Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 6785–6790, June 1998
Cell Biology

Caspase-dependent activation of cyclin-dependent kinases during Fas-induced apoptosis in Jurkat cells

BIN-BING ZHOU, HONGLIN LI, JUNYING YUAN, AND MARC W. KIRSCHNER

Department of Cell Biology, Harvard Medical School, Boston, MA 02115

Contributed by Marc W. Kirschner, April 7, 1998

ABSTRACT **The activation of cyclin-dependent kinases (cdks) has been implicated in apoptosis induced by various stimuli. We find that the Fas-induced activation of cdc2 and cdk2 in Jurkat cells is not dependent on protein synthesis, which is shut down very early during apoptosis before caspase-3 activation. Instead, activation of these kinases seems to result from both a rapid cleavage of Wee1 (an inhibitory kinase of cdc2 and cdk2) and inactivation of anaphase-promoting complex (the specific system for cyclin degradation), in which CDC27 homolog is cleaved during apoptosis. Both Wee1 and CDC27 are shown to be substrates of the caspase-3-like protease. Although cdk activities are elevated during Fas-induced apoptosis in Jurkat cells, general activation of the mitotic processes does not occur. Our results do not support the idea that apoptosis is simply an aberrant mitosis but, instead, suggest that a subset of mitotic mechanisms plays an important role in apoptosis through elevated cdk activities.**

Apoptosis, or programmed cell death, is an evolutionarily conserved process essential to the normal development and homeostasis of multicellular organisms. During apoptosis, members of the caspase protease family are activated and the cells undergo rapid death (1–3). Like mitotic cells, apoptotic cells also have condensed chromatin, rounded morphology, and dispersed nuclear membranes (1). However, it is unclear whether the morphological similarities between mitosis and apoptosis reflect common underlying mechanisms. Supporting such a linkage is the evidence that cdc2 may be a required component of the apoptosis pathway (4). Induction of apoptosis by various stimuli has been shown to require activation of either cdc2 or cdk2 (4–6), whereas forced expression of cyclin-dependent kinase (cdk) inhibitors during myocyte differentiation blocks apoptosis (7). Increased expression of a cdk activator, cdc25A, was also shown to be required for c-myc-induced apoptosis (8). Finally, it is of interest that $p57^{KIP2}$ knockout mice, defective in the cdk inhibitor $p57^{KIP2}$, show abnormalities caused by increased apoptosis (9, 10).

Several research groups have shown that cdks are activated during apoptosis (4–6), although the mechanism is unclear. During the cell cycle, cdc2 and cdk2 activities are regulated on different levels. On one level, cyclin synthesis activates cdk activity, whereas ubiquitin-dependent cyclin degradation inactivates cdk activity (11). The specific ubiquitin ligase, anaphase-promoting complex (APC) or cyclosome, catalyzes the rate-limiting step of cyclin A and B degradation (11). On a different level, Wee1 and Myt-1 introduce inhibitory phosphorylations, which can be removed by cdc25 (12). Additional negative regulation of cdks is achieved through association with cdk inhibitors (13). The role of these different levels of regulation during apoptosis is unknown.

To study the mechanism of cdc2 and cdk2 activation in apoptotic cells, we examined cyclin synthesis, cyclin degradation, and posttranslational modifications of cdc2 and cdk2 during Fas-induced apoptosis in Jurkat cells. We find that Fas induction activates cdc2 and cdk2, despite a potential loss of these proteins caused by the very rapid drop in the capacity for protein synthesis. Activation of these kinases seems to result from the maintenance of cyclin levels by rapid inactivation of APC, through a caspase-dependent cleavage of one of its subunits, and tyrosine dephosphorylation of cdks caused by a cleavage of the inhibitory kinase Wee1.

MATERIALS AND METHODS

Reagents. Staurosporine, 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and other molecular biology grade reagents were purchased from Sigma unless specified. *N*-Acetyl-Asp-Glu-Val-Asp-aldehyde (DEVD-CHO) and *N*-Acetyl-Tyr-Val-Ala-Asp-chloromethylketone (YVAD-CMK) were obtained from Bachem.

Immunoprecipitation and *In Vitro* Kinase Assays. Whole cell lysates were made in Nonidet P-40 lysis buffer (50 mM Tris, pH 7.7, containing 150 mM NaCl/0.5% Nonidet P-40/1 mM DTT/10% glycerol and protease inhibitors) and were immunoprecipitated with various antibodies (anti-cdc2, GIBCO/BRL; anti-cdk2, anti-cyclin A, and anti-cyclin B, Santa Cruz Biotechnology; and anti-Plk-1, Zymed). Plk-1 activity was measured as described (14). H1 kinase assays were performed on immunoprecipitates as described (15). Quantitation of H1 kinase activity was obtained by using IMAGEQUANT program (Molecular Dynamics), and the relative values were converted to activities normalized by total conversion of substrate.

[^{35}S]Methionine Labeling and Pulse–Chase Experiment. To measure protein synthesis, Jurkat cells were starved for 1 hr and then labeled with [^{35}S]methionine (DuPont/NEN) for 20 min. Labeled proteins were detected by phosphorimaging after SDS/PAGE.

