had no paclitaxel added to the medium. Radiolabeled cells were lysed in NET-N buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris, pH 8.0, 0.2% NP40; Ref. 18) supplemented with 1 mM each phenylmethylsulfonyl fluoride and vanadate, 25 mM sodium fluoride, 2 mg/ml aprotinin, and 2 μg/ml leupeptin. Lysates were incubated at 4°C and centrifuged as described, and postnuclear supernatants were precleared with a mixture of protein G/A-agarose (Life Technologies, Inc.) before one-half the sample had 1.5 ml of anti-Bcl-x<sub>L</sub> monoclonal antibody 7B2 (19) added. Identical samples without antibody addition served as controls, and all samples were further processed by the addition of protein G/A-agarose and washes in NET-N containing 0.1% NP40. Immunoprecipitates were solubilized prior to SDS-PAGE, and the gels were dried and placed on film to detect [32P]-labeled Bcl-x<sub>L</sub>.

## RESULTS

**Phosphorylation of Raf-1 and Bcl-2 Is Significantly Decreased in MCF7 Cells Treated with Tubulin-targeting Drugs at High Cell Density.** Previous studies have shown that both Bcl-2 and Raf-1 are phosphorylated following treatment of cells with microtubule-active agents (10). The observation that phosphorylation did not occur after paclitaxel exposure in paclitaxel-resistant cell lines exhibiting mutant tubulins and an impaired polymerization response to paclitaxel (10, 16, 20) prompted us to examine other conditions in which taxane responsiveness is diminished. It was reported that phosphorylation of Bcl-2 failed to occur in noncycling pre-B leukemia cells treated with tubulin-targeting drugs (12) and that G<sub>2</sub>-M arrest accompanied by paclitaxel-induced apoptosis was detected in monolayer but not spheroid cultures (21). Because microtubule-active drugs are schedule dependent and are more toxic to actively dividing cells, we sought to determine whether the replicative status influenced Bcl-2 and Raf-1 phosphorylation by treating cultures at different cell densities. As shown in Fig. 1A, the phosphorylated forms of Raf-1 and Bcl-2 were significantly more evident in MCF7 cells incubated with paclitaxel or vincristine at low cell density (e.g., approximately 40–60% confluent) than at high density (cells confluent for 48 h). In the absence of any drug treatment, Bcl-2 migrates as a single band of approximately 26 kDa; however, after exposing cells to paclitaxel or vincristine at low density, two slower-migrating forms of Bcl-2 were observed. A fraction of Raf-1 and Bcl-2 remained unmodified because the more rapidly migrating species were still evident following drug treatment (Fig. 1A).

MCF7 cells at both low and high cell density were treated with 150 ng/ml paclitaxel for 24 h and viewed by fluorescence microscopy (Fig. 1B). At low density in the absence of drug, MCF7 cells appeared flat and polygonal by phase contrast microscopy, whereas following paclitaxel treatment, 80% of the cells became rounded (data not shown). In contrast, the morphology of the cells at high density was similar before and after paclitaxel treatment. DAPI staining showed fully intact nuclei in the untreated cells at either low or high cell density (Fig. 1B, panels a and c), whereas following paclitaxel treatment, about 80% of the cells at low cell density displayed fragmented nuclei consistent with apoptosis (Fig. 1B, panel b). In contrast, only about 15% of the cells at high cell density treated with paclitaxel displayed lobulated nuclei, indicative of cell death (Fig. 1B, panel d). Consistent with these observations, when cells were analyzed by flow cytometry, in the absence of any drug, two-thirds as many cells were in S phase in the high cell density population as in the low, and only two-thirds as many cells were in G<sub>2</sub>-M phase following paclitaxel challenge (data not shown). Together, these observations suggest that phosphorylation of Bcl-2 and Raf-1 is dependent on active cell cycle and correlates with apoptotic induction by microtubule-active agents.
BCL-X<sub>L</sub> PHOSPHORYLATION AFTER MICROTUBULE DISRUPTION

