# Caspase-dependent Cdk Activity Is a Requisite Effector of Apoptotic Death Events

**Kevin J. Harvey, Dunja Lukovic, and David S. Ucker**

Department of Microbiology and Immunology, University of Illinois College of Medicine, Chicago, Illinois 60612

*Abstract.* The caspase-dependent activation of cyclin-dependent kinases (Cdks) in varied cell types in response to disparate suicidal stimuli has prompted our examination of the role of Cdks in cell death. We have tested the functional role of Cdk activity in cell death genetically, with the expression of dominant negative Cdk mutants (DN-Cdks) and Cdk inhibitory genes. Here we demonstrate that Cdk2 activity is necessary for death-associated chromatin condensation and other manifestations of apoptotic death, including cell shrinkage and the loss of adhesion to substrate. Susceptibility to the induction of the cell death pathway, including the activation of the caspase cascade, is unimpaired in cells in which Cdk2 activity is inhibited. The direct visualization of active caspase activity in these cells confirms that death-associated Cdk2 acts downstream of the caspase cascade. Cdk inhibition also does not prevent the loss of mitochondrial membrane potential and membrane phospholipid asymmetry, which may be direct consequences of caspase activity, and dissociates these events from apoptotic condensation. Our data suggest that caspase activity is necessary, but not sufficient, for the full physiological cell death program and that a requisite function of the proteolytic caspase cascade is the activation of effector Cdks.

Key words: physiological cell death • Cdk2 • caspase • mitochondria • apoptosis



## Introduction

Recent studies reveal that a thematically conserved pathway, comprised of the functionally ordered products of expanded gene families, effects physiological cell death, leading to the characteristic morphology of apoptosis (Ellis and Horvitz, 1986; Boise et al., 1993; Oltvai et al., 1993; Chinnaiyan et al., 1996; Enari et al., 1996; Harvey et al., 1998; Hirata et al., 1998). Among the most prominent hallmarks of physiological cell death are prelytic nuclear events, including chromatin condensation, genome digestion, and breakdown of the nuclear envelope (Russell et al., 1980; Wyllie, 1980; Ucker et al., 1992). Other markers of the physiological cell death response have been described, including plasma membrane reorganization associated with blebbing, shrinkage, and the loss of membrane phosphatidylserine asymmetry (Kerr et al., 1972; Fadok et al., 1992). Mitochondrial integrity is compromised during the death process as well, and involves the release of cytochrome *c* and the loss of membrane potential ($\Delta\Psi_m$; Susin et al., 1997; Yang et al., 1997). It remains still to be determined what specific molecular events are responsible for the demise of the cell and where, within the conserved pathway, the irreversible commitment to lethality occurs.

Address correspondence to David S. Ucker, Department of Microbiology and Immunology, University of Illinois College of Medicine, Rm. E803 (M/C 790), 835 South Wolcott, Chicago, IL 60612. Tel.: (312) 413-1102. Fax: (312) 996-6415. E-mail: duck@uic.edu

Nuclear events during cell death parallel processes that occur in viable cells during the mitotic cell cycle (Ucker, 1991). That many stimuli that induce cell proliferation also can trigger death suggests that the mechanisms that control the fundamental biological processes of mitosis and apoptosis are related. The induction of physiological cell death under conditions of trophic factor deprivation (Galaktionov et al., 1996; Luo et al., 1996) and in post-mitotic cells (Al-Ubaidi et al., 1992; Feddersen et al., 1992), and the activation-driven deletion of lymphocytes (Fournel et al., 1996; Radvanyi et al., 1996; Hakem et al., 1999) depend on the function of molecules of the productive cell cycle and exemplify this close interplay.

The molecular engines of the cell cycle, first defined genetically in yeast and now well characterized in mammalian cells, are composed of cyclin-dependent kinases (Cdks[1]; Riabowol et al., 1989; Meyerson et al., 1992). Multiple modes of regulation, especially on the posttransla-

[1]*Abbreviations used in this paper:* $\Delta\Psi_m$, mitochondrial membrane potential; Cdk, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; ClCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DEVD, Asp-Glu-Val-Asp; DN-Cdk, dominant negative cyclin-dependent kinase; EGFP-F, enhanced GFP that includes a sequence for farnesylation; GFP, green fluorescent protein; IETD, Iso-Glu-Thr-Asp; MCA, 4-methyl-coumaryl-7-amide; PE, phycoerythrin; TMRE, tetramethyl rhodamine ethyl ester; YVAD, Tyr-Val-Ala-Asp.


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tional level, pertain to the cell cycle–specific control of Cdk activity. Cdks are inactive unless complexed with their periodically synthesized cognate cyclins (Solomon et al., 1990). Reversible phosphorylations at distinct sites activate and inhibit kinase activity (Russell and Nurse, 1987; Strausfeld et al., 1991; Heald et al., 1993). Cyclin-dependent kinase inhibitors (CKIs), including members of the Cip/Kip and Ink4 families, are involved in assembling and inactivating Cdk complexes (Polyak et al., 1994; Toyoshima and Hunter, 1994; Brugarolas et al., 1995; Serrano et al., 1996). Finally, the subcellular localization of Cdks and their regulators restrict the activation of Cdks to appropriate temporal and spatial compartments (Heald et al., 1993; Diehl and Sherr, 1997; Jin et al., 1998).

In contrast, the identities of the molecular elements that drive the cell death process are not elaborated fully. Genetic studies of developmental cell death in the worm *C. elegans* have led to the identification and characterization of elements of a singular and conserved death pathway (Ellis and Horvitz, 1986). Cell death in *C. elegans* is dependent on the activation of Ced3, a member of the caspase family of aspartate-specific cysteine proteases (Yuan et al., 1993; Xue et al., 1996). Ced9, encoded by a homologue of the *bcl-2* family of human oncogenes, inhibits *ced3*-dependent death and acts upstream of Ced3 in the cell death pathway (Hengartner et al., 1992; Vaux et al., 1992; Hengartner and Horvitz, 1994). Ced4 also acts upstream of Ced3 and is required for death (Shaham and Horvitz, 1996). Mammalian proteins with partial similarity to Ced4 activate mammalian caspases; Apaf1, for example, activates caspase 9 in the presence of cytochrome *c* (Li et al., 1997; Zou et al., 1997). One of the limitations of these genetic studies is their reliance on mutations with unconditional death-resistance phenotypes. Screens for unconditional mutants preclude the identification of genes that are necessary both for viability and for death.

The basic molecular framework for regulating and executing cell death appears to be conserved in mammalian cells. In contrast to worms, however, death in mammalian cells is characterized by diverse initiating signals and multiple death-regulating members of both the caspase and *bcl-2* gene families (Oltvai and Korsmeyer, 1994; Minn et al., 1996; Salvesen and Dixit, 1997). Each caspase is synthesized as a pro-enzyme and activated by cleavage at internal sites, potentially by the same or another caspase class (Thornberry et al., 1992; Nicholson et al., 1995). Caspases function within a proteolytic cascade that is punctuated by members of the Bcl-2 family (Enari et al., 1995; Harvey et al., 1998). Bcl-2 seems to regulate the activation of downstream caspases, possibly through the compartmentalization of activating factors such as cytochrome *c* without directly affecting the activity of upstream caspases (Kluck et al., 1997; Yang et al., 1997; Harvey et al., 1998). Caspases that act upstream of the sparing function of Bcl-2 have been characterized as initiators of the cell death response; caspases that act downstream of Bcl-2 have been designated as effectors of cell death (Harvey et al., 1998). The functional assignments thus made have been supported generally by gene structure analysis, as well as by groupings based on substrate specificities (Thornberry et al., 1997; Van de Craen et al., 1997). Initiator pro-caspases contain long pro-domains that facilitate their oligomerization and activation by cell surface molecules, such as the TNF-$\alpha$ "death receptor", or by intracellular death regulators such as Apaf1 (Chinnaiyan et al., 1995; Fernandes-Alnemri et al., 1996). The effector pro-caspases, in contrast, possess only short pro-domains and are activated within the proteolytic cascade by upstream caspases (Li et al., 1997; Slee et al., 1999).

Although the activation of the caspase cascade has been recognized as necessary for the physiological cell death process, the essential death substrates and lethal biochemical events that are targeted have not been identified (Wang et al., 1995; Rao et al., 1996; MacFarlane et al., 1997; Zhang et al., 1998). We and others have described the activation of Cdks as a common, caspase-dependent attribute of the physiological cell death process, and have hypothesized that it is important for the cell death response (Harvey et al., 1998; Levkau et al., 1998; Zhou et al., 1998).

To explore the role of Cdk activity within the cell death process, we have examined death responses in cells in which we have manipulated Cdk activity genetically. Here we show that death-associated Cdk activity is necessary for a variety of cell death events including chromatin condensation, and that it functions downstream of the caspase cascade in a conserved death pathway. The proteolytic caspase cascade facilitates the activation of effector Cdks, but is not itself sufficient for the complete cell death program.

## Materials and Methods

### Strategy for the Tracking and Analysis of Transfected Cells

We have employed a green fluorescent protein (GFP) variant as a marker to visualize transfected cells directly. Soluble GFP leaks from dying cells (Harvey, K.J., unpublished observations); in addition, it is not useful in cytofluorimetric DNA analyses where ethanol fixation is required (Jiang and Hunter, 1998). In contrast, a membrane-targeted GFP, the genetic fusion of the enhanced green fluorescent protein and the farnesylation sequence of p21$^{Ras}$ (EGFP-F; Jiang and Hunter, 1998), is retained preferentially in unfixed dying cells (Harvey, K.J., unpublished data). Using this marker, we have been able to identify transfectants consistently, independent of their ultimate fate. Moreover, we find that the cell rounding and shrinkage associated with the loss of adhesion during cell death results in a condensed EGFP-F signal: an increased intensity of green fluorescence per cell surface area, presumably resulting from the retention and concentration of EGFP-F in dying cells with reduced volume (see Fig. 3). EGFP-F condensation serves as a quantitative and reliable correlate of cellular collapse.

