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Biotinylation is a natural, albeit rare, modification of human histones

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ABSTRACT

Previous studies suggest that histones H3 and H4 are posttranslationally modified by binding of the vitamin biotin, catalyzed by holocarboxylase synthetase (HCS). Albeit a rare epigenetic mark, biotinylated histones were repeatedly shown to be enriched in repeat regions and repressed loci, participating in the maintenance of genome stability and gene regulation. Recently, a team of investigators failed to detect biotinylated histones and proposed that biotinylation is not a natural modification of histones, but rather an assay artifact. Here, we describe the results of experiments, including the comparison of various analytical protocols, antibodies, cell lines, classes of histones, and radiotracers. These studies provide unambiguous evidence that biotinylation is a natural, albeit rare, histone modification. Less than 0.001% of human histones H3 and H4 are biotinylated, raising concerns that the abundance might too low to elicit biological effects in vivo. We integrated information from this study, previous studies, and ongoing research efforts to present a new working model in which biological effects are caused by a role of HCS in multiprotein complexes in chromatin. In this model, docking of HCS in chromatin causes the occasional binding of biotin to histones as a tracer for HCS binding sites.

1. Background

Chromatin comprises DNA, histones, and other chromatin proteins [1]. In each nucleosomal core particle, about 146 basepairs of DNA are wrapped around an octamer of core histones (one H3/H3/H4/H4 tetramer and two H2A/H2B dimers). Amino acids in the N-terminal tails of core histones and, to a lesser extent, amino acids in globular domains and C-termini, are exposed at the nucleosomal surface [2]. The N-terminal tails are subject to a multitude of posttranslational modifications, including acetylation and methylation [3]. These modifications play crucial roles in gene regulation. For example, acetylation of lysine (K)-9 in histone H3 (H3K9ac) is associated with transcriptionally active chromatin, whereas dimethylation and trimethylation of K9 (H3K9me2, H3K9me3) are associated with transcriptionally repressed chromatin [3]. Other, less abundant, modifications such as phosphorylation of serine-14 in histone H2B play critical roles in events such as programmed cell death [4].

Hymes et al. suggested that histones are also modified by covalent attachment of the vitamin biotin, mediated by the enzyme biotinidase [5]. While the original report was based on in vitro studies, evidence soon emerged that histone biotinylation is a natural phenomenon, based on probing and tracing biotinylation with streptavidin, anti-biotin, and [3H]biotin in primary human lymphoid cells [6]. While biotinidase undoubtedly has histone biotinyl ligase activity in vitro [5,7], it is now believed that holocarboxylase synthetase (HCS) is the enzyme responsible for biotinylation of histones in vivo. This conclusion is based on observations that HCS is a nuclear, chromatin-associated protein [8–11]; that phenotypes of HCS knockdown include shortened life span and decreased heat tolerance in *Drosophila melanogaster*, while phenotypes of biotinidase knockdown are relatively minor [9]; that both human recombinant HCS (rHCS) and its microbial ortholog BirA have enzymatic activity to biotinylate histones and histone-based peptides [12,13]; and that HCS knockdown causes aberrant gene regulation in human Jurkat lymphoblastoma cells and *D. melanogaster* [9,10].

Over the past several years, we have identified the following histone biotinylation sites: K4, K9, K18, and perhaps K23 in histone H3 [13,14] and K8 and K12 in histone H4 [7,15]. We provided evidence that K9, K13, K125, K127, and K129 in histone H2A also are targets for biotinylation albeit only in trace amounts [16]. We raised target-specific antibodies to many of these marks and to HCS [7,14–16]. Using these antibodies, new roles of biotin in gene regulation and genome stability have been discovered. In those studies, K12-biotinylated histone H4 (H4K12bio) was the most prominently featured mark, primarily due to the availability of an antibody of exceptional quality [7]. The anti-H4K12bio used in those studies does not cross-react with non-

Abbreviations: ACC, acetyl-CoA carboxylase; HCS, holocarboxylase synthetase; H3K9ac, histone H3, acetylated at lysine-9; H3K4bio, histone H3, biotinylated at lysine-4; H3K9bio, biotinylated at lysine-9; H3K18bio, biotinylated at lysine-18; H3K4me3, histone H3, trimethylated at lysine-4; H3K9me2, histone H3, dimethylated at lysine-9; H3K9me3, histone H3, trimethylated at lysine-9; H4K12bio, histone H4, biotinylated at lysine-12; HPLC, high-performance liquid chromatography; K, lysine; MCC, 3-methylcrotonyl-CoA carboxylase; MS, mass spectrometry; PBS, phosphate-buffered saline; PC, pyruvate carboxylase; PCC, propionyl-CoA carboxylase; TAU-PAGE, Triton-Acid-Urea gel electrophoresis; TPBS, 0.05% Tween-20 in PBS.