Fig. 1. Phosphorylation of Raf-1 and Bcl-2 in response to microtubule-disrupting agents is significantly decreased in MCF7 cells at high cell density. MCF7 cells incubated with paclitaxel show morphological changes when treated at low cell density (40–60% confluence) but not at high cell density. A, immunoblots of MCF7 cells, either untreated or treated for 24 h with 150 ng/ml paclitaxel (P) or vincristine (V). A comparison of Raf-1 and Bcl-2 phosphorylation in MCF7 cells at low or high cell density. Arrows in A, phosphorylated form(s) of each protein. Aliquots of cell lysates containing 100 µg of protein were loaded in each lane. B, fluorescence microscopy of DAPI-stained MCF7 cells. A comparison of MCF7 cells (a–d) grown in the absence of drug at low (a) or high (c) cell density to those treated for 24 h with 150 ng/ml paclitaxel at low (b) or high (d) cell density.

Relationship between Bcl-2 and Bcl-x<sub>L</sub>. Expression in Different Cell Types. We sought to reconcile the observation that Bcl-2 expression is undetectable in some cells with the hypothesis that phosphorylation of Bcl-2 following treatment with tubulin-disrupting agents plays a significant role in drug cytotoxicity. We speculated that in the absence of Bcl-2, a Bcl-2 homologue, such as Bcl-x<sub>L</sub>, might serve a similar role. Although some cell lines, such as MCF7, express both proteins, we had observed that across a spectrum of cell lines, the expression of Bcl-2 and Bcl-x<sub>L</sub> appeared to be inversely correlated. This impression was supported by data from the National Cancer Institute Anticancer Drug Screen, showing an inverse correlation (r values ranging from −0.5 to −0.8) between RNA or protein expression levels for Bcl-2 and Bcl-x<sub>L</sub> in 34 cell lines derived from colon, breast, ovary, central nervous system, and renal cancers.

Immunoblot analysis of nine cell lines, representing four different tissues (breast, lung, colon, and ovary) demonstrates marked variation in levels of both Bcl-2 and Bcl-x<sub>L</sub> protein expression. In this series, cell lines that had low-level or no Bcl-2 expression demonstrated easily detectable levels of Bcl-x<sub>L</sub>. In the absence of any drug treatment, Bcl-x<sub>L</sub> appeared as a doublet just under 30 kDa. Whereas MCF7 cells express both Bcl-2 and Bcl-x<sub>L</sub> and were used as a control for immunodetection, HS 578T, A549, HT29, COLO205, and SKOV3 expressed no or barely immunodetectable Bcl-2 protein but expressed Bcl-x<sub>L</sub> (Fig. 2). This result correlated with high mRNA expression levels observed for Bcl-x<sub>L</sub> in these cells. In addition, the ovarian carcinoma cell line A2780(1A9) and two paclitaxel-resistant cell lines derived from this single cell clone express similar levels of Bcl-x<sub>L</sub> but do not express Bcl-2 to a detectable level (Fig. 2).

3 M. S. Poruchynsky and T. Fojo, unpublished observations.
Retardation of Mobility of Bcl-xL and Raf-1 on SDS-PAGE following Tubulin-targeting Agents Depends on High Cell Density. For cells expressing Bcl-xL, we wanted to determine whether changes in the gel mobility of Bcl-xL, similar to those noted for Bcl-2, would be observed following drug treatment. Indeed, when A2780(1A9) or SKOV3 cells at 40–60% confluence were incubated with paclitaxel or vincristine for 24 h, slower migrating forms of both Raf-1 and Bcl-xL were noted (Fig. 3, A and B). However, such bands were less pronounced or entirely absent in the lysates derived from A2780(1A9) and SKOV3 cells at high cell density (cells confluent for 48 h). Not all of the Bcl-xL was evidently modified in the A2780(1A9) cells (Fig. 3A; see also Figs. 7 and 8) or the SKOV3 cells (Fig. 3B), whereas Raf-1 appeared more completely modified in both cell lines at low cell density and also decreased in overall amount (Fig. 3, A and B; see also Fig. 8B).

