Mitotic Phosphorylation of Bcl-2 during Normal Cell Cycle Progression and Taxol-induced Growth Arrest*

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There is increasing evidence that prolonged mitotic arrest initiates apoptosis; however, little is known about the signaling pathways involved. Several studies have associated deregulated Cdc2 activity with apoptosis. Herein, we report that the anti-apoptotic protein, Bcl-2, undergoes cell-cycle-dependent phosphorylation during mitosis when there is elevated Cdc2 activity. We found that paclitaxel (Taxol®) treatment of epithelial tumor cells induced a prolonged mitotic arrest, elevated levels of mitotic kinase activity, hyperphosphorylation of Bcl-2, and subsequent cell death. The Taxol-induced Bcl-2 phosphorylation was dose-dependent. Furthermore, phosphorylated Bcl-2 remained complexed with Bax in Taxol-treated cells undergoing apoptosis. Immunoprecipitation experiments revealed a Bcl-2-associated kinase capable of phosphorylating histone H1 in vitro. However, the kinase was likely not cyclin B1/Cdc2, since cyclin B1/Cdc2 was not detectable in Bcl-2 immunoprecipitates, nor was recombinant Bcl-2 phosphorylated in vitro by cyclin B1/Cdc2. The results of this study further define a link between mitotic kinase activation and the apoptotic machinery in the cell. However, the role, if any, of prolonged Bcl-2 phosphorylation in Taxol-mediated apoptosis awaits further definition of Bcl-2 mechanism of action. Taxol may increase cellular susceptibility to apoptosis by amplifying the normal downstream events associated with mitotic kinase activation.

Microtubule inhibitors such as Taxol have proven to be effective in the treatment of many cancers including metastatic breast, ovarian, and non-small cell lung cancer (1). The effects of Taxol are correlated with increased tubulin polymerization, tubulin bundling, and cell cycle arrest (2–5). Although Taxol binding to tubulin is well characterized and the ability of the drug to induce a mitotic arrest recognized, the biochemical mechanisms by which altered microtubule dynamics leads to cytotoxicity are not well defined. Several studies suggest that Taxol alters certain intracellular signal transduction events; however, few studies have focused on Taxol-mediated signaling during mitosis, the phase of the cell cycle in which Taxol-treated cells arrest.

In eukaryotic cells, progression from G2 into mitosis is dependent upon the activation of the cyclin-dependent kinase, Cdc2 (6). Cdc2 is tightly regulated by a series of phosphorylations and protein-protein interactions. Mitotic events, including chromosome condensation, nuclear membrane breakdown, and altered spindle dynamics, are stimulated by an active cyclin B1/Cdc2 complex (6). The activation of Cdc2 requires association with cyclin partners and phosphorylation by a Cdk-activating kinase (7, 8). During S or G2 phase, cells accumulate cyclin B1/Cdc2 in an inactive form in which the Cdc2 component is hyperphosphorylated (7, 9). Inhibitory phosphorylations on Cdc2 are thought to be due to the activities of Weel and Myt1 kinases (10–12). The conversion of Cdc2 from an inactive to active form is accomplished by the phosphatase, Cdc25C (13–15). During mitosis, Weel is hyperphosphorylated (16) and inactive (17) while Cdc25C is present in a hyperphosphorylated, active form (18). In metaphase, once the chromosomes align properly on the mitotic spindle, a series of biochemical reactions involved in spindle checkpoint function activate a proteolytic pathway that allows transition into anaphase (19, 20). If a single chromosome fails to attach properly to the spindle or microtubule dynamics are disrupted, the mitotic spindle checkpoint is engaged and cells arrest at metaphase (21). It has been proposed that prolonged mitotic arrest stimulates apoptosis (22), but the biochemical pathway linking these two events remains unclear.

