Effects of okadaic acid (OA), a protein phosphatase inhibitor, on chromatin structure and phosphorylation of histones were examined using HeLa and N18 cells. The chromatin condensation in HeLa cells was mild and resemble prometaphase nuclei, while the condensation in N18 cells was extensive and chromatin became a compact body. H2A in HeLa cells was extensively and consistently phosphorylated at the same site throughout the cell cycle, and H3 was demonstrated to be phosphorylated at the mitosis-specific site Ser10. In contrast, H1 phosphorylation was rapidly decreased in most sites within 3 h. The reduction of H1 phosphorylation was accompanied by a quantitative change in the set of H1 phosphopeptides. During the early phase of the OA treatment, H1 phosphorylation was transiently elevated in tandem, whereas H3 phosphorylation reached a maximum somewhat later. The results suggest that mitosis-specific events (cdc2/H1 kinase activation, H1 superphosphorylation, mitosis-specific H3 phosphorylation and chromatin condensation) induced by OA are sequentially associated. The changes appear to reflect a molecular mechanism similar to that operating in normal mitosis.

Alteration of Cell Cycle-dependent Histone Phosphorylations by Okadaic Acid

INDUCTION OF MITOSIS-SPECIFIC H3 PHOSPHORYLATION AND CHROMATIN CONDENSATION IN MAMMALIAN INTERPHASE CELLS*

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Kozo Ajiro‡§, Kinya Yoda†, Kazuhiro Utsumi, and Yasuhiro Nishikawa‡

From the †Aichi Cancer Center, Research Institute, Laboratory of Cell Biology and ¶Laboratory of Ultrastructure Research, Chikusa-ku and the ‡Nagoya University, Faculty of Science, Institute of Molecular Biology, Nagoya 464, Japan

The control of cell cycle progression is one of the most basic biological systems for which involvement of protein phosphorylation and dephosphorylation has been shown (1, 2). These processes are controlled by protein kinase and phosphatase activities. To understand the mechanisms of chromosome kinetochore formation, it is thus crucial to analyze the role of nuclear protein phosphorylations at the initiation of chromatin condensation.

Histones constitute a major component of chromosomes and construct nucleosomes as a basic chromatin element (3). During the cell cycle, three kinds of histones (H1, H2A, and H3)1 are extensively phosphorylated in a histone-specific manner (4). During the late G2 to M phase, the level of H1 phosphorylation reaches a maximum somewhat later. The results suggest that mitosis-specific events (cdc2/H1 kinase activation, H1 superphosphorylation, mitosis-specific H3 phosphorylation and chromatin condensation) induced by OA are sequentially associated. The changes appear to reflect a molecular mechanism similar to that operating in normal mitosis.

Okadaic acid (OA), a specific inhibitor of serine/threonine protein phosphatase (18–20) and a potential tumor promoter (21), is known to affect mammalian cells and induce mitosis-like processes. The chemical causes rounding of cells, PCC, spindle deconstruction and enhancement of H1 kinase activity (22–25). The chemical also induces apoptotic cells (26, 27). These phenomena accompany an elevation of overall cellular protein phosphorylations (28, 29). The inhibition of phosphatase activity by OA is associated with increased phosphorylation in which pp1 or pp2A phosphatases are involved. Thus it is possible to investigate the relationship between chromatin condensation and histone phosphorylation during cell proliferation using this agent (30).

In the present investigation, we examined critically the effects of okadaic acid on chromatin condensation and histone phosphorylations in both HeLa and N18 cells and found that OA induces H3 phosphorylation in a mitosis-specific site concomitantly with chromatin condensation in interphase cells.

MATERIALS AND METHODS

Chemicals—OA was provided by Dr. S. Yamada of Nagoya University or purchased from Wako Chemical Co. 6-Dimethylaminopurine (6-DMP) was purchased from Sigma Stock solution of OA was 1 mM dissolved in dimethyl sulfoxide.