Posttranslational Modification of Bcl-xL and Raf-1 Occurs in Cell Lines of Diverse Tumor Origin. To determine whether posttranslational modification of Bcl-xL and Raf-1 was a general phenomenon in response to microtubule disruptive agents, six other human cell lines of diverse tumor origin were examined. Following incubation of cells with paclitaxel or vincristine, more slowly migrating forms of Raf-1 and Bcl-xL were observed in lysates of all six cell lines examined (Fig. 4 and data not shown). Thus, the posttranslational modification of Bcl-xL following treatment of cells with tubulin-targeting agents is a general response in many tumor types.

The Posttranslational Modifications Observed for Raf-1, Bcl-2, and Bcl-xL Are Each Sensitive to Treatment with Protein Phosphatase. The electrophoretic retardation of Bcl-2 was shown to be due to phosphorylation at one or more sites (9); however, the evidence for Raf-1 phosphorylation was circumstantial (9, 10). To confirm that the mobility shifts observed for Bcl-xL, Bcl-2, or Raf-1 following drug treatment were dependent on phosphorylation, cell lysates were treated with the enzyme protein phosphatase, which has specificity for cleavage of phosphate groups appended to the amino acids serine, threonine, or tyrosine (22). When protein phosphatase was added to lysates of paclitaxel or vincristine treated MCF7 and A2780(1A9) cells and then incubated at 30°C, we observed that the more slowly migrating forms of Raf-1, Bcl-2, and Bcl-xL, were absent (Fig. 5). On the other hand, when lysates were incubated with enzyme in the presence of phosphatase inhibitors or without enzyme at either 4°C (Fig. 5) or 30°C (data not shown), the slowly migrating bands of each protein were unaltered. These results confirm that the slowly migrating forms of Raf-1, Bcl-2, and Bcl-xL, which appear following treatment of cells with microtubule-targeting drugs, are phosphorylated. In this and other experiments, the disappearance of the slower migrating form of Bcl-xL was accompanied by an increase in the intensity of the most rapidly migrating form.

The Modified Form of Bcl-xL Observed after Treatment of Cells with Paclitaxel Can Be Labeled with [32P]P. To further confirm that Bcl-xL is phosphorylated in malignant cells in response to treatment with tubulin targeted compounds, SKOV3 cells were incubated with paclitaxel and metabolically labeled with [32P]P, and Bcl-xL was immunoprecipitated using a Bcl-xL-specific mouse monoclonal antibody 7B2 (19). We observed a Bcl-xL-immunoprecipitated band (Fig. 6) migrating with an apparent molecular weight of 30 kDa in the position identical to that of the modified form of Bcl-xL observed on Western blots for cells that underwent paclitaxel treatment. This radiolabeled band, corresponding to phosphorylated Bcl-xL, was absent from cells that had not undergone paclitaxel treatment and from those that had the antibody omitted from the immunoprecipitation protocol (Fig. 6).

Phosphorylation of Bcl-xL Is Not Observed in Response to Paclitaxel Treatment in Paclitaxel-resistant Cells. To determine whether the interaction of microtubule disruptive agents with tubulin was a prerequisite for the phosphorylation of Bcl-xL, we performed studies using paclitaxel-resistant clones, derived from the ovarian carcinoma cell line A2780(1A9), which exhibit defects in tubulin polymerization in response to paclitaxel (16, 20). The paclitaxel-resistant clones, designated PTX18 and PTX22, are 20–30-fold more resistant to paclitaxel than the parental cell line (16) and are coltollarily sensitive to Vinca alkaloids (16). In the present study, we

Fig. 3. Modification of Raf-1 and Bcl-xL in response to microtubule-disrupting agents fails to occur in A2780(1A9) or SKOV3 cells at high cell density. A and B, a comparison of Raf-1 and Bcl-xL modification in either A2780(1A9) (A) or SKOV3 (B), cells at low density (40–60% confluence) or high cell density. Cells were either untreated or treated for 24 h with 150 ng/ml paclitaxel (P) or vincristine (V). Arrows in A and B, immunoreactive forms of Bcl-xL. Arrows in A and B, modified forms of each protein. Each blot was reprobed with antiactin.