The Bcl-2 family plays a central role in the control of apoptosis. The family includes a number of proteins which have amino acid sequence homology, including anti-apoptotic members such as Bcl-2 and Bcl-xL, as well as pro-apoptotic members including Bax and Bad (reviewed in Refs. 23 and 24). The family members form both homodimers and heterodimers (23, 24). While the functional significance of these protein-protein interactions is unclear, it is believed that the heterodimerization of Bcl-2 and Bax is critical in preventing Bax-mediated apoptosis (25, 26). The Bcl-2 family members reside primarily in the membranes of the mitochondria, but Bcl-2 has also been localized to the membranes of the endoplasmic reticulum and the nucleus (27). Overexpression of Bcl-2 and Bcl-xL has been shown to prevent chemotherapeutic agent-induced apoptosis associated with altered mitochondria transmembrane potential (28). In addition, Bcl-2 has been shown to prevent the release of cytochrome c (29) from the mitochondria while Bax promotes its release (30). Cytochrome c release stimulates an apoptotic protease cascade involving caspases (31), which are cysteine proteases active during the effector phase of apoptosis (32).

The role of post-translational modifications of Bcl-2 family members in promoting or inhibiting apoptosis remains controversial (33–35). In cells treated with microtubule inhibitors,
Bcl-2 has been shown to be phosphorylated on serine residues (35, 36). Several kinases, including c-Raf-1 (37–39), p54-SAPK\(\beta\) (40), and protein kinase A (34), have been implicated in this phosphorylation event, and it has been proposed that phosphorylation of Bcl-2 reduces heterodimer formation with Bax (34, 41). In this study, we determined that Bcl-2 is normally phosphorylated during mitosis and that Taxol-mediated Bcl-2 phosphorylation is a consequence of drug-induced cell cycle arrest in mitosis. Furthermore, the Taxol-induced cell cycle arrest led to elevated mitotic kinase activity, prolonged phosphorylation of Bcl-2, and subsequent onset of apoptosis in tumor-derived epithelial cells. The results of this study begin to temporally link mitotic events with modulation of proteins, such as Bcl-2, that regulate apoptosis.

**EXPERIMENTAL PROCEDURES**

**Cell Lines, Growth Conditions, and Taxol Treatment—**H460 and BT-474 cells were obtained from American Type Culture Collection. The H460 cells were maintained in RPMI (HyClone) supplemented with 10% fetal bovine serum. RKO and H460 cells were generated by transfection of 

- H460 protein lysates (25 μg) were incubated in 50 mM PIPES, pH 6.0, 1 mM DTT (final volume 100 μl) for 10 min at 30 °C, followed by addition of 0.75 unit of potato acid phosphatase (Boehringer Mannheim). The incubation was continued for 30 min at 30 °C. The phosphatase reaction was stopped by the addition of Laemmli SDS sample buffer. The control and phosphatase-treated lysates were analyzed by Western blot.

**Potato Acid Phosphatase Assay—**H460 protein lysates (25 μg) of total cell protein extract were pre-cleared for 1.5 h at 4 °C with 5 μg mouse IgG pre-bound to protein A-Sepharose (Amersham Pharmacia Biotech). Pre-cleared lysates were transferred to new microcentrifuge tubes and incubated with c-kinin B1 (GNSI, Santa Cruz Biotechnology) and protein A-Sepharose for 3 h at 4 °C. The immunoprecipitates were washed twice with KLB followed with kinase buffer (KLB: 50 mM Tris, pH 7.4, 20 mM MgCl\(\text{2}\), 2 mM DTT) prior to incubation with either five μg of histone H1 (Boehringer Mannheim), 2 μg of GST-Bcl-2 (Santa Cruz Biotechnology), or 1 μg of GST protein prepared as described (43) and 15 nM ATP for 10 min at 25 °C. Samples were incubated with 2 or 5 μCi of \(\text{[\gamma-32P]}\) ATP for 10 or 30 min at 30 °C and resolved by SDS-PAGE. For Bcl-2-associated kinase activity assays, 200 μg of total cell protein extract were pre-cleared for 1.5 h at 4 °C with 5 μg of mouse IgG pre-bound to protein G-Sepharose (Amersham Pharmacia Biotech). The precipitated c-kinin B1/Cdc2 activity assays, 150 μg of total cell protein extract was immunoprecipitated with either c-kinin B1 (clone B2, 100 μg) or protein G-Sepharose (Amersham Pharmacia Biotech) and protein G-Sepharose for 3 h at 4 °C. The immunoprecipitates were washed twice with KLB and twice with KB prior to incubation with 5 μg of histone H1 (Boehringer Mannheim) and 15 nM ATP for 10 min at 25 °C. Samples were incubated with 5 μCi of \(\text{[\gamma-32P]}\) ATP for 30 min at 30 °C and resolved by SDS-PAGE. Kinase assays were quantified using an Instant Imager (Packard Instruments) prior to autoradiography.

