The Involvement of Protein Phosphatases in the Activation of ICE/CED-3 Protease, Intracellular Acidification, DNA Digestion, and Apoptosis*

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Many events in apoptosis have been identified but their temporal relationships remain obscure. Apoptosis in human ML-1 cells induced by etoposide is characterized by intracellular acidification, enhanced Hoechst 33342 fluorescence, DNA digestion, chromatin condensation, and proteolysis of poly(ADP-ribose) polymerase. This proteolysis is a marker for the action of ICE/CED-3 proteases, which are critical activators of apoptosis. We observed that three serine/threonine protein phosphatase inhibitors, okadaic acid, calyculin A, and cantharidin, prevented all of these apoptotic characteristics. To determine which protein phosphatase was involved, we investigated the dephosphorylation of the retinoblastoma susceptibility protein Rb, a substrate for protein phosphatase 1 but not protein phosphatase 2A. Rb was dephosphorylated during apoptosis, and each inhibitor prevented this dephosphorylation at the same concentrations that prevented apoptosis. No increase in protein phosphatase 1 activity was observed in apoptotic cells suggesting that dephosphorylation of Rb may result from loss of Rb kinase activity in the presence of a constant level of protein phosphatase activity. Long term inhibition of protein phosphatase 1 (>8 h) also led to the appearance of dephosphorylated Rb, cleavage of poly(ADP-ribose) polymerase and apoptosis, suggesting these events are not solely dependent upon protein phosphatase 1. Rb dephosphorylation was also observed in several other models of apoptosis. Hence, an imbalance between protein phosphatase 1 and Rb kinase may be a common means to activate ICE/CED-3 proteases resulting in the subsequent events of apoptosis.

Maintaining tissue and organ homeostasis requires a delicate balance between the rate of cell division and the rate of cell death. Cells that are either metabolically compromised or no longer required are eliminated by a pathway commonly known as apoptosis. This process has been characterized by cell shrinkage, cytoplasmic blebbing, chromosome condensation, DNA digestion, and, finally, non-inflammatory removal of the cell from the tissue (1). Apoptosis occurs during development, immune regulation, and normal cell turnover, as well as being induced by many pharmacological insults. Decreased apoptosis can lead to cancer and autoimmune diseases, while increased apoptosis can result in neurodegenerative disorders and AIDS (2). Despite the importance of apoptosis, the signal transduction pathways responsible for the associated morphological and biochemical changes are poorly understood. Much recent emphasis has focused on the identification of proteases of the ICE/CED-3 family that appear essential to this pathway (3–5), but both the upstream and downstream events remain elusive.

While investigating endonucleases that might be involved in apoptosis, we detected and purified deoxyribonuclease II (DNase II), an endonuclease that is active at slightly acidic pH but inactive at pH 7.0 (6). To determine whether DNase II might be responsible for the DNA digestion, we measured intracellular pH in various cell models during apoptosis. In each model tested, the apoptotic cells were seen as a distinct population with an acidic shift of 0.5–0.8 pH units (7–11). Recently, other laboratories have confirmed that intracellular acidification occurs during apoptosis (12, 13). Hence, intracellular acidification appears to represent a common event in the pathway of apoptotic cell death.

Although intracellular acidification can activate DNase II (6), we have recently established that a low intracellular pH is not required for DNA digestion, suggesting that an additional means of activating DNase II can occur, or that an alternate endonuclease may be involved. Specifically, when CTL-L-2 cells were maintained at an extracellular pH of 8.0, DNA digestion and apoptosis still occurred upon removal of interleukin 2, but the intracellular pH only dropped to 7.2, a pH at which DNase II should be inactive (10). Hence, during apoptosis, cells always appear to undergo intracellular acidification, but the low pH that results is not required for the activation of an endonuclease. These observations suggest that both intracellular acidification and DNA digestion are regulated by a common upstream regulator. This paper presents the results of a search for such a regulator.

