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G₁ Phase-Dependent Expression of Bcl-2 mRNA and Protein Correlates with Chemoresistance of Human Cancer Cells

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This paper is available online at http://www.molpharm.org

ABSTRACT

Recent experiments suggest an interconnection between cell proliferation and programmed cell death (apoptosis), although the detailed molecular mechanisms remain unclear. We have hypothesized that expression of some apoptosis regulators is cell cycle-dependent, which in turn influences tumor cell chemosensitivity in a cell cycle-dependent fashion. To test these hypotheses, we synchronized human leukemia Jurkat T, Neo (using aphidicolin), breast cancer MCF-7, normal fibroblast, and simian virus 40-transformed cells (by aphidicolin or serum starvation), and measured levels of several Bcl-2 family proteins. The highest expression of Bcl-2 protein was found in the G₁ phase of all the five cell lines tested. In contrast, levels of Bax protein remained relatively unchanged in four of the cell lines, and levels of Bcl-X$_L$, Bcl-X$_S$, and Bak proteins showed little or no cell cycle-dependent changes in Jurkat T cells. Similar to the changes in Bcl-2 protein levels, its mRNA expression was also G₁ phase-specific, whereas the level of a Bcl-2 cleavage activity remained constitutive. When treated with an anticancer drug (etoposide or cisplatin) or the kinase inhibitor staurosporin, the cells containing a high G₁ population and a high Bcl-2 protein level were much more resistant to the induced apoptosis than the cells containing a high S phase population and a low Bcl-2 protein level. Constitutive overexpression of Bcl-2 protein in Jurkat T cells completely blocked the S phase-associated sensitivity to these apoptosis stimuli. The cell cycle-dependent Bcl-2 protein expression seems to contribute to the regulation of chemosensitivity and apoptotic commitment of human tumor cells.

Cell proliferation is tightly controlled in normal mammalian cells, but deranged in cancer cells (Pardee et al., 1978). The growth factor-mediated signals that drive the cell cycle progression and thereby cell proliferation have been linked to functions of several cell cycle-dependent regulators (Sherr and Roberts, 1999). Programmed cell death (apoptosis) is the process by which a cell will actively commit suicide under tightly controlled circumstances (Wyllie et al., 1980). Apoptosis occurs in two physiological stages, commitment and execution (Earnshaw, 1995; Martin and Green, 1995). It has been proposed that Bcl-2 family proteins are involved in the apoptotic commitment in mammalian cells (Green and Reed, 1998). Most recent experiments have demonstrated that several Bcl-2 family proteins are located in the outer mitochondrial membrane, where they control release of some caspase-activating proteins (such as cytochrome *c*) into the cytosol. Release of cytochrome *c* can be induced by proapoptotic Bcl-2 family proteins (such as Bax), but inhibited by antiapoptotic

Bcl-2 family proteins (such as Bcl-2). The radio of proapoptotic Bcl-2 family members (such as Bax) to antiapoptotic members (such as Bcl-2), therefore, determines whether a cell is committed to apoptotic death or not (Green and Reed, 1998). Apoptotic execution in mammalian cells is initiated by activation of specific caspase proteases (Earnshaw, 1995; Martin and Green, 1995), which cleave important cellular target proteins, including poly(ADP-ribose) polymerase (PARP) (Lazebnik et al., 1994) and retinoblastoma protein (An and Dou, 1996), resulting in disassembly of the cell.

Homeostasis of cell numbers is achieved by balancing cell proliferation and cell death, suggesting an accurate coordination between these two processes. Indeed, recent experiments have shown that signal transduction pathways controlling cell proliferation and cell cycle progression are also involved in mediating apoptosis and that dysregulation of cell cycle progression is an important event for the initiation of apoptosis (Lee et al., 1993; Dou et al., 1995; Linette et al., 1996; Dou, 1997). Furthermore, expression of a growth-promoting oncogene Ras or Myb in cells induces expression of the death suppressor Bcl-2 expression (Kinoshita et al., 1995; Grassilli et al., 1999). Recent work has also suggested involvement of apoptosis regulators in cell cycle progression.

ABBREVIATIONS: PARP, poly(ADP-ribose) polymerase; VP-16, etoposide; SV40, simian virus 40; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; p21, the cyclin-dependent kinase inhibitor Cip1 or Waf-1.

For example, overexpression of the apoptosis inhibitor Bcl-2 delayed entry of activated T cells into S phase (O'Reilly et al., 1996) while overexpression of the apoptosis inducer Bax increased the G_1 to S transition (Brady et al., 1996). In addition, overexpression of Bcl-2 in other cell systems either inhibited the transition of G_0 to S or accelerated cycling cells exit into quiescent stage (Vairo et al., 1996). However, how endogenous Bcl-2 protein and mRNA are regulated during the cell cycle and how Bcl-2 protein regulates chemoresistance of tumor cells remain largely unknown.

In the current study, we measured levels of several Bcl-2 family proteins during the cell cycle progression using synchronized cells. We found that in all the tested five cell lines, expression of Bcl-2 protein peaked in the G_1 phase, whereas levels of Bax protein remained relatively unchanged in most of the cell lines. There were also little changes in levels of Bcl-X_L, Bcl-X_S, and Bak proteins during the cell cycle in Jurkat T cells. In addition, expression of Bcl-2 mRNA was also G_1 phase-specific, whereas the level of Bcl-2 degradation activity was constitutive. Furthermore, S phase cells expressing low levels of Bcl-2 protein were much more sensitive to apoptosis induction than G_1 cells expressing high levels of Bcl-2 protein, and Bcl-2 overexpression completely blocked the S phase-specific sensitivity to induced apoptotic cell death.

Experimental Procedures

Materials. RPMI 1640, penicillin, and streptomycin were purchased from Life Technologies, Inc. (Rockville, MD). Fetal calf serum, aphidicolin, etoposide (VP-16), cisplatin, staurosporin, propidium iodide, RNase A, glucose, and salicylic acid were from Sigma Chemical Co. (St. Louis, MO). Monoclonal anti-human Bcl-2 antibody was obtained from Dako Co. (Glostrup, Denmark); polyclonal antibodies to human Bax and actin were from Santa Cruz Biotechnology (Santa Cruz, CA); polyclonal antibodies to human Bcl-X_L and Bcl-X_S, and monoclonal antibodies to human Bak and Caspase-3 were from Oncogene Research Products (Cambridge, MA); monoclonal antibodies to human p53 and p21^{Cip1} were from Pharmingen (San Diego, CA); polyclonal antibody to human PARP was from Boehringer Manheim (Indianapolis, IN). Anti-mouse IgG-horseradish peroxidase and anti-rabbit IgG-horseradish peroxidase were purchased from Santa Cruz Biotechnology. L-[^{35}S]Methionine was from Amersham (Piscataway, NJ) and [^{32}P]dCTP was from Boehringer Mannheim.

Cell Culture, Synchronization, and Treatment. Jurkat T cells transfected with bcl-2 cDNA (Bcl-2) or pcDNA3.0 alone (Neo) were gifts from Dr. Hong-gong Wang (Moffitt Cancer Center and Research Institute, Tampa, FL). MCF-7 cells, human Jurkat T cells, and Jurkat T cells overexpressing the human Bcl-2 oncoprotein or the vector alone were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. Normal (WI-38) and simian virus 40 (SV40)-transformed (VA-13) human fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. All cells were maintained in a 5% CO_2 atmosphere at 37°C.

Cells were synchronized in the G_0/G_1 phase of the cell cycle by serum starvation. Briefly, exponentially grown cells were cultured in the serum-free medium for 72 (for MCF-7 cells) or 96 h (for WI-38 and VA-13 cells), followed by restimulating the cells to proliferate by addition of the serum. Cells were synchronized in the G_1/S boundary by aphidicolin. Cells were incubated for 24 h with aphidicolin at a final concentration of 3 (for Jurkat T cells, Neo, and Bcl-2 cells) or 5 μg/ml (for MCF-7, WI-38, and VA-13 cells), followed by wash with

PBS and reculture in the growth medium. At each time point, one-third of the cells were harvested for Western blotting assay, one-third for cell cycle analysis, and one-third were treated for 3 or 24 h with either etoposide (50 μM/ml), cisplatin (20 μM/ml), or staurosporin (1 μM/ml). After each treatment, total cell populations (or a mixture of detached and attached cells) were collected, and used for assaying apoptotic cell death (see below).