**Immunoprecipitation: Western Analysis—**For immunoprecipitations, 150 μg of total cell protein extract was immunoprecipitated with either c-kinin B1 (clone B2, 100 μg) or protein G-Sepharose (Amersham Pharmacia Biotech) and protein G-Sepharose for 3 h at 4 °C. The immunoprecipitates were washed three times in KLB and separated by SDS-PAGE and subsequent Western analysis performed.

**RESULTS**

**Taxol-induced Changes in the Phosphorylation State of Bcl-2 and G2/M Regulatory Proteins—**To determine the relationship between Taxol-induced mitotic arrest, Cdc2 activation, and Bcl-2 phosphorylation, we initially examined the status of G2/M regulatory proteins in several epithelial cell lines after Taxol treatment. Human lung (H460), colon (RKO), and breast (BT-474) carcinoma cell lines were treated with Taxol (100 nM) and protein extracts analyzed by Western (Fig. 1A). After 24 h of Taxol treatment, slower migrating forms of Bcl-2, Cdc25C, and Wee1 proteins were present in cells, indicating changes in the phosphorylation state of these proteins. In addition, there was a shift to a faster migrating form of Cdc2 consistent with the hypophosphorylated, active form of the protein. To determine if the slower migrating forms of Bcl-2, Cdc25C, and Wee1 represented phosphorylated forms of the proteins, we exposed Taxol-treated H460 lysates to potato acid phosphatase. This treatment resulted in loss of the slower migrating forms of...
the proteins and a parallel increase in the faster migrating protein species (Fig. 1B). The Taxol-induced hyperphosphorylation of Bcl-2 coincided with the phosphorylation changes of Wee1, Cdc25C, and Cdc2 that typically accompany mitotic entry. Similar changes in protein phosphorylation were also observed in PC-3 prostate, MDA-MB468 breast, and HCT116 colon carcinoma cell lines (data not shown).

The observed Taxol-induced phosphorylation changes in Wee1, Cdc25C, Cdc2 and Bcl-2 were dose-dependent. H460 and RKO cells were treated with Taxol (0.1–100 nM) for 24 h and protein lysates analyzed by Western (Fig. 2A). Changes in G2/M regulatory protein phosphorylation and Bcl-2 hyperphosphorylation were evident after treatment of cells with 10 nM Taxol. As the dose of Taxol increased from 10 nM to 100 nM, there was a parallel increase in phosphorylation of Bcl-2, Cdc25C, and Wee1 and dephosphorylation of Cdc2 in both cell types. However, the protein mobility of the pro-apoptotic protein, Bax, remained unchanged (Fig. 2B). Recently, Poruchynsky et al. (44) and Fang et al. (45) have reported that Bcl-xL is phosphorylated in Taxol-treated cells. We also detected a subtle mobility shift in Bcl-xL after Taxol treatment (30 and 100 nM). This change in Bcl-xL migration may be due to increased phosphorylation of the protein.