The intracellular acidification that occurs during apoptosis results from selective loss of pH regulation. Furthermore, we have demonstrated that the acidification that occurs in apoptotic CTL-L-2 cells results from an alteration in the set point of the Na+/H+ antiport (10). Normally, the Na+/H+ antiport raises the intracellular pH to around neutrality. Although apoptotic CTL-L-2 cells still retained functional antiport, it was only able to raise intracellular pH to around 6.3 in apoptotic cells. The set-point of the Na+/H+ antiport has been shown to be regulated by phosphorylation (14–16). A wide variety of external signals, including growth factors and the extracellular...
matrix, activate a kinase cascade that leads to phosphorylation of the antiprot and intracellular alkalinization. These same stimuli are also known to enhance cell survival. Many intracellular signaling components, such as protein kinase C and Ha-Ras, also lead to enhanced survival and activation of the antiprot (17, 18). Furthermore, the protein phosphatase inhibitor okadaic acid has been shown to activate the antiprot (19). These observations all support the notion that protein kinase cascades protect cells through pathways that also regulate intracellular pH. Accordingly, we have investigated the role of protein phosphatases as potential mediators of intracellular acidification and apoptosis.

Previous results have suggested that the protein phosphatase inhibitors okadaic acid and calyculin A can prevent the onset of apoptosis (20, 21). Those results are confirmed and extended here. We demonstrate that apoptosis, as assessed by DNA digestion, intracellular acidification, changes in membrane permeability, and morphology, can be prevented by a number of protein phosphatase inhibitors. Furthermore, pro tease cleavage of poly(ADP-ribose) polymerase (PARP),1 a number of protein phosphatase inhibitors. Furthermore, pro

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EXPERIMENTAL PROCEDURES

Materials—Etoposide, okadaic acid, calyculin A, cantharidin, acridine orange, ethidium bromide, Percoll, other chemicals, and enzymes were purchased from Sigma. The acetoxyethyl ester of carboxy-SNARF-1 was obtained from Molecular Probes (Eugene, OR). Cell culture supplies and the protein phosphatase assay kit were purchased from Life Technologies, Inc. The G3-24S anti-Rb mouse monoclonal antibody used in initial studies was purchased from Pharmingen (San Diego, CA); the C-15 anti-Rb rabbit polyclonal antibody used in subsequent studies was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-PP1 polyclonal antibody was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). The C-2-10 monoclonal antibody to poly(ADP-ribose)polymerase was a gift of Dr. Guy Poirier (Laval University Hospital, Quebec, Canada).

Cell Culture—ML-1 myeloid leukemia cells were maintained in 5% CO2 at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. All cultures were maintained in logarithmic growth at a density (<1 × 106) cells/ml. Cells were incubated with etoposide for 30 min after which they were centrifuged, washed twice, and resuspended in completed medium without drug. The phosphatase inhibitors were added only for the incubation period following removal of etoposide. In most experiments, cells were harvested and analyzed 4 h after removal of etoposide.

DNA Fragmentation Assay—DNA digestion was measured by agarose gel electrophoresis as described previously (6, 24). Briefly, 106 cells were added directly to the wells of a 2% agarose gel, where they were lysed and digested with ribonuclease A and proteinase K. The gel was electrophoresed for 16 h, and the DNA was stained with ethidium bromide and visualized under ultraviolet light. In this method, high molecular weight DNA (>20 kilobase pairs) remains trapped in or near the well, while smaller fragments (down to 180 base pairs) are resolved in the gel.

Acridine Orange Staining—Chromatin condensation in apoptotic cells was assessed by staining with acridine orange, while membrane

