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Activation of cyclin A-dependent protein kinases during apoptosis

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ABSTRACT **Apoptosis was induced in S-phase-arrested HeLa cells by staurosporine, caffeine, 6-dimethylaminopurine, and okadaic acid, agents that activate M-phase-promoting factor and induce premature mitosis in similarly treated hamster cell lines. Addition of these agents to asynchronously growing HeLa cells or to cells arrested in early G_1 phase with lovastatin had little or no effect. S-phase arrest also promoted tumor necrosis factor α-induced apoptosis, eliminating the normal requirement for simultaneous cycloheximide treatment. For all of the apoptosis-inducing agents tested, the appearance of condensed chromatin was accompanied by 2- to 7-fold increases in cyclin A-associated histone H1 kinase activity, levels approximating the mitotic value. Where examined, both Cdc2 and Cdk2, the catalytic subunits known to associate with cyclin A, were activated. Stable overexpression of *bcl-2* suppressed the apoptosis-inducing activity of all agents tested and reduced the amount of Cdc2 and Cdk2 in the nucleus, suggesting a possible mechanism by which *bcl-2* inhibits the chromatin condensation characteristic of apoptosis. These findings suggest that at least one of the biochemical steps required for mitosis, activation of cyclin A-dependent protein kinases, is also an important event during apoptosis.**

Apoptosis is a form of cell death that can be induced in susceptible cells by a wide variety of normal physiological stimuli as well as by deleterious environmental conditions and toxic chemicals (1–4). Deregulation of apoptosis that follows Epstein–Barr virus infection or a t(14;18) chromosome translocation leads to lymphoma in humans (5, 6). In both of these cases, normal physiological death of B cells by apoptosis is blocked by activation of the *bcl-2* oncogene. *bcl-2* is a valuable molecular criterion for distinguishing apoptosis from other sorts of cellular pathology because its expression protects cell types of diverse lineages from apoptosis induced by numerous stimuli (7–11).

Apoptosis is associated with the activation of a number of genes that mediate the transition from quiescence to proliferative growth (4, 12). This activation frequently leads to an abortive cell cycle that fails to enter S phase (13, 14), although cases exist that demonstrate induction of apoptosis in S (15) or G_2 (16) phase. In some cell lines, treatment with agents that arrest cells early in G_1 phase offers protection from apoptosis (17), while transfection with molecules that stimulate DNA replication, such as adenovirus *E1A* or c-*myc*, promotes this response (9, 18–20). Similarities between apoptosis and mitosis have also been noted. The latter stages of apoptosis are characterized by highly condensed chromatin, cytoplasmic blebbing, rounding up of adherent cells, and nuclear lamina disassembly (21–23). Since many agents that induce apoptosis also perturb normal cell cycle progression (4, 24), it has been suggested that apoptosis may result from premature activation of a subset of the protein kinases normally only active at mitosis (25, 26). In fact, at least one apoptosis-inducing agent, the protein kinase inhibitor staurosporine, is known to activate cyclin-dependent protein kinases (CDKs) and induce premature mitosis in hamster cells (27).

We report here that tumor necrosis factor α (TNF-α), staurosporine, and three additional chemicals known to elicit premature mitosis all rapidly induced apoptosis in S-phase-arrested HeLa cells. The appearance of apoptotic chromatin condensation was accompanied in all cases by stimulation of cyclin A-associated histone H1 kinase activity. Stable expression of *bcl-2* suppressed the ability of these agents to induce apoptosis and reduced the abundance of Cdc2 and Cdk2 within the nucleus, suggesting a possible mechanism by which *bcl-2* inhibits apoptotic chromatin condensation.

MATERIALS AND METHODS

Apoptosis Induction. HeLa cells, cultured in monolayer (28), were incubated with 2.5 mM hydroxyurea (HU) until mitotic cells were no longer visible (≈5 h). Apoptosis-inducing agents were then added as indicated. At the indicated times during treatment, cells were fixed on the dishes with methanol and stained for 10 min with Hoechst 33258 (Calbiochem) at 0.5 μg/ml in H_2O. The percentage of cells containing apoptotic nuclei was determined by fluorescence microscopy at ×400 magnification (see Fig. 1*A*). From 500 to 700 cells were counted for each sample. Nuclear/chromosome spreads were prepared by swelling cells for 10 min in 0.55% KCl, fixing with methanol/acetic acid, 3:1 (vol/vol), and dropping onto wet slides. For electron microscopy, cells in 60-mm dishes were fixed, stained, and photographed as described (23).