Fig. 4. Posttranslational modification of Raf-1 and Bcl-xL occurs in response to tubulin-targeting agents in cell lines of diverse tumor origin. Aliquots of lysates from either HT29 (colon carcinoma), HS 578T (breast carcinoma), or A549 (non-small cell lung carcinoma) cells that were either untreated (—) or treated for 24 h with 150 ng/ml paclitaxel (P) or vincristine (V) were analyzed. Arrows, modified form of the Raf-1 and Bcl-xL proteins. Bracket, immunoreactive forms of Bcl-xL. For the purpose of bringing the apparent Raf-1 and Bcl-xL protein expression levels within range of each other for display, the HS 578T Raf-1 panel was taken from a shorter chemiluminescent exposure, whereas that of the HS 578T Bcl-xL was taken from a longer exposure.
observed that the phosphorylated form of Bcl-xL, appeared in the paclitaxel-sensitive parental cell line A2780(1A9) following either paclitaxel or vincristine treatment, but only after vincristine and not paclitaxel treatment of paclitaxel-resistant PTX18 and PTX22 cells (Fig. 7). These results suggest that the interaction between drug and tubulin must be uncompromised in order for phosphorylation of Bcl-xL to occur.

Bcl-xL is Phosphorylated in Response to Drugs That Affect Microtubule Structure. In addition to paclitaxel and vincristine, other microtubule-targeted drugs have been shown to result in phosphorylation of Raf-1 and Bcl-2 in MCF7 cells (10). These drugs include vinblastine, colchicine, nocodazole, and epothilones A and B, which display separate targets on tubulin but culminate in disruption of normal microtubule structures. Bcl-xL is phosphorylated in sensitive ovarian carcinoma A2780(1A9) cells following paclitaxel, vincristine, colchicine, and nocodazole treatment (Fig. 8A). A similar result was observed in the paclitaxel-resistant cell lines PTX22 (Fig. 8A) and PTX18 (data not shown) for each of these drugs except paclitaxel. In all of these studies, phosphorylation of Bcl-xL correlates with Raf-1 phosphorylation. Notably, we consistently observed that when Raf-1 was phosphorylated in A2780(1A9) cells and its derivatives, Raf-1 appeared to decrease in intensity, implying that this protein modification may enhance its turnover (Fig. 8B).

In general, the intensity of the phosphorylated form of Bcl-xL correlated with the concentration of drug used and was observed after a 24-h incubation even at very low drug concentrations. For example, when vinblastine was added to A2780(1A9) cells in increasing concentrations ranging from 5 to 150 ng/ml for 24 h, the phosphorylated form of Bcl-xL was observed in the presence of as little as 5 ng/ml vinblastine. This concentration is well within the therapeutic range, and the relative intensity of the upper band increased through the highest drug concentration examined, 150 ng/ml (Fig. 8C).

Reduced Raf-1 Expression and Inhibition of Raf-1 Phosphorylation by Geldanamycin Is Accompanied by Decreased Phosphorylation of Bcl-xL during Microtubule Disruption. As already stated, previous studies suggest that Raf-1 phosphorylation in response to microtubule disruption occurs concurrently with Bcl-2 phosphorylation (10). In an effort to examine the phosphorylation of Bcl-xL in response to tubulin-targeted drugs in the presence of reduced Raf-1 levels, cells were incubated with geldanamycin. Although there are likely to be other cellular effects of geldanamycin (23), Raf-1 levels have been shown to be reduced or depleted by geldanamycin treatment (9). When A2780(1A9) cells were pretreated with 1 μM geldanamycin for 26 h, in the absence of any other drug treatment, the amount of Raf-1 was reduced (Fig. 9). When the cells were treated with geldanamycin for 2 h prior to coincubation with paclitaxel for 24 h, a phosphorylated form of Raf-1 was not detected (Fig. 9). Paralleling the Raf-1 result, the fraction of the slower migrating phosphorylated form of Bcl-xL was significantly less than that seen in control cells treated only with paclitaxel (Fig. 9). Comparable results were also observed for Raf-1 and Bcl-xL in A549 cells treated identically (data not shown).

