Phosphorylation of Bcl-2 Is a Marker of M Phase Events and Not a Determinant of Apoptosis*

Yi-He Ling‡, Carmen Tornos§, and Roman Perez-Soler¶¶

From the †Department of Thoracic/Head and Neck Medical Oncology, Section of Experimental Therapy and §Department of Pathology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas, 77030

Phosphorylation of Bcl-2 protein is a post-translational modification of unclear functional consequences. We studied the correlation between Bcl-2 phosphorylation, mitotic arrest, and apoptosis induced by the antitubulin agent paclitaxel. Continuous exposure of human cervical carcinoma HeLa cells to 50 ng/ml paclitaxel resulted in mitotic arrest with a symmetrical bell-shaped curve over time. The number of mitotic cells was highest at 24 h (82%), then declined as arrested cells progressed into apoptosis, and barely no mitotic cells were present at 48–60 h. The time courses of paclitaxel-induced cyclin B1 accumulation and stimulation of Cdc2/cyclin B1 kinase activity were identical and superimposable to that of M phase arrest. In contrast, apoptosis was first detected at 12 h and steadily increased thereafter until the termination of the experiments at 48–60 h, when about 80–96% of cells were apoptotic. Bcl-2 phosphorylation was closely associated in time with M phase arrest, accumulation of cyclin B1, and activation of Cdc2/cyclin B1 kinase, but not with apoptosis. At 24 h, when about 82% of the cells were in mitosis, almost all Bcl-2 protein was phosphorylated, whereas at 48 h, when 70–90% of the cells were apoptotic, all Bcl-2 protein was unphosphorylated. Similar results were obtained with SKOV3 cells, indicating that the association of paclitaxel-induced M phase arrest and Bcl-2 phosphorylation is not restricted to HeLa cells. We used short exposure to nocodazole and double thymidine to synchronize HeLa cells and investigate the association of Bcl-2 phosphorylation with mitosis. These studies demonstrated that Bcl-2 phosphorylation occurs in tight association with the number of mitotic cells in experimental conditions that do not lead to apoptosis. However, a continuous exposure to nocodazole resulted in a pattern of Bcl-2 phosphorylation, M phase arrest, and apoptosis similar to that observed with paclitaxel. The phosphatase inhibitor okadaic acid was found to inhibit the dephosphorylation of phosphorylated Bcl-2 and to delay the progression of nocodazole M phase-arrested cells into interphase. In contrast, the serine/threonine kinase inhibitor staurosporine, but not the tyrosine kinase inhibitor genistein, led to rapid dephosphorylation of phosphorylated Bcl-2 and accelerated the progression of nocodazole M phase-arrested cells into interphase. Immune complex kinase assays in cell-free systems demonstrated that Bcl-2 protein can be a substrate of Cdc2/cyclin B1 kinase isolated from paclitaxel-treated cells arrested in M phase. Taken together, these studies suggest that Bcl-2 phosphorylation is tightly associated with mitotic arrest and fail to demonstrate that it is a determinant of progression into apoptosis after mitotic arrest induced by anti-tubulin agents.

Apoptosis is controlled by a complex interplay between regulatory proteins (1). Bcl-2, a 26-kDa integral membrane oncoprotein, was the first anti-apoptosis gene product discovered (2). Several reports have demonstrated that overexpression of Bcl-2 protein protects cells from undergoing apoptosis in some cell systems, although a recent report indicates that the level of this oncoprotein is not always correlated with an increased ability of the cell to resist death-promoting stimuli (3). Phosphorylation of Bcl-2 was first reported by Alnermi et al. (4) in SF9 cells, and its functional implications remain controversial. Haldar et al. (5, 6) recently reported that treatment with either okadaic acid, a potent inhibitor of phosphatase, or the antitubulin agent paclitaxel resulted in Bcl-2 protein phosphorylation and induction of programmed cell death in lymphoid cells, suggesting that Bcl-2 phosphorylation may abrogate its antiapoptotic function. Other studies, however, do not seem to support this hypothesis. May et al. (7) demonstrated that treatment of murine myeloid factor-dependent FDC-P1/ER cells with interleukin-3 and bryostatin-1, a protein kinase C activator, resulted in the induction of Bcl-2 protein hyperphosphorylation and prevention of apoptosis, indicating that Bcl-2 protein phosphorylation may not be associated with loss of function. Paclitaxel is an effective agent in the treatment of breast, ovarian, lung, and head and neck cancers (8). Paclitaxel promotes tubulin polymerization, thus altering the dynamic equilibrium of assembling and disassembling of microtubules and causing mitotic arrest of dividing cells (9). However, the precise mechanisms of paclitaxel-induced cytotoxicity and apoptosis have not been elucidated. Recently, several studies have suggested that paclitaxel-induced apoptosis may be mediated by loss of Bcl-2 function as a result of phosphorylation by activated Raf-1 kinase (10–12). Other studies, however, have not confirmed this observation. Ibrado et al. (13) reported that paclitaxel-induced apoptosis in human myeloid leukemia HL-60 cells was not associated with activation of Raf-1 kinase and Bcl-2 phosphorylation (13). These controversial findings prompted us to study more in detail the relationship between paclitaxel-induced Bcl-2 phosphorylation and apoptosis in HeLa cervical carcinoma cells and SKOV3 ovarian carcinoma cells. The results presented here indicate that hyperphosphorylation of Bcl-2 is temporally associated with paclitaxel-induced M phase arrest but not with apoptosis. Results with HeLa cells synchronized by either nocodazole or double thymidine blockade confirmed that the phosphorylation of Bcl-2 is a