Plasmids and Transfections. Plasmid pCΔj-bcl-2, containing the human *BCL-2* coding region under control of the simian virus 40 enhancer and promoter and carrying the neomycin resistance (neo) marker, and the identical plasmid pCΔj-SV2, lacking the *BCL-2* coding region (29), were used to create stable HeLa cell lines. HeLa cells (10^6 cells) were transfected with 2 μg of pCΔj-bcl-2 or pCΔj-SV2 by using Lipofectin (GIBCO/BRL) and were selected by growth in G418 (0.5 mg/ml). Twenty-four pCΔj-bcl-2-transformants and 12 pCΔj-SV2-transformants were screened for Bcl-2 expression by SDS/PAGE and Western blot analysis, using procedures as described (30) and a commercial Bcl-2 antiserum (PharMingen) at a dilution of 1:1000. Of the 24 neo$^+$ clones selected, 4 expressed high levels of Bcl-2; no expression was detected in any of the 12 control clones.

Immunoprecipitation and H1 Kinase Assays. Antibodies against C-terminal domains of Cdc2 (LDNQIKKM) and Cdk2 (DVTKPVPHLRL) and against full-length recombinant human cyclin A and cyclin B (gift of J. Ruderman, Harvard Medical School), prepared as described (31), were raised in rabbits (Immuno-Dynamics, La Jolla, CA). Monoclonal anti-cyclin E antibody (clone HE172) was a gift of E.

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Abbreviations: CDK, cyclin-dependent protein kinase; 6-DMAP, 6-dimethylaminopurine; TNF-α, tumor necrosis factor α; CHX, cycloheximide; HU, hydroxyurea; neo, neomycin resistance.

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Harlow (Massachusetts General Hospital, Boston). Cells were harvested for H1 kinase assays by trypsin treatment of monolayers 4 h after addition of apoptosis-inducing agents. Detached cells were collected and pooled with the monolayer cells prior to lysis and immunoprecipitation (30). Mitotic cells were collected by mechanical shake-off after a 16-h incubation in the presence of nocodazole (40 ng/ml). H1 kinase assays were performed on immunoprecipitates using [γ-^{32}P]ATP (6500 cpm/pmol; 100 μM) and Sigma type VII-S histone H1 (Sigma; 0.5 mg/ml) as substrate (27). H1 kinase activity was assayed by autoradiography after SDS/PAGE of substrate histone and by measuring radioactivity by Cerenkov counting of H1 bands excised from SDS/PAGE gels. Autoradiographs were scanned using a Mustek scanner equipped with UNIX VISAGE 4.6K software (Millipore); integrated band densities were then normalized to common baseline values using the activity of extracts from HU-arrested cells as the reference point.

Nuclear and Cytoplasmic Extracts and Western Blots. Cells were accumulated for 12 h in 2.5 mM HU and released from the monolayer by incubation with 0.5 mM EDTA. Nuclear and cytoplasmic fractions were prepared using 0.1% digitonin as the solubilizing agent (32), separated by SDS/PAGE, transferred to Immobilon membranes, and probed with antibody (30). Cyclin A, cyclin B, Cdc2, and Cdk2 antibodies were used at 1:1000 dilutions; anti-cyclin E hybridoma supernatant (clone HE12, a gift of E. Harlow) was used at a dilution of 1:5; anti-myc hybridoma supernatant (clone 9E10, Cell Culture Facility, Harvard University) was used at a dilution of 1:20.

