Histone H2B Phosphorylation in Mammalian Apoptotic Cells

AN ASSOCIATION WITH DNA FRAGMENTATION*

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Histone phosphorylation was investigated in several mammalian cells undergoing apoptosis (human HL-60 and HeLa, mouse FM3A and N18 cells, and rat thymocytes). Among the four nucleosomal core histones (H2A, H2B, H3, and H4), H2B, which is not usually phosphorylated in quiescent or growing cells, was found to be phosphorylated after treatment with various apoptotic inducers. The H2B was phosphorylated around the time when nucleosomal DNA fragmentation was initiated and, like this fragmentation, was completely blocked with Z-Asp-CH2-DCB, an inhibitor of ICE or ICE-like caspase. The involved single phosphopeptide of H2B proved to be phosphorylatable in vitro with a protein kinase C, and the site Ser-32 was tentatively identified. Despite typical apoptotic chromatin condensation, the H3 phosphorylation was at a low level, and the sites where phosphorylation did occur did not include any mitosis-specific phosphopeptides. Phosphorylation of H4 was increased, but the other two histone proteins (H1 and H2A) were not appreciably changed. These observations imply that 1) H2B phosphorylation occurs (H1 and H2A) were not appreciably changed. These observations imply that 1) H2B phosphorylation occurs throughout the cell cycle, whereas H4 phosphorylation is weak or negligible depending on the cell type. These two histones (H2A and H4) are also phosphorylated at serine residues of the N-terminal end.

In view of the specificity of histone phosphorylation during the cell cycle, it is clearly of interest to investigate in more detail whether apoptotic chromatin has a specific pattern of histone phosphorylation associated with nucleosomal DNA fragmentation or chromatin condensation. I have mainly concentrated attention on the human preleukemic cell line, HL-60, which can be readily induced to undergo apoptosis by various agents including anticancer drugs (27). Here, I report that most mammalian apoptotic cells demonstrate unique phosphorylation of H2B, which does not usually occur in interphase cells.

**EXPERIMENTAL PROCEDURES**

Induction of Apoptosis in HL-60 Cells by Various Agents—HL-60, human preleukemic cells, were routinely maintained in suspension culture with RPMI 1640 medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.) at 37 °C in the presence of 5% CO2. For the induction of apoptosis, the HL-60 cells (approximately 4 × 10^6) were treated with various apoptosis inducers and related chemicals including VP-16 (etoposide), cisplatin, A23187, ethanol, demecolcine, UV, and anti-Fas antibody. In addition, HeLa cells were treated with 0.1 µmol/L of either of the following: okadaic acid (28) and mouse FM3A cells with VP-16 (29). Mouse N18TG2 cells were grown (30) and cultured for 24 h in a serum-free medium. Rat thymocytes were incubated with corticosterone or dexamethasone (31), as indicated in Table I. Apoptotic cells were counted as those with apoptophores caused by blebbing (1), assessed under a phase contrast microscope.

Preparation of Phosphorylated Histones—For the preparation of 32P-labeled histones, cells (1 × 10^6) were labeled with [32P]orthophosphate (40 µCi/ml, ICN; [32P]orthophosphate, carrier-free) as described earlier (32) for the final 3 h of each period of treatment with an apoptosis-inducing agent. For pulse labeling, HL-60 cells (5 × 10^6) were treated with 20 µg/mL VP-16 and [32P]orthophosphate for different 2 h periods. 32P-Labeled histones were prepared as described previously (32), and the proteins (30 µg) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with Triton X-100-containing gels (33) at 120 V for 48 h. The proteins were stained with 0.2% Amido Black. For examination of the effects of a caspase inhibitor, cells were treated with Z-Asp-CH2-DCB (Peptide Inst, Inc) at the concentration of 25 to 100 µg/ml together with an apoptosis agent, and then 32P-labeled histones were extracted. Autoradiography of 32P-labeled proteins was conducted with an Image Analyzer (FujiX, BAS 2000).

Preparation of phosphorylated histones in vitro with protein kinase C was conducted as described (34). Phosphorylation was carried out at 30 °C in a 100-µl reaction mixture containing 100 µg of H2B, 300 units of
HL-60 cells were cultured with or without apoptotic induced by VP-16. Histones were extracted, and the proteins (30 μg) were resolved by acid-urea polyacrylamide gel electrophoresis containing Triton X-100 at 120 V for 48 h as described under “Experimental Procedures.” The gels were stained with 0.2% Amido Black, and autoradiography was conducted with an Image Analyzer (Fujix, BAS 2000). a, b, and c show results for control (Me2SO only), VP-16 (20 μg/ml for 6 h), and Colcemid (0.04% for 16 h) cases, respectively. Ab, Amido Black-stained proteins; 32P, 32P autoradiography.