### Cellular Procedures and Analyses

*Cell Culture and Synchronization.* Freshly cloned populations of HeLa cells were grown in DME medium (Mediatech) supplemented with heat inactivated fetal calf serum (10% v/v; HyClone Laboratories) and 2 mM L-glutamine (Mediatech). Two methods of synchronization were employed. We used the double thymidine block method of Rao and Engelberg, (1966). Cells were incubated in complete medium supplemented with 2.5 mM thymidine (Sigma Chemical Co.) for 16 h, released into medium without thymidine for 9 h, and then treated with thymidine for an additional 16 h. Cells also were starved of serum by culturing in media containing 0.2% fetal calf serum for 48 h. These treatments alone did not cause cell death.

*Transfections.* The constructs employed include: pcDNA3, CrmA/pcDNA3 and p35/pcDNA3 (Tewari et al., 1995), pSFFV-Neo/Bcl-2 (Hockenbery et al., 1990), pCMVp16-Ink4a (David Beach, Cold Spring Harbor Laboratory), pBabe-p21$^{Cip1/Waf1}$-GFP (Hiroaki Kiyokawa, University of Illinois, Chicago), pCMV5/Kip-p27 (Kiyokawa et al., 1996), pCMV-p21$^{Cip1/Waf1}\Delta$17-52-HA (Robles et al., 1998), and pCMV-Cdk{1-4}-DN (van den Heuvel and Harlow, 1993). 6 $\mu$g of each construct or empty vector was cotransfected into $10^5$ HeLa cells seeded the night before in six-well plates using a standard calcium phosphate precipitation method with 0.6 $\mu$g of EGFP-F (Jiang and Hunter, 1998; a kind gift of Eve Shinbrot, Clontech Laboratories, Palo Alto, CA) as a transfection marker. After 16 h cells were washed free of precipitate and incubated in fresh media. We routinely obtain transfection efficiencies with HeLa cells of ~35% (data not shown). The transfection procedure itself also induces a low level (<10%) of cell death in the entire population (including both EGFP-F$^+$ cells that have successfully expressed transfected DNA and those that have not). We have noted that the death-inhibitory effects of gene products such as Bcl-2 can be observed as a specific reduction of this background death among the EGFP-F$^+$ cells; conversely, death-inducing gene products, such as p16$^{Ink4a}$, enhance background death specifically among the EGFP-F$^+$ cells.

*Cell Cycle Analysis.* 48 h after transfection, cell cycle analysis was performed by staining with propidium iodide (Krishan, 1975). We have found that some farnesylated green fluorescent protein from transfectants is solubilized upon fixation in ethanol. Inclusion of a low concentration of BSA in all aqueous buffers is necessary to prevent EGFP-F from sticking nonspecifically to untransfected cells after ethanol fixation (Harvey, K.J., and D.S. Ucker; unpublished data). In brief, $10^6$ cells were washed twice with PBS supplemented with 0.2% BSA and fixed in one ml of 50% ethanol for 30 min on ice. Cells were pelleted and treated in the dark with 1 mg/ml of RNase A and 50 $\mu$g/ml of propidium iodide in 400 $\mu$l PBS/BSA for 30 min at 25°C. Stained cells were analyzed cytofluorimetrically on a FacsCalibur™ instrument (Beckton Dickinson). Cell cycle distributions were quantified using ModFit software (Verity Software House).

*Death Induction.* 48 h after transfection, cells were induced to die with 1 $\mu$M staurosporine (Sigma Chemical Co.), 10 ng/ml TNF-$\alpha$ (a generous gift of Dale Hales, University of Illinois, Chicago), 100 ng/ml of actinomycin D (Sigma Chemical Co.), or 200 $\mu$M carbonyl cyanide *m*-chlorophenylhydrazone (ClCCP; kindly provided by Dale Hales). Sensitivity of HeLa cells to TNF-$\alpha$ is enhanced in the presence of a nonlethal dose of cycloheximide; 3 $\mu$g/ml cycloheximide (Sigma Chemical Co.) was added simultaneously with TNF-$\alpha$ in all cases.

*Assessment of Chromatin Condensation.* After incubation with 1 $\mu$g/ml of Hoechst 33342 (Sigma Chemical Co.; Ex = 355 nm; Em = 465 nm) for 10 min at 37°C, chromatin was visualized in transfectant cells with a Nikon Diaphot 200 microscope with epi-fluorescence. EGFP-F fluorescence was detected in parallel (Ex = 490 nm; Em = 525 nm). Digital images were acquired with a SenSys CCD Camera (Photometrics). Images were analyzed using Image-Pro Plus (Media Cybernetics). Chromatin condensation was scored among EGFP-F$^+$ transfectants that exhibited at least a twofold increase in pixel intensity of the Hoechst 33342 signal. For each sample, 3 fields with at least 100 cells each were counted.

*Assessment of Cell Shrinkage, Rounding, and Condensation.* The manifestations of typical apoptotic morphology in adherent HeLa cells, which includes shrinkage and cell rounding, were visualized by phase contrast microscopy. In addition, dying transfectant cells reliably displayed a condensed pattern of EGFP-F fluorescence. EGFP-F condensation was quantified from digital images of transfected cells as an increase in pixel intensity. In all cases analyzed, EGFP-F condensation strictly correlated with chromatin condensation as measured by Hoechst staining of nuclei (Harvey, K.J., unpublished data).

*Analysis of Caspase Activity In Vivo.* HeLa cells were transfected as described above and replated after 16 h onto micro plates with a coverslip bottom (Fisher Scientific). After an additional 32 h, cells were treated for 4 h with death stimuli as above, or left untreated. The medium was replaced with a minimal volume (100 $\mu$l) supplemented with the cell permeant pro-fluorescent DEVD-specific caspase substrate PhiPhiLux-$G_2D_2$ (10 $\mu$M; Packard et al., 1996; Oncoimmunin) during the last hour of incubation. Hoechst 33342 was added during the last 10 min to stain chromatin, as above. Cells were washed gently three times in complete media. Signals from PhiPhiLux-$G_2D_2$ (Ex = 540 nm; Em = 580 nm), EGFP-F, and Hoechst were detected by fluorescence microscopy. For cytofluorimetric analysis, transfectant populations were treated with death stimuli for 3 h as usual. Cells then were harvested with PBS + 4 mM $Na_2$EDTA, washed once with complete medium, and resuspended for an additional 1 h of incubation at 37°C in 50 $\mu$l of medium supplemented with 10 $\mu$M PhiPhiLux-$G_2D_2$. Cells were diluted into 450 $\mu$l of cold flow cytometry buffer and analyzed within 30 min (Ex = 488 nm; Em = 585 nm).

*Annexin V Staining.* Cells were harvested with PBS + 4 mM $Na_2$EDTA and washed twice with cold PBS. Cells were resuspended in 100 $\mu$l of binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and incubated with 5 $\mu$l of phycoerythrin (PE)-conjugated annexin V (PharMingen) for 15 min in the dark at 25°C. After incubation, 400 $\mu$l of binding buffer was added per sample and cells were analyzed cytofluorimetrically (Ex = 488 nm; Em = 585 nm).

*Mitochondrial Membrane Potential.* Mitochondrial membrane potential was assessed simply with tetramethyl rhodamine ethyl ester (TMRE; Molecular Probes), a fluorescent dye rapidly responsive to mitochondrial membrane potential (Farkas et al., 1989). TMRE was stored as a stock solution at 25 mg/ml in DMSO; a fresh working solution (40 $\mu$M) was prepared in medium. Cells were incubated in medium with 100 nM TMRE for 10 min at 37°C. Cells were harvested and washed once with PBS supplemented with 0.2% BSA. The TMRE signal (Ex = 488 nm; Em = 585 nm) was analyzed either cytofluorimetrically or by fluorescence microscopy. The difference in staining for cells with polarized and depolarized mitochondria typically is at least one order of magnitude.

## In Vitro Procedures and Analyses

*Analysis of Caspase Activity.* Preparation of digitonin extracts in HKEB (100 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 5 mM EGTA, 100 $\mu$M PMSF, 1 mM DTT, and 50 $\mu$M $\gamma$-S-ATP) and assays of caspase and Cdk activities followed our previously described protocols (Harvey et al., 1998). Specific caspase activities were assayed using fluorogenic 4-methyl-coumaryl-7-amide (MCA)-derivatized reporter substrate peptides for caspase 1, Acetyl-Tyr-Val-Ala-Asp-4-methyl-coumaryl-7-amide (YVAD-MCA; Thornberry et al., 1992; Peninsula Laboratories) caspase 8, Acetyl-Iso-Glu-Thr-Asp-4-methyl-coumaryl-7-amide (IETD-MCA; Peptides International) and caspase 3, Acetyl-Asp-Glu-Val-Asp-4-methyl-coumaryl-7-amide (DEVD-MCA; Peptides International). Stock solutions of peptide reagents, including derivatized inhibitors (Peptides International), were prepared in anhydrous DMSO.

*Analysis of Cdk Activity.* Cdk activity was assayed essentially as described previously (Harvey et al., 1998), using a synthetic substrate peptide derived from the site of histone H1 phosphorylation mediated by Cdk1 (Cdc2; Promega). Reactions were stopped after 10 min by the addition of 5 $\mu$l of 5% $H_3PO_4$ and spotted on duplicate 1.5 × 1.5 cm squares of P-81 paper, washed four times for 5 min in 1% $H_3PO_4$ and one time in acetone, dried, and quantified by scintillation analysis. Immunodepletion was accomplished by incubation of 100 $\mu$g of extract with 1 $\mu$g of antibody specific for Cdk2 or other kinases (Santa Cruz Biotechnology) for 4 h at 4°C in 200 $\mu$l of HKEB. Immune complexes were cleared thrice by incubation for 2 h with 20 $\mu$l of protein A agarose (Sigma Chemical Co.). Kinase activity remaining in the supernatant was assayed as described above.

