Global Regulation of Post-translational Modifications on Core Histones*

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Scott C. Galasinski‡‡, Donna F. Louie¶¶, Kristen K. Gloor†, Katheryn A. Resing‡, and Natalie G. Ahrn¶¶**

From the ‡Howard Hughes Medical Institute, the ¶Department of Chemistry and Biochemistry, and the †Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, Colorado 80309

Full-length masses of histones were analyzed by mass spectrometry to characterize post-translational modifications of bulk histones and their changes induced by cell stimulation. By matching masses of unique peptides with full-length masses, H4 and the variants H2A.1, H2B.1, and H3.1 were identified as the main histone forms in K562 cells. Mass changes caused by covalent modifications were measured in a dose- and time-dependent manner following inhibition of phosphatases by okadaic acid. Histones H2A, H3, and H4 underwent changes in mass consistent with altered acetylation and phosphorylation, whereas H2B mass was largely unchanged. Unexpectedly, histone H4 became almost completely deacetylated in a dose-dependent manner that occurred independently of phosphorylation. Okadaic acid also partially blocked H4 hyperacetylation induced by trichostatin-A, suggesting that the mechanism of deacetylation involves inhibition of H4 acetyltransferase activity, following perturbation of cellular phosphatases. In addition, mass changes in H3 in response to okadaic acid were consistent with phosphorylation of methylated, acetylated, and phosphorylated forms. Finally, kinetic differences were observed with respect to the rate of phosphorylation of H2A versus H4, suggesting differential regulation of phosphorylation at sites on these proteins, which are highly related by sequence. These results provide novel evidence that global covalent modifications of chromatin-bound histones are regulated through phosphorylation-dependent mechanisms.

Histones H2A, H2B, H3, and H4 form the nucleosome core and are important targets for post-translational modification in eukaryotic cells. Acetylation, methylation, phosphorylation, ribosylation, and ubiquitination of histones have been implicated in transcription, DNA recombination, DNA repair, and chromosome condensation (reviewed in Refs. 1 and 2). Phosphorylation of histones, particularly H3, is likely to be involved in both transcriptional activation and chromatin condensation (3–7). Recent studies have revealed regulatory relationships between different modifications, for example, phosphorylation of H3 at serine 10 enhances recognition by GCN5p and thus acts as a signal to facilitate acetylation (8–10), whereas methylation of H3 can both activate and antagonize transcription (11–15). Thus, histone modifications collaborate to confer unique structural and nucleosome identifications. Less well understood is whether covalent regulation of histones may also occur on a global level, affecting total nucleosome populations. This aspect of global chromatin dynamics requires analyzing patterns of covalent modifications on bulk histones and monitoring their modulation in response to cell stimulation.

Mass spectrometry is well suited for the characterization of protein post-translational modifications (16–18). Direct coupling of reversed-phase HPLC1 (LC) with electrospray ionization mass spectrometry (MS) allows in-line separation of mixtures of protein or peptides to be analyzed as they elute from the column (19, 20). The identification of modifications by direct mass analysis is unbiased, lacking drawbacks of metabolic labeling or chemical derivatization, and enables multiple chemistries to be observed at the same time.

In this study, changes in post-translational modifications on chromatin-derived histones were examined in response to treatment of resting cells with the serine-threonine phosphatase inhibitor, okadaic acid (OA). Cellular responses to OA include the activation of signaling transduction pathways, nuclear envelope breakdown, premature chromatin condensation, and apoptosis (21–28). Here, we report that histones H2A, H3, and H4 become phosphorylated after treatment with OA, but with differential kinetics, suggesting distinct regulatory mechanisms for each histone. In addition, OA leads to global deacetylation of H4 in a manner independent of phosphorylation, whereas H2A undergoes deacetylation in a manner coupled to phosphorylation. These results demonstrate the regulation of acetylation on bulk nucleosomes by phosphatase inhibition and reveal mechanisms for global modulation of covalent modification patterns in chromatin.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatment—K562 human erythroleukemia cells were obtained from the American Type Culture Collection. The cells were grown in spinner cultures at 37 °C and 5% CO2 at a density of 5 × 10^5 cells/ml in RPMI (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen) and 100 μg/ml streptomycin and penicillin. The cells were treated with varying amounts of okadaic acid (Alexis) or trichostatin-A (Wako) dissolved in Me2SO, and controls received the same volume of Me2SO without drugs. UV irradiation (244 nm) was performed for 30 s using a handheld source over 2.5 × 10^6 cells suspended in 1 ml of medium in a 10-cm plate.