Morphological Observations—HeLa (S-3) or N18 (mouse neuroblastoma) cells were cultured in dishes (9-cm diameter, Falcon) for 2 days. The cells were treated with or without chemicals for 30 min to 2 h. For detailed observation of cell nuclei, cells were plated on coverslips and treated with 0.1–1.0 μM of OA or 0.5–1.0 mM 6-DMP. After washing twice with phosphate-buffered saline solution, the cells were fixed with Carnoy’s solution (methanol:acetic acid, 3:1) for 30 min and stained with 50 μg/ml Hoechst 33258 for 30 min, then washed twice with 90% ethanol and observed by immunofluorescence microscopy (Nikon, Fluophot).

Preparation of 32P-Labeled Histones—Cells were grown in plastic bottles (Coster 3275). For the collection of mitotic cells, logarithmically growing cells were treated with 0.02 μg/ml colcemid for 16 h. Then, loosely attached cells were shaken off and collected. For 32P labeling, cells (approximately 3.5 × 106) grown either in dishes or in suspension culture were resuspended in a phosphate-free medium (8) containing [32P]orthophosphate (40 μCi/ml) with or without chemicals. The cultures were incubated for 3 h or 30 min for pulse labeling at 37 °C. Cells were partially lysed with a solution containing 80 mM NaCl, 20 mM EDTA, and 1% Triton X-100 (pH 7.6). Total histones were extracted twice from chromatin with 0.4 M H2SO4, and the proteins precipitated with 4 volumes of ethanol described earlier (14).

Acid-Urea Triton X-100 Polyacrylamide Gel Electrophoresis—Samples containing 30 μg of histones were resolved by electrophoresis on 30-cm-long 12% polyacrylamide gel containing 7.5 M urea, 5% acetic acid, and 6 mM Triton X-100 (31). The proteins were run at 120 V for 48 h, and the gels were stained with 0.2% Amido Black and autoradiographed as described previously (14). For the quantitation of protein condensation (PCC) (13–15) at Ser10 (16, 17). The level of H2A phosphorylation has been observed to be constant throughout the cell cycle (4, 9).

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were then washed three times with beads buffer A. H1 kinase activity (36.6:11.4:45.3:56.7).

Chromatin condensation in the presence of OA. HeLa (S-3) cells were cultured at 4.5 × 10^6/ml on coverslips for 2 days, treated with 1 μM OA for 1 h, washed with saline solution, and then fixed in methanol/acetic acid (3:1) for 30 min. The cells were stained with 50 μg/ml Hoechst 33258 in 90% ethanol, washed twice with 90% ethanol, and dried. Observation was under an immunofluorescence microscope. A, untreated HeLa cells; B, cells treated with 1 μM OA for 1 h.

HeLa cells was mild and resembled prometaphase nuclei. The extent of condensation varied, suggesting that it depends on the cell stage at the time of administration. Most mitotic nuclei were overcondensed with reduction in size and rounding (Fig. 1B, indicated by an arrowhead). In nuclei pretreated with colcemid for 16 h prior to the addition of OA for 3 h, the number of prometaphase-like or compact nuclei was remarkably increased (data not shown). However, most nuclei of N18 cells were extensively condensed and showed round body with no chromatin fibers (data not shown).