**Mitic Phosphorylation of Bcl-2**—The Taxol-induced phosphorylation changes of Cdc25C, Wee1, and Cdc2 were consistent with cells accumulating in mitosis with an active cyclin B1/Cdc2 complex. Since Taxol-mediated Bcl-2 phosphorylation coincided with these protein phosphorylation changes, we hypothesized that the observed phosphorylation of Bcl-2 was M phase-specific. To test this hypothesis, we isolated control and Taxol-treated cells from various phases of the cell cycle and compared Bcl-2 phosphorylation state to cyclin B1/Cdc2 activity. RKO cells were grown in the presence or absence of Taxol (100 nM) for 3.5 h and size-fractionated by centrifugal elutriation. Relatively pure populations of G1 and G2/M cells were purified as assessed by flow cytometric analysis (Fig. 3A).

Matched cell fractions from control and Taxol-treated cells gave almost identical flow cytometric profiles (Fig. 3A, compare fractions 1–8 from control and Taxol-treated cultures). Protein lysates were prepared from each fraction and cyclin B1 and Bcl-2 protein levels were determined as well as cyclin B1/Cdc2 kinase activity using histone H1 as a substrate. Fraction 1 from control and Taxol-treated cells contained a relatively pure population of G1 cells with little, if any, detectable cyclin B1 protein, phosphorylated Bcl-2, or cyclin B1/Cdc2 activity (Fig. 3, B–D). Even though Fraction 2 was comprised of predominantly G1 cells (Fig. 3A), cyclin B1 immunoprecipitable kinase activity was readily apparent (Fig. 3D). The level of cyclin B1-associated kinase activity appears inconsistent with the low levels of cyclin B1 protein in this fraction (Fig. 3B). However, 6 times more protein lysate was used in the kinase assay as compared with that analyzed by Western. Fractions 5–7 were comprised of cells with predominantly a 4N DNA content that had significantly increased levels of cyclin B1 protein (Fig. 3B) and cyclin B1-immunoprecipitable kinase activity (Fig. 3D). The presence of cyclin B1/Cdc2 activity was consistent with cells that had entered mitosis. Phosphorylated Bcl-2 was readily detectable in control cells (fractions 4–7) and Taxol-treated cells (fractions 4–8); these fractions contained predominantly mitotic cells with elevated levels of cyclin B1-immunoprecipitable kinase activity. The increased ratio of phosphorylated to non-phosphorylated Bcl-2 in the Taxol-treated cells (fractions 4–8) correlated with significantly higher levels of cyclin B1/Cdc2 activity in these cells. These results indicate that phosphorylated Bcl-2 is present normally in mitosis during times of peak cyclin B1/Cdc2 activity. Thus, in asynchronously growing, Taxol-treated cell cultures, the observed Bcl-2 phosphorylation is a consequence of mitotic arrest.

**Elevated Levels of Cyclin B1/Cdc2 Activity Correlate with Prolonged Bcl-2 Phosphorylation and Cell Death**—To further examine and confirm the relationship between entry of cells into mitosis and Bcl-2 phosphorylation, H460 cells were synchronized in G1/S using mimosine and then released in the presence or absence of Taxol (100 nM). Cell cycle progression was monitored by flow cytometric analysis (Fig. 4A), and protein analyses performed (Fig. 4, B–E). Analysis of cyclin B1/Cdc2 immunoprecipitable kinase activity as well as the presence of mitotic phosphoproteins using the MPM-2 antibody allowed biochemical detection of 4N DNA-containing cells that had entered M phase. A previous study by Davis et al. (46)
showed that the MPM-2 antibody recognizes phosphorylated polypeptides found only in mitotic cells; thus, this antibody is a useful reagent for distinguishing 4N DNA-containing mitotic cells from those in G2 phase.

