H1 histones are involved in the formation of higher order chromatin structures and in the modulation of gene expression. Changes in chromatin structure are a characteristic initial feature of apoptosis. We therefore have investigated the histone H1 pattern of the human leukemic cell line HL60 undergoing programmed cell death, as induced by topoisomerase I inhibition. Histone H1 proteins were isolated and analyzed by high performance liquid chromatography and capillary zone electrophoresis. DNA fragmentation after apoptosis induction was monitored by agarose gel electrophoresis. The patterns of the three H1 histone subtypes extractable from apoptotic HL60 cells significantly differed from those of control cells in showing a decrease of phosphorylated H1 subtypes and an increase of the respective dephosphorylated forms. This dephosphorylation of H1 histones could be observed already 45 min after apoptosis induction and preceded internucleosomal DNA cleavage by approximately 2 h. We conclude that during apoptotic DNA fragmentation, the H1 histones become rapidly dephosphorylated by a yet unknown protein phosphatase.

During the last 5 years, our understanding of the process of apoptosis has dramatically increased. This highly regulated form of programmed death of a eukaryotic cell can either be induced by neighboring cells via receptor-mediated activation of an apoptotic cascade or by the cell itself, e.g. when irreversibly DNA damage has been detected by cell cycle checkpoint control (reviewed in Refs. 1 and 2). Both forms of cell death share most of the signaling cascade mediated by caspases, and both forms finally result in a characteristic chromatin condensation and later in the degradation of the cell into apoptotic bodies, subcellular particles that can be removed by neighboring cells. The signaling molecules that play major roles in the amplification and execution of cell death have been thoroughly investigated through the last years, and even first clinical applications have been described (10), we postulate a role for the phosphorylation state of certain histone H1 subtypes shortly after induction of apoptosis and before the onset of internucleosomal cleavage. Since the H1 histones are positioned at a candidate site for the initiation of enzymatic attack of an internucleosomally cleaving DNase. Thus, we were interested in determining whether the patterns of H1 histones and their posttranslationally modified forms change after induction of apoptosis and, if so, whether these changes might play a role in the regulation of apoptotic DNA cleavage.

In this study, we describe the rapid dephosphorylation of certain histone H1 subtypes shortly after induction of apoptosis and before the onset of internucleosomal cleavage. Since the H1 histones are positioned at a candidate site for the initiation of DNA fragmentation, and since it has been already demonstrated that H1 histones can greatly enhance CAD activity in vitro (10), we postulate a role for the phosphorylation state of H1 histone subtypes for the modulation of DNA recognition and/or DNA cleavage by this enzyme.

EXPERIMENTAL PROCEDURES

Purification of Histone H1 Proteins from Human Tumor Cell Lines—HL60 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum in a humidified air/CO2 (19:1) atmosphere at 37 °C and treated with different drugs as indicated in the figures. After the incubation, cell suspensions (usually 5 × 107 cells/incubation) were centrifuged (5 min, room temperature, 300 × g), and the cells were washed once with phosphate-buffered saline. 5% perchloric acid was added to the cell pellets, and cells were lysed and extracted by incubation for 1 h at room temperature under continuous stirring. Samples were centrifuged (30 min, 4 °C, 14,000 × g), and the supernatant was stored while the pellet was reextracted once with 5% perchloric acid. Supernatants were collected and centrifuged again. The acid-soluble proteins were precipitated by the addition of 7 volumes of acetone (−20 °C) and overnight incubation at −20 °C. After centrifugation (see above), pellets were washed twice with cold acetone and vacuum-dried. The histones were then dissolved in 30 mM HCl and subjected to further purification (see below). The HL60 cell line (ATCC no. CCL-240) was obtained from Cell Line Service (Heidelberg, Germany). Cell culture reagents and media were purchased from Biochrom (Berlin, Germany).

This paper is available on line at http://www.jbc.org
All biochemical reagents except topotecan (from SmithKline Beecham) were from Sigma.

**Induction of Apoptosis**—To induce apoptosis, the human leukemic cell line HL60 was treated with the topoisomerase I inhibitor topotecan (150 ng/ml) for various times. The process of ongoing apoptosis was monitored by light microscopy and also by isolation of fragmented DNA from apoptotic cells after a method previously described (11). The fragmented DNA from equal amounts of cells was applied onto a 1.5% agarose gel and separated electrophoretically.

To analyze the incorporation of phosphate into H1 histones after apoptosis induction, cells from parallel dishes were cultivated in phosphate-free RPMI for 2 h and then treated with [32P]orthophosphoric acid (9.25 MBq/ml of medium) 1 h prior to topotecan treatment. At various times, the cells were washed, and H1 histone subtypes were isolated as described (11). Protein amounts of H1 from equal cell numbers were separated on a 12% polyacrylamide gel. The gel was fixed and stained with Coomassie Blue and then scanned and subjected to autoradiography. The autoradiography was analyzed densitometrically, and the amount of incorporated [32P] was calculated as percentage of the unincorporated phosphate.