Fig. 2. Histone phosphorylations of apoptotic HL-60 and rat thymocyte cells. A, HL-60 cells were treated with 20 μg/ml VP-16 for 6 h (b) or 16 h (c) or 100 ng/ml anti-Fas antibody (Medical & Biological Laboratories Co., Ltd.) for 6 h (d) or 16 h (e) or no agent (a). B, rat thymocyte cells were incubated in vitro with saline (a), RPMI solution (b), 100 nm dexamethasone (c), or 100 nm corticosterone (d) for 7 h at 37 °C. 32P-Labeled core histones (A) or total histones (B) were prepared and run on an acid-urea polyacrylamide gel. The picture shows only results of autoradiography. The positions of the histones and their subtypes are indicated on the left.

RESULTS

Phosphorylated histones in various mammalian apoptotic cells were labeled with [32P]orthophosphate and analyzed with acid-urea polyacrylamide gels containing Triton X-100 (Fig. 1). Examination of total histone patterns in HL-60 cells (Fig. 1, Amido Black-stained protein (Ab)) treated with an apoptosis-inducing agent, VP-16, revealed no essential variation (Fig. 1b) between the control (Fig. 1A) and the mitotic cells (Fig. 1c). However, considerable differences were observed regarding histone phosphorylation, as shown by 32P autoradiography (Fig. 1, 32P). In the control cells, among the five histones, H1 and H2A were highly and H4 was weakly phosphorylated, whereas phosphorylation of H2B and H3 was negligible (Fig. 1a), as observed in most mammalian interphase cells. In mitotic arrest cells treated with Colcemid, H3 was highly phosphorylated, in line with chromosome condensation during the cell cycle (Fig. 1e). This H3 phosphorylation is known as a mitosis-specific phosphorylation at Ser-10. However, in the histones of apoptotic cells induced by VP-16, H2B and H4 were found to be highly phosphorylated. An increase of H3 phosphorylation in both H3.1 and H3.2 was also observed to a lesser extent (Fig. 1d). Comparison between Amido Black-stained proteins and autoradiograms demonstrated that the H2B phosphorylation occurred in a partial fraction (Fig. 1b). The amount of phosphorylated protein was approximately 5–10% of the total H2B estimated by the protein shift from the main peak in the elution profile of HPLC (data not shown).

Since separation of core histones is greater in samples without H1, the H1 in some histone samples was removed by extraction with 5% perchloric acid. H2B phosphorylation increased in parallel with prolonged duration of VP-16 treatment for 6 and 16 h (Fig. 2a, b, and c, respectively). Furthermore, H2B phosphorylation was identified with apoptosis induced by the anti-Fas antibody, increasing in parallel with the duration of treatment (Fig. 2a, d, and e). H2B phosphorylation was also consistently observed in apoptotic HL-60 cells after treatment with A23187, ethanol, or UV light (Table I) and in rat thymocyte cells exposed to two kinds of steroid hormones, dexamethasone and corticosterone (Fig. 2b, c, and d). Similarly, H2B phosphorylation was found in mouse apoptotic FM3a cells induced by VP-16 and in N18TG2 cells cultured in a serum-free medium. The extent of H2B phosphorylation approximately corresponded to the level of induction of apoptosis in most cases, as shown quantitatively with the + and − symbols in Table I. The evidence thus indicates that 1) H2B phosphoryl-

