A Nonenzymatic Modification of the Amino-terminal Domain of Histone H3 by Bile Acid Acyl Adenylate*

Received for publication, August 11, 2004, and in revised form, September 30, 2004
Published, JBC Papers in Press, October 1, 2004, DOI 10.1074/jbc.M409205200

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Although it has been proposed that the secondary bile acids, deoxycholic acid and lithocholic acid, increase the number of aberrant crypt foci in the colon and may act as colon tumor promoters, there is little evidence detailing their mechanism of action. Histones play an important role in controlling gene expression, and the posttranslational modification of histones plays a role in regulation of intracellular signal transduction. In particular, the amino-terminal tail domain of histone H3 is sensitive to several posttranslational modifications, and acetylation of this domain changes its electrostatic environment and results in the loss of native folding. Therefore, we studied the modification of ε-amino groups on human histone H3 by deoxycholyl adenylate, which is an active intermediate in deoxycholyl thioester biosynthesis. After incubation of recombinant human histone H3 with a smaller amount of acyl adenylate, followed by enzymatic digestion, the peptide fragment mixtures were analyzed by matrix-assisted laser desorption ionization mass spectrometry. These data showed the formation of only one adduct fragment, which corresponded to amino acids 3–8 with a deoxycholate adduct, suggesting that the ε-amino group of Lysε had the highest reactivity. This novel modification, formation of a bile acid adduct on the histone H3 amino-terminal tail domain through an active acyl adenylate, may relate to the carcinogenesis-promoting effects of secondary bile acids.

In eukaryotic cells, the chromatin complex manages diverse intracellular signals and functions as a biological relay point (1–4). The nucleosome, the repeating unit of the chromatin, is formed by ~200 base pairs of DNA, a histone octamer, which consists of two molecules each of the core histones H2A, H2B, H3, and H4 and histone H1 as a linker (5). The eight histones in the core are arranged into a H3(H2A·H2B)4 tetramer and a pair of H2A-H2B heterodimers. The tetramer, in cooperation with two other dimers, forms a left-handed superhelical structure with a diameter of 11 nm, around which ~146 base pairs of DNA wrap in two turns (5). Each nucleosome bead is linked with a variable length of linker DNA and a linker histone H1. All of these histones are very basic proteins, and 25% of the amino acid residues are arginine or lysine. In addition, each histone has an amino-terminal tail that extends outward from the core structure. These tails are flexible, contain a greater number of lysine and arginine residues than the core domain, and undergo a variety of posttranslational modifications (1–4). Histone amino-terminal modifications control interaction affinities for chromatin-associated proteins and thereby participate in the transformation of chromatin between transcriptionally active and silent states (4). Considering the electrostatic requirements for folding the chromatin polymer, histone amino-terminal modifications, such as acetylation and phosphorylation, can prevent the formation of chromatin fibers. The length of the amino-terminal tail domains varies from 16 to 44 amino acids, and, in particular, the H3 tail domain is necessary for stabilization of the three-dimensional structure of the nucleosomes (6).

During the S phase of the cell cycle, histones, which are synthesized in the cytoplasm (7, 8), must be imported into the nucleus to form nucleosomes on newly replicated DNA. The nuclear import of histones occurs via an active transport mechanism. The core histones are transported by several members of the importin β superfamily, and histone H1 is transported by a heterodimer consisting of importin β and importin 7 (9). Importin β1 has affinity for the nuclear localization sequences of H2A, H3, and H4 (10). It has been reported that recognition of histone H3 and H4 by importin β1 differs depending on whether these histones are acetylated (10). The acetylation of amino-terminal histone tail domains may function to inhibit the nuclear transport of acetylated core histones by masking the nuclear localization sequence, and this may suppress unnecessary signal transductions.