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biotinylated histones, does not cross-react with biotinylated histones other than histone H4, and does not cross-react with biotinylation sites other than K12. Evidence was provided that H4K12bio is enriched at repressed loci and at repeat regions in the human genome [10,17,18], and, further, that a low abundance of H4K12bio coincides with de-repression of retrotransposons and increased frequency of chromosomal abnormalities in biotin- and HCS deficient humans, human and murine cell lines, and *D. melanogaster* [11].

In the early stages of these investigations, we proposed that biotinylation of histones is a rare event; binding of biotin to histones is in the order of only attomoles of biotin incorporated into histones isolated from 10^6 human lymphocytes [6]. Subsequent studies by independent investigators confirmed that <0.03% of histones are biotinylated in human cell cultures [19]. In a recent report, yet another laboratory failed to detect histone biotinylation marks by using streptavidin, antibodies, and mass spectrometry (MS); based on their negative results those investigators proposed that histone biotinylation is an artifact caused by non-specific probes and purification procedures [20]. Here, we conducted a thorough examination of probe specificity and purification procedures to provide an unambiguous answer to the question of whether biotinylation of histones is a rare event or simply an artifact. In view of our findings that biotin is a natural although rare histone modification, we propose a mechanism to explain the rarity as well as the biological significance.

2. Methods

2.1. Cell culture

Jurkat human lymphoblastoma cells, IMR-90 human fibroblasts, HepG2 human hepatocellular carcinoma cells, and U-937 human monocytic cells were obtained from American Type Culture Collection (Manassas, VA). HeLa human cervical cancer cells and MCF-7 human breast cancer cells were gifts by Drs. Jennifer Wood and Angela Pannier at the University of Nebraska-Lincoln. Cells were cultured using media and conditions as recommended by the American Type Culture Collection. In some experiments, cells were cultured in biotin-defined media (0.025 nM, 0.25 nM, or 10 nM), representing biotin concentrations in plasma from biotin-deficient, biotin-normal, and biotin-supplemented individuals [21,22]. Biotin-defined media were prepared using customized culture media and biotin-depleted fetal bovine serum as previously described [23]. Where indicated, [3H] biotin (specific activity = 2.0535 TBq/mmol; Perkin Elmer, Boston, MA) or custom-made [14C]biotin (specific activity = 2.2052 GBq/mmol; Moravek, Inc., Brea, CA) were substituted for unlabeled biotin in culture media.

2.2. Purification of histones

Cell nuclei were released by treatment with detergent and collected by gradient centrifugation [6]; histones were extracted overnight with 1 M HCl at 4 °C and the pH in the supernatant was adjusted to ~7.0 with 10 M NaOH. For comparison, we used the H_2SO_4-based extraction protocol by Healy et al. [20]. For some experiments, histones were further purified by high-performance liquid chromatography (HPLC). Briefly, histones were desalted by using PD MidiTrap G-10 columns (GE Healthcare, Piscataway, NJ) and further purified by using a C8 HPLC column (250 mm × 4.6-mm inner diameter, 10 μm particle size; Grace Vydac, Hesperia, CA). Proteins were eluted using the following binary gradient (flow rate = 0.7 mL/min) at room temperature (solvent A = 0.1% trifluoroacetic acid in water; solvent B = 0.1% trifluoroacetic acid in acetonitrile): 0% solvent B for 10 min; linear increase to 30.5% solvent B over 10 min; linear increase to 39% solvent B over 10 min; 39% solvent B held for 5 min; linear increase to 46.7% solvent B over 65 min; linear increase to 100% solvent B over 1 min; 100% solvent B held for 5 min; linear decrease to 0% solvent B over 1 min; and re-equilibration of the column with 0% solvent B for 6 min. Elution of proteins was monitored at 214 nm using a UV/Vis spectrometer, and HPLC fractions were collected at 1-min intervals. The identities of histones in HPLC fractions were confirmed by MS (data not shown) [7].

