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Histones are modified post-translationally, e.g. by methylation of lysine and arginine residues, and by phosphorylation of serine residues. These modifications regulate processes such as gene expression, DNA repair, and mitosis and meiosis. Recently, evidence has been provided that histones are also modified by covalent binding of the vitamin biotin. The aims of this study were to identify biotinylation sites in histone H3, and to investigate the crosstalk among histone biotinylation, methylation and phosphorylation. Synthetic peptides based on the sequence of human histone H3 were used as substrates for enzymatic biotinylation by biotinidase; biotin in peptides was probed using streptavidin peroxidase. These studies provided evidence that K4, K9 and K18 in histone H3 are good targets for biotinylation; K14 and K23 are relatively poor targets. Antibodies were generated to histone H3, biotinylated either at K4, K9 or K18. These antibodies localized to nuclei in human placental cells in immunocytochemistry and immunoblotting experiments, suggesting that lysines in histone H3 are biotinylated *in vivo*. Dimethylation of R2, R8 and R17 increased biotinylation of K4, K9 and K18, respectively, by biotinidase; phosphorylation of S10 abolished biotinylation of K9. These observations are consistent with crosstalk between biotinylation of histones and other known modifications of histones. We speculate that this crosstalk provides a link to known roles for biotin in gene expression and cell proliferation.

Histones are small proteins (11–22 kDa) that mediate the folding of DNA into chromatin. The following five major classes of histones have been identified in eukaryotic cells: H1, H2A, H2B, H3 and H4 [1]. DNA is wrapped around octamers of core histones, each consisting of one H3–H3–H4–H4 tetramer and two H2A–H2B dimers, to form the nucleosomal core particle. Histone H1 associates with the DNA connecting nucleosomal core particles. Nucleosomes are stabilized by electrostatic interactions between negatively charged phosphate groups in DNA and positively charged ε-amino groups (lysine residues) and guanidino groups (arginine residues) in histones.

Histones consist of a globular C-terminal domain and a flexible N-terminal tail [1]. The amino terminus of histones protrudes from the nucleosomal surface;

Abbreviation
DAPI, 4′,6-diamidino-2-phenylindole.

lysines, arginines, serines, and glutamates in the amino terminus are targets for acetylation, methylation, phosphorylation, ubiquitination, poly(ADP-ribosylation) and sumoylation [1–5]. These modifications play important roles in chromatin structure, regulating processes such as transcriptional activation or silencing of genes, DNA repair, and mitotic and meiotic condensation of chromatin.

Recently, a novel covalent modification of histones has been identified in human cells, namely the biotinylation of lysine residues [6,7]. Two enzymes can independently catalyze biotinylation of histones: biotinidase [8] and holocarboxylase synthetase [9]. Biotinidase belongs to the nitrilase superfamily of enzymes [10]; biotinylation of histones by biotinidase depends on the hydrolytic cleavage of biocytin (biotinyl-ε-lysine), coupled to the transfer of the biotinyl residue to free amino groups in histones [11]. In contrast, biotinylation of histones by holocarboxylase synthetase depends on ATP and biotin [9]. Preliminary studies suggest that biotinylation of histones might play a role in processes such as gene silencing [12], cell proliferation [6,9], and DNA repair or apoptosis [12,13]. These observations could have important implications for human health, based on the following lines of reasoning. First, preliminary evidence has been provided that biotinylation of K12 in histone H4 decreases rapidly in response to double-stranded DNA breaks caused by the cancer drug etoposide [13]. This observation is consistent with the hypothesis that alterations in the biotinylation pattern of histones might be an early signaling event in response to DNA damage. Second, mutations of the genes encoding biotinidase [14–16] and holocarboxylase synthetase [17] have been documented; some of these mutations are fairly common [18,19]. Fibroblasts from individuals with mutated holocarboxylase synthetase are deficient in histone biotinylation [9]. Likewise, *in vitro* studies provided evidence that mutated biotinidase is not capable of catalyzing biotinylation of histones [8]. Future studies might unravel abnormal patterns of gene silencing [12], cell proliferation [6,9], and DNA repair or apoptosis [12,13] in individuals carrying mutations of genes coding for biotinidase and holocarboxylase synthetase.

Although all five major classes of histones appear to be biotinylated in human cells [6], only two biotinylation sites have been identified so far: K8 and K12 in histone H4 [7]. This gap in our understanding of histone biotinylation has created a significant obstacle for investigating roles of biotinylated histones in cell biology, based on the following lines of reasoning. As long as biotinylation sites remain unknown, no site-specific antibodies to biotinylated histones can be

generated. Such antibodies are invaluable tools to: (a) study the cross-talk among modifications of histones, e.g. biotinylation and acetylation of lysine residues [7]; (b) investigate cellular distribution patterns of biotinylated histones by using immunocytochemistry; and (c) investigate roles for biotinylation of histones in the regulation of transcriptional activity of genes by using chromatin immunoprecipitation assays.

Recently we have developed a peptide-based procedure to identify biotinylation sites in histones [7]. In this study we applied this procedure to identify biotinylation sites in human histone H3. As a secondary goal we investigated interactions among histone biotinylation, methylation and phosphorylation. Histone H3 was chosen as a model because of its pivotal role in regulating gene expression [20–22].

