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Cell Cycle-Dependent Cytotoxicity, G2/M Phase Arrest, and Disruption of $p34^{cdc2}$/Cyclin B_1 Activity Induced by Doxorubicin in Synchronized P388 Cells

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SUMMARY

We studied the effect of doxorubicin (Dox) on cell cycle progression and its correlation with DNA damage and cytotoxicity in p53-mutant P388 cells. P388 cells synchronized in S and G2/M phases were >3-fold more sensitive to Dox than were cells in G1 phase (Dox ID_{50} = 0.50 ± 0.16 μM in cells synchronized in S phase versus 1.64 ± 0.12 μM in asynchronized cells; drug exposure, 1 hr). Treatment of synchronized cells in early S phase with 1 μM Dox (2 × ID_{50}) for 1 hr induced a marked cell arrest at G2/M phase at 6–12 hr after drug incubation. We then studied the effect of Dox on the $p34^{cdc2}$/cyclin B_1 complex because it plays a key role in regulating G2/M phase transition. In untreated control P388 cells, $p34^{cdc2}$ kinase localizes in the nucleus and cytoplasm, particularly in the centrosomes, and $p34^{cdc2}$ kinase activity is dependent on cell cycle progression, with the enzyme activity increasing steadily from G1/S to G2/M and markedly declining thereafter. Treatment of synchronized P388 cells in early S phase with 1 μM Dox for 1 hr did not affect the pattern of subcellular distribution of the enzyme but completely abrogated its function for ≥10 hr. In a cell-free system, Dox did not inhibit $p34^{cdc2}$ kinase activity, indicating that it has no direct effect on the enzyme function. In whole cells, Dox treatment prevented $p34^{cdc2}$ kinase dephosphorylation without altering its synthesis, and this effect was due to neither down-regulation of cdc25C nor inhibition of protein-tyrosine phosphatase activity. In contrast, Dox treatment was found to induce cyclin B_1 accumulation as a result of stimulating its synthesis and inhibiting its degradation. A good correlation was found between extent of DNA double-strand breaks and $p34^{cdc2}$ kinase activity inhibition. Our results suggest that anthracycline-induced cytotoxicity is cell cycle dependent and is mediated, at least in part, by disturbance of the regulation of $p34^{cdc2}$/cyclin B_1 complex, thus leading to G2/M phase arrest.

Dox is the most widely used anthracycline antibiotic in cancer therapy because of its broad spectrum of antineoplastic activity, which includes solid tumors and hematological malignancies (1–3). Although several investigations have suggested that (i) anthracycline compounds exert their cytotoxicity by inducing DNA strand breaks via interaction with the targeted enzyme topoisomerase II and (ii) the extent of DNA strand breaks directly correlates with cytotoxicity in a variety of tumor cell lines (4, 5), the precise mechanism of action for these compounds remains to be defined.

Recently, the mechanisms that control cell cycle progression have been intensively investigated. $p34^{cdc2}$, initially defined in the fission yeast *Schizosaccharomyces pombe*, has been found to play a very important role in the regulation of the cell cycle (6–8). The cdc2 gene product, $p34^{cdc2}$, is a protein kinase that interacts with a family of cellular cyclins. $p34^{cdc2}$ kinase phosphorylates a variety of cellular proteins, including oncogene products and the tumor-suppressor gene products Rb and p53, in a cell cycle-dependent manner (9–11). Activation of $p34^{cdc2}$/cyclin B protein kinase is needed to trigger G2/M transition (12, 13). Furthermore, a number of reports have shown that a variety of DNA-damaging agents and irradiation are capable of arresting cell cycle progression at G2 phase (14–17). These findings suggest that there is a coupling relationship among DNA damage, arrest at G2/M, and inhibition of cell division, although the molecular mechanism of such relationship remains to be defined.

In this study, we used synchronized P388 cells as a model system to determine whether Dox delays cell cycle progression and whether DNA damage induced by Dox is coupled to the disruption of cell cycle-associated events, such as inhibition of the activation of $p34^{cdc2}$ kinase, alteration in the phosphorylation status of $p34^{cdc2}$ kinase, and alteration in the expression of cdc2 and cyclin B_1.

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ABBREVIATIONS: Dox, doxorubicin; PBS, phosphate-buffered saline; BSA, bovine serum albumin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PTPase, protein-tyrosine phosphatase; SDS, sodium dodecyl sulfate.

Materials and Methods

Chemicals. Dox was purchased from Ben Venue Laboratories (Bradford, OH), and a stock solution (2 mM) was prepared in PBS, pH 7.4. p34^{cdc2} kinase substrate peptide and anti-cdc2 antibody were purchased from GIBCO-BRL (Gaithersburg, MD). Monoclonal anti-cyclin B_1 antibody was purchased from Oncogene Science (Uniondale, NY). Polyclonal anti-cdc25C antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). [γ-^{32}P]ATP (specific activity, >7000 Ci/mmol) and [*methyl*-^{14}C]thymidine (specific activity, 60 mCi/mmol) were purchased from Amersham International Co. (Arlington Heights, IL), and [^{35}S]methionine (specific activity, >1000 Ci/mmol) was obtained from ICN (Irvine, CA). Tetra-*n*-propyl ammonium hydroxide was obtained from RSA Corp. (Ardsley, NY). Other chemicals were obtained from Fisher Co. (Pittsburgh, PA).

Cell synchronization and treatment. P388 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum. The cell synchronization was performed as described by Stein *et al.* (18). In brief, cells (5×10^5 cells/ml) were synchronized with a double thymidine block (two 16-hr periods of exposure to 2 mM thymidine separated by a 10-hr period without thymidine). Two hours after release, cells at early S phase were exposed to various concentrations of Dox for 1 hr, washed twice with cold PBS solution, pH 7.4, and then recultured in drug-free medium.

Cell cycle analysis through flow cytometry. After synchronization and incubation in fresh medium at indicated periods of time, aliquots of cells were taken, washed in cold PBS solution, pH 7.4, fixed with 75% ethanol, and stored at 4° until analysis. Fixed cells were treated with 500 units/ml of RNase at 37° for 1 hr, and then the cellular DNA was stained with 50 μg/ml propidium iodide. The cell cycle was determined with a Becton Dickinson fluorescence-activated cell analyzer.

Cytotoxicity assay. Synchronous or asynchronous P388 cells (0.1×10^6 cells/ml) were exposed to various concentrations of Dox at 37° for 1 hr. After being washed three times with cold PBS solution, pH 7.4, cells were resuspended in drug-free medium and plated onto 96-microwell plates. After a 72-hr incubation, cell survival was determined based on the reduction of MTT dye as previously described (19). The drug effects were expressed as the percentage survival of treated cells versus control cells. ID_{50} values were determined graphically.

Assessment of p34^{cdc2} kinase activity. The assay of p34^{cdc2} kinase activity was performed as described by Marshak *et al.* (20). In brief, extracts from synchronized P388 cells (~50 μg of protein) were incubated at 30° in a reaction mixture containing 100 mM Tris·HCl, pH 8.0, 2 mM EDTA, 20 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM P34^{cdc2} kinase substrate peptide, and 10 nM [γ-^{32}P]ATP for 15 min. The reaction was stopped by the addition of ice-cold 10% trichloroacetic acid. After centrifugation at 15,000 × *g* for 5 min, the aliquots of supernatant were spotted onto a phosphocellulose disc (25-mm diameter), washed three times with 100 mM phosphoric acid, and air-dried. The radioactivity on the disc was determined through liquid scintillation counting.