**Purification of H1 Histones by Gel Filtration**—H1 histones were purified from histone solutions in 30 mM HCl (see above) by gel filtration (12, 13). Briefly, solubilized H1 histones were applied onto a Bio gel P60 column (Bio-Rad). The application volume was 2 ml, and the total amount was ~100 µg. The column length was 350 mm, and column width was 18 mm. After application, the column was eluted with 30 mM HCl at a flow rate of ~0.3 ml/min. 3.5-min fractions were collected, 20-µl aliquots of all fractions were applied onto a 12.5% polyacrylamide gel, and, after running the gel, it was stained with Coomassie Blue for analysis of the elution profile. Fractions containing H1 histones were pooled and, in the case of HL60 histone, concentrated by vacuum-drying and subjected to H1 subtype separation by high-performance liquid chromatography (HPLC).

**HPLC of H1 Histone Subtypes**—The HPLC purification method was modified after a method described by Talazaz et al. (14) to get a better separation of the migrating human H1 subtypes H1.4 and H1.2. However, we have not achieved quantitative separation of human H1.4 from H1.2 using reversed phase HPLC. To increase the separation efficiency, we used a relatively flat acetonitrile gradient and manual fraction collection. The HPLC unit was a Kontron 450 MT2 system, equipped with an M 420 pump, an M 425 gradient former, and an M 432 variable wavelength detector. 100 µl of total P60-purified H1 histones with a concentration of 100–1000 µg/ml solubilized in 30 mM HCl were loaded onto a Zorbax 300SB-C18 column (4.6 × 250 mm, 5-µm particle size; Agilent Technologies, Waldbronn, Germany). Samples were eluted at a constant flow of 1 ml/min, and absorbance of the effluent was monitored at 210 nm. Solvent A was water containing 0.1% trifluoroacetic acid; solvent B was 59.5% acetonitrile in water containing 0.1% trifluoroacetic acid. The gradient run was as follows: 0–15 min, 0% B; 15–82.5 min, 34–64% solvent B. Manually collected samples were freeze-dried, resolubilized in 30 mM HCl, and subjected to further analysis by polyacrylamide gel electrophoresis (PAGE) or capillary zone electrophoresis (CZE) (see below).

**CZE of H1 Histones**—CZE was performed on a Perkin-Elmer/Applied Biosystems 270A-HT system. The separation conditions were optimized after a method of Lindner et al. (15). The capillary type was fused silica, length was 122 cm, and inner diameter was 50 µm. Separations were performed at the following conditions: temperature, 30 °C; voltage, 20 kV; current, ~35 µA; separation time, 50 min; sample injection by pressure; injection time, 2 s; sample protein concentration, ~0.5 mg/ml; absorbance detection at 200 nm. Separation buffer was 30 mM H2PO4, 60 mM HCO3, 0.02% hydroxypropylmethylcellulose (HPMC), pH 2.00, adjusted with trifluoroacetic acid. Absorbance data were recorded and analyzed by a PC-based Kontron Integration System.

**In Vitro Protein Kinase Assays with Recombinant H1 Histone H1.2**—Recombinant human H1 histone H1.2 was prepared from transformed yeast as described (11, 21) and purified by P60 gel filtration as described above. H1.2 was incubated either with 1 µM of human recombinant cyclin-dependent kinase 1 (CDK1) (New England Biolabs, Frankfurt, France), 1 µM PKCa (both from Sigma), or with 0.5 µM of human recombinant protein kinase Cα (Calbiochem, Schwalbach, Germany). The incubation buffer for CDK1 was 50 mM Tris/HCl, pH 7.2, 20 mM MgCl2, 1 mM EGTA, 2 mM dithiothreitol, 200 µM ATP. Incubation buffer for PKCa was 50 mM Tris/HCl, pH 7.2, 20 mM magnesium acetate, 1 mM CaCl2, 200 µM ATP with 30% lipid mix (1 mg/ml phosphatidylserine, 200 µM dl diocatoylglycerol (both from Sigma) in 10 mM Hepes, pH 7.5, 0.3% Triton X-100). Incubation times were as indicated in the figure legends. Total incubation volume was 13 µl. Incubations were stopped by the addition of 1 µl of 1 M HCl and then subjected to analysis by CZE.

**Analysis of Fragmented DNA by Agarose Gel Electrophoresis**—For the preparation of fragmented DNA, cells were harvested and washed as described for the purification of histone H1 proteins. Preparation of fragmented DNA has been described in Ref. 11. Briefly, cell pellets were incubated on ice for 30 min in 450 µl of lysis buffer (0.2% Triton X-100 in 10 mM Tris, 10 mM EDTA, pH 8.0). Samples were microcentrifuged (11, 600 × g for 10 min at 4 °C), and the nonsedimentable DNA fragments of the supernatants were precipitated by the addition of 450 µl of 2-propanol and 100 µl of 10 mM LiCl and incubation at ~20 °C overnight. After centrifugation as above, the pellets were washed twice with ice-cold 70% (v/v) ethanol and vacuum-dried. The pellets were then resolubilized in 50 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Samples were extracted twice for DNA with chloroform/isooamyl alcohol (24:1), and the purified DNA was again precipitated by the addition of 100 µl of ethanol and 15 µl of 3 M sodium acetate (pH 4.8) and incubation for at least 2 h at ~70 °C. After centrifugation as above, pellets were vacuum-dried and resolubilized in 30 µl of TE buffer. Extracted DNA was resolved in 1.2% agarose gels using a constant current of 70 mA and visualized by staining with ethidium bromide.