RESULTS

Multiple Forms of Apoptosis Are Potentiated by S-Phase Arrest. When staurosporine is added to S-phase-arrested hamster cells, premature mitosis is induced (27). In contrast, similarly treated HeLa cells undergo apoptosis. Fluorescent staining of DNA revealed patches of irregularly dispersed brightly staining condensed chromatin (Fig. 1*A*). Chromosome spreads of apoptotic nuclei stained homogeneously, indicating a loss of normal nuclear structure, and typically contained one or more nonstaining "holes" (Fig. 1*B*) that have been noted previously during glucocorticoid-induced apoptosis in thymocytes (33). Electron microscopy revealed the presence of highly condensed chromatin localized to the inner surface of an intact nuclear envelope (Fig. 1*C*) and extensive cytoplasmic blebbing (Fig. 1*D*). By using the distinctive appearance of apoptotic nuclei as a measure of response, the effectiveness of various doses of staurosporine was assessed in asynchronously growing and S-phase-arrested cells after an 8-h exposure to the drug. As seen in Fig. 2*A*, staurosporine doses as low as 1 ng/ml showed some apoptosis-inducing ability when cells were arrested in S phase (4 vs. 1% in the presence of HU alone; $P < 0.01$ by binomial t test), whereas at the maximum effective dose, 20 ng/ml, approximately one-third of the cells were apoptotic. In contrast, asynchronously growing HeLa cells continued to proliferate in the presence of staurosporine at 20 ng/ml. Only at doses $\geq$200 ng/ml did the fraction of cells showing apoptotic nuclei begin to approach 15%. Very high doses of staurosporine (466 ng/ml) have been reported (10) to induce apoptosis in asynchronous cultures of other human cell lines. Staurosporine-induced apoptosis was also potentiated by aphidicolin (1 μg/ml) and a high concentration of thymidine (2.5 mM), indicating that this response was common to other DNA synthesis inhibitors (data not shown). Arrest earlier in the cell cycle by lovastatin treatment, which produces a reversible G_1-phase arrest (34), did not potentiate staurosporine-induced apoptosis (data not shown), suggesting that the stage of cell cycle arrest is important for this response.

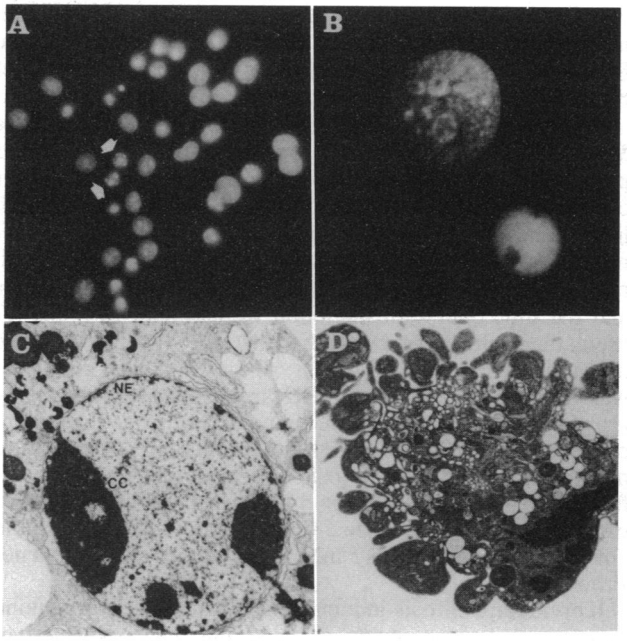

FIG. 1. Fluorescence and electron micrographs of staurosporine-induced apoptosis. HeLa cells were arrested in S phase with HU for 5 h followed by treatment with staurosporine (20 ng/ml) for an additional 8 h. (*A*) Cells fixed *in situ* with methanol and stained with Hoechst 33258. Arrowheads, representative apoptotic nuclei. (×170.) (*B*) Chromosome spread showing a normal nucleus (upper) and an apoptotic nucleus (lower), stained with Hoechst 33258. (×420.) (*C*) Electron micrograph showing condensed chromatin (CC) within an intact nuclear envelope (NE). (×7800.) (*D*) Electron micrograph showing extensive cytoplasmic blebbing. (×6200.)

To determine whether arrest in S phase would potentiate other apoptosis-inducing agents, the effect of HU on TNF-α-induced apoptosis was examined. HeLa cells that were asynchronously growing, arrested in S phase with HU, or cotreated with CHX were exposed to TNF-α (1–100 ng/ml). TNF-α alone did not induce apoptosis at doses up to 100 ng/ml (Fig. 2*B*), whereas CHX cotreatment led to apoptosis starting at 10 ng/ml, with 100 ng/ml causing $\approx$30% of the cells to respond within 8 h. This dependence on CHX cotreatment has been reported (35). Interestingly, arrest in S phase with

