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Cleavage of p21$^{Cip1/Waf1}$ and p27^{Kip1} Mediates Apoptosis in Endothelial Cells through Activation of Cdk2: Role of a Caspase Cascade

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Summary

Apoptosis of human endothelial cells after growth factor deprivation is associated with rapid and dramatic up-regulation of cyclin A–associated cyclin-dependent kinase 2 (cdk2) activity. In apoptotic cells, the C termini of the cdk inhibitors p21$^{Cip1/Waf1}$ and p27^{Kip1} are truncated by specific cleavage. The enzyme involved in this cleavage is CPP32 and/or a CPP32-like caspase. After cleavage, p21$^{Cip1/Waf1}$ loses its nuclear localization sequence and exits the nucleus. Cleavage of p21$^{Cip1/Waf1}$ and p27^{Kip1} results in a substantial reduction in their association with nuclear cyclin–cdk2 complexes, leading to a dramatic induction of cdk2 activity. Dominant-negative cdk2, as well as a mutant of p21$^{Cip1/Waf1}$ resistant to caspase cleavage, partially suppress apoptosis. These data suggest that cdk2 activation, through caspase-mediated cleavage of cdk inhibitors, may be instrumental in the execution of apoptosis following caspase activation.

Introduction

Apoptosis is a fundamental mechanism involved in development and differentiation of multicellular organisms. Growing evidence suggests that apoptosis is frequently associated with cells in the G1 phase of the cell cycle (King and Cidlowski, 1995; Meinkrantz and Schlegel, 1995), and arrest in late G1 or S phase can accelerate or potentiate apoptosis (Meikrantz et al, 1994). This implies the existence of molecules in late G1 and S whose activities facilitate execution of the apoptotic process. Progression through G1 and entry into S phase are regulated by the activation of cyclin-dependent kinase 2 (cdk2), which is complexed with cyclin A and cyclin E (Sherr, 1993), together with proteins that bind to the cyclin–cdk complex and inhibit its activity—the cdk inhibitors (Hunter and Pines, 1994; Morgan, 1995; Sherr and Roberts, 1995). Apoptosis appears linked in some cases to aberrations in the activity of cdks: in HeLa cells, induction of apoptosis through a variety of agents is associated with the activation of cyclin A–associated kinases (Meikrantz et al., 1994), and dominant-negative mutants of cdc2, cdk2, and cdk3 suppress apoptosis (Meikrantz and Schlegel, 1996); apoptosis in leukemic T cell lines correlates with cdk1 and cdk2 activation (Wang et al., 1995), and pharmacologic inhibition of cdks prevents growth factor deprivation–induced apoptosis in PC12 cells (Park et al., 1996); granzyme B–induced apoptosis requires cyclin A–cdc2 and cyclin A–cdk2 activation (Shi et al., 1994, 1996; Chen et al., 1995b), and apoptosis mediated through the HIV-1 Tat protein is associated with enhanced activation of cyclin A–associated cdk2 and cdc2 (Li et al., 1995). Although these studies provide evidence for a potentially essential role for cdks in apoptosis, the mechanism for activation of cdks and their role in apoptosis has not been identified. Furthermore, all of the aforementioned studies used transformed cell lines that, by their nature, are aberrant in cell cycle regulation, and it is therefore unclear whether similar associations hold for normal diploid cells.

In the present study, we examined the participation and regulation of cell cycle molecules in the process of apoptosis induced by growth factor deprivation of cultured human umbilical vein endothelial cells (HUVEC). In HUVEC undergoing apoptosis, we identify specific cleavage of two cyclin A–associated cdk inhibitors, p21$^{Cip1/Waf1}$ and p27^{Kip1}, which coincides with rapid up-regulation of cyclin A–cdk2 activity. We characterize the enzymes responsible for this cleavage as members of the caspase family of proteases, critically involved in the execution of the apoptotic program (Salvesen and Dixit, 1997). Further, we provide evidence that cdk inhibitor cleavage contributes to activation of cdk2 and that cdk2 activation has a significant impact on apoptosis.

Results

Cyclin E– and Cyclin A–Associated Kinase Activities Are Up-Regulated in Apoptotic HUVEC

To evaluate cdk regulation in the process of apoptosis, we characterized the activities of cdks in HUVEC undergoing apoptotic cell death following growth factor deprivation. After growth factor removal, individual cells exhibit typical morphologic features of apoptosis, such as membrane blebbing and nuclear condensation/fragmentation, and loose contact with the matrix. These apoptotic cells detach and appear progressively in the culture supernatant as "floaters," which display the biochemical features (DNA laddering, caspase activation, poly[ADP-ribose] polymerase [PARP] cleavage) characteristic of apoptosis (data not shown; for caspases and PARP cleavage, see Figure 5). Apoptotic floaters are observed as early as 4 hr after growth factor withdrawal and after 12–16 hr make up 40%–45% of the total cell population, while the remaining adherent cells are "via-

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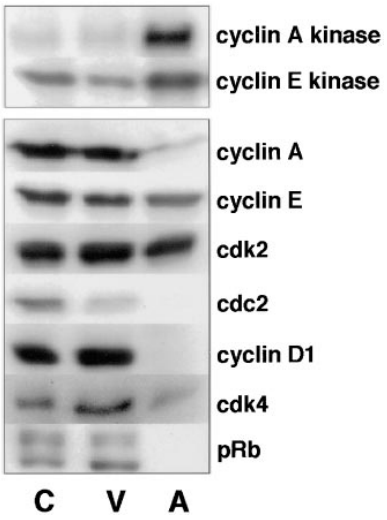

Figure 1. Induction of Cyclin A– and Cyclin E–Associated Kinase Activities and Regulation of Cell Cycle Molecules in Apoptotic HUVEC

Cyclin A– and cyclin E–associated kinase activities were determined in control cells (C) and, 12 hr after growth factor deprivation, in the surviving viable cells (V) and apoptotic floaters (A) using histone H1 as a substrate. Cell lysates were immunoblotted sequentially with antibodies to cyclin A, cyclin E, cdk2, cdc2, cyclin D1, cdk4, and Rb.

ble" and would survive and proliferate if resupplemented with growth factors.

In this experimental system, cyclin A–associated kinase activity is dramatically up-regulated in the apoptotic HUVEC, as compared with the viable and control, growth factor–supplemented cells (Figure 1). Cyclin E–associated kinase activity is also substantially up-regulated (Figure 1). There are no changes in the total protein levels of cyclin E or cdk2, whereas cyclin A protein is reduced in the apoptotic cells, and cdc2 is absent (Figure 1). The increased cyclin A– and cyclin E–associated kinase activities do not appear to be due to a change in cdk2 phosphorylation (Gu et al., 1992) because there is no apparent increase in T160 phosphorylation or a measurable decrease in Y15 phosphorylation (data not shown). We also find that the abundance of the cdk-activating tyrosine phosphatase cdc25A (Galaktionov and Beach, 1991) does not increase; rather, it is down-regulated in growth factor–deprived cells (both in the floaters and the viable cells) compared with controls (data not shown). In the apoptotic cells, cdk4 protein levels are slightly diminished, whereas cyclin D1 and the retinoblastoma protein (Rb) are not detected (Figure 1), and proliferating cell nuclear antigen (PCNA) levels are unchanged (data not shown). Despite having significantly increased cyclin A–cdk2 and cyclin E–cdk2 activities, HUVEC undergoing apoptosis do not enter the S phase of the cell cycle, as BrdU-labeling for the entire period of growth factor deprivation does not result in BrdU incorporation into apoptotic cells (data not shown). These observations suggest that, after growth factor deprivation, the cells may be exiting the cell cycle prior to S phase to undergo apoptosis.