*Western Blot Analysis.* Immunodepletion of Cdk components was confirmed by Western blot analysis of both supernatants and pellets. 20 $\mu$l of the depleted extract and 1/10 of the resuspended pellet from the first round of depletion were run on 15% polyacrylamide gels and transferred to Immobilon *P* (Millipore). Antibodies specific for Cdk2 and cyclin A (Santa Cruz Biotechnology) were used to detect depleted proteins. Immunoreactive bands were visualized by the luminol reaction (ECLplus; Amersham). Cleavage of epitope-tagged p21$^{Cip1/Waf1}$ also was analyzed by Western blot, using an HA-epitope-specific antibody (HY11, Santa Cruz Biotechnology) and antibody directed to a p21 $NH_2$-terminal peptide (amino acids 58–77; Oncogene Research Products), after electrophoresis of extract proteins on 15% polyacrylamide gels.

# Results

## Cdk2 Activity Is Activated in a Caspase-dependent Manner during HeLa Cell Death

The constitutive expression of viral caspase inhibitors CrmA and p35 in transfected cells, which confers pleiotropic cell death resistance, has demonstrated the indispensable role of the proteolytic caspase cascade in the physiological cell death process (Enari et al., 1995; Tewari et al., 1995; Harvey et al., 1998). We have sought to test the role of Cdks in cell death similarly, through the genetic interference of specific Cdk activity with dominant negative Cdk mutants (DN-Cdks) and Cdk inhibitors (CKIs). Since unconditional interference with Cdk activity would abrogate proliferation and preclude the recovery of stably transfected cells (see below), we have expressed these inhibitory constructs transiently. To mark transfected cells, we chose a green fluorescent protein (GFP) variant that allows direct visualization without disruption or fixation. Because soluble GFP leaks from dying cells (KJH, unpublished data), we made use of a membrane-anchored GFP, derived from a construct that includes a sequence for farnesylation (EGFP-F; Jiang and Hunter, 1998; see Materials and Methods).

For these analyses, we selected a cell line that is both readily transfectable and susceptible to a variety of physiological death stimuli. HeLa cells meet these criteria: they can be transfected at high efficiency (we typically have obtained efficiencies of 35% or greater; see Materials and Methods) and they are susceptible to death induced by TNF-α, staurosporine, and high doses of inhibitors of macromolecular synthesis, such as actinomycin D. When induced to die, the cells undergo a typical physiological cell death, exhibiting characteristic chromatin condensation, cell shrinkage, and loss of adhesion (see below).

A common process of cell death, similar to that characterized previously in T and B lymphocytes (Harvey et al., 1998), is induced in HeLa cells by each of these suicidal stimuli. It involves the ordered induction of upstream initiator caspase activity, downstream effector caspase activity, and Cdk activity (Fig. 1, A and B, and data not shown). While YVAD-specific initiator caspase activity (Harvey et al., 1998) is not detectable in cytoplasmic extracts from

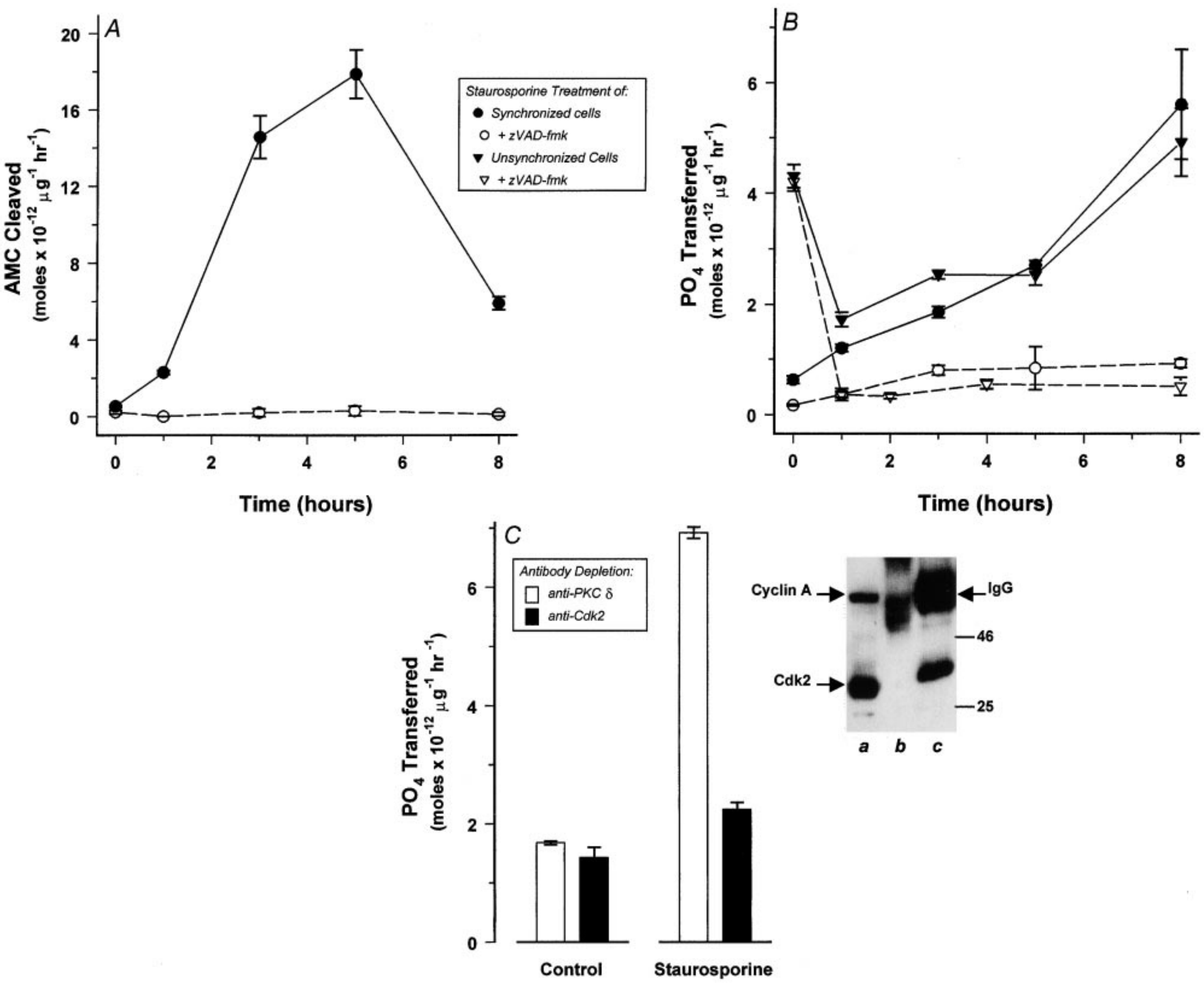


*Figure 1.* Activation of effector caspase and caspase-dependent Cdk2 activities in dying HeLa cells. The kinetics of DEVD-specific caspase activity (A) and Cdk activity (B) were monitored in extracts of synchronized HeLa cells (after a double thymidine block) after the addition of 1 μM staurosporine in the absence (●) and presence (○) of the pan-caspase inhibitor z-VAD-fmk (100 μM). The appearance of death-associated Cdk activity in unsynchronized HeLa cells in the absence (▼) and presence (▽) of the pan-caspase inhibitor z-VAD-fmk (100 μM) also is presented. The death-associated caspase-dependent Cdk activity from HeLa cells treated with staurosporine for 8 h was characterized by immunodepletion analysis (C); only the results of depletion with one Cdk-2–specific antibody (■) and one irrelevant antibody specific for protein kinase C-δ (PKC-δ, □; see Emoto et al., 1995) are presented here. A Western blot analysis of the staurosporine-induced extract, using Cdk2- and cyclin A-specific antibodies, is presented in C: (a) mock-depleted extract, (b) extract depleted with Cdk2-specific antibody, (c) precipitated material from Cdk-2 depleted extract. Cdk2 and cyclin A bands are indicated. Note that the cyclin A band in the right lane is masked by precipitating IgG heavy chain.

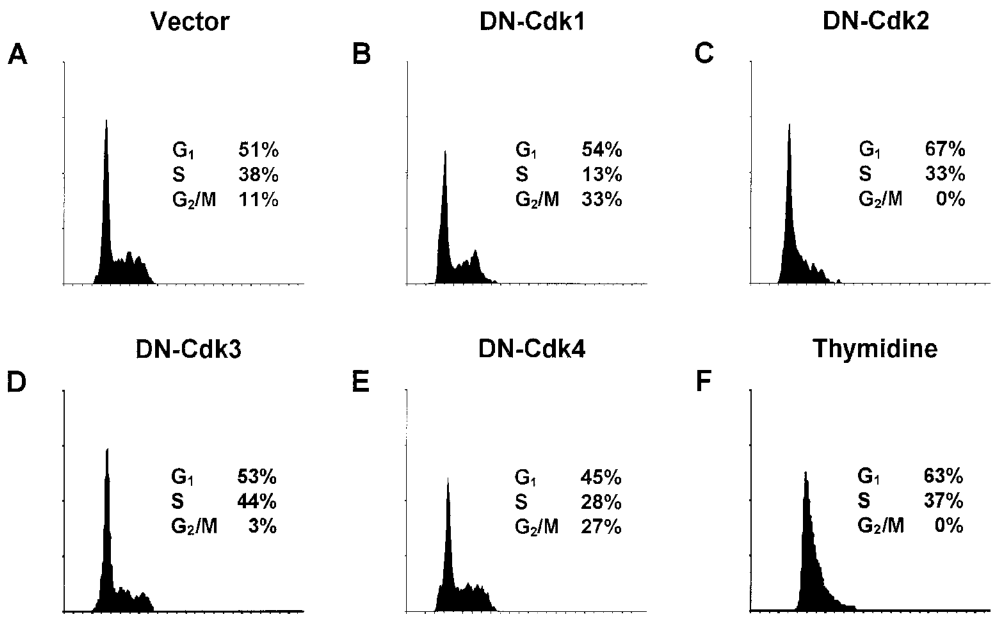


*Figure 2.* DN-Cdk mutants arrest HeLa cells in the cell cycle compartment in which the endogenous wild-type Cdk acts. HeLa cells were cotransfected with EGFP-F and empty vector (A), DN-Cdk1 (B), DN-Cdk2 (C), DN-Cdk3 (D), and DN-Cdk4 (E). Cells were harvested, fixed and stained with propidium iodide to assess DNA content. At least 10,000 EGFP-F$^+$ cells were analyzed. In parallel, untransfected cells were subjected to a double thymidine block and analyzed (F).

dying HeLa cells, we observe other upstream caspase activities, such as cleavage of IETD-MCA, a specific substrate for apical caspase 8 (data not shown). The induction of death-associated Cdk activity in HeLa cells follows the kinetics of downstream effector caspase activity (Fig. 1, A and B). Chromatin condensation and other terminal events of death also follow these kinetics.