To measure the half-life of cyclins, pulse–chase experiment was performed. Jurkat cells were labeled with [^{35}S]methionine (0.08 mM methionine) for 20 min and quenched with cold methionine to 5 mM final concentration. At different time points after quenching, total cell extracts were made in Nonidet P-40 lysis buffer. Anti-cyclin A and anti-cyclin B immunoprecipitates were subjected to SDS/PAGE, and the labeled cyclin A or B were detected by phosphorimaging. The relative level of labeled cyclin A or B was quantitated by using the IMAGEQUANT program. The half-life of cyclin A and B was estimated by fitting data to first-order kinetics. We observed

Abbreviations: APC, anaphase-promoting complex; Cdks, cyclin-dependent kinases; MTT, 3-(4, 5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide; DEVD-CHO, *N*-Acetyl-Asp-Glu-Val-Asp-aldehyde; Plk-1, polo-like kinase-1; YVAD-CMK, *N*-Acetyl-Tyr-Val-Ala-Asp-chloromethylketone; PARP, poly(ADP-ribose) polymerase; GST, glutathione *S*-transferase;

*To whom reprint requests should be addressed. e-mail: marc@hms.harvard.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

that ≈10–20% of cyclin A and B are relatively stable, presumably a result of using asynchronous cells; therefore, time points later than 160 min were not included in the quantitations.

Polysome Profile. Approximately 2×10^7 Jurkat cells were collected by centrifugation, quickly placed in ice–ethanol bath for 30 sec, and then placed on ice. The pellet was resuspended in ≈200 μl of extract buffer (0.05 M Tris·HCl, pH 7.4/0.25 M KCl/0.05 M $MgCl_2$/0.5 unit/μl RNAsin (Promega)/0.2% Triton X-100) and vortexed for 5 sec, returned on ice for 2 min, and then vortexed again. After centrifugation at $20,000 \times g$ for 10 min, supernatants were layered on 12 ml of linear gradients (0.5–1.5 M sucrose in the extract buffer), and centrifuged for 1.5 hr at 4°C in SW40 rotor (Beckman) at 40,000 rpm and collected with an ISCO gradient fractionator. Polysome profiles were recorded by using the UV analyzer in a Pharmacia fast protein liquid chromatography (FPLC) system.

Immunoblot Analysis. Primary antibodies used in our immunoblot analysis include anti-poly(ADP ribose) polymerase (PARP) (PharMingen), anti-CDC27 (generous gift of P. Hieter, University of British Columbia), anti-Wee1 (UBI), DM1α anti-tubulin, MPM-2 antibody (generous gift of J. Kung, M. D. Anderson Cancer Center), anti-phosphorylated tyrosine antibody (UBI) and a phospho-specific cdc2 (cdk2) antibody against Tyr-15 (NEB). Most of the other primary antibodies used in the paper were purchased from Santa Cruz Biotech.

***In Vitro* Cleavage Assays.** *In vitro* translations of ^{35}S-labeled proteins were carried out by using the TNT-coupled transcription/translation kit (Promega) in the presence of [^{35}S]methionine (Amersham). ^{35}S-labeled proteins or immunopurified proteins were incubated with either bacterial lysates or purified caspase in reaction buffer (20 mM Tris·HCl, pH 7.5/10 mM DTT/0.1 mM EDTA/1 mM phenylmethylsulfonyl fluoride/0.5 μg/ml aprotinin) for 2 hr at 30°C.

APC Activity Assay. To assess APC activities from various extracts, APC was immunopurified and assayed as described (16).

Cdc25 and Wee1 Assays. Cdc 25 activity was measured as described (17). The Thr161 phosphorylated cdc2/glutathione *S*-transferase (GST) cyclin B substrate for the Wee1 assay was prepared by adding 20 μg of GST cyclin B to 400 μl of cycloheximide treated *Xenopus* interphase extract for 30 min at room temperature and isolating the complex on glutathione agarose beads. The Thr161 monophosphorylated cdc2/cyclin B beads were further treated with unfertilized *Xenopus* egg extract (15) in the presence of 1 μM okadaic acid to remove residual phosphorylations at Tyr15 and Thr14 positions. The beads were then washed extensively as described (17). To measure Wee1 activity, 45-μl samples were incubated with 30 μl of active cdc2/cyclin B beads for 30 min at room temperature in the presence of ATP.

Immunofluoresence. Control and apoptotic Jurkat cells were fixed with 3% formaldehyde in PBS for 15 min and washed three times with PBS containing 0.1% Nonidet P-40. Fixed cells were then spun down to the slide surface by using a Cytospin (Shandon, Pittsburgh) at 12,000 rpm for 5 min. Microtubules were stained with anti-tubulin antibody (DM1α) followed by a rhodamine-conjugated secondary antibody (Jackson ImmunoResearch). Images were obtained by using confocal microscopy (Axiovert 135 TV microscope, Zeiss).

RESULTS

Increase in cdc2 and cdk2 Activity During Fas-Induced Apoptosis in Jurkat Cells. As shown in Table 1, both cdc2 and cdk2 are activated in the early stage of Fas-induced apoptosis in Jurkat cells. Cyclin A/cdc2 and cyclin A/cdk2 are responsible for the largest increase in cdk activities. Cyclin A-dependent kinases have been implicated in apoptosis because such kinases have been shown previously to be stimulated by many apoptosis-inducing agents (18, 19). Reciprocally, recombinant cyclin A1/cdk2 induces the formation of apoptotic nuclei in *Xenopus* egg extracts (21). Cyclin B/cdc2 activity also increases during Fas-induced apoptosis, but not as dramatically as during mitosis, presumably reflecting the much higher level of cyclin B in mitosis (Table 1). During apoptosis, there is no increase in cyclin A or B levels (Fig. 4*C*). It has been shown previously that cdk activation is required for Fas-induced apoptosis in Jurkat cells (6). Jurkat cells transiently transfected with Wee1 kinase or dominant mutants of cdc2 and cdk2 have decreased level of apoptosis after Fas induction (6).

Table 1. H1 kinase activities of various immunoprecipitations from Jurkat cell extracts

Treatment	H1 kinase activity			
	cdc2	cdk2	Cyclin A	Cyclin B
−αFas	15 ± 6	24 ± 5	27 ± 6	4 ± 2
+αFas*	97 ± 22	51 ± 9	110 ± 27	10.1 ± 2
Nocadazole arrested†	91 ± 21	60 ± 10	121 ± 32	275 ± 53

Activities are reported in units of picomol of Pi per 30 min per milligram of cell extract at room temperature and presented as mean ± SD of triplicate determinations.
*Jurkat cells were treated with 10 ng/ml anti-Fas (7C11) for 6 hr, and the cell death percentage was 91% measured by MTT assay (20).
†Approximately 50% cells in mitosis as indicated by FACS analysis.