Results

Biotinylation sites in histone H3

The N-terminal tail of histone H3 was efficiently biotinylated by biotinidase. The binding of biotin was substantially greater in peptide N_{1-25} than in peptide N_{15-39}, if equal amounts of both peptides were incubated with biotinidase and biocytin for 45 min (Fig. 1;
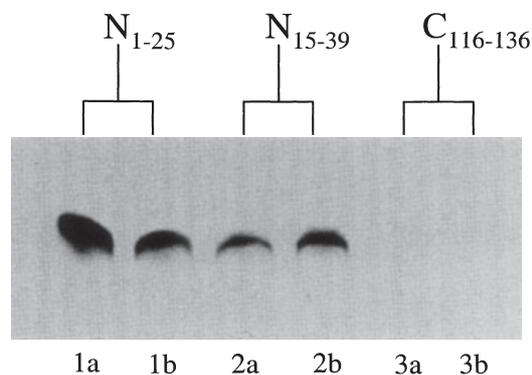
N_{1-25} = ARTKQTARKSTGGKAPRKQLATKAA
N_{15-39} = APRKQLATKAARKSAPATGGVKKPH
$C_{116-136}$ = KRVTIMPKDIQLARRIRGERA

Fig. 1. Biotinylation targets amino acids in the N-terminal tail of human histone H3. Synthetic peptides based on the N- and C-terminal region of histone H3 were biotinylated enzymatically, and biotin was probed using gel electrophoresis and streptavidin peroxidase. N_{1-25}, peptide spanning amino acids 1–25 in histone H3 (lanes 1a and b); N_{15-39}, peptide spanning amino acids 15–39 in histone H3 (lanes 2a and b); $C_{116-136}$, peptide spanning amino acids 116–136 in histone H3 (lanes 3a and b). Duplicate analyses are depicted.

compare lanes 1a and 1b with lanes 2a and 2b). The peptide ($C_{116-136}$) based on the C-terminus of histone H3 was not biotinylated if incubated with biotinidase (lanes 3a and 3b). This is consistent with previous observations that biotinylation and other modifications of histones cluster in the N-terminal region [2,7]. Also these findings suggest that the primary targets for biotinylation are located in the region spanning the 25 N-terminal amino acids. Thus, subsequent studies focused on this region in the histone H3 molecule.

Previous studies suggested that lysine residues in histones are targets for biotinylation [7]. Thus, we subdivided the N-terminal 25 amino acids into four synthetic peptides to allow for easier identification of biotinylated lysines in histone H3: N_{1-9} (including K4 and K9), N_{9-16} (including K9 and K14), N_{16-23} (including K18 and K23), and N_{18-25} (including K18 and K23); subscripts denote the amino acid residues in the histone H3 sequence. These peptides were incubated with biotinidase and biocytin for up to 45 min; at timed intervals aliquots were collected and biotinylated peptides on transblots were probed using streptavidin peroxidase. Apparently, peptide N_{18-25} was a better substrate for biotinylation than peptides N_{1-9}, N_{9-16} and N_{16-23} (Fig. 2). The minor apparent differences in biotinylation signal among the peptides loaded in Fig. 2B lanes 2–4 are caused by intra-assay variation, and are not observed if biotinylation of the same peptides is quantified by gel densitometry using multiple independent gels (Fig. 2A). Peptide N_{1-25} was used as a reference and was heavily biotinylated (Fig. 2B, lane 1): 100% relative biotinylation after 45 min of incubation. Peptide $C_{116-136}$ was used as a negative control and was not biotinylated after 45 min (lane 6). These findings suggest that either K18, K23, or both are targets for biotinylation (see below). However, further below we provide evidence that modifications of arginines may substantially enhance the biotinylation of histone H3 by biotinidase, and that K4 and K9 may also be targets for biotinylation *in vivo*. All subsequent enzymatic biotinylations were conducted for 45 min.

The next series of experiments focused on K4, K9 and K14. Peptide N_{1-25} was used as a positive control and was heavily biotinylated (Fig. 3, lane 1). As expected, if both lysines (K4 and K9) in a peptide spanning amino acids 1–9 in histone H3 were substituted by alanine ($K4,9A_{1-9}$), no binding of biotin was detectable (lane 2). This is consistent with previous studies, suggesting that lysines rather than other amino acids are targets for biotinylation [7]. If K4 was substituted with alanine ($K4A_{1-9}$), biotinylation of K9 was barely detectable (lane 3). In contrast, if K9 was substituted with alanine ($K9A_{1-9}$), K4 was biotinylated
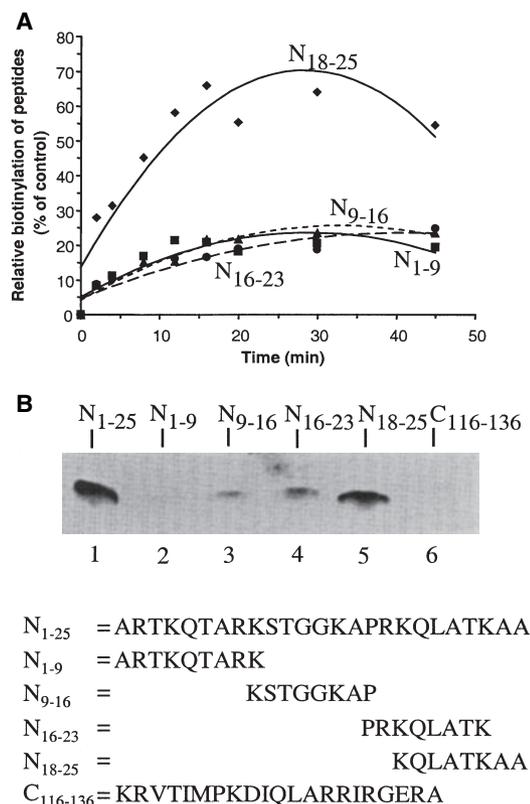

Fig. 2. Biotinylation of peptides based on the N-terminal tail in histone H3. (A) Synthetic peptides were biotinylated enzymatically, and biotin was probed using gel electrophoresis and streptavidin peroxidase. N_{1-9}, peptide spanning amino acids 1–9 in histone H3; N_{9-16}, peptide spanning amino acids 9–16 in histone H3; N_{16-23}, peptide spanning amino acids 16–23 in histone H3; and N_{18-25}, peptide spanning amino acids 18–25 in histone H3. Each data point represents the mean of three independent measurements. (B) Representative gels, depicting peptides that were incubated with biotinidase and biocytin for 45 min. N_{1-25} (lane 1), peptide spanning amino acids 1–25 in histone H3; N_{1-9}, N_{9-16}, N_{16-23} and N_{18-25} (lanes 2–5) are as described for panel A; $C_{116-136}$ (lane 6), peptide spanning amino acids 116–136 in histone H3.

considerably (lane 4). These findings suggest that K4 is a target for biotinylation.