2.3. Streptavidin blots and western blots of carboxylases and histones

The following biotinylated carboxylases are markers for cellular biotin status [24]: acetyl-CoA carboxylases (ACC) 1 and 2; 3-methylcrotonyl-CoA carboxylase (MCC); propionyl-CoA carboxylase (PCC); and pyruvate carboxylase (PC). Whole cell extracts were prepared as previously described [23], using a buffer that contains protease inhibitors and DNase. If the goal of an experiment was to analyze biotinylated carboxylases (80–250 kDa) and histones (11–20 kDa) on one single gel, whole cell extracts were resolved using 4–12% Bis–Tris gels (Invitrogen, Carlsbad, CA); if the goal of an experiment was to analyze only carboxylases, 3–8% Tris Acetate gels (Invitrogen) were used to improve resolution of individual carboxylases. Transblots of gels were probed with Immuno Pure Streptavidin Horseradish Peroxidase Conjugate (Thermo Scientific, Waltham, MA), diluted 4000-fold in phosphate-buffered saline (PBS) containing 0.05% Tween-20 (TPBS) to detect protein-bound biotin [23]. Total (apo + holo) PCC and PC were probed using an "in-house" rabbit anti-human PCC antibody (antigen = keyhole limpet hemocyanin-conjugated KAGDTVGEGDLLVELE) diluted 250-fold in 0.637 M NaCl/TPBS, and a commercial rabbit anti-human PC antibody (Santa Cruz, Inc., Santa Cruz, CA) diluted 200-fold in 0.537 M NaCl/TPBS before use.

Histones in nuclear extracts were typically resolved using 18% Tris-Glycine gels (Invitrogen) [7]; histones in HPLC fractions were lyophilized and dissolved in distilled water prior to gel electrophoresis. HPLC fractions were resolved using Triton-Acid-Urea gel electrophoresis (TAU-PAGE) and electroblotted using *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS) transfer buffer (25 mM CAPS, pH 10.0, and 20% methanol). Transblots were probed with streptavidin peroxidase; polyclonal goat anti-biotin peroxidase conjugate (30,000- to 200,000-fold dilution in TPBS, Sigma); polyclonal rabbit anti-biotin (1000-fold dilution in 0.637 M NaCl/TPBS, Abcam); polyclonal rabbit antibodies to K9-biotinylated histone H3 (H3K9bio), K18-biotinylated histone H3 (H3K18bio) [14], K12-biotinylated histone H4 (H4K12bio) (250-fold dilution in 0.637 M NaCl/TPBS) [7], and commercial rabbit anti-H4K8bio (500-fold dilution in 0.637 M NaCl/TPBS, Abcam); commercial antibodies to the C-termini in histones H3 (17,000-fold dilution in 0.637 M NaCl/TPBS, Santa Cruz, Inc; Santa Cruz, CA) and H4 (5000 fold dilution in 0.637 M NaCl/TPBS, Abcam); anti-acetyl lysine (250-fold dilution in 0.637 M NaCl/TPBS, Abcam); and anti-pan methyl lysine (250-fold dilution in 0.637 M NaCl/TPBS, Abcam). In addition, a new polyclonal antibody to H4K8bio was raised in rabbits as described before [7]. The secondary antibodies used were goat anti-rabbit IgG horseradish peroxidase conjugate (100,000-fold dilution; Sigma), donkey anti-goat IgG horseradish peroxidase conjugate (100,000-fold dilution; Sigma), goat anti-rabbit IgG IRDye800CW conjugate (100,000-fold dilution; LI-COR, Lincoln, NE), and donkey anti-goat IgG IRDye800CW conjugate (100,000-fold dilution; LI-COR) in 0.237 M NaCl/TPBS. Controls included pre-immune serum, horseradish peroxidase-conjugated streptavidin (Thermo Scientific), and IRDye 800CW Streptavidin (LI-COR). Bands were visualized using an Odyssey infrared imaging system (LI-COR) or a chemiluminescence-based film developer, depending on the antibodies used.

The target specificities of anti-H3K4bio, anti-H3K9bio, anti-H3K18bio, and anti-H4K8bio were tested as described before [7], using the following antigens: (i) synthetic peptides H3K4bio (denoted N_{1-13}bioK4), H3K9bio (N_{1-13}bioK9), H3K18bio (N_{13-25}bioK18), and a non-biotinylated peptide spanning amino acids 1–25 in histone H3 (N_{1-25}) [14]; (ii) synthetic peptides H4K8bio (N_{6-15}bioK8), H4K12bio (N_{6-15}bioK12), and a non-biotinylated peptide spanning amino acids