Protein Synthesis Shutdown Is an Early Event During Fas-Induced Apoptosis in Jurkat Cells. One potential distinction between mitosis and apoptosis is that apoptosis is independent of protein synthesis in many cases (22), whereas mitosis always requires *de novo* synthesis of cyclins to activate cdks. For this reason, we examined protein synthesis and particularly cyclin synthesis during Fas-induced apoptosis in Jurkat cells. As shown in Fig. 1*A*, the level of newly synthesized cyclin A and B labeled by [^{35}S]methionine decreased >95% in the early stages of apoptosis, although total cyclin A or B level was unchanged (data not shown). The decreased cyclin synthesis seems to have resulted from a decrease in general protein synthesis because the level of newly synthesized total proteins labeled by [^{35}S]methionine also declined dramatically (Fig. 1*A*). However, the measurement of protein synthesis rates from labeling kinetics is beset with difficulty in cells undergoing major physiological changes. During the first 2 hr after Fas induction, we found methionine uptake also decreased (data not shown); therefore, the observed decrease in labeling could be caused by decreased methionine uptake and increased amino acid turnover. To circumvent these problems and confirm the decline in general protein synthesis, we examined the profile of polysomes before and after Fas induction by using sucrose gradients. As shown in Fig. 1*B*, the polysome profile collapsed during apoptosis, indicating an almost total shutdown of protein synthesis. The kinetics of polysome disappearance was very rapid, occurring even before caspase-3 activation (Fig. 1*C*). Interestingly, the prior addition of caspase inhibitor YVAD-CMK (300 μM) did not prevent Fas-induced polysome disappearance, although it was quite effective in suppressing apoptosis (data not shown).

If there is a requirement of cdc2 or cdk2 activity during apoptosis (4–6), then the rapid and nearly complete loss of cyclin A and cyclin B synthesis may pose problems, particularly in the G_1 phase when continued APC activity maintains cyclin turnover (23, 24). We measured the half-life of cyclin A and cyclin B in asynchronous Jurkat cells by pulse–chase experiment and found that majority of cyclin B (>80%) has a half-life of ≈77 min whereas majority of cyclin A (>75%) has a half-life of ≈115 min. Although this result is an aggregate rate for all phases of the cell cycle, fluorescence-activated cell sorting (FACS) analysis revealed that 65% of the cells in the sample are in G_1 phase whereas <5% are in M phase. If cyclin A- or cyclin B-dependent activity was required for apoptosis, deg-

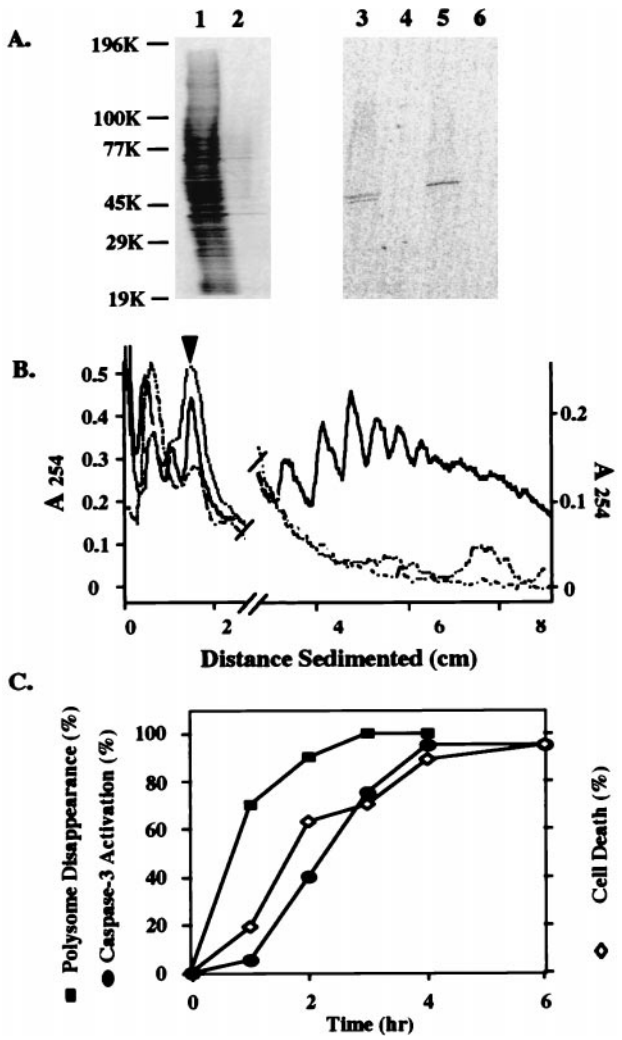

FIG. 1. Inhibition of protein synthesis during Fas-induced apoptosis in Jurkat cells. (*A*) Protein synthesis measured by [^{35}S]methionine labeling. Lanes: 1, control extract; 2, apoptotic extract (4-hr anti-Fas treatment); 3, anti-cyclin A immunoprecipitate from control extract; 4, anti-cyclin A immunoprecipitate from apoptotic extract; 5, anti-cyclin B immunoprecipitate from control extract; and 6, anti-cyclin B immunoprecipitate from apoptotic extract. (*B*) Polysome profiles. Solid line, control extract; long- and short-dashed line, control extract treated with staphylococcal nuclease (0.05 unit/ml); dashed line, apoptotic extract (4-hr anti-Fas treatment). The arrow head indicates the position of the monosome. (*C*) Kinetics of polysome disappearance during anti-Fas treatment compared with those of cell death rate measured by MTT assay (20) and caspase-3 activation (measured by disappearance of procaspase-3 and appearance of PARP cleavage product). Polysome disappearance was estimated by the total area underneath the polysome peaks.

radation of cyclins should be inhibited or cdk activity should be activated posttranslationally to compensate for the decreased synthesis of proteins.