Analysis of Acid-soluble Nuclear Proteins and Their Phosphorylations in the Presence of OA or DMAP—Since protein phosphorylations are associated with chromosome condensations, histone phosphorylations were examined. HeLa cells were labeled with ^32P in the presence of OA or DMAP, and the histones were extracted and analyzed by acid-urea-Triton X-100 gel electrophoresis (Fig. 2). Electrophoretic patterns of the proteins were the same for both control and inhibitor-treated cells, except for additional H2A bands caused by phosphorylation (Fig. 2A, lanes 2 and 3, H2A.1 and H2A.2). H2A and H1 are extensively phosphorylated as observed in most growing cells (Fig. 2A, lane 7). In the OA-treated cells, H2A phosphorylation was remarkably increased (Fig. 2A, lanes 8 and 9) with two subtypes, H2A.1 and H2A.2, incorporating 2 and 2.5 times more of ^32P, respectively (Fig. 2A and Table I). It was unexpected that the level of H1 phosphorylation was less than half of that in control cells. H3 was rapidly phosphorylated in all three subtypes (H3.1, H3.2/H3.3) and further increased with an increase in the OA concentration, whereas H2B and H4 remained unphosphorylated. With the N18 cells (Fig. 2B), similar results to OA were obtained, although the phosphorylation rate was lower than HeLa (Fig. 2B, lanes 8 and 9). The data therefore indicated that OA affected histone phosphorylation in a histone-specific manner in the two mammalian interphase cells. Thus, 1) H2A phosphorylation was increased, 2) H1 phosphorylation was extensively decreased, 3) H3 phosphorylation was newly induced in interphase cells, and 4) H2B and H4 remained in an unphosphorylated state as in the control cells, D-DMAP inhibited both H2A and H1 histone phosphorylations of HeLa cells (Fig. 2A, lanes 11 and 12). The chemical also suppressed the mitotic H3 phosphorylation induced by colcemid (Fig. 2A, lane 10). The HeLa cells with colcemid contained approximately 48% mitotic cells. The decrease of these phosphorylations corresponded to the treatment dose (0.5 μM in lane 11 and 1.0 μM in lane 12) and also to the extent of chromatin decondensation (data not shown). A similar influence with histone dephosphorylation by D-DMAP was observed (Fig. 2B, lanes 11 and 12 in the N18 cells. Quantitative data for histone phosphorylations in both HeLa and N18 cells in the presence of these two chemicals are summarized in Table I.

Critical examination of the effective dose of OA for induction of H3 phosphorylation and decreased H1 phosphorylation revealed clear differences between N18 and HeLa cells (Fig. 3). The minimum concentration of OA for induction of H3 phosphorylation in the HeLa case was 0.2 μM (Fig. 3, lane 8), whereas the induction in N18 cells at 0.2 μM was very low (Fig. 3, lane 3). The extent of H3 phosphorylation in N18 cells rapidly increased with low concentrations of OA (0.1–0.5 μM) (Fig. 3, lanes 2–4), to a greater extent than with colcemid (data not shown). H3 phosphorylation reached maximum levels at 0.5 μM in HeLa cells (Fig. 3, lane 9), against 1 μM in N18 cells (Fig. 3, lane 10). In contrast, H1 phosphorylation decreased with an increasing dose of OA in both cell types.

Comparison of Histone Phosphopeptides between OA-treated Cells and M Cells—To analyze whether the changes of histone...
Okadaic Acid and Histone H3 Phosphorylation

Incorporation of \([^{32}P]\)phosphate into histones in cells in the presence of OA or DMAP. HeLa or N18 cells were labeled with \([^{32}P]\)phosphate (40 \(\mu\)Ci/ml) for 3 h, and histones were extracted as described under “Materials and Methods.” Forty \(\mu\)g (\(\sim\)3500 cpm) aliquots of the proteins were run on acid-urea-Triton gel electrophoresis at 120 V for 48 h. The gels were stained with 0.2% Amido Black and then autoradiographed. A, HeLa; B, N18. Lanes 1–3 show the effects of OA on histone phosphorylations, and lanes 4–6, the effects of 6-DMAP. Lane 1, control; lane 2, 0.5 \(\mu\)M OA; lane 3, 1 \(\mu\)M OA; lane 4, colcemid only; lane 5, colcemid with 0.5 mg/ml 6-DMAP; lane 6, colcemid with 1 mg/ml 6-DMAP. Lanes 1–6, Amido Black stain and lanes 7–12, \(^{32}P\) autoradiography corresponding to lanes 1–6, respectively.