Assessment of PTPase activity. The assay of PTPase activity was carried out as described by Keyse and Emslie (21). In brief, extracts from synchronized P388 cells (~15 μg of protein) were assayed for their ability to hydrolyse *p*-nitrophenol phosphate in the reaction mixture containing 50 mM imidazole, pH 7.5, 0.1% β-mercaptoethanol, and 20 mM *p*-nitrophenol phosphate, at 30° for 30 min. The reaction was stopped by the addition of 0.25 M NaOH, and the absorbance at 410 nm was determined.

Immunocytochemistry. P388 cells were loaded onto cell slides by cytospin, fixed with 4% paraformaldehyde in PBS solution, pH 7.4, for 20 min, and washed three times with 0.1% BSA in PBS solution at room temperature. After permeabilization of cells with 0.1% Triton X-100 and 0.1% BSA in PBS solution for 10 min at room temperature, 10 μl of anti-cdc2 antibody was dropped on the cell slide for 60 min in a moist chamber. After being washed with 0.1% BSA in PBS solution, cells were incubated with fluorescein isothiocyanate-conjugated anti-rabbit IgG in a moist, dark chamber for 30 min. After being washed five times with 0.1% BSA in PBS, cells were examined through a Nikon fluorescence microscope with objective lenses at 100× magnification.

Analysis of Western blot. Extracts from P388 cells were obtained by using lysis buffer containing 50 mM Tris·HCl, pH 7.4, 0.1% Triton X-100, 250 mM NaCl, 15 mM $MgCl_2$, 1 mM dithiothreitol, 20 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 2 mM EDTA, 25 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 20 μg/ml aprotinin. The protein content was determined according to the method of Bradford (22). Cell extracts (50 μg of protein) from both control and treatment cultures were subjected to electrophoresis in a 0.1% SDS-10% acrylamide gel. After electrophoresis, proteins were transferred onto a nitrocellulose membrane; the cdc2, cyclin B_1, or cdc25C protein was detected by using anti-cdc2, anti-cyclin B_1, or anti-cdc25C antibody as primary antibody and then probed with anti-mouse or anti-rabbit IgG conjugated peroxidase as a secondary antibody. The specific bands were detected with an enhanced chemiluminescence method conducted as commercially recommended. The relative density of each band was determined with a laser scanning densitometer (Molecular Dynamics, Sunnyvale, CA)

Protein labeling and immunoprecipitation. P388 cells (2×10^6 cells) were labeled with 125 μCi [^{35}S]methionine in methionine-free medium containing 10% dialyzed fetal bovine serum at 37° for 1 hr. The [^{35}S]methionine labeled cells were lysed with 0.5 ml of lysis buffer in an ice bath for 15 min. After centrifugation at 15,000 × *g* for 15 min, the supernatant was collected, and 5 μg of anti-cdc2 or anti-cyclin B_1 antibody was added. After gentle mixing, 0.1 ml of protein G/Sepharose was added and incubated at 4° overnight. After centrifugation at 15,000 × *g* at 4° for 15 min, the precipitated beads were collected and washed several times with lysis buffer. After the boiling of beads in 50 μl of SDS-polyacrylamide gel electrophoresis buffer, the samples were analyzed on a 0.1% SDS-10% acrylamide gel. The labeled protein bands were detected with autoradiography and exposure to X-AR film (Eastman Kodak, NY) at −70° for 3 days.

Measurement of DNA damage. DNA double-strand breaks caused by Dox were determined by alkaline elution as described by Kohn *et al.* (23). In brief, cells were prelabeled with [^{14}C]thymidine (0.1 μCi/ml) for 24 hr, chased for 3 hr in fresh medium, and then exposed to various concentrations of Dox for 1 hr at 37°. Then, after removal of drugs, cells were loaded onto polycarbonated filters (2-μm pore size), lysed with 5 ml of 2% SDS lysis buffer containing 25 mM disodium EDTA, pH 9.6,, and incubated with 0.5 mg/ml proteinase K. The damaged DNA on the filter was eluted with 25 ml of alkaline elution solution, pH 9.6, containing 0.1% SDS, 20 mM EDTA, and tetra-*n*-propyl ammonium hydroxide at a flow rate of 0.03–0.04 ml/min. The frequency of DNA double-strand breaks was expressed as rad-equivalents.

Results

Effect of Dox on cell cycle progression. Initially, we synchronized murine leukemia P388 cells by using a double thymidine block as described in Materials and Methods. As shown in Fig. 1A, ~80% of the cell population was synchronized in the G1 phase after double thymidine block (two periods of exposure with a 10-hr interval of incubation in normal medium). The peak of S phase occurred at 6 hr after release of block, and the highest population of cells in the G2/M phase was seen at 6–8 hr after release of block. A 1-hr treatment with Dox in the early S phase, ~2 hr after release of block, resulted in a significant blockade of cells at the G2/M phases. For example, at 12 hr after incubation, ~58% cells were at the G2/M Phase (Fig. 1B) versus 14% in the control

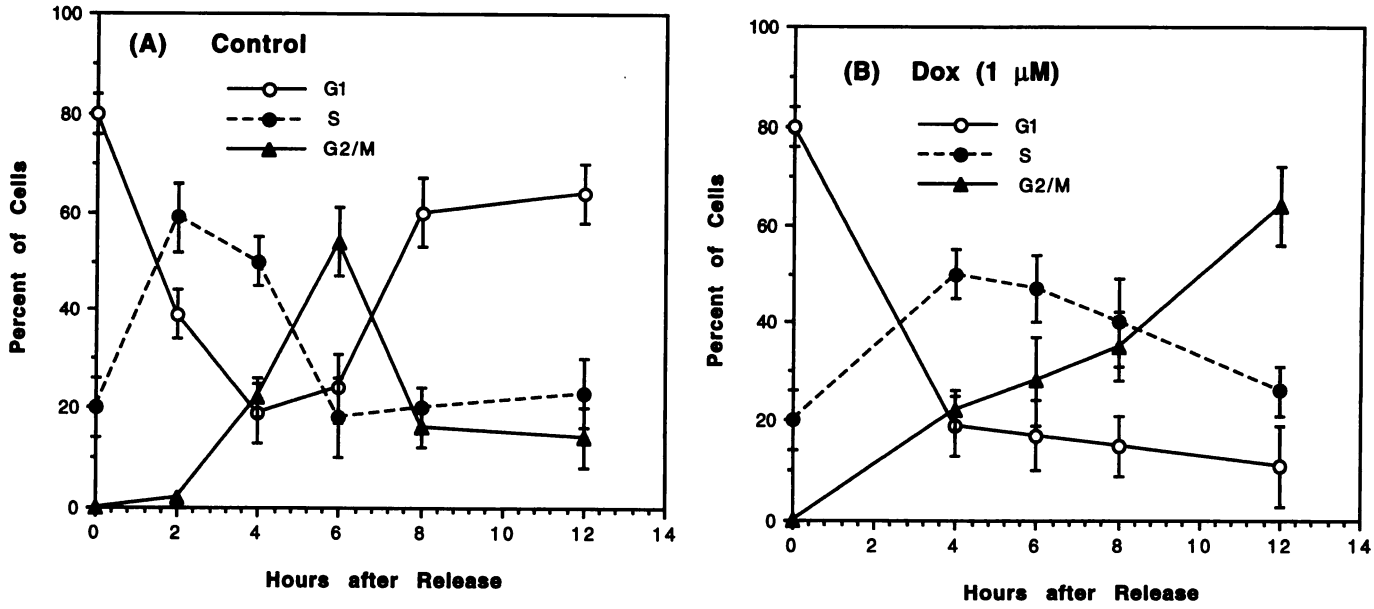


Fig. 1. Flow cytometric analysis of cell cycle progression in synchronized P388 cells treated with Dox. Cells were synchronized with a double thymidine block as described in Materials and Methods. At 2 hr after incubation from block, cells were briefly treated with 1 μM Dox or the same volume of medium as control at 37° for 1 hr. After removal of drug and being washed twice with PBS solution, cells were continuously incubated in fresh medium. At the indicated times, aliquots of culture were sampled for flow cytometric analysis as described in Materials and Methods. *Points*, mean ± standard deviation of three independent experiments.