A Subunit of APC and Wee1 Are Caspase Substrates. We investigated the posttranslational regulation of cdks during apoptosis by immunoblotting for various cell cycle regulators, namely: p57^{KIP2}, p27^{KIP1}, cdc25A, cdc25C, Myt-1, Wee1, CDC27 (APC3), CDC16 (APC6), Plk-1, APC7, APC2, APC5, BimE (APC1), p55 CDC, cyclin A, cyclin B, cdc2, and cdk2. Among them, only a subunit of APC, the human CDC27 homolog, and Wee1 showed evidence of complete cleavage during Fas-induced apoptosis in Jurkat cells (Fig. 2). These cleavages occurred early in apoptosis and paralleled the cleavage of PARP. The same cleavage patterns were obtained in staurosporine-induced apoptosis in Jurkat cells (Fig. 2) and in

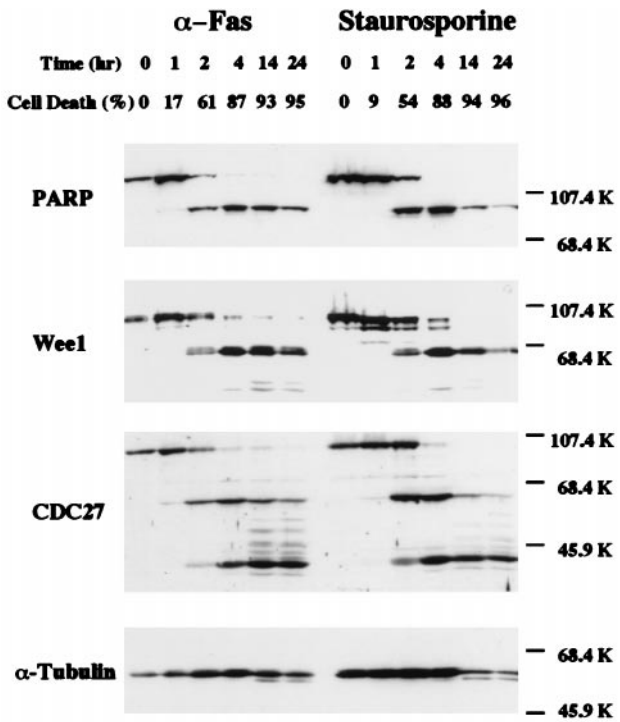

FIG. 2. The temporal profiles of PARP, Wee1 and CDC27 cleavages during apoptosis in Jurkat cells, with tubulin serving as a negative control. The percentage of cell death was measured by MTT assay (20); 2×10^6 Jurkat cells were treated with either 10 ng/ml anti-Fas antibody or 0.1 μM staurosporine for various time periods as indicated. Aliquots of the total cell lysates were subjected to SDS/PAGE, and immunoblotting was performed by using anti-PARP, anti-CDC27, anti-Wee1, and anti-tubulin antibodies.

tumor necrosis factorα-induced apoptosis in HeLa cells (data not shown). The cleavages of both CDC27 and Wee1 *in vivo* can be inhibited by DEVD-CHO, a specific inhibitor of caspase-3 (Fig. 3*A*). Similar cleavages were obtained *in vitro* when immunopurified APC and Wee1 were exposed to recombinant caspase-3 (Fig. 4*B* and data not shown) or when *in vitro*-translated CDC27 and Wee1 were exposed to recombinant caspase-3 (Fig. 3*B*). *In vitro*-translated Wee1 has a faster mobility, presumably caused by differences in phosphorylations with the *in vivo* protein (Fig. 3*B*, 25). *In vitro*-translated Wee1 also was cleaved by caspase-7 and caspase-8 to give the same products as those by caspase-3. Although caspase-1 can cleave *in vitro*-translated CDC27, it gives different cleavage products from those *in vivo* (Fig. 3*B*).

APC Is Inactivated During Fas-Induced Apoptosis in Jurkat Cells. Cleavage of CDC27 did not disrupt APC as measured by the coprecipitation of several APC subunits (namely, BimE, APC2, APC5, CDC16, and APC7) with cleaved CDC27 (data not shown); however, the ubiquitin ligase activity of APC decreased in proportion to the amount of cleaved CDC27 (Fig. 4 *A* and *B*). Late in apoptosis, the BimE homolog and APC2 were partially cleaved (data not shown), but these cleavages occurred late and long after the loss of ubiquitin ligase activity. When 95% of CDC27 in immunopurified APC from apoptotic extract was cleaved with a loss of >90% of ubiquitin ligase activity (Fig. 4 *A* and *B*), only 5% of BimE in immunopurified APC was cleaved (data not shown). The other APC subunits, human CDC16, APC4, APC5, APC7, and APC8 (CDC 23 homolog) were not cleaved at all (data not shown). To check whether the caspase cleavage could directly cause the inactivation of APC, immunopurified APCs from *Xenopus* interphase and mitotic extracts were incubated with recombinant caspase-3. It was previously shown that mitotic APC is active and phosphorylated (16, 26). As shown in Fig. 4*B*, CDC27 was