Histone Isolation—Histones were isolated from 2.5 × 10^6 cells by acid extraction. The cells were harvested by centrifugation (2,000 × g, 5 min, 4 °C), washed in ice-cold phosphate-buffered saline, and resuspended in 1 ml of buffer A (20 mM HEPES, pH 7.2, 1% sodium deoxycholate, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 10 μM Na4MoO4, 100 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml OA, 10 μg/ml trichostatin-A).

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‡ Present address: Howard Hughes Medical Inst., Dept. of Biochemistry and Molecular Biology, Pennsylvania State University, University Park, PA 16802-4500.

** To whom correspondence should be addressed: Howard Hughes Medical Inst., Dept. of Chemistry and Biochemistry, Campus Box 215, University of Colorado, Boulder CO 80309. Tel.: 303-492-4799; Fax: 303-492-2439; E-mail: Natalie.Ahrn@colorado.edu.

The abbreviations used are: HPLC, high pressure liquid chromatography; LC, reversed-phase HPLC; MS, mass spectrometry; OA, okadaic acid; TSA, trichostatin-A.
aprotinin, 2 μg/ml pepstatin A, 1 mM benzamidine). Lysates were prepared by 20 strokes in a tight fitting glass/glass Dounce homogenizer, followed by three 20-s pulses of sonication on ice. Insoluble material was collected by centrifugation (17,000 × g for 10 min), and the pellets were resuspended in buffer B (8 M urea, 100 mM dithiothreitol, 1% Triton X-100) and incubated 10 min at room temperature. After centrifugation (17,000 × g for 10 min), the loose, viscous pellet was transferred to a new tube, and 1/10 volume of 0.3 N HCl was added, followed by incubation for 1 h at 4 °C with vortexing. The insoluble pellet was separated from the supernatant by centrifugation (17,000 × g for 10 min). Histones and chromatin-associated proteins were precipitated from the supernatant by the addition of 10 volumes of cold acetone and incubation overnight at −20 °C. The proteins were collected by centrifugation (17,000 × g for 10 min), and the pellets were lyophilized and resuspended in 100 mM HEPES, pH 8.0, plus protease inhibitors. Isolated histones were analyzed on 15% SDS-PAGE gels with Coomassie staining to verify protein integrity. In some experiments, isolated histones were treated with 20 units of calf alkaline intestinal phosphatase (Promega, with no additional buffers). Phosphatase reactions were run for 30 min at 30 °C prior to LC/MS.

Mass Spectrometric Analysis—The samples were acidified to 1.0% (v/v) with formic acid and loaded onto POROS R120 perfusion chromatography (PerSeptive) packed in fused silica capillary columns (500 μm × 20 cm). The column eluate was directly coupled to a Perkin-Elmer Sciex API-III triple quadrupole mass spectrometer equipped with a nebulization assisted electrospray source (LC/electrospray ionization/MS). The proteins were eluted from the column with a gradient of

![FIG. 1. Contour plot of core histones detected by mass spectrometry. The x axis represents the time of elution from HPLC and scan number, and the y axis represents the m/z ratio for each protein detected in multiple m/z states. The order of elution for each histone and the scan regions used to generate the mass spectrum for each protein are indicated.](http://www.jbc.org/)

![FIG. 2. Ion spectra of core histones.](http://www.jbc.org/)