1The abbreviations used are: PARP, poly(ADP-ribose) polymerase; CHI, Chihua hamster ovary; ICE, interleukin 1α-converting enzyme; pH, intracellular pH; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A.
The kinetics of DNA fragmentation were determined following incubation of cells with 20 \( \mu \text{g/ml} \) etoposide (Fig. 1B). At the time of removal of etoposide (30 min), there was an increase in high molecular weight DNA fragments migrating about 1 cm into the gel. These fragments result from the cleavable complexes formed by etoposide and topoisomerase II, but disappear rapidly upon removal of the drug. The majority of internucleosomal DNA fragmentation appeared 3–4 h after removal of etoposide. For HL-60 cells, we previously showed that the cells most susceptible to apoptosis were those in S phase at the time of incubation with etoposide; the remaining cells eventually progressed to, and arrested in, the G2 phase of the cell cycle before undergoing apoptosis (8). Similar experiments in ML-1 cells also showed that the S phase cells were most susceptible (shown below with regard to Fig. 8), but the remaining surviving cells stayed arrested in the G1 phase (data not shown). This is consistent with the ML-1 cells expressing the wild-type p53 tumor suppressor protein, in contrast to HL-60 cells. Hence, the apoptosis observed in this paper is independent of p53 status, although expression of p53 does contribute to cell cycle perturbations in the surviving cells.

To determine the number of cells undergoing apoptosis at each time or drug concentration, cells were analyzed by flow cytometry for increased fluorescent staining with Hoechst 33342. A number of reports have shown that apoptotic cells accumulate Hoechst 33342 at an increased rate, and this can be assessed by a short incubation (<5 min) with the dye (10, 26, 29). Simultaneously, cells were analyzed for intracellular pH with the pH-sensitive fluorescent ratio dye carboxy-SNARF-1. The results are visualized as a scatter plot in which Hoechst fluorescence is shown on the ordinate and intracellular pH (expressed as the emission ratio of 545/640 nm) is shown on the abscissa (Fig. 2). Interestingly, we have found that these two fluorescent dyes detect exactly the same population of cells and, as a result, the combination of dyes helps to discriminate otherwise overlapping populations (10). The normal cells appear in the lower left quadrant and the majority of apoptotic cells in the upper right quadrant; the number of apoptotic cells is recorded in each panel. The number of apoptotic cells increases from about 2% at 5 \( \mu \text{g/ml} \) etoposide to almost 80% at 80 \( \mu \text{g/ml} \). With respect to kinetics following incubation with 20 \( \mu \text{g/ml} \) etoposide, the number of apoptotic cells increases rapidly between 3 and 4 h after treatment in concert with the time of appearance of DNA digestion. The flow cytometry analysis was performed while the extracellular pH was maintained at 7.1, leading to an intracellular pH of 7.15 in the normal cells and 6.5 in the apoptotic cells.

Cells were also stained with acridine orange and scored under a microscope for chromatin condensation and alterations in nuclear morphology that occur during apoptosis (25). Control samples consistently showed less than 5% apoptotic cells, whereas 4 h after incubation with etoposide, 30–41% of the cells exhibited apoptotic morphology. These values are of a similar order as the results obtained by flow cytometry. In two other models, we have previously sorted cells by flow cytometry on the basis of intracellular pH and established that only the acidic cells exhibit these morphological changes (8, 10). The current experiments further confirm that enhanced Hoechst fluorescence and intracellular acidification are valid markers of apoptosis. In all subsequent experiments, apoptosis was induced with 20 \( \mu \text{g/ml} \) etoposide, with DNA fragmentation and other parameters of apoptosis measured after an additional 4-h incubation.

Protein Phosphatase Inhibitors Prevent Etoposide-induced DNA Fragmentation and Apoptosis—The serine/threonine phosphatase inhibitor okadaic acid has been shown to activate
the Na\textsuperscript{+}/H\textsuperscript{+}-antiport involved in intracellular pH regulation (19). Okadaic acid, as well as another serine/threonine phosphatase inhibitor calyculin A, have also been reported to inhibit apoptosis (20, 21). Hence, we investigated the effect of these inhibitors on DNA fragmentation in our model of apoptosis. ML-1 cells were induced to undergo apoptosis with etoposide, and each inhibitor was then added at various concentrations during the subsequent incubation. We found that 1 \mu M okadaic acid and 10 nM calyculin A were the minimum concentrations required to markedly inhibit DNA digestion (Figs. 3 and 4). We also investigated the effect of another protein phosphatase inhibitor, cantharidin, on DNA fragmentation; this inhibitor was found to prevent DNA fragmentation at 20–30 \mu M (Fig. 5).

