Sequential Phosphorylation of Ser-10 on Histone H3 and Ser-139 on Histone H2AX and ATM Activation During Premature Chromosome Condensation: Relationship to Cell-Cycle Phase and Apoptosis

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Background: Histone H1 and H3 phosphorylation associated with chromatin condensation during mitosis has been studied extensively. Less is known on histone modifications that occur during premature chromosome condensation (PCC). The aim of the present study was to reveal the status of histone H3 and H2AX phosphorylation on Ser-10 and Ser-139, respectively, as well as ATM activation through phosphorylation on Ser-1981, during PCC, and relate these events to cell-cycle phase and to initiation of apoptosis.

Materials and Methods: To induce PCC, A549 and HL-60 cells were exposed to the phosphatase inhibitor calyculin A (Cal A). Phosphorylation of histone H3 and H2AX as well as ATM activation were detected immunocytochemically concurrent with analysis of cellular DNA content and activation of caspase-3, a marker of apoptosis. The intensity of cellular fluorescence was measured by flow- or laser scanning cytometry.

Results: Induction of PCC led to rapid histone H3 phosphorylation, followed by activation of ATM and then H2AX phosphorylation in both, HL-60 and A549 cells. All these events occurred sequentially, prior to caspase-3 activation, and affected cells in all phases of the cell cycle. ATM activation and H2AX phosphorylation was seen during mitosis of A549 but not HL-60 cells.

Conclusions: Because the Cal A-induced phosphorylation of histone H3 and H2AX, and of ATM, precede caspase-3 activation these modifications are pertinent to PCC and not to apoptosis-associated chromatin condensation. The sequence of histone H3 and H2AX phosphorylation and ATM activation during PCC is compatible with a role of ATM in mediating phosphorylation of H2AX but not H3. Mitosis in some cell types may proceed without ATM activation and H2AX phosphorylation.

Key terms: PCC; DNA damage; DNA replication; DNA double-strand breaks; ATM activation dosimetry; cell cycle; S phase; chromatin; laser scanning cytometry; iCyte

Histone proteins undergo perpetual covalent modifications in living cells. The modifications predominantly affect their N-termini and involve acetylation and methylation of lysines and arginines, phosphorylation of serines and threonines as well as polyribosylation and ubiquitinylation. These modifications are associated with rearrangement of chromatin structure as the cell progresses through the cell-cycle, as well as with regulation of the changeable repertoire of DNA transcription. It was proposed that the combinatorial nature of histone-terminal modifications generates a “histone code” and this code extends the information potential provided by the genetic code (1,2).

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Phosphorylations are among the most extensively studied histone modifications. Most studies have been done to relate histone phosphorylation with chromatin changes that occur during the cell-cycle, particularly involving chromatin condensation in mitosis (reviews: 3,4). Phosphorylation of histone H1 during entrance of cell to mitosis was reported over three decades ago (5), and the role of this modification in chromatin condensation has been postulated (6). More recent studies, however, have shown that histone H1 is not required for chromatin to undergo mitotic condensation (7,8).

Phosphorylation of the flexible N-termini of nucleosome core histones appears to play a more significant role in chromatin condensation than that of the linker histone H1. Among modifications of the core histones, phosphorylation of Ser-10 of histone H3 is a hallmark of chromatin condensation during mitosis (9,10). In fact, the immunocytochemical detection of Ser-10 phosphorylated histone H3 has been used as a marker of mitotic cells by cytometry (11,12). It should be noted, however, that despite the fact that Ser-10 histone H3 phosphorylation is a highly specific event occurring during mitosis, the function of this modification in the mechanism of chromosome condensation is still contentious (13). Ser-10 phosphorylation of histone H3 appears to also play a role in the regulation of transcriptional activity (14,15). Aurora kinases mediate histone H3 phosphorylation on Ser-10, at least during mitosis (16).

It has been recently reported that histone H2AX, one of several variants of the nucleosome core histone H2A (17,18) is phosphorylated on Ser-139 in mitotic cells (19-22). The Ser-139 phosphorylated form of H2AX is being defined as γ-H2AX. Although H2AX phosphorylation is known to occur in response to DNA damage that involves formation of double-strand breaks (DSBs) (23,24), the H2AX phosphorylation during mitosis appears not to be related to DNA damage (19-22). It is likely, thus, that as in the case of phosphorylation of other histones, it may be associated with chromatin condensation.