The induction of death-associated Cdk activity is most clearly evident in cells synchronized at the $G_1$/S boundary after a double thymidine block (Figs. 1 B and 2 F). In unsynchronized HeLa cell populations, the initiation of a death response triggers a rapid drop in basal Cdk activity that precedes the induction of death-associated Cdk activity (Figs. 1 B and 2 A). In previous studies, we have described post-mitotic cell cycle arrest as an early and common event of cell death (Ucker, 1991; Harvey et al., 1998). Death responses in HeLa cells involve similar cell cycle arrest (data not shown). We interpret the initial depletion of Cdk activity to reflect this cessation of cell cycle progression.

Cdk2 has been implicated as the death-associated Cdk in a variety of cells, including HeLa cells (Meikrantz et al., 1994; Meikrantz and Schlegel, 1996; Shi et al., 1996; Harvey et al., 1998; Levkau et al., 1998; Zhou et al., 1998). We find that the death-associated Cdk activity in HeLa cells is fully depleted from crude extracts using an anti-Cdk2 antibody (Fig. 1 C) and exhibits other diagnostic characteristics of Cdk2, including complete inhibition by 50 $\mu$M olomoucine (Glab et al., 1994) and a $K_m$ for peptide substrate of 12 $\mu$M (vs. 8 $\mu$M for purified Cdk1; data not shown). The induction of Cdk2 activity (Fig. 1 B), like caspase activity (Fig. 1 A) and morphological manifestations of death (see below), is inhibited when cells are cultured in the presence of the pan-caspase peptide inhibitor, z-VAD-fmk. These data confirm that HeLa cells rely on the caspase-dependent pathway of death and, most significantly, demonstrate that death-associated Cdk2 activity is dependent on, and downstream of, the caspase cascade.

### *Death-associated Chromatin Condensation Is Dependent on Cdk Activity*

Dominant negative Cdk mutants have been shown to arrest transfectant cells in the specific phase of the cell cycle in which the functional Cdk normally acts (van den Heuvel and Harlow, 1993). These mutants lack kinase activity (due to substitution of the active site aspartate with aspargine [$D^{145}\rightarrow N$ for huCdk2]) and act in a dominant negative manner due to their ability to compete with endogenous wild-type Cdks for binding of necessary partner cyclins (van den Heuvel and Harlow, 1993).

We find that DN-Cdks exert appropriately similar phenotypes in transfected HeLa cells (Fig. 2). Expression of DN-Cdk1, a mutant of the mitotic Cdk whose primary activating partner is cyclin B, significantly increases the number of cells in the $G_2$/M compartment (Fig. 2 B). DN-Cdk2, a mutant of the Cdk that acts at the $G_1$/S transition or during S phase when complexed with cyclins E or A respectively, arrests cells efficiently at the $G_1$/S boundary (Fig. 2 C). Expression of DN-Cdk3, a mutant of a less well characterized Cdk that interacts with cyclin A (Meikrantz and Schlegel, 1996), has only a modest effect on cell cycle progression (Fig. 2 D). DN-Cdk4, a mutant of a Cdk that has been implicated recently in $G_2$ phase control (Gabrielli et al., 1999), expands the number of cells in that phase of the cycle (Fig. 2 E). The primary role of Cdk4 in regulating exit from $G_1$ through the cyclin D-dependent Rb pathway is not evident in HeLa cells, consistent with the inactivation of Rb proteins by the HPV viral oncogene E7 (Munger et al., 1989; Matsushime et al., 1994).

This demonstration of functional expression of DN-Cdks in HeLa cells allowed us to probe the specific roles of Cdks in the cell death process. After transfection, cells were treated with TNF-$\alpha$ or staurosporine, or left untreated (see Materials and Methods). Our hypothesis that Cdks might be involved especially in the nuclear events of cell death led us first to examine death-associated chromatin condensation. Chromatin condensation, as indicated by

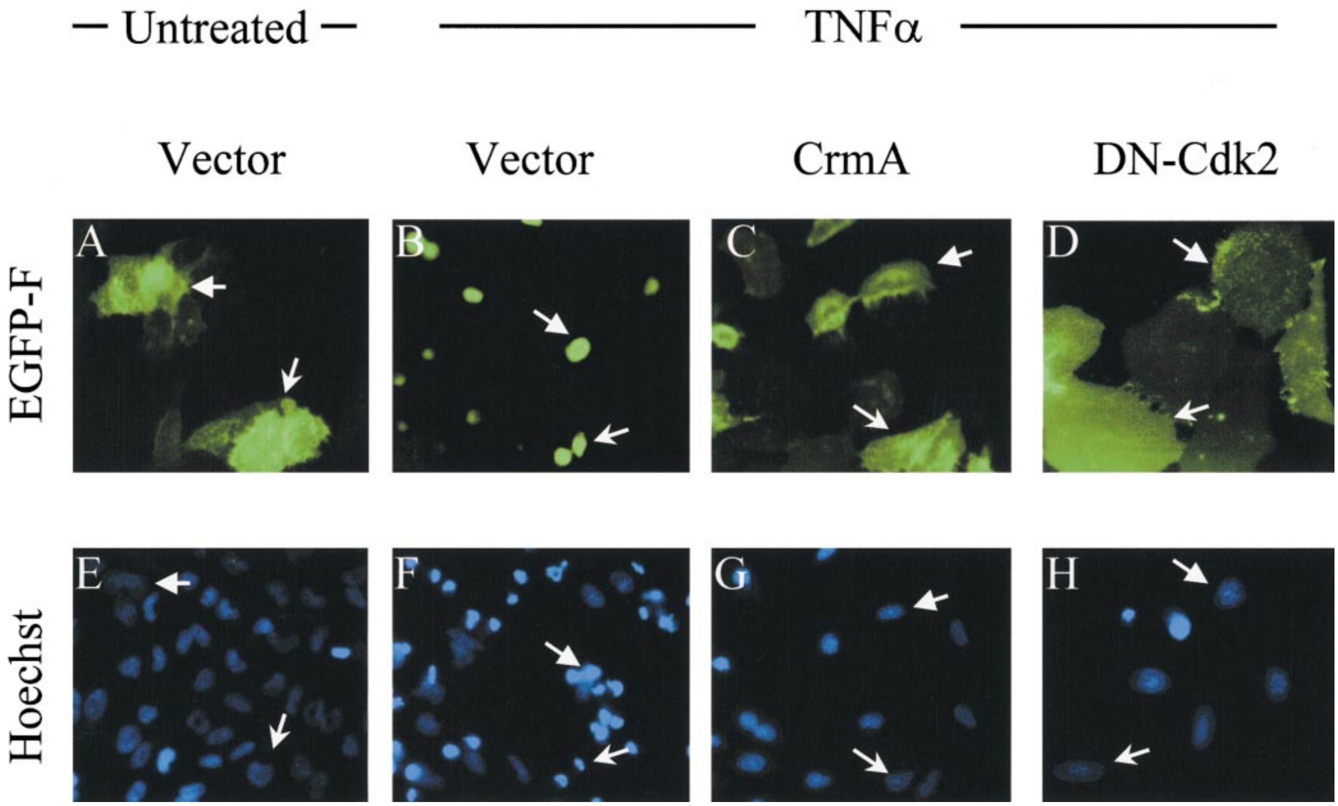


*Figure 3.* Caspase and Cdk activities are necessary for death-associated chromatin condensation and cell shrinkage. Transfectants were identified by fluorescence microscopy, by virtue of expression of the cotransfection marker EGFP-F, which is retained in dying cells (A–D). Chromatin condensation, assessed microscopically by the intensity of Hoechst 33342 staining, was visualized microscopically (E–H). Cell shrinkage and the loss of adhesion are revealed here by the condensation and rounding of EGFP-F staining (A–D). These manifestations of death were assessed in untreated cells previously transfected with empty vector (A and E) and in cells transfected with empty vector (B and F), CrmA (C and G), and DN-Cdk2 (D and H) and subsequently treated for 7 h with 10 ng TNF-α in the presence of a nonlethal dose (3 μg/ml) of cycloheximide. The evaluation of cellular morphology by EGFP-F staining yields results identical to those derived from analysis by phase contrast microscopy (see Fig. 5).

enhanced Hoechst 33342 staining intensity, was assessed in EGFP-F$^+$ cells (see Fig. 3).