**TABLE I**

| Histone | Swiss Protein Database Accession number | Calculated mass | Observed mass | S.D. | Number of peptides | Peptide identification number (average observed peptide mass) |
|---------|----------------------------------------|----------------|--------------|------|-------------------|-------------------------------------------------------------|
| H2A.1   | (P02261)                               | 14,001         | 14,004       | 1.3  | 3                 | 2 (2186.6), 3 (4304.1)                                      |
| H2A.2   | (P28001)                               | 14,045         |              |      | 3                 | 2 (2186.6)                                                  |
| H2A.M   | (P04908)                               | 14,082         |              |      | 3                 | 2 (2186.6)                                                  |
| H2A.X   | (P16104)                               | 15,054         |              |      | 3                 | 2 (2186.6)                                                  |
| H2A.Z   | (P17317)                               | 13,462         |              |      | 2                 | 2 (3193.7), 3 (1772.0), 4 (6401.3)                         |
| H2B.1   | (P06899)                               | 13,773         | 13,773       | 1.5  | 1                 | 2 (2475.9), 3 (1772.0), 4 (6585.3)                         |
| H2B.1A  | (P02728)                               | 13,775         |              |      | 2                 | 2 (2461.9), 3 (3194.7), 4 (1772.0), 5 (6401.3)             |
| H2B.2   | (P25527)                               | 13,776         |              |      | 2                 | 2 (2476.0), 3 (1772.0), 4 (6401.3)                         |
| H2B.F   | (P33778)                               | 13,819         | 13,817       | 1.5  | 4                 | 2 (2319.3), 3 (1772.0), 4 (6401.3)                         |
| H3.1    | (P16106)                               | 15,273         | 15,272       | 1.5  | 5                 | 2 (509.6), 3 (1911.3), 5 (1553.8)                           |
| H3.3    | (P06351)                               | 15,197         |              |      | 2                 | 2 (509.6), 3 (1911.3), 5 (1553.8)                           |
| H4      | (P02304)                               | 11,278         | 11,278       | 1.5  | 4                 | 2 (5006.9), 3 (1922.2), 4 (2000.3)                          |

*a* After removal of methionine-1; *b* N-terminal acetylation for H2A and H4.

* Expected number of peptides after complete theoretical digest with endoproteinase Asp-N.

* Peptide number and observed mass matched to histone theoretical digest with endoproteinase Asp-N.

* Peptides corresponding to H2A.Z were observed, but the full-length protein mass for this variant was not, suggesting minor abundance of H2A.Z.
with 20% (w/w) endoproteinase Asp-N (Roche Molecular Biochemicals). The digestions were performed at 30 °C for 18 h, loaded onto a C-18 column, desalted, and analyzed by LC/MS. Eluted peptides were analyzed as described for proteins.

**RESULTS**

**Identification of Histones: Mass Determination and Peptide Mapping—**Histones were enriched by acid extraction and analyzed by reversed-phase chromatography in-line with ion spray mass spectrometry (LC/MS). Detection of histones is