Premature chromosome condensation (PCC) in many respects mimics changes in chromatin otherwise seen during mitosis (25). Because PCC can be induced by okadaic acid (OA) or calyculin A (Cal A), inhibitors of phosphatases (26-28), it is likely that histone phosphorylation may be the causative factor for PCC. Namely, in the presence of OA or Cal A the equilibrium between histone phosphorylation and dephosphorylation may be shifted towards accumulation of the phosphorylated histone forms. Indeed, induction of phosphorylation of histone H3 on Ser-10 has been observed during PCC (29,30). We were unable, however, to find reports as to whether histone H2AX also undergoes phosphorylation during PCC. The aim of the present study, therefore, was to compare phosphorylation of histone H3 at Ser-10 and histone H2AX at Ser-139 in mitotic cells with those in cells induced to PCC. Because ATM kinase is one of the key enzymes phosphorylating H2AX on Ser-139 (31), we also assessed whether ATM undergoes activation during mitosis as well as during PCC. In addition, we concurrently measured activation of caspase-3, a marker of apoptosis, to exclude the possibility that the observed changes in histone modifications are related to apoptosis and not to induction of PCC. Multiparameter flow- and laser scanning cytometry (32) was used to correlate histone modifications during PCC with the cell-cycle phase and with cell morphology. The studies were carried out on human leukemic HL-60 and pulmonary carcinoma A549 cells as representative of cell lines that grow in suspension and attached to slides, respectively. It should be noted that Cal A is a very effective inducer of PCC, causing chromatin condensation rapidly (1-3 h after administration) and affecting cells in all phases of the cell-cycle (16).

**MATERIALS AND METHODS**

**Cells, Cell Treatment**

HL-60 and A549 cells were obtained from American Type Culture Collection (ATCC; Manassas, VA). HL-60 cells were grown in 25-ml Falcon flasks (Becton Dickinson, Franklin Lakes, NJ) in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine (all from Gibco/BRL Life Technologies, Grand Island, NY) at 37°C in an atmosphere of 5% CO2 in air. At the onset of the experiments, there were fewer than 5 × 10⁵ cells/ml in culture and the cells were at an exponential and asynchronous phase of growth. A549 cells were grown in Dulbecco’s minimum essential medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine (Gibco/BRL Life Technologies, Grand Island, NY) at 37°C in an atmosphere of 5% CO2 in air. The cultures were diluted and replated every 4 days to maintain them in an asynchronous and exponential phase of growth. For experiments, the cells were trypsinized and seeded at low cell density (about 5 × 10⁴ cells per chamber) in a 2-chambered Falcon CultureSlide (Becton Dickinson Labware, Franklin Lakes, NJ). To induce PCC, the cultures were treated with 10 nM of calyculin A (Biomol, Plymouth, PA) for various time intervals as described in the figure legends. Control cultures were treated with the equivalent volumes of dimethylsulfoxide (DMSO, Sigma Chemical, St Louis, MO) that was used to prepare stock solutions of Cal A. The cells were then fixed either in suspension or by transferring the slides into Coplin jars containing 1% methanol-free formaldehyde (Polysciences, War-ington, PA) in PBS for 15 min on ice followed by suspension in 70% ethanol where they could be stored at −20°C for 2-24 h.

**Immunocytochemical Detection of Histone γ-H2AX, Histone H3 Phosphorylated on Ser-10 (H3-P), Activated ATM (ATM-S1981P) and Activated (Cleaved) Caspase-3**

The cells were washed twice in PBS and suspended in 0.2% Triton X-100 (Sigma) in a 1% (w/v) solution of bovine serum albumin (BSA; Sigma) in PBS for 30 min to suppress nonspecific antibody (Ab) binding. The cells were then incubated in 100 μl of 1% BSA containing either a combination of 1:200 diluted antiphospho-histone H2AX (Ser-
Detection of H3-P and Caspase-3 Activation: Samples Prepared for Fluorescence Microscopy

Cells were treated with Cal A as described above, rinsed with PBS, and then fixed in 1% methanol-free formaldehyde followed by 70% ethanol. After washing with PBS, the cells were immersed in 0.2% Triton X-100 (Sigma) in a 1% (w/v) solution of PBS for 30 min. The cells were then incubated in 100 μl volume of 1% BSA containing both 1:100 diluted antiphospho-histone H3 (Ser10) (6G3) mouse mAb (Cell Signaling Technology) and a 1:100 dilution of anticleaved (activated) caspase-3 rabbit polyclonal Ab (Cell Signaling Technology). The cells were then incubated overnight at 4°C, washed twice with PBS, and resuspended in 100 μl of 1:100 diluted Alexa Fluor 633 conjugated F(ab’)2 fragment of goat antimouse IgG (H + L) (Molecular Probes, Eugene, OR) and 1:200 diluted Alexa Fluor 488 conjugated goat antirabbit IgG (H + L) (Molecular Probes) for 30 min at room temperature in the dark. The cells were then counterstained with 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI, Molecular Probes) in PBS for 5 min. Cellular fluorescence was measured using a iCys Research Imaging Cytometer (CompuCyte, Cambridge, MA).