Expression of DN-Cdk2 inhibited chromatin condensation induced by TNF-α and by staurosporine (Figs. 3, 4, and 5 A). DN-Cdk3 expression was equally effective (Figs. 4 and 5 A). In contrast, the expression of DN-Cdk1 or DN-Cdk4 had no significant inhibitory effect (Figs. 4 and 5 A). These data are consistent with the biochemical identification of death-associated Cdk2 activity (Fig. 1 C) and implicate Cdk2 as required for death-associated chromatin condensation. An independent role of Cdk3 in this cell death process cannot be excluded (see Discussion). The overexpression of p21$^{Cip1/Waf1}$ or p27$^{Kip1}$, CKIs that target Cdk2, also abrogated staurosporine- and, to a lesser degree, TNF-α–induced chromatin condensation (Figs. 4 and 5 A, and data not shown). On the other hand, the relative inability of p16$^{Ink4a}$, a specific inhibitor of Cdk4, to inhibit chromatin condensation is consistent with DN-Cdk4 data in suggesting no significant role for Cdk4 in HeLa cell death (Figs. 4 and 5 A; see below).

The blockade of chromatin condensation afforded by Cdk2 inhibition is comparable to that resulting from caspase inhibition. Overexpression of the viral serpin CrmA, a specific inhibitor of initiator caspase activity, prevented death-associated chromatin condensation (Figs. 3, 4, and 5 A). We obtained similar results with the broader spectrum caspase inhibitor, p35 (data not shown). Interestingly, the human oncogene Bcl-2 inhibited chromatin condensation induced by staurosporine but not by TNF-α (Figs. 4 and 5 A). This is consistent with other reports of the inability of Bcl-2 to interfere with death signaled via receptors of the TNF-α superfamily in a manner independent of mitochondrial events (Memon et al., 1995; Strasser et al., 1995; Dbaibo et al., 1997). Together, these data confirm that caspases are necessary generally for cell death–associated chromatin condensation, although distinct caspase cascades are activated by different stimuli, and extend this conclusion to suggest that, independent of the specific death stimulus, it is the caspase-dependent activation of Cdks that drives this nuclear manifestation of the death process.

### *Other Apoptotic Events Are Dependent on Cdk Activity*

We explored the death response more completely in these transfectants by examining other manifestations of cellular demise. We observed that cells with condensed chromatin typically exhibited a shrunken, rounded cellular morphol-

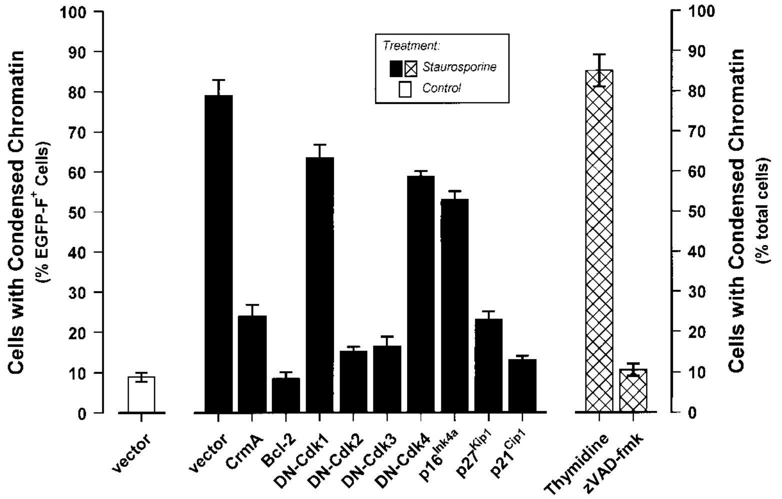


*Figure 4.* The inhibition of Cdk2 activity prevents staurosporine-induced chromatin condensation. Chromatin condensation was assessed in cells transfected with the indicated vectors (□, ■) or in untransfected cells (▩) synchronized by double thymidine block or incubated with the pan-caspase inhibitor z-VAD-fmk (100 μM; see Fig. 1). Cells were treated with staurosporine (1 μM; ■, ▩) or left untreated (□), and chromatin condensation was assessed after 7 h. The percentage of transfected (EGFP-F$^+$) or untransfected cells with condensed chromatin was assessed microscopically (see Fig. 3). The data shown represent one of three replicate experiments where each sample is the average of three microscopic fields of at least 100 cells of interest each.

ogy and the loss of substrate adhesion, as assessed by phase contrast microscopy. In addition, we noted that one consequence of cell shrinkage and rounding in EGFP-F$^+$ transfectants is a condensed EGFP-F signal (see Fig. 3 and Materials and Methods). We find that this EGFP-F condensation is a quantitative and reliable correlate of cellular collapse (see Materials and Methods).

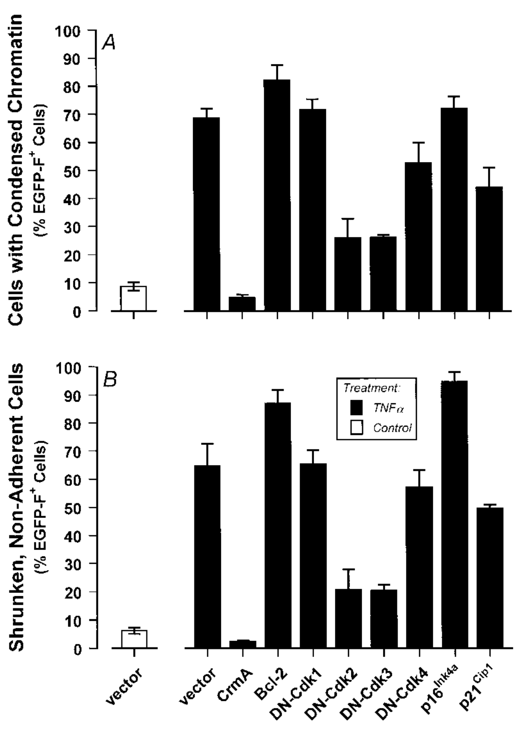


*Figure 5.* The inhibition of Cdk2 activity prevents TNF-α–induced cell death manifestations. Cells transfected with the indicated vectors were treated with 10 ng TNF-α in the presence of 3 μg/ml of cycloheximide (as in Fig. 3; ■), or left untreated (□). After 7 h, the percentages of transfected (EGFP-F$^+$) cells with condensed chromatin (A) and with shrunken, nonadherent morphology, as assessed both by phase contrast microscopy and the condensation of EGFP-F staining (B) were quantified. The data shown represent one of three replicate transfections, where each sample is the average of three microscopic fields of at least 100 EGFP-F$^+$ cells each.

Remarkably, not only chromatin condensation but also cellular rounding, EGFP-F condensation, and the loss of adherence are blocked by Cdk inhibition (see Fig. 3). Fig. 5 demonstrates the concordance of these distinct aspects of the cell death process. In this case, where cells were treated with TNF-α, CrmA but not Bcl-2 prevented cellular as well as chromatin condensation. The inhibition of caspases by the expression of p35 also abolished death and condensation (data not shown). Inhibition of Cdk2 activity comparably inhibited nuclear events and cell shrinkage equivalently, while the expression of either DN-Cdk1 or DN-Cdk4 exerted only minor effects. The expression of CKIs targeting Cdk2 similarly was effective in preventing condensation. In contrast, and as suggested in the analysis of chromatin condensation (Figs. 4 and 5 A), transfection of p16$^{Ink4a}$ itself elicits cell death in these transformed cells (Sandig et al., 1997; Schreiber et al., 1998). In these transient experiments, expression of EGFP-F could be followed for at least 5 d. DN-Cdk2 and DN-Cdk3 transfectants remained intact, adherent, and uncondensed throughout this period; their continued ability to exclude propidium iodide (data not shown) confirmed their integrity. Indeed, as long as EGFP-F expression was detected, the ability of the DN-Cdks to prevent nuclear and cellular condensation was unabated. In this regard, the blockade of death-associated events by DN-Cdk2 and DN-Cdk3 was no less complete than the death-sparing effect of CrmA.

We have observed the strict correlation of chromatin condensation and cellular collapse, and the sparing of both by Cdk inhibition, after treatment with other suicidal stimuli including staurosporine (data not shown). That Cdk activity plays a requisite role in death-associated chromatin condensation provides support for the hypothesis that parallels may exist between the cell death– and cell cycle–related processes. These data, moreover, are consistent with the notion that the induction of chromatin condensation in response to a death signal is intimately linked to other manifestations of cellular disintegration, and extend our hypothesis that caspase-dependent Cdk activity may be an effector of death.

