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K8 and K12 are biotinylated in human histone H4

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Folding of DNA into chromatin is mediated by binding to histones such as H4; association of DNA with histones is regulated by covalent histone modifications, e.g. acetylation, methylation, and biotinylation. We sought to identify amino-acid residues that are biotinylated in histone H4, and to determine whether acetylation and methylation of histones affect biotinylation. Synthetic peptides spanning fragments of human histone H4 were biotinylated enzymatically using biotinidase. Peptide-bound biotin was probed with streptavidin–peroxidase. Peptides based on the N-terminal sequence of histone H4 were effectively recognized by biotinidase as substrates for biotinylation; in contrast, peptides based on the C-terminal sequences were not biotinylated. Substitution of K8 or K12 with alanine or arginine decreased biotinylation, suggesting that these lysines are targets for biotinylation; K8 and K12 are also known targets for acetylation. Chemical acetylation or methylation of a given lysine decreased subsequent enzymatic biotinylation of neighboring lysines, consistent with cross-talk among histone modifications. Substitution of a given lysine (positive charge) with glutamate (negative charge) abolished biotinylation of neighboring lysines, providing evidence that the net charge of histones has a role in biotinylation. An antibody was generated that specifically recognized histone H4 biotinylated at K12. This antibody was used to detect biotinylated histone H4 in nuclear extracts from human cells. These studies suggest that K8 and K12 in histone H4 are targets for biotinylation, that acetylation and biotinylation compete for the same binding sites, and that acetylation and methylation of histones affect biotinylation of neighboring lysines.

Keywords: acetylation; biotin; histone H4; lysine; methylation.

Histones are small proteins (11–22 kDa) which mediate the folding of DNA into chromatin. The following five major classes of histones have been identified in humans: H1, H2A, H2B, H3, and H4 [1]. Histones contain a large number of lysine and arginine residues, resulting in a net positive charge of these proteins. In mammals, DNA is wrapped around an octamer of core histones, consisting of one H3–H3–H4–H4 tetramer and two H2A–H2B dimers, to form the nucleosomal core particle. This complex is stabilized by electrostatic interactions between the negatively charged phosphate groups in DNA and the positively charged ε-amino groups (lysine residues) and guanidino groups (arginine residues) in histones. Histone H1 associates with the DNA between two nucleosomal core particles to complete nucleosomal assembly. In chromatin, nucleosomes are arranged like beads on a string; these repetitive sequences are folded into structures of higher order.

Histones consist of a globular C-terminal domain and a flexible N-terminal tail [1]. The N-terminus of histones protrudes from the nucleosomal surface; lysines, arginines, and serines in the N-terminus are targets for covalent modifications such as acetylation, methylation, phosphorylation, ubiquitination, poly(ADP-ribosylation) [1–3], and most recently by sumoylation [4]. These modifications affect the binding of DNA to histones and, thus, play important parts in maintaining chromatin structure. For example, covalent modifications of histones regulate transcriptional activity of DNA [5], DNA repair [6,7], cell proliferation [1], and spermatogenesis [1].

Evidence has been provided for a novel modification of histones: covalent binding of the vitamin biotin [8,9]. Binding of biotin is catalyzed by biotinidase (EC 3.5.1.12) using biocytin (biotin-ε-lysine) as a substrate [8]. Recently, evidence has been provided that holocarboxylase synthetase may also catalyze biotinylation of histones [10]. Likely, all five classes of histones are biotinylated in human cells [9]. Biotinylation of histones may have a role in cell proliferation [9], gene silencing [11], and cellular response to DNA damage [11]. The amino-acid residues that are targets for biotinylation have not yet been identified.

The different post-translational modifications of histones can influence each other in synergistic or antagonistic ways, thereby mediating gene regulation. For example, phosphorylation of S10 inhibits methylation of K9 in histone H3, but is coupled with K9 and/or K14 acetylation during mitogenic stimulation in mammalian cells [5]. Conversely, deacetylation of K14 in histone H3 facilitates subsequent methylation of K9, leading to transcriptional silencing. Ultimately, modifications of histones affect the access of enzymes such as RNA polymerases and DNA repair enzymes to DNA. Identification of biotinylation sites in histones is the first step in deciphering the cross-talk between

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biotinylation and other covalent modification of histones that regulate gene expression.

In this study, we chose to work with histone H4 over other histones because (a) H4 plays a central role in organizing the DNA–histone complex, (b) post-translational modifications of H4 appear to be essential for cell cycle progression, and (c) the amino-acid sequence of H4 is highly conserved among species [1]. Using synthetic peptides, we sought to identify residues that are biotinylated in histone H4 and to determine whether acetylation and methylation of histone H4 cross-talk with biotinylation.