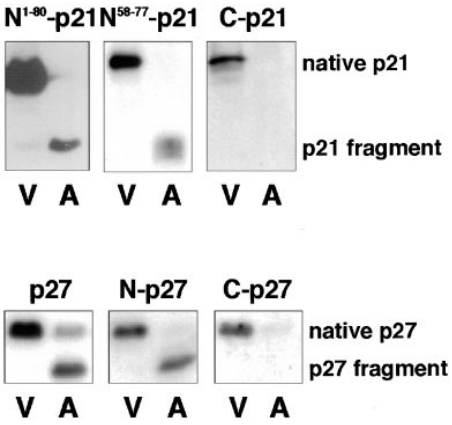

Figure 2. C-Terminal Cleavage of $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ in Apoptotic HUVEC

Cell lysates from viable (V) and apoptotic (A) cells 12 hr after growth factor deprivation were subjected to Western blot analysis with monoclonal antibodies to amino acids 1-80 (N^{1-80}-p21) and 58-77 of $p21^{Cip1/Waf1}$ (N^{58-77}-p21), an antibody to the C terminus of $p21^{Cip1/Waf1}$ (aa 146-164, C-p21), a polyclonal antibody to $p27^{Kip1}$ (p27), and antibodies to the N terminus (aa 2-21, N-p27) and C terminus (aa 181-198, C-p27) of $p27^{Kip1}$.

$p21^{Cip1/Waf1}$ and $p27^{Kip1}$ Are Truncated at Their C Termini in Apoptotic HUVEC

To understand the mechanism behind the up-regulation of cyclin A–cdk2 and cyclin E–cdk2 activities in apoptotic cells, we examined two cdk inhibitors, $p21^{Cip1/Waf1}$ and $p27^{Kip1}$, known to play major roles in the regulation of cdk2 activity (Hunter and Pines, 1994; Sherr and Roberts, 1995). Western blot analysis with an antibody against amino acids 58-77 at the N terminus of $p21^{Cip1/Waf1}$ reveals that the native protein is absent in the apoptotic cells (Figure 2). However, this antibody cross-reacts with a protein running at approximately 14 kDa on SDS gels, while an antibody against the C terminus of $p21^{Cip1/Waf1}$ fails to detect this protein (Figure 2). Another antibody against amino acids 1-80 of $p21^{Cip1/Waf1}$ confirmed that the N terminus is intact (Figure 2), suggesting that the lower molecular weight band corresponds to a $p21^{Cip1/Waf1}$ molecule that is truncated at the C terminus. The amount of this truncated $p21^{Cip1/Waf1}$ molecule is substantially lower than that of the native protein. When we examined $p27^{Kip1}$ in the same samples, there was a significant decrease in the native $p27^{Kip1}$ protein in the apoptotic cells. As in the case of $p21^{Cip1/Waf1}$, an additional $p27^{Kip1}$–cross-reactive band appears, running this time at approximately 22 kDa (Figure 2). Again, antibodies against the N terminus, but not the C terminus, of $p27^{Kip1}$ recognize this band (Figure 2). These experiments demonstrate that truncation of the C-terminal portions of both $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ is associated with the process of apoptosis. Truncation of $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ is not limited to growth factor deprivation–induced apoptosis; in HUVEC undergoing apoptosis through prevention of cell adherence (Re et al., 1994), as well as in staurosporine-induced apoptosis, we observe the same $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ fragments (data not shown).

CPP32-like Caspase Activity in Apoptotic Cell Lysates Cleaves p21$^{Cip1/Waf1}$

Analysis of the amino acid sequence of p21$^{Cip1/Waf1}$ reveals that the C-terminal region of the molecule contains the sequence DHVD^{112}L, a potential cleavage site for CPP32 (Talanian et al., 1997; Thornberry et al., 1997), a member of the caspase family of apoptotic proteases. During HUVEC apoptosis, CPP32 is activated, and PARP, a known substrate for this enzyme, is cleaved (Figure 5). Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (ZVAD-fmk), a general inhibitor of caspases known to inhibit apoptosis induced by diverse stimuli in many cell types (Livingston, 1997), prevents apoptosis in our system in a concentration-dependent manner, with complete inhibition at 100–200 μM. Treatment of HUVEC with ZVAD-fmk also prevents the appearance of truncated p21$^{Cip1/Waf1}$ and p27^{Kip1} (Figure 3A).

These observations suggest that proteolytic cleavage by activated CPP32 or CPP32-like caspases may be involved in the C-terminal truncations of the cdk inhibitors during endothelial cell apoptosis. To characterize the p21$^{Cip1/Waf1}$ cleavage activity in apoptotic HUVEC, we incubated recombinant human p21$^{Cip1/Waf1}$ with lysates from apoptotic cells, as described for in vitro cleavage of other substrates of CPP32 (Casciola-Rosen et al., 1996). In this cell-free system, recombinant p21$^{Cip1/Waf1}$ is cleaved in a time-dependent manner, generating a fragment that corresponds in molecular weight to the endogenous p21$^{Cip1/Waf1}$ cleavage fragment present in apoptotic cells (Figure 3B). Lysates from viable cells fail to cleave p21$^{Cip1/Waf1}$ (Figure 3C).

To test the hypothesis that CPP32 or a homologous caspase with similar substrate specificity is involved in p21$^{Cip1/Waf1}$ cleavage, we incubated apoptotic cell lysates with inhibitors of different caspases and tested their ability to prevent cleavage of recombinant p21$^{Cip1/Waf1}$. The specific inhibitor of CPP32-like caspases Ac-DEVD-CHO (Nicholson et al., 1995) inhibits p21$^{Cip1/Waf1}$ cleavage in vitro in a concentration-dependent manner with complete inhibition at 10 nM (Figure 3B) and an IC_{50} of approximately 1–2 nM (Figure 3C). In contrast, two inhibitors of ICE-like caspases, Ac-YVAD-CHO and Ac-YVAD-CMK, have no effect on p21$^{Cip1/Waf1}$ cleavage at concentrations known to completely prevent ICE activity (200 nM and 250 nM, respectively) (Thornberry, 1992) (Figure 3B). The cleavage products are also observed when the cells are directly lysed in boiling SDS-sample buffer. This helps exclude the possibility that cleavage could be occuring after cell lysis and suggests that cleavage is associated with apoptosis.