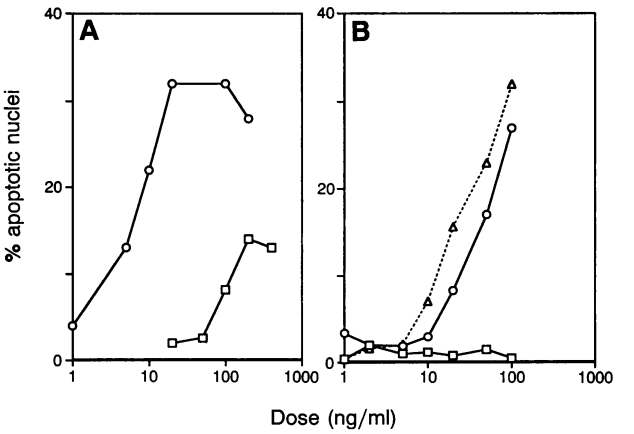

FIG. 2. Effect of S-phase arrest on staurosporine- and TNF-α-induced apoptosis in HeLa cells. Apoptosis dose–response curves for staurosporine administered to asynchronously growing (□) and HU-arrested (○) cells (*A*) and TNF-α administered to asynchronously growing cells (□), cells arrested in S phase with HU (○), and asynchronously growing cells cotreated with cycloheximide (CHX, 30 μg/ml; △) (*B*).

HU was able to substitute for CHX. These results imply that inhibition of protein synthesis *per se* by CHX is not critical for TNF-α-induced apoptosis, but rather some other effect that can be mimicked by S-phase arrest.

Apoptosis Is Induced by Agents That Promote Premature Mitosis. The ability of staurosporine to induce apoptosis prompted us to test the apoptosis-inducing ability of other chemicals known to induce premature mitosis in hamster cell lines. As shown in Fig. 3, caffeine (5 mM), 6-dimethylaminopurine (6-DMAP, 5 mM), and okadaic acid (0.5 μM) all rapidly induced apoptosis in HeLa cells. The effective doses and the time course of induction were similar to those for premature mitosis in hamster cells (28, 36–38), with the maximum response seen by 8 h. Ability to induce apoptosis was strongly dependent on HU arrest, as asynchronously growing cells displayed little or no response at these concentrations. As is the case with induction of premature mitosis, induction of apoptosis by the methylxanthines caffeine and 6-DMAP was not due to their ability to inhibit cAMP phosphodiesterase, since isobutylmethylxanthine concentrations as high as 5 mM were without effect (data not shown).

If premature mitosis-inducing agents are triggering genuine apoptosis, overexpression of *bcl-2* might be expected to block the induced chromatin condensation. Therefore, HeLa cells were transfected with a plasmid expressing human *BCL-2* (29) or an identical control plasmid lacking the *BCL-2* coding region. All four Bcl-2$^+$ clones displayed a greatly reduced frequency of staurosporine- and TNF-α-induced apoptosis when compared with the control (neo$^+$) clones. A representative Bcl-2$^+$ clone (HB-14 Bcl-2$^+$) and control clone (H-E neo$^+$) were further examined for their response to all of the apoptosis-inducing agents (Fig. 4). For each agent examined, the response of the neo$^+$ control clone was similar to that of the parental cells (see Figs. 2 and 3), whereas the percentage of cells showing apoptotic nuclei in the Bcl-2$^+$ clone was reduced to $\leq$2%. Bcl-2 expression for these two clones is shown in Fig. 5. These results confirm earlier morphological evidence that premature mitosis-inducing agents, as well as TNF-α, promote genuine apoptosis in HeLa cells.

Activation of CDKs During Apoptosis. Cyclin A, cyclin B, and cyclin E immunoprecipitates were prepared and assayed for associated H1 kinase activity. As shown in Fig. 6, cyclin A-dependent H1 kinase activity was greatly increased by treatment of HU-arrested cells with okadaic acid, caffeine,