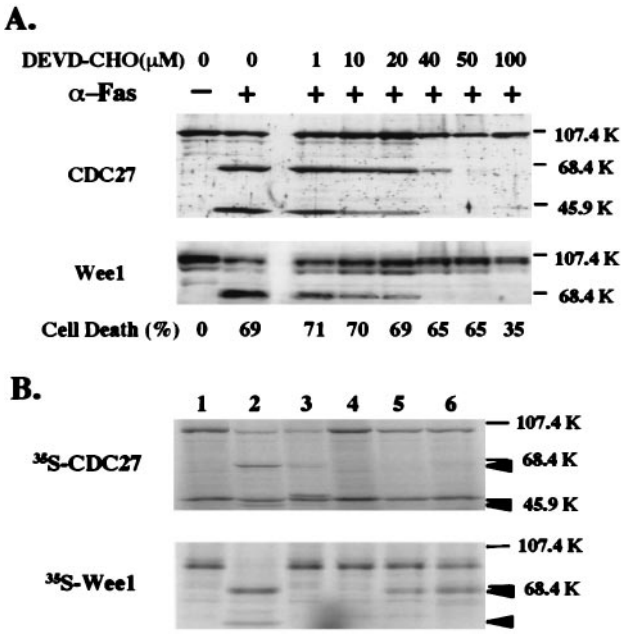

FIG. 3. Both Wee1 and CDC27 are substrates of the caspase-3 like protease. (*A*) Inhibition of Wee1 and CDC27 cleavage by DEVD-CHO; 2×10^6 Jurkat cells were treated with 10 ng/ml anti-Fas antibody for 4 hr in the presence of various concentrations of DEVD-CHO. The percentage of cell death was measured by MTT assay (20). Cleavages of CDC27 and Wee1 were examined by Western blotting by using anti-CDC27 and anti-Wee1 antibodies. (*B*) Cleavages of *in vitro* translated, ^{35}S-labeled Wee1 and CDC 27 by various caspases. Lanes: 1, inactive caspase; 2, caspase-3; 3, caspase-1; 4, caspase-2; 5, caspase-7; and 6, caspase-8. The arrows indicate the cleavage products.

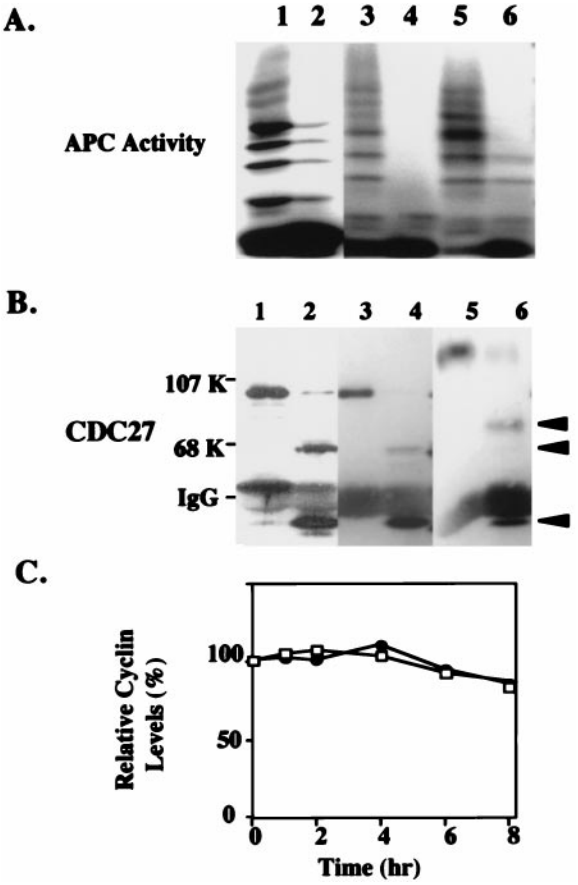

FIG. 4. Changes in APC activity and cyclin levels during apoptosis. (*A*) APC activity assay. Lanes: 1, APC from control Jurkat cell extract; 2, APC from apoptotic Jurkat cell extract (88% cell death); 3, purified *Xenopus* interphase APC with mock treatment; 4, purified *Xenopus* interphase APC treated with caspase-3; 5, purified *Xenopus* mitotic APC with mock treatment; and 6, purified *Xenopus* mitotic APC treated with caspase-3. (*B*) Samples used for ubiquitin ligase activity were analyzed by anti-CDC27 immunoblotting. The arrows indicate the cleavage products of CDC27. Lane description is the same as in *A*. (*C*) Relative levels of cyclin A (□) and cyclin B (●) during the time course of Fas-induced apoptosis, as measured by immunoblotting and quantitated by IMAGEQUANT program.

cleaved in both the interphase (unphosphorylated) and mitotic (phosphorylated) forms of CDC27. Cleavage led to the loss of ubiquitin ligase activity (Fig. 4*A*). In addition to CDC27, the BimE homolog (APC1) also was cleaved by caspase-3 *in vitro* (data not shown).

Although APC is active in late mitosis and inactive in S phase, it maintains some activity in G_1 (23, 24). Because both cyclin A and cyclin B have short half-lives *in vivo* as discussed earlier, loss of protein synthesis without loss of cyclin degradation should result in a rapid decline in cyclin levels. However, during apoptosis, both cyclin A and cyclin B are stabilized (Fig. 4*C*), presumably as a consequence of inhibiting APC activity.

Loss of the Inhibitory Tyrosine Phosphorylation on cdc2 and cdk2 During Apoptosis in Jurkat Cells. Although APC inactivation could prevent cyclin levels from declining, it does not explain the increase in cdc2 and cdk2 activity seen in apoptosis. It had been previously shown that the activation of cdc2 is associated with decreased tyrosine phosphorylation during Granzyme B-triggered apoptosis (4). We have found that increased H1 kinase activity of both cdc2 and cdk2 is associated with dephosphorylation of the inhibitory Tyr-15 site during Fas-induced apoptosis in Jurkat cells. As shown in Fig. 5*A*, levels of cdc2 and cdk2 remain the same during apoptosis induced by either Fas or staurosporine, whereas there is a dramatic decrease in the level of tyrosine phosphorylation at Tyr-15. The marked decrease in the Tyr-15 phosphorylation during Fas-induced apoptosis also was shown by anti-phosphorylated tyrosine immunoblotting of cdc2 and cdk2 immunoprecipitates (data not shown).