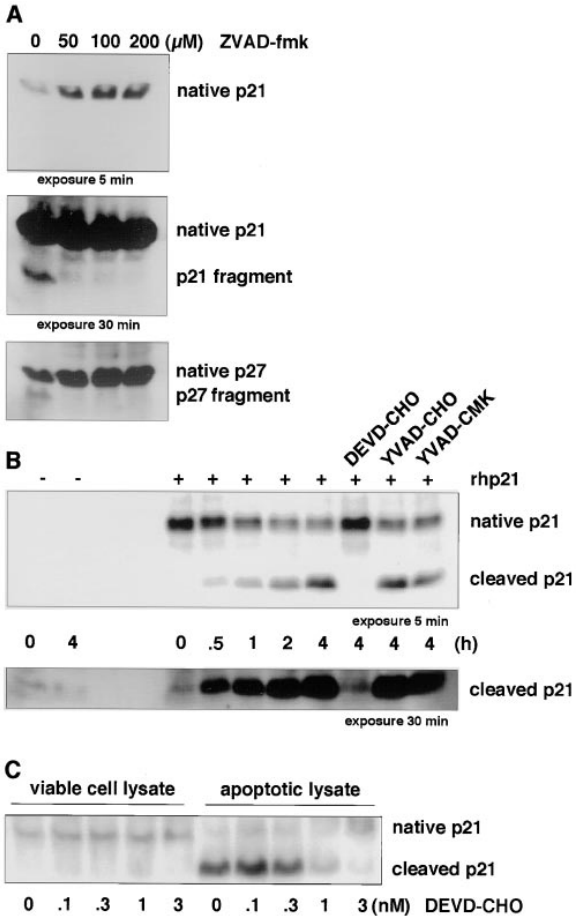

Figure 3. CPP32-like Caspases Are Involved in the Cleavage of p21$^{Cip1/Waf1}$ at DHVD^{112}L In Vivo and In Vitro

(A) The caspase inhibitor ZVAD-fmk prevents the appearance of p21$^{Cip1/Waf1}$ and p27^{Kip1} proteolytic fragments. Cells were deprived of growth factors in the presence of increasing concentrations of ZVAD-fmk, and lysates of total cell populations at 16 hr were immunoblotted for p21$^{Cip1/Waf1}$ and p27^{Kip1}.

(B) Recombinant human p21$^{Cip1/Waf1}$ is cleaved by lysates of apoptotic HUVEC, and cleavage is inhibited by the CPP32 inhibitor Ac-DEVD-CHO. Apoptotic cell lysates (12 hr after growth factor deprivation) were incubated with rhp21$^{Cip1/Waf1}$ at 37°C for the indicated times in the presence or absence of Ac-DEVD-CHO (10 nM) and the ICE inhibitors Ac-YVAD-CHO (200 nM) and Ac-YVAD-CMK (fully inhibits ICE but not CPP32 at 250 nM), and cleavage was detected by immunoblotting. The first two lanes (without rhp21$^{Cip1/Waf1}$) show the relative level of endogenous p21$^{Cip1/Waf1}$ cleavage fragment present in the cell-free system, which can only be seen with very long exposures of the same Western blot.

(C) Lysates of viable cells do not cleave rhp21$^{Cip1/Waf1}$, and cleavage by apoptotic cell lysates is inhibited by the CPP32 inhibitor Ac-DEVD-CHO. Cell lysates from viable and apoptotic cells (12 hr) were incubated with rhp21$^{Cip1/Waf1}$ as described in (B) in the presence of increasing concentrations of Ac-DEVD-CHO, and cleavage fragments were detected by immunoblotting.

CPP32 and ICE LAP3 Induce Cleavage of Recombinant and Endogenous p21$^{Cip1/Waf1}$ and p27^{Kip1} but Not of a p21$^{Cip1/Waf1}$ Mutant Altered at the Putative Cleavage Site

To identify individual caspases capable of cleaving p21$^{Cip1/Waf1}$ in vitro, we incubated recombinant human p21$^{Cip1/Waf1}$ with three recombinant caspases, CPP32, ICE LAP3, and Mch2, in the presence of extracts from control cells. Incubation with CPP32 results in complete cleavage of p21$^{Cip1/Waf1}$, with generation of a fragment with the same molecular weight as the one generated by the CPP32-like activity present in apoptotic cell lysates (Figure 4A). In the presence of ICE LAP3, which has the same substrate specificity as CPP32, p21$^{Cip1/Waf1}$ is also substantially cleaved, whereas Mch2, which prefers an

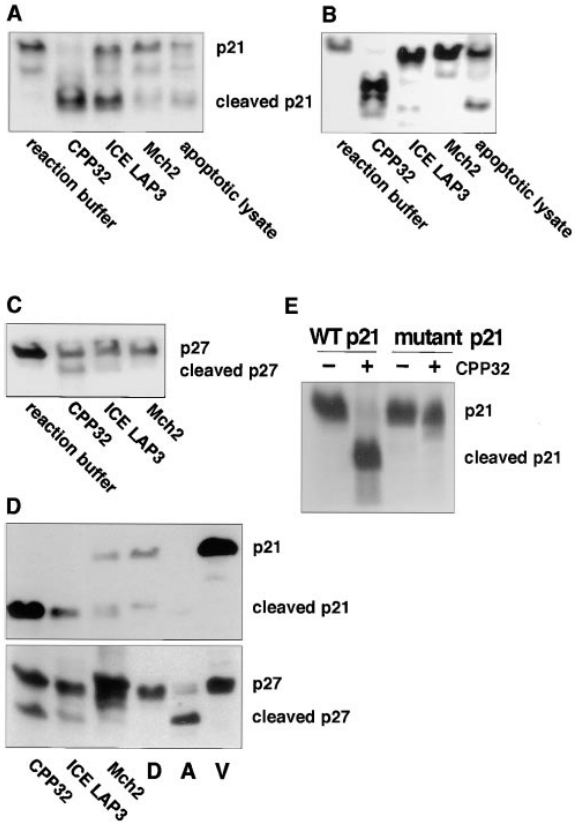

Figure 4. CPP32 and ICE LAP3 Are Involved in the Cleavage of $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ In Vitro, and Mutation of the CPP32 Cleavage Site of $p21^{Cip1/Waf1}$ Prevents Its Cleavage

Two nanograms of $rhp21^{Cip1/Waf1}$ were incubated with 500 ng each of the indicated recombinant human caspases for 2 hr at 37°C in the presence (A) or absence (B) of 10 μg control cell extract (HUVEC with growth factors). Lane 5 (apoptotic lysate) shows $rhp21^{Cip1/Waf1}$ cleaved by endogenous caspases present in 10 μg of apoptotic cell lysate in 2 hr. (C) 0.5 ng of $rhp27^{Kip1}$ was incubated with 500 ng of each caspase and cleavage fragments detected by immunoblotting. (D) Endogenous $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ are cleaved by recombinant caspases. Fifty micrograms (for $p21^{Cip1/Waf1}$) and 10 μg (for $p27^{Kip1}$) of control cell lysates were incubated with diluent (D) or with caspases, and the resulting cleavage fragments were compared with those present in cell lysates from viable (V) and apoptotic (A) HUVEC by immunoblotting. (E) In vitro translated wild-type $p21^{Cip1/Waf1}$ and a $p21^{Cip1/Waf1}$ mutant carrying a 109EhvG112 substitution in the CPP32 substrate consensus sequence 109DhvD112 in $p21^{Cip1/Waf1}$ were incubated with 25 ng/μl CPP32 in the presence of 1 μg/μl control cell extract for 2 hr. $p21^{Cip1/Waf1}$ and the cleavage fragments were immunoprecipitated and radiolabeled products visualized by autoradiography.

(IVL)EXD motif in its substrates, has only a minimal effect under the same conditions. When we incubate recombinant $p21^{Cip1/Waf1}$ with the same caspases in the absence of control cell extracts, we observe a different cleavage pattern in both the size of the fragments and in the efficiency of cleavage (Figure 4B). Incubations of caspases with $p21^{Cip1/Waf1}$ in the presence of recombinant cyclin A and cdk2 also give the same cleavage pattern as in Figure 4B, which is distinct from the one observed in apoptotic cells (data not shown). Incubation of recombinant human $p27^{Kip1}$ with the same three caspases but in the absence of control cell extracts results in proteolytic cleavage and generation of the same molecular weight fragment as the one observed in apoptotic cells (Figure 4C).

