

RESEARCH ARTICLES

Lysine residues in N-terminal and C-terminal regions of human histone H2A are targets for biotinylation by biotinidase

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Abstract

In eukaryotic cell nuclei, DNA associates with the core histones H2A, H2B, H3 and H4 to form nucleosomal core particles. DNA binding to histones is regulated by posttranslational modifications of N-terminal tails (e.g., acetylation and methylation of histones). These modifications play important roles in the epigenetic control of chromatin structure. Recently, evidence that biotinidase and holocarboxylase synthetase (HCS) catalyze the covalent binding of biotin to histones has been provided. The primary aim of this study was to identify biotinylation sites in histone H2A and its variant H2AX. Secondary aims were to determine whether acetylation and methylation of histone H2A affect subsequent biotinylation and whether biotinidase and HCS localize to the nucleus in human cells. Biotinylation sites were identified using synthetic peptides as substrates for biotinidase. These studies provided evidence that K9 and K13 in the N-terminus of human histones H2A and H2AX are targets for biotinylation and that K125, K127 and K129 in the C-terminus of histone H2A are targets for biotinylation. Biotinylation of lysine residues was decreased by acetylation of adjacent lysines but was increased by dimethylation of adjacent arginines. The existence of biotinylated histone H2A *in vivo* was confirmed by using modification-specific antibodies. Antibodies to biotinidase and HCS localized primarily to the nuclear compartment, consistent with a role for these enzymes in regulating chromatin structure. Collectively, these studies have identified five novel biotinylation sites in human histones; histone H2A is unique among histones in that its biotinylation sites include amino acid residues from the C-terminus.

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1. Introduction

Histones are quantitatively and qualitatively important DNA-binding proteins in eukaryotic cells. The following five major classes of histones have been identified: H1, H2A, H2B, H3 and H4 [1]. Histones carry a positive net charge due to the great abundance of arginine and lysine

residues in these proteins [1]. The binding of negatively charged DNA to positively charged histones is mediated by electrostatic interactions. In chromatin, stretches of 146 base pairs of DNA are wrapped around octamers of core histones (one H3–H3–H4–H4 tetramer and two H2A–H2B dimers) to form nucleosomal core particles. Linker histone H1 associates with the DNA connecting two core particles to complete the nucleosomal assembly [1].

N-terminal tails of histones are exposed at the nucleosomal surface [1]. Lysine, arginine, serine, threonine and glutamate residues in these tails are targets for posttranslational modifications such as acetylation, methylation, ubiquitination, phosphorylation, sumoylation and poly(ADP-ribosylation) [2]. These covalent modifications play important roles in the epigenetic control of chromatin structure,

Abbreviations: DAPI, 4', 6-diamidino-2-phenylindole; Fmoc, *N*-fluorenylmethoxycarbonyl; HCS, holocarboxylase synthetase; MALDI, matrix-assisted laser desorption ionization.

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genomic stability and gene expression [3–5]. Some regions in C-terminal domains (e.g., hinge regions) are also exposed at the nucleosomal surface and are potential targets for covalent modifications [1]. For example, K120 in histone H2B is a target for ubiquitination [6], and K108, K116, K120 and K125 in histone H2B are targets for acetylation [7]. Histone H2A is unique among core histones in having its C-terminal tail exposed at the nucleosomal surface [1,8]. Consistent with this observation, the following modifications have been identified in the C-terminus of histone H2A and its variant H2AX: ubiquitination of K119 [6,9] and phosphorylation of S139 [10,11], respectively.

Recently, a novel posttranslational modification of histones has been identified: biotinylation of lysine residues [12]. The covalent binding of biotin to histones is mediated by biotinidase and holocarboxylase synthetase (HCS) [13,14]. Biotinidase uses biocytin (biotinyl- ϵ -lysine) as a substrate for biotinylation of histones [13], whereas HCS uses biotin and ATP [14]. The following biotinylation sites have been identified in human histones: K4, K9 and K18 in histone H3 [15] and K8 and K12 in histone H4 [16]. Biotinylation of histones plays a role in the regulation of gene expression [17], cell proliferation [12,14] and the cellular response to DNA damage [17,18].

Previous studies suggested that histone H2A contains streptavidin-reactive material [12], consistent with biotinylation of this protein. In this study, we tested the hypothesis that specific lysine residues in histone H2A and its variant H2AX are targets for biotinylation by biotinidase. Histones H2A and H2AX were chosen as models for the following reasons: First, both histones H2A and H2AX contain biotinylation motifs in their N-terminal and C-terminal domains (A. Kueh and J. Zemleni, unpublished observations). Second, the N-terminal and C-terminal regions of histone H2A have important functions in telomeric silencing in yeast [19]. Third, phosphorylation of histone H2AX plays a role in the cellular response to DNA damage [11]. Fourth, various posttranslational modifications are known to occur in histone H2A (e.g., phosphorylation of S1 [20], acetylation of K5, K9 [21] and K13 [7], ubiquitination of K119 [6,9], phosphorylation of T120 [22] and methylation of K125 or K127 [7]). Likely, these modifications affect subsequent biotinylation [16]. Collectively, identification of biotinylation sites in histones H2A and H2AX is likely to produce valuable insights into roles of these histones in chromatin structure and genomic stability.