By 9 h after mimosine release, a percentage of the control H460 cells began to enter mitosis, as evidenced by the elevated levels of cyclin B1 protein and a 3-fold increase in cyclin B1/Cdc2 activity (Fig. 4, B and E). The Taxol-treated H460 cells had slower kinetics of mitotic entry with a 3-fold increase in cyclin B1/Cdc2 activity and significant detectable MPM-2 positivity beginning 14 h after mimosine release. There was very low level Bcl-2 phosphorylation in the control cells (Fig. 4C, 18–24-h time points). However, significant levels of phosphorylated Bcl-2 were readily detectable at 14 h after Taxol treatment and coincided with the elevated cyclin B1/Cdc2 activity and the appearance of MPM-2 phosphoepitopes.

At 18 h after mimosine release, flow cytometric profiles of the control and Taxol-treated cell populations were almost identical (Fig. 4A). However, there were significant elevations in the levels of phosphorylated Bcl-2 (Fig. 4C), cyclin B1/Cdc2 activity (Fig. 4E), and MPM-2 phosphoepitopes (Fig. 4D) in the Taxol-treated cells as compared with control cultures. By 22 h after mimosine release, the control cultures had returned to G1 phase, as evidenced by the increase in 2N DNA-containing cells (Fig. 4A); however, the Taxol-treated cultures remained arrested in mitosis with elevated levels of cyclin B1-associated kinase activity and hyperphosphorylated Bcl-2. The highest levels of phosphorylated Bcl-2 protein were found in samples...
Levels of cyclin B1 (for protein analysis at the indicated time points. A, to determine cell cycle position, DNA content was determined by flow cytometric analysis. Levels of cyclin B1 (B), Bcl-2 (C), and MPM-2 epitopes (D) in total cell lysates from control and Taxol-treated cells were analyzed by Western. E, cyclin B1 was immunoprecipitated from total cell lysates of control and Taxol-treated cells, and the activities of the immunoprecipitates were evaluated using histone H1 as the substrate. Fold increase, the increase in kinase activity in Taxol-treated cells relative to the 0-h time point.

containing 7–12-fold increases in cyclin B1/Cdc2 activity and significant elevations in the level of MPM-2 phosphoepitopes.

Between 39 and 45 h after Taxol treatment of the H460 cells, the elevated levels of mitotic kinase activity were followed by the appearance of sub-diploid cells in the cultures (Fig. 4A). This sub-diploid DNA content was accompanied by the appearance of nuclear blebbing and chromatin condensation in the Taxol-treated RKO cells (data not shown).

To determine if Taxol-induced apoptosis was preceded by elevated levels of mitotic kinase activity and prolonged phosphorylation of Bcl-2 in another cell type, the RKO cells were mimosine-synchronized and released in the presence or absence of Taxol (100 nM). Cell cycle progression was monitored by flow cytometric analysis (Fig. 5A) and protein analyses performed (Fig. 5, B–F).

Elevations in cyclin B1/Cdc2 kinase activity were apparent at 16 h in the control cultures and at 14–16 h in the Taxol-treated cultures. Similar to the results with the H460 cells, the kinetics of Bcl-2 phosphorylation in the Taxol-treated RKO cells coincided with the appearance of MPM-2 phosphoepitopes and significant increases in cyclin B1/Cdc2 activity. By 16 h, a percentage of control cells had re-entered G1 phase, as evidenced by the appearance of cells with a 2n DNA content; however, the Taxol-treated cultures remained arrested with a 4n DNA content and significant elevations in mitotic kinase activity through 24h (Fig. 5, C and D).

After 24 h, the Taxol-treated cells underwent gross morphological changes consistent with apoptosis (data not shown) and the number of viable cells decreased significantly (Fig. 5A).

Analysis of protein lysates from apoptotic cells demonstrated that the cells still had significant levels of cyclin B1/Cdc2 activity, phosphorylated Bcl-2, and high levels of MPM-2 phosphoepitopes (Fig. 5, 38- and 45- time points). Thus, elevated mitotic kinase activity and Bcl-2 phosphorylation preceded cell death in both H460 and RKO cells.