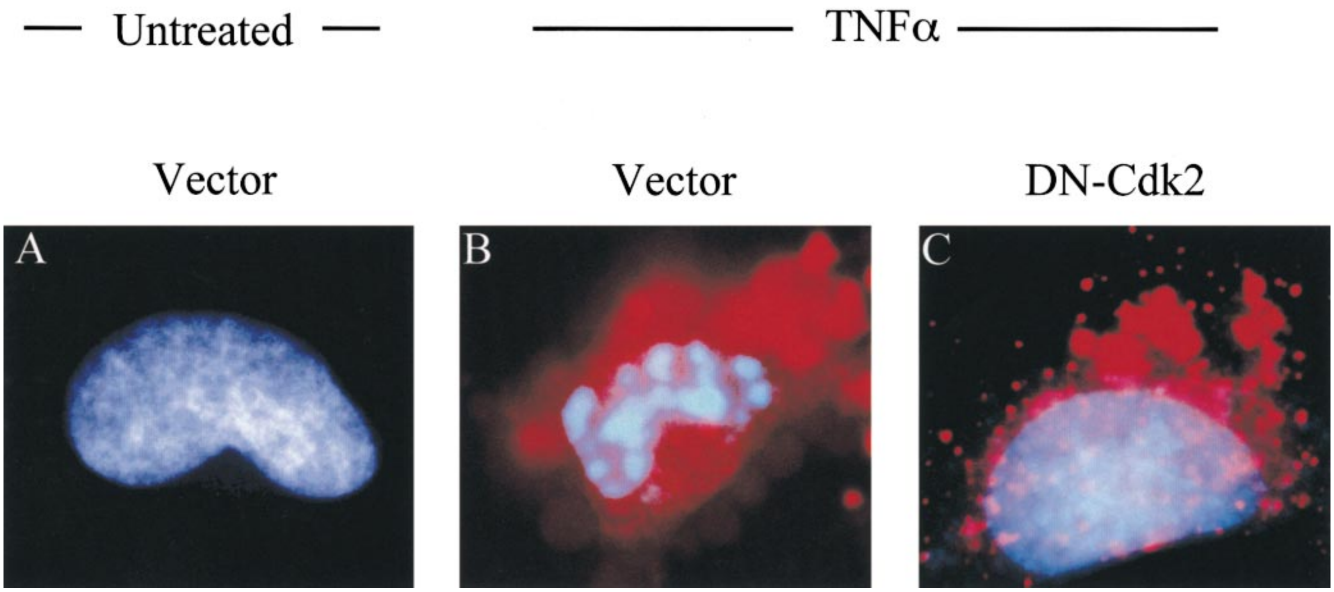


*Figure 6.* Death-associated caspase activity and chromatin condensation, visualized in vivo, are dissociated by DN-Cdk2 expression. Cells cotransfected with EGFP-F and empty vector (A and B) or DN-Cdk2 (C) were left untreated (A) or treated for 4 h with TNF-α (B and C). Cells then were analyzed microscopically for nuclear condensation (Hoechst 33342 staining; blue), effector caspase activity (in vivo fluorogenic DEVD peptide substrate, PhiPhiLux-$G_2D_2$; red), and expression of EGFP-F (green, not shown). Each of the cells shown was EGFP-$F^+$.

### *Cdks Act Downstream of Caspase Activation As Requisite Effectors of Cell Death Events*

That Cdks, acting to promote cell cycle progression, can modulate cellular susceptibility to death (Galaktionov et al., 1996; Luo et al., 1996; Hakem et al., 1999) raises the possibility that the abolition of cellular condensation resulting from the inhibition of Cdk activity might reflect resistance to the induction of death conferred by cell cycle arrest. If so, alternative means of arresting cells should mimic this death resistance. HeLa cells were arrested at the $G_1$/S boundary using a double thymidine block (Fig. 2 F) or at an earlier stage in $G_1$ by serum starvation (data not shown). Unlike transfectants that are arrested by DN-Cdk2 expression (Fig. 2 C), thymidine-arrested and serum-starved cells retained susceptibility to TNF-α– and staurosporine-induced chromatin condensation and death (Figs. 4 and 5, and data not shown). These results demonstrate that cell cycle arrest per se is not sufficient to inhibit death and suggest that the sparing effects of DN-Cdks do not relate simply to the restriction of cells in an insensitive compartment of the cell cycle.

The more definitive question is whether DN-Cdks exert their sparing effects by targeting caspase-dependent Cdk activity. If Cdk inhibition alters late steps of the death pathway but not its initiation, events upstream of Cdk activation should ensue unimpaired. We took two different approaches to test whether effector caspases are activated in transfectants expressing DN-Cdks. We employed an in vivo fluorogenic DEVD peptide substrate (PhiPhiLux-$G_2D_2$; Packard et al., 1996) to probe caspase 3–like caspase activity microscopically and cytofluorimetrically in EGFP-$F^+$ cells (see Materials and Methods). We also tested biochemically whether effector caspase activity was able to cleave a cotransfected substrate protein.

The in vivo fluorogenic substrate revealed effector caspase activity in cells induced to die by TNF-α and by staurosporine, but not in untreated controls (Figs. 6 and 7). The mean fluorescence intensity of the PhiPhiLux-$G_2D_2$ signal (580 nm) in caspase-positive cells typically was seven to eightfold greater than that of caspase-negative control cells; this compares with the ~20-fold peak induction in total DEVD-specific activity seen in vitro (Fig. 1 A). The reduction in the number of caspase-positive cells resulting from the transfection of caspase inhibitors CrmA (Fig. 7) and p35 (data not shown) confirms the validity of this assay, and the cytoplasmic localization of the fluorescent signal (Fig. 6) agrees with our previous biochemical fractionation studies (Harvey et al., 1998). Strikingly, death stimuli still induced caspase activity in cells transfected with DN-Cdk2 or DN-Cdk3 (Figs. 6 and 7). Caspase activity in these transfectants, assessed by PhiPhi-Lux-$G_2D_2$ signal intensity, was unaltered relative to control (e.g., vector only) transfectants.

The analysis of caspase-dependent protein cleavage gave results entirely consistent. Several recent reports have documented the caspase-dependent cleavage in dying human cells of the CKIs, $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ (Gervais et al., 1998; Levkau et al., 1998; Zhang et al., 1999). The cleavage by caspase 3 of $p21^{Cip1/Waf1}$ at $AEEDHVD^{112}LSL$ results in 14- and 7-kD peptides that separate the $NH_2$-terminal cyclin/Cdk interacting domain from the COOH-terminal PCNA interacting domain (Chen et al., 1995; Adams et al., 1996). We have observed that deletions within the $NH_2$-terminal region of $p21^{Cip1/Waf1}$ abrogate the death-inhibitory activity of the molecule without altering its susceptibility to caspase cleavage. A $p21^{Cip1/Waf1}$ mutant, deleted for amino acids 17 through 52, and fused in-frame to a hemagglutinin (HA) epitope tag,

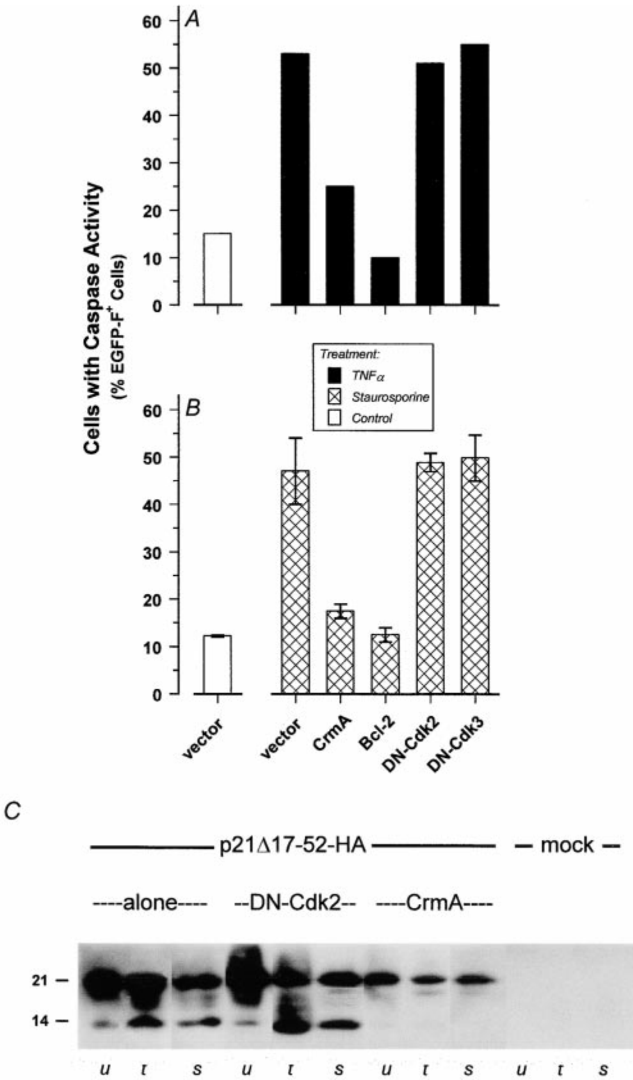


*Figure 7.* DN-Cdk2 blocks cell death events through its action downstream of caspase activation. The induction of effector caspase activity was assessed with the in vivo fluorogenic DEVD peptide substrate PhiPhiLux-$G_2D_2$ in EGFP-$F^+$ transfectants by dual color cytofluorimetry. At least 5,000 EGFP-$F^+$ cells were analyzed after 4 h of treatment with TNF-α (■; A) or staurosporine (▩; B) and in untreated controls (□). PhiPhiLux-$G_2D_2$ was included during the last hour of incubation (see Materials and Methods). Independently, caspase cleavage of cotransfected and epitope-tagged p21$^{Cip1/Waf1}$ mutant was monitored by Western blotting of cellular extracts (C). Extracts were prepared from cells transfected with p21$^{Cip1/Waf1}$Δ17-52-HA alone or together with DN-Cdk2 or CrmA, and from mock-transfected cells. Cells were left untreated (*u*) or were treated for 4 h with TNF-α (*t*) or staurosporine (*s*). Both the intact p21$^{Cip1/Waf1}$Δ17-52-HA molecule and its $NH_2$-terminal 14-kD caspase cleavage fragment are visualized here with an antibody specific for a p21$^{Cip1/Waf1}$ peptide (amino acids 58–77). Note that endogenous wild-type p21$^{Cip1/Waf1}$ is not visible at this level of sensitivity.

provided an excellent probe with which to test caspase function in transfected cells. While p21$^{Cip1/Waf1}$Δ17-52-HA itself did not prevent cell death, it had no effect on the ability of DN-Cdk2 to spare cells from death-associated events induced by TNF-α or by staurosporine (Harvey, K.J., D. Lukovic, and D.S. Ucker, manuscript in preparation).