This study addressed the following specific aims: First, we sought to identify the amino acid residues in histones H2A and H2AX that are targets for biotinylation by biotinidase. Second, we sought to determine whether acetylation, methylation and phosphorylation of histone H2A affect its subsequent biotinylation by biotinidase. Third, we sought to determine whether

biotinidase and HCS localize to the nucleus in human cells, consistent with a role of these enzymes in histone biotinylation *in vivo*.

2. Methods and materials

2.1. Identification of biotinylation sites

In previous studies, we developed a procedure to identify amino acid residues in histones that are targets for biotinylation [23]. Briefly, this procedure is based on the following analytical sequence: (1) short peptides (<20 amino acids in length) are synthesized chemically; amino acid sequences in these peptides are based on the sequence in a given region of a histone; (2) peptides are incubated with biotinidase or HCS to conduct enzymatic biotinylation; and (3) peptides are resolved by gel electrophoresis and peptide-bound biotin is probed using streptavidin peroxidase. Amino acid substitutions (e.g., lysine-to-alanine substitutions) in synthetic peptides are used to corroborate identification of biotinylation sites. In addition, amino acid modifications (e.g., acetylation of lysines) in peptides can be used to investigate the cross-talk between biotinylation of histones and other known modifications of histones.

In this study, peptides were synthesized based on the amino acid sequences in human H2A.1 (GenBank accession number M60752) and H2AX (GenBank accession number P16104). Peptides were synthesized using *N*-fluoren-9-ylmethoxycarbonyl (Fmoc)-activated L-isomers of amino acids [16]. One-letter annotation is used for denoting amino acids in this article [24]. Chemically modified peptides were synthesized by using biotinylated, acetylated and dimethylated ϵ -NH₂-derivatives of Fmoc-lysine, dimethylated guanidino derivatives of Fmoc-arginine and phosphorylated derivatives of Fmoc-serine. Peptides were quantified as described [16]. Identities of peptides were confirmed by matrix-assisted laser desorption ionization (MALDI)—time of flight—and by quadrupole—time of flight mass spectrometry—at the Nebraska Center of Mass Spectrometry (University of Nebraska–Lincoln, Lincoln, NE, USA). Amino acid sequences of synthetic peptides are provided in Results.

Peptides from both the N-terminal and C-terminal regions of histones H2A and H2AX were included in the analysis of biotinylation sites. Synthetic peptides were biotinylated enzymatically as described previously [16] with the following modifications: 5 μ g of a given peptide was dissolved in 100 μ l of a mixture containing 15 μ l of human plasma (as a source of biotinidase), 10 μ l of biocytin solution (75 μ mol/L final concentration, as a source of biotin) and 75 μ l of Tris buffer (50 mmol/L final concentration, pH 8.0). Samples were incubated at 37°C for up to 45 min. Reactions were quenched by adding an equal volume of Tricine gel loading buffer (Invitrogen, Carlsbad, CA, USA). Peptides were resolved by gel

electrophoresis and peptide-bound biotin was probed by using streptavidin peroxidase [16].

2.2. Polyclonal antibodies

The following biotinylation sites were identified in histone H2A in the experiments described below: K9 and K13 in the N-terminal region and K125, K127 and K129 in the C-terminal region. We generated antibodies against K9-biotinylated histone H2A and K13-biotinylated histone H2A. In addition, we generated antibodies against the two human enzymes that mediate biotinylation of histones: biotinidase and HCS. Polyclonal antibodies were produced using a commercial facility (Cocalico Biologicals, Reamstown, PA, USA). The following peptides were synthesized by AnaSpec (San Jose, CA, USA) and the University of Virginia Biomolecular Research Facility (Charlottesville, VA, USA) for injection into rabbits: (1) N_{1–12}bio-K9=SGRGKQGGK(biotin)ARAC (amino acids 1–12 in histone H2A plus a cysteine); (2) N_{10–24}bioK13=ARAK(biotin)AKTRSSRAGLQC (amino acids 10–25 in histone H2A plus a cysteine); (3) biotinidase (GenBank accession number NM_000060)=CLRKSRLSSGLVTAALYGR-LYERD (amino acids 520–542 in biotinidase plus one cysteine); and (4) HCS (GenBank accession number NM_000411)=EHVGRDDPKALGEEPQRRGC (amino acids 58–77 in HCS plus one cysteine). Identities and purities of these peptides were confirmed by using high-performance liquid chromatography and MALDI (data not shown). Peptides were conjugated to keyhole limpet hemocyanin before injection into White New Zealand rabbits [16]. Rabbit serum was collected before (preimmune serum) and after three injections with peptides mixed with Freund's adjuvant over a period of 49 days. Immunoglobulin G was purified from serum by using an ImmunoPure (A) IgG Purification Kit (Pierce, Rockford, IL, USA) according to the manufacturer's protocol. Antibody specificities were investigated by using synthetic peptides and histone extracts from human cells as described [16].

2.3. Cell culture

Human-derived Jurkat lymphoma cells and JAr choriocarcinoma cells (ATCC, Manassas, VA, USA) were cultured as described [25,26]. Acid extracts from Jurkat cell nuclei [12] were used for Western blot analysis of biotinylated histone H2A [16], whereas JAr cells were used for analysis of biotinylated histone H2A by immunocytochemistry.