Phosphorylated Bcl-2 Associates with Bax—It has been proposed that Taxol-mediated phosphorylation of Bcl-2 decreases its ability to heterodimerize with Bax, thus abrogating the ability of Bcl-2 to prolong cell survival (34, 41). To determine if the Taxol-induced phosphorylation of Bcl-2 altered its ability to heterodimerize with Bax, we evaluated the amount and isoform of Bcl-2 bound to Bax in the RKO protein lysates (Fig. 5). Analysis of Bcl-2-Bax protein binding in synchronized RKO populations allowed a direct comparison of Bcl-2-Bax complex formation in control and Taxol-treated RKO cells during different phases of the cell cycle. Western analysis indicated that similar levels of Bax protein were immunoprecipitated from control and Taxol-treated cells (Fig. 5E) and that Bcl-2 was complexed with Bax under both conditions (Fig. 5F). Phosphorylated Bcl-2 proteins were still able to form a complex with Bax, even in cells undergoing apoptosis (Fig. 5, A and F, 38- and 45-h time points). Similar results were obtained with the H460 cells (data not shown).

Cyclin B1/Cdc2 Neither Associates with Nor Phosphorylates Bcl-2—The parallel increases in Bcl-2 phosphorylation and Cdc2 activity prompted us to determine whether cyclin B1/Cdc2 was the kinase directly involved in mitotic phosphorylation of Bcl-2. In one approach, we investigated whether cyclin B1/Cdc2 was associated with Bcl-2 in vivo. For these experiments, stable RKO transfectants that expressed either endogenous Bcl-2 (RKO-pCEP4) or 30-fold higher levels of Bcl-2 protein (RKO-Bcl-2) were used (Fig. 6A). Taxol treatment of RKO-Bcl-2 cells resulted in hyperphosphorylation of a significant portion of the over-expressed Bcl-2 protein, suggesting that the kinase involved was highly abundant and/or highly active (Fig. 6A, RKO-Bcl-2). In addition, the high levels of overexpressed Bcl-2 in these cells allowed for detection of Bcl-2 phosphorylation in control cultures. The significant Bcl-2 overexpression in the RKO-Bcl-2 cells did not alter the sensitivity of the RKO cells to Taxol-mediated cytotoxicity. The relative kinetics of apoptosis were the same in RKO-pCEP4 as compared with RKO-Bcl-2 cells (data not shown). Bcl-2 was immunoprecipitated from protein lysates prepared from control and Taxol-treated RKO-pCEP4 and RKO-Bcl-2 cells and the immunoprecipitates analyzed by Western for the presence of cyclin B1 or Cdc2. In both RKO-pCEP4 and RKO-Bcl-2 cell lines, phosphorylation of Bcl-2 by mitotic kinase activity could not be detected. This result is consistent with the lack of association between Bcl-2 and cyclin B1/Cdc2 in the control cultures.
Mitotic Phosphorylation of Bcl-2

In this study, we demonstrate that Bcl-2 is normally phosphorylated during mitosis and that Taxol-mediated phosphorylation of Bcl-2 is a consequence of prolonged drug-induced mitotic arrest. It is likely that phosphorylated Bcl-2 is readily detectable in Taxol-treated cells as compared with asynchronously growing untreated cells due to the increased number of cells in mitosis and the prolonged duration of this cell cycle phase after drug treatment. We evaluated whether cyclin B1/Cdc2 was the mitotic kinase involved in direct phosphorylation of Bcl-2, and our results suggest that another kinase is responsible for direct phosphorylation of Bcl-2 in mitosis. Furthermore, we demonstrate that phosphorylated Bcl-2 can still form heterodimeric complexes with Bax. Thus, if phosphorylation of Bcl-2 inhibits its anti-apoptotic function, it is unlikely due to changes in complex formation with Bax protein.