Cell Cycle Analysis. Cell cycle analysis based on DNA content was performed as follows. Cells were harvested, counted, and washed twice with PBS. Cells (5×10^6) were then suspended in 0.5 ml of PBS, pipetted, and fixed in 5 ml of 70% ethanol for at least 2 h at −20°C. Cells were centrifuged, resuspended in 1 ml of propidium iodide staining solution (50 μg propidium iodide, 1 mg RNase A, and 1 mg of glucose per ml of PBS) and incubated at room temperature for 30 min. The cells were then analyzed with FACScan (Becton Dickinson Immunocytometry, Mountain View, CA) and ModFit LT cell cycle analysis software (Verity Software, Topsham, ME). The cell cycle distribution is shown as the percentage of cells containing G_1, S, G_2, and M DNA judged by propidium iodide staining. The apoptotic population (Ap) is the percentage of cells with $<G_1$ DNA content.

Whole-Cell Extract and Western Blot Assay. To prepare a whole cell extract, cells were lysed in a protein lysis buffer (50 mM Tris·HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, and 0.5% Nonidet P-40) containing a freshly added cocktail of protease inhibitors (Erickson et al., 1998). The lysates were centrifuged at 20,000g for 30 min and the supernatant was collected. Equal amounts of protein (30–60 μg) were resolved by SDS-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) using a SemiDry Transfer System (Bio-Rad, Hercules, CA). The membrane was blocked with 5% nonfat dry milk in PBS-Tween (v/v, 0.2%) for 1 h at room temperature and then incubated overnight at 4°C with the specific antibody to Bcl-2 (1: 500), Bax (1:300), Bcl-X_L (1:100), Bcl-X_S (1:100), Bak (1:100), Caspase-3 (1:500), actin (1:500), p53 (1:500), p21 (1:500), or PARP (1:3000). The membrane was washed, blotted with secondary antibody conjugated with horseradish peroxidase (1:2000) at room temperature for 1 h, and then washed again. The protein bands were visualized with the enhanced chemiluminescence system (Amersham) according to the manufacturer's instructions.

Human bcl-2 cDNA Cloning and Bcl-2 Protein Degradation Assay. Human, full-length, bcl-2α cDNA was amplified by reverse transcription-polymerase chain reaction using total RNA derived from Jurkat T cells. The primer pairs used for reverse transcription-polymerase chain reaction are: 5′-TACTCGAGAAGGATGGCG-CACGCTGGGA-3′ (forward) and 5′-GCAAGCTTCTTCACTTGTG-GCTCAGA-3′ (reverse). The obtained bcl-2 cDNA was confirmed by sequencing and cloned into pcDNA3.1(−) (Invitrogen, Carlsbad, CA). ^{35}S-labeled Bcl-2 protein was prepared with pcDNA3.1(−)-bcl-2 as a template by coupled in vitro transcription/translation using TNT-coupled reticulocyte lysate systems (Promega, Madison, WI) according to the manufacturer's instructions. To prepare a protein extract for Bcl-2 degradation assay, cells were lysed by Dounce homogenization in a buffer containing 20 mM HEPES, pH 7.4, 1.5 mM $MgCl_2$, 5 mM KCl, and 1 mM dithiothreitol. The lysates were centrifuged at 20,000g for 30 min and the supernatants were collected. For the Bcl-2 degradation assay, ^{35}S-labeled Bcl-2 (1 μl) was incubated with 70 μg of the above cell extracts in an assay buffer (10 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 1 mM dithiothreitol, 0.1 mg/ml creatine kinase, 100 mM creatine phosphate, and 5 mM ATP) for 4 h at 37°C. The reactions were stopped by the addition of the same volume of 2× SDS sample buffer. After electrophoresis, the gel was treated with 1 M sodium salicylate and dried, followed by autoradiography.

Northern Blot Assay. Total RNA was extracted from 5×10^6 synchronized cells using TRIzol (Life Technologies) according to the manufacturer's instructions. RNA was electrophoresed (30 μg/lane) in a 1.2% agarose gel, blotted onto a Zeta-Probe nylon membrane (Bio-Rad), and hybridized to ^{32}P-labeled bcl-2 cDNA according to

standard procedures (Hu et al., 1996). Bcl-2 mRNA was detected by autoradiography. After stripping residual radioactivity, membranes were hybridized with a glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA probe. G3PDH mRNA was detected by autoradiography and used for normalization of RNA loading and hybridization efficiency. The bcl-2 and G3PDH cDNA probes were radiolabeled with [^{32}P]dCTP using random primer DNA labeling kit (Boehringer Mannheim).

DNA Fragmentation Assay. After drug treatment, cells were harvested, washed twice with ice-cold PBS, resuspended in a DNA lysis buffer (10 mM Tris·HCl, pH 7.4, 10 mM NaCl, 10 mM EDTA, 1% SDS, and 0.5 mg proteinase K), and incubated for 24 h at 37°C. RNA was digested by adding 0.2 mg/ml of DNase-free RNase and incubating at 37°C for 1 h. DNA was precipitated by isopropanol, washed once with 75% ethanol, and dissolved in Tris-EDTA buffer (10 mM Tris·HCl, pH 7.4, 1 mM EDTA). Fifteen micrograms of DNA from each sample was subjected to electrophoresis on 1.2% agarose containing 0.5 μg/ml of ethidium bromide and visualized under UV light.

Results

G₁ Phase-Dependent Expression of Bcl-2 Protein in Human Tumor, Transformed, and Normal Cell Lines. We investigated levels of Bcl-2 family proteins during the cell cycle progression. Human Jurkat T cells were synchronized at the G₁/S boundary by using aphidicolin, an inhibitor of DNA polymerase α (Huberman, 1981), followed by removal of the drug and further incubation of the cells in fresh growth medium. At each time point, cells were harvested and used for measurement of the cell cycle distribution (by flow cytometry) and Bcl-2-like protein expression (by Western blot assay) (Fig. 1).

Treatment with aphidicolin for 24 h accumulated 69% of the Jurkat cells in G₁ and 30% in early S phase (Fig. 1A, 0 h). Immunoblot with a combination of Bcl-2 and Bax antibodies revealed a high level of Bcl-2 and a low level of Bax protein in these cells (Fig. 1B, lane 1). At 6 h after release from the aphidicolin block, the G₁ cell population was decreased by 37%, associated with 25% increase in S population and 12% increase in G₂/M population (Fig. 1A). At this time, the Bcl-2 protein level was significantly decreased but Bax level remained unchanged (Fig. 1, B and C, lane 2 versus lane 1), resulting in a decrease in the Bcl-2/Bax ratio. At 10 h after removal of aphidicolin, both G₁ and S populations were decreased, associated with a marked increase in G₂/M cell population (Fig. 1A). This was accompanied by a further decrease in the level of Bcl-2 protein (Fig. 1B, lane 3), indicating that Bcl-2 expression is not G₂/M phase-dependent. At this time, the level of Bcl-2 became even lower than that of Bax that remained unchanged (Fig. 1, B and C, lane 3 versus lane 2). At 18 h, 70% of these cells had reentered into G₁ and 27% even into early S phase (Fig. 1, A), accompanied by reappearance of highly expressed Bcl-2 protein and a slight decrease in Bax expression (Fig. 1, B and C, lane 4 versus lane 3).