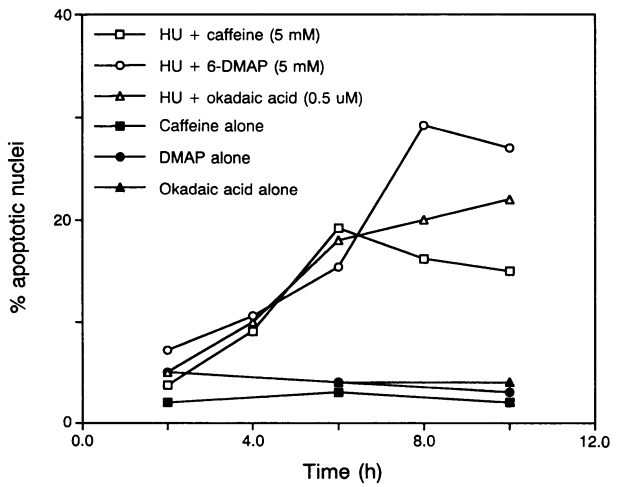

FIG. 3. Time course for apoptosis induction by premature mitosis-inducing agents. HeLa cells were asynchronously growing or arrested in S phase with HU for 5 h and then treated for an additional 8 h with the indicated chemicals.

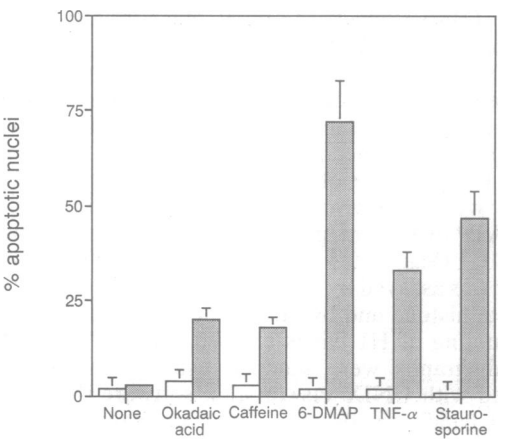

FIG. 4. Effect of stable *bcl-2* overexpression on apoptosis. Bcl-2$^+$ (clone HB14, open bars) or control neo$^+$ (clone H-E, shaded bars) cells were arrested with HU for 5 h and incubated with inducing agents for an additional 8 h. Concentrations were as in Fig. 3, plus staurosporine (20 ng/ml) and TNF-α (100 ng/ml). Values are the mean ± SEM for two determinations. From 400 to 500 cells were counted for each sample.

6-DMAP, and staurosporine, as well as TNF-α, which, to our knowledge, has not previously been recognized as a CDK activator. CHX treatment alone also caused an increase in cyclin A-dependent H1 kinase activity, which was further stimulated by addition of TNF-α, suggesting a mechanism by which CHX may potentiate TNF-α-induced apoptosis. Table 1 presents the specific activities obtained for all treatment groups. Each agent tested activated cyclin A-associated activity 2- to 7-fold, or to 40–150% of the mitotic value. Since in HeLa cells cyclin A is known to bind both Cdc2 and Cdk2

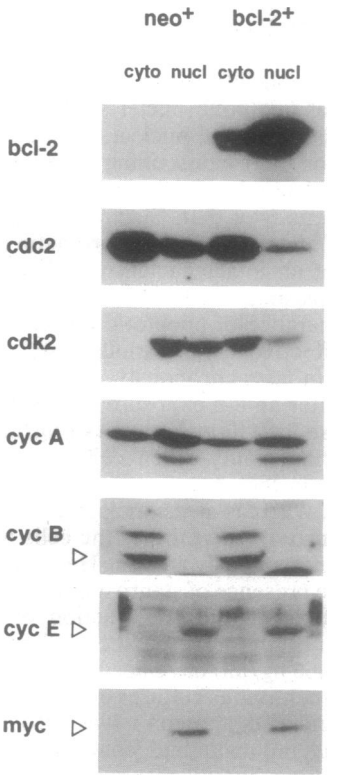

FIG. 5. Effect of Bcl-2 overexpression on the amount of Cdc2 and Cdk2 in the nucleus. Identical cytoplasmic (cyto) and nuclear (nucl) extracts from neo$^+$ and Bcl-2$^+$ cells were examined by immunoblot analysis for the proteins indicated. Each lane was loaded with 5×10^5 cell equivalents. cyc, Cyclin.