Next, variations of a peptide spanning amino acids 9–16 in histone H3 (i.e. including K9 and K14) were tested. If both K9 and K14 were substituted with alanine ($K9,14A_{9-16}$), no binding of biotin was detectable (lane 5). If K14 was substituted with alanine ($K14A_{9-16}$), K9 was heavily biotinylated (lane 6). This is in contrast to the findings described above, which suggested that K9 is a poor target for biotinylation (peptide $K4A_{1-9}$ in lane 3). We offer the following explanation for these apparently contradictory observations: peptide $K14A_{9-16}$ is lacking the

N_{1-25} = ARTKQTARKSTGGKAPRKQLATKAA

$K4,9A_{1-9}$ = ARTAQTARA

$K4A_{1-9}$ = ARTAQTARK

$K9A_{1-9}$ = ARTKQTARA

$K9,14A_{9-16}$ = ASTGGAAP

$K14A_{9-16}$ = KSTGGAAP

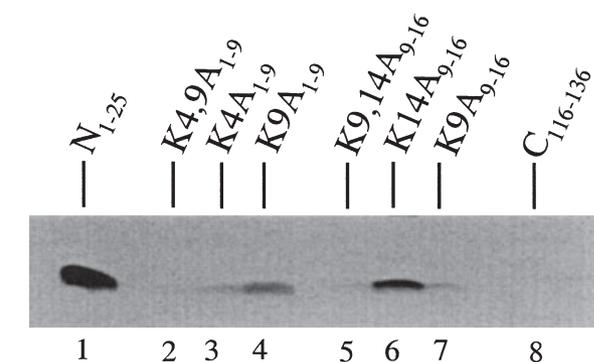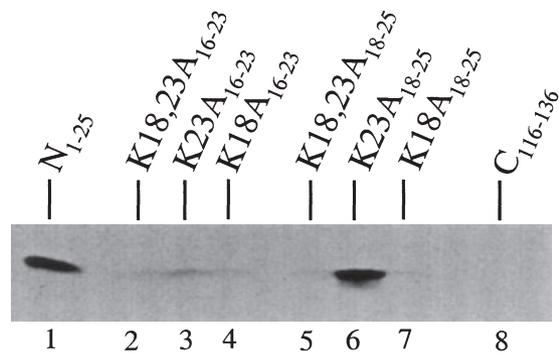$K9A_{9-16}$ = ASTGGKAP

$C_{116-136}$ = KRVTIMPKDIQLARRIRGERA

Fig. 3. Biotinylation of K4, K9 and K14 in the N-terminal tail in histone H3. Synthetic peptides were biotinylated enzymatically, and biotin was probed using gel electrophoresis and streptavidin peroxidase. N_{1-25}, peptide spanning amino acids 1–25 in histone H3 (lane 1); $K4,9A_{1-9}$, $K4A_{1-9}$, $K9A_{1-9}$, $K9,14A_{9-16}$, $K14A_{9-16}$ and $K9A_{9-16}$ are substitutions of K4, K9 and K14 in histone H3; $C_{116-136}$, peptide spanning amino acids 116–136 in histone H3.

N_{1-25} = ARTKQTARKSTGGKAPRKQLATKAA

$K18,23A_{16-23}$ = PRAQLATA

$K23A_{16-23}$ = PRKQLATA

$K18A_{16-23}$ = PRAQLATK

$K18,23A_{18-25}$ = AQLATAAA

$K23A_{18-25}$ = KQLATAAA

$K18A_{18-25}$ = AQLATKAA

$C_{116-136}$ = KRVTIMPKDIQLARRIRGERA

Fig. 4. Biotinylation of K18 and K23 in the N-terminal tail in histone H3. Synthetic peptides were biotinylated enzymatically, and biotin was probed using gel electrophoresis and streptavidin peroxidase. N_{1-25}, peptide spanning amino acids 1–25 in histone H3 (lane 1); $K18,23A_{16-23}$, $K23A_{16-23}$, $K18A_{16-23}$, $K18,23A_{18-25}$, $K23A_{18-25}$ and $K18A_{18-25}$, substitutions of K18 and K23 in histone H3; and $C_{116-136}$, peptide spanning amino acids 116–136 in histone H3.

positively charged and bulky arginine residue in position 8; in contrast peptide $K4A_{1-9}$ includes R8. Biotinylation of $K14A_{9-16}$ cannot be explained by biotinylation of K14, given that K14 is a poor target for biotinylation (peptide $K9A_{9-16}$, lane 7). These findings are consistent with the hypothesis that K9 might be a good target for biotinylation if R8 is modified covalently; this hypothesis was further tested in dimethylation experiments described below. Peptide $C_{116-136}$ was used as a negative control; no biotinylation was detectable (lane 8).

The following series of experiments focused on K18 and K23. Peptide N_{1-25} was used as a positive control and was heavily biotinylated (Fig. 4, lane 1). As expected, if both lysines (K18 and K23) in a peptide based on amino acids 16–23 in histone H3 were substituted with alanine (peptide $K18,23A_{16-23}$), no binding of biotin was detectable (lane 2). Likewise, biotinylation of K18 was weak if K23 was substituted with alanine ($K23A_{16-23}$; lane 3), and biotinylation of K23 was weak if K18 was substituted with alanine ($K18A_{16-23}$; lane 4). This is in apparent contrast to the findings