2.4. Immunocytochemistry

K9-biotinylated histone H2A, K13-biotinylated histone H2A, biotinidase and HCS were visualized in JAr cells by using immunocytochemistry as described [27]. Primary antibodies were as described above. We used donkey anti-rabbit Cy2-labeled antibody (Jackson ImmunoResearch, West Grove, PA, USA) as a secondary antibody. Cytoplasmic and nuclear compartments were stained with rhoda-

mine phalloidin and 4', 6-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO, USA) as described [27]. Images were obtained by using an Olympus FV500 confocal microscope (Microscopy Core Facility, University of Nebraska–Lincoln).

3. Results

3.1. Biotinylation sites in histones H2A and H2AX

Both the N-terminal and C-terminal of histone H2A contain targets for biotinylation by biotinidase. We synthesized the following five peptides based on the N-terminal and C-terminal of histone H2A: N_{1–9}=amino acid sequence SGRGKQGGK; N_{7–14}=GGKARAKA; N_{12–20}=AKAKTRSSR; C_{113–121}=AVLLPKKTE; and C_{122–129}=SHHKAKGK; subscript numbers denote the position of amino acid residues in histone H2A. These peptides were subjected to enzymatic biotinylation and peptide-bound biotin was probed using gel electrophoresis and streptavidin peroxidase. Both N_{1–9} and N_{7–14} were good targets for biotinylation by biotinidase but N_{12–20} was not a good target (Fig. 1, Lanes 1–3). Moreover, peptide C_{113–121} was not but C_{122–129} was a good target for biotinylation (Lanes 4 and 5). Previous studies are consistent with the hypothesis that lysine residues in these peptides are the most likely targets for biotinylation [16].

We verified that peptide biotinylation approached maximal levels under the conditions described in Methods and materials. First, the time course of biotinylation of peptide N_{1–9} was monitored at timed intervals for up to 45 min; concentrations of peptide, biocytin and biotinidase were kept constant as described above. Biotinylation of peptide N_{1–9} was detectable 15 min after starting the incubation with biotinidase and reached maximal levels after 45 min (data

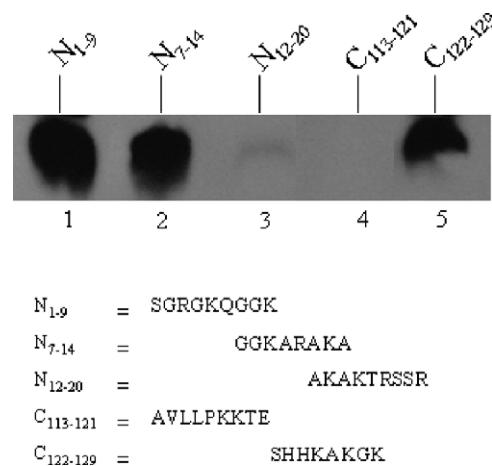


Fig. 1. Both N-terminal and C-terminal regions of histone H2A are targets for biotinylation by biotinidase. Synthetic peptides based on histone H2A were incubated with biotinidase and biocytin for enzymatic biotinylation. Peptides were resolved by gel electrophoresis and peptide-bound biotin was probed using streptavidin peroxidase.

not shown), consistent with previous studies [15]. Second, we tested the effects of substrate (biocytin) availability. Peptide N_{1-9} was incubated with biotinidase at various concentrations of biocytin (7.5, 37.5, 75, 112.5 and 150 $\mu\text{mol/L}$) for 45 min. Biotinylation of N_{1-9} reached a plateau at 75 $\mu\text{mol/L}$ of biocytin (data not shown), consistent with previous studies [13]. Finally, we varied the concentration of peptide N_{1-9} in the biotinylation reaction. The biotinylation signal paralleled the amount of N_{1-9} added to incubation mixtures (data not shown).

Next, we identified biotinylation targets in the N-terminus of histone H2A. A first series of experiments suggested that K9 is a biotinylation target based on the following lines of evidence. Peptide N_{1-9} (containing both K5 and K9) was heavily biotinylated in response to incubation with biotinidase (Fig. 2, Lane 1). When K9 was substituted with alanine (peptide $K9A_{1-9}$), no biotinylation was detectable (Lane 2). In contrast, substitution of K5 with alanine residues (peptide $K5A_{1-9}$) did not decrease biotinylation (Lane 3). When both lysine residues in peptide N_{1-9} were substituted with alanines (peptide $K5,9A_{1-9}$), no biotinylation was detectable (Lane 4). Biotinylation of K9 by biotinidase was further corroborated using the following control. Peptide N_{7-14} contains both K9 and K13 from histone H2A and was heavily biotinylated in response to incubation with biocytin and biotinidase (data not shown). When K13 in N_{7-14} was substituted with alanine, the biotinylation signal decreased only moderately; in contrast, when K9 was substituted with an alanine, the biotinylation signal decreased substantially (data not shown).

A second series of experiments suggested that K13 in the N-terminus of histone H2A becomes a target for biotinylation if the neighboring K15 is modified. This notion is based on the following lines of evidence. Peptide N_{12-20} contains both K13 and K15 and was a poor target for biotinylation by

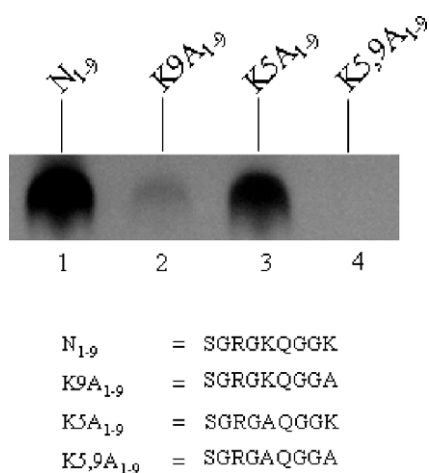


Fig. 2. K9 in histone H2A is a good target for biotinylation by biotinidase. Synthetic peptides based on histone H2A were incubated with biotinidase and biocytin for enzymatic biotinylation. Peptides were resolved by gel electrophoresis and peptide-bound biotin was probed using streptavidin peroxidase.