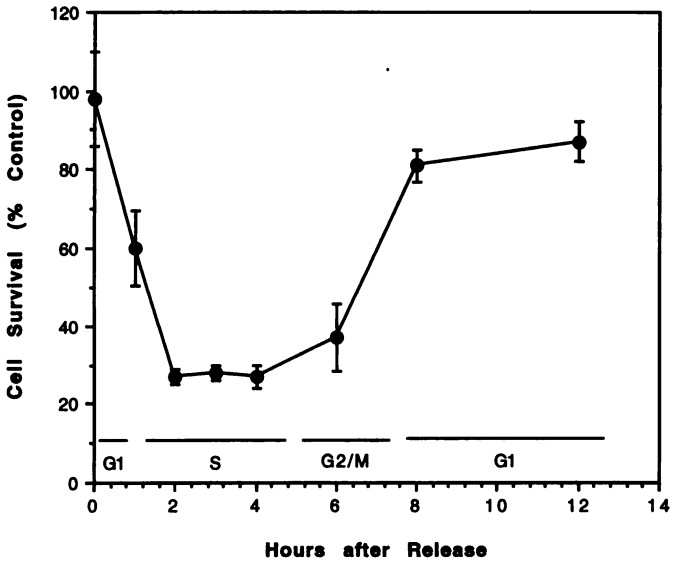


Fig. 2. Cell cycle-dependent cytotoxicity of Dox against synchronized P388 cells. P388 cells were synchronized with a double thymidine block; then at indicated time points after release from block, the aliquots (~0.1 × 10^6 cells/ml) of cultures were taken and treated with 1 μM Dox at 37° for 1 hr. After removal of Dox and usual washing, cells were continuously incubated in drug-free medium for 3 days. The cell survival was determined by MTT assay as described in Materials and Methods. *Points*, mean ± standard deviation of three independent experiments.

cells (Fig. 1A), indicating that Dox treatment resulted in arrest of synchronized P388 cells at G2/M phase. These results are consistent with a report by Traganos *et al.* that Dox and *N*-alkyl analogues of Dox arrested the cell cycle at the G2/M phase in L1210 cells (24).

Cell cycle-dependent cytotoxicity of Dox. To determine whether anthracycline-induced cytotoxicity is cell cycle dependent, we used synchronized P388 cells to determine the effects of Dox on cell growth in the different phases of the cell cycle. P388 cells were synchronized with a double thymidine block as described above, and at the indicated time point after release of block, aliquots of cells were taken from culture and exposed to 1 μM Dox or the same volume of medium as the control at 37° for 1 hr. After removal of drug and being washed three times with cold PBS solution, pH 7.4, cells were refed with fresh medium and continuously incubated at 37° for 3 days. The cell growth was determined with MTT assay as described in Materials and Methods. As shown in Fig. 2, Dox-induced cytotoxicity followed a typical cell cycle-dependent pattern; i.e., cells in the G1 phase were the most resistant to Dox, whereas cells in the S and G2/M phases were markedly sensitive to it. To further confirm these results, we also compared Dox-induced cytotoxicity in S phase of synchronized cells with that in asynchronized cells. As shown in Table 1, Dox had a more potent activity against the cell growth in S phase synchronized cells than in asynchronized cells. The ID_{50} value for Dox against S-phase cells was ~3.28-fold lower than that in asynchronized cells. All of these findings further confirm that Dox-induced cytotoxicity is indeed dependent on the cell cycle.

Inhibition of $p34^{cdc2}$ kinase activity by Dox. Several reports have indicated that $p34^{cdc2}$ kinase plays a central

TABLE 1

Cytotoxicity of Dox in asynchronized and S phase P388 cells

S-phase and asynchronized cells were exposed to various concentrations of Dox at 37° for 1 hr, washed three times with cold PBS, and then recultured in drug-free medium for 3 days.

Cells	ID_{50}	Sensitization index
	μM[b]	
Asynchronized	1.60 ± 0.12	
S phase	0.50 ± 0.16	3.28 ± 0.29

[a] ID_{50} values were determined with the use of MTT assay as described in Materials and Methods. All data are mean ± standard deviation of three independent experiments.

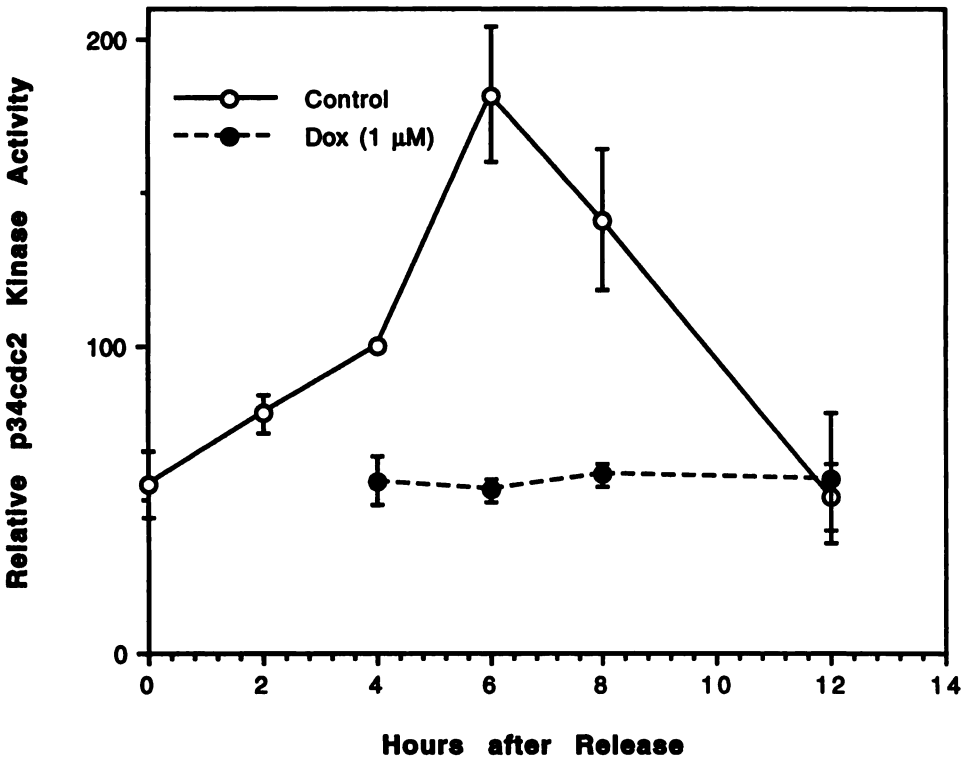


Fig. 3. Effect of Dox on p34^{cdc2} kinase activity in synchronized P388 cells. P388 cells were synchronized with a double thymidine block as described above. Cells at early S phase (2 hr after release incubation) were briefly treated with 1 μM Dox or the same volume of medium as control. After a 1-hr treatment, cells were washed twice with cold PBS, pH 7.4, and incubated in drug-free medium. At the indicated time points, aliquots of cells were sampled, and the p34^{cdc2} kinase activity was determined as described in Materials and Methods. The relative activity of p34^{cdc2} kinase was expressed compared with the control value at 4 hr after incubation. *Points*, mean ± standard deviation of three independent experiments.