Next, we investigated the effect of each inhibitor on the increased Hoechst fluorescence and intracellular acidification that occurs during apoptosis (Fig. 6). Incubation of cells with 1 \mu M okadaic acid for 4 h neither enhanced Hoechst fluorescence nor caused intracellular acidification. However, when added to cells following incubation with etoposide, 1 \mu M okadaic acid completely prevented both enhanced Hoechst fluorescence and intracellular acidification. Incubation with 0.1 \mu M okadaic acid was unable to prevent acidification or increased Hoechst fluorescence consistent with its inability to inhibit DNA fragmentation (Fig. 3).

Results obtained with calyculin A and cantharidin were slightly different from okadaic acid in that each inhibitor alone
induced a slight acidic shift (Fig. 6). The intracellular acidification, and dephosphorylation of Rb.

The experimental protocol was identical to Fig. 3, with the exception that the cells were incubated with indicated concentrations of cantharidin rather than okadaic acid.

The morphological changes detectable by staining with acridine orange were also investigated for each of these conditions. Etoposide treatment alone induced chromatin condensation in 30–41% of the cells, whereas the addition of either okadaic acid, calyculin A, or cantharidin to etoposide-treated cells reduced this value to 12.6, 7.0, or 5.0%, respectively (average of three separate experiments). Hence, all three inhibitors were able to prevent apoptosis as assessed by chromatin condensation, increased Hoechst fluorescence, and intracellular acidification, and this protection occurred at the same concentrations that also prevented DNA fragmentation.

Protein Phosphatase Inhibitors Prevent Proteolysis of Poly-(ADP-ribose) Polymerase—Considering the recent reports that demonstrate a role for ICE/CED-3 proteases in apoptosis, we investigated whether the inhibitors used here functioned at a step upstream or downstream of this protease. PARP has been shown to be proteolytically cleaved from a 116-kDa protein to a 85-kDa fragment by ICE/CED-3 proteases (30). Most recently, this cleavage has been associated with the CPP32/Yama/apoptosis protease family (5,31). This cleavage can be used as an endogenous marker for the action of these proteases. We first confirmed that the cleavage of PARP occurred with the same dose and time dependence as all of the other indicators of apoptosis used here. Cleavage of PARP was faintly visible following 10 μg/ml etoposide, consistent with this concentration inducing faint DNA fragmentation (Fig. 1A). Cleavage of PARP was faintly visible 2.5 h after incubation with 20 μg/ml etoposide, which was 30 min before the first detectable DNA fragmentation (Fig. 1B). At 4 h following 20 μg/ml etoposide, at which about 50% of the cells were apoptotic according to the other indicators, approximately 50% of the PARP protein appeared to be cleaved; this suggests that complete cleavage of the protein was probably occurring in each apoptotic cell.

We next determined whether the protein phosphatase inhibitors prevented cleavage of PARP. In each case, the same concentration of each inhibitor that prevented apoptosis was able to prevent the cleavage of PARP (Figs. 3–5). These results demonstrate that the action of a protein phosphatase is an event upstream of proteolysis in the pathway of apoptosis.

De-phosphorylation of the Rb Protein Is Associated with Apoptosis—Okadaic acid, calyculin A, and cantharidin are inhibitors of both protein phosphatases 1 and 2A; in vitro okadaic acid and cantharidin are more effective against PP2A, whereas calyculin A is equally effective against both PP1 and PP2A (32, 33). The fact that low concentrations of calyculin A, but high concentrations of okadaic acid and cantharidin, were required to protect cells suggested the involvement of PP1 in apoptosis. Since these concentrations cannot be extrapolated directly to cells, we selected an intracellular marker of PP1 activity to establish its involvement. PP1 is involved in the de-phosphorylation of the retinoblastoma susceptibility protein Rb at the conclusion of mitosis (22); Rb is then dephosphorylated by cyclin-dependent kinases during G1 and S phases (34). In contrast, PP2A appears inactive toward phosphorylated Rb.