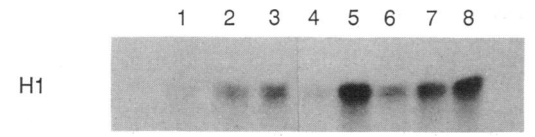

FIG. 6. Cyclin A-associated H1 kinase activity during apoptosis. HeLa cells were arrested in S phase with HU for 5 h followed by treatment for an additional 4 h with the indicated agents. The concentrations used were the same as those in Figs. 3 and 4. Autoradiograph shows ^{32}P-labeled H1 after SDS/PAGE. Lanes: 1, HU alone; 2, HU/caffeine; 3, HU/6-DMAP; 4, HU/okadaic acid; 5, HU/staurosporine; 6, HU/TNF-α; 7, CHX alone; 8, CHX/TNF-α.

catalytic subunits (39), the relative contribution of each of these kinases was assessed. As seen in Table 1, Cdc2-associated H1 kinase activity approximately doubled after staurosporine treatment, and Cdk2-associated activity increased 3-fold. Thus, it seems that the observed increase in cyclin A-associated H1 kinase activity is due to the activation of both cyclin A–Cdk2 and cyclin A–Cdc2 complexes.

In contrast to cyclin A, cyclin B-associated activity was very low, remaining at ≤1% of the activity seen at mitosis regardless of treatment (Table 1). This is not surprising, since cyclin B synthesis in S-phase HeLa cells is minimal (28, 32, 40, 41). While some treatments (addition of staurosporine to HU-arrested cells and addition of CHX to asynchronously growing cells) led to a doubling of cyclin E-associated activity, increases were not evident after other treatments, indicating a poor correlation between cyclin E-associated kinase activation and apoptosis.

In vitro, CDKs tend to show little substrate specificity, and it is thought that cyclin subunits help target CDKs to specific subcellular sites *in vivo* (42). In an initial attempt to understand the mechanisms by which *bcl-2* suppresses apoptosis, we examined the subcellular distribution of cyclins and CDKs in Bcl-2$^+$ and control cells (Fig. 5). In agreement with results from other laboratories (32, 39, 43), cyclin A was found in both the nucleus and cytoplasm, existing in two electrophoretically distinct forms in the nucleus. Cyclin B, which is known in HeLa cells to be entirely confined to the cytoplasm until the end of G_2 phase (32), was found only in cytoplasmic fractions, and cyclin E appeared to be exclusively localized to the nucleus. No differences in cyclin localization were seen between neo$^+$ and Bcl-2$^+$ cells. The localization of Myc, a nuclear protein whose ectopic expression can induce apoptosis in serum-deprived rat fibroblasts (9, 15), was also unaffected. In contrast, the amount of Cdc2 and Cdk2 in the nuclei of Bcl-2$^+$ cells was reduced 3- to 4-fold when compared with neo$^+$ control cells, as determined by densitometry. Corresponding 3- to 4-fold decreases in nuclear cyclin-dependent histone H1 kinase activity were also found in Bcl-2$^+$ cells when compared with neo$^+$ cells (data not shown). We note that, in the Bcl-2$^+$ cells, Bcl-2 was found mainly in the nuclear fraction, a result confirmed by immunofluorescence (data not shown) and in agreement with other investigators (44).

DISCUSSION

Apoptosis shares a number of morphological features with mitosis, including cell rounding, surface blebbing, lamin disassembly, and chromatin condensation (23, 26). Here we have shown that apoptosis can be induced in HeLa cells by the same chemical agents known to induce premature mitosis in hamster cells and that overexpression of *bcl-2* blocks this response. Prior arrest in S phase enormously potentiated their effect. The induction of apoptosis was accompanied in all cases by activation of cyclin A-dependent protein kinases.

The apoptosis-promoting effect of S-phase arrest and the activation of cyclin A-dependent protein kinases were not peculiar to the class of premature mitosis-inducing chemicals, as similar effects were seen with TNF-α, which induces apoptosis by a receptor-mediated pathway (45). Although caffeine, staurosporine, and okadaic acid are known to activate cyclin B–Cdc2 in S-phase-arrested hamster cells (27, 28, 38), to our knowledge, stimulation of cyclin A-dependent kinase activity has not been recognized as a consequence of TNF-α binding. We have also found that arrest in S phase can substitute for CHX in sensitizing HeLa cells to TNF-α. It is likely, therefore, that the sensitizing effect of CHX is due more to its ability to increase cyclin A-associated kinase activity than to inhibit the synthesis of apoptosis-suppressing proteins. The finding in other cell types that cell killing by TNF-α is potentiated by staurosporine (46) and caffeine (47), chemicals shown in the present study to induce cyclin A-associated kinase activity, is consistent with this idea.