Materials and methods

Peptide synthesis

Previous studies have suggested that lysine residues in histone H4 are likely targets for biotinylation [12]. Here, synthetic peptides spanning fragments of human histone H4 (GenBank accession number NM_175054) were used to identify lysines that are targets for biotinylation. Peptides were synthesized using Fmoc chemistry by a standard solid-phase method [13]. One-letter annotation is used to denote amino acids throughout this paper [14]. All solvents were purchased from EM Science (Gibbstown, NJ, USA) unless noted otherwise. L-isomers of Fmoc-amino acids (25 mg per coupling; Ana Spec Inc, San Jose, CA, USA) were used for peptide synthesis unless noted otherwise. Chemically modified peptides were synthesized by using biotinylated, acetylated, dimethylated, or formylated ε-NH_2 derivatives of Fmoc-lysine. For pilot studies, the following two peptides were synthesized using a Pioneer peptide synthesizer (ABI Inc, Foster City, CA, USA) using manufacturer recommended protocols: (a) N-terminus of histone H4, spanning amino acids 1 through 19 (SGRGKGGKGLGKGGAKRHR); the N-terminus contains lysines in position 5, 8, 12 and 16; (b) C-terminus of histone H4, spanning amino acids 82 through 102 (TAMDVVYALKRQGRTLYGFGG). For peptide analogs, a base peptide with the sequence Fmoc-GGABBRC-amide was assembled on PAL resin (ABI Inc; B = β-alanine) using a Pioneer peptide synthesizer. Aliquots of $\approx$ 25 mg of the base resin ($\approx$ 20 µmol peptide) were used to manually synthesize the different H4 peptide analogs using established procedures [15,16]. Most studies described below focused on the N-terminus in histone H4 because: (a) pilot studies suggested that the N-terminus of histone H4 is a good target for biotinylation whereas the C-terminus is not (see below); (b) lysine residues in the N-termini of histones are likely targets for biotinylation [12]; (c) K8 and K12 in histone H4 are less likely to be occupied by acetylation than K16 [17], which is consistent with the availability of K8 and K12 for biotinylation. Thus, most of our studies were based on the use of the following H4 fragment and variations thereof: GGK(8)GLGK(12)GGA ('K' denotes lysines in positions 8 and 12, respectively, in the H4 molecule). Modifications were introduced in positions 8 and 12 during peptide synthesis (Table 1).

Table 1. Synthetic peptides based on histone H4. One-letter amino acid code and abbreviations: A, L-alanine; Ac-G, acetyl-α-NH_2-L-glycine; Ac-K, acetyl-ε-NH_2-L-lysine; bio-K, biotin-ε-NH_2-L-lysine; D-K, D-lysine; Dme-K, dimethyl-ε-NH_2-L-lysine; E, L-glutamate; For-K, formyl-ε-NH_2-L-lysine; G, L-glycine; K, L-Lysine; L, L-leucine; Q, L-glutamine; R, L-arginine. Deviations from the native sequence (peptide 1) are in bold. Position numbers refer to the positions of amino acids in human histone H4 (GenBank accession number NM_175054) after removal of the N-terminal methionine.

	Position									
Peptide	6	7	8	9	10	11	12	13	14	15
1	Ac-G	G	K	G	L	G	K	G	G	A
2	Ac-G	G	**A**	G	L	G	K	G	G	A
3	Ac-G	G	K	G	L	G	**A**	G	G	A
4	Ac-G	G	**A**	G	L	G	**A**	G	G	A
5	Ac-G	G	**Ac-K**	G	L	G	K	G	G	A
6	Ac-G	G	K	G	L	G	**Ac-K**	G	G	A
7	Ac-G	G	**Dme-K**	G	L	G	K	G	G	A
8	Ac-G	G	K	G	L	G	**Dme-K**	G	G	A
9	Ac-G	G	K	G	L	G	**For-K**	G	G	A
10	Ac-G	G	**Bio-K**	G	L	G	K	G	G	A
11	Ac-G	G	K	G	L	G	**Bio-K**	G	G	A
12	Ac-G	G	**R**	G	L	G	K	G	G	A
13	Ac-G	G	K	G	L	G	**R**	G	G	A
14	Ac-G	G	**R**	G	L	G	**R**	G	G	A
15	Ac-G	G	**E**	G	L	G	K	G	G	A
16	Ac-G	G	K	G	L	G	**E**	G	G	A
17	Ac-G	G	**Q**	G	L	G	K	G	G	A
18	Ac-G	G	K	G	L	G	**Q**	G	G	A
19	Ac-G	G	**Q**	G	L	G	**Q**	G	G	A
20	Ac-G	G	K	G	L	G	**D-K**	G	G	A

Peptide quantification

Lyophilized peptides were dissolved in 2 mL distilled water, and quantified on the basis of their cysteine residue using Ellman's reagent [18]. Briefly, aliquots (20 µL) of peptide solutions were mixed with 178 µL 1.0 M Tris/HCl (pH 8.2) containing 0.02 M EDTA, and with 2 µL 0.01 M 5,5′-dithiobis-2-nitrobenzoic acid in methanol. Cysteine standard curves (0–1.14 M) were used for calibration. Samples were incubated for 10 min at room temperature, and absorbance was measured at 405 nm. Equal amounts of peptides were used in subsequent biotinylation experiments.