presented in Fig. 2, which suggested that K18 or K23 are good targets for biotinylation (peptide N_{18-25} in Fig. 2). Based on the following lines of reasoning we hypothesize that R17 in peptide $K23A_{16-23}$ interfered with biotinylation of K18 in the experiments depicted in Fig. 4: (a) Peptide N_{18-25} (Fig. 2) starts with K18, i.e. does not include R17; (b) peptide $K23A_{16-23}$ (Fig. 4) starts with A16, i.e. this peptide includes R17; (c) experiments involving K9 suggested that arginine residues may interfere with biotinylation (see above). This hypothesis was tested as follows. Peptides were synthesized that started with K18 in histone H3; hence, these peptides did not include R17 but did include both K18 and K23 unless noted otherwise. No biotinylation was detected if both K18 and K23 were substituted with alanine ($K18,23A_{18-25}$; lane 5). If K23 was substituted with alanine ($K23A_{18-25}$), K18 was heavily biotinylated (lane 6). In contrast, if K18 was substituted with alanine ($K18A_{18-25}$), biotinylation of K23 was barely detectable (lane 7). Peptide $C_{116-136}$ was used as a negative control; no biotinylation was detectable (lane 8). These findings are consistent with the
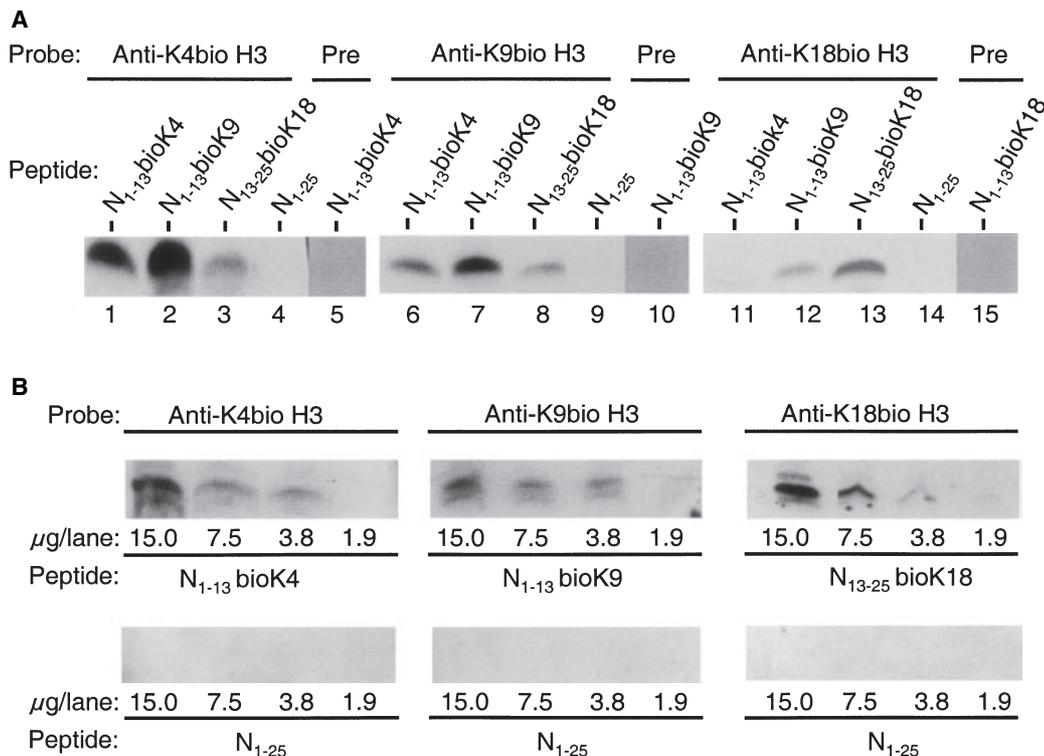
hypothesis that K18 is a target for biotinylation if R17 is modified; this hypothesis was further tested as described below. Also, these findings suggest that K23 is a poor target for biotinylation.

Arginine residues such as R2 and R17 in human histone H3 are modified by mono- and di-methylation; various lysine residues in histones are modified by mono-, di-, or tri-methylation [2,23]. Here we determined whether naturally occurring modifications of arginines render lysines a better target for biotinylation in histone H3. Peptide N_{16-23} was used as a control; this peptide includes K18 and K23, and an arginine residue (R17) that is not dimethylated. Peptide N_{16-23} was a moderate target for biotinylation by biotinidase (Fig. 5, lane 1), confirming findings presented above. Likewise, peptides N_{1-9} (including K4 and K9) and N_{9-16} (including K9 and K14) were relatively poor targets for biotinylation (data not shown; see also Fig. 2). Dimethylation of R2 and R8 (combined or

individually) moderately increased the enzymatic biotinylation of K4 and K9 by biotinidase (compare lanes 2–4 to lane 1). Dimethylation of R17 (peptide dmeR17$_{16-23}$) substantially increased the enzymatic biotinylation of K18 (compare lanes 1 and 5). Note that peptide dmeR17$_{16-23}$ also contains K23; however, studies presented above suggested that K23 is a poor target for biotinylation.

Effects of arginine residues on biotinylation of lysines were further corroborated in the following series of experiments. The synthetic peptide N_{6-13} (including R8 and K9) was used as a control; this peptide was a moderate target for biotinylation (Table 1). If R8 was substituted with an alanine (peptide R8A$_{6-13}$) biotinylation increased considerably, suggesting that unmodified arginines interfere with biotinylation of lysines by biotinidase. Substitution of arginine with ornithine leaves intact the positive charge in position 8. If R8 was substituted with an ornithine (peptide R8O$_{6-13}$) biotinylation increased considerably, suggesting that the positive charge of arginine is not responsible for inhibiting biotinylation of lysines. If a negative charge was introduced by phosphorylation of S10 during peptide synthesis [S10S(p)$_{6-13}$], K9 became a poor target for biotinylation. This suggests that the naturally occurring phosphorylation of S10 [2] may play a role in decreasing the availability of K9 for biotinylation. If K9 was substituted with an alanine (peptide K9A$_{6-13}$), no biotinylation was observed (negative control). Finally, changing the sequence of amino acids 7 and 8 from AR to RA did not substantially affect biotinylation of K9.