To test the ability of the recombinant caspases to cleave endogenous $p21^{Cip1/Waf1}$ and $p27^{Kip1}$, we incubated extracts from control cells with each of the three caspases. After 2 hr, all endogenous $p21^{Cip1/Waf1}$ is cleaved in the presence of CPP32 and ICE LAP3, while Mch2 is again less effective (Figure 4D). Endogenous $p27^{Kip1}$ is also cleaved by the caspases with similar specificities to $p21^{Cip1/Waf1}$, generating a fragment corresponding in molecular weight to the one we observe in apoptotic cells (Figure 4D). However, whereas $p21^{Cip1/Waf1}$ is completely cleaved, endogenous $p27^{Kip1}$ is only partially cleaved in vitro, consistent with the incomplete $p27^{Kip1}$ cleavage in apoptotic cells.

To determine whether $DHVD^{112}L$ is indeed the cleavage site in $p21^{Cip1/Waf1}$, we mutated the two conserved aspartic acids in the CPP32-cleavage site consensus sequence DXXD into glutamic acid and glycine, respectively. Incubation of in vitro translated wild-type and mutant $p21^{Cip1/Waf1}$ with CPP32 reveals that only the wild-type, but not the mutant $p21^{Cip1/Waf1}$ protein, is cleaved by the caspase (Figure 4E).

Cleavage and Exit of Truncated $p21^{Cip1/Waf1}$ from the Cell Nucleus Precede Induction of Cyclin A–cdk2 Activity

In kinetic studies, the N-terminal $p21^{Cip1/Waf1}$ fragment is observed as early as 2 hr after growth factor deprivation, with substantial loss of the signal for the native $p21^{Cip1/Waf1}$ protein by 12 hr (Figure 5). The appearance of the $p21^{Cip1/Waf1}$ fragment precedes the appearance of the $p27^{Kip1}$ fragment by 4–6 hr and correlates closely with the activation kinetics of both CPP32 and ICE LAP3 (Figure 5). The appearance of the $p21^{Cip1/Waf1}$ fragment is followed closely by an increase in cyclin A–cdk2 activity, which is detected as early as 4 hr after growth factor deprivation (Figure 5). Subsequently, we observe cleavage of $p27^{Kip1}$ and PARP.

The C termini of both $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ contain a nuclear localization sequence. Therefore, we examined the intracellular distribution of these cdk inhibitors during apoptosis. Four hours after growth factor deprivation, immunocytochemistry with an antibody to the C terminus of $p21^{Cip1/Waf1}$ shows that the immunoreactivity for $p21^{Cip1/Waf1}$ has disappeared from the nuclei of approximately 50%–60% of the cells (Figure 6A). Immunostaining with an antibody to the N terminus of $p21^{Cip1/Waf1}$ also shows a substantial decrease in nuclear $p21^{Cip1/Waf1}$ (data not shown). These observations suggest that $p21^{Cip1/Waf1}$ is lost from the nucleus after cleavage. Immunostaining for $p27^{Kip1}$ at the same time point demonstrates comparable nuclear staining in control and growth factor–deprived cells (Figure 6A), consistent with the lack of significant cleavage of $p27^{Kip1}$ by Western analysis, compared with $p21^{Cip1/Waf1}$ 4 hr after growth factor deprivation (Figure 5). To test the possibility that the loss of nuclear $p21^{Cip1/Waf1}$ immunostaining seen 4 hr after growth factor deprivation results from exit of cleaved $p21^{Cip1/Waf1}$ from the nucleus, HUVEC extracts were fractionated into nuclear and cytoplasmic fractions and immunoblotted for

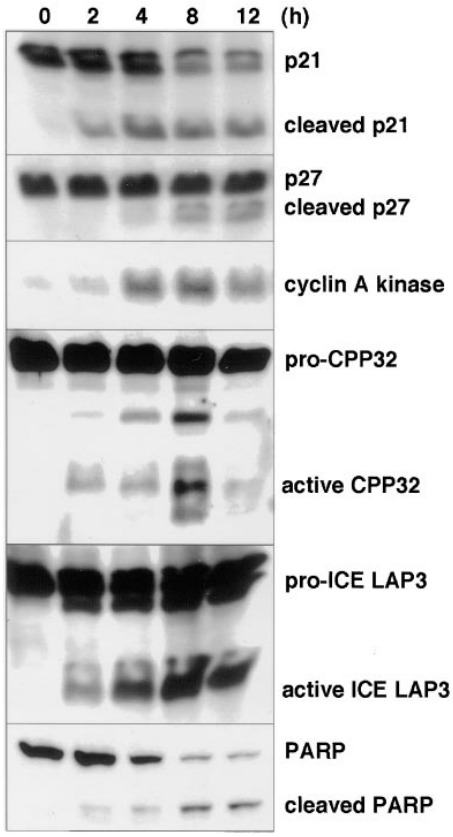

Figure 5. Cleavage of $p21^{Cip1/Waf1}$ Precedes Induction of Cyclin A–cdk2 Activity and Cleavage of $p27^{Kip1}$

HUVEC were exposed to growth factor deprivation for the indicated times, and cyclin A–cdk2 activity, as well as protein levels of $p21^{Cip1/Waf1}$, $p27^{Kip1}$, PARP, CPP32, and ICE LAP3 were determined in pooled cell populations. Active caspase indicates the migration position of the large subunit of the active heterotetrameric caspase complex (p17 for CPP32 and p20 for ICE LAP3), generated after proteolytic processing of the proenzyme.

$p21^{Cip1/Waf1}$. Full-length $p21^{Cip1/Waf1}$ is present almost entirely in the nuclear fraction of control cells and cells deprived of growth factors for 5 hr, while the N-terminal cleavage fragment of $p21^{Cip1/Waf1}$ appears in both the nuclear and cytoplasmic fraction of growth factor–deprived cells (Figure 6B). At the same time the relative level of intact nuclear $p21^{Cip1/Waf1}$ is diminished as seen on shorter exposures. Subcellular localization of a control, PCNA, shows it is primarily restricted to the nuclear fraction. Thus, truncation of $p21^{Cip1/Waf1}$ at its C terminus leads to its early exit from the nucleus and may decrease its availability in the nucleus to bind and inhibit the activity of nuclear cyclin–cdk complexes. To test this, we examined the association of cdk inhibitors with cyclins and cdk2 in apoptosis.

Levels of $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ Associated with Cyclin E– and Cyclin A–Cdk2 Complexes Are Greatly Reduced in Apoptotic HUVEC

The kinase inhibitory domains of both cdk inhibitors are located in their N termini, and truncations of $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ at their C termini have no effect on the ability of the remaining molecules to suppress cyclin–cdk activities (Luo et al., 1995). To understand the role of C-terminal truncations of $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ in regulating cyclin E– and cyclin A–cdk2 activities during apoptosis, we immunoprecipitated cyclin A and cyclin E from apoptotic cell lysates 12 hr after growth factor deprivation and determined the levels of $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ associated with the cyclin–cdk complexes. We detect neither $p21^{Cip1/Waf1}$ nor $p27^{Kip1}$ in cyclin A immunoprecipitates in apoptotic cells (Figure 7A). In cyclin E immunoprecipitates, the N-terminal 14 kDa fragment of $p21^{Cip1/Waf1}$ can still be detected, but its level is dramatically lower than that of intact $p21^{Cip1/Waf1}$ in viable cells (Figure 7A). Similar to $p21^{Cip1/Waf1}$, much less N-terminal $p27^{Kip1}$ is bound to cyclin E in apoptotic cells compared with viable cells.