role in the regulation of cell cycle transition from G2 to M phase (6–8). Accordingly, we used a specifically synthesized peptide as a substrate to assess the p34^{cdc2} kinase activity in synchronized P388 cells. As shown in Fig. 3, the p34^{cdc2} kinase activity in control cells oscillated with the cell cycle progression; i.e., the relative activity of p34^{cdc2} kinase gradually increased after release of thymidine block; the maximum value of p34^{cdc2} kinase activity was observed at 6 hr after release (which corresponds to the cell cycle in the G2/M phase transition); and the enzyme activity was markedly decreased between 6 and 12 hr after incubation (which corresponds to the cell cycle transition from M phase to G1 phase). In contrast, a 1-hr treatment of early-S-phase cells with 1 μM Dox led to a dramatic inhibition of p34^{cdc2} kinase activity. Between 4 and 12 hr after incubation, the relative activity of p34^{cdc2} kinase was low and did not fluctuate. To

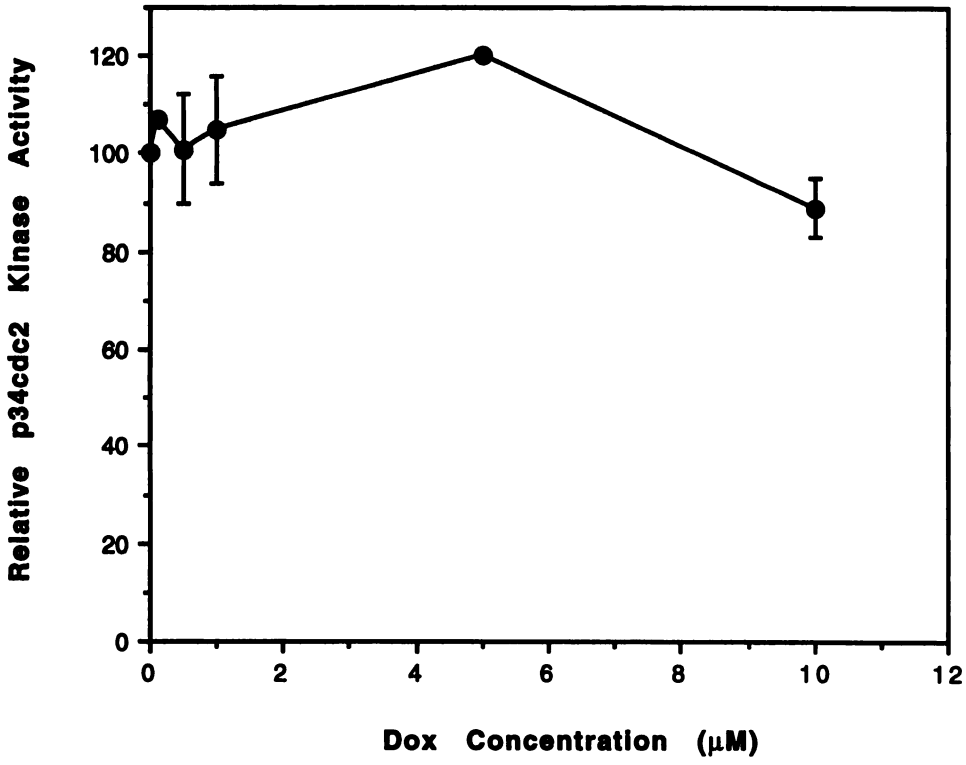


Fig. 4. Effects of Dox on activity of p34^{cdc2} kinase from lysate extracts of P388 cells. Dox at various concentrations was added into the lysate extracts of P388 cells. After a 1-hr incubation at 37°, the p34^{cdc2} kinase activity was determined as described in Materials and Methods. The relative activity of p34^{cdc2} kinase was expressed as percentage of control. *Points*, mean ± standard deviation of three independent experiments.

understand whether Dox could directly inhibit the enzyme itself, Dox at various concentrations was added into lysates prepared from P388 cells. The results revealed that even at a high concentration (10 μM), Dox did not affect the activity of $p34^{cdc2}$ kinase (Fig. 4), indicating that the Dox-induced reduction of $p34^{cdc2}$ kinase activity is not a direct effect on $p34^{cdc2}$ kinase *per se*.

Effect of Dox on $p34^{cdc2}$ subcellular distribution. Using indirect immunocytochemistry, we assessed the subcellular distribution of $p34^{cdc2}$ kinase in synchronized P388 cells. Fig. 5A shows a typical subcellular distribution of $p34^{cdc2}$ kinase in control P388 cells in the interphase. The fluorescence signal of $p34^{cdc2}$ kinase was localized in both nucleus and cytoplasm, and interestingly, a highly intense $p34^{cdc2}$ fluorescence signal was seen in the centrosomes. In the M phase, the fluorescence signal of $p34^{cdc2}$ kinase was observed in the mitotic spindle (data not shown). All of these results are in agreement with the report by Bailly *et al.*, who used immunocytochemical and fractional approaches to detect the subcellular distribution of $p34^{cdc2}$ kinase in HeLa and lymphoblastic cells (KE37) (25). The signal intensity and the subcellular distribution of $p34^{cdc2}$ kinase showed no differences between Dox-treated and control cells (Fig. 5B), indicating that Dox did not affect the level and subcellular distribution of $p34^{cdc2}$ kinase.

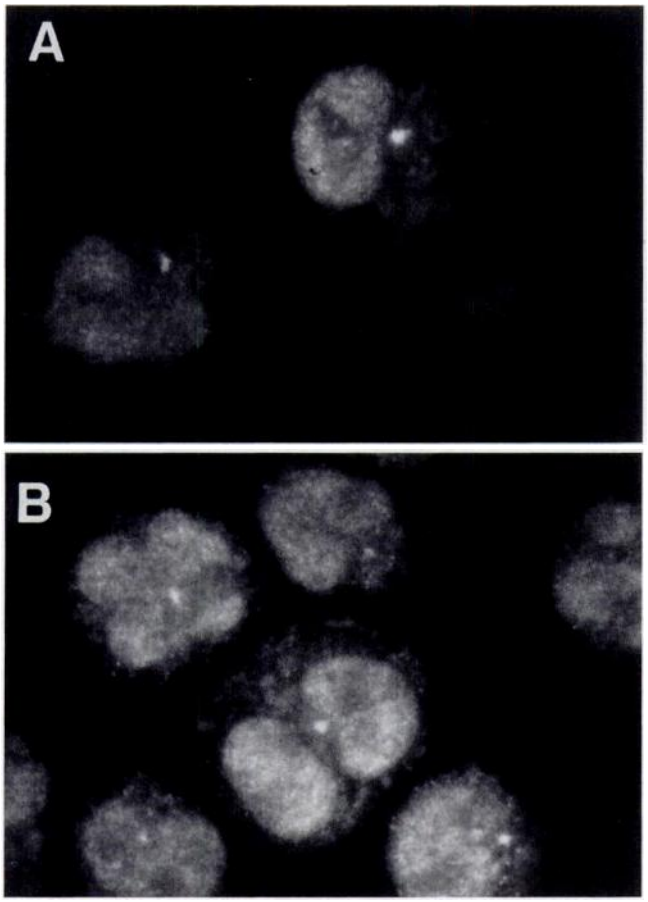


Fig. 5. Immunocytochemical localization of cdc2 in interphase of P388 cells. After treatment with 1 μM Dox (B) or with the same volume of medium as control (A) at 37° for 1 hr, P388 cells were loaded onto a slide by cytospin, fixed with 4% paraformaldehyde in PBS solution, pH 7.4, and then incubated in polyclonal anti-cdc2 antibody at room temperature for 1 hr. After the usual washing, cells were reincubated in fluorescein isothiocyanate-conjugated goat anti-rabbit IgG at room temperature for 1 hr. The cdc2 localization in P388 cells were detected through a Nikon fluorescence microscope (magnification ×100).