Cell lysates were separated by electrophoresis, and Rb was detected by Western blotting; the phosphorylated forms of Rb exhibit retarded electrophoretic mobility. In undamaged ML-1 cells, the majority of Rb was detected as a single band that represents the hyperphosphorylated form (Fig. 1). Following incubation of ML-1 cells with etoposide, the Rb protein electrophoresed faster indicating dephosphorylation; this dephosphorylation occurred with an etoposide dose dependence that correlated with both the appearance of DNA digestion and the cleavage of PARP (Fig. 1A). With respect to kinetics, the dephosphorylation of Rb was clearly visible 2.5 h after removal of etoposide, whereas the cleavage of PARP and DNA digestion were first detected at 3 h (Fig. 1B). The time of Rb dephosphorylation was also prior to any significant increase in intracellular acidification or increased Hoechst fluorescence. Hence, it appears that Rb dephosphorylation may precede cleavage of PARP and DNA fragmentation by at least 30 min, suggesting that Rb dephosphorylation is an upstream event in the pathway. By 5 h after etoposide treatment, the majority of Rb was dephosphorylated, even though only about 50% of the cells had undergone apoptosis. As mentioned earlier, the remaining surviving cells were arrested predominantly in the G1 phase, at which point in the cell cycle dephosphorylation of Rb would be expected.

The phosphorylation status of Rb was assessed following incubation of the cells with the three protein phosphatase inhibitors. The minimum concentration of each inhibitor that prevented DNA fragmentation and proteolysis was also the minimum concentration that prevented de-phosphorylation of Rb (Figs. 3–5). These results suggest the involvement of PP1 as a mediator of apoptosis.

We also used an antibody to PP1 to assess any potential proteolysis or other change in electrophoretic mobility. PP1 protein appeared as a single band with a molecular mass of 37 kDa in both undamaged and apoptotic cells (Figs. 3–5). Although PP1 is regulated by phosphorylation (35), presumably this does not alter its electrophoretic mobility. In most cases, no significant change was seen in the intensity of the PP1 protein. However, incubation of cells with calyculin A led to a dose-de-
pendent loss of detectable PP1 protein (Fig. 4), as well as loss of measurable PP1 activity (data not shown). This loss was observed both in etoposide-treated cells and in otherwise undamaged cells. The concentration of calyculin A that caused loss of PP1 protein and activity correlated with the concentration that prevented apoptosis. Based on the report that another protein phosphatase inhibitor, microcystin, irreversibly inhibits PP1 by direct alkylation (36), it is possible that calyculin A may bind to PP1 and either block antibody detection or target it for destruction. Whatever the reason, the loss of PP1 at exactly the same concentration as calyculin A that blocks apoptosis is further evidence to support the hypothesis that PP1 is involved in mediating apoptosis.

We also determined whether the intracellular acidification caused by incubation of cells with calyculin A alone (Fig. 6) might be due to residual PP2A activity. This is possible as calyculin A is about equally effective as an inhibitor of PP1 and PP2A. In contrast, okadaic acid at a relatively low concentration inhibits only PP2A. Cells were incubated with the combination of 10 nM calyculin A and either 0.1 or 1 μM okadaic acid. The results were identical to those observed if okadaic acid had been omitted, i.e. the cells still showed the calyculin A-mediated intracellular acidification, as well as the suppression of etoposide-mediated apoptosis. Accordingly, we could not demonstrate any role for PP2A in the regulation of intracellular acidification or apoptosis. The calyculin A-mediated acidification is probably due to a purported direct interaction with ion channels (37).