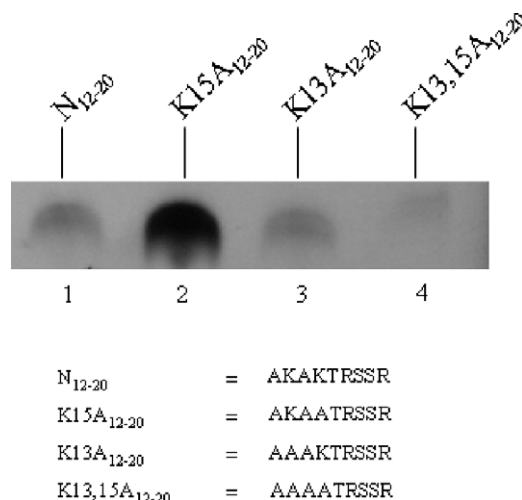
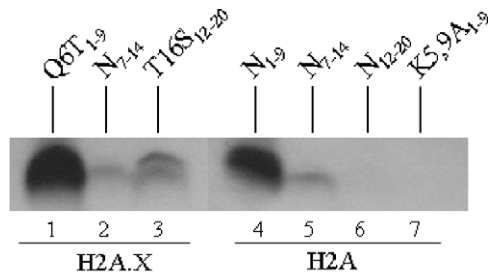


Fig. 3. Substitution of K15 in histone H2A with alanine renders K13 a good target for biotinylation by biotinidase. Synthetic peptides based on histone H2A were incubated with biotinidase and biocytin for enzymatic biotinylation. Peptides were resolved by gel electrophoresis and peptide-bound biotin was probed using streptavidin peroxidase.

biotinylation (Fig. 3, Lane 1). However, when K15 was substituted with an alanine (peptide $K15A_{12-20}$), K13 became a good target for biotinylation (Lane 2). Substitution of K13 with an alanine (peptide $K13A_{12-20}$) did not render K15 a good target for biotinylation (Lane 3). When both lysine residues in peptide N_{12-20} were substituted with alanine residues (peptide $K13,15A_{12-20}$), no biotinylation was detectable (Lane 4). Note that the lysine-to-alanine substitutions used here are an artificial system that does not necessarily represent histones from human cells. However, findings described below suggest that naturally occurring variations in amino acid sequences (see histone variant H2AX) and posttranslational modifications of amino acids (see cross-talk among histone modifications) render K13 a good target for biotinylation.

The N-terminal tail of human histone H2AX differs from the tail in histone H2A in two positions [19]: glutamine in Position 6 is substituted with threonine and threonine in Position 16 is substituted with serine in histone H2AX. First, we synthesized the following two peptides based on the N-terminus of histone H2AX: $Q6T_{1-9}$ = amino acid sequence SGRGKTGGK and $T16S_{12-20}$ = AKAKSRSSR. Both peptides were good targets for biotinylation by biotinidase (Fig. 4, Lanes 1 and 3). Peptide N_{7-14} represents a moderate target for biotinylation (see above) and was used as a control (Lanes 2 and 5). In fact, the N-terminus of histone H2AX was a better target for biotinylation than the N-terminus of histone H2A (compare Lanes 1–3 with Lanes 4–6). Specifically, a peptide containing both K9 and K13 ($Q6T_{1-9}$) was a good target for biotinylation (Lane 1), whereas a peptide containing K13 and K15 ($T16S_{12-20}$) was biotinylated only moderately in response to incubation with biotinidase (Lane 3). Peptide $K5,9A_{1-9}$ does not contain any lysine residue and was used



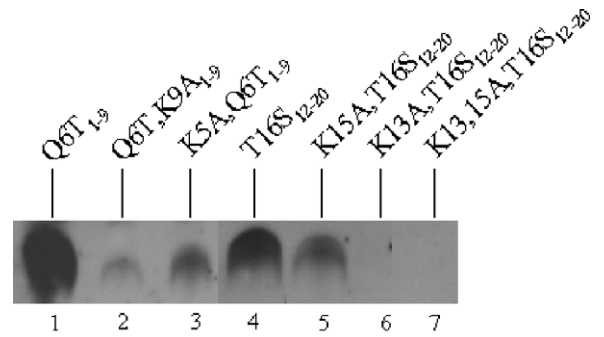
K5,9A₁₋₉ = SGRGAQGGA
 Q6T₁₋₉ = SGRGKTGGK
 N₇₋₁₄ = GGKARAKA
 T16S₁₂₋₂₀ = AKAKSRSSR
 N₁₋₉ = SGRGKQGCK
 N₇₋₁₄ = GGKARAKA
 N₁₂₋₂₀ = AKAKTRSSR

Fig. 4. The N-terminus of H2AX is a good substrate for biotinylation by biotinidase. Synthetic peptides based on histone H2AX were incubated with biotinidase and biocytin for enzymatic biotinylation. Peptides were resolved by gel electrophoresis and peptide-bound biotin was probed using streptavidin peroxidase.

as a negative control (Lane 7); no biotinylation was detectable after incubation with biotinidase.