* This work was supported in part by National Institutes of Health Grant CA50270. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom requests for reprints should be addressed: Dept. of Thoracic/Head and Neck Medical Oncology, Box 080, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030. Tel.: 713-792-6363; Fax: 713-796-8655.

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marker of M phase events and failed to demonstrate a direct linkage between Bcl-2 phosphorylation and induction of apoptosis. Additional studies with inhibitors of phosphatase and kinase in nocodazole-blocked M phase cells further confirmed that the phosphorylation of Bcl-2 is tightly linked to the regulation of M phase events.

MATERIALS AND METHODS

Chemicals and Antibodies—Paclitaxel, nocodazole, and thymidine were purchased from Sigma. Okadaic acid, staurosporine, genistein, and histone H1 were purchased from Boehringer Mannheim. Cell culture medium was obtained from Life Technologies, Inc. Monoclonal anti-Bcl-2 (Ab-1) antibody, anti-Cdc2 (Ab-1), and anti-cyclin B1 (Ab-2) were purchased from Calbiochem. Polyclonal anti-Raf-1 antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell Culture and Synchronization—HeLa and SKOV3 cells were obtained from American Type Culture Collection (Rockville, MD) and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 250 \( \mu \)M L-glutamine. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO\(_2\), 95% air incubator. Exponentially growing cells were exposed to paclitaxel at the indicated concentrations for different lengths of time. Tissue culture samples were collected by the gentle shakeoff method and washed three times by centrifugation at 4,000 rpm for 10 min at 4 °C. The protein content in each sample was determined as described above using a DC protein assay kit and adjusted accordingly before the assay. Lysate (0.5 ml) was incubated with 5 \( \mu \)g monoclonal anti-Cdc2 and anti-cyclin B1 antibodies and 50 \( \mu \)l of protein A/protein G conjugated agarose (Calbiochem) at 4 °C overnight. After washing three times with lysis buffer and once with reaction buffer, the immunoprecipitate complex was collected and incubated at 30 °C in 50 \( \mu \)l of kinase reaction mixture containing 50 mM Tris-HCl (pH 7.4), 10 mM MgCl\(_2\), 1 mM dithiothreitol, 10 mM ATP, 5 \( \mu \)Ci of \([\gamma-32P]ATP\), and 0.5 mg/ml of histone H1 for 15 min. The reaction was terminated by the addition of 20 \( \mu \)l of 4\( \times \) Laemmli’s sample buffer and boiling for 5 min. The \( 32P \) incorporation into the immunoprecipitated histone H1 was separated by centrifugation at 4,000 rpm for 10 min at 4 °C. The protein amount in each sample was determined by a DC protein assay kit and stored at -90 °C until use.

Immunoblotting—Cells were lysed with lysis buffer containing 50 mM Tris-HCl (pH 7.4), 0.1% Triton X-100, 1% SDS, 250 mM NaCl, 15 mM MgCl\(_2\), 1 mM dithiothreitol, 2 mM EDTA, 2 mM EGTA, 25 mM NaF, 1 mM phenylmethanesulfonyl fluoride, 10 \( \mu \)g/ml leupeptin, and 10 \( \mu \)g/ml aprotenin. The protein amount in each sample was determined by a DC protein assay kit (Bio-Rad). Equal amounts of lysate were subjected to electrophoresis in a 0.1% SDS, 10% polyacrylamide gel. The proteins were transferred onto nitrocellulose membrane. After blocking with 5% nonfat milk in Tris-buffered saline-Tween buffer at room temperature for 1 h, Bcl-2, Cdc2, and cyclin B1 were probed with monoclonal anti-Bcl-2, anti-Cdc2, or anti-cyclin B1 antibodies. The immunoblots were analyzed using an ECL detection system according to the manufacturer’s recommendation (Amersham Pharmacia Biotech). The relative amounts of Cdc2, cyclin B1, and phosphorylated and unphosphorylated Bcl-2 protein were quantitatively measured by laser scanning densitometry (Molecular Dynamics, Sunnyvale, CA).