Enzymatic biotinylation of peptides

It has been proposed that the following catalytic sequence leads to biotinylation in histones [8,19]. First, biocytin is cleaved by biotinidase to form an intermediate, cysteine-bound biotin. Second, the biotinyl moiety from the cysteine residue is transferred to the ε-amino group of lysines in histones. In this study, synthetic peptides were biotinylated enzymatically using human plasma (as source of biotinidase) and biocytin (as source of biotin) as described previously [8]. Peptide concentrations in stock solutions were adjusted to 50 $mg \cdot L^{-1}$; 20 µL peptide solution was mixed with 1.88 mL 50 mM Tris/HCl (pH 8.0), 40 µL 0.75 mM biocytin, and 60 µL human plasma. Samples were incubated at 37 °C for 45 min and stored at −70 °C unless stated otherwise.

Gel electrophoresis

After enzymatic biotinylation, peptides were electrophoresed using tricine/16% polyacrylamide gels according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Peptides were electroblotted on to poly(vinylidene difluoride) membranes (Millipore, Bedford, MA, USA), which were blocked with 50 mL 30 $g \cdot L^{-1}$ BSA. Peptide-bound biotin was probed with streptavidin–peroxidase [9].

HPLC analysis

Peptides were chromatographed by HPLC (Shimadzu, Columbia, MD, USA) to (a) determine purity of synthetic peptides, (b) prepare samples for analysis by MS, and (c) confirm enzymatic biotinylation of peptides. Synthetic peptides were subjected to HPLC (0.46 × 25 cm C_{18} column) using a binary gradient system (buffer A = 1 mL trifluoroacetic acid/1 L water; buffer B = 1 mL trifluoroacetic acid/0.9 L acetonitrile/0.1 L water: 85% A and 15% B for 2 min; linear increase to 100% buffer B over 12 min; 100% buffer B for 3 min; linear decrease to 15% buffer B over 3 min; 85% buffer A and 15% buffer B for 5 min). Flow rate was 1.0 $mL \cdot min^{-1}$. Peptides in the eluate were monitored at 220 nm, using a diode array detector (SPD-M10Avp; Shimadzu).

Mass spectrometry

Purified peptides were analyzed by MALDI-TOF and quadrupole-time of flight MS at the University of Nebraska-Lincoln MS facility.

Polyclonal antibody

A polyclonal antibody to human H4 (biotinylated at K12) was generated using a commercial facility (Cocalico Biologicals, Reamstown, PA, USA). This antibody was used to detect biotinylated histone H4 in human cells. Briefly, a conjugate of a synthetic peptide, biotinylated at K12 (Table 1, peptide 11), and keyhole limpet hemocyanin, was injected into New Zealand White rabbits. Booster injections were given after 14, 21, and 49 days. Serum was collected before immunization (preimmune serum) and 2 days after each booster injection. Serum collected after the second and third booster injection were used for assays described below; preimmune serum (control) was used as a control. First, we determined whether the antibody was specific for biotinylation sites. Electroblots of synthetic peptides (biotinylated at either K8 or K12) were probed with the antibody to histone H4 (biotinylated at K12) and a polyclonal goat anti-(rabbit IgG)–peroxidase conjugate [20]. Secondly, we determined whether human cells contain histone H4, biotinylated at K12. Nuclear histones were extracted from human lymphoid (Jurkat) cells [11] using HCl [9]. Extracts were electrophoresed using 18% Tris/glycine/polyacrylamide gels (Invitrogen) as described [9]. Biotinylated histone H4 was probed using the antibody to histone H4 (biotinylated at K12) using standard procedures [20]. As specificity controls, nuclear histone extracts (30 µg) from Jurkat cells were probed with antibody to histone H4 (biotinylated at K12) in the presence of excess peptides (up to 180 µg), biotinylated at K8 or K12 (competition studies).

In analogous experiments, peptide 10 (Table 1) was used to generate a polyclonal antibody to histone H4 biotinylated at K8. This antibody lacked specificity and cross-reacted with histone H4 biotinylated at K12 (data not shown). Thus, the K8 antibody was excluded from experiments described below.

Biotin-free controls for Western blot analysis were prepared as follows: 0.1 mL histone extract (≈ 0.5 mg histones) was incubated with 0.05 mL avidin beads (Pierce, Rockford, IL, USA) at 4 °C for 1 h. The supernatant did not contain detectable quantities of biotinylated histones, as judged by probing with streptavidin–peroxidase [9]; treatment with avidin beads decreased the amount of total histones in the extract by ≈ 50%, as judged by staining with Coomassie blue [9].