In contrast to the dramatic changes in expression of Bcl-2 during the cell cycle progression, levels of the antiapoptotic Bcl-X$_L$, the proapoptotic Bcl-X$_S$, and the proapoptotic Bak proteins changed only slightly during this process (Fig. 1, D–F). Therefore, Bcl-2 is the only member of the family we examined that demonstrated a significant cell cycle-dependent protein expression in Jurkat T cells. When Jurkat cells transfected with an empty vector (for the following Bcl-2

study) were treated by aphidicolin, followed by release for up to 24 h, we again observed a G₁ phase-associated Bcl-2 expression and an unchanged Bax expression (Fig. 2, A, D, and E, lanes 1–6).

To further study the G₁ phase-dependent expression of Bcl-2 protein, we synchronized several other human cell lines by either aphidicolin treatment or serum starvation. When human normal fibroblasts (WI-38) were treated with aphidicolin for 24 h, about 80% of the cells were accumulated in G₁ phase, which contained a very high level of Bcl-2 protein (Fig. 3A, lane 1). After the aphidicolin-pretreated WI-38 cells were incubated in fresh growth medium for up to 14 h, the G₁ population was dramatically decreased (by 32–48%), associated with a decrease of Bcl-2 expression to an undetectable level (Fig. 3A, lanes 2–4). After released for 18 to 24 h, the G₁ population of the cells was increased by 20 to 25%, accompanied by a significant increase in the levels of Bcl-2 protein (Fig. 3A, lanes 5 and 6). In contrast, very little change was observed in Bax protein levels in the treated WI-38 cells (Fig. 3A). When SV40-transformed WI-38 (VA-13; Fig. 3B) or breast carcinoma MCF-7 cells (Fig. 3C) were synchronized with aphidicolin and then released, the G₁ phase-dependent Bcl-2 protein expression was again observed.
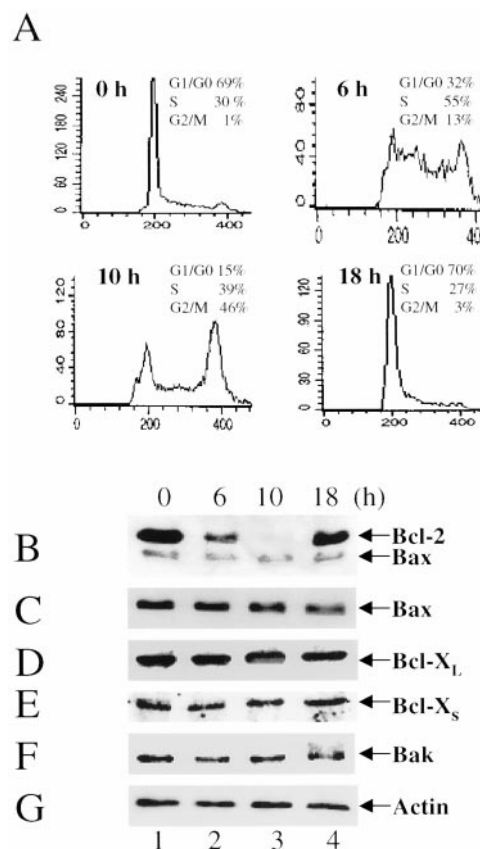
Fig. 1. Expression of Bcl-2 family members in human Jurkat T cells during the cell-cycle progression. Human Jurkat T cells were treated with aphidicolin at 3 μg/ml for 24 h (0 h), followed by release for up to 18 h (see *Experimental Procedures*). At each time point, one-third of the cells were harvested for flow cytometry analysis of the cell cycle (A), one-third were for whole cell extraction and Western blot assay (B–G), and one-third were incubated with VP-16, followed by measurement of apoptosis (see Fig. 7). B, the filter was first incubated with a specific antibody to Bcl-2, rinsed, and then incubated with a Bax antibody. C, anti-Bax antibody alone. D to G, results using specific antibodies to Bcl-X$_L$, Bcl-X$_S$, Bak, and Actin, respectively.

To demonstrate that the observed G_1 phase-associated Bcl-2 protein expression (Figs. 1–3) is not an artifact from the aphidicolin treatment, human breast cancer MCF-7 cells were synchronized in G_0/G_1 phase by serum starvation and then restimulated to proliferate by the addition of fresh serum. About 95% of MCF-7 cells were synchronized in G_0/G_1 phase by serum starvation for 72 h, which expressed high levels of Bcl-2 protein (Fig. 4A, lane 1). Addition of serum for 6 h resulted in ~10% of these cells across the G_1/S boundary, which was accompanied by a slight decrease in the level of Bcl-2 protein (Fig. 4A, lane 2 versus lane 1). After 16 to 20 h of stimulation, 50 to 60% of the originally synchronized G_1 cells entered into S phase, and at these time points, the levels of Bcl-2 protein were significantly decreased (lanes 3 and 4). After 24 h, ~15% of cells entered into G_2/M phase, and the level of Bcl-2 protein still remained low (Fig. 4A, lane 5). When SV40-transformed WI-38 cells were serum-starved and then released, the G_1 phase-dependent Bcl-2 expression was again detected (Fig. 4B). These data confirmed that expression of Bcl-2 protein is G_1 phase-dependent.

We also measured levels of Bax in these experiments. Expression of Bax protein remained relatively constitutive during the cell cycle progression in aphidicolin-synchronized WI-38 (Fig. 3A) and VA-13 cells (data not shown from the experiment in Fig. 3B) as well as in serum starvation-synchronized VA-13 cells (Fig. 4B), which were similar to the results obtained from aphidicolin-synchronized Jurkat T (Fig. 1C) or Jurkat T cells transfected with a vector (Fig. 2E, lanes 1–6). In contrast to all of these four cell lines, expression of Bax in MCF-7 cells, synchronized by either aphidicolin treatment (Fig. 3C) or serum starvation (Fig. 4A), showed a cell cycle-dependent pattern. Low levels of Bax protein were detected in MCF-7 cells with high percentage of G_1 population (Fig. 3C, lanes 1, 5, and 6, and Fig. 4A, lanes 1 and 2), whereas very high Bax expression was found in the cells containing high S, G_2, and M populations (Fig. 3C, lanes 2–4, and Fig. 4A, lanes 3–5).