**RESULTS**

Dephosphorylation of H1 Histone Subtypes from HL60 Cells after Induction of Apoptosis—Exponentially growing HL60 cells were pretreated with [32P]orthophosphoric acid for 1 h and then treated with the topoisomerase I inhibitor topotecan at a concentration of 150 ng/ml of medium. At different times after treatment, H1 histones and fragmented DNA were prepared...
FIG. 2. HPLC and CZE separation of H1 histone subtypes from HL60 cells: comparison with CZE electropherograms from 
*in vitro* phosphorylated H1.2. Either untreated HL60 cells or cells that had been treated with topotecan for 8 h were harvested, and H1 histones were
from the cells as described under “Experimental Procedures.” The H1 histone proteins were analyzed by polyacrylamide gel electrophoresis and subsequent autoradiography and also by capillary electrophoresis. The fragmented DNA was separated by agarose gel electrophoresis and then stained and imaged. The data show that topotecan induces DNA fragmentation, becoming visible 3–4 h after treatment of the cells (Fig. 1A). This apoptotic response of HL60 cells to topoisomerase I inhibitors has already been demonstrated by us and other groups (Ref. 11; reviewed in Ref. 16). The analysis of the H1 histones by polyacrylamide gel electrophoresis revealed that the total amounts of H1 histones extractable from 10⁶ cells did not significantly vary after induction of apoptosis (Fig. 1B). However, regarding the phosphorylation state of the H1 histones, there was a more than 50% reduction in total H1 phosphorylation, becoming evident already 2 h after topotecan treatment (Figs. 1, C and D). This reduction remained constant throughout the observation time of 8 h.

HPLC Separation of H1 Histones and Subsequent CZE Analysis Allows Quantification of Phosphorylation Rates of Individual H1 Histone Subtypes—The CZE analysis of H1 histones from untreated versus topotecan-treated HL60 cells had revealed that modified and unmodified forms of H1 subtypes contribute to the total H1 pattern (11). We therefore separated the major H1 histone subtypes present in HL60 cells by reversed phase HPLC before CZE (see “Experimental Procedures”). With this technique, we could quantitatively separate H1.5 from H1.4 and H1.2 (Fig. 2A). Moreover, we achieved a substantial separation of H1.2 from H1.4, as demonstrated by PAGE in Fig. 2B. This HPLC fractionation allowed us to reanalyze the individual H1 subtypes by CZE (Fig. 2C) as a prerequisite for the interpretation of the electropherograms that we obtained with total H1 histone preparations (e.g. in Fig. 3A).

Since the data shown in Fig. 1 indicate a dephosphorylation of H1 histones after topotecan treatment, we tried to prove whether the additional peaks of the H1 subtypes shown in Fig. 2C indeed reflect phosphorylated forms of the individual subtypes. Therefore, we performed in vitro protein kinase assays using recombinant human CDK1 and PKCo and recombinant human histone H1.2 as a substrate. The subsequent analysis of the phosphorylation products was done by CZE. As shown in Fig. 2D, phosphorylation of H1.2 by CDK1 or PKCo resulted in a decrease of the unphosphorylated form. Simultaneously, several additional peaks appeared migrating slower than the unmodified H1.2. They had the same peak distances from the unmodified form as the additional peaks we observed in CZE electropherograms of H1 subtypes from HL60 cells (Fig. 2C, control). Thus, we conclude that these peaks indeed reflect phosphorylated forms of H1 subtypes. This ability of the capillary zone electrophoresis technique to separate phosphorylated H1 variants has already been demonstrated by Lindner et al. (17). Other groups have independently shown that CZE is capable of separating differentially phosphorylated forms of single proteins because of the total net charge of the molecule as the dominant separation force in CZE (18–20).

Unexpectedly, the H1 subtypes of untreated cells strongly differed in their phosphorylation state, in such a way that H1.5 and H1.4 were up to 3 times phosphorylated, with a maximum of once and twice phosphorylated forms, whereas H1.2 seemed to be only phosphorylated once. Whether this phosphorylated form of H1.2 is phosphorylated preferentially at a defined serine or threonine residue or whether it represents a mixture of differently, but only once, phosphorylated forms, remains to be determined by tryptic digestion and mass spectrometry analysis of the resulting peptides (see “Discussion”).