In a next series of experiments, we confirmed that K9 and K13 in variant H2AX are specifically targeted by biotinylation in analogy to the findings described for histone H2A. Overall, we observed the same trends for peptides based on histone H2AX compared with histone H2A. Peptide Q6T₁₋₉ contains both K9 and K13 from histone H2AX and was biotinylated in response to incubation with biotinidase (Fig. 5, Lane 1). Substitution of K9 with alanine (peptide Q6T,K9A₁₋₉) substantially decreased biotinylation (Lane 2), whereas substitution of K5 with alanine (peptide Q6T,K5A₁₋₉) decreased biotinylation only moderately (Lane 3). Peptide T16S₁₂₋₂₀ contains both K13 and K15 and was biotinylated in response to incubation with biotinidase (Lane 4). When K15 was substituted with alanine (peptide K15A,T16S₁₂₋₂₀), biotinylation decreased moderately (Lane 5). No biotinylation was detectable when K13 was substituted with alanine (peptide K13A,T16S₁₂₋₂₀; Lane 6). When both lysine residues in peptide T16S₁₂₋₂₀ were substituted with alanine (peptide K13,15A,T16S₁₂₋₂₀), no biotinylation was detectable (Lane 7).

Lysines in the C-terminus of histone H2A were targeted for biotinylation by biotinidase. The C-terminus of histone H2A contains three lysine residues in positions 125, 127 and 129. A synthetic peptide including all three of these lysines (C₁₂₂₋₁₂₉) was a good substrate for biotinylation by biotinidase (Fig. 6, Lane 1). Biotinylation decreased only moderately when K125 and K127 were substituted with alanine residues (peptide K125,127A₁₂₂₋₁₂₉; Lane 2), suggesting that K129 is a good target for biotinylation. Consistent with this hypothesis, substitution of K125 and K129 (peptide K125,129A₁₂₂₋₁₂₉) and K127 and

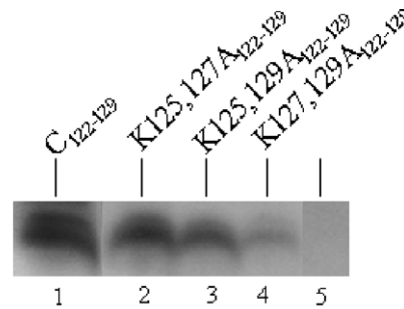


Q6T₁₋₉ = SGRGKTGGK
 Q6T,K9A₁₋₉ = SGRGKTGGA
 K5A,Q6T₁₋₉ = SGRGATGGK
 T16S₁₂₋₂₀ = AKAKSRSSR
 K15A,T16S₁₂₋₂₀ = AKAASRSSR
 K13A,T16S₁₂₋₂₀ = AAAKSRSSR
 K13,15A,T16S₁₂₋₂₀ = AAAASRSSR

Fig. 5. K9 and K13 in histone H2AX are targets for biotinylation by biotinidase. Synthetic peptides based on histones H2A and H2AX were incubated with biotinidase and biocytin for enzymatic biotinylation. Peptides were resolved by gel electrophoresis and peptide-bound biotin was probed using streptavidin peroxidase.

K129 (peptide K127,129A₁₂₂₋₁₂₉) with alanine residues caused a considerable decrease of biotinylation (Lanes 3 and 4, respectively). When all lysine residues in peptide C₁₂₂₋₁₂₉ were substituted with alanine residues (peptide K125,127,129A₁₂₂₋₁₂₉), no biotinylation was detectable (Lane 5).

The C-terminus of histone H2AX was not a good target for biotinylation. Note that N-terminal sequences are



C₁₂₂₋₁₂₉ = SHHKAKGK
 K125,127A₁₂₂₋₁₂₉ = SHHAAAGK
 K125,129A₁₂₂₋₁₂₉ = SHHAAKGA
 K127,129A₁₂₂₋₁₂₉ = SHHAAAGA
 K125,127,129A₁₂₂₋₁₂₉ = SHHAAAGA

Fig. 6. K125, K127 and K129 in the C-terminus of histone H2A are targets for biotinylation by biotinidase. Synthetic peptides based on histone H2A were incubated with biotinidase and biocytin for enzymatic biotinylation. Peptides were resolved by gel electrophoresis and peptide-bound biotin was probed using streptavidin peroxidase.

highly conserved between histones H2A and H2AX but that the C-terminal sequences of these two histones are unique [19]. In this study, we synthesized the following three peptides based on the C-terminus of histone H2AX: C_{113–121}=AVLLPKKTS; C_{122–131}=ATVGPKAPSG; and C_{132–142}=GKKATQASQEY. These peptides were not biotinylated in response to incubation with biotinidase (data not shown).