The cells were then counterstained with DAPI in PBS for 5 min for DNA content analysis.

Fluorescence Measurement Using iCys

Cellular green, long red, and blue fluorescence emission was measured simultaneously in the same cells using an iCys research imaging cytometer (LSC; CompuCyte, Cambridge, MA), utilizing standard filter settings; fluorescence was excited with a 488-nm argon ion, helium neon, and violet diode lasers, respectively. The intensities of maximal pixel and integrated fluorescence were measured and recorded for each cell. At least 3,000 cells were measured per sample.

Each experiment was run in duplicate or triplicate. All experiments were repeated at least three times.

RESULTS

Expression of Ser-10 phosphorylated histone H3 and Ser-139 phosphorylated histone H2AX in the untreated (Ctrl) or Cal A treated HL-60 cells, in relation to the cell-cycle phase. HL-60 cells were untreated (Ctrl) or exposed to 10 nM Cal A for 1, 3, or 5 h. Phosphorylation of histones H3 and H2AX was detected immunocytochemically and cellular DNA was counterstained with DAPI. The dashed lines on the scattergrams represent the upper limit of expression of H3-P or H2AX IF, respectively, for at least 95% cells from the untreated cultures. Note that histone H3 phosphorylation was transient, maximal at 1 h, and preceded phosphorylation of H2AX, which was seen after 5 and 5 h. Among the untreated cells only M and immediate postmitotic (pM) cells are H3-P positive. Percent of immunopositive cells is shown in the respective panels.
However, 1 h after administration of Cal A, a distinct subpopulation of HL-60 cells reactive with this Ab, distributed in all phases of the cell-cycle, becomes apparent (Fig. 1). The intensity of Ser-10 phosphorylated histone H3-related IF of this subpopulation was distinctly lower than that of M-phase cells from the untreated cultures. Also, there was no evidence of the presence of the strongly labeled M-phase cells in all cultures treated with Cal A. Morphologically, the cells that become labeled with H3-P Ab after 1 h exposure to Cal A showed early signs of PCC (Figs. 2C and 2D). After treatment with Cal A for 2 h the cells with phosphorylated histone H2AX become apparent in the cultures. However, more cells exhibited phosphorylated histone H3 than phosphorylated H2AX, while all cells showing phosphorylated H2AX were positive for phosphorylated histone H3 (Figs. 2E and 2F). Compared with cells exposed to Cal A for 1 h, the intensity of cell labeling with H3-P Ab declined after 3 and 5 h incubation with this inhibitor (Fig. 1).

The decline in the intensity of cell labeling with H3-P Ab after 3 and 5 h treatment with Cal A was paralleled by the rise in IF representing Ser-139 phosphorylated histone H2AX (Fig. 1). Thus, significant proportion (21.5%) of ATM-positive cells was apparent.
already after 3 h, and most cells were ATM-P positive after 5 h. The cells that were ATM-P-negative after 5 h were predominantly in G1 and G2M. There was no significant evidence of caspase-3 activation during 3 h incubation with Cal A, but a subpopulation of cells (<30%) with activated caspase-3 became apparent after 4 and 5 h. Most cells with activated caspase-3 were histone H3-P-negative (Fig. 5).

The kinetics of induction of histone H3 and H2AX phosphorylation, as well as of ATM and caspase activation during cell treatment with Cal A is presented in Figure 5. It is quite evident from these data that histone H3 phosphorylation was the earliest event, distinctly preceding activation of ATM. Activation of ATM, on the other hand, when measured at 1 h after administration of Cal A, preceded H2AX phosphorylation. Between 1 and 4 h, however, the percentage of ATM-P- and γ-H2AX-positive cells was increasing concurrently. All these events affected over 60% of the cells following 4 h of treatment and occurred before caspase-3 activation. A decline in percentage of H3-P-positive cells was seen after 4 h of treatment with Cal A.

Attempts have also been made to measure phosphorylation of histone H3 and H2AX as well as activation of ATM and caspase-3 upon the induction of PCC in the cells that grow adherent to culture vessels. Towards this end, pulmonary adenocarcinoma A549 cells growing in chambers on microscope slides were treated with Cal A, fixed and immunostained for Ser-10 and Ser-139 phosphorylated histone H3 and H2AX, respectively, as well as for activated ATM and caspase-3. However, shortly (>20 min) after administration of Cal A, a large number of cells began to detach. Thus, following fixation of the slides, relatively few cells that remained attached could be analyzed. Because the detachment was selective, affecting the cells with the most advanced degree of chromatin condensation, there was no point in attempting to quantitatively analyze the slides because of the bias introduced by the detachment. Among the cells that remained attached, however, we have seen a similar sequence of events as in the case of HL-60 cells, namely that histone H3 was phosphorylated prior to H2AX and prior to ATM activation. No caspase-3 activation was apparent, most likely because all the apoptotic cells had already detached from the slides (data not shown).