MCF-7 cells express wild-type p53 gene (Runnebaum et al., 1991). It has been shown that p53 plays an essential role in down-regulation of Bcl-2 expression (Haldar et al., 1994; Miyashita et al., 1994) and up-regulation of Bax transcription (Miyashita and Reed, 1995) in several cell systems. We then investigated whether the cell cycle-dependent expression of Bcl-2 and Bax correlated to any change in p53 levels in MCF-7 cells synchronized by either aphidicolin treatment (Fig. 3C) or serum starvation (Fig. 4A). Expression of p53 protein was low in G_1 phase, but significantly increased when the cells progressed into S and G_2/M phases (Figs. 3C and 4A). Expression of the cyclin-dependent kinase inhibitor p21, a well-known downstream target of p53 (El-Deiry et al., 1993), was also cell cycle-dependent in these MCF-7 cells. More importantly, the periodic expression pattern of p21 was almost identical with that of p53 (Figs. 3C and 4A), suggesting that p53 is functional as a transcription factor under these conditions. Although the cell cycle-dependent pattern of Bax expression was similar to that of p53, the G_1 phase-associated expression of Bcl-2 was inversely comparable with levels of p53 (Figs. 3C and 4A). These data are consistent
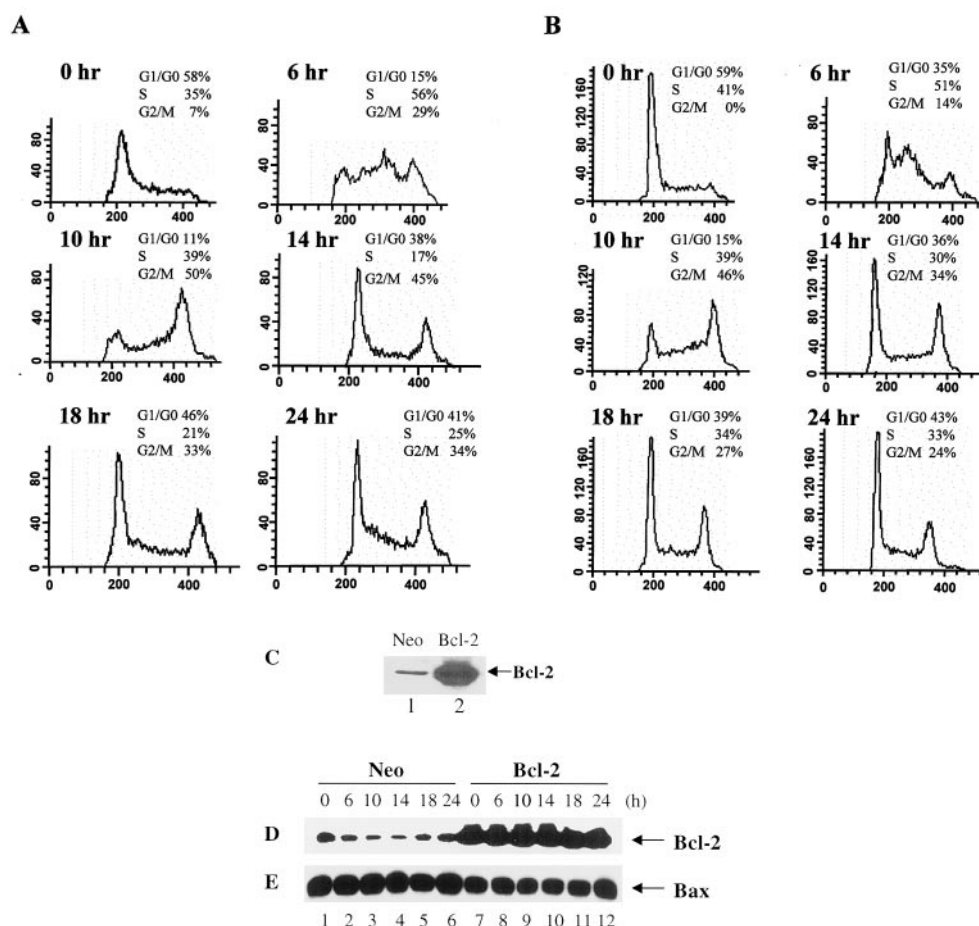

Fig. 2. Cell cycle-dependent expression of Bcl-2 and Bax protein in human Jurkat T cells transfected with an empty vector (Neo) or bcl-2 cDNA-containing vector (Bcl-2). Exponentially grown Neo- and Bcl-2-expressing Jurkat cells (C) were treated with aphidicolin at 3 μg/ml for 24 h (0 h), followed by release for 6, 10, 14, 18, or 24 h, as indicated. At each time point, one-third of the cells were harvested for cell cycle analysis (A, B), one-third were for Western blot assay using specific antibodies to Bcl-2 (D) or Bax (E), and one-third were incubated with VP-16 or staurosporin, followed by measurement of apoptosis (see Fig. 8).

with the hypothesis that p53 up-regulates Bax and down-regulates Bcl-2 during the cell cycle progression of MCF-7 cells. Interestingly, although normal human WI-38 cells also contain wild-type p53 gene, these cells expressed unchanged levels of p53 during the cell cycle (data not shown from the experiment in Fig. 3A).

G₁ Phase-Dependent Bcl-2 Protein Expression Is Regulated on the Level of Bcl-2 mRNA but Not Bcl-2 Proteolysis. The high expression of Bcl-2 protein in G₁ phase could be caused by either a low level of a Bcl-2 proteolytic activity or a high level of Bcl-2 mRNA expression. To investigate these two possibilities, we measured levels of Bcl-2 degradation activity and Bcl-2 mRNA expression in the cell cycle. Aphidicolin-synchronized human Jurkat T (Fig. 5) and MCF-7 cells (Fig. 6) were used for extraction of proteins (for proteolysis assay) and mRNAs (for Northern blot assay). A [³⁵S]methionine-labeled Bcl-2 protein, generated by in vitro transcription and translation of a full-length human bcl-2α cDNA, was used as a substrate in a cell-free Bcl-2 proteolysis assay (see Fig. 6A, lane 1). When incubated with a protein extract prepared from the aphidicolin-treated Jurkat cells, a portion of the labeled Bcl-2 was cleaved, as evidenced by appearance of a labeled band with a faster mobility

(indicated by an arrowhead; compare Fig. 5A, lane 1, with Fig. 6A, lane 1), suggesting the presence of a Bcl-2 proteolytic activity in the G₁/S cells. The same Bcl-2 protein cleavage product was observed in a similar intensity when protein extracts prepared from other time points were used (Fig. 5A), which corresponded to different phases of the cell cycle (see Figs. 1A and 2A). This result indicates that the level of the Bcl-2 protein cleavage activity is constitutive during the cell cycle progression and, therefore, should not be responsible for the G₁ phase-dependent expression of Bcl-2 protein (Figs. 1–4).

When the RNA samples, prepared from the synchronized Jurkat T cells, were analyzed by Northern blot assay using a specific bcl-2 cDNA probe, a cell cycle-dependent pattern of Bcl-2 mRNA expression was observed (Fig. 5B). Jurkat cells, which were accumulated at G₁/S by aphidicolin treatment (see 0 h in Figs. 1A and 2A), expressed a high level of Bcl-2 mRNA (Fig. 5B, lane 1). At 6 h after removing aphidicolin, ~40% of the original G₁ cells entered into S, G₂, and M phase (Figs. 1A and 2A), associated with a decrease in the level of Bcl-2 mRNA (Fig. 5B, lanes 2 versus 1). At 10 and 14 h, ~50% of cells were accumulated in G₂/M phase (Figs. 1A and 2A), where the lowest level of Bcl-2 mRNA was detected (Fig. 5B, lanes 3, 4). At 18 h and 24 h, the cells reentered into G₁ and later G₁/S phase (Figs. 1A and 2A), where the levels of Bcl-2 mRNA were significantly increased (Fig. 5B, lanes 5, 6). By stripping and rehybridizing the filter with a human G3PDH probe, a constitutive expression of G3PDH mRNA was observed (Fig. 5B). Therefore, expression of endogenous Bcl-2
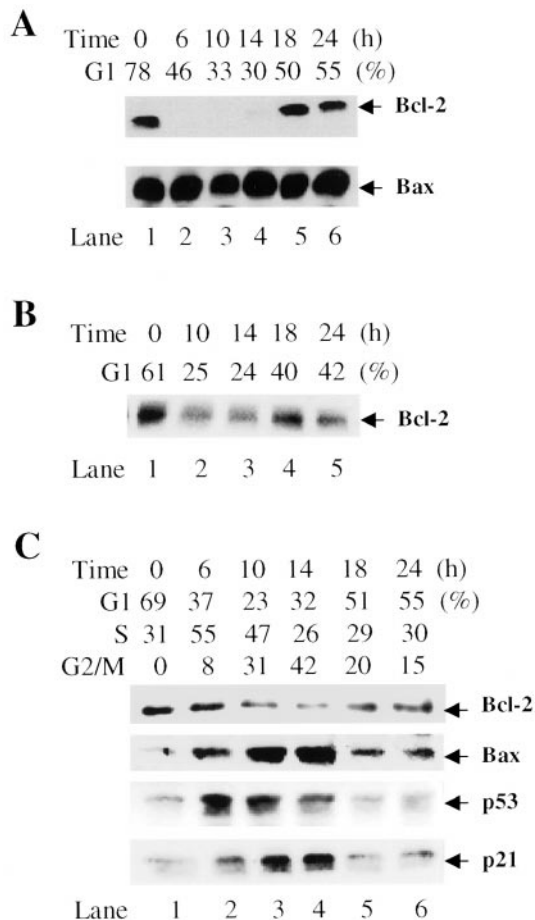
Fig. 3. G₁ phase-specific expression of Bcl-2 protein in aphidicolin-synchronized cells. Exponentially grown WI-38 (A), VA-13 (B), or MCF-7 cells (C) were treated with aphidicolin at 5 µg/ml for 24 h (lane 1, 0 h), followed by release for up to 24 h, as indicated. At each time point, cells were harvested for cell cycle analysis (indicated by percentage of cells in each phase of the cell cycle) and Western blotting using specific antibodies to Bcl-2, Bax, p53, or p21 protein.
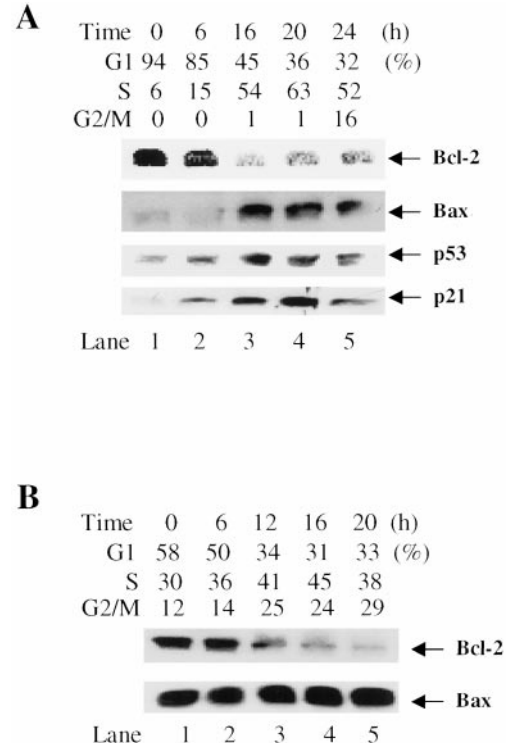
Fig. 4. G₁ phase-specific expression of Bcl-2 protein in serum starvation-synchronized cells. Exponentially grown MCF-7 cells were cultured in the serum-free medium for 72 h (0 h in A) or exponentially grown VA-13 cells were cultured in the serum-free medium for 96 h (0 h in B), followed by addition of the serum for up to 24 h as indicated. At each time point, cells were harvested for cell-cycle analysis (indicated by percentage of cells in each phase of the cell cycle) and Western blotting using specific antibodies to Bcl-2, Bax, p53, or p21 protein.