The dephosphorylation of Rb normally occurs at mitosis, and it is rephosphorylated as a cell passes through G1 (34). We have already established that the cells preferentially undergoing apoptosis following etoposide are in the S phase. Therefore, we needed to establish whether the cells undergoing apoptosis were the same as those dephosphorylating Rb, thus implicating aberrant activity of PP1 in apoptosis. Apoptotic cells, which have an increased buoyant density, were separated from the remaining surviving cells by fractionation on a Percoll density gradient (Fig. 7). The fractionated cells were then analyzed for cell cycle distribution and Rb phosphorylation. Greater than 95% of the undamaged cells banded at a single density, showed no DNA digestion, and exhibited hyperphosphorylated Rb. In contrast, approximately 35% of the etoposide-treated cells banded at a higher density, and these cells contained digested DNA and dephosphorylated Rb. The surviving cells were predominantly in G1 and G2 phases, with a marked reduction in S phase cells compared to untreated cells. In contrast, the apoptotic cells exhibited only a sub-G1 population as is commonly observed for cells with fragmented DNA. Hence, apoptosis is occurring preferentially in S phase cells, and these are the cells that contain dephosphorylated Rb, supporting the hypothesis that PP1 is inappropriately activated in the S phase cells.

Protein Phosphatase Activity in Cell Lysates during Apoptosis—The phosphorylation status of Rb is dependent upon the rate of both phosphorylation and dephosphorylation. To determine whether protein phosphatases were activated during apoptosis, we assayed the activity of PP1 as well as PP2A in cell lysates. About 80% of the observed protein phosphatase activity was attributed to PP1 as judged by its inhibition by PP1 inhibitor-2 peptide, and the remainder was attributed to PP2A as judged by its inhibition by 2 nM okadaic acid (Fig. 8). During apoptosis, there was no detectable increase in either total phosphatase activity or in the activity of either PP1 or PP2A. These results suggest that the observed dephosphorylation of Rb is probably due to inhibition of a protein kinase (see "Discussion").

Induction of Apoptosis by Protein Phosphatase Inhibitors—These protein phosphatase inhibitors are unable to provide long term protection from etoposide as they eventually induce apoptosis themselves. Therefore, we determined whether dephosphorylation of Rb and proteolysis of PARP would still
occur at later times even though PP1 and PP2A were inhibited.
ML-1 cells were incubated with calyculin A and cells analyzed at various times thereafter. DNA fragmentation began around 8 h, and was visible as a distinct nucleosome ladder by 16 h (Fig. 9). At this time, both PARP cleavage and dephosphorylated Rbwere observed. Assay of the cell lysates confirmed that PP1 activity remained undetectable throughout this time course. In the absence of PP1, we assume that the apparent Rb dephosphorylation results from loss of kinase activity required to phosphorylate de novo synthesized Rb. This same kinase activity is presumably responsible for the suppression of ICE/CED-3 and the subsequent events of apoptosis.

Dephosphorylation of Rb in Other Models of Apoptosis—The observations made in this paper are not unique to ML-1 cells and etoposide. We have obtained identical results with HL-60 cells treated with etoposide: when incubated for 30 min with 40 \( \mu \)g/ml etoposide, intracellular acidification, enhanced Hoechst fluorescence, DNA digestion, cleavage of PARP, and dephosphorylated Rb were observed, and each of the protein phosphatase inhibitors was equally effective as in ML-1 cells. The HL-60 cells are deficient for p53, in contrast to ML-1 cells, suggesting these results are independent of p53 status. Dephosphorylation of Rb is also not unique to etoposide-treated cells; we have also observed dephosphorylation of Rb in ML-1 cells induced to undergo apoptosis with 400 \( \mu \)M staurosporine. These cells also undergo intracellular acidification, cleave PARP, and dephosphorylate Rb, and each of the protein phosphatase inhibitors prevent these changes at the same concentrations as in ML-1 cells (data not shown). Finally, we have also observed Rb dephosphorylation in Chinese hamster ovary (CHO) cells induced to undergo apoptosis following incubation with either staurosporine or cisplatin, and in the cytotoxic T cell line, CTTL-2, following withdrawal of interleukin 2.