**Table I**

Incorporation of \(^{32}P\) into individual histones (cpm/\(\mu\)g) in the presence of OA or 6-DMAP with colcemid

|            | OA 0 \(\mu\)M | OA 0.5 \(\mu\)M | OA 1.0 \(\mu\)M | 6-DMAP with colcemid 0 \(\mu\)M | 6-DMAP with colcemid 1.0 \(\mu\)M | 6-DMAP with colcemid 2.0 \(\mu\)M |
|------------|--------------|---------------|---------------|-------------------------------|--------------------------------|--------------------------------|
| HeLa       |              |               |               |                               |                                |                                |
| H2A.1      | 149.0        | 305.3 (2.04)  | 294.3 (1.98)  | 125.3                         | 15.4 (0.12)                    | 10.6 (0.08)                    |
| H2A.2      | 137.5        | 352.0 (2.56)  | 331.5 (2.41)  | 125.3                         |                                |                                |
| H3.1       | 6.4          | 200.3 (31.30) | 245.2 (38.30) | 38.1                          |                                |                                |
| H1         | 179.5        | 72.5 (0.40)   | 94.5 (0.53)   | 201.4                         | 117.9 (0.59)                   | 67.6 (0.33)                    |
| H3.2/3     | 4.2          | 133.2 (31.71) | 194.6 (46.30) | 23.6                          |                                |                                |
| N18        |              |               |               |                               |                                |                                |
| H2A.1      | 81.5         | 100.5 (1.23)  | 104.6 (1.28)  | 80.0                          | 49.1 (0.61)                    | 26.0 (0.33)                    |
| H2A.2      | 69.3         | 98.6 (1.31)   | 90.5 (1.42)   | 70.4                          | 52.5 (0.75)                    | 26.5 (0.38)                    |
| H3.1       | 10.5         | 15.7 (1.5)    | 15.9 (2.8)    | 12.5                          |                                |                                |
| H1         | 90.6         | 31.6 (0.35)   | 19.9 (0.22)   | 127.1                         | 98.8 (0.78)                    | 37.2 (0.29)                    |
| H3.2/3     | 8.3          | 10.6 (1.28)   | 25.6 (3.08)   | 11.0                          |                                |                                |

Phosphorylations by OA reflect any alteration in the number of phosphorylation sites. \(^{32}P\) phosphopeptide maps of the three kinds of histones were prepared and compared with those of control cells (Fig. 4). The phosphopeptide of H2A in the OA-treated cells was single (Fig. 4b) and migrated to the same position as with H2A in untreated cells (Fig. 4a). The H2A spot in OA-treated cells was more extensively labeled than that in the control H2A. The positions of individual H1 phosphopeptides with OA (Fig. 4d) were also similar to those in untreated cells (Fig. 4c), but the intensity of the individual phosphopeptide differed considerably. The \(^{32}P\) radioactivity in most spots was reduced and one spot demonstrated intensification (Fig. 4d, indicated by an arrow). The two major H3 phosphopeptides with OA (Fig. 4f) were the same as the two mitotic H3 phosphopeptides (3-1 and 3-2 in Fig. 4e). The amino acid sequences of the phosphopeptides, 3-1 and 3-2 were shown previously to contain the same residue Ser(P)\(^{10}\), the peptide 3-2 is a product of incomplete proteolysis (17). The results indicate that OA does not induce an extra phosphorylation site in both H2A and H1, but does induce H3 phosphorylation in interphase cells exactly at the same site as in mitotic cells and some minor sites.

Relationship between H1 Kinase Activity and H1 and H3 Phosphorylations in HeLa Cells in the Presence of OA—Since OA-induced chromatin condensation in interphase cells, H1 superphosphorylation and mitosis-specific H3 phosphorylation were investigated simultaneously (Fig. 5). The H1 and H3 phosphorylations in OA-treated cells were examined by pulse labeling with \(^{32}P\) (Fig. 5a). The H1 kinase activity in the 0.4 M NaCl-soluble nuclear fraction was examined in vitro using HeLa histone H1 as a substrate (Fig. 5a). At the same time, H1 and H3 phosphorylations in aliquots of OA-treated cells were examined by pulse labeling with \(^{32}P\) (Fig. 5b). During the first 30 min after the OA treatment, the incorporation of \(^{32}P\) into H1 did not essentially differ from that of untreated cells (Fig. 5, a and b), and H3 phosphorylation was negligible. H1 phosphorylation was elevated and peaked after 1 h, and H3 phosphorylation reached a maximum level at around 1.5 h. Thereafter, H1 phosphorylation returned to the normal level by 2 h, whereas H3 phosphorylation was maintained at a relatively high level. The data indicate that the kinetics of H3 phosphorylation differed from that of H1 phosphorylation. Quantitative