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**TABLE II**

Interpretation of observed masses

| Histone | Mass observed (Da) | S.D. | Interpretation |
|---------|--------------------|------|----------------|
| H2A     | 14,004             | 1.3  | H2A.1 + N-Ac   |
|         | 14,046             | 1.3  | H2A.1 + N-Ac + 1K-Ac |
|         | 14,083             | 1.3  | H2A.1 + N-Ac + 1P |
|         | 14,127             | 1.3  | H2A.1 + N-Ac + 1K-Ac + 1P |
| H2B     | 13,759             | 1.5  | Unknown        |
|         | 13,773             | 1.5  | H2B.1          |
|         | 13,788             | 1.5  | H2B.1 + 1Me    |
|         | 13,817             | 1.5  | H2B.F          |
|         | 13,845             | 1.5  | H2B.F + 2Me    |
|         | 13,699             | 1.5  | H2B.F + 1P     |
| H3      | 15,272             | 1.5  | H3.1           |
|         | 15,302             | 1.5  | H3.1 + 2Me     |
|         | 15,315             | 1.5  | H3.1 + 3Me or 1K-Ac |
|         | 15,328             | 1.5  | H3.1 + 4Me or 1K-Ac + 1Me |
|         | 15,344             | 1.5  | H3.1 + 5Me or 1K-Ac + 2Me |
|         | 15,355             | 1.5  | H3.1 + 6Me or 1K-Ac + 3Me or 2K-Ac |
|         | 15,368             | 1.5  | H3.1 + 7Me or 1K-Ac + 4Me or 2K-Ac + 1Me |
|         | 15,382             | 1.5  | H3.1 + 8Me or 1K-Ac + 5Me or 2K-Ac + 2Me or 2Me + 1P |
|         | 15,399             | 1.5  | H3.1 + 9Me or 1K-Ac + 6Me or 2K-Ac + 3Me or 3K-Ac |
|         | 15,425             | 1.5  | H3.1 + 11Me or 1K-Ac + 8Me or 2K-Ac + 5Me or 3K-Ac + 2Me or 1K-Ac + 2Me + 1P |
|         | 15,509             | 1.5  | H3.1 + 17Me or 1K-Ac + 14Me or 2K-Ac + 11Me or 3K-Ac + 11Me or 4K-Ac + 3Me or 5K-Ac |
|         | 15,574             | 1.5  | H3.1 + 22Me or 1K-Ac + 18Me or 2K-Ac + 16M or 3K-Ac + 13Me or 4K-Ac + 8Me or 5K-Ac + 2Me or 15,368 (Da) + 1P + 3K-Ac |
| H4      | 11,278             | 1.5  | H4 + N-Ac     |
|         | 11,292             | 1.5  | H4 + N-Ac + 1Me |
|         | 11,306             | 1.5  | H4 + N-Ac + 2Me |
|         | 11,317             | 1.8  | H4 + N-Ac + 1K-Ac |
|         | 11,334             | 1.5  | H4 + N-Ac + 1Me + 1K-Ac |
|         | 11,348             | 1.5  | H4 + N-Ac + 2Me + 1K-Ac |
|         | 11,359             | 1.8  | H4 + N-Ac + 2K-Ac |
|         | 11,376             | 1.5  | H4 + N-Ac + 1Me + 2K-Ac |
|         | 11,386             | 1.5  | H4 + N-Ac + 2Me + 1P |
|         | 11,390             | 1.5  | H4 + N-Ac + 2Me + 2K-Ac |
|         | 11,401             | 1.8  | H4 + N-Ac + 3K-Ac |
|         | 11,431             | 1.5  | H4 + N-Ac + 2Me + 3K-Ac |
|         | 11,444             | 3     |                   |
|         | 11,475             | 1.8  | H4 + N-Ac + 2Me + 4K-Ac |
|         | 11,516             | 1.8  | H4 + N-Ac + 2Me + 5K-Ac |

*This peak was detected with variable masses ranging between 11,440 and 11,449 Da in different experiments after treatment with OA and could not be assigned to specific covalent modifications.*

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**Fig. 3.** Deconvolution plots of core histones from control cells. Plots were summed from intensities of ions shown in Fig. 2 and show mass windows for H4 (A), H2A and H2B (B), and H3 (C). Interpretations of mass forms are indicated in Tables I and II.
represented in a contour plot (Fig. 1), showing elution of H4, followed by co-elution of H2A and H2B (which are distinguishable by mass) and finally elution of H3. The selection of scans shown in Fig. 1 reveals a series of ions resulting from multiple charging of each protein (Fig. 2).