Effect of Dox on synthesis and phosphorylation of cdc2 protein. Because the activation of $p34^{cdc2}$ kinase is associated with its synthesis and phosphorylation status (26, 27), we investigated whether the inhibition of $p34^{cdc2}$ kinase activity in Dox-treated cells could be due to reduction in its synthesis or to alteration of its phosphorylation. To determine the rate of cdc2 synthesis, aliquots of synchronized cells were taken from the culture at the indicated time point after release of thymidine block and labeled with 125 μCi [^{35}S]methionine in the methionine-free medium containing dialyzed fetal bovine serum at 37° for 1 hr. After immunoprecipitation with polyclonal anti-cdc2 antibody, the [^{35}S]methionine-labeled proteins were electrophoresed, and the cdc2 band was detected with autoradiography as described in Materials and Methods. As shown in Fig. 6, there is no pronounced differences of cdc2 protein synthesis between control and Dox treatment, indicating that drug-induced reduction of enzyme activity may not be due to inhibition of cdc2 protein synthesis. Next, we determined the effects of drugs on the phosphorylation status of $p34^{cdc2}$ kinase. Using Western blot analysis, we found that the intensity of hypophosphorylated lower band of $p34^{cdc2}$ was increased at 6–8 hr after incubation, whereas that of the hyperphosphorylated upper band

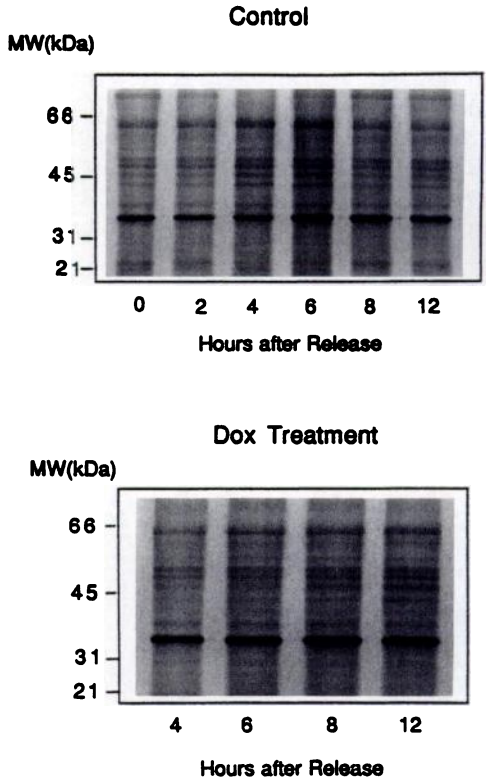


Fig. 6. Effect of Dox on cdc2 protein synthesis in synchronized P388 cells. Synchronized cells at early S phase (2 hr after incubation) were briefly exposed to 1 μM Dox at 37° for 1 hr. After removal of drug and being washed, cells were incubated in drug-free medium. At the indicated time points of postincubation, aliquots of cells (2×10^6) were taken and labeled with 125 μCi [^{35}S]methionine for 1 hr. The cells were lysed with lysis buffer and then immunoprecipitated with polyclonal anti-cdc2 antibody. Then, after 12% SDS-polyacrylamide gel electrophoresis, the cdc2 bands were detected with autoradiography as described in Materials and Methods.

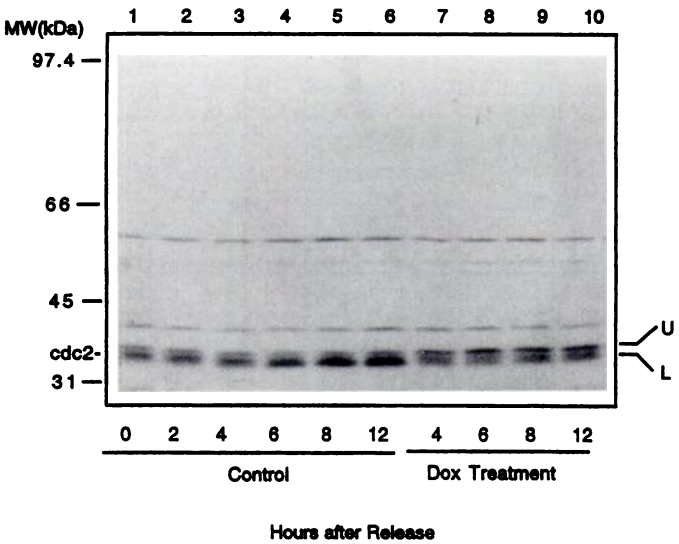


Fig. 7. Effect of Dox on phosphorylation of p34^{cdc2} kinase in synchronized P388 cells. P388 cells were synchronized with a double thymidine block. After release from the block at various time points, aliquots of cells were taken and lysed with lysis buffer. The lysates (50 μg of protein/lane) were then subjected to the 10% SDS-polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred onto a nitrocellulose membrane. The membrane was then reacted with polyclonal anti-cdc2 antibody followed by alkaline phosphatase-conjugated goat anti-rabbit IgG. The bands were detected using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates. *U* (*top bands*), hyperphosphorylated status of p34^{cdc2} kinase; *L* (*bottom bands*), hypophosphorylated status of p34^{cdc2} kinase.

was decreased (Fig. 7, *lanes 1–6*). In the drug-treated cells, the hyperphosphorylated bands remained constant at 4–12 hr after incubation (Fig. 7, *lanes 7–10*), indicating that inhibition of p34^{cdc2} kinase activity in Dox-treated cells is associated with the prevention of dephosphorylation of this enzyme. Because PTPase is involved in the dephosphorylation of tyrosine residues in p34^{cdc2} kinase, we investigated the effect of Dox on this enzyme. Synchronized P388 cells were treated with 1 μM Dox for 1 hr, and then aliquots of cells were sampled, and PTPase activity was determined as described in Materials and Methods. As shown Fig. 8, the PTPase activity in Dox-treated cells is not different from that in control cells, indicating that the prevention of dephosphorylation of p34^{cdc2} kinase caused by Dox may not be related with the inhibition of PTPase. Millar and Russell reported that the dephosphorylation and activation of p34^{cdc2}/cyclin B are controlled by cdc25 protein (28). We therefore determined whether Dox treatment could alter the expression of cdc25C protein in P388 cells. Western blot analysis reveals that the level of cdc25C in synchronized P388 cells displays a constant pattern throughout the cell cycle, and this result is consistent with the report by Millar *et al.* (29). In Dox-treated cells, the cdc25C levels are similar to those in control cells (Fig. 9), indicating that treatment with Dox does not affect the cdc25C protein expression and that drug-induced inhibition of dephosphorylation of cdc2 may not be due to down-regulation of this protein.

Effect of Dox on cyclin B_1 synthesis and degradation. Because the activation of p34^{cdc2} kinase requires interaction with B-type cyclins (30, 31), we further investigated the effect of Dox on cyclin B_1 level and synthesis. Through Western blot analysis of the total cell lysates, the levels of cyclin B_1 in synchronized P388 cells treated with or without Dox were detected with monoclonal anti-cyclin B_1 antibody. As shown in Fig. 10, the level of cyclin B_1 in synchronized P388 cells shows a cell cycle-dependent manner; i.e., cyclin B_1 level at G1 phase is low and then increases as cell cycle progresses from S and G2 phase, with the protein level dramatically declining at exit from M phase and entrance into G1 phase. In contrast, the cyclin B_1 levels in Dox-treated cells remain at higher and more constant levels compared with control cells,