**DISCUSSION**

The experiments presented here were stimulated by two observations relating to the regulation of apoptosis. First, it is known that cells require survival factors such as growth factors or extracellular matrix, and that these function by activating intracellular protein kinase cascades (38). Furthermore, some of these kinases, when constitutively activated, enhance survival and lead to cancer (39, 40). Second, we have consistently observed intracellular acidification during apoptosis, and this can occur by an alteration in the set-point of the Na\(^+\)/H\(^+\)-antiport, a protein known to be phosphorylated and activated by the same survival pathways (10). This evidence for the role of protein kinases in protecting cells led to our hypothesis that protein phosphatases might be activators of the apoptotic pathway. Accordingly, we investigated the possibility that protein phosphatase inhibitors might prevent apoptosis.

Three protein phosphatase inhibitors were used in these studies and all were found to inhibit DNA digestion, chromatin condensation, cleavage of PARP, increased Hoechst fluorescence, and intracellular acidification. The experiments established a minimum concentration of each inhibitor that was required for this protection; these concentrations were 1 \( \mu \)M okadaic acid, 10 \( nM \) calyculin A, and 20 \( \mu M \) cantharidin. These three agents preferentially inhibit PP1 and PP2A, but are relatively ineffective on other protein phosphatases. The efficacy of each inhibitor has been established on purified phosphatases; PP2A is inhibited by 1 \( nM \) okadaic acid, 1 \( nM \) calyculin A, and 40 \( nM \) cantharidin, whereas PP1 is inhibited by 60–500 \( nM \) okadaic acid, 2 \( nM \) calyculin A, or 2 \( \mu M \) cantharidin...
these experiments is that apoptosis appears to be regulated
by a yet uncharacterized role in apoptotic cell death.

Hence, the relative concentrations of each inhibitor
required to prevent apoptosis is most consistent with the
involvement of PP1.

The in vitro efficacy of any inhibitor does not extrapolate
directly to the concentrations required to inhibit an enzyme in
intact cells. Therefore, to test the possible involvement of PP1
in apoptosis more directly, we investigated the phosphorylation
status of Rb, a protein that is known to be dephosphorylated by
PP1, but not by PP2A (22). We observed that Rb was dephos-
phorylated during apoptosis, and that the minimum concentra-
tion of each protein phosphatase inhibitor that prevented
apoptosis was the same as that effective at preventing dephos-
phorylation of Rb. Furthermore, we observed loss of detectable
PP1 protein at concentrations of calyculin A that prevented
apoptosis. Hence, the dephosphorylation observed here repre-
sents abnormal activity of PP1, suggesting that PP1 is an
activator of apoptosis.

In this etoposide-induced model of apoptosis, the cells prefer-
entially die in the S phase, a period of the cell cycle in which
Rb should normally be hyperphosphorylated. However, analy-
sis of PP1 in cell lysates did not show increased activity during
apoptosis. This suggests that Rb dephosphorylation during
apoptosis may result from inhibition of Rb kinase, while the
level of PP1 activity remains unchanged. Alternately, the
standard assay for PP1 used here may be unable to detect
changes in phosphatase activity either because the critical PP1
activity may be compartmentalized (for example, bound to Rb),
or that the binding of an endogenous inhibitor may be dis-
rupted during extraction and assay. Hence, the conclusion of
these experiments is that apoptosis appears to be regulated by
an imbalance between PP1 and Rb kinase, although the molec-
ular basis still needs to be established. Rb is phosphorylated by
cyclin-dependent kinases, predominantly cyclin D/Cdk4 or
cyclin D/Cdk6 in the G1 phase and possibly cyclin E/Cdk2 in
late G1/S phase (34). Phosphorylation of Rb in G1 is required for
passage of cells into S phase, but the reason for continued
phosphorylation during the S phase has remained elusive. The
current results suggest that this continuing phosphorylation
may be necessary to suppress apoptosis.

Several other reports have demonstrated that okadaic acid
and calyculin A can prevent apoptosis (20, 21), while other
reports show that these agents can induce apoptosis (20, 41).
This discrepancy can be explained on the basis of differences in
concentration and time. A report by Song et al. (20) and this
work have shown that longer incubations with these agents can
indeed induce apoptosis. This suggests an alternative, PP1-
dependent mechanism for the activation of apoptosis. The
discussion above suggests that loss of a protein kinase may be
more important than activation of a protein phosphatase, and
this is consistent with our observation that unphosphorylated
Rb appears in cells even when PP1 is inhibited. This may be
due to lack of phosphorylation of de novo synthesized Rb. Al-
ternately, the eventual dephosphorylation of Rb may result
from a low level of some other protein phosphatase.