N_{16-23} = PRKQLATK

dmeR2R8$_{1-9}$ = ARTKQTARK
      (CH$_3$)$_2$ (CH$_3$)$_2$

dmeR8$_{1-9}$ = ARTKQTARK
                    (CH$_3$)$_2$

dmeR2$_{1-9}$ = ARTKQTARK
      (CH$_3$)$_2$

dmeR17$_{16-23}$ = PRKQLATK
                       (CH$_3$)$_2$

Fig. 5. Effects of arginine dimethylation on the biotinylation of lysine residues in the N-terminal tail in histone H3. Synthetic peptides were biotinylated enzymatically, and biotin was probed using gel electrophoresis and streptavidin peroxidase. N_{16-23}, peptide spanning amino acids 1–23 in histone H3 (lane 1); dmeR2R8$_{1-9}$, dmeR8$_{1-9}$, dmeR2$_{1-9}$ and dmeR17$_{16-23}$, dimethylation of R2, R8 or R17 in histone H3 (lanes 2–5).

Polyclonal antibody

Polyclonal antibodies were generated to determine whether histone H3 is biotinylated at K4, K9 and K18 *in vivo*. First, we determined whether the antibodies were specific for biotinylation sites. Transblots of the following biotinylated peptides were probed with the

Table 1. Amino acid modifications affect biotinylation of K9 by biotinidase. TARKSTGG represents the native unmodified peptide, based on the amino acid sequence in position 6–13 in histone H3.

Identifier	Amino acid sequence	Relative biotinylation
N_{6-13}	TARKSTGG	+ +
R8A$_{6-13}$	TAAKSTGG	+ + +
R8O$_{6-13}$	TAOKSTGG	+ + +
S10S(p)$_{6-13}$	TARKS(p)TGG	–
K9A$_{6-13}$	TARASTGG	–
AR7,8RA$_{6-13}$	TRAKSTGG	+

Fig. 6. Biotinylation site specificity of antibodies against histone H3, biotinylated at K4, K9 or K18. Synthetic peptides based on histone H3 were chemically biotinylated either at K4 (denoted N_{1-13}bioK4), K9 (N_{1-13}bioK9), or K18 (N_{13-25}bioK18); a nonbiotinylated peptide spanning amino acids 1–25 was used as a negative control (N_{1-25}). (A) Peptides were probed using antibodies to histone H3, biotinylated at K4 (lanes 1–4), biotinylated at K9 (lanes 6–9), biotinylated at K18 (lanes 11–14), or preimmune sera (denoted Pre, lanes 5, 10 and 15) to test for cross-reactivity. (B) Peptides were titrated using antibodies against biotinylated K4, K9, and K18; equal amounts of the nonbiotinylated peptide N_{1-25} were used as negative control.

newly developed antibodies in all possible combinations: N_{1-13}bioK4, N_{1-13}bioK9 and N_{13-25}bioK18 (see Experimental procedures for sequence information). The following observations were made with regard to antibody specificities. The antibody raised against histone H3 (biotinylated at K4) reacted with N_{1-13}bioK4 and cross-reacted with N_{1-13}bioK9, but bound only very weakly to N_{13-25}bioK18 (Fig. 6A, lanes 1–3). No signal was detectable if nonbiotinylated peptide (N_{1-25}) was used as a target (lane 4), or if N_{1-13}bioK4 was probed using preimmune serum (lane 5). The antibody raised against histone H3 (biotinylated at K9) reacted with N_{1-13}bioK9, but cross-reacted only very weakly with N_{1-13}bioK4 and N_{13-25}bioK18 (lanes 6–8). No signal was detectable if nonbiotinylated peptide (N_{1-25}) was used as a target (lane 9), or if N_{1-13}bioK9 was probed using preimmune serum (lane 10). The antibody raised against histone H3 (biotinylated at K18) reacted with N_{13-25}bioK18, but did not bind to N_{1-13}bioK4 and cross-reacted only very weakly with N_{1-13}bioK9 (lanes 11–13). No signal was detectable if

nonbiotinylated peptide (N_{1-25}) was used as a target (lane 14), or if N_{13-25}bioK18 was probed using preimmune serum (lane 15). Peptides N_{1-13}bioK4, N_{1-13}bioK9 and N_{13-25}bioK18 produced equal signals if biotin was probed with streptavidin–peroxidase (data not shown). This is consistent with the notion that equal amounts of peptide were loaded per lane in specificity experiments.

Finally, we titrated biotinylated and nonbiotinylated peptides by using antibodies to biotinylated histones. If biotinylated peptides were used as targets, the chemiluminescence signal paralleled the mass of peptide loaded per lane (Fig. 6B, top row). In contrast, no signal was detectable if nonbiotinylated peptides were used as target (Fig. 6B, bottom row). These findings suggest that the affinities of antibodies for biotinylated peptides were at least seven times greater than for nonbiotinylated peptides: detection limits were about 1.9–3.8 μg·lane^{-1} for biotinylated peptides vs. > 15 μg·lane^{-1} for nonbiotinylated peptides if the autoradiography films were exposed to membranes for about 5 s.

Analysis of biotinylated histone H3 in human cells by immunoblotting and immunocytochemistry

Histone H3 in human JAr cell nuclei is biotinylated at K4, K9 and K18, as judged by western blot analysis (Fig. 7A, top row). Recombinant (nonbiotinylated) histone H3 was used as a negative control and did not produce a signal (bottom row). If human histone extracts or recombinant histone H3 were probed with preimmune sera no signal was detectable (data not shown).

Biotinylation of histone H3 depended on biotinidase and holocarboxylase synthetase. For example, biotinylation of K18 in histone H3 was less abundant in fibroblasts from biotinidase- and holocarboxylase synthetase-deficient individuals compared with normal fibroblasts (Fig. 7B, lanes a–c); equal loading of lanes was confirmed by staining with Coomassie blue (lanes d–f). Of note, the antibodies against biotinylated histone H3 did not show significant cross-reactivity with histones H1, H2A, H2B and H4.