mRNA in Jurkat T cells is G_1 phase-specific. The cell cycle-dependent pattern of Bcl-2 mRNA (Fig. 5B) was almost identical with that of Bcl-2 protein (Fig. 2D, lanes 1–6).

We also measured levels of Bcl-2 proteolytic enzyme activity and Bcl-2 mRNA in MCF-7 cells synchronized by aphidicolin (Fig. 6). Again, we observed constitutive levels of the Bcl-2 cleavage activity (Fig. 6A) and G_1 phase-specific expression of Bcl-2 mRNA (Figs. 6B versus 3C). The cell cycle dependent pattern of Bcl-2 mRNA expression was again very similar to that of Bcl-2 protein under these conditions (compare Figs. 6B and 3C). From these data, we concluded that the G_1 phase-specific expression of Bcl-2 mRNA is responsible for the G_1 phase-dependent expression of Bcl-2 protein during the cell cycle.

S Phase Cells Containing Low Bcl-2 Levels Are Most Sensitive to Apoptosis Induction. Many human cancers are resistant to apoptosis induced by chemotherapy, which is at least partially caused by overexpression of the Bcl-2 oncoprotein (Reed, 1995; Simonian et al., 1997). We hypothesized that the periodic expression of Bcl-2 protein should influence tumor cellular chemosensitivity in a cell cycle-dependent manner. To test this idea, human Jurkat, Neo, MCF-7, or VA-13 cells were synchronized by either exposure to aphidicolin, followed by release, or withdrawal of serum, followed by addition of fresh medium, as described in Figs. 1 to 4. At

each time point, an aliquot of the synchronized cells was incubated for additional 3 or 24 h with either a chemotherapeutic agent (VP-16 or cisplatin) or the kinase inhibitor staurosporin (Tamaoki, 1991). This was followed by collecting total cell population (for MCF-7 and VA-13 cells, the detached and the attached fractions were combined) and measuring cell death by apoptotic peak, PARP cleavage, Caspase-3 processing/activation, DNA fragmentation (from the 3-h treatment) and cell viability (from the 24 h treatment).