Immunoprecipitation and Kinase Assay—Cells (1 \( \times 10^6 \) cells) were aliquoted from the cultures at the indicated time points and solubilized in 0.5 ml of lysis buffer containing 50 mM Tris-HCl (pH 7.4), 250 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 10 \( \mu \)g/ml leupeptin, 10 \( \mu \)g/ml aprotenin, and 1% Triton X-100. After incubation at 4 °C for 15 min, the lysate was separated by centrifugation at 4,000 rpm for 10 min at 4 °C. The protein content in each sample was determined as described above using a DC protein assay kit and adjusted accordingly before the assay. Lysate (0.5 ml) was incubated with 5 \( \mu \)g monoclonal anti-Cdc2 and anti-cyclin B1 antibodies and 50 \( \mu \)l of protein A/protein G conjugated agarose (Calbiochem) at 4 °C overnight. After washing three times with lysis buffer and once with reaction buffer, the immunoprecipitate complex was collected and incubated at 30 °C in 50 \( \mu \)l of kinase reaction mixture containing 50 mM Tris-HCl (pH 7.4), 10 mM MgCl\(_2\), 1 mM dithiothreitol, 10 \( \mu \)M ATP, 5 \( \mu \)Ci of \([\gamma-32P]ATP\), and 0.5 mg/ml of histone H1 for 15 min. The reaction was terminated by the addition of 20 \( \mu \)l of 4\( \times \) Laemmli’s sample buffer and boiling for 5 min. The \( 32P \) incorporation into histone H1 was separated by centrifugation at 4,000 rpm for 10 min at 4 °C. The radioactive incorporation was quantified by autoradiography using Kodak X-Omat film or by liquid scintillation counting.

To assay Bcl-2 phosphorylation by Cdc2/cyclin B1 kinase, Bcl-2 and Cdc2/cyclin B1 were immunoprecipitated from 1 \( \times 10^7 \) HeLa cells by monoclonal anti-Bcl-2 and anti-Cdc2/anti-cyclin B1 antibodies as described above. The reaction of Bcl-2 phosphorylation was carried out in 50 \( \mu \)l of reaction mixture containing 50 mM Tris-HCl (pH 7.4), 10 mM Tris-HCl (pH 8.3), 250 mM NaCl, 1 mM MgCl\(_2\), 1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 10 \( \mu \)g/ml leupeptin, 10 \( \mu \)g/ml aprotenin, 10 \( \mu \)g/ml aprotinin, 1% Triton X-100, and 10 \( \mu \)Ci of \([\gamma-32P]ATP\). The reaction was terminated by the addition of 20 \( \mu \)l of 4\( \times \) Laemmli’s sample buffer and boiling for 5 min. The \( 32P \) incorporation into histone H1 was separated by centrifugation at 4,000 rpm for 10 min at 4 °C. The radioactive incorporation was quantified by autoradiography using Kodak X-Omat film or by liquid scintillation counting.
MgCl₂, 1 mM dithiothreitol, 10 μM ATP, 5 μCi of [γ-³²P]ATP, and Bcl-2 and Cdc2/cyclin B1 immune complex. After incubation at 30 °C for 15 min, the reaction was terminated by addition of 4× Laemmli’s sample buffer. After boiling for 5 min, the samples were subjected to a 10% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to the nitrocellulose membrane and probed with monoclonal anti-Bcl-2 antibody with ECL detection as described above. The ³²P-incorporated Bcl-2 protein was analyzed by autoradiography.