Results

Biotinylation sites in histone H4

First, we determined whether the N-terminus or the C-terminus of histone H4 is a better substrate for biotinylation by biotinidase. Peptides spanning the N-terminal 19 amino acids (SGRGKGGKGLGKGGAKRHR) and the C-terminal 21 amino acids (TAMDVVYALKRQGRT LYGFGG) of histone H4 were incubated with biotinidase and biocytin for enzymatic biotinylation. The N-terminal peptide was biotinylated by biotinidase, whereas the C-terminal peptide was not biotinylated; controls incubated without biocytin and biotinidase did not produce a detectable signal (Fig. 1A). This is consistent with the hypothesis that the N-terminal tail of histone H4 contains a biotinylation motif that is not present in the C-terminal domain. Subsequent studies focused on peptides derived from the N-terminus of histone H4.

A time course was conducted to determine when biotinylation of peptides reaches maximal levels. The N-terminal peptide (SGRGKGGKGLGKGGAKRHR) was incubated with plasma and biocytin for 0 (control), 2, 4, 8, 12, 16, 20, 30, 40, 50, and 60 min. Abundance of biotinylated peptide reached a plateau 20–60 min after the start of the reaction (Fig. 1B). All subsequent enzymatic biotinylations were conducted for 45 min.

For reasons described above, we focused on K8 and K12 in histone H4 when investigating biotinylation sites. The following peptide spans amino acids 6 through 15 in histone H4, and was used as a native control: GGKGLGKGGA (Table 1, peptide 1). This peptide was efficiently biotinylated by biotinidase, suggesting that lysines in position 8 or 12 (or both) are targets for biotinylation (Fig. 1C, peptide 1, 'K/K'). If one of the lysines in position 8 or 12 was replaced by an alanine (Table 1, peptides 2 and 3, respectively), the covalent binding of biotin decreased substantially (Fig. 1C; 'A/K' and 'K/A'); deletion of K8 had a greater effect than deletion of K12. When both lysines were replaced by alanines (Table 1, peptide 4), the synthetic peptide did not undergo biotinylation (Fig. 1C; 'A/A'). Collectively, these data suggest that both K8 and K12 are targets for