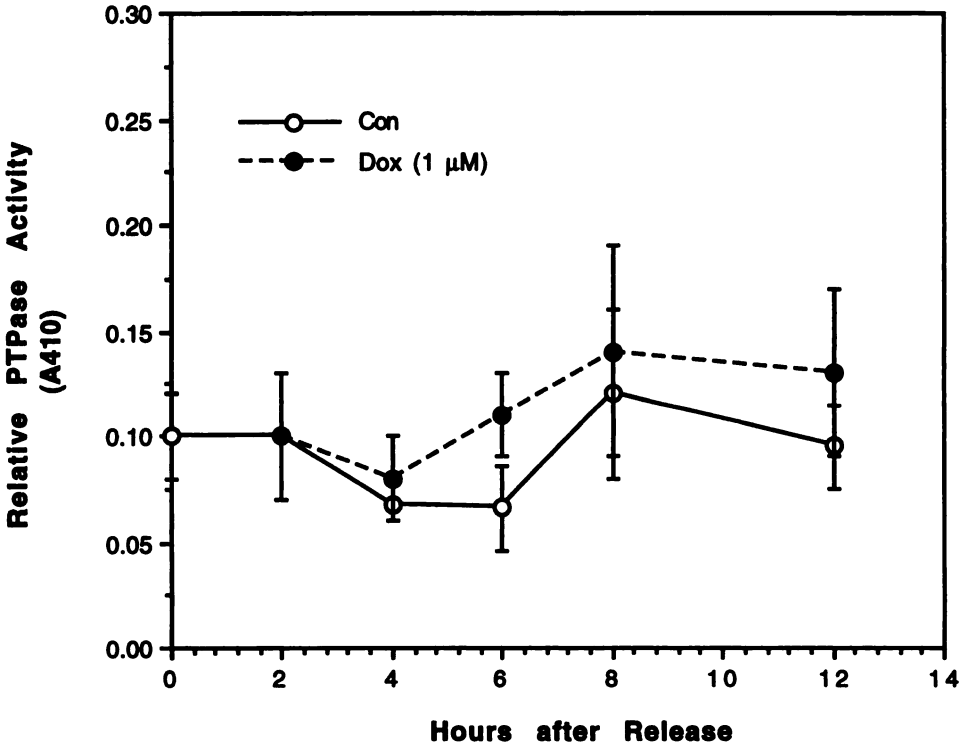


Fig. 8. Effect of Dox on PTPase activity. Synchronized P388 cells at S phase were briefly treated with 1 μM Dox for 1 hr and incubated in drug-free medium. At indicated time points, cell aliquots were taken from the culture, and the extracts (15 μg of protein) were assayed for their ability to hydrolyse *p*-nitrophenol phosphate. PTPase activity was measured at 410 nm absorbance as described in Materials and Methods. *Points*, mean ± standard deviation of three independent experiments.

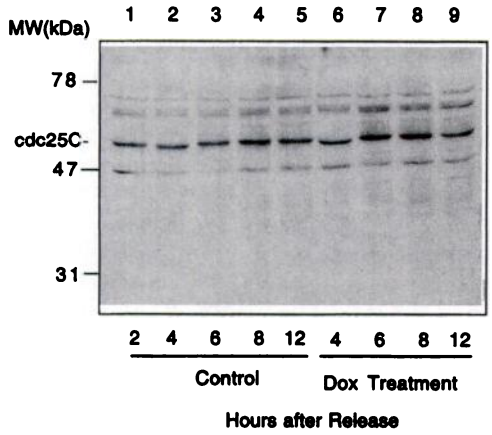


Fig. 9. Effect of Dox on cdc25C levels in synchronized P388 cells. Synchronized P388 cells at S phase were briefly treated with 1 μM Dox for 1 hr and then incubated in drug-free medium. At indicated time points, aliquots of cells were sampled, and the cdc25C levels in each time point were analyzed with Western blotting as described in Materials and Methods.

indicating that Dox treatment disturbs the regulation of cell cycle-dependent cyclin B_1 expression and induces cyclin B_1 accumulation. To investigate whether the accumulation of cyclin B_1 caused by Dox treatment is associated with changes in protein synthesis, synchronized P388 cells at different time point were labeled with [^{35}S]methionine at 37° for 1 hr in methionine-free medium containing dialyzed fetal bovine serum, and the newly synthesized cyclin B_1 was analyzed with immunoprecipitation as described above. As shown in Fig. 11, the rate of cyclin B_1 synthesis displays a cell cycle-dependent manner. In Dox-treated cells, the rate of cyclin B_1 synthesis was higher than that in control cells at 4–6 hr after incubation. Furthermore, we studied the effect of Dox treatment on cyclin B_1 degradation in synchronized P388 cells. Synchronized cells were prelabeled with [^{35}S]methionine at S phase (3 hr after release of block) in methionine-free medium containing dialyzed fetal bovine serum at 37° for 1 hr and then chased in fresh medium in the absence or presence of 1 μM Dox. At the indicated time point, aliquots of cells were sampled, and the labeled cyclin B_1 was immunoprecipitated with monoclonal cyclin B_1 antibody. The amounts of cyclin B_1 were monitored with autoradiography and measured with a laser scanning densitometer as described in Materials and Methods. As shown in Fig. 12, Dox treatment was found to markedly prevent the cyclin B_1 degradation compared with control. These results suggest that Dox-induced disturbance of regulation of cyclin B_1 levels may be, at least in part, associated with both an increase in cyclin B_1 synthesis and prevention of its degradation.

Correlation of DNA damage with reduction of p34^{cdc2} kinase activity. A number of investigations have demonstrated that DNA-damaging agents such as DNA topoisomerase I and II inhibitors, alkylating agents, or irradiation induce the inhibition of p34^{cdc2} kinase activity. We have shown that Dox treatment results in arrest at the G2/M phase and inhibition of p34^{cdc2} kinase. We subsequently attempted to investigate whether there is a relationship between DNA double-strand breakage and inhibition of p34^{cdc2} kinase activity caused by Dox. Cells were exposed to various concentrations of Dox for 1 hr, and then aliquots of the cells were sampled to determine the number of DNA double-strand breaks by alkaline elution; the remainder of each cell culture was taken for assay of p34^{cdc2} kinase activity as described in Materials and Methods. As shown in Fig. 13, the extent of reduction in p34^{cdc2} activity was plotted on a linear scale against the frequency of DNA double-strand breaks expressed as rad-equivalents on the log scale, revealing a correlation between the inhibition of p34^{cdc2} kinase activity and the increase in the frequency of DNA damage induced by Dox.