Mitosis and Apoptosis Assay—The number of mitotic cells was determined in cells stained with Wright-Giemsa dye solution. Apoptotic cells were detected using a Tunel reaction kit according to the manufacturer’s recommendation (Boehringer Mannheim). After the reaction, the fluorescence-labeled cells were determined by flow cytometry (Epics Profile Analyzer, Coulter Co., Miami, FL). For determination of paclitaxel-induced DNA fragmentation, cells were solubilized with 0.5 ml of lysis buffer containing 10 mM Tris-HCl (pH, 8.0), 200 mM NaCl, and 0.2% Triton X-100 at room temperature for 30 min. After centrifugation at 14,000 rpm for 5 min, the supernatant fraction was collected and fragmented DNA was precipitated with 100 mM NaCl and equal volume isopropanol at −20 °C overnight. DNA was dissolved in Tris-EDTA buffer containing 20 units of RNase and incubated at 60 °C for 60 min. After electrophoresis in 1% agarose gel, DNA was stained with ethidium bromide, and resulting DNA fragmentation was visualized by UV illumination (15).

RESULTS

Effect of Paclitaxel on Mitotic Arrest, Cyclin B1 Accumulation, Activation of Cdc2/Cyclin B1 Kinase, Apoptosis, and Phosphorylation of Bcl-2 Protein—Initially, we treated HeLa cells continuously with 50 ng/ml paclitaxel (a concentration that corresponds to the ID₉₀) and determined drug-induced M phase arrest by using Wright-Giemsa dye staining. About 2% cells were in M phase at baseline. Paclitaxel treatment resulted in a steady increase of cells arrested at M phase which was maximum at 24 h and decreased thereafter, i.e. 82% in M phase at 24 h and barely no cells in M phase at 48–60 h. Because M phase is associated with accumulation of B type cyclins and activation of Cdc2/cyclin B complex kinase (16, 17), we determined the effect of paclitaxel on the levels of Cdc2 and cyclin B1 and activity of Cdc2/cyclin B1 kinase. Similar to M phase arrest, paclitaxel treatment caused a gradual increase in cyclin B1 amounts that peaked at 24 h and markedly decreased at 48–60 h. Under the same experimental conditions, the Cdc2 levels were relatively constant. As expected, the time-course of drug-induced stimulation of Cdc2/cyclin B1 kinase activity was similar to those of drug-induced M phase arrest and induction of cyclin B1 (Fig. 1). In summary, paclitaxel-induced M phase arrest, accumulation of cyclin B1, and stimulation of Cdc2/cyclin B1 kinase exhibited a bell shape time-course with the peak of the curve at 24 h.

Next, we determined the effect of paclitaxel in inducing

Fig. 2. Paclitaxel-induced apoptosis in HeLa cells. Cells were continuously exposed to 50 ng/ml paclitaxel for different times. Apoptotic cells were determined by observation of morphological changes, Tunel reaction, and DNA fragmentation assay as described under “Materials and Methods.” A, apoptotic cells induced by paclitaxel for different times and determined by Tunel reaction assay; B, apoptotic cells determined by microscopic observation (○—○) or Tunel assay (●—●); C, DNA fragmentation determined by 1% agarose gel electrophoresis.

Fig. 3. Paclitaxel-induced Bcl-2 phosphorylation and M phase arrest in HeLa cells. Cells were continuously exposed to 50 ng/ml paclitaxel. Mitotic cells were determined by counting Wright-Giemsa-stained cells and Bcl-2 phosphorylation was determined by Western blot analysis with ECL detection as described under “Materials and Methods.” A, Bcl-2 phosphorylation induced by paclitaxel after exposure for different times. Bands representing hyperphosphorylated (U), phosphorylated (M), and unphosphorylated Bcl-2 protein (B) were detected with ECL reaction; B, the correlation between M phase arrest and Bcl-2 phosphorylation after exposure to paclitaxel.
apoptosis in HeLa cells. Drug treatment was the same as described above. Aliquots of cells were taken at different time points and apoptotic cells determined by morphology and Tunel reaction assay. As shown in Fig. 2, A and B, no apoptotic cells were observed at 6 h; about 8–18% of cells were apoptotic by 12–24 h. The number of apoptotic cells markedly increased after 24 h, with 60–80% apoptotic cells at 36–48 h and 96% at 60 h. To confirm that drug-induced cell death was due to apoptosis, DNA obtained from cells at different time points was subjected to a 1% agarose gel electrophoresis, stained with ethidium bromide, and visualized by UV illumination. As shown in Fig. 2C, a typical apoptotic hallmark, cleavage of genomic DNA into multiple internucleosome (≈200 base pairs), appeared at 24 h, and the extent of DNA fragmentation increased with time. These data further confirm our previous findings in KB and SKOV3 cells that paclitaxel-induced mitotic arrest precedes apoptosis (18), and are consistent with the report by Jordan et al. (19).