1 The abbreviations used are: HPLC, high performance liquid chromatography; ICE, interleukin-1β convertase enzyme.
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The number of apoptotic cells with apoptophores was counted under a phase contrast microscope (%). As indicated in Fig. 3, the presence of Z-Asp-CH2-DCB, a specific inhibitor for ICE or caspase-1, decreased the extent of apoptosis. The effect of the peptide on histone phosphorylation was also completely inhibited by Okadaic acid, a protein phosphatase inhibitor that specifically binds protein kinase C. The H2B phosphopeptide observed here may be a substrate for protein kinase C and to a limited extent by protein kinase A. H2B phosphorylation was only phosphorylated significantly by protein kinase, protein kinase A, and protein kinase C. The data indicate that phosphorylation of H2B is most closely associated with apoptotic cells, especially apoptosis-specific nucleosomal DNA fragmentation. These data indicate that phosphorylation of H2B is most closely associated with apoptotic cells, especially apoptosis-specific nucleosomal DNA fragmentation in VP-16-induced apoptotic HL-60 cells. To access the sites of histone phosphorylation in apoptotic HL-60 cells, 32P-labeled histones were purified by HPLC and digested with trypsin. The products were analyzed by two-dimensional thin layer chromatography and autoradiographed for 32P activity (Fig. 5). With regard to the H2B phosphopeptide, a principal single spot migrated into the highly basic, less polar region in the peptide map (Fig. 5, inset). H2B phosphopeptides were phosphorylated in vitro with available protein kinases including cyclin/cdc2 kinase, mitogen-activated protein kinase, protein kinase A, and protein kinase C. The H2B phosphopeptide was only phosphorylated significantly by protein kinase C and to a limited extent by protein kinase A. Fig. 5b is a tryptic peptide map for H2B phosphorylated by protein kinase C. The H2B phosphopeptides observed here may contain some phosphopeptides that are incompletely cleaved at Lys or Arg residues by trypsin. A mixture of both H2B phosphopeptides (Fig. 5, a and b) showed that the H2B spot in

### Table I

| Cells          | Inducing agents | Apoptotic cells 32P-H2B |
|---------------|----------------|------------------------|
| HL60          | Blank          | (3.6)                  |
|               | VP-16, 20 μg/ml| ++ (34.4)              |
|               | VP-16, 20 μg/ml| + (6.1)                |
|               | Ethanol, 5.5%  | ++ (62.8)              |
|               | Colcemid, 0.04%| + (4.5)                |
|               | UV, 250 J/m²   | ++ (50.0)              |
|               | Anti-Fas, 100 ng/ml| + (18.7)              |
| HL-60         | Blank          | (3.6)                  |
|               | Okadaic acid, 100 nm| + (94.0)              |
| FM3A          | Blank          | (2.3)                  |
|               | VP-16, 20 μg/ml| ++ (36.2)              |
| N18TG2        | Blank          | (3.1)                  |
|               | Serum-free     | ++ (34.2)              |
| Thymocytes (rat)| Blank         | + (2.4)                |
|               | Corticosterone, 100 nm| + (61.0)              |
|               | Dexamethasone, 100 nm| + (68.9)              |

Fig. 3. Prevention of both DNA fragmentation and histone H2B phosphorylation with a caspase inhibitor. A. HL-60 cells were treated with 10 μg/ml VP-16 for 24 h in the absence or presence of Z-Asp-CH2-DCB, and DNA was prepared and analyzed with 2% agarose gels. M, DNA size marker. a, untreated cells; b, c, d, and e are cells treated with VP-16 in the presence of Z-Asp-CH2-DCB at 0, 25, 50, and 100 μg/ml, respectively. B, cells were treated with VP-16 as above or with ethanol for the last 3 h with 40 μCi/ml 32Porthophiate. Histones were extracted and analyzed with acidi-urea polyacrylamide gel electrophores and autoradiographed. a, untreated cells; b, c, and d are cells treated with 10 μg/ml VP-16 for 24 h in the presence of Z-Asp-CH2-DCB at 0, 25, and 50 μg/ml, respectively. e, f, and g are cells treated with 5.5% ethanol for 5 h with Z-Asp-CH2-DCB at 0, 25, and 50 μg/ml, respectively.
apoaptotic cells comigrated with one of the protein kinase C phosphopeptides (Fig. 5c, indicated by an arrow). The data demonstrated an involvement of the same H2B phosphopeptide among the several spots targeted by protein kinase C in vitro.

The major sites of H3 phosphorylation in apoptotic cells with VP-16 (Fig. 5d) or A23187 (Fig. 5e) were clearly different from those associated with mitosis (Fig. 5f, indicated by the two arrows). Thus, the two spots usually demonstrated very faint or no labeling in normal interphase cells. Phosphopeptide maps of the three other histones (H1, H2A, and H4) did not demonstrate differences in sites from those of the control, whereas phosphorylation of a single H4 phosphopeptide was extensively enhanced (data not shown).

**DISCUSSION**

The present data for HL-60 cells are consistent with those of other laboratories in indicating that 1) histone H3 phosphorylation at mitosis-associated sites does not occur in apoptosis and 2) H1 and H2A do not show any considerable increase or changes in any spots. In this report, I document that 1) H2B is phosphorylated in most mammalian apoptotic cells, and 2) H3 is weakly phosphorylated at a few sites that are different from Ser-10, 3) H4 was enhanced in the phosphorylation level. It is quite likely that H2B phosphorylation is apoptosis-specific, since it was observed in many mammalian apoptotic cells induced by various apoptotic agents and increased parallel with both treatment duration and dose, 2) H2B started to be phosphorylated around the time when nucleosomal DNA fragmentation was initiated, 3) the phosphorylation was inhibited completely by Z-Asp-CH2-DCB, a broad inhibitor for ICE or ICE-associated caspase at concentrations less than that inhibiting apoptosis (37). The evidence thus strongly implies that 1) phosphorylation of H2B is tightly associated with apoptosis in most mammalian cells, and 2) phosphorylation of H2B is related to the mechanism of caspase-initiated apoptosis.