As shown in Fig. 7 C, caspase-dependent cleavage of the mutant p21$^{Cip1/Waf1}$ molecule ensued even when cells were spared by DN-Cdk2 expression. The ~14-kD fragment, revealed with an antibody specific for an $NH_2$-terminal p21$^{Cip1/Waf1}$ peptide, is diagnostic of this cleavage. This cleavage product was visible even in untreated transfectants, reflecting the background of dead cells in transfectant populations (see Materials and Methods). Death stimuli triggered a substantial increase in the relative abundance of the ~14-kD product, and a corresponding diminution of the full-length molecule. Cleavage of p21$^{Cip1/Waf1}$ was triggered identically in the DN-Cdk2 transfectants (Fig. 7 C). In contrast, cell death inhibitors that act within the caspase cascade, such as CrmA, eliminated even the background of p21$^{Cip1/Waf1}$ cleavage (Fig. 7 C). We observed a similar correspondence between the full-length p21$^{Cip1/Waf1}$Δ17-52-HA band and the complementary 7-kD COOH-terminal cleavage fragment in parallel blots developed with an antibody specific for the HA epitope (data not shown). The 7-kD fragment is much less abundant, however, reflecting apparent degradation by non-caspase proteases at COOH-terminal sites. These data argue that requisite Cdk function is downstream of the caspase cascade, consistent with the view that it is the Cdk activity we characterized as caspase dependent whose function is essential for death-associated cellular condensation. The proteolytic caspase cascade, while necessary, is not sufficient for the full physiological cell death program.

### *Dissociation of Mitochondrial and Other Direct Effects of Caspases from Cdk-dependent Death Events*

Just as caspases remain active in cells in which down-

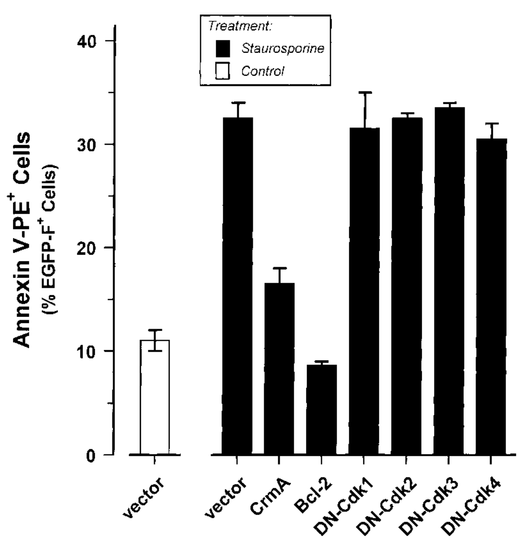


*Figure 8.* The caspase-dependent loss of membrane phospholipid asymmetry is not blocked by Cdk inhibition. The loss of plasma membrane phosphatidylserine asymmetry was assessed as the extent of binding of annexin V-PE to EGFP-$F^+$ transfectants by dual color cytofluorimetry. At least 5,000 EGFP-$F^+$ cells were analyzed after 4 h of staurosporine treatment (■) and in untreated controls (□).

stream Cdk activity is inhibited, caspase-proximal events that are upstream or independent of Cdks would be predicted to proceed unimpaired in DN-Cdk transfectants. The loss of the asymmetric distribution of phosphatidylserine between the inner and outer leaflet of the cytoplasmic membrane is associated with cell death, and may be involved in the recognition of dying cells by phagocytic macrophage (Fadok et al., 1992). We examined the loss of plasma membrane phosphatidylserine asymmetry induced by staurosporine (Fig. 8) in DN-Cdk transfectants. Externalization of phosphatidylserine was measured cytofluorimetrically by the extent of binding of PE-conjugated annexin V on EGFP-F$^{+}$ cells. Bcl-2 and CrmA transfectants were not induced to become annexin V-positive after treatment with staurosporine (Fig. 8), consistent with the action of these death inhibitors to block caspase activity (Fig. 7). In contrast, and as seen with the induction of caspase activity, cells spared from death-associated condensation by transfected DN-Cdk2 or DN-Cdk3 were unimpeded in their ability to expose phosphatidylserine on the outer leaflet of the cytoplasmic membrane (Fig. 8).

The disruption of mitochondrial integrity has been implicated as a central event in the physiological cell death process (Marchetti et al., 1996; Zamzami et al., 1996; Susin et al., 1997). The release of cytochrome *c* from the intramembrane space may be an early determinative step, and the loss of mitochondrial membrane potential ($\Delta\Psi_m$) and liberation of another death mediator, apoptosis inducing factor (AIF), have been reported to occur subsequently, possibly in a caspase-dependent manner (Marchetti et al., 1996; Zamzami et al., 1996; Susin et al., 1997, 1999). We measured $\Delta\Psi_m$ by monitoring the intensity of fluorescence of the mitochondrial membrane potential-specific probe tetramethylrhodamine ethyl ester (TMRE; Farkas et al., 1989) in EGFP-F$^{+}$ transfectants.

We observed profound mitochondrial depolarization in HeLa cells dying in response to treatment with staurosporine or TNF-$\alpha$. The magnitude of this drop in mitochondrial membrane potential was equal in intensity to that caused by pharmacologic depolarization triggered by the mitochondria-specific ionophore carbonyl cyanide *m*-chlorophenylhydrazone (ClCCP; data not shown). Transfectants expressing caspase inhibitors CrmA and p35, that were spared from TNF-$\alpha$– and staurosporine-mediated death, did not suffer this loss of membrane potential (Fig. 9, A and B, and data not shown). In contrast, the loss of mitochondrial membrane potential induced by staurosporine or TNF-$\alpha$ was not prevented in DN-Cdk2 and DN-Cdk3 transfectants (Fig. 9, A and B). As seen in the analysis of caspase activity, the inhibition of caspase-dependent Cdk activity by DN-Cdk transfection prevents cellular condensation without affecting upstream events.

Direct depolarization of mitochondria with ClCCP also results in a typical apoptotic cell death (Zamzami et al., 1996). That this death response is not due simply to $\Delta\Psi_m$ is suggested by the observation that death is induced only at a dose of ClCCP (30–100 $\mu$M) at least 10-fold higher than the minimal dose needed for full depolarization (data not shown), and by the ability of Bcl-2 to inhibit ClCCP-induced death (Zamzami et al., 1996; Fig. 9 C). As demonstrated by the death-sparing effect of p35 (Fig. 9 C), ClCCP induced a caspase-dependent death process. In contrast to p35, CrmA did not spare ClCCP-induced death (Fig. 9 C); the ability of this mitochondrial agent to initiate a death response appears to obviate the requirement for upstream caspase activity. Here too, inhibition of Cdk activity downstream of the caspase cascade attenuated chromatin condensation, even in the absence of mitochondrial membrane potential (Fig. 9 C and data not shown). These

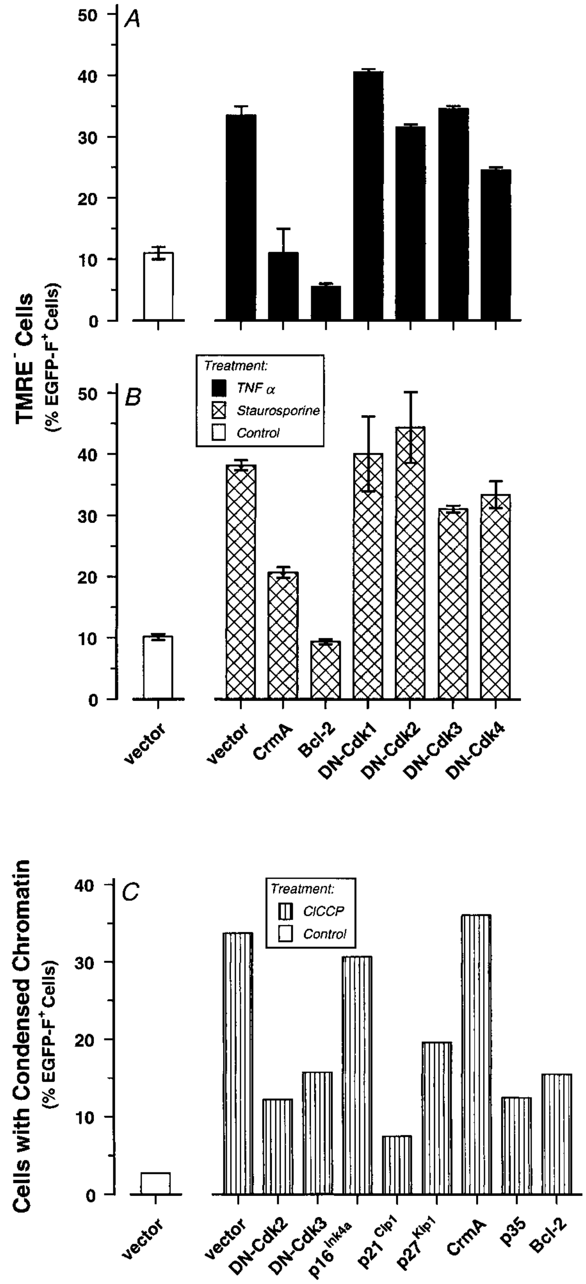


*Figure 9.* The inhibition of Cdk2 blocks chromatin condensation independent of mitochondrial disruption. Loss of mitochondrial membrane potential ($\Delta\Psi_m$) was assessed by the intensity of TMRE fluorescence in EGFP-F$^{+}$ transfectants by dual color cytofluorimetry. At least 5,000 EGFP-F$^{+}$ cells were analyzed after 4 h of treatment with TNF-$\alpha$ (■; A) or staurosporine (▩; B) and in untreated controls (□). Chromatin condensation was assessed after 9 h of treatment with 200 $\mu$M of ClCCP (▥) and in untreated controls (□) as described in Fig. 3 (C).

data reveal that the action of Cdks downstream of caspases represents a conserved phase of the cell death process, closely linked with actual lethality.