Finally, we determined whether paclitaxel-induced apoptosis is associated with Bcl-2 phosphorylation. We treated cells after 24 h, with 60–80% apoptotic cells at 36–48 h and 96% at 60 h. To confirm that drug-induced cell death was due to apoptosis, DNA obtained from cells at different time points was subjected to a 1% agarose gel electrophoresis, stained with ethidium bromide, and visualized by UV illumination. As shown in Fig. 2C, a typical apoptotic hallmark, cleavage of genomic DNA into multiple internucleosome (≈200 base pairs), appeared at 24 h, and the extent of DNA fragmentation increased with time. These data further confirm our previous findings in KB and SKOV3 cells that paclitaxel-induced mitotic arrest precedes apoptosis (18), and are consistent with the report by Jordan et al. (19).
Bcl-2 phosphorylation is associated in time with M phase arrest and activation of Cdc2/cyclin B kinase, thus preceding apoptosis, and that at the time of massive apoptosis the Bcl-2 protein is fully unphosphorylated (Fig. 4).

To confirm that paclitaxel-induced Bcl-2 phosphorylation is associated with mitosis in a different cell line, we treated human ovarian carcinoma SKOV3 cells continuously with 50 ng/ml paclitaxel and determined Bcl-2 phosphorylation, mitosis and apoptosis at different time points. As shown in Fig. 5, the patterns of paclitaxel-induced Bcl-2 phosphorylation, M phase arrest and apoptosis in SKOV3 cells are similar to those in HeLa cells described above. Thus it indicates that the association of drug-induced Bcl-2 phosphorylation with M phase arrest, not with apoptosis is not restricted to HeLa cells.

Bcl-2 Phosphorylation in Synchronized HeLa Cells—To further confirm that Bcl-2 protein phosphorylation is associated with M phase arrest, we used synchronized HeLa cells to determine Bcl-2 phosphorylation at different phases of the cell cycle. First, we treated cells with 100 ng/ml nocodazole for 16 h and collected M phase blocked cells as described in Materials and Methods. At different times post-release, bands representing phosphorylated (U), phosphorylated (M), and unphosphorylated Bcl-2 protein (B) were detected with ECL reaction; B, time curves of mitotic and apoptotic cells and Bcl-2 phosphorylation after exposure to nocodazole.

continuously with 50 ng/ml paclitaxel, collected cell aliquots from culture at the indicated time-points, and lysed cells with lysis buffer. An equal amount of lysate (50 μg protein) from each sample was subjected to a 10% SDS-polyacrylamide gel electrophoresis, and Bcl-2 protein was probed by monoclonal anti-Bcl-2 antibody with ECL detection. The results presented in Fig. 3A, a typical and reproducible Western blot analysis, revealed that at the time of massive apoptosis the Bcl-2 protein is fully unphosphorylated (Fig. 4), that at the time of massive apoptosis the Bcl-2 protein is fully unphosphorylated (Fig. 4), and Bcl-2 phosphorylation was determined by morphological changes and Bcl-2 phosphorylation was determined by Western blot analysis with ECL detection as described under “Materials and Methods.” A, Bcl-2 phosphorylation induced by continuous exposure to nocodazole for different times. Bands representing hyperphosphorylated (U), phosphorylated (M), and unphosphorylated Bcl-2 protein (B) were detected with ECL reaction; B, time curves of mitotic and apoptotic cells and Bcl-2 phosphorylation after exposure to nocodazole.

FIG. 7. Bcl-2 phosphorylation, M phase arrest and apoptosis induced by continuous exposure to nocodazole in HeLa cells. Cells were continuously exposed to 100 ng/ml nocodazole at 37 °C for the indicated periods of time. Mitotic cells were determined by counting Wright-Giemsa stained cells, apoptosis was determined by morphological changes and Bcl-2 phosphorylation was determined by Western blot analysis with ECL detection as described under “Materials and Methods.” A, Bcl-2 phosphorylation induced by continuous exposure to nocodazole for different times. Bands representing hyperphosphorylated (U), phosphorylated (M), and unphosphorylated Bcl-2 protein (B) were detected with ECL reaction; B, time curves of mitotic and apoptotic cells and Bcl-2 phosphorylation after exposure to nocodazole.