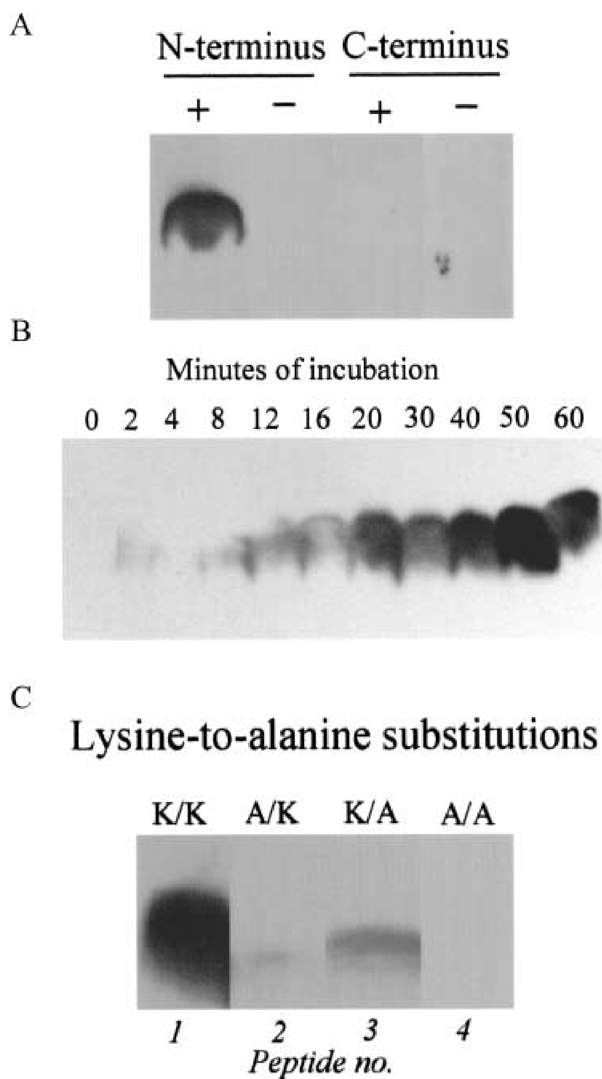

Fig. 1. K8 and K12 in histone H4 are targets for enzymatic biotinylation. All panels: peptide-bound biotin was probed using streptavidin–peroxidase. (A) N-terminal (SGRGKGGKGLGKGGAKRHR) and C-terminal (TAMDVVYALKRQGRTLYGFGG) peptides from histone H4 were biotinylated enzymatically using biotinidase and biocytin (' + '); controls were incubated without biotinidase and biocytin ('–'). (B) Time course of enzymatic biotinylation of the N-terminal peptide derived from histone H4. The peptide was incubated with biotinidase and biocytin at 37 °C; aliquots were collected at timed intervals. (C) K to A substitutions in peptides affect their enzymatic biotinylation. 'K/K' (Table 1, peptide 1) = native peptide (amino acids 6–15 in histone H4) containing K8 and K12; 'A/K' (Table 1, peptide 2) = K8A; 'K/A' (Table 1, peptide 3) = K12A; 'A/A' (Table 1, peptide 4) = K8,12A.

biotinylation, and that K8 seems to be a better target for biotinylation by biotinidase than K12.

Effects of amino-acid modifications in positions 8 and 12

Biotinylation of K8 and K12 decreased when neighboring lysine residues were covalently modified by acetylation, formylation, or dimethylation. If K8 was acetylated (Table 1, peptide 5), biotinylation was barely detectable (Fig. 2A, lane b) compared with the native peptide (Fig. 2A, lane a). Likewise, acetylation of K12 (Table 1, peptide 6) decreased biotinylation of the peptide (Fig. 2A, lane c). If one of the lysines in position 8 or 12 was dimethylated (Table 1, peptides 7 and 8, respectively), covalent modification by biotin decreased substantially (Fig. 2A, lanes d and e). When K12 was replaced with formyl-lysine (Table 1, peptide 9), biotinylation of K8 decreased compared with the native peptide (Fig. 2A, lane f). Lanes g and h in Fig. 2A depict synthetic peptides that were chemically biotinylated in position 8 or 12 (Table 1, peptides 10 and 11, respectively).

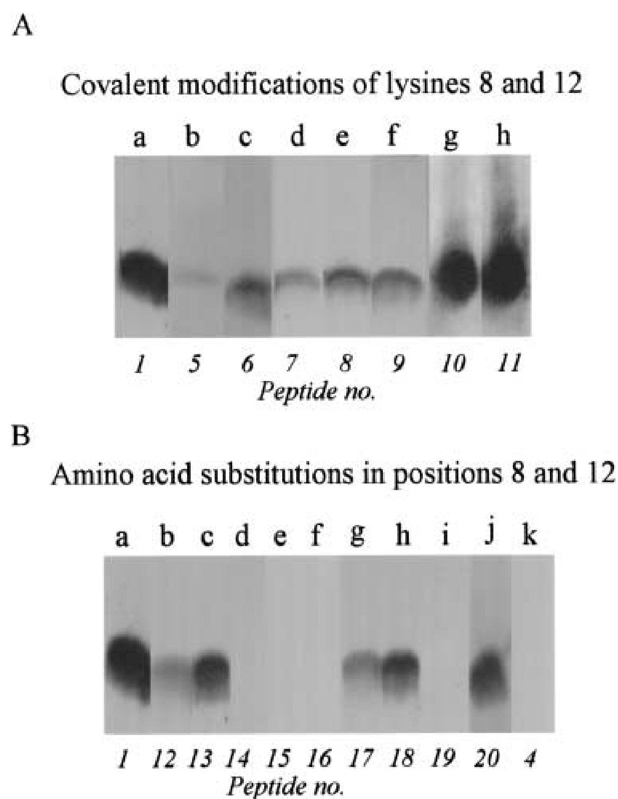

Fig. 2. Amino-acid modifications affect the biotinylation of histone H4. Synthetic peptides were incubated with biotinidase and biocytin for enzymatic biotinylation. Peptide-bound biotin was probed with streptavidin–peroxidase. Equimolar amounts of peptides were used except for the chemically biotinylated peptides, which were diluted 100-fold. (A) Lane a, native peptide; lane b, peptide acetylated at K8; lane c, peptide acetylated at K12; lane d, peptide dimethylated at K8; lane e, peptide dimethylated at K12; lane f, peptide formylated at K12; lane g, peptide biotinylated chemically at K8; lane h, peptide biotinylated chemically at K12. (B) Lane a, native peptide; lane b, K8R; lane c, K12R; lane d, K8,12R; lane e, K8E; lane f, K12E; lane g, K8Q; lane h, K12Q; lane i, K8,12Q; lane j, L-K12 was substituted with D-lysine; lane k, K8,12A.

Previous studies provided preliminary evidence that guanidino groups in arginine residues are not good targets for biotinylation [12]. This was confirmed in the present study: if K8 was replaced with arginine (Table 1, peptide 12), the efficiency of enzymatic biotinylation decreased substantially (Fig. 2B, compare lanes a and b). Similarly,

if K12 was replaced with arginine (Table 1, peptide 13), efficiency of biotinylation decreased moderately (Fig. 2B, compare lanes a and c). Finally, if both K8 and K12 were replaced with arginines (Table 1, peptide 14), biotinylation was not detected (Fig. 2B, lane d).