We performed the same experiment with topotecan-treated cells to analyze the rate of dephosphorylation of individual histone H1 subtypes as indicated by the labeling experiments shown in Fig. 1. H1 histones from 4 × 10⁶ HL60 cells treated for 8 h with 150 ng/ml topotecan were extracted and purified by gel chromatography, and the H1 subtypes were separated by HPLC (Fig. 2A, right). The peaks eluting from the HPLC were collected, and the individual H1 subtypes were analyzed by PAGE (Fig. 2B, bottom) and capillary electrophoresis (Fig. 2C, bottom). As expected from the experiment shown in Fig. 1, all three histone H1 subtypes showed a massive reduction of their phosphorylated subtypes upon topotecan treatment, leading to a relative increase of the respective unphosphorylated forms. The HPLC peak representing H1.4 showed two additional peaks when analyzed by CZE. However, since the separation of H1.4 from H1.2 by HPLC is incomplete, there is an overlay of residual H1.2 with twice phosphorylated H1.4 within the peak eluting last in the electropherogram of H1.4 (see overlay in Fig. 2C, bottom), making it impossible to quantify the amount of twice phosphorylated H1.4 within the preparation. However, the phosphorylation seen in untreated HL60 cells appears to be, at least partially, H1 subtype-specific (see above), whereas the dephosphorylation differs only in its extent with respect to individual histone H1 subtypes.

H1 Subtype Dephosphorylation Precedes Nucleosomal DNA Fragmentation—HL60 cells were incubated in the presence of 150 ng/ml topotecan for different times. After incubation, the cell cultures were split, and half the cells were used for the extraction of H1 histones, while the other half served as a source for the preparation of fragmented DNA. H1 histones were then purified as described under “Experimental Procedures” and further analyzed by capillary zone electrophoresis, as described in Ref. 11. The identification of unmodified histone H1 subtypes had been previously shown by mixing the preparations from cell lines with recombinant proteins purified from transformed yeast (21). Mixing e.g. H1.2 with the H1 preparation from human cell lines had resulted in the selective increase of one single peak, indicating that this peak represents H1.2 within the mixed preparation. These mixing experiments had been done with all human recombinant subtypes except H1t, which is exclusively expressed in the testis (22). The identities of the peaks with the respective H1 histone subtypes are indicated in the upper (control cells) and lower panel (6-h topotecan-treated cells) of Fig. 3A. Isolation of fragmented DNA was done by treatment of the cells with a buffer containing 0.2% Triton X-100. Experimental details are given under “Experimental Procedures.” The fragmented DNA was purified prepared by perchloric acid extraction as described under “Experimental Procedures.” H1 histones were then solubilized in 30 mM HCl and purified by gel filtration. Purified H1 histones were subjected to HPLC separation using a reversed phase C18 column and a water plus 0.1% trifluoroacetic acid to 60% acetonitrile, water, 0.1% trifluoroacetic acid gradient (see “Experimental Procedures”). Absorbance of the effluents at 210 nm was continuously recorded. The HPLC chromatograms of H1 from untreated (control) as well as topotecan-treated cells (topo) are shown in A. The peaks representing the three different H1 subtypes present in HL60 cells (H1.5, H1.4, and H1.2) were manually collected, dried, and resolubilized in 30 mM HCl. Identical aliquots of each three samples were subjected to PAGE, and then the gels were stained with Coomassie Blue (B). Other aliquots were subjected to CZE using a fused silica capillary with a total length of 122 cm and an inner diameter of 50 μm. Absorbance data at 200 nm were continuously recorded. C, electropherograms of the separated H1 subtypes as well as overlays of the individual subtype electropherograms from untreated (control; upper four panels) as well as topotecan-treated (topo; lower four panels) cells. D, electropherograms of recombinant human histone H1.2 purified from transformed yeast (see “Experimental Procedures”; D, top) that was phosphorylated in vitro either by incubation with recombinant human CDK1 for 15 min (middle) or with human recombinant PKCo for 30 min (bottom).
by organic extraction and electrophoretically separated on a 1.25% agarose gel. The gel was stained with ethidium bromide and digitally imaged (Fig. 3B).

The CZE analysis revealed that already 45 min after topotecan treatment, a significant change in the total H1 subtype pattern could be observed, leading to an increase in the amounts of unmodified H1 subtypes H1.4 and H1.5, whereas other peaks presumably representing phosphorylated H1 subtypes simultaneously decreased (Fig. 3A). However, nucleosomal DNA fragments could not be detected before 3 h of topotecan treatment (Fig. 3B). This indicates that the putative protein phosphatases or protein kinase inhibitors that are responsible for the dephosphorylation of H1 histones after apoptosis induction may be activated by a mechanism that acts by organic extraction and electrophoretically separated on a 1.25% agarose gel. The gel was stained with ethidium bromide and digitally imaged (Fig. 3B).