PP1 dephosphorylates many proteins in addition to Rb, any
of which might be the critical target. The Na+/H+-antiport is an
example of a potential target for PP1, particularly as high
concentrations of okadaic acid (1 M) are required to prevent its
dephosphorylation (19). Therefore, the imbalance between PP1
and protein kinase provides a potential mechanistic explana-
tion for the change in antiport activity that is associated with
intracellular acidification and apoptosis. Our results also allow
the interesting possibility that untimely S-phase dephospho-
phorylation of Rb might cause abnormal sequestration of Rb-asso-
ciated proteins such as E2F, c-Myc, and c-Abl. Since these
nuclear proteins are required for normal DNA synthesis and
cell proliferation, it is possible that their withdrawal may play
a yet uncharacterized role in apoptotic cell death.

It is also important to consider where the protein phosphatase
may be located in the pathway of apoptosis. One possibility
is that it is a consequence of apoptosis, perhaps activated as a
consequence of DNA digestion or intracellular acidification.
However, this possibility could only be true if aberrant Rb
dephosphorylation and proteolysis of PARP were also a conse-
quence of DNA digestion. All the evidence presented here
shows that the inhibition of protein phosphatase activity, as
judged by the inhibition of Rb dephosphorylation, prevents all
of the biochemical and morphological indicators of apoptosis.
Hence, the protein phosphatase inhibitors must act on an up-
stream regulator in the pathway, i.e. at a step that is a cause
rather than a consequence of the onset of apoptosis.

We have also observed that these protein phosphatase inhib-
itors prevent the cleavage of PARP, a recognized substrate for
the CPP32 protease, a member of the ICE family (5, 31).
Activation of CED-3, the Caenorhabditis elegans member of this
family of proteases, is required for programmed cell death in
this nematode (42), and its mammalian homologs are also
considered to be required for apoptosis. Hence, these results
suggest that PP1 functions, directly or indirectly, as an activa-
tor of a protease that is essential for the induction of apoptosis.

Clearly, apoptosis is not an obligate consequence of PP1
activity. PP1 is a normal constituent of cells and plays a role in
normal cell cycle regulation. For example, PP1 is normally
activated as cells pass through mitosis and is inactivated again
in G1, which correlates with the cycle of Rb dephosphorylation
and rephosphorylation (22, 34). Presumably, other signals
must suppress the apoptotic action of PP1 during its normal

![Fig. 9. Calyculin A induces DNA digestion, PARP cleavage, and dephosphorylation of Rb during extended incubations.](Image)
activation in G1; survival signals from the extracellular environment and the associated kinase cascades represent likely means to mediate this suppression, and may explain the need for such survival signals in the G1 phase of the cell cycle. The results presented here also suggest that an imbalance between these kinase and phosphatase activities regulates the decision between cell survival and apoptosis. Although the majority of experiments reported here were performed with etoposide as the inducer of apoptosis, these observations are not unique to this model. Experiments with HL-60, CTLL-2, and CHO cells confirmed that Rb was dephosphorylated in these cells as they underwent apoptosis. Very recently, another group also reported the dephosphorylation of Rb during apoptosis in human lymphoid cells incubated with etoposide and another anticancer drug cytosine arabinoside (43, 44). Those reports concluded that the cells arrested in G1 prior to undergoing apoptosis, but analysis of their results shows a miscalculation of the number of cells in the G1 phase; our interpretation of their results is that a preferential loss of S phase cells occurred as reported here. In conclusion, an imbalance between PP1 and Rb kinase may be a common regulator in the induction of apoptosis. It remains to determine the critical substrates for dephosphorylation, how this activates the ICE/CED-3 protease, and finally, to determine the identity of the critical substrates for proteolysis.

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