3.2. Cross-talk among histone modifications

Previous studies suggested that acetylation and methylation of histone H4 affect subsequent biotinylation [16]. Of note, K5, K9, K13 and other lysine residues in human histone H2A are targets for acetylation [7]. We provide evidence that acetylation and methylation of histone H2A are likely to affect subsequent biotinylation. Peptide N_{1–9} (containing both K5 and K9) was heavily biotinylated in response to incubation with biotinidase and was used as a positive control (Fig. 7, Lane 1). Acetylation of K5 caused a moderate decrease in the biotinylation of K9 (Lane 2). A peptide containing acetylated K9 and free K5 was not a target for biotinylation (Lane 3). This is consistent with our observation that K9 but not K5 is a target for biotinylation (see above). Moreover, dimethylation of R3 did not cause a change in the biotinylation signal (data not shown) because the adjacent K5 is not a biotinylation target. Peptide N_{7–14} (containing both K9 and K13) was a good target for biotinylation (Lane 4). Again, acetylation of K9 decreased the biotinylation signal substantially (data not shown). Moreover, both dimethylation

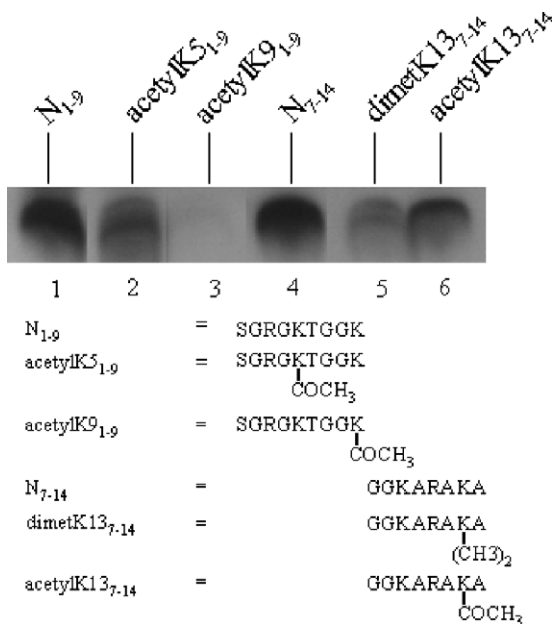


Fig. 7. Methylation and acetylation of amino acids in the N-terminus of histone H2A affect the subsequent biotinylation of adjacent lysine residues by biotinidase. Synthetic peptides based on histone H2A were incubated with biotinidase and biocytin for enzymatic biotinylation. Peptides were resolved by gel electrophoresis and peptide-bound biotin was probed using streptavidin peroxidase.

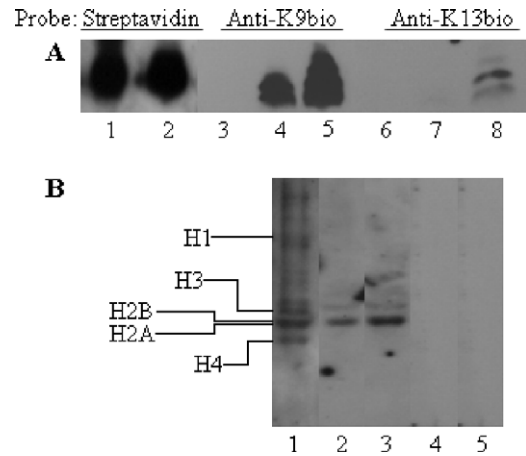


Fig. 8. Western blot analysis of biotinylated histones. (A) Synthetic peptides based on histone H2A were probed with streptavidin peroxidase or antibodies to histone H2A, biotinylated in position K9 or K13. Lanes 1, 4 and 7=peptide N_{1–12}bioK9 [SGRGKQGGK(biotin)ARAC]; Lanes 2, 5 and 8=peptide N_{10–24}bioK13 [ARAK(biotin)AKTRSSRAGLQC]; and Lanes 3 and 6=peptide N_{1–20} (SGRGKQGGKARAKAKTRSSR). (B) Jurkat cells contain biotinylated histone H2A. Nuclear histones were purified by acid extraction. Histones were resolved by gel electrophoresis and probed as follows: Lane 1, streptavidin peroxidase; Lane 2, antibody anti-K9bio; Lane 3, antibody anti-K13bio; Lane 4, preimmune serum for antibody anti-K9bio; and Lane 5, preimmune serum for antibody anti-K13bio.

and acetylation of K13 decreased the biotinylation of K9 (Lanes 5 and 6). On the other hand, dimethylation R11 considerably increased the enzymatic biotinylation of K9 or K13 (or both) by biotinidase (data not shown). Peptide N_{12–20} (containing both K13 and K15) was a poor target for biotinylation, but dimethylation of R17 substantially increased the biotinylation of K13 or K15 (or both) (data not shown). Increased biotinylation of lysine residues in response to biotinylation of adjacent arginine residues is consistent with previous studies [16].

3.3. Biotinylation of histone H2A in human cells

Human cells contain biotinylated histone H2A as judged by using novel biotinylation site-specific antibodies. In a first series of experiments, we raised antibodies to K9-biotinylated and K13-biotinylated histone H2A and validated the specificity of these antibodies by using synthetic peptides. We ran the same two peptides that were used for injections into rabbits (N_{1–12}bioK9 and N_{10–25}bioK13) on a polyacrylamide gel and probed the biotin tag by using streptavidin peroxidase. The two peptides produced a similar signal (Fig. 8A, compare Lanes 1 and 2), suggesting that biotinylation of peptides and loading of peptides on gels were similar. Second, the two peptides and a nonbiotinylated control (N_{1–20}) were probed with antibodies to K9-biotinylated and K13-biotinylated histone H2A. Anti-K9bio antibody did not bind to peptide N_{1–20} (Lane 3) but cross-reacted with both biotinylated peptides N_{10–25}bioK9 and N_{10–25}bioK13 (Lanes 4 and 5); preimmune serum did not produce a detectable signal (data