The observed protein masses were computationally deconvoluted from each ion series and compared with the masses predicted for histone variants (Table I). Assignments were confirmed by peptide mapping, in which individual histones were purified by reversed-phase chromatography, digested with endoproteinase Asp-N, and analyzed by LC/MS. Masses of peptides detected in digests were compared with predicted peptide masses in histone variants (Table I). In some cases, histones were found in only one form. For example, the single isoform of H4 was verified by protein and peptide mass identification. H3 exists as variants H3.1 and H3.3, both of which were consistent with observed peptide masses; however, only H3.1 was present, based on observed protein mass. Peptide masses corresponding to H2A were observed, with variant H2A.1 detected as the predominant form. H2B variants were more difficult to distinguish because of overlap in predicted peptide mass; however, the unique assignments of peptides of 2475.9 and 6385.3 Da to the protein mass of 13,773 Da and the observed protein mass of 13,817 indicate the presence of H2B.1 and H2B.F.

Histone Covalent Modifications—Post-translational modifications of histones were apparent from the MS spectra of full-length proteins (Fig. 2). For example, the spectra show ions with charges from +8 to +18 representing the “charge envelope” of H4 (Fig. 2A). An expanded view of one charge form (+11 ion; Fig. 2A, inset), shows four major ions (m/z = 1257.4,
1261.9, 1266.5, and 1274.3), indicating multiple mass forms of H4. This is confirmed by deconvoluting the MS spectrum (Fig. 3A), revealing full-length masses of each form ranging from 11,200 to 11,500 Da. We interpret these data as follows. H4 exists as a single isoform, with the observed mass 11,278 Da identical to that calculated from the primary sequence, including removal of the N-terminal methionine and N-terminal acetylation of serine 1. Additional covalently modified forms of H4 are detected as methylated (11,292 Da), dimethylated (11,306 Da), methylated /N-acetylated (11,334 Da), dimethylated /N-acetylated (11,348 Da), methylated /diacetylated (11,376 Da), dimethylated /diacetylated (11,390 Da), and dimethylated /triacetylated (11,431 Da) (Fig. 3A and Table II). By noting the intensity of the major isoforms, we conclude that H4 in resting cells exists primarily in a dimethylated state (11,306 Da) and a dimethylated + monoacetylated state (11,348 Da).

Multiple ions at individual charge states were also seen with H2A (m/z = 1274.0, 1277.9, 1281.4), and H2B (m/z = 1251.8, 1253.2, 1254.4, 1257.3, 1261.9, 1264.5) (Fig. 2B, inset). As described above the major form of H2A was assigned to H2A.1 modified by N-terminal acetylation (Table II). Secondary forms of H2A.1 are acetylated or phosphorylated. H2B was detected as both H2B.1 and H2B.F isoforms by inspection of peptide and full-length protein masses. Deconvolution plots (Fig. 3B, 13,759–13,899 Da) suggest that H2B.1 is the predominant form (13,773 Da) and is also methylated (13,788 Da), whereas H2B.F (13,817 Da) is less abundant and observed in unmodified, dimethylated, and phosphorylated forms (Table II). A predominant peak observed at 13,759 Da
cannot be matched to any known H2B variant and may represent a unique variant present in this cell line or a stable degradation product.

Modified forms of H3 were also apparent from mass spectra and deconvolution plots (Figs. 2C and 3C). The mass of 15,272 Da corresponded to the unmodified variant H3.1 and was verified by peptide mapping (Table I). In general, full-length masses increased in increments of 14 Da (Table II) and most likely reflect lysine methylation, although acetylation cannot be excluded. Mass increases were also observed that corresponded to phosphorylation of methylated and acetylated forms (15,382, 15,425, and 15,574 Da). As shown in Table II, the observed masses suggest complex patterns of covalent modification for H3.