**DISCUSSION**

Several studies have reported that paclitaxel has cellular consequences that extend beyond its direct effect on microtubules (24–26).

Fig. 7. Phosphorylation of Bcl-xL is not observed in response to paclitaxel treatment in paclitaxel-resistant cells. Immunoblot of cell lysates probed with anti-Bcl-xL. Bcl-xL phosphorylation in paclitaxel-sensitive parental A2780(1A9) cells is compared to that of paclitaxel-resistant sublines, PTX22 and PTX18. Each cell line was either cultured without drug (−) or treated for 24 h with 150 ng/ml paclitaxel (P) or vincristine (V). Brackets, immunoreactive forms of Bcl-xL; arrow, phosphorylated form of Bcl-xL.
response, and was absent from cell lysates incubated with A protein tubulin-active drugs, occurred at low drug dose, followed a dose phosphorylation of Bcl-xL occurred after treatment of malignant cells with demonstrating high Bcl-2 expression. Phosphorylation of Raf-1 and comparison, we also examined one breast carcinoma cell line (MCF7) examined in the present study. We examined cells lines derived from multiple tissues, all of which demonstrated easily detectable expres- sion of Bcl-xL and negligible or absent Bcl-2 protein expression. For Paclitaxel and other agents that exert diverse effects on microtubule structure, and the phosphorylation of Bcl-xL follows a dose response to increasing drug concentration. A and B, duplicate aliquots from paclitaxel-sensitive A2780(1A9) or paclitaxel-resistant PTX22 cells, which were either untreated (−) or treated for 24 h with 150 ng/ml paclitaxel (P), vincristine (V), or colchicine (C), or with 500 ng/ml nocodazole (N). Lysates of PTX18 were also separated on the latter gel. C, Lysates of A2780(1A9) cells that were untreated (0) or treated for 24 h with increasing concentrations (5, 15, 50, and 150 ng/ml) of vinblastine were probed with anti-Bcl-xL. Brackets, immunoreactive forms of Bcl-xL; arrows, phosphorylated forms of Bcl-xL and Raf-1. Immunoblots were probed with either anti-Bcl-xL or anti-Raf-1, and then each was reprobed with antiaction.

Fig. 8. Bcl-xL and Raf-I are phosphorylated in response to drugs that affect microtubule structure, and the phosphorylation of Bcl-xL follows a dose response to increasing drug concentration. A and B, duplicate aliquots from paclitaxel-sensitive A2780(1A9) or paclitaxel-resistant PTX22 cells, which were either untreated (−) or treated for 24 h with 150 ng/ml paclitaxel (P), vincristine (V), or colchicine (C), or with 500 ng/ml nocodazole (N). Lysates of PTX18 were also separated on the latter gel. C, Lysates of A2780(1A9) cells that were untreated (0) or treated for 24 h with increasing concentrations (5, 15, 50, and 150 ng/ml) of vinblastine were probed with anti-Bcl-xL. Brackets, immunoreactive forms of Bcl-xL; arrows, phosphorylated forms of Bcl-xL and Raf-1. Immunoblots were probed with either anti-Bcl-xL or anti-Raf-1, and then each was reprobed with antiaction.

Paclitaxel and other agents that exert diverse effects on microtubule structure have been shown to result in the phosphorylation of Raf-I kinase, accompanied by Bcl-2 phosphorylation and cell death (8, 9, 10). This phosphorylation was not observed using alkylating agents, DNA-damaging compounds, or antimetabolites (10), suggesting that initiation of this signaling cascade requires disruption of microtubule architecture (12). We sought to determine whether, in cells with low or undetectable Bcl-2 expression, a Bcl-2 homologue is similarly modified. The putative role of Bcl-xL as a component of an analogous apoptotic pathway and its potential for posttranslational modification via phosphorylation in response to microtubule-targeting agents were examined in the present study. We examined cells lines derived from multiple tissues, all of which demonstrated easily detectable expres- sion of Bcl-xL and negligible or absent Bcl-2 protein expression. For comparison, we also examined one breast carcinoma cell line (MCF7) demonstrating high Bcl-2 expression. Phosphorylation of Raf-I and Bcl-2 or Bcl-xL was observed in all cell lines examined when grown at low cell density and treated with paclitaxel or vincristine. Phosphorylation of Bcl-xL occurred after treatment of malignant cells with tubulin-active drugs, occurred at low drug dose, followed a dose response, and was absent from lysates incubated with λ protein phosphatase. Moreover, using a Bcl-xL-specific monoclonal antibody, the phosphorylated form of Bcl-xL was immunoprecipitable from cells treated with paclitaxel and metabolically labeled with [32P]P. Our results suggest that the phosphorylation of Bcl-xL via upstream signals that include the disruption of microtubules may provide a means for regulating the onset and execution of a cell death program.