The CZE analysis revealed that already 45 min after topotecan treatment, a significant change in the total H1 subtype pattern could be observed, leading to an increase in the amounts of unmodified H1 subtypes H1.4 and H1.5, whereas other peaks presumably representing phosphorylated H1 subtypes simultaneously decreased (Fig. 3A). However, nucleosomal DNA fragments could not be detected before 3 h of topotecan treatment (Fig. 3B). This indicates that the putative protein phosphatases or protein kinase inhibitors that are responsible for the dephosphorylation of H1 histones after apoptosis induction may be activated by a mechanism that acts...
rapid Histone H1 Dephosphorylation after Apoptosis Induction

**DNA Fragmentation as Mediated by Topoisomerase I Inhibition Is Diminished by Simultaneous Protein Phosphatase Inhibition**—To determine if the dephosphorylation of H1 histones in the course of apoptosis induction could be influenced by phosphatase inhibition, we treated the cells with the phosphatase inhibitor calyculin A, a substance that is about equally potent in inhibiting type 1 and 2 protein phosphatases. Incubation of HL60 cells with a concentration of 30 nM calyculin A for 8 h resulted in a significant hyperphosphorylation of H1 histones (Fig. 4F). We then were interested in determining whether protein phosphatase inhibition could influence the sensitivity of HL60 cells toward apoptotic inducers such as topotecan. We therefore treated the cells for 8 h with increasing concentrations of calyculin A (3, 10, and 30 nM) and simultaneously with a constant concentration of topotecan (150 ng/ml). After treatment, the cells were washed and subjected to the extraction of H1 histones on one hand as well as analysis of DNA fragmentation on the other hand. We observed that the lower molecular weight DNA fragmentation caused by topotecan treatment was diminished in the presence of 3 nM calyculin A, and higher molecular weight fragments remained in the upper parts of the respective lanes (Fig. 4G). Moreover, with higher concentrations of calyculin A we observed a reduction in the total amount of fragmented DNA per given cell number, suggesting that internucleosomal DNA fragmentation is reduced by simultaneous protein phosphatase inhibition.

The pattern of H1 histones showed an increase in peaks putatively representing phosphorylated H1 histone subtypes after treatment with topotecan and calyculin A at the same time (Fig. 4, C–E). This hyperphosphorylation could be observed with 3 nM of calyculin A but was more pronounced with higher concentrations. The pattern observed with 30 nM of calyculin A and 150 ng/ml topotecan (Fig. 4E) was very similar to that with 30 nM of calyculin A alone (Fig. 4F), implying that the effect of protein phosphatase inhibition, leading to a dramatic hyperphosphorylation of H1 histones, was dominant over the dephosphorylating effect of topotecan treatment.

**Activation of PKC by PMA Treatment Also Inhibits Apoptotic DNA Fragmentation**—The role of protein kinase C activation within the signaling process of apoptosis has been discussed in the literature rather controversially (reviewed in Ref. 23). Pro-apoptotic as well as a series of antiapoptotic effects have been reported, depending on the different tissue model systems (24, 25). We therefore were interested in the effects of protein kinase C activation on DNA fragmentation and apoptosis as induced by the inhibition of topoisomerase I. HL60 cells were treated with 100 ng/ml PMA, 150 ng/ml topotecan, or both substances (D) for 6 h. After incubation, H1 histones were isolated and purified as described above. H1 histones were separated by CZE and the absorbance at 200 nm was recorded. A–D show 6-min intervals (from 40 to 46 min) of 50-min capillary electrophoresis separations. Fragmented DNA was purified from cells treated in the same way and analyzed on a 1.2% agarose gel (Fig. 5E). The marker bands are the same as shown in Fig. 1A.

**The PKC Inhibitor Staurosporine Reduces H1 Phosphorylation and Induces Apoptosis and DNA Cleavage**—The protein kinase C inhibitor staurosporine has been reported to be a potent apoptosis inducer in several cell lines, including HL60 cells (26). We were therefore interested in the effects of staurosporine on the histone H1 phosphorylation pattern as well as on the DNA fragmentation pattern. As shown in Fig. 6F, a 2-h treatment with 0.3 μM staurosporine was capable of inducing DNA fragmentation in HL60 cells. At the same time, the phosphorylation levels of all H1 subtypes were greatly reduced (Fig. 6B). At 6 h of staurosporine treatment, the peak representing mainly once phosphorylated histone H1.2 became very small (Fig. 6F). This suggests that in the HL60 promyelocytic cell line the induction of protein kinase C as the main target of PMA has antiapoptotic effects that are not correlated with the capacity to increase histone H1 phosphorylation.

It is obvious that PMA treatment resulted in an overall increase of the relative amount of phosphorylated H1 subtypes, leading to a decrease of the peaks representing the unphosphorylated H1 histones H1.4 and H1.5, while the latest peak representing mainly monophosphorylated H1.2 increased (Fig. 5B). Treatment of the cells with both PMA and topotecan (Fig. 5D) led to a CZE pattern, which highly resembled the pattern obtained with topotecan alone (Fig. 5C), indicating that in this case, the dephosphorylating effect of topotecan is dominant over the induction of H1 phosphorylation by PMA. Regarding the DNA fragmentation pattern (Fig. 5E), PMA alone did not induce DNA fragment formation by itself but instead was capable of reducing the DNA fragmentation capacity of topotecan. This suggests that in the HL60 promyelocytic cell line the upstream of the caspase 3-dependent activation of CAD/DFF40.