The phosphorylation state of Tyr-15 is regulated during the cell cycle by the opposing activities of cdc25 and Wee1 (12). To assess whether the decrease in tyrosine phosphorylation is caused by up-regulation of cdc25 or down-regulation of Wee1 activity, or both, an *in vitro* assay was used to measure these two activities. Cdc25 activity was assayed as the phosphatase activity removing inhibitory phosphorylation of inactive GST-cyclin B/cdc2 (17). As shown in Fig. 5*B*, cdc25 activity and mobility on SDS/PAGE remains unchanged during Fas-induced apoptosis in Jurkat cells. As a positive control, extracts from cells arrested in mitosis by nocodazole show an increase in cdc25 activity, as well as the appearance of a form of cdc25 with decreased mobility on SDS/PAGE. Wee1 activity was assayed by using active GST-cyclinB/cdc2 beads, in which cdc2 was monophosphorylated at the activating site Thr 161. Wee1 kinase activity was detected as an increase in phosphorylation of Tyr15 position in cdc2 and a corresponding decrease in the H1 kinase activity of GST-cyclin B/cdc2. As shown in Fig. 5*D*, Wee1 activity decreases >20-fold during apoptosis, measured by both tyrosine phosphorylation and H1 kinase activity of GST-cyclin B/cdc2 beads. The loss of activity parallels the amount of cleaved Wee1; 70–80% immunodepletion of Wee1 from the Jurkat cell extract also removed ≈80% of the Tyr15 phosphorylation activity, suggesting that the Wee1 gene product is responsible for most of the tyrosine kinase activity (data not shown). The major cleavage product of Wee1 can be recognized by a polyclonal antibody against C terminus of Wee1. The C-terminal antibody can immunoprecipitate intact Wee1 but not the cleavage product, indicating a possible

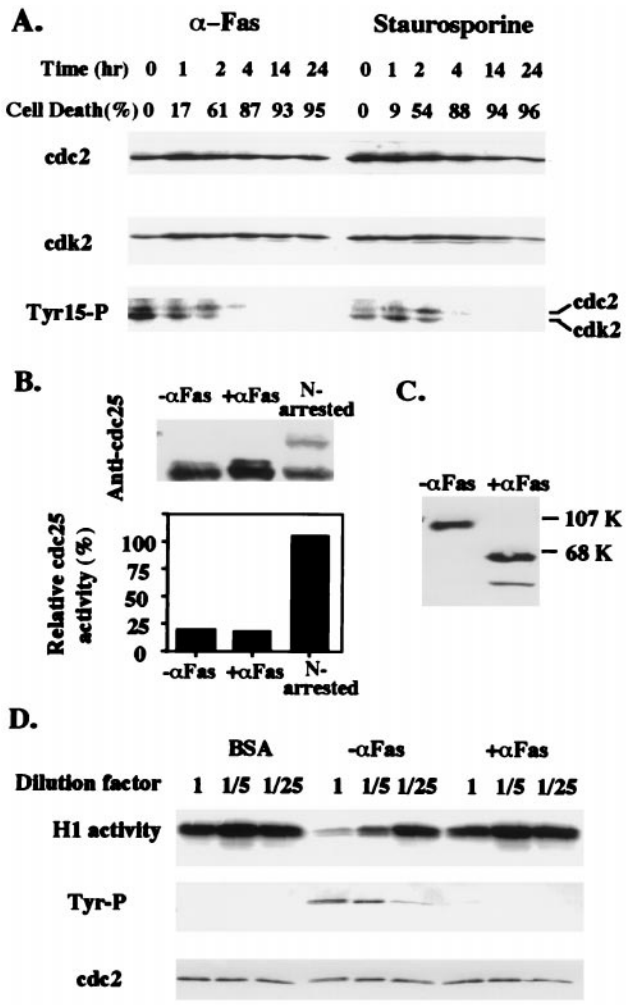

FIG. 5. (*A*) Changes in Tyr-15 phosphorylation of cdc2 and cdk2 during Fas- and staurosporine-induced apoptosis. The same filter was blotted with anti-cdc2, anti-cdk2, and a phospho-specific cdc2 (cdk2) antibody against Tyr-15. The cell death percentage was measured by MTT assay (20). (*B*) Cdc25 activity during Fas-induced apoptosis. Samples from control extract ($-\alpha$ Fas), apoptotic extract ($+\alpha$ Fas), and nocodazole arrested extract (N-arrested, ≈50% in mitosis) were analyzed with anti-cdc25 immunoblotting (*Upper*). The relative cdc25 activities are shown graphically (*Lower*). (*C*) Anti-Wee1 blot of the extracts. (*D*) Wee1 activity during apoptosis. (*Middle* and *Bottom*) Western blot analysis of the active cyclin B/cdc2 beads treated with different extracts. (*Top*) H1 kinase activity of the same corresponding beads. Each extract (≈5 mg/ml total proteins) made in Nonidet P-40 lysis buffer was analyzed at three different dilutions: 1, 1/5, and 1/25. Five milligrams per milliliters BSA was used as a negative control.

conformational change in C-terminal domain caused by the cleavage. Because of the unstable nature of the Wee1 cleavage product in apoptotic extracts, we have not been able to purify it. These measurements of enzymatic activities confirm the protein assays for Wee1 and cdc25 (Fig. 5) that show a cleavage in Wee1 and no change in cdc25.