Covalent modifications of histones can change the net charge of the molecule, e.g. phosphorylation and poly(ADP-ribosylation) introduce negative charges and subsequently influence other post-translational modifications of nearby residues [1,5,21]. Theoretically, localized changes in charge could affect biotinylation of histones. To verify this scenario, lysine residues were substituted with glutamates to introduce negative charges into synthetic peptides. If K8 (Table 1, peptide 15) or K12 (Table 1, peptide 16) was replaced with glutamate, enzymatic biotinylation was not detectable (Fig. 2B, lanes e and f, respectively). Next, we sought to formally exclude the possibility that effects of glutamate were caused by steric hindrance rather than by charge effects. Glutamine is of similar size to glutamate but does not carry a net charge. Thus, K8 or K12 was replaced with glutamine (Table 1, peptide 17 and 18, respectively). Enzymatic biotinylation of glutamine-substituted peptides decreased compared with the native peptide (Fig. 2A, compare lanes g and h with lane a), but the effects of glutamine substitution were smaller than the effects of glutamate substitution. If both K8 and K12 were replaced with glutamine (negative control), no enzymatic biotinylation was detectable (Fig. 2B, lane i; Table 1, peptide 19). These results suggest that charge interactions between histones and biotinidase are important for enzymatic biotinylation.

Biotinylation of lysine residues in histones is not stereospecific. If L-lysine in position 12 was replaced with D-lysine, enzymatic biotinylation decreased only moderately (Fig. 2B, lane j) compared with the native peptide (Fig. 2B, lane a). Lane k in Fig. 2B depicts a peptide in which both lysines were replaced with alanine (negative control).

Identification of biotinylated peptides by HPLC/MS

Analysis of peptides incubated with biotinidase and biocytin by HPLC/MS confirmed that biotinidase mediated covalent biotinylation. First, an HPLC method was developed to separate nonbiotinylated peptides from biotinylated peptides. Nonbiotinylated peptide derived from the N-terminus in histone H4 (e.g. Table 1, peptide 1) was eluted at $t =$ 6.0 min; peptides that were chemically biotinylated at either K8 (Table 1, peptide 10) or K12 (Table 1, peptide 11) were eluted at $t = 9.5$ min (Fig. 3). This is consistent with a decreased polarity of biotinylated peptides compared with nonbiotinylated controls. HPLC fractions eluted at 6 min (native peptide) and 9.5 min (biotinylated peptide) were analyzed by MS at the Nebraska Center for Mass Spectrometry, University of Nebraska-Lincoln. Molecules of the following masses were detected: 1243.4 Da for the native, nonbiotinylated peptide (expected mass = 1242.6 Da) and 1469.8 Da for the chemically biotinylated peptides (expected mass = 1468.6 Da). These data confirmed the identities of the synthetic peptides.

Next, the native, nonbiotinylated peptide derived from the N-terminus in histone H4 (Table 1, peptide 1) was incubated with biocytin and biotinidase before separation by HPLC. The HPLC fraction eluted at 9.5 min was collected and subjected to MS as described above. A molecule with a mass of 1469.7 Da was detected, confirming enzymatic biotinylation of the peptide.

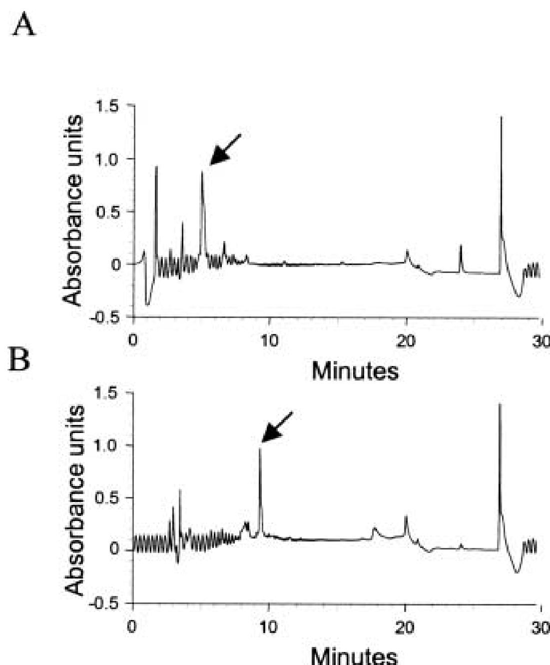

Fig. 3. Biotinylation of peptides causes a shift in retention times in HPLC. (A) Chromatogram of peptide 1 (as per Table 1), GGKGLGKGGA. (B) Chromatogram of peptide 11 (as per Table 1), GGKGLGK(bio)GGA (biotinylated at K12).