not shown). In contrast, anti-K13bio antibody did not bind to N_{1–20} and N_{10–25}bioK9 (Lanes 6 and 7) but was specific for peptide N_{10–25}bioK13 containing biotinylated K13 (Lane 8); preimmune serum did not produce a detectable signal (data not shown). Moreover, antibodies to biotinylated histone H2A did not cross-react with biotinylated peptides based on histone H4: N_{6–15}bioK8=GGK(biotin)GLGKGGA and N_{6–15}bioK12=GGKGLGK(biotin)GGA (data not shown). Collectively, these data suggest that both anti-K9bio and anti-K13bio are specific for biotinylated histone H2A peptides and are unlikely to cross-react with other biotinylated histones (see below). These data also suggest that antibody anti-K13bio is biotinylation site specific, whereas antibody anti-K9bio cross-reacts with both biotinylated K9 and K13.

Next, histone extracts from Jurkat cell nuclei were probed with antibodies to biotinylated histone H2A. The histone extracts contained biotinylated histones H1, H2A, H2B, H3 and H4 as judged by staining with streptavidin peroxidase (Fig. 8B, Lane 1). The polyclonal antibodies raised in this study were specific for histone H2A and did

not cross-react with other classes of histones (Lanes 2 and 3). When biotinylated histones were probed with preimmune serum, no detectable signal was produced (Lanes 4 and 5).

Biotinylated histone H2A localized to the nucleus in Jar choriocarcinoma cells as judged by confocal microscopy and antibodies against biotinylated histone H2A. First, the subcellular localization of K9-biotinylated histone H2A was visualized using antibody anti-K9bio (Fig. 9A). Nuclear and cytoplasmic compartments were stained with DAPI and rhodamine phalloidin, respectively (Fig. 9A). Merged images are consistent with nuclear localization of K9-biotinylated histone H2A. Preimmune serum did not generate a detectable signal. Analogous experiments were conducted for K13-biotinylated histone H2A. Antibody anti-K13bio localized primarily to the cell nucleus (Fig. 9B).

Both biotinidase and HCS showed considerable nuclear localization in Jar cells. First, we validated the specificity of antibodies against biotinidase and HCS using synthetic peptides as described for histone antibodies (data not

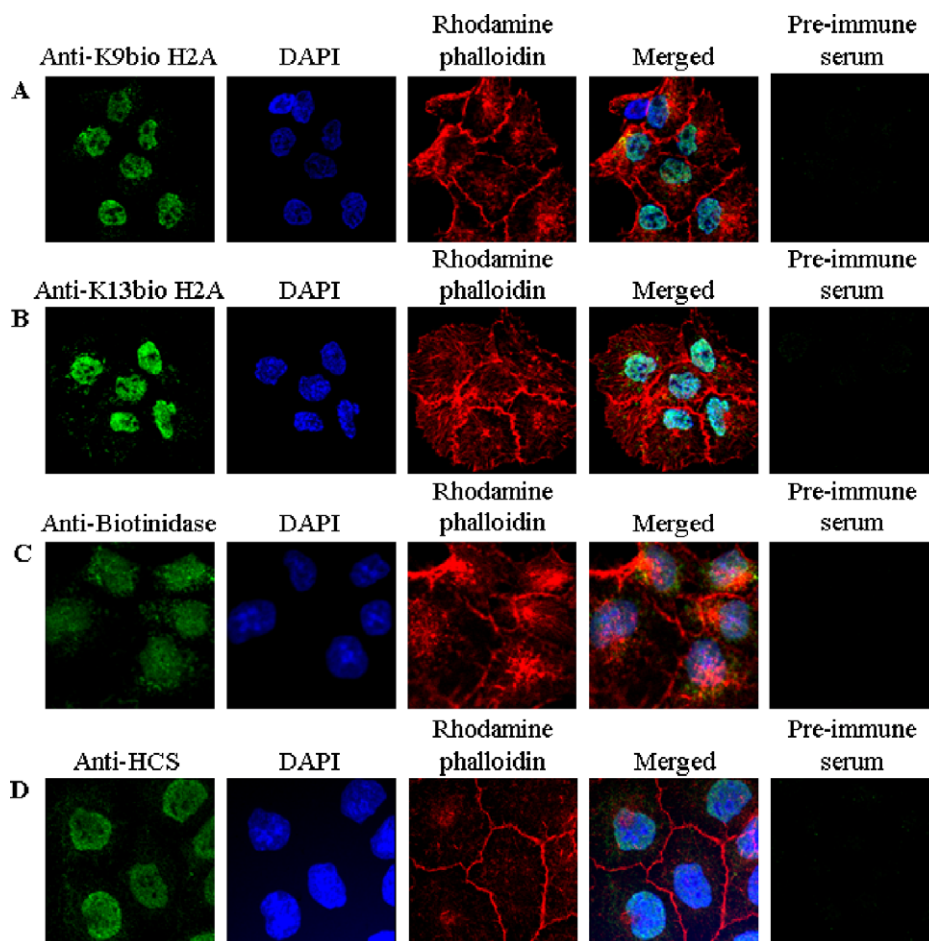


Fig. 9. Biotinylated histone H2A, biotinidase and HCS localize primarily to the nucleus in Jar choriocarcinoma cells. Cells were stained with antibodies to K9-biotinylated histone H2A (A), K13-biotinylated histone H2A (B), biotinidase (C) and HCS (D). The nuclear and cytoplasmic compartments were stained using DAPI and rhodamine phalloidin, respectively. Images entitled *Merged* were created by overlaying images obtained by staining with antibody, DAPI and rhodamine phalloidin. Preimmune sera were used as negative controls.