To evaluate shifts in cdk inhibitors in cyclin–cdk complexes independently, we examined the amounts of $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ associated with total cdk2, since cdk2 protein levels are the same in control, viable, and apoptotic cells (Figure 1). No native $p21^{Cip1/Waf1}$ is associated with cdk2 in apoptotic cells compared with control and viable cells, while minor amounts of the N-terminal $p21^{Cip1/Waf1}$ cleavage fragment are detectable in the complex (Figure 7B). Similar results were obtained for $p27^{Kip1}$ except that only very low levels of the cleavage fragment are associated with cdk2 in apoptotic cells. Immunodepletion of $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ also shows that substantial amounts of cdk2 are associated with $p21^{Cip1/Waf1}$ (less with $p27^{Kip1}$) in control and viable cells (Figure 7C), and more than 75%–80% of cdk2 and cyclin A are associated with both $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ (data not shown), arguing for the role for cleavage and loss of both cdk inhibitors in the induction of cdk2 activity during apoptosis.

Dominant-Negative Cdk2 and an Uncleavable $p21^{Cip1/Waf1}$ Mutant Suppress Endothelial Apoptosis

To examine the significance of up-regulation of cyclin A–cdk2 and cyclin E–cdk2 activities in HUVEC apoptosis, we cotransfected cells with either a dominant-negative mutant of cdk2 (dnk2), lacZ, cdk2, or $p16^{INK4A}$, an inhibitor of cdk4 and cdk6, but not of cdk2 (Serrano et al., 1993), and enhanced green fluorescence protein (EGFP) 36 hr prior to induction of apoptosis. We observe a dramatic increase in the survival of dnk2-transfected cells compared with lacZ-, cdk2-, and $p16^{INK4A}$-transfected cells evaluated 24 hr after growth factor deprivation (Figure 8A). Dnk2 induces an approximately 70%–80% rescue effect compared with lacZ controls, whereas both cdk2 and $p16^{INK4A}$ appear to augment the apoptotic potential of growth factor deprivation in this system.

To test whether cleavage of $p21^{Cip1/Waf1}$ plays a role in the initiation and/or progression of the apoptotic program and is required for apoptosis, we transfected HUVEC with wild-type $p21^{Cip1/Waf1}$ and the uncleavable $p21^{Cip1/Waf1}$ mutant. We observe that the uncleavable $p21^{Cip1/Waf1}$ mutant was more effective than wild-type $p21^{Cip1/Waf1}$ in protecting the cells from apoptosis (50% vs. 10% above lacZ-controls, respectively; Figure 8B). Control experiments demonstrated that the wild-type and uncleavable

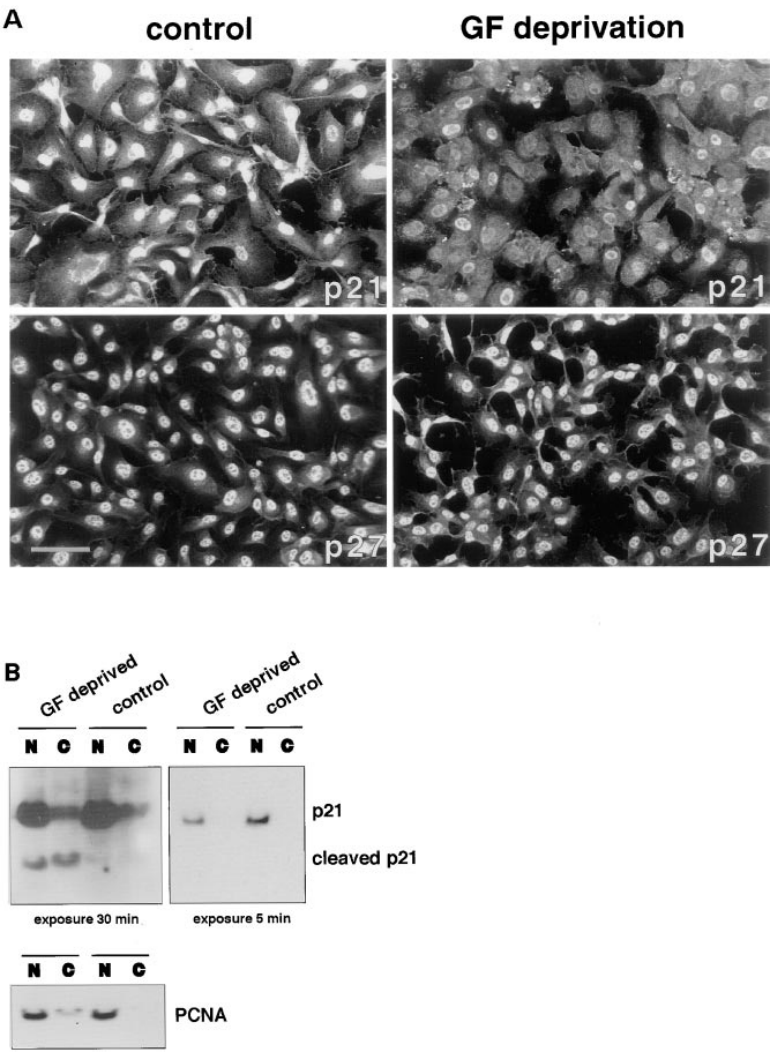

Figure 6. p21$^{Cip1/Waf1}$ Exits the Nucleus after Cleavage, and the N-Terminal p21$^{Cip1/Waf1}$ Fragment Appears in the Cytoplasm

(A) HUVEC exposed to growth factor deprivation for 4 hr and control cells (growth factor–supplemented) were immunostained for p21$^{Cip1/Waf1}$ and p27^{Kip1} with C-terminal antibodies. Staining was evaluated by confocal microscopy. Bar, 150 μm. (B) Cell fractionation analysis of p21$^{Cip1/Waf1}$ in control cells and cells deprived of growth factors for 5 hr. The nuclear and cytoplasmic fractions of 200,000 cells were loaded per lane and immunoblotted for p21$^{Cip1/Waf1}$ and PCNA.

p21$^{Cip1/Waf1}$ mutant were expressed at equivalent levels in viable, proliferating cells (data not shown). These results argue for the direct involvement of cdk inhibitor cleavage in the activation of cdk2 during apoptosis and its contribution to the execution of the apoptotic program.

Discussion

The Cdk Inhibitors p21$^{Cip1/Waf1}$ and p27^{Kip1} Are Early Targets of CPP32-like Caspases during Endothelial Apoptosis

Determination of whether defined molecular changes in the cell mediate the apoptotic process or simply represent its end points is critical to identification of the molecular mechanisms responsible for apoptosis. In endothelial cells undergoing growth factor deprivation–induced apoptosis, we show rapid cleavage of p21$^{Cip1/Waf1}$, which precedes up-regulation of cyclin A–associated kinase activity. Induction of apoptosis by a number of different stimuli is accompanied by up-regulation of cyclin A–associated kinase activity, and inhibition of this activity suppresses apoptosis (Shi et al., 1994; Chen et al., 1995b; Li et al., 1995; Wang et al., 1995; Meikrantz and Schlegel, 1996). In our experiments, cyclin A–cdk2 activation occurs as early as 4 hr after growth factor deprivation, a time when the majority of the cells are still viable.