To further evaluate Bcl-2-associated mitotic kinase activity, Bcl-2 was immunoprecipitated from control and Taxol-treated RKO cells. Associated kinase activity was measured by the ability of the Bcl-2 immunoprecipitates to phosphorylate histone H1 substrate. After Taxol treatment, the Bcl-2-associated kinase activity increased in a time-dependent manner that correlated with the increase in Bcl-2 phosphorylation observed in the same protein lysates (Fig. 6C). However, the kinetics of the Taxol-induced increase in Bcl-2-associated kinase activity was delayed as compared with that of cyclin B1/Cdc2 isolated from the same lysates. The cyclin B1/Cdc2 activity increased 1.4-fold over control at 4 h, whereas the Bcl-2-associated kinase activity did not increase to the same extent until 8 h. Taken together, the results presented in Fig. 6 suggest that cyclin B1/Cdc2 is likely not the kinase directly involved in Bcl-2 phosphorylation in mitosis.

**DISCUSSION**

In this study, we demonstrate that Bcl-2 is normally phosphorylated during mitosis and that Taxol-mediated phosphorylation of Bcl-2 is a consequence of prolonged drug-induced mitotic arrest. It is likely that phosphorylated Bcl-2 is readily detectable in Taxol-treated cells as compared with asynchronously growing untreated cells due to the increased number of cells in mitosis and the prolonged duration of this cell cycle phase after drug treatment. We evaluated whether cyclin B1/Cdc2 was the mitotic kinase involved in direct phosphorylation of Bcl-2, and our results suggest that another kinase is responsible for direct phosphorylation of Bcl-2 in mitosis. Furthermore, we demonstrate that phosphorylated Bcl-2 can still form heterodimeric complexes with Bax. Thus, if phosphorylation of Bcl-2 inhibits its anti-apoptotic function, it is unlikely due to changes in complex formation with Bax protein.

There are several candidate kinases thought to be involved in Bcl-2 phosphorylation. Maundrell et al. (40) have correlated activation of p54-SAPK\(\delta\) with increased phosphorylation of Bcl-2 and demonstrated that p54-SAPK\(\delta\) can phosphorylate Bcl-2 in vitro. Other studies have correlated increased phosphorylation of Raf-1 (38) with elevated Bcl-2 phosphorylation. However, Wang et al. (39) were not able to demonstrate a direct phosphorylation of Bcl-2 by Raf-1, even though the two proteins were co-precipitable out of 32D.3 and Sf9 cells. Recently, cAMP-dependent protein kinase was demonstrated to bind to and phosphorylate Bcl-2 in vitro (34). In this study, chemical inhibitors of cAMP-dependent protein kinase were used to implicate...
involvement of this kinase pathway in Bcl-2 phosphorylation in vivo. However, interpretation of experiments using kinase inhibitors is confounded by the cell cycle perturbations these inhibitors induce. Lack of Taxol-induced Bcl-2 phosphorylation after inhibitor addition could be due to cell cycle arrest and the inability of cells to progress into mitosis where Taxol-mediated Bcl-2 phosphorylation occurs. From our results, we predict that the kinase(s) involved in Taxol-mediated Bcl-2 phosphorylation will be part of a mitotic kinase cascade likely activated by Cdc2.