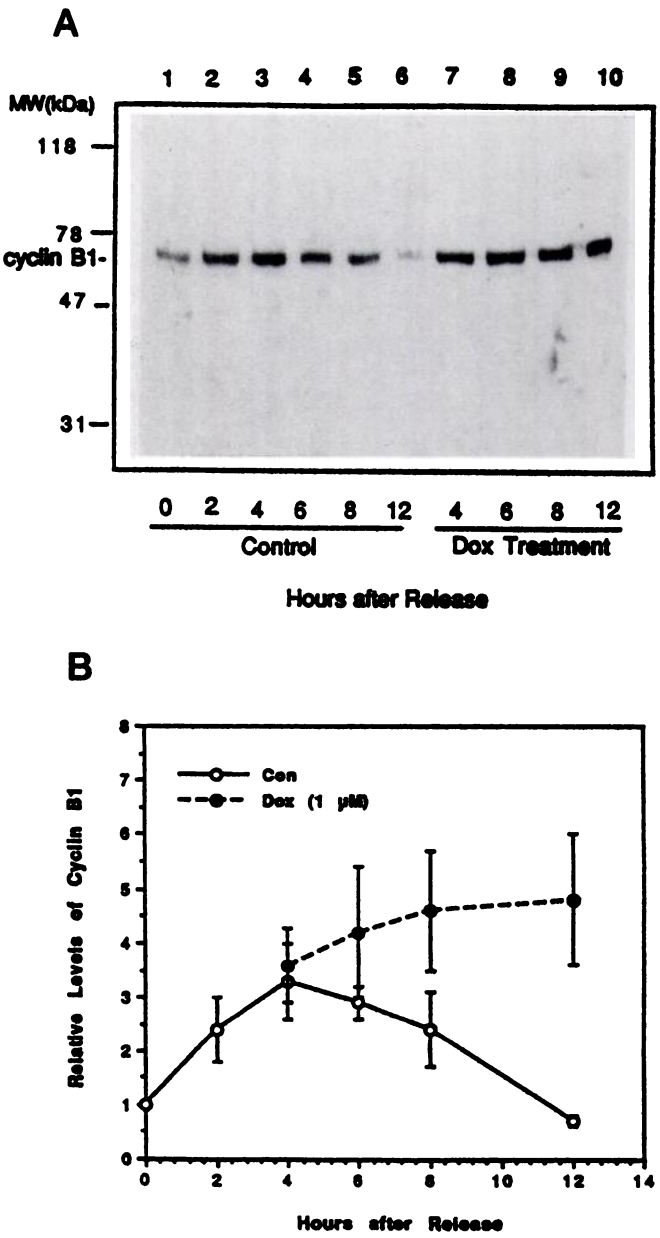
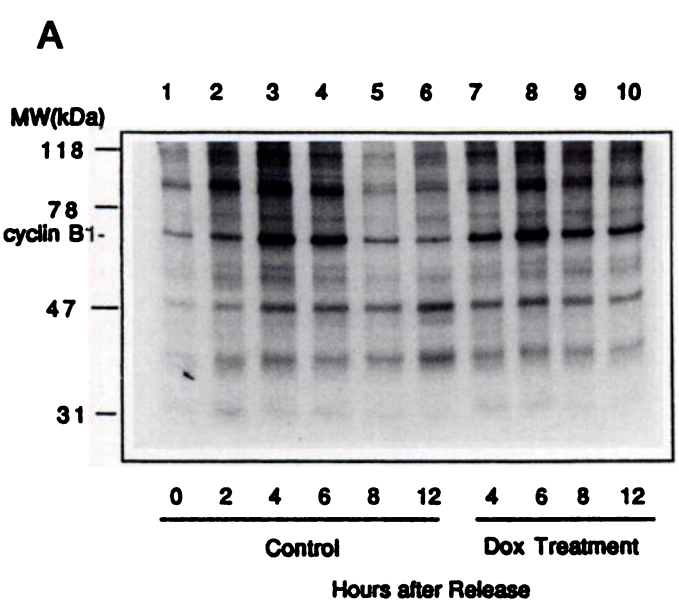


Fig. 10. Effect of Dox on cyclin B_1 level in synchronized P388 cells. Synchronized cells at early S phase were briefly treated with 1 μM Dox for 1 hr and then incubated in drug-free medium. At indicated time points, aliquots of cells were sampled, cyclin B_1 levels at each time point were analyzed with Western blotting (A), and relative amounts of cyclin B_1 were quantified with a laser scanning densitometer (B) as described in Materials and Methods.

Discussion

We briefly treated synchronized P388 cells at early S phase with 1 μM Dox for 1 hr and found that Dox induced cell arrest at G2/M phase. In addition, we showed that Dox-induced



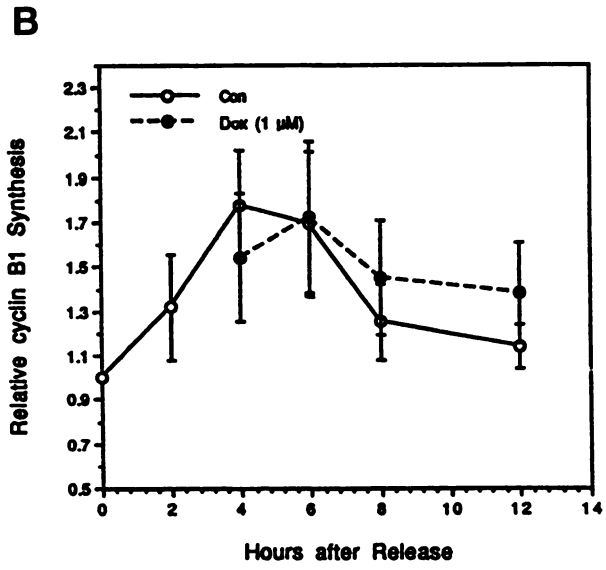
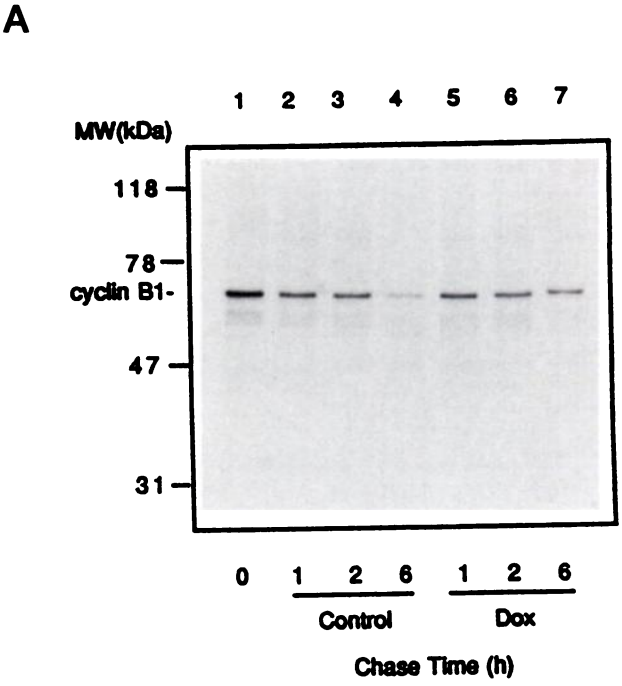


Fig. 11. Effect of Dox on cyclin B_1 synthesis in synchronized P388 cells. Synchronized cells at early S phase were briefly treated with 1 μM Dox for 1 hr. After removal of drugs, cells were recultured in drug-free medium, aliquots of culture at indicated time points were sampled, the cells were labeled with [^{35}S]methionine at 37° for 1 hr, the labeled cells were lysed, and cyclin B_1 protein was immunoprecipitated as described in Materials and Methods. A, Autoradiography of cyclin B_1 in control and drug treatment at the indicated time points. B, The relative intensity of cyclin B_1 measured with a laser scanning densitometer. *Points*, mean ± standard deviation of three independent experiments.



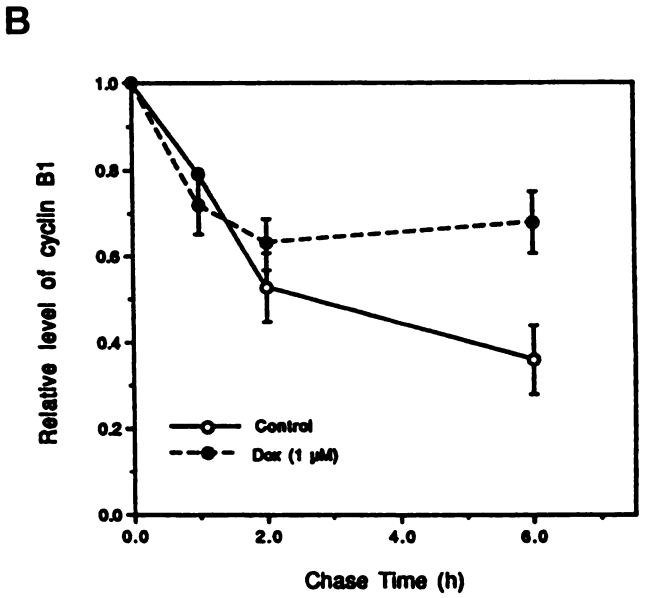


Fig. 12. Effect of Dox on cyclin B_1 degradation in synchronized P388 cells. Synchronized cells at S phase (3 hr after release of block) were prelabeled with [^{35}S]methionine in methionine-free medium containing 10% dialyzed fetal bovine serum at 37° for 1 hr and then chased in fresh medium in the absence or presence of 1 μM Dox. At indicated time points, aliquots of cells were sampled, and labeled cyclin B_1 was immunoprecipitated with monoclonal anti-cyclin B_1 antibody. The labeled cyclin B_1 bands were detected with autoradiography (A), and the relative intensity of cyclin B_1 was measured by a laser scanning densitometer (B) as described in Materials and Methods. *Points*, mean ± standard deviation of three independent experiments.

cytotoxicity was cell cycle dependent; i.e., cells at G1 phase were more resistant to Dox than were those at S and G2/M phase. It is well known that cells that express a mutant p53 protein, like P388 cells, have a deficient cell cycle "checkpoint" control at G1/S. Therefore, the studies did not address the potential effect of doxorubicin on G1/S checkpoint control. This effect is currently studied in MCF-7 breast carcinoma cells, which express wild-type p53.