The process of apoptosis or programmed cell death occurs during the development of all animals thus far examined (27) and can be initiated by a wide variety of stimuli that cause cells to proceed to a preapoptotic common effector stage (1, 3, 28). Prior to the onset of the final and irreversible degradation stage, the effector phase is subject to regulation via pro- and antiapoptotic members of the Bcl-2 family (1, 3, 28). Although some of the proteins are located in the cytosol, others have hydrophobic transmembrane domains and can localize to specific organelles, most notably to mitochondria (1, 2). The early cas- cade of events leading to cell death includes the release of caspase activators, such as cytochrome c, from the mitochondrial intermembrane space (1, 3, 28) and/or the disruption of mitochondrial mem- brane function. Both Bcl-xL and Bcl-2 have been shown to inhibit the release of cytochrome c from mitochondria (29–31). Interestingly, recent in vitro evidence shows that Bcl-xL, Bcl-2, and Bax all have pore-forming capabilities (32–34) and exhibit different properties (34), with Bax forming pores that Bcl-2 was able to block at physiological pH (34). Although the precise mechanism of action of Bcl-2 family members in mitochondria and the impact of their phosphoryl- ation have not been elucidated, their presence in this compartment appears to regulate cell survival.

Bcl-2 family members can form homo- or heterodimers (2, 4), including complexes between proteins with opposing actions. Het- erodimers may allow family members that are otherwise unable to spontaneously associate with membranes to do so, which may be relevant to their mechanisms of action (2). Phosphorylation of Bad inhibits its heterodimerization with Bcl-xL and alters its intracellular location (15). Other studies have shown retargeting of Bcl-2 family proteins from one cellular compartment to another following a death stimulus (35). Thus, both Bax and Bcl-xL have been reported to redistribute from a mostly cytosolic location to mitochondria following treatment of cells with dexamethasone (35), illustrating the poten- tial for function at another site or site-dependent function. The impact of protein phosphorylation on the ability to alter protein conformation, function, or location should be considered following

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**Fig. 8.** Bcl-xL and Raf-I are phosphorylated in response to drugs that affect microtubule disruption. A and B, duplicate aliquots from paclitaxel-sensitive A2780(1A9) or paclitaxel-resistant PTX22 cells, which were either untreated (−) or treated for 24 h with 150 ng/ml paclitaxel (P), vincristine (V), or colchicine (C), or with 500 ng/ml nocodazole (N). Lysates of PTX18 were also separated on the latter gel. C, Lysates of A2780(1A9) cells that were untreated (0) or treated for 24 h with increasing concentrations (5, 15, 50, and 150 ng/ml) of vinblastine were probed with anti-Bcl-xL. Brackets, immunoreactive forms of Bcl-xL; arrows, phosphorylated forms of Bcl-xL and Raf-1. Immunoblots were probed with either anti-Bcl-xL or anti-Raf-1, and then each was reprobed with antiaction.