After the aphidicolin-treated Jurkat T cells (containing 69% of G_1 population and a high level of Bcl-2 protein; Fig. 1, A and B) were exposed to VP-16 for 3 h, no apoptosis occurred, as demonstrated by lack of $preG_1$ population (Fig. 7A) and apoptosis-specific p85/PARP fragment, p17/Caspase-3 fragment (Fig. 7, B and C, lane 1) and DNA ladder (data not shown but see Fig. 8B). However, progression of these cells from G_1 to $S/G_2/M$ phases was apparent after the treatment (compare 0 h in Fig. 7A with 0 h in Fig. 1A), indicating that 3-h exposure to VP-16 had little or no effect on the cell cycle progression. When VP-16 was incubated for 3 h with the Jurkat cells, which were released from the aphidicolin block for 6 and 10 h and that contained decreased G_1 population and Bcl-2 expression (Fig. 1, A and B), apoptosis occurred, as evidenced by 20 to 30% increase in the $preG_1$ apoptotic population (Fig. 7A) and a significant amount of the p85/PARP cleavage fragment, the active p17/Caspase-3 fragment (Fig. 7, B and C, lanes 2 and 3) and fragmented DNA (data not shown, but see Fig. 8B). Along with increased pre-G_1 population, the 3-h VP-16 treatment also decreased cell populations mainly from $S/G_2/M$ phases of the cell cycle (compare 6 h and 10 h in Fig. 7A with 6 h and 10 h in Fig. 1A). No
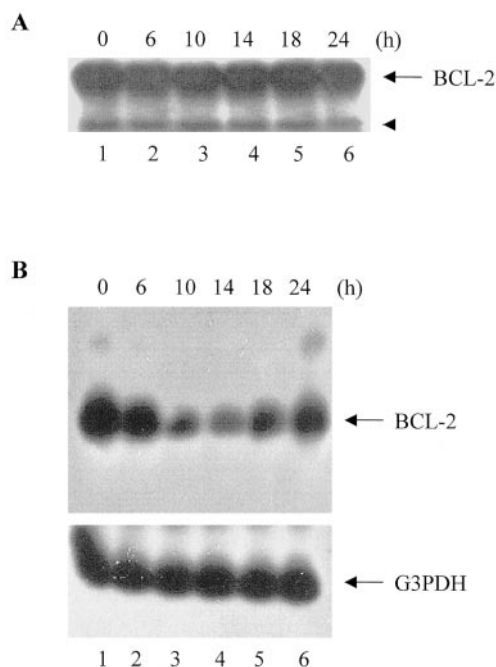
Fig. 5. Levels of Bcl-2 proteolytic activity and mRNA during the cell cycle progression in human Jurkat T cells. Human Jurkat T cells were treated by aphidicolin for 24 h (0 h), followed by release for up to 24 h. At each time point, protein (A) and mRNA (B) samples were prepared. A, Bcl-2 proteolytic activity assay. ^{35}S-labeled Bcl-2 protein (1 μl; see lane 1 in Fig. 6A) was incubated at 37°C for 4 h with 70 μg of a protein extract prepared from aphidicolin-synchronized cells, followed by SDS-polyacrylamide gel electrophoresis and autoradiography. An arrow and an arrowhead, respectively, indicate the uncleaved Bcl-2 protein and a cleavage fragment. B, Northern blot assay. The extracted total RNA samples were electrophoresed, blotted, and hybridized to a ^{32}P-labeled bcl-2 cDNA. Levels of Bcl-2 mRNA were detected by autoradiography (upper). After stripping, the membrane was hybridized with a G3PDH cDNA probe. The G3PDH mRNA was measured for normalization of RNA loading (lower). The Bcl-2 and G3PDH mRNAs are approximately 6.5 and 1.4 kilobase pairs, respectively.
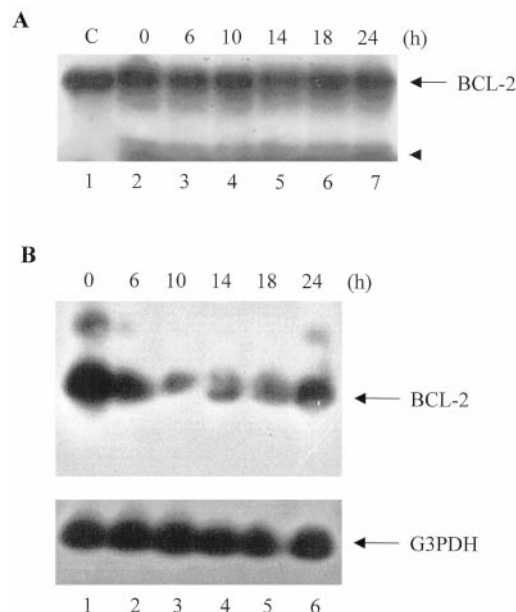
Fig. 6. Levels of Bcl-2 proteolytic activity and mRNA during the cell cycle progression in human breast carcinoma MCF-7 cells. MCF-7 cells were treated by aphidicolin for 24 h (0 h), followed by release for up to 24 h. At each time point, protein (A) and mRNA (B) samples were prepared. A, Bcl-2 proteolytic activity assay. ^{35}S-labeled Bcl-2 protein (as a control, C, in lane 1) was incubated at 37°C for 4 h with 70 μg of a protein extract prepared from aphidicolin-synchronized MCF-7 cells. An arrow and an arrowhead, respectively, indicate the uncleaved and cleaved Bcl-2. B, Northern blot assays. The mRNA levels of Bcl-2 (top) and G3PDH (bottom) are shown.
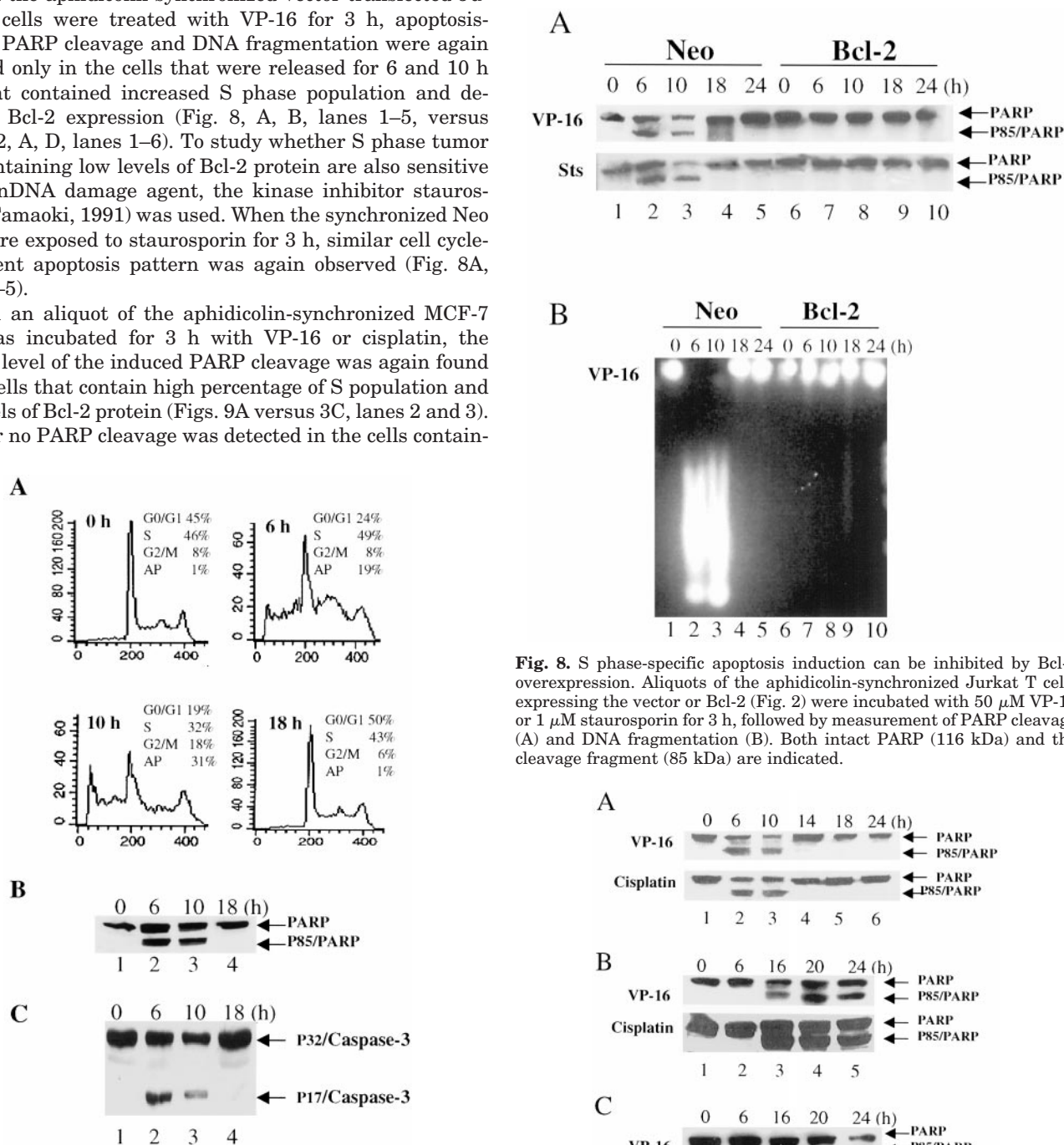
apoptotic cell death was observed when VP-16 was added into the aphidicolin-pretreated cells that had been released for 18 h (Fig. 7, A, B, and C, lane 4), which contained significantly increased G_1 population and Bcl-2 protein expression (Fig. 1, A and B). The 3-h VP-16 treatment did not block the cell cycle progression in these cells, either (compare 18 h in Figs. 7A and 1A).

When the aphidicolin-synchronized vector-transfected Jurkat T cells were treated with VP-16 for 3 h, apoptosis-specific PARP cleavage and DNA fragmentation were again detected only in the cells that were released for 6 and 10 h and that contained increased S phase population and decreased Bcl-2 expression (Fig. 8, A, B, lanes 1–5, versus Figure 2, A, D, lanes 1–6). To study whether S phase tumor cells containing low levels of Bcl-2 protein are also sensitive to a nonDNA damage agent, the kinase inhibitor staurosporin (Tamaoki, 1991) was used. When the synchronized Neo cells were exposed to staurosporin for 3 h, similar cell cycle-dependent apoptosis pattern was again observed (Fig. 8A, lanes 1–5).
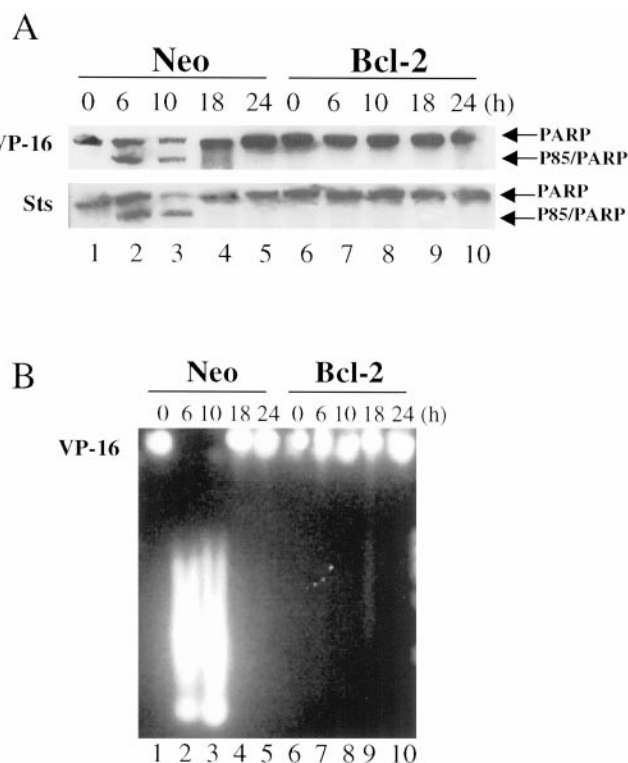
When an aliquot of the aphidicolin-synchronized MCF-7 cells was incubated for 3 h with VP-16 or cisplatin, the highest level of the induced PARP cleavage was again found in the cells that contain high percentage of S population and low levels of Bcl-2 protein (Figs. 9A versus 3C, lanes 2 and 3). Little or no PARP cleavage was detected in the cells contain-