FIG. 8. Bcl-2 phosphorylation in HeLa cells synchronized by double thymidine block. Cells were synchronized with double thymidine block as described under “Materials and Methods.” A, cell cycle distribution determined by flow cytometry; B, changes in Bcl-2 protein phosphorylation at different times post-release, bands representing phosphorylated (U) and unphosphorylated (B) Bcl-2 detected with ECL reaction.
protein was dephosphorylated. After 3–5 h of release, almost all cells were in interphase and only a 26-kDa dephosphorylated band was displayed in the gel. We also determined the apoptotic cells at different time points after release and only found a few apoptotic cells (10%) up to 48 h after release.

To compare side by side the effects of nocodazole and paclitaxel under the same experimental conditions, we treated HeLa cells with 50 ng/ml paclitaxel for 16 h and collected the M phase cells as described above. Cell aliquots were collected at different times after drug incubation. The number of mitotic and apoptotic cells and the extent of Bcl-2 phosphorylation were determined as described above. As shown in Fig. 6B, the M phase blockade caused by paclitaxel was very slowly reversible. For example, about 90% cells were in mitosis during the first 6 h post-incubation, decreasing to 80–60% at 12–24 h post-incubation. Again, the extent of Bcl-2 phosphorylation was tightly associated with the number of mitotic cells, i.e. about 90% of Bcl-2 protein was phosphorylated during the first 6 h post-incubation, decreasing to 80–50% at 12–24 h. In contrast, only 3–5% cells were in mitosis and there was no detectable phosphorylated Bcl-2 24 h after a 16 h exposure, and resulted in massive (>80%) apoptosis at 60 h, with a pattern very similar to that observed in cells continuously exposed to paclitaxel (Fig. 4). Altogether, these results confirm a tight temporal association between Bcl-2 phosphorylation and M phase arrest, and indicate that with either agent apoptosis is only induced under experimental conditions that result in prolonged mitotic arrest and Bcl-2 phosphorylation.

Next, we used double thymidine to synchronize HeLa cells and determined the presence of phosphorylated Bcl-2 at different phases of the cell cycle. As shown in Fig. 8, about 70% of cells were synchronized at G1 phase after double thymidine block (two periods of exposure with a 10-h interval of incubation in normal medium). Accumulation of cells at G2/M was started to be observed at 6 h post-block release, peaked (about 60% G2/M cells) at 10 h, and still remained significant (about 40% G2/M cells) at 12 h. Interestingly, Western blot analysis revealed that the slower mobility band, representing phosphorylated Bcl-2 protein, appeared at 6 h after release and lasted until 12 h after release. The phosphorylated band disappeared at 24 h, when 80% of cells were in G1 phase and only 8% of cells in G2/M phase. These results further confirm that Bcl-2 protein phosphorylation is associated with cells at G2/M phase.

**Effects of Phosphatase Inhibitor and Protein Kinase Inhibitor on Bcl-2 Phosphorylation and Mitosis**—Although the function of phosphorylated Bcl-2 and the sites of phosphorylation on the Bcl-2 protein remain to be defined, it has been speculated that serine/threonine kinases and phosphatases may be involved in the regulation of phosphorylation and dephosphorylation of Bcl-2 protein (20). To further study the association between Bcl-2 phosphorylation and mitosis, we used inhibitors of phos-
phosphatase and protein kinase to examine whether the alteration in Bcl-2 phosphorylation by these inhibitors could alter the exit of cells from mitosis. First, we synchronized HeLa cells in M phase with nocodazole and then replated the M phase cells in fresh medium in the absence or presence of 1 μM okadaic acid, a phosphatase inhibitor. After incubation for the indicated periods of time, cell aliquots were taken from the culture, and the number of mitotic cells and extent of Bcl-2 phosphorylation were determined as described above. As shown in Fig. 9A, treatment with okadaic acid prevented or delayed the exit of the cells from mitosis and the dephosphorylation of phosphorylated Bcl-2. For example, 69% cells remained in M phase and the hyperphosphorylated or phosphorylated Bcl-2 bands were still predominant at 4–6 h post-release in the presence of okadaic acid, versus only 4–9% cells and barely no phosphorylated Bcl-2 bands in the absence of inhibitor. Next, we used staurosporine, a serine/threonine kinase inhibitor, to examine whether the inhibition of Bcl-2 phosphorylation could facilitate the exit of cells from mitosis and entry into interphase. Cells arrested at M phase after exposure to 100 ng/ml nocodazole for 16 h were used for these experiments. As shown in Fig. 9B, treatment of cells arrested at M phase by nocodazole with 100 nM staurosporine for 60 min resulted in a massive entry of mitotic cells into interphase, whereas 80% cells remained in M phase in the absence of staurosporine. As expected, the presence of staurosporine for 60 min resulted in complete dephosphorylation of the Bcl-2 protein, whereas the Bcl-2 protein remained at the hyperphosphorylation state in the absence of inhibitor. Finally, we used genistein, a tyrosine kinase inhibitor, to determine the effect of this inhibitor on cell entry into interphase and Bcl-2 dephosphorylation. Genistein did not alter the number of mitotic cells nor the extent of Bcl-2 phosphorylation in cells treated with 25 μM genistein for 90 min after release (Fig. 9C). These findings suggest that serine/threonine kinases, but not tyrosine kinases, are involved in the regulation of Bcl-2 phosphorylation and M phase transition. These results are consistent with those reported by Chen and Faller (21).