Our studies demonstrate by a number of criteria that the caspase family of enzymes (especially CPP32), thought to be the final executioner of the cell, is involved in the cleavage of p21$^{Cip1/Waf1}$ and p27^{Kip1}: first, Ac-DEVD-CHO, a specific inhibitor of CPP32-like caspases, prevents in vitro cleavage of recombinant p21$^{Cip1/Waf1}$ by active endogenous caspases in apoptotic cell lysates; second, recombinant CPP32 and ICE LAP3 cleave recombinant p21$^{Cip1/Waf1}$ and p27^{Kip1} in vitro to fragments that correspond to those generated during HUVEC apoptosis; and third, mutation of the putative CPP32-cleavage site in p21$^{Cip1/Waf1}$ rendered it resistant to caspase cleavage. The initial cleavage of p21$^{Cip1/Waf1}$, followed by cleavage of p27^{Kip1}, coincides with the activation kinetics of both CPP32 and ICE LAP3 in apoptotic endothelial cells and precedes significant cleavage of PARP.

Several possible explanations exist for the inability of caspases to cleave p21$^{Cip1/Waf1}$ in vitro to fragments corresponding in molecular weight to those observed

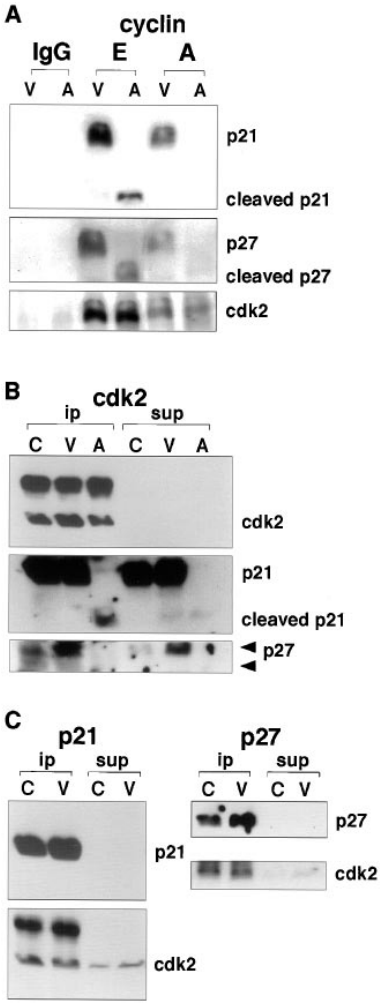

Figure 7. Levels of the Cdk Inhibitors $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ Associated with Cyclin A, Cyclin E, and Cdk2 Are Substantially Reduced in Apoptotic HUVEC

(A) Cyclin E and cyclin A were immunoprecipitated from viable (V) and apoptotic (A) cell lysates 12 hr after growth factor deprivation, and $p21^{Cip1/Waf1}$, $p27^{Kip1}$, and cdk2 associated with the cyclins were evaluated by immunoblotting. Normal rabbit IgG was used as a control.

(B) Cdk2 was immunodepleted from control (C), viable (V), and apoptotic cell lysates (A), and both the immunoprecipitates (ip) and 25 μg of the supernatants (sup) were immunoblotted for $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ associated with cdk2 or free of cdk2, respectively.

(C) $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ were immunodepleted from control (C) and viable (V) cells, and the relative amounts of cdk2 associated with or free of $p21^{Cip1/Waf1}$ and $p27^{Kip1}$, respectively, were determined in immunoprecipitates and supernatants by immunoblotting.

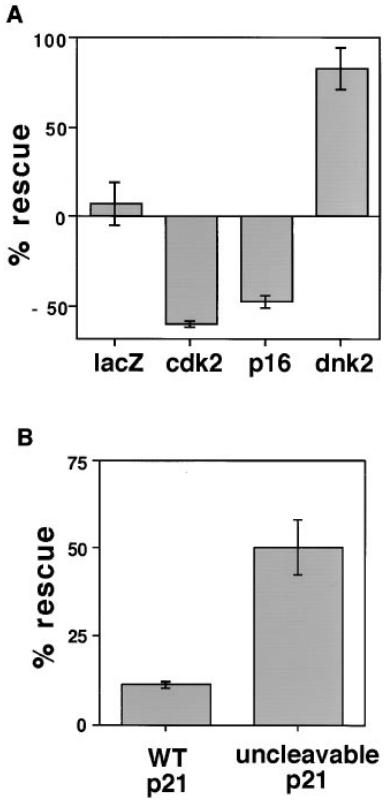

Figure 8. Dominant-Negative Cdk2 and a $p21^{Cip1/Waf1}$ Mutant Resistant to CPP32-Mediated Cleavage Suppress Apoptosis

(A) HUVEC were cotransfected with EGFP and either lacZ, cdk2, $p16^{INK4A}$, or dominant-negative cdk2 (dnk2) and exposed to growth factor deprivation for 24 hr. The surviving cells were analyzed for EGFP positivity by flow cytometry and the results calculated as the difference between the percentage surviving EGFP-positive cells following growth factor deprivation and the EGFP-positive cells before growth factor withdrawal. These have then been expressed relative to the 100% survival represented by the EGFP-positive cells before growth factor withdrawal.

(B) HUVEC were transfected with lacZ, wild-type $p21^{Cip1/Waf1}$, and the uncleavable mutant of $p21^{Cip1/Waf1}$, survival was calculated as in (A), and results expressed as percent survival above lacZ-control. Each column in (A) and (B) represents mean ± SD (n = 3).

in apoptotic cells in the absence of control cell extracts. Binding of $p21^{Cip1/Waf1}$ to cdk–cyclin complexes within the cell may be required to expose the putative CPP32 cleavage site. Alternatively, cellular modifications of $p21^{Cip1/Waf1}$, such as phosphorylation described to occur on Ser98 and Ser130 of $p21^{Cip1/Waf1}$ (Zhang et al., 1994), may be required. Another possibility is that the exogenously added recombinant caspases activate CPP32-like enzymes present in the control cell lysates, which subsequently cleave $p21^{Cip1/Waf1}$—a scenario similar to the one described for the indirect cleavage of U1-70 kDa and PARP in vitro by Mch2 through activation of CPP32 (Orth et al., 1996).

Cleavage of $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ Alters Intracellular Localization and Results in Cdk2 Activation through Their Loss from Cyclin A–cdk2 and Cyclin E–cdk2 Complexes

During apoptosis, the C-terminal regions of both $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ are lost, leaving the N-terminally located kinase inhibitory domain of each (Chen et al., 1995a; Luo et al., 1995; Fotedar et al., 1996) intact. C-terminal deletion mutants of $p21^{Cip1/Waf1}$ that contain only the first 72 N-terminal amino acids (compared to 112 in the cleavage fragment we observe), are able to bind and inhibit cyclin–cdk2 complexes with similar efficiency as full-length $p21^{Cip1/Waf1}$ in vitro and can arrest cells in G1 (Luo et al., 1995). However, cleavage of $p21^{Cip1/Waf1}$ at the CPP32

cleavage site, also present at the C terminus of $p27^{Kip1}$ ($DPSD^{139}S$), separates a C-terminal portion that includes the nuclear localization sequence. Loss of this sequence could result in exit of cdk inhibitors from the nucleus, generating cyclin–cdk complexes that are inhibitor-free. Indeed, $p21^{Cip1/Waf1}$ immunoreactivity declines rapidly in the nucleus only 4 hr after growth factor withdrawal, which correlates closely with the appearance of the $p21^{Cip1/Waf1}$ cleavage fragment in the cytoplasmic fraction and the dramatic induction of cyclin A–cdk2 activity. Consistent with the delay in the appearance of $p27^{Kip1}$ cleavage, there is no significant change in nuclear $p27^{Kip1}$ immunoreactivity at 4 hr. However, we cannot exclude the possibility that cleavage of $p27^{Kip1}$ is also involved in the up-regulation of cyclin A–cdk2 activity: a clear disappearance and substantial reduction, respectively, in the levels of both $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ associated with cyclin A–cdk2 and cyclin E–cdk2 is observed in apoptotic cells, causing a dramatic induction in cdk2 activities.