The induction of apoptosis in Taxol-treated tumor cells has been proposed to result from the inability of Bcl-2 to heterodimerize with Bax and inhibit its pro-apoptotic function (34, 41). However, in our studies we demonstrate that phosphorylated Bcl-2 is able to remain complexed with Bax in cells even after 38–45 h of Taxol treatment. Recently, Otter et al. (48) proposed that Bcl-2 inhibition of apoptosis did not correlate with the number of Bcl-2/Bax heterodimers, but rather with the amount of Bcl-2 that is not bound to Bax. This theory is consistent with reports that have proposed that Bax overexpression increases the sensitivity of a cell to Taxol due to the lack of free Bcl-2 in the cell (49). In addition, in cells where Bcl-2 (50) and Bcl-xL (51) are overexpressed, there is decreased sensitivity to chemotherapeutic agents including Taxol; it is likely that the anti-apoptotic protein is in excess and, thus, not associated with Bax in the cell. In our studies, Taxol treatment of RKO and H460 cells did not significantly change the total levels of Bcl-2 protein or the amount of Bcl-2 complexed with Bax. Perhaps Taxol-induced prolonged phosphorylation of Bcl-2 promotes association with other protein(s) or non-proteinaceous targets in the cell, thus reducing the levels of free Bcl-2 in the cell.

The Bcl-2 family members localize to the membranes of the mitochondria (27). Recently Bax, Bcl-2, and Bcl-xL have been shown to form channels/pores in lipid vesicles in vitro (52–55). The release of cytochrome c from the mitochondria activates caspases (31), and in vitro, Bax promotes cytochrome c release from mitochondria (30) while Bcl-2 and Bcl-xL prevent its release (29, 56). Perhaps Bax forms a channel/pore in mitochondria, which, when open, facilitates cytochrome c release (57). When Bax is heterodimerized with the anti-apoptotic Bcl-2 family members, the channel remains closed. In Taxol-treated cells, prolonged phosphorylation of Bcl-2 could inactivate its ability to negatively regulate Bax-mediated release of cytochrome c. Alternatively, phosphorylation of Bcl-2 may not be important in regulating the anti-apoptotic function of the protein, but rather just a reflection of mitotic events. During mitosis, the mitochondria are arranged on the metaphase plate to allow equal segregation of these organelles into daughter cells. Thus, after Taxol treatment, Bcl-2 localized in mitochondrial membranes may be phosphorylated by virtue of prolonged...
presence at the metaphase plate in opposition to numerous kinases that are associated with the mitotic spindle.

The Taxol-mediated apoptosis that we observe after elevated Cdc2 activity is consistent with numerous studies that suggest a link between activation of Cdc2 and apoptosis. Shi et al. (58) demonstrated that loss of Cdc2 in a mouse mammary tumor cell line decreased sensitivity to some apoptotic stimuli, and in lymphoma cells, fragmentin-2-induced apoptosis was associated with an increase in Cdc2 activity. Similarly, in Jurkat cells, granzyme B-induced apoptosis was correlated with increased cyclin A-associated cyclin dependent kinase activity (59), and activation of Cdc2 in this cell line was demonstrated to occur during Fas receptor-mediated apoptosis (60). In several mammalian cell lines, the expression of either Wee1 kinase (61) or dominant negative Cdc2 mutants (60, 62) inhibited both Fas receptor-mediated and granzyme B-mediated apoptosis, as well as TNF-α and staurosporine-induced apoptosis (62).

Recently, caspases have been shown to cleave Wee1 and Cdc27 (a component of the anaphase promoting complex) in vitro (63). The inactivation of these proteins would allow for increased Cdc2 activity in the cell, depending in which phase of the cell cycle caspasies are activated. Finally, Leach et al. (64) have recently shown that p53-induced apoptosis in a murine T- lymphoma cell line was associated with activation of cyclin B1/Cdc2.

Clearly there is much to be learned regarding the role of mitotic kinases in the regulation of apoptosis. Our studies demonstrate that Bcl-2 is phosphorylated in a cell cycle-dependent manner during times of peak Cdc2 activity. Which kinase is responsible for Bcl-2 phosphorylation and whether Bcl-2 hyperphosphorylation plays a role in Taxol-mediated apoptosis remain to be determined. The latter depends on further definition of Bcl-2 mechanism of action. However, we predict that the kinase involved in mitotic phosphorylation of Bcl-2 is regulated by the Cdc2 signaling cascade and that Taxol induces apoptosis by prolonging the signaling events associated with mitotic kinase activation.

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