At present, the function of H2B phosphorylation in the apoptotic chromatin structure is not known. However, since only a proportion of H2B was phosphorylated, the present data suggest an association with the early phase of DNA fragmentation (Figs. 3 and 4) rather than chromatin condensation. In fact, nucleosomes are released by apoptosis-specific nucleosomal fragmentation (38), and H2B phosphorylation might occur in free nucleosomes or oligonucleosomes. The N-terminal domain of H2B is reported to lie outside the nucleosomes associated with linker DNA (39, 40). Zunino et al. (41) describe...
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Detection of a histone H2B epitope by an immunological method in the early stage of apoptosis in T lymphocytes. Whether the phosphorylation of H2B in chromatin facilitates its partial dissociation from linker DNA and makes it accessible to endonuclease(s) remains unclear. The H2B phosphorylation site was here tentatively identified as Ser-32. Interestingly, this is located in the inner globular region near the border with the N-terminal H2B tail. The site may be inside the nucleosome structure and therefore not exposed to protein kinases. This would explain why the site is usually not phosphorylated. In earlier studies by Nishizuka and co-workers, it was found that H2B at Ser-32 is phosphorylatable in vitro by a protein kinase C, which was identified earlier as protein kinase M (42) or protein kinase G (43). There is some evidence that H2B phosphorylation is stimulated by phorbol esters in quiescent Reuber H35 hepatoma cells (44). Circumstantial support for a role for protein kinase C, an isomer of protein kinase C, has emerged with the observation that it contains a possible cleavage site at Gln-Asp-Asn of the caspase family (ICE) and the finding that the protein kinase C isozyme is proteolytically activated in apoptotic U937 cells exposed to ionizing radiation (45). It should now be determined whether H2B is phosphorylated in the nuclei or degraded free nucleosomes exposed to the cytoplasm where activated kinases are abundant.

The available data in the literature indicate that apoptotic cells have no histone phosphorylation at Ser-10, which is specific for mitosis (24) and premature chromosome condensation (21), but the present findings indicate that apoptotic HL-60 cells have a low level of H3 phosphorylation at different sites. Direct evidence has been reported recently by Hendzel et al. (46) using a Ser-10 antibody in which apoptotic cells do not demonstrate mitotic H3 phosphorylation. I proposed earlier the idea that a high level of H1 phosphorylation was necessary for the onset of mitosis-specific H3 phosphorylation in vivo (25, 26). My data and those from other laboratories indicate that apoptotic chromatin condensation does not appear to be linked to H1 phosphorylation (46–48). This suggests that apoptotic chromatin condensation differs from that occurring during mitosis or premature chromosome condensation, despite the reported similarities (49, 50). Apoptotic chromatin condensation appears irreversible with formation of chromatin clumps undergoing degradation, whereas mitotic chromatin condensation is reversed with progression through the cell cycle.

Variation in apoptotic chromatin structure may also reflect other histone modifications dependent on the cell species or agents, such as the absence of ubiquitinated histone H2A in gliomas and neurinomas (47). Using inhibitors or activators of protein kinases, apoptosis can be induced by staurosporine without considerable histone H2A in gliomas and neurinomas (47). Using inhibitors or activators of protein kinases, apoptosis can be induced by staurosporine without considerable histone H2A in gliomas and neurinomas (47). Using inhibitors or activators of protein kinases, apoptosis can be induced by staurosporine without considerable histone H2A in gliomas and neurinomas (47). Using inhibitors or activators of protein kinases, apoptosis can be induced by staurosporine without considerable histone H2A in gliomas and neurinomas (47). Using inhibitors or activators of protein kinases, apoptosis can be induced by staurosporine without considerable histone H2A in gliomas and neurinomas (47). Using inhibitors or activators of protein kinases, apoptosis can be induced by staurosporine without considerable histone H2A in gliomas and neurinomas (47). Using inhibitors or activators of protein kinases, apoptosis can be induced by staurosporine without considerable histone H2A in gliomas and neurinomas (47).