shown). The subcellular localization of biotinidase in JAR cells was visualized using confocal microscopy and anti-biotinidase (Fig. 9C). Nuclear and cytoplasmic compartments were stained with DAPI and rhodamine phalloidin, respectively (Fig. 9C). Merged images were consistent with nuclear localization of biotinidase. Preimmune serum did not generate a detectable signal (Fig. 9C). Analogous experiments were conducted for HCS. Anti-HCS also localized to the cell nucleus; preimmune serum (negative control) did not generate a detectable signal (Fig. 9D).

4. Discussion

This study provides evidence that (1) K9 and K13 in the N-terminus of histones H2A and H2AX are targets for biotinylation by biotinidase; (2) K125, K127 and K129 in the C-terminus of histone H2A are targets for biotinylation by biotinidase; (3) K9-biotinylated and K13-biotinylated histone H2A reside in human cell nuclei; (4) acetylation and dimethylation of lysine residues in histones decrease subsequent biotinylation of adjacent lysine residues; (5) dimethylation of arginine residues increases subsequent biotinylation of adjacent lysine residues; and (6) both HCS and biotinidase reside primarily in the nuclear compartment.

The findings presented here are likely to generate novel insights into biologic functions of histone biotinylation. Ongoing studies in our laboratory have provided evidence that biotinylation of distinct lysine residues in various classes of histones might play unique roles in the cellular response to double-strand breaks of DNA, chromatin condensation, telomere structure and nucleoli structure (unpublished observations). Specifically, we propose that biotinylation of histone H2A might participate in the following processes: First, biotinylation of histone H2A targets K9, which is also a target for acetylation; these two modifications are mutually exclusive. It has been proposed that acetylation of K9 in histone H2A might be associated with transcriptionally active chromatin [28]. We speculate that biotinylation of K9 decreases the transcriptional activity of chromatin, mediated by decreased acetylation of K9. Second, phosphorylation of histone H2AX is known to participate in DNA repair events, mediated by accumulation at sites of DNA damage [11]. Biotinylation of histones is known to change in response to DNA damage [17,18], but it remains to be determined whether biotinylation of K9 and K13 in histone H2A plays a role in repair events. Third, biotinylation of lysines in the C-terminus of histone H2A might affect histone–histone interactions in nucleosomes based on the following lines of reasoning. Histone H2A is unique among core histones in having its C-terminal tail exposed at the nucleosomal surface [1,8]. However, the larger part of the C-terminal domain of histone H2A and other histones is buried inside the nucleosomes [1]. The C-terminal histone fold domain is predominantly α -helical with a long central helix bordered on each side by a loop segment (β -bridge, hinge region) and a shorter helix [1].

The long helix acts as a dimerization interface between histones [1]. We speculate that the biotinylation of C-terminal lysine residues in histone H2A affects the dimerization of histones. Note that K125 and K127 are also targets for methylation [7]. The effects of lysine methylation in the C-terminus of histone H2A are uncertain, but interactions between biotinylation and methylation are likely to occur *in vivo*.

We shall point out the following uncertainty of the studies presented here. Whereas the existence of K9-biotinylated and that of K13-biotinylated histone H2A have been confirmed by using antibodies, no such antibodies have yet been raised against histone H2A biotinylation in the C-terminus. Hence, while this study suggests that K125, K127 and K129 are targets for biotinidase, the existence of these modifications *in vivo* awaits formal confirmation.

Previous studies suggested that dimethylation of arginine residues in histone H4 increases biotinylation of adjacent lysine residues [16]. We observed a similar pattern for histone H2A: dimethylation of R11 increased the biotinylation of K9 or K13 (or both) by biotinidase and dimethylation of R17 increased the biotinylation of K13 or K15 (or both). Note that arginine residues in histones can be converted to citrulline and ornithine by deimination [29,30]. Citrulline and ornithine residues are good targets for biotinylation by biotinidase (Y.C. Chew and J. Zemleni, unpublished observation). Collectively, posttranslational modifications of arginine residues are likely to play important roles in histone biotinylation.

Finally, this study provides evidence that significant fractions of cellular biotinidase and HCS localize to the nuclear compartment, consistent with a role of these enzymes in chromatin structure. Previous studies are consistent with a nuclear localization of HCS [14]. These previous studies suggested that most HCSs localized to the nuclear periphery rather than to the nucleoplasm. The functional significance of this observation is currently being investigated. The amino acid residues in histones that are targets for biotinylation by HCS await identification, whereas targets for biotinidase have been characterized [15,16]. Unlike that for HCS, the cellular distribution of biotinidase is controversial. This study and a previous study [31] are consistent with nuclear localization of biotinidase; in contrast, Stanley et al. [32] suggested that biotinidase localizes to the cytoplasm but not to the nucleus. The reasons for these apparently conflicting observations are unknown.

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