Finally, biotinylated species of histone H3 were visualized in JAr cells by using immunocytochemistry. Antibody to K4-biotinylated histone H3 localized primarily to the cell nucleus (Fig. 8, image a–d); preimmune serum did not generate a detectable signal (image e). Likewise, staining with antibodies to K9-biotinylated and K18-biotinylated histone H3 was

consistent with nuclear localization of biotinylated histones (images f–o). No signal was detectable if cells were stained with secondary antibody alone (data not shown). Staining with an antibody to K12-biotinylated histone H4 [7] also produced a nuclear signal (positive control; data not shown). Collectively, these findings suggest that human cells contain histone H3, biotinylated at K4, K9 and K18.

Discussion

This study provides evidence (a) that K4, K9 and K18 in histone H3 are good targets for biotinylation by human biotinidase; (b) that K14 and K23 are relatively poor targets for biotinylation; (c) that human cells contain histone H3, biotinylated in positions K4, K9 and K18; and (d) that dimethylation of arginine residues in histone H3 enhances biotinylation of adjacent lysine residues, whereas phosphorylation of serine residues is likely to abolish biotinylation of adjacent lysine residues.

The following observations suggest that biotinylation of K4, K9 and K18 in histone H3 is physiologically important. First, evidence has been provided that biotinylation of histones might play a role in the cellular response to DNA damage [12,13]. Second, biotinylation of histones might be associated with gene silencing [12]. Third, K4 and K9 are targets for both methylation [2] and biotinylation; methylation and
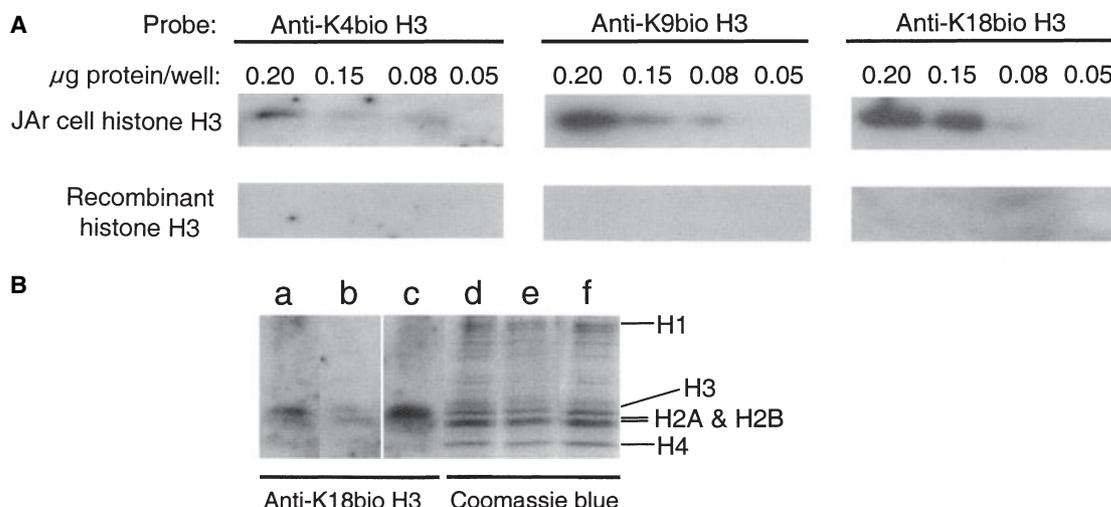