Role of Cdc2/Cyclin B Kinase in Bcl-2 Phosphorylation—Because Bcl-2 phosphorylation is temporally associated with activation of Cdc2/cyclin B1 kinase, we investigated the potential role of Cdc2/cyclin B1 complex kinase in phosphorylating Bcl-2. To determine whether Bcl-2 phosphorylation may be associated with activation of Cdc2/cyclin B1 kinase, we used monoclonal anti-Bcl-2 antibody to immunoprecipitate Bcl-2 protein from HeLa cells. Equal amounts of Bcl-2 detected with ECL were added to a reaction mixture consisting of 50 mM Tris-HCl, 10 mM MgCl₂, 10 μM ATP, 5 μCi of [γ-32P]ATP and Cdc2/cyclin B1 complex immunoprecipitated from untreated and paclitaxel-treated cells. The paclitaxel-induced stimulation of Cdc2/cyclin B1 kinase activity was determined by using histone H1 as a substrate as described above. As shown in Fig. 10, the Bcl-2 protein immunoprecipitated from untreated cells was markedly phosphorylated by the Cdc2/cyclin B1 kinase complex precipitated from paclitaxel-treated cells, although the extent of such phosphorylation was lower than that using histone H1 as a substrate. In contrast, no Bcl-2 protein was phosphorylated by the Cdc2/cyclin B1 kinase complex precipitated from untreated cells. Under the same experimental conditions, other cell cycle related kinases precipitated from paclitaxel-treated cells, such as Cdc2/cyclin A and Cdc2/cyclin E, failed to phosphorylate Bcl-2 protein, indicating that phosphorylation of Bcl-2 may be at least in part associated with the activation of M phase-related kinase (data not shown).

**DISCUSSION**

The results of the present study demonstrate that the antitubulin agents paclitaxel and nocodazole induce transient Bcl-2 phosphorylation in close association with mitotic arrest and cyclin B1 kinase activation. Bcl-2 phosphorylation of variable duration was observed in HeLa cells synchronized with double thymidine when they reach G₂/M phase, HeLa cells transiently arrested in mitosis by nocodazole, and HeLa cells arrested in mitosis prior to apoptosis after continuous exposure to paclitaxel and nocodazole. The duration of Bcl-2 phosphorylation was about 3–6 h in cells synchronized with double thymidine or transiently arrested in mitosis after a 16-h exposure to nocodazole. In both cases, no apoptosis was observed. In contrast, Bcl-2 phosphorylation lasted >24 h in cells continuously exposed to paclitaxel and nocodazole and in these situations massive apoptosis was observed 24–36 h after maximum mitotic arrest. However, at the time of massive apoptosis all Bcl-2 was dephosphorylated. Altogether, these results do not appear to support the recently stated hypothesis that inactivation of Bcl-2 by phosphorylation is a major potential mechanism of paclitaxel-induced apoptosis (4, 7, 11, 12). If Bcl-2 phosphorylation was a major determinant of paclitaxel-induced apoptosis, one would have expected to observe a similar apoptotic effect in nocodazole-synchronized mitotic cells in which >90% of Bcl-2 protein was phosphorylated. However, we can not rule out nor prove from our studies that it is not Bcl-2 phosphorylation per se but a persistent (>24 h) Bcl-2 inactivation through phosphorylation the important determinant of paclitaxel-induced apoptosis.

Another possible explanation for the discrepant conclusions between ours and previous studies may be related to the time frame of the experiments, 24 h in previous studies as opposed to 48–60 h in our study. If our studies had not included deter-
minations at 48–60 h, we would have not been able to demonstrate that paclitaxel-induced Bcl-2 phosphorylation peaks at 24 h, as well as other markers of mitotic arrest such as accumulation of cyclin B1 and activation of Cdc2/cyclin B1 kinase, and that all the mitotic markers are completely reversed by 48–60 h, when massive apoptosis is paramount (Fig. 3). Interestingly, our results are consistent with the recent report by Ibrado et al. (13) in HL-60 human leukemia cells demonstrating complete dephosphorylation of Bcl-2 at the time of paclitaxel-induced DNA degradation.