In addition to the present findings, other data support a role for cyclin A in apoptosis. Arresting L929 fibroblast cells early in G_1 phase, prior to the onset of cyclin A synthesis, protects them from TNF-α-induced apoptosis (17). Conversely, two genes whose ectopic expression promotes apoptosis, *myc* and adenovirus *E1A*, are both positive regulators of cyclin A transcription (48, 49). When apoptosis is found in nonproliferating tissues, it seems to be associated with reentry of cells into an abortive cell cycle. Castration-induced regression of the rat ventral prostate results in the induction of cyclin A mRNA (R. Buttyan, personal communication) and other markers of proliferation (12). Cyclin A mRNA is also induced during apoptosis elicited by serum withdrawal in Rat 1a cells constitutively overexpressing c-*myc* (A. Hoang and C. Dang, personal communication).

It is possible that cyclin A targets activated Cdc2 and Cdk2 to substrates necessary for chromatin condensation and other morphological changes during both apoptosis and mitosis.

Table 1. H1 kinase activity of immunoprecipitates from HeLa cells

Treatment	H1 kinase activity				
	Cdc2	Cdk2	Cyclin A	Cyclin B	Cyclin E
HU arrest	40 ± 5	20 ± 7	23 ± 5.6	≤1	4.9 ± 1.0
Nocodazole arrest	119 ± 7	59 ± 8	110 ± 23	240 ± 32	
HU/staurosporine	80 ± 7	61 ± 6	164 ± 30	≤1	12.5 ± 3.5
HU/caffeine			66 ± 8	≤1	7.7 ± 2.8
HU/6-DMAP			102 ± 20	≤1	3.6 ± 1.9
HU/okadaic acid			42 ± 21		
HU/TNF-α			65 ± 9	≤1	9.6 ± 3.1
CHX alone			103 ± 21	≤1	11.4 ± 3.4
CHX/TNF-α			135 ± 22	≤1	5.7 ± 2.4

Immunoprecipitates with precipitating antibodies to the indicated proteins were collected and activities were measured. Specific activity is pmol of P_i per 20 min per mg of cell extract; data are the mean ± SEM of two or three determinations; each done in triplicate.

bcl-2 may act not by directly inhibiting cyclin A-dependent protein kinases, but by preventing the interaction of these kinases with critical target substrates. The strategic location of Bcl-2 in the nuclear envelope could facilitate this activity by directly affecting nuclear transport (44). Our finding that *bcl-2* expression reduced the amount of Cdc2 and Cdk2 in the nucleus is consistent with such a hypothesis. Since Bcl-2 has also been found associated with intracellular membranes other than the nuclear envelope, it is likely that this oncoprotein has other effects as well. For example, Bcl-2 has been shown to suppress the formation of reactive oxygen species and thus may act in an antioxidant capacity (50).

Finally, we propose that these data might have some clinical relevance. While cyclin A is not expressed in quiescent cells (51), overexpression or deregulated expression of cyclin A has been linked to hepatocellular and breast carcinomas (52–54) and seems to be a general characteristic of cells immortalized either spontaneously (55) or by DNA tumor virus oncoproteins (31, 56). TNF-α and chemicals related to staurosporine, okadaic acid, caffeine, or 6-DMAP might, therefore, be used in conjunction with an S-phase-arresting agent such as HU to selectively kill actively dividing tumor cells. The use of HU would in theory permit the use of much lower doses of the apoptosis-inducing agent, thereby reducing harmful side effects.

We are grateful to F. Hall for a gift of antibodies to cyclin A and cdk2 that were used in preliminary studies, J. Yao for preliminary characterization of the response of the HeLa Bcl-2$^+$ clones to TNF-α, Y. Tsujimoto for pCΔj-bcl-2 and pCΔj-SV2 plasmids, and C. Cooke for electron microscopy. This work was supported in part by a grant from the Human Frontier Science Program (R.S.), by PHS National Research Service Award 1F32CA61626-01 from the National Cancer Institute (W.M.), and by National Institutes of Health/National Institute of Environmental Health Sciences Graduate Training Grant T32 ES07155.

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