Previously, we have reported that bile acid acyl adenylate (Fig. 1), which is formed during activation of the carboxyl group at the C-24 position into the acyl-CoA thioester (11), easily reacts with the amino groups on the side chains of lysine residues and at the amino termini of proteins and peptides. Such nonenzymatic reactions result in the production of protein-bile acid adducts (12, 13). Bile acids are major cholesterol metabolites, assist in lipolysis and absorption of fats by forming mixed micelles in the intestinal lumen, and are localized in enterohepatic circulation because of their efficient hepatic uptake. During these processes, the epithelial cells lining the colon are exposed to a large quantity of unconjugated bile acids absorbed from the intestine. It has been reported that deoxycholic acid and lithocholic acid, which are secondary bile acids formed by the action of microbial flora in the colonic environment, may act as colon tumor promoters in high-risk populations (14, 15). In conventional and germ-free rats, both secondary bile acids promote the formation of colonic adenocarcinoma. However, the mechanism of the tumor-promoting activity of secondary bile acids remains to be elucidated. Here we demon-
The acyl adenylate is chemically active and readily soluble in physiological solutions (13). Recombinant histone H3, which contains 13 lysine residues, was incubated with a 10-fold molar excess of DCA-AMP at 4 °C in 40 mM potassium phosphate buffer (pH 6.8). A peptide fragment mixture, which was obtained by digestion with endoprotease Arg-C, was analyzed by MALDI-TOF MS (Fig. 2A); the peptide fragments detected by MALDI-TOF MS are listed in Table I. Seventeen different peptide peaks were observed on the mass spectra. Furthermore, nine peptide fragments possessed a mass shift of 374, which corresponds to one DCA molecule. Such modified peptides included peptides corresponding not only to the amino-terminal tail domain but also to the core of the histone H3 molecule. Affinity extraction using the anti-deoxycholate monoclonal antibody was quite effective for purification of the peptide-DCA adducts, and all nine peptide fragments modified with DCA could be extracted from the reaction mixture as shown in Fig. 2B.

When using equimolar amounts of DCA-AMP and histone H3, the adduct formation phenomenon was considerably different from the results mentioned above. The MALDI-TOF mass spectrum obtained from this reaction mixture is illustrated in Fig. 3A. In this case, all but one of the peptides lacked the mass shift corresponding to the DCA molecule. After immunooaffinity extraction, only one peptide peak, which corresponded to a fragment of amino acids 3–8 bound to a DCA molecule, was observed on the mass spectra shown in Fig. 3B. In addition, similar results were obtained when one-tenth or one-hundredth the molar amount of DCA-AMP was incubated with recombinant histone H3 (data not shown).