Okadaic Acid Modulates the Patterns of Histone Covalent Modifications—OA is a potent serine-threonine phosphatase inhibitor that alters phosphorylation of many intracellular proteins, including histones (24–26, 28). K562 cells were treated with 0, 10, 100, and 1000 nM OA, spanning a lower concentration range (10–100 nM) expected to inhibit protein phosphatase 2A in vivo and a higher range (100–1000 nM), which inhibits both protein phosphatase 2A and protein phosphatase 1 (25,
Histones were harvested after 1 and 18 h of treatment with 0, 1, 10, 100, and 1000 nM OA (Fig. 4). As mentioned above, H4 from control cells was predominantly dimethylated and N-terminal acetylated (11,306 Da). Additional forms were apparent, corresponding to further modifications by monoacetylation and diacetylation (11,348 and 11,390 Da). No significant changes in post-translational modification of H4 were seen after 1 h of OA treatment at any concentration. However, treatment for 18 h resulted in a dramatic loss of H4 acetylation, apparent at concentrations of 10 nM and higher (Fig. 4, D, F, and H). At 1000 nM OA, H4 was primarily detected in its dimethylated (11,306 Da) or dimethylated + phosphorylated forms (11,386 Da), with loss of acetylation. No peaks corresponding to monoacetylated + monophosphorylated (11,428 Da) was observed, indicating that the loss of acetylated H4 was not caused by conversion to a phosphorylated form. Furthermore, at 100 nM, deacetylation is clearly apparent prior to formation of the phosphorylated species. This indicates that deacetylation and subsequent phosphorylation events are uncoupled, such that phosphorylation occurs independently and at a later time after deacetylation.

The first five residues of H4 and H2A are identical in the region surrounding the site of phosphorylation; thus it was surprising that these proteins differed with respect to dose and time dependence of phosphorylation. H2A accumulated as a 80-Da phosphorylated form after 1 h at 1000 nM OA (Fig. 5, G), whereas H4 was unmodified under these conditions. Increased H2A phosphorylation was observed at all concentrations of OA after 18 h (Fig. 5, B, D, F, and H, compare 14,004 Da with 14,083 Da). We also noted loss of the acetylated form (14,046 Da) and a corresponding increase in an acetylated + phosphorylated form (14,127 Da) at 10 and 100 nM OA, indicating that the acetylated species of H2A are substrates for phosphorylation. These changes suggest a faster response for H2A phosphorylation and greater sensitivity to OA, compared with H4.

Next, histones from OA-treated cells were dephosphorylated in vitro with alkaline phosphatase. Phosphatase treatment led to a significant reduction in the 11,386-Da species of H4 and the 14,083- and 14,132-Da species of H2A (Fig. 6). Little change was observed using heat-inactivated phosphatase, confirming their assignments as phosphorylated forms of H4 and H2A.

Post-translational modification of H2B changed little throughout the varying dosages of OA at either 1 h (data not shown) or 18 h (Fig. 7). After 18 h at 1000 nM OA, the 13,817-Da species decreased, with no detectable increase that would suggest redistribution because of covalent modification. Thus, H2B F might be more susceptible to degradation or preferentially lost during isolation from cells treated with OA.

Deconvolution plots of H3 showed major changes in post-
translational modification after treating cells with 1000 nM OA for 18 h (Fig. 8). A nearly identical pattern was observed after 1 h (data not shown). Major changes in H3 included loss of intensities at 15,328, 15,344, and 15,355 Da and increased intensities at 15,472 and 15,488 Da. As described earlier, the apparent modifications on H3 were complex and difficult to interpret. Most likely, the overall shift in peak intensities to greater mass reflects H3 phosphorylation in response to phosphatase inhibition. For example, the mass at 15,488 Da may correspond to diphosphorylation of the 15,328-Da species.

**H4 Modifications Are Not Caused by Apoptosis**—Many cell lines, including K562, undergo cell death after several hours of treatment with OA; thus, it was possible that the effects on histone post-translational modifications reflect chromatin modifications caused by apoptotic signaling. To examine this, histone H4 was analyzed after UV irradiation of K562 cells, which induces apoptosis without inhibiting cellular phosphatase activity. UV-induced apoptosis was monitored by microscopy, observing extensive cellular fragmentation at 10 h. The mass of H4 was unchanged after irradiation for 30 s and further incubation for 4–24 h (Fig. 9). This indicates that the deacetylation and phosphorylation events in response to OA are unlikely to occur as secondary responses to apoptosis and are more likely regulated by phosphorylation-dependent events.