Fas-Induced Apoptosis in Jurkat Cells Is Not Simply an Aberrant Form of Mitosis. Inactivation of Wee1 in *S. pombe* induces mitotic catastrophe, in which the mitotic process is prematurely activated (27). Mitotic catastrophe also can be induced in mammalian cells by overexpression of cdc2 together with either cyclin A or cyclin B (28). Thus, inactivation of Wee1 and subsequent activation of cdks during Fas-induced apoptosis could support a mitotic catastrophe view of some steps in apoptosis. To assess whether mitosis is initiated, we used the MPM-2 antibody (29) to measure general mitotic phosphorylation. During Fas-induced apoptosis in Jurkat cells, we did not see major increases in mitotic phosphorylation (Fig. 6*B*), nor did we see the typical mitotic elevation of another key mitotic kinase, polo-like kinase-1 (Plk-1) (Fig. 6*A*). During mitotic catastrophe, cells arrest in mitosis with a biopolar spindle (28). However, mitotic spindles do not form during Fas-induced apoptosis in Jurkat cells (Fig. 6*C*). These results support the view that apoptosis is not simply a form of mitotic catastrophe, rather it may be a unique state characterized by caspase-dependent cdk activation, that fails to activate the core mitotic mechanisms.

DISCUSSION

Apoptosis is a highly regulated process that seems to proceed in an orderly fashion. During apoptosis, critical molecular components are disassembled or cleaved, permitting apoptotic cells to vanish with minimum effect on the surrounding tissues. We find that, during Fas-induced apoptosis in Jurkat cells, protein synthesis is shutdown before caspase-3 activation (Fig. 1) and cannot be inhibited by the caspase inhibitor YVAD-CMK (300 μM) (data not shown). The early shutdown of protein synthesis might play an important role in apoptosis. Support for such an idea comes from evidence that the protein synthesis inhibitor cycloheximide can cause or potentiate apoptosis (22). Furthermore, activation of insulin-like growth factor I receptor and phosphatidylinositol 3-kinase, which stimulates general protein synthesis, also prevents cells from programmed death (30, 31). These results are consistent with the hypothesis that protein synthesis inhibition can inhibit cell survival pathways such as that involving NF-κ B (22). It also is possible that the shutdown of protein synthesis reduces the level of short-lived apoptosis inhibitors.

Our results suggest that cdc2 and cdk2 activation are associated with early steps in Fas-induced apoptosis in Jurkat cells. Experiments perturbing cdc2 and cdk2 activity have argued

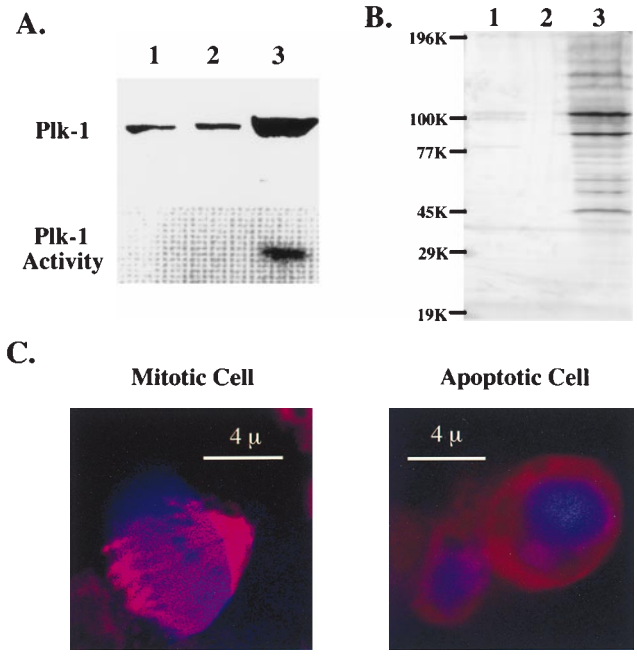

FIG. 6. Changes in mitotic parameters during Fas-induced apoptosis in Jurkat cells. Lanes: 1, control extracts; 2, apoptotic extracts (88% cell death); 3, nocodazole arrested cell extracts (≈50% in mitosis). (*A*) Plk-1 is not activated during apoptosis. (*Upper*) anti-Plk-1 Western blot. (*Lower*) Plk-1 activity was measured as described (14). (*B*) Mitotic phosphorylation as shown by MPM-2 immunoblotting (29). (*C*) Absence of mitotic spindle in apoptotic cells. Microtubules stained with anti-tubulin antibody appears orange-red, whereas DNA labeled with Hoechst dye (1 μg/ml) appears blue. The mitotic cell was chosen among control Jurkat cells.

previously for their necessity (4–6). The strongest evidence that cdc2 kinase activation is on a direct pathway to apoptosis is the prevention of apoptosis in cells not expressing cdc2 (4) and in cells transiently transfected with Wee1 kinase, which inactivates cdc2 and cdk2 (9, 32). Activation of cdks in Jurkat cells must occur in the face of a very early and nearly complete shutdown of protein synthesis in an unusual pathway insensitive to the caspase inhibitor YVAD-CMK (300 μM). Compensating for the loss of cyclin synthesis is an early inhibition of cyclin degradation by a caspase-dependent cleavage of the CDC27 subunit of APC. Further activation of cdks is caused by a loss of tyrosine phosphorylation on both cdc2 and cdk2 caused by a decrease of cdc2 tyrosine kinase activity, without any measurable change in the tyrosine dephosphorylation activity (cdc25 activity). Although we have demonstrated the inactivation of APC, we have not yet established the activity of the Wee1 cleavage product. Caspase cleavage of $p21^{CIP1}$ and $p27^{KIP1}$ has been reported to mediate apoptosis in endothelial cells (33). However, we did not observe $p27^{KIP1}$ cleavage during the early stage of Fas-induced apoptosis in Jurkat cells, which could suggest different mechanisms in different systems.