**Figure 5.** The protein kinase C activator PMA also inhibits topotecan-mediated DNA fragmentation but does not attenuate topotecan-mediated H1 dephosphorylation. 10^6 HL60 cells were treated with 100 ng/ml PMA (B), 150 ng/ml topotecan (C), or both substances (D) for 6 h. After incubation, H1 histones were isolated and purified as described above. H1 histones were separated by CZE and the absorbance at 200 nm was recorded. A–D show 6-min intervals (from 40 to 46 min) of 50-min capillary electrophoresis separations. Fragmented DNA was purified from cells treated in the same way and analyzed on a 1.2% agarose gel (Fig. 5E). The marker bands are the same as shown in Fig. 1A.
or less constant. However, by using capillary electrophoresis alone, it was not possible to identify the nature of histone H1 subtype modifications (11).

Radioactive labeling of HL60 cells with [32P]orthophosphate prior to the analysis of H1 histones clearly demonstrated that the demodification we observed by CZE after apoptosis induction correlated with a strong reduction in the amount of incorporated phosphate, indicating that these proteins are dephosphorylated upon onset of apoptosis. Yet, the CZE electropherograms showed an overlay of the peaks of multiply phosphorylated forms of H1.5 and H1.4 with the peaks representing unphosphorylated and once phosphorylated forms of H1.2. To overcome this problem, we established a combined method, first separating the H1 subtypes present in HL60 cells by reversed phase HPLC and second separating the differentially modified forms of each subtype by capillary electrophoresis. This allowed us to differentially analyze the phosphorylation state of H1 subtypes from normally growing cells and to compare it with the extent of phosphorylation of subtypes purified from apoptotic cells (see Fig. 2).

We show here that there is a very strong correlation between the occurrence of internucleosomal DNA fragmentation in the course of apoptosis of human leukemic cells and a massive dephosphorylation of all H1 histone subtypes that could be isolated from these cells. Since the dephosphorylation precedes the DNA fragmentation and since inhibitors of H1 dephosphorylation also inhibit DNA fragmentation, the activation of a putative protein phosphatase responsible for the H1 dephosphorylation after apoptosis induction could be located upstream of the caspase 3-mediated activation of CAD, the enzyme putatively responsible for the generation of nucleosomal fragments. On the other hand, our data show that although activation of PKC by PMA was able to reduce the DNA fragmentation-inducing effect of topotecan, the histones were nonetheless dephosphorylated, indicating that the dephosphorylation of H1 histones is not a sufficient prerequisite in the activation process of CAD.

Within the sequences of H1 histones, there is a variety of putative phosphorylation sites; e.g. histone H1.4 has one potential phosphorylation site for Ca2+/calmodulin-dependent kinase II, seven sites for casein kinase I, two for glycogen-stimulated kinase 3, five sites for kinases of the CDK family, two sites for protein kinase A, six sites for protein kinase C, and two sites for protein kinase G (computationally predicted phosphorylation sites, partially overlapping; source: Center for Biological Sequence Analysis (Technical University of Denmark) on the World Wide Web). Evidently, not all these sites are phosphorylated in vivo. Our data with the broad specificity protein kinase C activator PMA as well as with the protein kinase C inhibitor staurosporine suggest a participation of members of the protein kinase C family in the process of H1 histone phosphorylation. However, it is unclear whether this is a direct or indirect involvement. Moreover, it is unknown whether the strong proapoptotic effects of staurosporine (26) and the protective effect of PMA in HL60 cells are directly correlated with the abilities of these substances to modulate protein kinase C activity (33). Additional experiments showed that more specific PKC inhibitors (e.g. Goe 6976, which is specific for the inhibition of the conventional PKC isoforms) had no proapoptotic effect and also no inhibitory effect on H1 phosphorylation, indicating that members of the novel PKC or the atypical PKC subfamilies or even unrelated protein kinases could be involved in these pathways (data not shown).

The identities of specific physiological H1 phosphatases have also not been ruled out yet. Although several reports describing H1 phosphatase activities have been published, the results are
rather controversial. Some authors have indications for a protein phosphatase 2A activity in postmitotic (34) or in apoptotic (35) cells, whereas other groups have shown evidence for phosphatase type 1 being at least the protein phosphatase responsible for the H1 dephosphorylation at the end of mitosis (36). In radiation-treated human cells, also a rapid dephosphorylation of H1 histones has been observed, although the authors could not discriminate between phosphatase 1 and phosphatase 2A being responsible for this dephosphorylation (37).

There are also evident differences in the degree of phosphorylation of individual H1 subtypes of control HL60 cells as well as in the degrees of their dephosphorylation after apoptosis induction. The subtype H1.2 has four potential phosphorylation sites for protein kinases of the CDK family (motif: (S/T)XK). Although the subtypes H1.4 and H1.5 have only one more CDK phosphorylation site (located at the C terminus of the peptides), they are phosphorylated in vivo up to three times, whereas H1.2 is only phosphorylated once. Consequently, this single phosphorylation site of H1.2 is nearly completely dephosphorylated upon apoptosis induction, leading to a strong reduction of the latest peak in electropherograms of total H1 preparations from apoptotic cells (see Fig. 3A), whereas the other two subtypes still show a moderate phosphorylation level. In addition to these in vivo studies, we have performed in vitro phosphorylation assays with recombinant human histone H1.2 and with CDK1 and PKCα as protein kinases. Under these conditions, H1.2 could be phosphorylated at multiple sites (Fig. 2D). The reason for this discrepancy between in vitro and in vivo phosphorylation levels is unclear. However, one could speculate that in vivo, regulatory proteins selectively interact with parts of the H1.2 molecule, thereby masking some of the phosphorylation sites for protein kinases of the CDK family, resulting in a single phosphorylation event. Experiments with mass spectrometric analysis of tryptic peptides from H1.2, H1.4, and H1.5 are planned to prove this hypothesis. In Tetracyclina, the phosphorylation sites of macronuclear H1 have been mapped and also mutated. Even when all five hydroxyamino acid sites were changed to alanines, the viability of this organism was not significantly altered, indicating that the phosphorylation seems to be dispensable in this protozoan organism (38).