Our observations that $p21^{Cip1/Waf1}$ is lost from the nucleus early in the apoptotic process suggest that cleavage occurs in the nucleus and that truncated $p21^{Cip1/Waf1}$ exits from the nucleus to the cytoplasm. This would require an altered affinity of $p21^{Cip1/Waf1}$ for the cyclin–cdk complex. The size of the N-terminal $p21^{Cip1/Waf1}$ fragment we observe is only 14 kDa, and it could theoretically enter the nucleus by passive diffusion, bind to cyclin–cdk complexes and, given a sufficiently high affinity for the complex, remain there. Reduced affinity of $p21^{Cip1/Waf1}$ following cleavage as observed in this study is supported by the recent identification of a second cyclin-binding site near the C terminus of $p21^{Cip1/Waf1}$ (Adams et al., 1996; Chen et al., 1996; Ball et al., 1997) that is lost in the cleaved $p21^{Cip1/Waf1}$ fragment. Peptides derived from this site can compete with full-length $p21^{Cip1/Waf1}$ for binding to the cyclin–cdk complex and can inhibit cdk4 activity, Rb phosphorylation by cyclin A–associated kinases, and induce G1/S arrest (Ball et al., 1997). Nuclear localization of $p21^{Cip1/Waf1}$ may also be mediated by its association with PCNA via its PCNA-binding domain (amino acids 133-164) at the very C terminus (Chen et al., 1995a; Luo et al., 1995; Warbrick et al., 1995), which is also absent in the truncated $p21^{Cip1/Waf1}$ we observe. PCNA together with the C terminus of $p21^{Cip1/Waf1}$ forms a trimeric ring around DNA strands and anchors the PCNA/$p21^{Cip1/Waf1}$ complex (Gulbis et al., 1996). Thus, loss of the PCNA binding domain through cleavage may also affect the ability of the $p21^{Cip1/Waf1}$ fragment to localize to the nucleus.

A marked reduction is observed in the total amounts of intact $p21^{Cip1/Waf1}$ in viable cells compared to the $p21^{Cip1/Waf1}$ cleavage fragment in apoptotic cells, suggesting that after cleavage, the $p21^{Cip1/Waf1}$ fragment is degraded or processed. A maximum in the amount of $p21^{Cip1/Waf1}$ fragment is seen 4 hr after induction of apoptosis, with a subsequent decrease paralleling the decrease of full-length $p21^{Cip1/Waf1}$. Thus, reduction in the levels of cleaved $p21^{Cip1/Waf1}$ in apoptotic cells and lack of the nuclear localization signal, as well as the second cyclin- and PCNA-binding domains, may together contribute to the loss of $p21^{Cip1/Waf1}$ from cyclin A– and cyclin E–cdk2 complexes and cdk2 activation.

Cdk2 Is a Potential Facilitator of the Execution of Apoptosis, and Uncleavable $p21^{Cip1/Waf1}$ Is a Protective Factor

Our studies demonstrate that transiently transfected dominant-negative cdk2 can suppress apoptosis of endothelial cells after growth factor deprivation, and they suggest that activation of cdk2 through cdk inhibitor cleavage may facilitate execution of the apoptotic process. Staurosporine- and TNFα-induced apoptosis of HeLa cells is also inhibited by dominant-negative cdk2 (Meikrantz and Schlegel, 1996), and PC12 cells are protected from growth factor deprivation–induced apoptosis by pharmacologic cdk2 inhibitors (Park et al., 1996). Thus, cdk2 activation may be generally necessary for apoptosis. One possible explanation for the anti-apoptotic effect of dnk2 in our system is that it may exert its protective effect by arresting the cells in G0/G1 and thus decrease the susceptibility of the cells to apoptosis (King and Cidlowski, 1995; Meinkrantz and Schlegel, 1995). This interpretation does not appear likely in our system because expression of $p16^{INK4A}$, a cdk inhibitor that blocks entry into S phase through inhibition of cdk4 and cdk6 (Serrano et al., 1995), does not prevent apoptosis, and the apoptotic HUVEC have not entered S phase by BrdU-staining, indicating that S phase entry is not required for apoptosis in our system.

Recent observations, together with the present data, suggest that $p21^{Cip1/Waf1}$ is a generally protective factor against apoptosis, and they raise the intriguing possibility that part of this protective function may be through prevention of cdk2 activation: first, overexpression of $p21^{Cip1/Waf1}$ blocks apoptosis during myogenic differentiation (Wang and Walsh, 1996), and, conversely, antisense oligonucleotides to $p21^{Cip1/Waf1}$ promote apoptosis during neurogenic differentiation (Poluha et al., 1996), although it is not clear whether cdk activities were dysregulated during apoptosis in these experimental systems; second, adenoviral overexpression of $p21^{Cip1/Waf1}$ protects against prostaglandin A2–induced apoptosis in human colorectal carcinoma cells (Gorospe et al., 1996); and third, although $p21^{Cip1/Waf1}$ alone is not always sufficient to counter the proapoptotic effect of p53 (Polyak et al., 1996), the ability to induce $p21^{Cip1/Waf1}$ appears to be required for resistance to p53-induced death in some cell types, as overexpression of p53 leads to apoptosis only when $p21^{Cip1/Waf1}$ fails to increase proportionally, and overexpression of $p21^{Cip1/Waf1}$ protects against p53-induced apoptosis (Gorospe et al., 1997).

Our results support a protective role of $p21^{Cip1/Waf1}$ in apoptosis. Expression of wild-type $p21^{Cip1/Waf1}$ could partially protect HUVEC from apoptosis, but a far more pronounced antiapoptotic effect was achieved by expression of a $p21^{Cip1/Waf1}$ mutant resistant to caspase cleavage. However, the uncleavable $p21^{Cip1/Waf1}$ mutant is less effective in protecting the cells than dominant-negative cdk2, and one possible explanation is that a cleavage-resistant $p27^{Kip1}$ acting together with the uncleavable $p21^{Cip1/Waf1}$ mutant to inhibit cdk2 is necessary to achieve the full protection effect of dnk2. Besides its effect on cdk2 activation, cleavage of $p21^{Cip1/Waf1}$ and its loss from the nucleus may also impair DNA repair, as $p21^{Cip1/Waf1}$ has been proposed to coordinate DNA replication and postreplicative DNA repair during S phase

(Li et al., 1994) due to its ability to bind PCNA and inhibit its function in DNA replication, while allowing gap-filling DNA repair synthesis. It will be important to examine the function of potential $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ cleavage in apoptotic systems such as granzyme B–induced apoptosis, where granzyme B activates both cyclin A–associated kinases required for apoptosis (Shi et al., 1994, 1996; Chen et al., 1995b) and caspases like CPP32 (Darmon et al., 1995).