Despite cdc2 activation and Wee1 inactivation, a typical mitotic catastrophe does not occur during Fas-induced apoptosis in Jurkat cells. Spindles do not form, widespread MPM-2 phosphorylations are not found, and Plk-1 is not activated. There must be other substrates of cdks that play a specific role in apoptosis; these have not been identified. Potential substrates include those contributing to the morphological similarities between apoptotic and mitotic cells. In summary, stimulation of cdc2 and cdk2 kinase activities seem to be an important early step in Fas-induced apoptosis in Jurkat cells. To activate these kinases in the face of a loss of protein synthesis, specific proteolysis and tyrosine phosphorylation are shut off. In some undetermined manner, the entire mitotic processes is not initiated. It will be especially interesting to identify the target of cyclin-dependent kinases during apoptosis. Such an identification may connect cell division with cell death directly.

We thank Guowei Fang for many insightful suggestions, Vince Cryns for apoptotic extract, Todd Stukenberg for various cell cycle reagents, and Tony Hunter for pΔ 213wee1. We also would like to thank members of Kirschner's laboratory and Yuan's laboratory for helpful discussions. B.-B.Z. is supported by a National Institutes of Health postdoctoral fellowship, and H. L. is supported by a postdoctoral fellowship from the National Institute of Aging. J.Y.'s laboratory is supported in part by grants from American Heart Association and National Institute of Aging. M.W.K.'s laboratory is supported by grants from the National Institute of General Medical Sciences.

1. Wyllie, A. H., Kerr, J. F. R. & Currie, A. R. (1980) *Int. Rev. Cytol.* 251–305.
2. Kumar, S. (1995) *Trends Biochem. Sci.* **20,** 198–202.
3. Yuan, J., Shaham, S., Ledoux, S., Ellis, H. M. & Horvitz, H. R. (1993) *Cell* **75,** 641–652.
4. Shi, L., Nishioka, W. K., Th'ng, J., Bradbury, E. M., Litchfield, D. W. & Greenberg, A. H. (1994) *Science* **263,** 1143–1145.
5. Meikrantz, W. & Schlegel, R. (1996) *J. Biol. Chem.* **271,** 10205–10209.
6. Yao, S., McKenna, K. A., Sharkis, S. J. & Bedi, A. (1996) *Cancer Res.* **56,** 4551–4555.
7. Wang, J. & Walsh, K. (1996) *Science* **273,** 359–361.
8. Galaktionov, K., Chen, X. & Beach, D. (1996) *Nature (London)* **382,** 511–517.
9. Yan, Y., Frisén, J., Lee, M., Massagué, J. & Barbacid, M. (1997) *Genes Dev.* **11,** 973–983.
10. Zhang, P., Liégeois, N. J., Wang, C., Finegold, M., Hou, H., Thompson, J. C., Silverman, A., Harper, J. W., DePinho, R. A. & Elledge, S. (1997) *Nature (London)* **387,** 151–158.
11. King, R. W., Deshies, R. J., Peters, J. M. & Kirschner, M. W. (1996) *Science* **274,** 1652–1659.
12. Morgan, D. O. (1995) *Nature (London)* **374,** 131–134.
13. Harper, J. W. & Elledge, S. J. (1996) *Curr. Opin. Genet. Dev.* **6,** 56–64.
14. Goslteyn, R. M., Mundt, K. E., Fry, E. A. & Nigg, E. A. (1995) *J. Cell Biol.* **129,** 1617–1628.
15. Murray, A. W. (1991) *Methods Cell Biol.* **36,** 581–605.
16. King, R. W., Peters, J. M., Tugendreich, S., Rolfe, M., Hieter, P. & Kirschner, M. W. (1995) *Cell* **81,** 279–288.
17. Shen, M., Stukenberg, P. T., Kirschner, M. W. & Lu, K. P. (1998) *Genes Dev.* 706–720.
18. Meikrantz, W., Gisselbrecht, S., Tam, S. W. & Schlegel, R. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 3754–3758.
19. Shi, L., Chen, G., He, D., Bosc, D. G., Litchfield, D. W. & Greenberg, A. H. (1996) *J. Immunol.* **157,** 2381–2385.
20. Mosmann, T. (1983) *J. Immunol. Methods* **65,** 55–63.
21. Anderson, J. A., Lewellyn, A. L. & Maller, J. L. (1997) *Mol. Biol. Cell* **8,** 1195–1206.
22. Wallach, D. (1997) *Trends Biochem. Sci.* **22,** 107–109.
23. Amon, A., Irniger, S. & Nasmyth, K. (1994) *Cell* **77,** 1037–1050.
24. Brandeis, M. & Hunt, T. (1996) *EMBO J.* **15,** 5280–5289.
25. Watanabe, N., Broome, M. & Hunter, T. (1995) *EMBO J.* **14,** 1878–1891.
26. Peters, J. M., King, R. W., Hoog, C. & Kirschner, M. W. (1996) *Science* **274,** 1199–1201.
27. Russell, P. & Nurse, P. (1987) *Cell* **49,** 559–567.
28. Heald, R., McLoughlin, M. & McKeon, F. (1993) *Cell* **74,** 463–474.
29. Davis, F. M., Tsao, T. Y., Fowler, S. K. & Rao, P. N. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 2926–2930.
30. Mendez, R., Kollmorgen, G., White, M. F. & Rhoads, R. E. (1997) *Mol. Cell. Biol.* **17,** 5184–5192.
31. Kulik, G., Klippel, A. & Weber, M. J. (1997) *Mol. Cell. Biol.* **17,** 1595–1606.
32. Chen, G., Shi, L., Litchfield, D. W. & Greenberg, A. H. (1995) *J. Exp. Med.* **181,** 2295–2300.
33. Levkau, B., Koyama, H., Raines, E. W., Clurman, B. E., Herren, B., Orth, K., Roberts, J. M. & Ross, R. (1998) *Mol. Cell* **1,** 553–563.