Recently, a series of reports from different groups described the initial phosphorylation of distinct core histones after apoptosis induction in different cellular model systems. The first observation was that treatment with the fungal toxin gliotoxin leads to a rapid phosphorylation of histone H3 at Ser-10 in thymocytes (39). In this case, the authors showed evidence for protein kinase A being involved in that apoptotic phosphorylation step. The phosphorylation of the core histone subtype H2A.X has also been reported to be closely correlated with the initiation of DNA fragmentation in Jurkat and HL60 cells (40, 41). This phosphorylation is mainly restricted to one position at Ser-139 and can be inhibited by simultaneous transfection of the cells with the inhibitor of CAD, ICAD. The authors postulate that the phosphorylation of H2A.X could be mechanistically correlated with the initiation of apoptotic DNA cleavage.

In addition, an apoptosis-correlated phosphorylation of histone H2B has been demonstrated in a series of human cell lines, including HL60 cells (42). However, the author could not find any major changes in the phosphorylation state of other histones, including the histones H3, H2A.X, and H1. This is in contrast to the data presented here as well as to the data concerning changes in the phosphorylation state of other core histones mentioned above.

In conclusion, several groups have reported an increase in core histone phosphorylation during onset of apoptosis in human cell lines. Our own data on changes of the phosphorylation state of H1 histones during apoptosis show for the first time a dramatic decrease in H1 histone phosphorylation at an early stage of apoptotic DNA fragmentation. A similar decrease in H1 phosphorylation has been observed by Guo et al. (37) after treatment of human cell lines (RKO and Jurkat) with high doses of ionizing radiation. Although the authors did not investigate apoptosis induction in their cell systems, it is likely that there is an overlap in the DNA strand break-inducing effects of ionizing radiation and topoisomerase I inhibition, making their results comparable with what we have observed in HL60 cells.

A stimulatory role for H1 histones in the process of CAD-mediated DNA fragmentation in vitro has already been shown twice (10, 43). However, it is unclear whether different H1 subtypes and/or differentially phosphorylated forms of H1 histones have specific influences on CAD activity. On the other hand, a role of changes in the phosphorylation state of H1 histones in another process of chromatin reorganization, namely in mitotic chromatin condensation has been proposed for a long time. Several reports provide evidence for kinases of the CDK family being involved in mitosis-specific phosphorylation (44), which in turn seems to be a prerequisite for the correct compaction of metaphase chromosomes (45). Thus, the structural changes within the nucleosomal organization of chromatin seem to depend, at least in part, on the location and numbers of hydroxyamino acid phosphorylations within the H1 molecule. It is not clear, however, which sites of individual H1 histone subtypes are obligatorily phosphorylated during mitosis and, consequently, which sites have to dephosphorylated in order to correctly complete anaphase and cytokinesis.

We have shown here that there is a strong correlation between initial apoptotic H1 dephosphorylation and the onset of apoptotic DNA fragmentation, implying that indeed the phosphorylation state of H1 histones in situ located within the chromatin might also be important for the processes of chromatin condensation and/or chromatin fragmentation. Further studies have been initiated to bring more light into the role of H1 histones for the biochemical execution of cell death.

Acknowledgments—We are grateful to SmithKline Beecham Inc. for providing topotecan and to Natalie Obermeyer and Christa Bode for assistance.