In apoptotic endothelial cells, there is a particular enhancement of cyclin A–cdk2 activity. Cyclin A, together with cdks, colocalizes with discrete sites of DNA replication in S phase nuclei, which are associated with the nuclear matrix and require an intact nuclear envelope for function (Cardoso et al., 1993). cdk2 has been shown to have DNA unwinding activity that is blocked by $p21^{Cip1/Waf1}$ (Adachi and Laemmli, 1994). Does decreased association of $p21^{Cip1/Waf1}$ with the cyclin–cdk complex after cleavage result in enhanced DNA unwinding activity of the cdks in apoptosis? With the concomitant caspase-mediated breakdown of the nuclear structure, bringing DNA synthesis and cell cycle progression to a halt, could this DNA unwinding activity of the cdks potentiate DNA fragmentation by apoptotic endonucleases? And is p53, a major regulator of apoptotic pathways and a phosphorylation substrate for cdk2 (Price et al., 1995; Wang and Prives, 1995), a target for the induced cdk activities in apoptosis?

The selective cleavage by caspases of specific substrates appears critical to commitment of the cell to apoptosis (Ashkenas and Werb, 1996), but it is currently unclear whether prevention of cleavage of any single caspase substrate will lead to permanent resistance to a process as complex as cell death. However, the kinetics and the extent of delay of apoptosis may help evaluate the differential importance of particular substrates in initiation versus execution of apoptosis. Our studies clearly support this perspective, and we provide the data in a normal diploid cell. It will be important to compare similarities and differences in the regulatory mechanisms of cdk activation during apoptosis between normal and transformed cells to identify possible alterations of apoptotic pathways in cancer cells.

Experimental Procedures

Reagents

The following antibodies and reagents were generously provided as indicated or purchased: MAB for human cyclin A (BF683) and cyclin E (HE172 and HE12) from Dr. E. Harlow and Dr. N. Dyson, Massachusetts General Hospital Cancer Center, Charlestown, MA; PAB for human cdk2, Rb, PCNA, C and N terminus of p21 and p27 (Santa Cruz); MAB for PSTAIR sequence from Dr. M. Yamashita, Hokkaido University, Sapporo, Japan; PAB for cdk4 from Dr. D. Beach, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY; PAB for cylcin D1 and for aa 1-80 of p21 (UBI); MAB for cdk2 (Transduction Labs); MAB for aa 58-77 of p21 (Oncogene); PAB for p21 immunoprecipitations (Pharmingen); antibody for PARP (Enzyme Systems Products); FITC-labeled goat anti-rabbit antibody (Cappel); ZVAD (Alexis); recombinant CPP32, ICE LAP3, and Mch2, and antibodies to these proteins from Dr. V. M. Dixit, University of Michigan, Ann Arbor, MI; recombinant human p21 and p27, PAB for human cyclin A, cyclin E, p27, and plasmids for cdk2 (pCS2+cdk2), dominant-negative cdk2 (pCMVdnk2), $p16^{INK4A}$ (pCMVp16), p21 (pWT p21), and the uncleavable p21 (pExxGp21) from two of the authors (J. M. R. and B. E. C); pcDNA3.1/His/lacZ (lacZ) from Invitrogen.

Cell Culture, Induction, and Assessment of Apoptosis

HUVEC were cultured in RPMI 1640, supplemented with 15% calf serum, 3% endothelial cell growth supplement, and 50 μg/ml heparin. Apoptosis was induced in confluent cell monolayers by incubating the cells in RPMI without growth factors. Apoptotic cells are found in the supernatant as floaters and were collected at the indicated times. The remaining adherent cells are viable and show no characterictic features of apoptosis. Apoptosis was assessed by staining with Hoechst 33342 for nuclear morphology, DNA laddering, PARP cleavage and CPP32, ICE LAP3, and Mch2 activation. BrdU incorporation after labeling for the entire period of induction of apoptosis was determined by flow cytometry analysis with an antibody to BrdU (Becton Dickinson) and 4,6-diamidino-2-phenylindole (Sigma) as DNA stain.

Protein Analysis, Immunoprecipitations, and Cdk Activity

Cells were rinsed with PBS and lysed in 50 mM Tris/HCl (pH 7.5), 0.5% NP-40, 10% glycerol, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.5 mM Na_3VO_4, 10 mM β-glycerophosphate, 0.5 mM PMSF, 5 μg/ml leupeptin and aprotinin. Cell lysates were separated on 15%–17% SDS-PAGE, proteins transferred to Immobilon membrane (Millipore), immunoblotted, and visualized by ECL (Amersham). Cyclin-associated kinase activities and cdk inhibitors associated with cyclin–cdk complexes were determined as in Koyama et al (1996). Cdk2 was immunodepleted from 200 μg cell lysate using 2.5 μg PAB (Santa Cruz), and the equivalent of 25 μg was loaded as supernatant. Western blotting for caspases was performed as in Orth et al. (1996) and cell fractionation as described for cyclin A in Pines and Hunter (1991).

Immunocytochemistry

HUVEC were plated on gelatin-coated chamber-slides, fixed in 4% paraformaldehyde for 20 min at RT, permeabilized in 0.5% NP-40 in PBS for 10 min, and washed in PBS. Incubation with primary antibodies (5 μg/ml) was performed in 0.1% BSA/PBS for 1 hr at RT followed by incubation with a FITC-labeled secondary antibody.

In Vitro Cleavage of $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ and Generation of $p21^{Cip1/Waf1}$ Mutants

Cell lysates were prepared as in Casciola-Rosen et al. (1996), and 10 μg viable and apoptotic cell lysate were incubated at 37°C with 2 ng of rhp21 in 10 μl for the indicated times. Ac-DEVD-CHO, Ac-YVAD-CHO, and Ac-YVAD-CMK (Bachem Bioscience) were added 20 min prior to the addition of p21, reactions were terminated by addition of sample buffer and analyzed by Western blotting. When recombinant caspases were used, 500 ng of each enzyme was incubated with 2 ng rhp21 in the presence or absence of 10 μg control cell extract for 2 hr at 37°C as described (Orth et al., 1996). In vitro cleavage experiments for p27 were performed as for p21 but using 0.5 ng rhp27 and in 1.5 μg/μl BSA. In experiments where endogenous cdk inhibitors were used as substrate, 500 ng recombinant caspase was incubated with 10 or 50 μg of control cell lysate for p27 and p21, respectively, for 2 hr. The CPP32 cleavage site substitution mutant of p21 was generated by site-directed mutagenesis (Kunkel et al., 1987) from pCS2hp21 (a gift from Dr. M. Ohtsubo, FHCRC, Seattle) by converting the sequence DHVD to EHVA using the primer 5′-acagcagaggaagaacatgtggccttaagcctgtcttgtaccctt-3′. In vitro translation was performed using the TNTR Coupled Reticulocyte Lysate System (Promega) and [35]Smethionine (1000 Ci/mmol, Amersham). Following incubation with or without CPP32, p21 and fragments were immunoprecipitated and the radiolabeled proteins visualized by autoradiography.

Transfections and Analysis of Transfected Cells

HUVEC were plated at 0.45×10^6 in 60 mm dishes, cultured overnight, and cotransfected with 1.1 μg EGFP (Clontech) and 9.9 μg of either pcDNA/His/lacZ, pCS2+cdk2, pCMVp16, or pCMVdnk2, and pcDNA/His/lacZ, pCS2hp21, or the pCS2hp21EhvG substitution mutant, respectively, using Pfx-7 (Invitrogen) in Opti-MEM I (Gibco) according to the manufacturer's instructions. Thirty-six hours after transfection, apoptosis was induced by growth factor deprivation

for 24 hr. The surviving cells were trypsinized and fixed in 2% paraformaldehyde/PBS containing 0.2% BSA. Cells positive for EGFP were quantified by flow cytometry.

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