Polyclonal antibody

A polyclonal antibody was generated to determine whether histone H4 is biotinylated at K12 in human cell nuclei. First, we determined whether the antibody was specific for biotinylation sites. Transblots of biotinylated peptides 10 and 11 (Table 1) were probed with the newly synthesized antibody. The antibody bound to the peptide that was chemically biotinylated at K12, but did not bind to the peptide biotinylated at K8 (Fig. 4A, compare lanes a and b); preimmune serum did not produce a detectable signal (lanes c and d). The K8 and K12 peptides showed similar reactivities when biotin was probed with streptavidin–peroxidase (Fig. 4A, compare lanes e and f). These observations suggest that the two peptides contained biotin and that the antibody would specifically recognize histone H4, biotinylated at K12.

Next, nuclear extracts from Jurkat cells were probed with the antibody. The nuclear extract contained biotinylated histones H1, H2A, H2B, H3 and H4, as judged by staining with streptavidin–peroxidase (Fig. 4B, lane a). The polyclonal antibody bound to histone H4 but did not cross-react with other classes of histones (Fig. 4B, lane b). If biotinylated histones were removed by using avidin beads before electrophoresis, the antibody did not bind to the remaining nonbiotinylated histones (Fig. 4B, lane c). If biotinylated histones were probed with preimmune serum, no detectable signal was produced (Fig. 4B, lane d). In control experi-

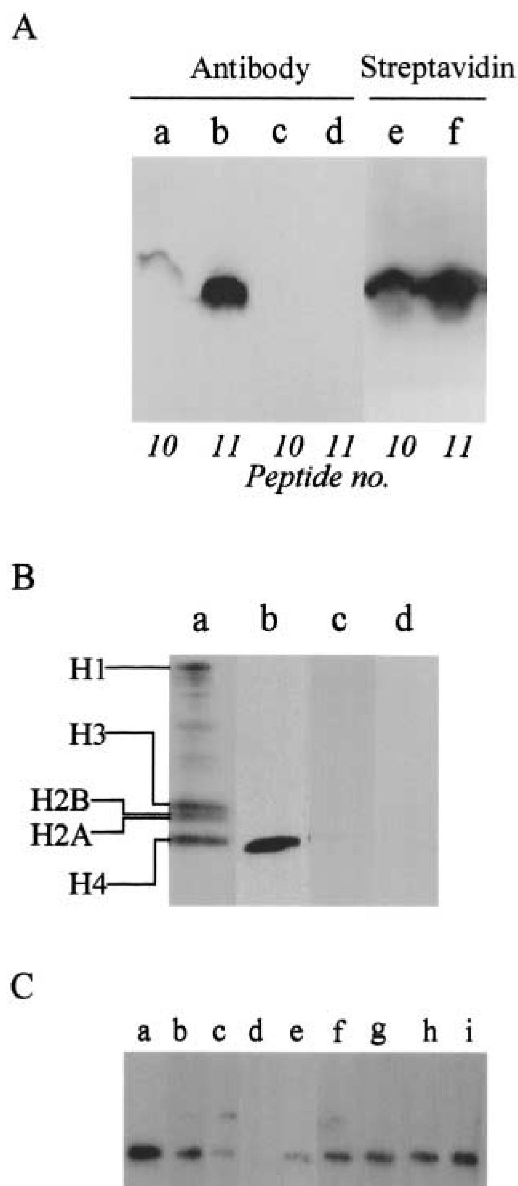

Fig. 4. Nuclear extracts from Jurkat cells contain histone H4, biotinylated at K12. (A) Synthetic peptides (GGKGLGKGGA) spanning amino acids 6 through 15 in histone H4 were biotinylated chemically either at K8 (lanes a, c, and e) or at K12 (lanes b, d, and f). Peptides were probed using an antibody to histone H4, biotinylated at K12 (lanes a and b), preimmune serum (lanes c and d), or streptavidin–peroxidase (lanes e and f). (B) Nuclear histones from Jurkat cells were analyzed as follows. Lane a, biotin in Jurkat cell histones was probed with streptavidin–peroxidase; lane b, biotin in Jurkat cell histones was probed with antibody to histone H4, biotinylated at K12; lane c, as in lane b, but biotinylated histones were removed by treatment with avidin beads before electrophoresis; lane d, as in lane b, but biotinylated histones were probed with preimmune serum. (C) Nuclear histones (30 μg) from Jurkat cells were resolved by gel electrophoresis; histone H4 was probed using the K12 antibody in the presence of excess synthetic peptides, biotinylated at K8 or K12: lane a, without competing peptide (control); lanes b–e, 4, 18, 70, and 180 μg, respectively, of K12-biotinylated peptide (Table 1, peptide 11); lanes f–i, 4, 18, 70, and 180 μg, respectively, of K8-biotinylated peptide (Table 1, peptide 10).

ments, the histone H4 band was excised from the gel for analysis by MS. The protein in the band was identical with histone H4; no other proteins were detected (data not shown).