Fig. 2. Incorporation of \([^{32}P]\)phosphate into histones in cells in the presence of OA or DMAP. HeLa or N18 cells were labeled with \([^{32}P]\)phosphate (40 \(\mu\)Ci/ml) for 3 h, and histones were extracted as described under “Materials and Methods.” Forty \(\mu\)g (\(\sim\)3500 cpm) aliquots of the proteins were run on acid-urea-Triton gel electrophoresis at 120 V for 48 h. The gels were stained with 0.2% Amido Black and then autoradiographed. A, HeLa; B, N18. Lanes 1–3 show the effects of OA on histone phosphorylations, and lanes 4–6, the effects of 6-DMAP. Lane 1, control; lane 2, 0.5 \(\mu\)M OA; lane 3, 1 \(\mu\)M OA; lane 4, colcemid only; lane 5, colcemid with 0.5 mg/ml 6-DMAP; lane 6, colcemid with 1 mg/ml 6-DMAP. Lanes 1–6, Amido Black stain and lanes 7–12, \(^{32}P\) autoradiography corresponding to lanes 1–6, respectively.

Fig. 3. Incorporation of \([^{32}P]\)phosphate into H1 and H3 histones in cells treated with various concentrations of OA. \(^{32}P\). Labeled histone preparation and analysis were conducted as described in the legend to Fig. 3. Histones (30 \(\mu\)g) were loaded on acid-urea-Triton gels and autoradiographed after electrophoresis. The figure shows only the H1 and H3 regions. Numbers 1–6 indicate HeLa histones for 0, 0.1, 0.2, 0.5, 1.0, and 1.5 \(\mu\)M of OA, respectively, and numbers 7–11, those from N18 cells treated with 0, 0.1, 0.2, 0.5, and 1.0 \(\mu\)M of OA, respectively.

4d, indicated by an arrow). The two major H3 phosphopeptides with OA (Fig. 4f) were the same as the two mitotic H3 phosphopeptides (3-1 and 3-2 in Fig. 4e). The amino acid sequences of the phosphopeptides, 3-1 and 3-2 were shown previously to contain the same residue Ser(P)\(^{10}\), the peptide 3-2 is a product of incomplete proteolysis (17). The results indicate that OA does not induce an extra phosphorylation site in both H2A and H1, but does induce H3 phosphorylation in interphase cells exactly at the same site as in mitotic cells and some minor sites.

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The present study confirmed that OA causes interphase cells to demonstrate mitosis-like characteristics in terms of both morphological and biochemical parameters. Thus, 1) most OA-treated interphase cells became round in shape with condensed chromosomes, and 2) mitosis-specific histone H3 phosphorylation was induced, although the other cell cycle-dependent histone phosphorylations (H1 and H2A) were also influenced. Our data imply that OA inhibition of phosphatase results in accumulation of phosphorylated H3 proteins, so condensation of chromatin was induced without the completion of DNA synthesis. 6-DMAP, in contrast, decondenses chromatin and concurrently dephosphorylates H3 even in cells pretreated with colcemid. The chemical is known to inhibit both MPF kinase activity and protein phosphorylations during the maturation of oocytes (35, 36). Therefore, 6-DMAP inactivates both H1 and H3 kinases and thereby dephosphorylates H1 and H3 phosphorylations, which are accumulated in the presence of colcemid, leading to chromatin decondensation. This result is consistent with that of Th'ng et al. (37), who used staurosporine, another protein kinase inhibitor, and found it induced both H1 and H3 dephosphorylations and chromatin decondensation in FM3A cells. This evidence suggests that chromatin condensation must be regulated by a protein kinase/protein phosphatase related mechanism.