Fig. 7. Biotinylated histones H3 are present in extracts from human cell nuclei, but biotinylation is reduced in biotinidase- and holocarboxylase synthetase-deficient cells. (A) Histones were extracted from JAr cell nuclei and biotinylated histones H3 were titrated using antibodies against biotinylated K4, K9 and K18; equal amounts of recombinant (nonbiotinylated) histone H3 were used as negative control. (B) Histones were extracted from biotinidase- and holocarboxylase synthetase-deficient human skin fibroblasts (lanes a and b, respectively); IMR-90 fibroblasts from a healthy human were used as a control (lane c). Histones were probed using an antibody to K18-biotinylated histone H3. Equal loading was confirmed by staining with Coomassie blue (lanes d–f).
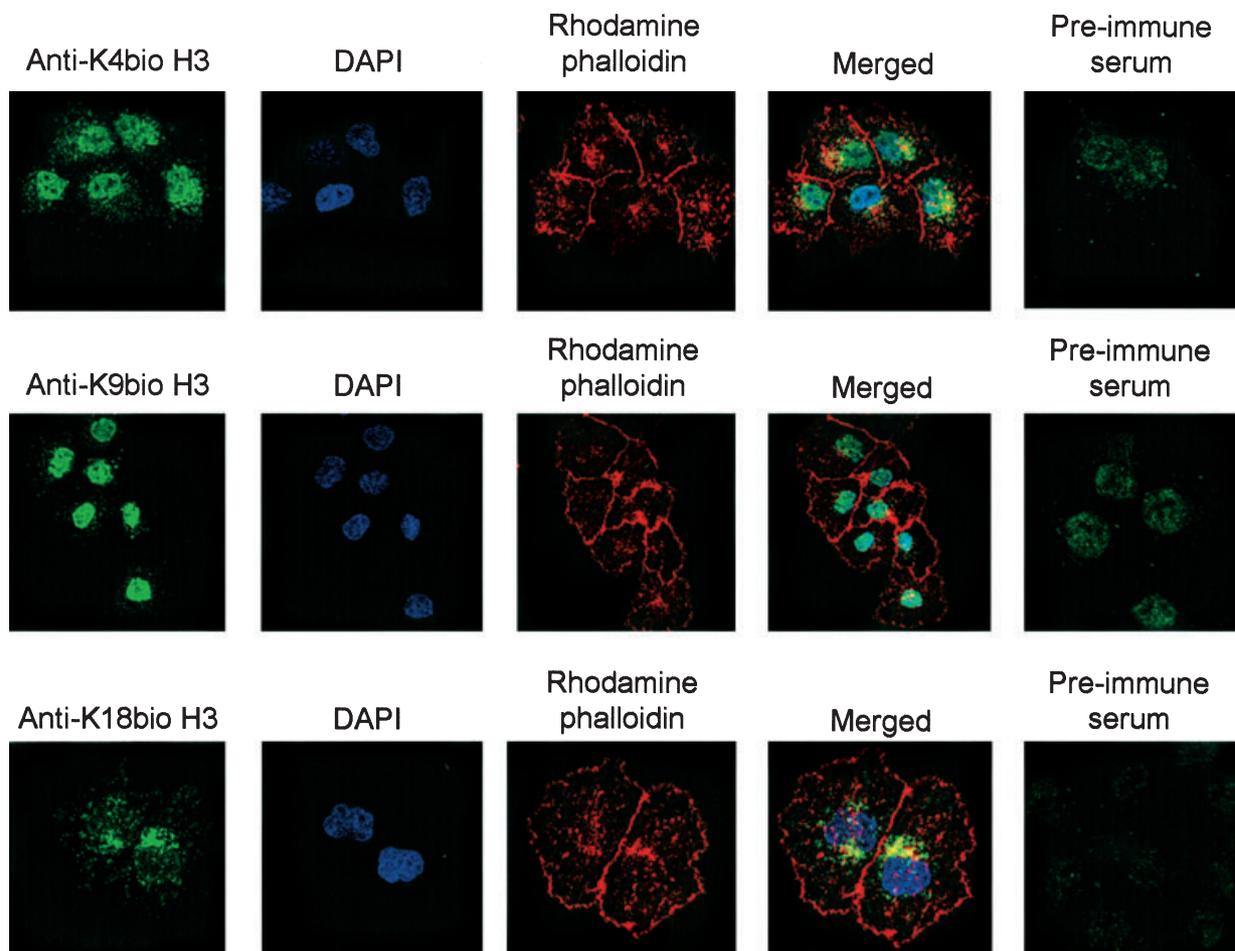
Fig. 8. Biotinylated histones H3 localize to the nucleus in JAr cells. Cells were stained with antibodies to K4-biotinylated histone H3 (top panel), K9-biotinylated histone H3 (middle panel) and K18-biotinylated histone H3 (lower panel). The nuclear compartment was stained using DAPI, and the cytoplasm was stained using rhodamine phalloidin. Images entitled 'Merged' were created by overlaying images obtained by staining with antibody, DAPI and rhodamine phalloidin. Pre-immune sera were used as negative controls.

biotinylation of the same lysine residue are mutually exclusive. Methylation of K4 is associated with transcriptionally active chromatin whereas methylation of K9 is associated with transcriptionally silent chromatin [3,24]. Thus, biotinylation of K4 and K9 is likely to affect transcriptional activity of chromatin. Fourth, K18 is a target for both acetylation [2,23] and biotinylation. Acetylation of K18 is associated with transcriptionally active chromatin [23]. It is unknown whether biotinylation of K18 affects acetylation-dependent activation of chromatin.

Modifications of arginine residues in histones affect biotinylation of adjacent lysine residues. The following lines of evidence support this notion. (a) Dimethylation of R2, R8 and R17 increased biotinylation of K4, K9 and K18, respectively, by biotinidase. Dimethylation of R2 and R17 in histone H3 has been shown to

occur *in vivo* [2,23], suggesting that the findings presented here are physiologically relevant. (b) Substitution of R8 with ornithine was associated with increased biotinylation of K9. This is of potential physiological significance, given that monomethyl- and dimethyl-arginines in histones can be hydrolyzed to produce citrulline and, perhaps, ornithine [25]. Formally, we cannot exclude the possibility that free amino groups in ornithine and citrulline are substrates for biotinylation rather than enhancing biotinylation of adjacent lysines. However, our investigations of biotinylation motifs suggested that ornithine is not biotinylated by biotinidase, and that citrulline is only a relatively poor target for biotinylation (A. Kueh and J. Zampleni, unpublished data).

Finally, the present study provides evidence that phosphorylation of serine residues may prevent

biotinylation of adjacent lysine residues. This may be important for processes such as mitotic and meiotic chromosome condensation (phosphorylation of S10 and S28 in histone H3), transcriptional activation of chromatin (phosphorylation of S10 and S28 in histone H3) and DNA repair (phosphorylation of S14 in histone H2B) [23,26].

What are the limitations of the studies presented here? First, it is unknown whether post-translational modifications such as acetylation and methylation coexist with biotinylation on the same histone molecule. For example, does methylation of K9 coexist with biotinylation of K4? These uncertainties are currently being addressed in our laboratory by MS analysis of histone extracts from human cells. Second, we have identified some modifications that affect biotinylation of histones, e.g. dimethylation of arginines and phosphorylation of serines. It is unknown whether this is a bidirectional interaction. For example, does biotinylation of K9 prevent phosphorylation of S10? Third, both biotinidase and holocarboxylase synthetase have biotinyl histone transferase activity [8,9]. In this study only biotinidase was used to identify biotinylation sites in histone H3. Theoretically, holocarboxylase synthetase might target distinct amino acid residues for biotinylation.

Taken together, the present study has revealed three new modifications of human histone H3: biotinylation of K4, K9 and K18. Previous studies suggested that K8 and K12 in histone H4 are also biotinylated [7], bringing to a total of five the known biotinylation sites in human histones. Undoubtedly, additional biotinylation sites will be identified in future studies, given that all five major classes of human histones contain streptavidin-reactive material [6]. The availability of site-specific antibodies to biotinylated histones H3 and H4 [7] is likely to generate novel insights into roles for histone biotinylation in eukaryotic cells.