**Okadaic Acid Influences Both Acetyltransferase and Deacetylase Enzyme Activities**—Fig. 4 shows H4 deacetylation at 10–100 nM OA, prior to the appearance of phosphorylated H4. Intensities of unmodified, acetylated, and phosphorylated forms were quantified by summing areas under each peak, averaging results of three separate experiments (Fig. 10). The observed dose response for H4 deacetylation (IC_{50} = 20 nM) is 5–10-fold lower than that of H4 phosphorylation (EC_{50} = 200 nM), confirming that deacetylation precedes phosphorylation (Fig. 10A). In contrast, H2A globally redistributed to phosphorylated and acetylated + phosphorylated forms with little net deacetylation (Fig. 10B).

This result suggested that enzymes that catalyze global acetylation and/or deacetylation of H4 are controlled directly or indirectly by phosphorylation mechanisms. To examine whether either or both of these activities were affected, the cells were treated with OA in the presence or absence of the deacetylase inhibitor trichostatin-A (TSA) (31). After treatment with TSA in the absence of OA, mass forms corresponding to mono-, di-, tri-, and tetra-acetylated H4 were observed (Fig. 11C, 11,348, 11,390, 11,431, and 11,475 Da), with the tetra-acetylated form in greatest abundance. A ladder of masses at 11,317, 11,359, 11,401, and 11,444 Da was also observed that corresponded to differentially acetylated forms of the 11,278-Da species (N-terminal acetylated and unmethylated H4). Treatment with both OA and TSA resulted in a unique pattern in which the stoichiometry of acetylation was intermediate between cells treated with either TSA or OA (Fig. 11D).

Thus, OA induces global H4 deacetylation in part through inhibition of acetyltransferases. In contrast, TSA did not shift H2A to maximally acetylated forms (Fig. 11G), implying that a significant amount of H2A deacetylase activity may be insensitive to this drug. Thus, effects of OA on acetylase versus deacetylase activities toward H2A could not be distinguished.

Finally, no peaks corresponding to the phosphorylated H4 species were observed after OA (Fig. 11D), suggesting that phosphorylation occurs only on forms of H4 that are completely unoccupied at all N-terminal lysine residues. In contrast, H2A species show differing behavior, in which phosphorylation is preferential for deacetylated forms but still occurs to a measurable level on the monoacetylated forms (Fig. 11H).
Mass Spectrometric Identification of Histone Modifications

Mass spectrometry allows chemistries and stoichiometries of protein modifications to be readily characterized on intact proteins. Our study identifies acetylation, phosphorylation, and methylation as predominant modifications of bulk H2A, H2B, H3, and H4, in agreement with literature reports (reviewed in Refs. 1 and 2). Significantly, we observe complex changes in chemical modification of H2A, H3, and H4 in response to treatment of cells with OA, indicating that global covalent modifications of histones respond to regulation of cellular serine/threonine phosphatases. In particular, marked changes in histone acetylation provide evidence that mechanisms facilitating acetylation and deacetylation of chromatin-bound histones can be globally regulated by phosphorylation.

Core histone subtypes were assigned to each of the observed full-length polypeptide masses by peptide mapping. By matching unique peptide masses with intensities of full-length masses in deconvolution plots, we identified H4 and variants H2A1, H2B1, H2B.F, and H3.1 in K562 cells. N-terminal peptides from histones H2A1, H3, or H4 were not detected, indicating that the N termini of these histones are likely to be covalently modified.

Previous mapping studies have shown that the acetylation of nucleosomal H4 occurs at lysines 5, 8, 12, and 16 (1). Therefore, it is reasonable to assume that these residues represent the major sites for lysine acetylation in our samples that primarily represent monoacetylated and diacetylated species under control conditions. Likewise, previous mapping identified serine 1 as the main phosphorylated residue on H4, and this site is most likely occupied in the 11,083-Da form. H4 acetylation at lysines 5 and 12 and phosphorylation of serine 1 have been suggested to be cytoplasmic events occurring on free histones (32–35). However, our isolation procedure involves precipitation of DNA-associated proteins; therefore the phosphorylation and deacetylation events we observe occur on chromatin.