OA-induced chromatin condensation of HeLa cells was relatively weak as compared with the case with N18 or other cells (22). Steinmann et al. (38) reported that chromatin in human and mouse cells is hard to condense by OA. The different extent of chromatin condensation among cell types may derive from different rates of H1 kinase induction. We found that induction of H1 kinase by OA was only two times enhanced in HeLa, against 10 times in baby hamster kidney cells (22). This might be due to a lower content of factors supporting H1 kinase activity, like cyclin (38).

It is known that the Ser/Thr-protein phosphatase inhibitor,
OA, specifically inactivates both protein phosphatases, pp1 and pp2A. The pp2A is inhibited completely with 1–2 nm OA, whereas pp1 requires 1 µM (18, 20). At the low concentration of OA (2 nM) giving pp2A inhibition, no appreciable effect was observed in terms of H2A and H3 phosphorylations or chromatin structure. Therefore, the data indicate that H2A or H3 dephosphorylations during the cell cycle involve pp1 rather than pp2A.

The mechanism by which OA induces such a burst increase of H3 phosphorylation remains unclear, and the question of whether H3 can be phosphorylated in its natural state or whether the phosphorylation requires some structural change in chromatin still awaits an answer. The H3 phosphorylation itself was probably accelerated by OA inhibition of the relevant phosphatase rather than by H3 kinase activation, since Ser\(^{10}\) in nucleosomes associated with H1 is hardly phosphorylated in vitro (17, 39).

In the presence of OA, the H1 phosphorylation level decreased after 2–3 h in the present case. Paulson et al. (40) similarly found that mitotic H1 phosphorylation of HeLa cells was inhibited by OA using retardation of phosphorylated proteins in gel electrophoresis. The present data indicate that 1) dephosphorylation of H1 occurs not only in M cells but also in interphase cells and 2) the dephosphorylation occurs only in H1 but not in H2A or H3. One possible explanation for this might be that H1 kinase is inactivated by OA with longer treatment as reported by Schonthal and Feramisco (41). The possibility is supported by the evidence that H2A and H3 phosphorylations are not inhibited by protein kinases other than cdc2/H1 kinase. Alternatively, the protein phosphatase responsible for H1 phosphorylation in vivo is neither pp1, pp2A, or the other new protein phosphatase, as discussed by Paulson et al. (40). Although it has been reported that pp2A (42) or pp1 (43) is involved, the phosphatase responsible in mammalian cells has not been determined yet. The remaining phosphorylated H1 protein demonstrated that the dephosphorylation of a specific peptide was very slow or unaffected by OA. This indicates that the efficiency of dephosphorylation by a protein phosphatase may depend on the primary structure of the protein (44).

The present study revealed that H3 phosphorylation associated with OA occurs at the mitosis-specific site (Ser\(^{10}\)). H3 phosphorylation is the most closely associated with chromatin condensation among the three phosphorylatable histones during the cell cycle. Extensive H3 phosphorylation has been observed thus far with various kinds of chromatin condensation; 1) normal mitosis from prophase to anaphase (4), 2) PCC in a hybrid cell between Sand M phase cells (13), 3) PCC in interphase cells (14, 15), and 4) PCC in the cell cycle. Extensive H3 phosphorylation has been observed since in amphibian embryonic system in vitro (45, 46), whereas it is in growing organisms (47). On the other hand, it is known that OA phosphorylated on 80-kDa proteins binding at AT-rich and on nuclear matrix-associated 70-kDa proteins (48). The effect of these phosphorylated non-histone proteins is not rule out for the chromatin condensation.

The H1 kinase activated by OA is known to be Cdc2-type protein kinase associated with cyclin B, which is same as in mitotic cells (22). The present evidence that transient H1 and mitotic H3 phosphorylations occur in the presence of OA indicates that chromatin condensation with OA in interphase cells is induced by a biochemical mechanism similar to that operating in normal mitosis. The causal relationship between chromatin condensation and H3 phosphorylation now requires elucidation, as well as determination of H3 kinase(s).

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