## Discussion

### Mapping Cdk Function Identifies a Point of Cell Death Commitment Downstream of Caspases

The results of our experiments testing the necessity of Cdk activity in cell death are striking. As in other cells, death-associated Cdk2 activity is induced in a caspase-dependent manner in HeLa cells. We imagined that the inhibition of Cdk2 activity might prevent apoptotic chromatin condensation. Indeed, transfection of DN-Cdk2 does prevent the chromatin condensation triggered by different suicidal stimuli, including TNF-α and staurosporine. CKIs that bind Cdk2, such as $p27^{Kip1}$ and $p21^{Cip1/Waf1}$, exert a similar inhibitory effect. Cdk2 inhibition prevents death-associated chromatin condensation as effectively as the established death-inhibitory gene products Bcl-2, CrmA, and p35. The inhibition afforded by DN-Cdk3 may indicate an additional role for Cdk3 within the cell death process. Alternatively, DN-Cdk3 may inhibit Cdk2 indirectly by virtue of its ability to bind cyclin A (Meikrantz and Schlegel, 1996). This would be consistent with the participation of cyclin A in the death-associated Cdk2 complex (see Fig. 1 C; Meikrantz et al., 1994; Harvey et al., 1998).

A necessary role for Cdks in the physiological cell death process had been suggested by a number of studies, especially using anti-sense and dominant negative approaches, to functionally ablate specific Cdk activity (Shi et al., 1994; Fotedar et al., 1995; Meikrantz and Schlegel, 1996; Park et al., 1998). However, those studies could not distinguish where within a death pathway required Cdks might function. Cell cycle transit is a necessary component of several death responses, including *myc*- and Cdc25A-dependent factor deprivation (Galaktionov et al., 1996) and activation-driven lymphocyte deletion (Fournel et al., 1996; Radvanyi et al., 1996; Hakem et al., 1999). In these particular cases of cell death, Cdks serve an essential function in a modulatory capacity, upstream of Bcl-2 and the caspase cascade. The recent characterization of the mammalian inhibitor of apoptosis (IAP), *survivin*, as a mitotic checkpoint protein suggests that cellular sensing mechanisms exist to signal and initiate the physiological cell death process directly upon detection of aberrant Cdk activation or irregular cell cycle progression (Li et al., 1998).

This modulatory Cdk function is distinct from the role of Cdks that we have characterized here. The results of our experiments indicate that transient ablation of Cdk activity does not alter the induction steps of the cell death pathway that precede Cdk activation, including downstream effector caspase activity. That DN-Cdk transfectants still exhibit a subset of the caspase-dependent features typically associated with cell death indicates that caspases indeed are active, and that the activation of caspases alone is not sufficient to effect the full physiological cell death program.

We previously attributed a nonlethal regulatory function to upstream caspase activity, based on the ability of Bcl-2 to spare cells from death without interfering with that activity (Harvey et al., 1998). Our new data similarly ascribe an essential role to downstream caspase activity that is not entirely sufficient. That Cdk activity is dependent on downstream caspase activity and is necessary for death-associated events suggests that nuclear Cdk activity may be a critical lethal effector of the process, and that its caspase-dependent activation represents a point of commitment in the process of cell death. In recapitulating the functional ordering of death activities we accomplished previously with stable transfectant clones, these data confirm the efficacy of the transient transfection strategy employed here and demonstrate that the thematically conserved pathway of death pertains in non-lymphoid cells as well.

### The Mechanism of Caspase-dependent Cdk Activation

Recent data support the view that the induction of death-associated Cdk activity may depend on the proteolytic destruction of negative regulators of Cdk activity by caspases. The caspase-dependent cleavage of Cdc27, a necessary component of the complex that targets cyclins for ubiquitin-dependent elimination, has been described in human Jurkat T cells (Zhou et al., 1998). Cdc27 destruction could be responsible for the elevated levels of cyclin A that we have described previously in dying lymphocytes (Harvey et al., 1998); it is notable that forced cyclin A overexpression is lethal (Meikrantz and Schlegel, 1996; Harvey, K.J., unpublished observations). That we have not observed changes in the gross levels of cyclin A or Cdk2 mRNA levels in dying cells (Harvey et al., 1998) suggests, more generally, that the induction of death-associated Cdk activity occurs primarily on a posttranslational level (Chang, S.H., K.J. Harvey, and D.S. Ucker, manuscript in preparation).

The caspase-dependent destruction of Wee1, the Cdk-inhibitory kinase, also has been noted in Jurkat cells (Zhou et al., 1998). However, we have not observed corresponding phosphorylation-specific mobility changes in death-associated Cdk2 in dying lymphocytes or HeLa cells (Harvey et al., 1998; Harvey, K.J., unpublished observation). The suggestion that caspase-mediated cleavage of the CKIs $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ (Gervais et al., 1998; Levkau et al., 1998; Zhang et al., 1999) could be an important mechanism for death-associated Cdk activation is not inconsistent with the abilities of overexpressed $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ to inhibit cell death responses. We note that the human $p21^{Cip1/Waf1}$ cleavage site is not present in the murine $p21^{Cip1/Waf1}$ (APEDHVA$^{110}$LSL).

The sparing effects exerted in HeLa cells by the overexpression of $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ contrasts with results described by Levkau et al. (1998), in which factor-deprived HUVEC cells were spared only by ectopic expression of a noncleavable mutant of $p21^{Cip1/Waf1}$. In our experiments, we have observed that caspase-cleavable CKIs are effective death inhibitors. We have found, moreover, that these cleavable CKIs are able to competitively inhibit caspase activity in certain cases, suggesting that CKIs may act at multiple levels to inhibit cell death (Harvey, K.D., D. Lukovic, and D.S. Ucker, manuscript in preparation). It is intriguing that the death-sparing efficacy of $p21^{Cip1/Waf1}$ correlates with that of Bcl-2; this may pertain to the relative involvement of receptor-coupled and mitochondria-dependent caspase cascades in different death responses. p21$^{Cip1/Waf1}$ is more effective at inhibiting death induced by staurosporine than by TNF-α, although the inhibition of these two responses by DN-Cdk2 is equivalent. That Bcl-2 blocks TNF-α–induced mitochondrial depolarization and caspase 3–specific activity but not death suggests that, in HeLa cells, TNF-α triggers a lethal caspase cascade directly through its receptor (presumably coupled to caspase 8), and that a secondary, mitochondria-dependent and Bcl-2 inhibitable cascade that involves caspases 9 and 3 is initiated but is not essential. In all cases, however, the activation of caspases is insufficient to complete the full cell death program.

### *The Role of Cdks in Effecting Cell Death*

The precise function of caspase-dependent Cdk activity in effecting apoptotic events remains to be determined. Whereas the link of death-associated Cdk activation and chromatin condensation fulfills the hypothesis that the processes of cell death and division may be related mechanistically, cell death is not simply an out-of phase mitotic catastrophe: mitotic cyclin B/Cdk1 complexes are not active (Harvey et al., 1998) and other markers of mitosis, including typical mitotic histone H3 phosphorylation, have not been detected in dying cells (Hendzel et al., 1998; Zhou et al., 1998). On the other hand, the insufficiency of caspases in this regard suggests that neither is chromatin condensation, simply the consequence of the proteolytic activation of endonucleases such as CAD (Enari et al., 1998; Sakahira et al., 1998), for instance. Of course, genome digestion is dispensable for physiological cell death (Ucker et al., 1992; Samejima et al., 1998; Zhang et al., 1998), and many cells do not degrade their genomes extensively when they die (Gromkowski et al., 1986; Howell and Martz, 1987; Ucker et al., 1992). We have not detected extensive DNA degradation in HeLa cells, as assessed by the appearance of a population of cells with sub-diploid DNA content or by measures of DNA strand breaks (Harvey, K.J., unpublished data). It remains to be determined whether caspase-dependent genome digestion can ensue in the absence of chromatin condensation, and whether other Cdk-regulated processes are necessary.

More generally, the control of the intracellular localization, particularly to the nucleus, of relevant molecules during the cell death process (Yasuhara et al., 1997) may be dependent on Cdk activity. The nuclear recruitment of cyclin A itself appears to be a caspase-dependent phenomenon (Harvey et al., 1998). We hypothesize that the dissolution of the nuclear envelope, including the solubilization of the lamin meshwork that resides within that envelope (Ucker et al., 1992; Lazebnik et al., 1995; Rao et al., 1996), depends primarily on the activity of nuclear Cdks and not on extranuclear caspases. It will be of value to examine further the involvement of other cell cycle components within the cell death process. Condensins, Cdk regulable complexes both necessary and sufficient for mitotic chromatin condensation in cell free assays (Hirano et al., 1997; Kimura et al., 1998), are of obvious interest. Sites for activating phosphorylation by Cdk1 that have been identified in one condensin subunit (Kimura et al., 1998) may be targeted by death-associated Cdk2, for example. Finally, the insufficiency of caspases suggests that alternative means of activating Cdks might be able to trigger physiological cell deaths that are caspase-independent (Heald et al., 1993; Blasina et al., 1997).

### *Defining Death*

We have refined our mapping of the cell death pathway using end-point markers that include chromatin condensation and the loss of cellular adhesion to substrate. In this context, caspase-proximal events are not sufficient to assure the full physiological cell death program. An operational definition of lethality, and the point of death commitment, is circumscribed by the measures of death employed, of course; it is clear that the demise of a cell must be inevitable, albeit not necessarily apoptotic, when its mitochondria are depolarized and abundant proteolytic activity is present. Still, the precise issue is whether cellular disintegration in the absence of caspase-dependent Cdk activity represents a concerted physiological process. If the externalization of phosphatidylserine were to be all that is needed for phagocytic recognition, for example, then activation of caspases upstream of Cdks would represent death commitment. Our experiments to date indicate that the loss of asymmetry of phosphatidylserine distribution is not sufficient for macrophage recognition (Cocco, R.E., and D.S. Ucker, manuscript in preparation). The ultimate purpose of physiological cell death is to facilitate the noninflammatory clearance of inappropriate cells (Ucker, 1997). This must be the criterion by which the point of death commitment is assessed finally.

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