REFERENCES
1. Nagata, S. (1997) Cell 88, 355–365
2. Nagata, S. (2000) Exp. Cell Res. 256, 12–18
3. Sobott, C. A., and Lowe, S. W. J. (1999) Pathology 187, 127–137
4. Bamford, M., Walkinshaw, G., and Brown, B. (2000) Exp. Cell Res. 256, 1–11
5. Wylie, A. H. (1980) Nature 284, 555–556
6. Oi, D. E., Oi, A. L. (1978) Am. Biochem. 66, 704–711
7. Liu, X., Zou, H., Slaughter, C., and Wang, X. (1997) Cell 89, 175–184
8. Enari, M., Sakairi, H., Yokoyama, H., Okawa, K., Iwamata, A., and Nagata, S. (1998) Nature 391, 43–50
9. Wolfe, A. P. (1997) Int. J. Biochem. Cell Biol. 12, 1463–1466
10. Liu, X., Zou, H., Widlak, P., Garrard, W., and Wang, X. (1999) J. Biol. Chem. 274, 13836–13840
11. Kratzeimer, M., Albig, W., Meersang, T., and Donnecke, D. (1999) Biochem. J. 337, 319–327
12. Huang, H. C., and Cole, R. D. (1985) FEBS Lett. 183, 270–274
13. von Holt, C., Brandt, W. F., Greyling, H. J., Lindsey, G. G., Retief, J. D., Rodrigues, D. J., Schwager, S., and Sewell, B. (1989) Methods Enzymol. 170, 431–523
14. Talazs, H., Sajnovics, N., Heilliger, W., Lindner, H., and Puschendorf, B. (1990) Adv. Biochem. 273, 32236–32243
15. Lindner, H., Heilliger, W., Sarg, B., and Meraner, C. (1995) Electrophoresis 16, 604–610
16. Kantarjian, H. (1999) Hematol. 36, Suppl. 8, 16–25
17. Lindner, H., Heilliger, W., Dirschmayer, A., Talazs, H., Wurm, M., Sarg, B., Jaquemar, M., and Puschendorf, B. (1992) J. Chromato. 598, 211–216
18. Dawson, J. F., Boland, M. P., and Holmes, C. F. (1994) Anal. Biochem. 220, 340–345
19. Wei, J., Yang, L., Harrata, A. K., and Lee, C. S. (1998) Electrophoresis 19, 2356–2360
20. Gamble, T. N., Ramachandran, C., and Bateman, K. P. (1999) Anal. Biochem. 271, 3469–3476
21. Albig, W., Runge, D. M., Kratzeimer, M., and Donnecke, D. (1998) FEBS Lett. 435, 245–250
Rapid Histone H1 Dephosphorylation after Apoptosis Induction

22. Drabent, B., Bode, C., and Doenecke, D. (1993) *Biochim. Biophys. Acta** 1216, 311–313
23. Cross, T. G., Scheel-Toellner, D., Henriquez, N. V., Deacon, E., Salmon, M., and Lord, J. M. (2000) *Exp. Cell Res.* 256, 34–41
24. Zhu, W. H., and Loh, T. T. (1996) *Biochem. Pharmacol.* 51, 1229–1236
25. Kaneko, Y. S., Ieda, K., Nakanishi, M. (1999) *Life Sci.* 65, 2251–2258
26. Finucane, D. M., Waterhouse, N. J., Amarante-Mendes, G. P., Cotter, T. G., and Green, D. R. (1997) *Exp. Cell Res.* 231, 166–174
27. Doenecke, D., Albig, W., Bouterfa, H., and Drabent, B. (1994) *J. Cell Biochem.* 54, 423–431
28. Meergans, T., Albig, W., and Doenecke, D. (1997) *DNA Cell Biol.* 16, 1041–1049
29. Allan, J., Mitchell, T., Harborne, N., Bohm, L., and Crane-Robinson, C. (1984) *J. Mol. Biol.* 187, 591–601
30. Thoma, F., Koller, T., and Klug, A. (1979) *J. Cell Biol.* 83, 403–427
31. Wolfe, A. P. (1999) *Chromatin: Structure and Function*, Academic Press, London
32. Spencer, V. A., Davie, J. R. (1999) *Gene (Amst.)* 240, 1–12
33. Masuda, Y., Yoda, M., Ohizumi, H., Aiuchi, T., Watabe, M., Nakajo, S., and Nakaya, K. (1997) *Int. J. Cancer.* 71, 691–697
34. Sola, M. M., Langan, T., and Cohen, P. (1991) *Biochim. Biophys. Acta** 1094, 211–216
35. Mills, J. C., Lee, V. M., and Pittman, R. N. (1998) *J. Cell Sci.* 111, 625–636
36. Paulson, J. R., Patzlaff, J. S., and Vallis, A. J. (1996) *J. Cell Sci.* 109, 1437–1447
37. Guo, C. Y., Wang, Y., Brautigan, D. L., and Larner, J. M. (1999) *J. Biol. Chem.* 274, 14533–14536
38. Mizzen, C. A., Dou, Y., Liu, Y., Cook, R. G., Gorovsky, M. A., and Allis, C. D. (1999) *J. Biol. Chem.* 274, 17929–17936
39. Waring, P., Khan, T., and Sjaarda, A. (1997) *J. Biol. Chem.* 272, 17929–17936
40. Rogakou, E. P., Pilch, D. R., Orr, A. H., Ivanova, V. S., and Bonner, W. M. (1998) *J. Biol. Chem.* 273, 5858–5868
41. Rogakou, E. P., Nieves-Neira, W., Boon, C., Pommier, Y., and Bonner, W. M. (2000) *J. Biol. Chem.* 275, 9390–9395
42. Ajiri, K. (2000) *J. Biol. Chem.* 275, 439–443
43. Widlak, P., Li, P., Wang, X., and Garrard, W. T. (2000) *J. Biol. Chem.* 275, 6220–6226
44. Swank, R. A., Tring, J. P., Guo, X. W., Valdez, J., Bradbury, E. M., and Gurley, L. R. (1997) *Biochemistry* 36, 13761–13768
45. Jerzmanowski, A., and Cole, R D. (1992) *J. Biol. Chem.* 267, 8514–8520