ing high G_1 populations and high Bcl-2 levels (Figs. 9A versus 3C, lanes 1, 5, and 6). No PARP cleavage was found in the cells that contained a low percentage of S population and also a low level of Bcl-2 (Figs. 9A versus 3C, lane 4), suggesting that the decrease in Bcl-2 expression is not sufficient to sensitize tumor cells (see under *Discussion*).
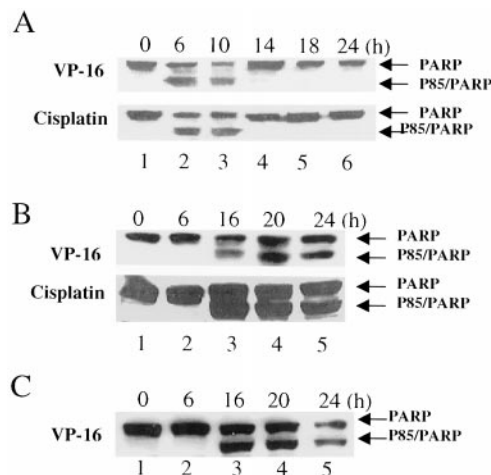
When aliquots of serum starvation-synchronized MCF-7

Fig. 8. S phase-specific apoptosis induction can be inhibited by Bcl-2 overexpression. Aliquots of the aphidicolin-synchronized Jurkat T cells expressing the vector or Bcl-2 (Fig. 2) were incubated with 50 μM VP-16 or 1 μM staurosporin for 3 h, followed by measurement of PARP cleavage (A) and DNA fragmentation (B). Both intact PARP (116 kDa) and the cleavage fragment (85 kDa) are indicated.

Fig. 7. Cell cycle-dependent apoptosis induction by VP-16 in human Jurkat T cells. Human Jurkat T cells were synchronized as described in Fig. 1. At each time point after the release from aphidicolin, a portion of cells was treated with 50 μM VP-16 for additional 3 h and were then harvested for measurement of cell cycle distribution and sub-G_1 population by flow cytometry (A), PARP cleavage (B), and Caspase-3 processing/activation (C). The intact PARP (116 kDa), a PARP cleavage fragment (85 kDa), pro-Caspase-3 (32 kDa), and an active form of Caspase-3 (17 kDa) are indicated.

Fig. 9. S phase-specific, apoptosis-associated PARP cleavage in solid tumor cell lines. Aliquots of (A) the aphidicolin-synchronized MCF-7 cells (from Fig. 3C) (A), the serum starvation-synchronized MCF-7 cells (from Fig. 4A) (B), or the serum starvation-synchronized VA-13 cells (Fig. 4B) (C) were incubated with 50 μM VP-16 or 20 μM cisplatin for 3 h, followed by collecting both detached and attached cells for assaying PARP cleavage.

(Fig. 4A) or VA-13 cells (Fig. 4B) were incubated with VP-16 or cisplatin, a tight correlation between induced apoptosis, cell cycle distribution and endogenous Bcl-2 protein levels was again obtained. When cells containing high percentages of S population and low levels of Bcl-2 protein (Fig. 4, A and B, lanes 3–5) were used, a 3-h drug treatment induced apoptosis-specific PARP cleavage (Fig. 9, B and C, lanes 3–5) and 24-h drug treatment induced loss of cell viability (60–85%). In contrast, when cells containing high G_1 populations and high Bcl-2 protein levels (Fig. 4, A and B, lanes 1, 2) were used, much less cell death was detected: a 3-h treatment of these G_1 cells induced no PARP cleavage (Fig. 9, B and C, lanes 1, 2), and a 24-h treatment induced loss of membrane permeability in a much lower percentage of cells (10–20%). These data suggest that tumor cell sensitivity to apoptosis induction is associated with both high S phase population and low Bcl-2 expression.

Overexpression of Bcl-2 Protein Partially Inhibits the G_1 to S Transition and Completely Blocks the S Phase-Specific Chemosensitivity. To study the functional significance of the cell cycle-dependent Bcl-2 protein expression, we used human Jurkat T cells that were transfected with the human bcl-2 gene. Overexpression of Bcl-2 protein in these cells was confirmed by Western blot analysis (Fig. 2C). Both Bcl-2- and vector-expressing cells were synchronized by aphidicolin, followed by release. At each time point, one-third of the cells were used for measurement of cell cycle distribution (Fig. 2, A and B), one-third for determination of Bcl-2 and Bax protein expression (Fig. 2, D and E), and one-third were incubated with VP-16 or staurosporin for an additional 3 h, followed by measurement of PARP cleavage and DNA fragmentation (Fig. 8).

As reported previously (Mazel et al., 1996; O'Reilly et al., 1996), overexpression of Bcl-2 protein slowed down the G_1 to S transition: after 6-h release from the aphidicolin block, only 24% of G_1 population entered into S/G_2/M phases in Bcl-2-expressing cells (Fig. 2B, 6 h versus 0 h), compared with 43% in the vector-expressing cells (Fig. 2A, 6 h versus 0 h). More importantly, at each time point, addition of VP-16 or staurosporin into Bcl-2 cells failed to induce PARP cleavage and DNA fragmentation (Fig. 8, A and B, lanes 6–10 versus 1–5). This was not caused by different cell cycle distribution of two cell lines, which is supported by the following comparison. At 10 h after release from the aphidicolin block, both Bcl-2- and vector-expressing cells contained similar percentages of G_1, S, and G_2/M populations (Fig. 2, B versus A), but different levels of Bcl-2 protein (Fig. 2D, lanes 9 versus 3). Addition of VP-16 or staurosporin at this time induced apoptosis only in the vector-, but not in Bcl-2-, expression cells (Fig. 8, A and B, lane 3 versus lane 8). Therefore, overexpression of Bcl-2 protein blocks the tumor cell S phase-associated sensitivity to apoptosis induction. Our data suggest that the cell cycle-dependent expression of endogenous Bcl-2 protein at least contributes to the cell cycle-dependent regulation of tumor cell chemoresistance.

Discussion

Recently, it has been reported that some cell-cycle regulators, such as p53, RB, E2F, and Myc, play a role in the process of apoptosis (Lee et al., 1993; Kastan et al., 1995; Adams and Kaelin, 1996; Dou, 1997; Thompson, 1998). In addition, it has also been reported that overexpression of cell death regulator Bcl-2 or Bax influences cell cycle progression (Brady et al., 1996; Mazel et al., 1996; O'Reilly et al., 1996; Gil-Gomez et al., 1998). However, how Bcl-2 and Bax are regulated under physiological conditions and how their regulation affects tumor cell chemosensitivity are unclear. In the current study, we have reported the following novel findings. First, expression of endogenous Bcl-2 mRNA and protein is G_1 phase-dependent in the cell cycle. Second, high Bcl-2-containing G_1 cell population is much more resistant to apoptosis induction by chemotherapeutic agents or staurosporin than low Bcl-2-containing S phase population. Finally, overexpression of Bcl-2 partially prevented the G_1 to S transition and, more importantly, completely inhibits the S phase-associated tumor cell sensitivity to induction of apoptosis.