Finally, competition experiments were conducted to provide additional evidence that the newly developed antibody to histone H4 is specific for biotinylation site K12. In these experiments, nuclear histones (30 μg) from Jurkat cells were resolved by gel electrophoresis; histone H4 was probed using the K12 antibody in the presence of excess synthetic peptides, biotinylated at K8 or K12. The peptide biotinylated at K12 showed dose-dependent competition with histone H4 for antibody binding (Fig. 4C, lanes a–e); in contrast, the peptide biotinylated at K8 did not compete with histone H4 for antibody binding (Fig. 4C, lanes a, f–i). Collectively, these findings suggest that (a) human cells contain histone H4, biotinylated at K12, (b) our antibody is specific for histone H4 (biotinylated at K12) and does not cross-react with other classes of histones, and (c) our antibody does not cross-react with nonbiotinylated histone H4.

Discussion

This study provides the first evidence that: (a) K8 and K12 in histone H4 are targets for biotinylation by biotinidase; (b) the C-terminal region of histone H4 is not a target for biotinylation; (c) arginine residues are not likely to be biotinylated; (d) charge interactions play an important part in biotinylation; (e) acetylation and dimethylation of histones decrease biotinylation of neighboring lysine residues.

Biotinylation of histones is probably physiologically meaningful. For example, peripheral blood mononuclear cells respond to proliferation with increased biotinylation of histones compared with quiescent cells [9]. Moreover, biotinylation of histones increases in response to DNA damage caused by UV light in human lymphoid cells [11]. Finally, biotinylated histones are enriched in transcriptionally silent chromatin [11]. These previous studies were limited to using streptavidin–peroxidase as a probe for biotin. The present study is an important first step in developing antibodies that are specific for biotinylation sites in a given class of histones. The availability of such antibodies will foster future studies of biological functions of biotinylated histones.

This study provides evidence that biotinylation occurs at the N-terminus of histone H4 rather than at the C-terminus. The N-terminus of histone H4 contains lysine residues in positions 5, 8, 12, and 16. These lysines are known to be targets for covalent acetylation also, mediating transcriptional activation of genes [22,23]. Of the four lysine residues in the N-terminus of histone H4, K16 is acetylated more abundantly than K12 and K5; the abundance of acetylated K8 is relatively small [17]. On the basis of the evidence provided here, some of the same lysines are also targets for biotinylation, namely K8 and K12. Preliminary studies suggested that K5 is also biotinylated (data not shown). Currently, no data are available on whether K16 is a target for biotinylation. Biotinylated histones are enriched in transcriptionally silent heterochromatin [11], whereas acetylated histones are enriched in transcriptionally active euchromatin [1]. Competition between biotin and acetate for the same binding sites is consistent with the mutually

exclusive effects of these modifiers on transcriptional activity of chromatin.

Modifications other than acetylation may also play a part in biotinylation regulation. This study provides evidence that methylation of histones may downregulate biotinylation. The *in vivo* relevance of this observation is uncertain, given that this study did not investigate classical methylation sites in histone H4. Finally, preliminary evidence suggests that phosphorylation of serine residues decreases biotinylation in histone H3 (K. Kobza, B. Rueckert, Y. C. Chew, G. Camporeale, G. Sarath, J. Zempleni, unpublished observation). We hope to provide a more elaborate analysis of the cross-talk among histone modifications in future studies.

Does enzymatic biotinylation of K8 and K12 also occur *in vivo*? Previous studies suggested that all five major classes of histones are biotinylated in human cells [9] and in chicken erythrocytes [11]. The value of these previous studies was limited by the fact that biotinylated histones were probed using streptavidin–peroxidase. This probe is neither specific for a given class of histones, nor is it specific for biotinylation sites within a class. The present study for the first time provides evidence that biotinylation of K12 in human histone H4 occurs *in vivo*. This conclusion is based on probing nuclear extracts from human lymphoid cells with a novel antibody against biotinylated histone H4.

Human cells maintain normal biotinylation of histones if the biotin concentration in culture medium is low [24]; under these conditions, biotinylation of carboxylases is barely detectable. It was proposed that biotin-deficient cells maintain normal biotinylation of histones by increasing the nuclear import of biotinidase [24]. Alternatively, nuclear accumulation of holocarboxylase synthetase [10] or slow turnover of biotinylated histones [25] may contribute to maintaining biotinylation of histones in biotin-deficient cells. Studies are in progress in our laboratory in which we knock down expression of the genes encoding biotinidase and holocarboxylase synthetase.

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