The concept of checkpoints at G1 and G2 that control cell cycle progression was introduced by Hartwell and Weinert (32). Kohn *et al.* proposed that the G2 phase arrest caused by DNA-damaging agents may be a mechanism of negative feedback control by which cells are arrested at G2/M phase and gene products that facilitate the repair of DNA lesions are induced. Therefore, the arrest at G2/M phase is thought to ensure that DNA replication will proceed with fidelity to avoid segregation of defective chromosomes (33). Some inves-

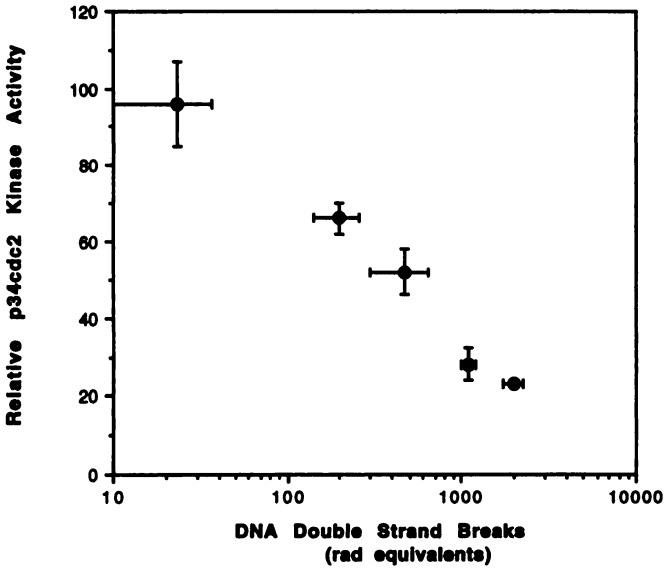


Fig. 13. Correlation of inhibition of $p34^{cdc2}$ kinase activity with DNA damage caused by Dox in P388 cells. After a 1-hr treatment with various concentrations of Dox, aliquots of cells were taken from culture and lysed with lysis buffer for assay of $p34^{cdc2}$ kinase activity. The remaining aliquots were used to determine DNA double-strand breaks through alkaline elution as described in Materials and Methods. *Points*, mean ± standard deviation of three independent experiments.

tigators have demonstrated that cells deficient in DNA repair are hypersensitive to DNA-damaging agents (34). In yeast, the gene *RAD9* has been confirmed to control the cell cycle response to DNA damage, and a mutation in this gene has been shown to result in lack of G2 arrest despite DNA damage (35). In this work, we provide evidence that Dox treatment results in cell cycle arrest at G2/M phase and decreases $p34^{cdc2}$ kinase activity in synchronized P388 cells. In addition, we investigated the relationship between DNA damage and the inhibition of $p34^{cdc2}$ kinase activity induced by various concentrations of Dox. The results support the hypothesis that Dox-induced DNA damage correlates with its induced suppression of $p34^{cdc2}$ kinase. Furthermore, we determined that in P388 cells at concentrations that induced DNA strand breaks, different kinds of DNA-damaging agents, such as camptothecin, etoposide, actinomycin D, and cisplatin, also led to marked inhibition of $p34^{cdc2}$ kinase activity. In contrast, we noticed that the microtubuline-disturbing agents vinblastine (a microtubuline polymerization inhibitor) or paclitaxel (a polymerization promoter) were able to arrest the cell cycle at the M phase and to stimulate $p34^{cdc2}$ kinase activity (data not shown).

Because the activity of $p34^{cdc2}$ kinase depends on the synthesis and phosphorylation status of the enzyme, we determined the effect of Dox on the $p34^{cdc2}$ protein synthesis and phosphorylation in synchronized P388 cells. We found that Dox treatment did not alter the synthesis of enzyme but rather prevented the dephosphorylation of $p34^{cdc2}$ kinase. Some reports have demonstrated that the dephosphorylation of tyrosine at residue 15 of $p34^{cdc2}$ is required for the activation of kinase activity and for its function at the G2/M checkpoint during the cell cycle progression (36). Because the major protein phosphatase that dephosphorylates the cdc2 inhibitory sites is cdc25 (37, 38), we determined whether Dox-induced hyperphosphorylation of $p34^{cdc2}$ kinase was the consequence of a down-regulation of cdc25C protein. The Western blotting analysis did not reveal changes in cdc25C protein expression (Fig. 9). In addition, we tested whether Dox-induced hyperphosphorylation of $p34^{cdc2}$ kinase could be linked to a reduction in PTPase activity. The results showed that the PTPase activity in the cells treated with 1 μM Dox for 1 hr was not decreased (Fig. 8). These results suggest that neither the reduction of cdc25 gene product nor the inhibition of PTPase was implicated in the drug-induced prevention of dephosphorylation of $p34^{cdc2}$. There is evidence indicating that Cdk activating kinases can phosphorylate cdc2 if it is associated with a cyclin partner (39, 40). In addition, Lundgren *et al.* reported that cdc2 phosphorylation and dephosphorylation is suggested to be mediated by wik1 and mee1, which cooperate in the inhibitory tyrosine phosphorylation of cdc2 (41). Therefore, it is necessary to further define whether Dox-induced prevention of dephosphorylation of $p34^{cdc2}$ kinase may be the result of interaction with these gene products.

It is well known that newly translated cyclin B_1 accumulates during S and G2 phases and is then abruptly degraded at the metaphase/anaphase transition. Cyclin B_1 degradation and dephosphorylation of the tyrosine on cdc2 are suggested to be necessary for activation of maturation promoting factor, which plays an important role in the initiation of mitosis, and such a mechanism seems to be linked to the G2/M transition checkpoint by which the completion of DNA synthesis is checked and the mitosis is triggered (42). In this work, we demonstrated that Dox treatment caused accumulation of cyclin B_1, by increasing its synthesis and preventing its degradation in synchronized P388 cells. Thus, our results suggest that DNA damage and cell cycle arrest at G2/M phase correlate with drug-induced disturbance of cell cycle-dependent regulation of cyclin B_1.

Previously, we demonstrated that Dox-induced DNA fragmentation and programmed cell death occurred after 12 hr of drug treatment in P388 cells (43). Here, we showed that a 1-hr treatment with Dox led to DNA strand breakage and striking suppression of $p34^{cdc2}$ kinase, indicating that these events occurred before apoptosis and suggesting that the continuously low activity of $p34^{cdc2}$ kinase, accumulation of cyclin B_1, and/or DNA damage may trigger the mechanisms of apoptosis.

In conclusion, the data presented in this article are consistent with the facts that DNA-damaging agents induce G2/M phase arrest, inhibition of $p34^{cdc2}$ kinase activity, and alteration in function of $p34^{cdc2}$/cyclin B_1 complex.

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