By using five different mammalian cell lines (Jurkat T, Jurkat T-expressing the vector, WI-38, VA-13, and MCF-7) that had been synchronized by aphidicolin treatment or serum starvation, we observed that expression of Bcl-2 protein is G_1 phase-specific (Figs. 1–4). The maximal expression of Bcl-2 protein seems to occur in mid- to late G_1 phase, supported by the following evidence. First, an immediate reentry into G_1 phase of the next cycle from G_2/M phase is not associated with an immediate increase in the level of Bcl-2 protein (14 h versus 10 h in Fig. 2, A and D, and Fig. 3C). In addition, after cells just crossed the G_1/S boundary, the level of Bcl-2 protein was further increased (24 h versus 18 h in Fig. 2, A and D). Furthermore, when more G_1 cells entered into S, the levels of Bcl-2 protein were significantly decreased (Figs. 1–4). Because neither aphidicolin treatment nor serum starvation could synchronize all the cells, our data did not rule out the possibility that Bcl-2 is also expressed in early S phase.

Our results are consistent with previous reports in which Bcl-2 overexpression either delayed entry into S phase (O'Reilly et al., 1996) or accelerated cycling cells exit into quiescent stage (Vairo et al., 1996). In addition, our investigation further expanded the recent findings that Bcl-2 expression was increased upon serum withdrawal from H9C2 cardiac muscle cells (Wang et al., 1998) and that endogenous Bcl-2 protein levels correlated with sensitivity of human T cells to dexamethasone-induced apoptosis (Montani et al., 1999). It should also be noted that the half-life of Bcl-2 mRNA in B cells was found to be ~2.5 h (Seto et al., 1988) whereas the half-life of Bcl-2 protein in HL-60 cells was ~20 h (Blagosklonny et al., 1996). It seems that half-lives of Bcl-2 mRNA and protein in a cell might differ, both of which may also vary in different cell systems.

We investigated the molecular mechanism responsible for the G_1 phase-dependent expression of Bcl-2 protein. We found that expression of Bcl-2 mRNA peaked in mid to late G_1 (Fig. 5B versus Fig. 2A, and Fig. 6B versus Fig. 3C), suggesting that the periodic change in Bcl-2 protein expression is caused by the cell cycle-dependent change in Bcl-2 mRNA levels. This argument is further supported by the observation that levels of Bcl-2 cleavage activity were constitutive during the cell cycle progression (Figs. 5A and 6A).

It has been reported that expression of Bcl-2 can be downregulated by the tumor suppressor protein p53 through either a negative responsive element in the bcl-2 gene (Miyashita et al., 1994) or an alternative unidentified mecha-

nism (Haldar et al., 1994). In the current study, three cell lines, Jurkat (Iwamoto et al., 1996), Jurkat expressing the vector and VA-13 (Zhu et al., 1991), contain inactive p53 protein. Therefore, a p53-independent mechanism must regulate the G_1 phase-specific expression of Bcl-2 mRNA and protein in these cells (Figs. 1–5). The current studies also included normal WI-38 and breast cancer MCF-7 (Runnebaum et al., 1991) cell lines, both of which contain wild-type p53 gene. Although levels of p53 remained unchanged in WI-38 cells (data not shown in the experiment presented in Fig. 3A), levels of p53, as well as that of p21, changed in a cell cycle-dependent manner in MCF-7 cells (Figs. 3C and 4A). Furthermore, there was an inverse relationship between the levels of Bcl-2 and p53 (as well as p21) in these cells (Figs. 3C and 4A), suggesting that in MCF-7 cells, p53 plays a role in down-regulating Bcl-2 expression in a cell cycle-dependent fashion.

A previous study also reported that p53 directly activates transcription of Bax gene (Miyashita and Reed, 1995). In MCF-7 cells, Bax expression was low in G_1 phase, dramatically increased in S, G_2, and M phases, and decreased again in G_1 of the next cell cycle (Figs. 3C and 4A). This cell cycle-dependent pattern matched very well to that of p53 expression (Figs. 3C and 4A), suggesting that p53 up-regulates Bax in MCF-7 cells. Interestingly, levels of p53 in normal WI-38 cells remained unchanged, associated with constitutive expression of Bax (Fig. 3A and data not shown).

It has also been reported that intracellular levels of Bcl-2 in MCF-7 cells can be up-regulated by estrogen (Teixeira et al., 1995; Huang et al., 1997b), which is present in the fetal bovine serum. We found high levels of Bcl-2 in the serum-starved MCF-7 cells; these levels were dramatically decreased after addition of the fresh serum (Fig. 4A). These data are inconsistent with the involvement of estrogen in up-regulation of Bcl-2. In addition, the periodic changes of Bcl-2 expression were also observed in aphidicolin-synchronized MCF-7 cells (Fig. 3C), in which serum was present all the time. Therefore, the periodic Bcl-2 expression in our MCF-7 system is cell cycle-dependent and is probably not regulated by estrogen.

Most recent experiments have suggested that the ratio of proapoptotic Bcl-2 family members (such as Bax) to antiapoptotic Bcl-2 members (such as Bcl-2) determines whether a cell is committed to apoptotic death or not (Green and Reed, 1998). We have found that levels of Bcl-2 peaked in G_1 phase in all the tested cell lines, whereas levels of Bax remained relatively unchanged in all the cell lines except MCF-7 and levels of Bcl-X_L, Bcl-X_S, and Bak showed little change in synchronized Jurkat cells (Figs. 1–4). Our data suggest that the ratio of Bax to Bcl-2 changes in a cell cycle-dependent manner: low in G_1 and high in S (super high in S phased MCF-7 cells because of an increase in Bax protein expression). Furthermore, we found that the cell cycle-dependent Bax/Bcl-2 ratio is associated with periodic changes in tumor cell sensitivity to apoptosis induction. The S phase tumor cells were much more sensitive to apoptosis induction by chemotherapeutic agents or staurosporin than the G_1 phase tumor cells (Figs. 7–9 versus 1–4). In addition, S phased Jurkat cells overexpressing Bcl-2 protein, which had a decreased ratio of Bax to Bcl-2, became resistant to apoptosis induced by either VP-16 or staurosporin (Fig. 8A). Our data argue that the apoptotic commitment is regulated by the

ratio of proapoptotic Bcl-2 family members (such as Bax) to antiapoptotic Bcl-2 members (such as Bcl-2) in a cell cycle-dependent manner.

We also observed that Bcl-2 overexpression delayed the G_1 to S transition (Fig. 2), consistent with the previous reports (O'Reilly et al., 1996; Mazel et al., 1996). However, it is unclear whether this cell cycle-inhibitory effect is related to the anti-apoptotic function of Bcl-2. It has been suggested that these two inhibitory effects of Bcl-2 can be genetically separated (Huang et al., 1997a).

Many chemotherapeutic drugs kill cancer cells via induction of apoptosis. Previous studies have demonstrated that under certain experimental conditions, the G_1 to S transition of the cell cycle is the most susceptible point for some cell systems to implement a death program (Meikrantz et al., 1994; Meikrantz and Schlegel, 1995; Dou, 1997), although the involved molecular mechanisms remain unknown. Our current studies have suggested that G_1 phase-dependent expression Bcl-2 mRNA and protein is a possible molecular mechanism that is involved in the cell cycle-associated tumor cell chemosensitivity. We also noticed that cells containing low percentages of S population and low levels of Bcl-2 protein were resistant to the drug treatment (Figs. 3C and 9A, lane 4), suggesting that tumor cell chemosensitivity is controlled not only by decreased Bcl-2 levels but also by some other S phase-specific factor(s). This S phase factor may not be the tumor suppressor p53, because most of the cell lines used in this study contain inactive p53 protein. Although the nature of this S phase factor is unclear, the presence of the candidate S phase-specific factor controlling tumor cell chemosensitivity further argues that cell cycle regulation influences apoptotic death decision-making. Our future studies will focus on molecular mechanisms responsible for G_1 phase-specific Bcl-2 mRNA expression and the function of Bcl-2 protein in the cell cycle-dependent tumor chemoresistance.

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