Experimental procedures

Peptide synthesis

Synthetic peptides were used as substrates for biotinidase to identify biotinylation sites in histone H3; the amino acid sequences in these peptides were based on human histone H3 (GenBank accession number NP_066403). Peptides were synthesized using Fmoc chemistry by a standard solid-phase method [27] as described previously [7]; L-isomers of amino acids were used in all syntheses. One-letter annotation is used for denoting amino acids throughout this paper [28]. Chemically modified peptides were synthesized by using biotinylated, dimethylated, and phosphorylated

Fmoc-ε-NH₂-D-biotinyl-L-lysine, Fmoc-dimethyl-L-arginine, and Fmoc-phospho-L-serine. Identities of synthetic peptides were confirmed by MS [7].

Post-translational modifications

Post-translational modifications of histone H3 cluster in the N-terminal region of the molecule (amino acids 1–36), e.g. methylation of K4 and K9, acetylation of K9, K18, K23 and K36, phosphorylation of S10, and mono- or dimethylation of R17 [2]. In pilot studies we used the following synthetic peptides to determine whether biotinylation of histone H3 also takes place in the N-terminal region: (a) N-terminus of histone H3, spanning amino acids 1–25 (ARTKQTARKSTGGKAPRKQLATKAA; this peptide was denoted N_{1-25}), and (b) a peptide based on amino acids 15–39 in histone H3 (APRKQLATKAARKSAPA-TGGVKKPH; denoted N_{15-39}). As a negative control we used a peptide spanning the C-terminus of histone H3, i.e. amino acids 116–136 (KRVTIMPKDIQLARRIRGERA; denoted $C_{116-136}$). Pilot studies using these peptides and previous studies of histone H4 [7] suggested that lysines located in the N-terminus of histone H3 are the primary targets for biotinylation (see below). Thus, the studies presented below focused on lysine residues in the N-terminal region; the amino acid sequences of the synthetic peptides used to identify biotinylation sites are provided in Results.

Enzymatic biotinylation of peptides

Synthetic peptides were incubated with biotinidase for enzymatic biotinylation as described previously [7,8]; biocytin (biotinyl-ε-lysine) was used as a biotin donor.

Gel electrophoresis

After enzymatic biotinylation, peptides were resolved using 16% tricine/polyacrylamide gels according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Peptides were electroblotted onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA); peptide-bound biotin was probed with streptavidin–peroxidase [6,7]. In previous studies both HPLC and MS were used to confirm covalent biotinylation of peptides [7].

Polyclonal antibodies

The following polyclonal antibodies to human histone H3 were generated using a commercial facility (Cocalico Biologicals, Reamstown, PA, USA): anti-H3 (biotinylated at K4), anti-H3 (biotinylated at K9) and anti-H3 (biotinylated at K18). To raise these antibodies, the following peptides were custom-synthesized by the University of Virginia

Biomolecular Research Facility (Charlottesville, VA, USA): (a) N_{1-13}bioK4, ARTK(biotin)QTARKSTGGC (amino acids 1–13 in histone H3); (b) N_{1-13}bioK9, ARTK-QTARK(biotin)STGGC (amino acids 1–13); and (c) N_{13-25}bioK18, GKAPRK(biotin)QLATKAAC (amino acids 13–25). Peptide identities were confirmed by MS. Peptides were conjugated to keyhole limpet hemocyanin by utilizing the C-terminal cysteine [7]; these peptide conjugates were injected into white New Zealand rabbits, following NIH and USDA guidelines for animal care. All possible measures were taken to minimize pain and discomfort to animals. 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 third booster injection was used for the assays described below; preimmune serum was used as a control. For assessment of antibody specificities, electroblots of peptides N_{1-13}bioK4, N_{1-13}bioK9 and N_{13-25}bioK18 were probed with the anti-(histone H3) Igs and a monoclonal mouse anti-rabbit IgG peroxidase conjugate as described previously [7]; nonbiotinylated peptide (N_{1-25}) was used as a control.

Analysis of biotinylated histone H3 in human cells by immunoblotting and immunocytochemistry

JAr human choriocarcinoma cells were cultured as described [29]. For western blot analysis, nuclear histones were extracted by using hydrochloric acid as described [6]. Recombinant (nonbiotinylated) histone H3 was purchased from Upstate Inc. (Lake Placid, NY, USA) and served as negative control. Equal amounts of JAr cell histone H3 and recombinant histone H3 (as judged by staining with Coomassie blue and gel densitometry) were loaded onto 18% Tris/glycine gels (Invitrogen) for electrophoresis. Proteins were electroblotted and probed with antibodies to biotinylated histone H3 as described [7]. Primary antibodies (rabbit serum) were diluted 250-fold, and the secondary antibody (mouse monoclonal anti-rabbit IgG peroxidase conjugate; Sigma, St. Louis, MO, USA) was diluted 20 000-fold.

Biotinylation of histones is mediated by biotinidase and holocarboxylase synthetase [8,9]. We obtained biotinidase-deficient human skin fibroblasts (strain code WG1371) and holocarboxylase synthetase-deficient human skin fibroblasts (strain code WG2215) from the Cell Repository at Montreal Children's Hospital (Quebec, Canada) to determine whether deficiency is associated with decreased biotinylation of histone H3. Human IMR-90 fibroblasts were used as control (ATCC clone CCL-186; Manassas, VA, USA). Nuclear histones from fibroblasts were analyzed by immunoblotting as described above.

Finally, biotinylated histones H3 in JAr cells were visualized by standard procedures of immunohistochemistry [26]. Primary antibodies (serum) were diluted 250-fold. Preimmune sera were used as negative controls. As secondary antibody we used Cy2-conjugated AffiniPure donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) at an 80-fold dilution. The nuclear compartment was stained using 4',6-diamidino-2-phenylindole (DAPI), and the cytoplasm was stained using rhodamine phalloidin (Molecular Probes, Eugene, OR, USA). Images were obtained using Olympus FV500 confocal microscope equipped with an oil immersion lens.

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