Treatment of cells with OA resulted in phosphorylation of H4, reaching stoichiometries of nearly 0.5 mol/mol. An unexpected finding was that OA also caused a substantial loss of H4 acetylation. This implies that global histone deacetylation can be regulated in vivo by phosphorylation-dependent pathways. The absence of H4 species that were both phosphorylated and acetylated indicated that phosphorylation only occurs on deacetylated H4; therefore deacetylation precedes phosphorylation. Furthermore, deacetylation of H4 is 10-fold more sensitive to OA than phosphorylation (Fig. 10), indicating that the two events are uncoupled and that the deacetylation event is not driven by a shift in equilibrium to phosphorylated H4. Thus, OA appears to increase the net rate of deacetylation of bulk histone. Conceivably this could be caused by decreased action of histone acetyltransferases, increased action of histone deacetylases, or both. Therefore the possibility that OA influences histone deacetylase activity was examined by treating cells with TSA, which inhibits most nucleosomal histone deacetylase enzymes. TSA shifted nearly all of the H4 protein to a tetra-acetylated form, which we ascribe to full occupancy at lysines 5, 8, 12, and 16. This implies that most if not all HDAC enzymes responsible for global H4 deacetylation are TSA-sensitive. Importantly, OA treatment reduced the stoichiometry of TSA-induced H4 acetylation to approximately equal distributions of 0, 1, 2, 3, and 4 mol/mol. This provides information about enzymatic regulation, because if the sole effect of OA were to enhance deacetylase activity with no effect on acetyltransferase activity, then the degree of acetylation induced by TSA should be unaffected by OA. The accumulation of intermediately acetylated forms suggests that at least part of the OA effect is due to phosphatase-dependent inhibition of H4 acetyltransferase activity.

Acetylated forms of H2A were also observed in resting cells, which decreased following OA treatment. However, the behavior of H2A and H4 differed significantly. First, mass forms corresponding to phosphorylated as well as acetylated + phosphorylated H2A appeared after OA treatment, with little net deacetylation of H2A in response to OA. This indicates that acetylated H2A is a substrate for phosphorylation, whereas acetylated H4 is not. Second, the kinetics of H2A versus H4 modification differed, such that phosphorylated forms of H2A were apparent 1 h after OA, whereas at the same drug concentration, phosphorylated H4 was observed only after 18 h. Fi-
nally, under conditions where H2A becomes completely phosphorylated in response to OA, H4 is only 50% phosphorylated.

The differences in behavior between H2A and H4 was unexpected given the sequence conservation between the two proteins at their known sites of phosphorylation. The sites of phosphorylation in H2A and H4 reported in vitro occur at serine 1 (34). This residue occurs within the sequence Ac-NH-Ser-Gly-Arg-Gly-Lys-5, which is conserved in H4 and H2A.1. It is thus likely that H2A and H4 are targeted by the same kinases. Thus, the more rapid response of H2A phosphorylation could be linked to the faster phosphorylation of acetlated or methylated histones. For example, all acetylated forms of H2A may be accessible to protein kinases, whereas deacetylation events with H4. For example, all acetylated forms of H2A may be accessible to protein kinases, whereas deacetylation events with H4. For example, all acetylated forms of H2A may be accessible to protein kinases, whereas deacetylation events with H4.

Thus, the more rapid response of H2A phosphorylation could reflect a greater accessibility of H2A in chromatin, compared with H4. For example, all acetylated forms of H2A may be accessible to protein kinases, whereas deacetylation events may be necessary to increase accessibility of H4. In summary, distinct patterns of post-translational modifications on nucleosomal histones reveal mechanisms for phosphorylation-dependent control of global acetyltransferase and deacetylase activities, as well as differences in accessibility of individual histones to protein kinases.

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