The fact that Bcl-2 is dephosphorylated at the time of massive apoptosis does not rule out in principle the possibility that a preceding Bcl-2 phosphorylation lasting more than a certain critical period of time might be sufficient to trigger the apoptotic pathway. However, the proposed mechanism of loss of Bcl-2 antiapoptotic activity appears to be mediated by a decreased ability of the phosphorylated protein to interact with proapoptotic proteins such as bax, in which case one would expect a persistently phosphorylated and non-functional Bcl-2 protein at the time of paclitaxel-induced massive apoptosis (22, 23).

The mechanism of Bcl-2 phosphorylation in mitotic cells has not been fully elucidated. There is evidence that Raf-1 can phosphorylate the Bcl-2 protein in the outer mitochondrial membrane (24, 25). Blagosklonny et al. (10, 11) reported that paclitaxel-induced apoptosis was associated with the activation of Raf-1 kinase and phosphorylation of Bcl-2 in MCF-7 cells. However, some investigators have shown that depletion of Raf-1 kinase activity by the specific inhibitor geldanamycin did not affect paclitaxel-induced Bcl-2 phosphorylation and apoptosis (13). To further investigate whether Raf-1 is responsible for phosphorylating Bcl-2 in mitotic cells, we used the Jurkat cells and the mutant cells J. CaM.1, which are deficient in Lck gene and lack Raf-1 kinase activation in M phase (26). Our preliminary results indicate that the pattern of Bcl-2 phosphorylation induced by paclitaxel in Jurkat cells is similar to that in J. CaM.1 cells, thus suggesting that Raf-1 kinase may not be involved in paclitaxel-induced Bcl-2 phosphorylation (data not shown). Another possibility would be that Bcl-2 is phosphorylated by Cdc2/cyclin B1 kinase since Cdc2/cyclin B1 kinase activation and Bcl-2 phosphorylation occur at the same time. It is well known that several proteins are phosphorylated in M phase by Cdc2/cyclin B kinase, and that these phosphorylated proteins play crucial roles in regulating the mitotic process, including chromatin condensation, nuclear envelope breakdown, and formation of the mitotic spindle. We were able to demonstrate using immune complex kinase assays in cell free systems that in fact the Cdc2/cyclin B1 complex can phosphorylate Bcl-2, although the extent of phosphorylation was lower than with histone H1 as a substrate (Fig. 10). It remains to be determined whether this is also the case in whole cell systems since the Cdc2/cyclin B1 complex is located in the nucleus and the Bcl-2 protein in the mitochondrial membrane. However, in favor of the possibility that Bcl-2 may be phosphorylated by Cdc2/cyclin B1 kinase is the observation by Lu et al. that the subcellular distribution of Bcl-2 protein determined by either immunocytchemistry or immunoelectron microscopy was dependent on cell cycle, i.e. Bcl-2 protein was localized in the cytoplasm at interphase and in the nucleus, in particular in chromosomes, at M phase (27).

Our results indicate that a phosphorylated Bcl-2 protein is a landmark of mitotic cells. Like in the case of M phase-related cyclins and kinases, it is reasonable to assume that Bcl-2 phosphorylation and dephosphorylation may play an important role in regulating M phase specific events, such as facilitation of mitotic-related kinase into nuclei, breaking down of nuclear envelope, condensation of chromatin, formation of the mitotic spindle, or chromosome segregation, and determining the progression of cells from G2 into mitosis and from mitosis into interphase, respectively. The elucidation of the mechanisms by which Bcl-2 phosphorylation and dephosphorylation affect cell cycle progression deserves, therefore, further investigation. In our studies, Bcl-2 phosphorylation was found to be regulated by unidentified phosphatases and serine/threonine kinase since treatment with either okadaic acid or staurosporine strikingly altered Bcl-2 phosphorylation and delayed or enhanced, respectively, exit from mitosis and entry into interphase. The observation that kinase and phosphatase inhibitors may disrupt the equilibrium between Bcl-2 phosphorylation and dephosphorylation and impair the normal progression of the cell cycle in mitosis could provide a therapeutic opportunity and be exploited to potentiate the effects of paclitaxel or other chemotherapeutic agents.

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