There was a striking difference, however, between the untreated HL-60 and A549 cells. Namely, among HL-60 cells there was no evidence of the presence of a discernible cell subpopulation with a G2/M DNA content that
would have phosphorylated H2AX and/or activated ATM (Figs. 1 and 3, Ctrl). In contrast, A549 cells from the untreated, control cultures presented quite distinct G2M cell subpopulations that showed elevated expression of γ-H2AX (7%) and of activated ATM (3%; Fig. 6). When activated ATM and γ-H2AX were immunostained with fluorescent chromosomes of different color emission and the cells plotted as the bivariate distribution ATM-P vs. γ-H2AX IF, it was apparent that some cells were ATM-P positive/γ-H2AX negative (14%), some were both ATM-P and γ-H2AX positive (30%), and some ATM-P negative/γ-H2AX positive (56%). Among the G2M cells expressing γ-H2AX there were also some nonmitotic cells. Examination of the G2M ATM-P positive cells relocated by LSC for imaging (32) revealed that large majority of them were mitotic cells (Fig. 6, right panels).

**DISCUSSION**

The present data indicate that sequential changes that involve phosphorylation of histone H3, activation of ATM, and phosphorylation of histone H2AX take place in the chromatin of cells undergoing PCC. All these changes occur in live cells prior to initiation of apoptosis. The latter was detected by measurement of caspase-3 activation. Our data are consistent with the published reports describing histone H3 phosphorylation during PCC (29,30). It has also been reported that during PCC histone H1 undergoes phosphorylation as well (29,30). The novelty of the present study is the observation that histone H3 phosphorylation and ATM are activated in response to DNA DSBs. Thus, for example, we presently observed that concomitant with ATM activation, H2AX was phosphorylated during mitosis of A549 cells in the untreated cultures (Fig. 6). This observation confirms the earlier reports that H2AX is phosphorylated during mitosis and that ATM mediates its phosphorylation (20–22). Yet, there is...
no conclusive evidence in the literature that DSBs are generated during normal mitosis. Rather the changes in chromatin structure during mitosis that may be associated with the induction of torsional stress on the DNA double helix that make it more sensitive to single-strand nucleases (45) or susceptible to denaturation (46,47) may be a signal triggering H2AX phosphorylation in mitotic cells. Phosphorylation of H2AX during mitosis, thus, provides an example that this event can occur in absence of the induction of DSBs. Likewise, H2AX phosphorylation during PCC may also be unrelated to the formation of DSBs.

It should be stressed, however, that it depends very much on the cell type as to whether or not H2AX is phosphorylated during mitosis. Thus, while mitotic A549 cells showed the presence of phosphorylated H2AX (Fig. 6), no evidence of H2AX phosphorylation was apparent in mitotic HL-60 cells (Figs. 1 and 3). We had also noticed that some other cell lines (e.g., T24, Jurkat) did not demonstrate the presence of phosphorylated H2AX during mitosis (not shown). Likewise, McManus and Hendzel (22) observed variability in the extent of H2AX phosphorylation during mitosis among the cell lines they studied. These observations indicate that H2AX phosphorylation, while detected in some cell types or lines, is not an ever-present feature of mitotic cells and thus is not essential for chromatin condensation during mitosis, in general. This is in contrast to histone H3 whose phosphorylation is considered the marker of mitotic chromatin condensation (9–12).

As mentioned, our data are compatible with a role of ATM as a mediator of H2AX phosphorylation during mitosis in A549 cells. We observed, however, that fewer G2/M cells expressed activated ATM than had phosphorylated H2AX (Fig. 6). Furthermore, among the G2/M cells subpopulation there were cells that had activated ATM with no evidence of H2AX phosphorylation, and vice versa, cells that showed phosphorylated H2AX in the absence of activated ATM. All these data combined point out that activation of ATM is a transient event, and of shorter duration compared to the length of time during which H2AX remains phosphorylated.

It has been reported that following DNA damage H2AX remains phosphorylated for prolonged time if Cal A is being added into cultures (33,48). This finding was explained by the inhibitory effect of Cal A on phosphatase(s) that otherwise dephosphorylate γ-H2AX (47). In the present study, however, we observed that Cal A by inducing PCC triggered H2AX phosphorylation. It is likely, therefore, that the observed effects of Cal A (33,48) were not a result of prevention of γH2AX de-phosphorylation, but in fact induction of H2AX phosphorylation by the initiation of PCC.

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