**Fig. 9.** Reduced Raf-1 expression and inhibition of Raf-1 phosphorylation by geldanamycin is accompanied by decreased phosphorylation of Bcl-xL during microtubule disruption. Immunoblots of aliquots of lysates from A2780(1A9) cells that were either untreated (−), treated for 24 h with 150 ng/ml paclitaxel (P), or incubated with 5 μM geldanamycin for 2 h prior to cotreatment with paclitaxel and separated by SDS-PAGE. Immunoblots were probed with anti-Raf-1 or anti-Bcl-xL, and then reprobed with antiaction. Bracket, immunoreactive forms of Bcl-xL; arrows, phosphorylated form of each protein.
treatment of cells with microtubule-active agents. Bcl-2 phosphorylation has been shown to play a role in modulating its antiapoptotic function (12, 36). It is not known whether the differentially phosphorylated forms of Bcl-2 and Bcl-xL are localized to different cellular compartments or if modification affects their function at the mitochondrial membrane. The influence of Raf-1 association with either Bcl-2 or Bcl-xL on the phosphorylation state of these proteins at the mitochondrial membrane (6) is also not known, but the capacity for regulation and signal transduction by this protein kinase may be significant.

The phosphorylation of Bcl-2 and Raf-1 in MCF7 cells following treatment with cytotoxic microtubule-disruptive agents (10) provided the paradigm to study a potentially analogous response in malignant cells not expressing Bcl-2. The paclitaxel-resistant ovarian carcinoma sublines used in this study have negligible levels of Bcl-2 protein but strongly express Bcl-xL and provide a unique system in which to examine the posttranslational modification of Bcl-xL because the cells have tubulin, which does not polymerize following paclitaxel treatment (16, 20).1 The PTX22 subline has an acquired mutation in the M40 isotype of β-tubulin (16), and PTX18 appears to have a variation in its α-tubulin as well.2 Whereas in parental A2780(1A9) cells, the phosphorylation of Bcl-xL and Raf-1 occurred in response to paclitaxel, vincristine, colchicine, nocodazole, or vinblastine, in the paclitaxel-resistant sublines, these proteins were phosphorylated in response to all microtubule-active drugs except paclitaxel. A cell that has developed resistance to the action of a particular drug by mutation of its intracellular target may fail to trigger downstream events, including posttranslational modifications that contribute to apoptotic induction. The phosphorylated forms of Bcl-2, Bcl-xL, and Raf-1 were susceptible to λ protein phosphatase but were uncleaved in the presence of phosphatase inhibitors. The total amounts of Bcl-2 or Bcl-xL did not vary significantly between control and drug-treated samples; however, Raf-1 levels appeared to decrease in several cell types following its phosphorylation. This was especially evident in the ovarian A2780(1A9), PTX18, and PTX22 cells and may indicate that Raf-1 is turned over rapidly once it is phosphorylated, thus providing another possible mechanism of regulation of the cell death pathway. Our results using cells treated with both geldanamycin and paclitaxel showed diminished phosphorylation of Bcl-xL and an overall reduction in the protein level of Raf-1. This raises speculation about the participation of other kinases in the phosphorylation of Bcl-xL/Bcl-2 family members, especially in light of the recent report that Bcl-2 phosphorylation occurs in response to PTX in those studies. Additional experimentation will help to distinguish whether phosphorylation of Raf-1 and Bcl-xL/Bcl-2 occurs in series or in parallel.

We also observed that phosphorylation of Bcl-2, Bcl-xL, and Raf-1 could be inhibited by high cell density. The capacity for Bcl-xL/Bcl-2 phosphorylation was correlated with apoptotic induction by DAPI staining. Cells treated with paclitaxel at low cell density showed a much higher percentage of fragmented nuclei than those at high cell density. Our observations also provide a possible explanation for the schedule dependence of paclitaxel, which manifested as increased cytotoxicity in dividing cells where phosphorylation occurs readily. It appears that growth status (i.e., entry into G2-M) and susceptibility of the microtubule structure are crucial determinants of the cellular response to microtubule-targeting agents. This is consistent with the report that Bcl-2 phosphorylation in 697 pre-B leukemia cells occurs specifically during G2-M following treatment of cells with drugs affecting microtubule integrity (12).

The existence of multiple steps in the transmission of a signal provides several potential points of intervention. The ability to define vulnerable targets that interface with mediators of cell death pathways in malignant cells may enable the rational identification of compounds that can alter protein function and in turn promote cellular demise.

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XL PHOSPHORYLATION AFTER MICROTUBULE DISRUPTION

Bcl-xL Is Phosphorylated in Malignant Cells following Microtubule Disruption

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