To determine the amino acid sequence of the peptide fragment covalently bound to DCA, it was subjected to nano-electrospray ionization tandem MS analysis using a hybrid quadrupole-time-of-flight mass analyzer. The nomenclature for the fragment ions is based on that proposed by Roepstorff and Fohlman (17), as modified by Biemann (18). Three product ion spectra of the DCA-bound amino-terminal tail peptides are shown in Fig. 4, and all tandem MS results are summarized in Table II. As shown in Fig. 4A, the product ion spectrum of doubly charged ions presented doubly charged ions corresponding to molecules that had eliminated either one or two water molecules at m/z 530.8 and 521.8, respectively. Because the same phenomenon was observed in our previous report (13), these ions may be produced by elimination of the 3α- and 12α-hydroxyl groups on the steroid nucleus. We observed y5 ions with both a mass shift of 374, corresponding to one DCA molecule, and the elimination of one or two water molecules at m/z 959.6 and 941.6. Clear peaks representing unmodified y1 to y5 ions were also observed. In addition, b8 and b9 ions and their dehydrated ions with mass shifts of 374 were clearly observed at m/z 568.4, 586.4, 604.3, 696.4, and 714.4 as singly charged ions. These results indicate that one DCA molecule was conjugated to the ε-amino group of the lysine residue at position 4 of the peptide fragment of amino acids 3–8 (TKQTAR) of histone H3. The product ion spectrum of m/z 638.4 corresponding to the doubly charged ion of the fragment of amino acids 9–17 (KSTGKAPR) is shown in Fig. 4B.
FIG. 2. MALDI mass spectrum of the reaction mixture resulting from the incubation of a 10-fold molar excess of deoxycholyl adenylate with human histone H3. A, before immunoaffinity extraction; B, after immunoaffinity extraction. MS conditions: instrument, Voyager DE-STR (reflector mode); matrix, α-cyano-4-hydroxycinnamic acid; accelerating voltage, 25 kV; grid voltage, 18 kV; guide wire voltage, 50 V.
doubly charged ions corresponding to the dehydrated fragment ions were observed at m/z 620.4 and 629.4. Analysis based on the y ion series (y1, y2, y3, y4, y6-H2O, y6-2H2O, y7, y7-H2O, y7-2H2O, y8, y8-H2O, and y8-2H2O) demonstrated that the modification was at Lys4 because of the mass shift of 502 between y3 and y4. The fragment ions at m/z 445 or 503, which may be produced as an unmodified KAPR and a b1 ion of DCA-bound y3 and y4. The fragment ions at m/z 471 and 503, which may be produced as an unmodified KAPR and a b1 ion of DCA-bound amino-terminal lysine fragment, respectively, were not observed. This observation, hence, suggests that the lysine residue at the amino terminus was not modified with DCA. The amino-terminal peptide fragments were predominantly modified, and its core domain was not wrapped with DNA. A theoretical three-dimensional structure of recombinant histone H3 was constructed using SWISS-MODEL Version 36.0003 (26–28), which is available at www.expasy.org/swissmod/. This structure may be similar to that of the nucleosome bead as shown in Fig. 5, and the core domain may fold in an α-helical conformation, explaining the difference in reactivity between the core and the amino terminus.

Bile acid acyl adenylate, which is an anhydrous mixed acid consisting of a carboxylic acid and a phosphoric acid, is very chemically active in a physiological environment (11) and reacts easily with amino groups at the amino termini of protein molecules and on lysine side chains (12, 13). We have demonstrated that the reactivity of the adenylate depends on the pH of the solution and that, in weakly alkaline solutions, the adenylate reacts rapidly with amino groups to produce the corresponding adducts (12). Histone H3 is only slightly soluble in alkaline solution; therefore, the reaction has been carried out under neutral conditions (pH 6.8). When excess amounts of deoxycholyl adenylate were used, many peaks corresponding to peptide fragments bound to deoxycholic acid were observed on the MALDI-TOF mass spectrum. Peptide fragments corresponding to the core domain were detected as both adduct and nonadduct peaks in the mass spectrum obtained without immunoffinity extraction. In contrast, amino-terminal peptide fragments were predominantly modified with deoxycholate. This may suggest that, because of their flexibility, the e-amino groups in the amino-terminal domain are more exposed to acylation by electrophilic reagents than those in the core domain. The commercially available recombinant histone H3 used in this study was not modified, and its core domain was not wrapped with DNA. Human histone H3, which is a small protein consisting of 135 amino acid residues, is very basic due to the presence of 13 lysine and 17 arginine residues in the molecule. The NH2-terminal tail domain of histone H3, which corresponds to one-thirteenth the molar amount of lysine residues in histone H3, was constructed using SWISS-MODEL Version 36.0003 (26–28), which is available at www.expasy.org/swissmod/. This structure may be similar to that of the nucleosome bead as shown in Fig. 5, and the core domain may fold in an α-helical conformation, explaining the difference in reactivity between the core and the amino terminus.

The exposure of histone H3 to lower amounts of deoxycholyl adenylate allowed us to clarify the differences in reactivity of each e-amino group in the amino-terminal domain. With the use of equimolar amounts of deoxycholyl adenylate and histone H3 (which corresponds to one-thirteenth the molar amount of lysine residues in histone H3), we only detected one adduct peak on the mass spectrum, and the other fragments were unmodified. After immunoffinity extraction, a peptide fragment peak corresponding to amino acids 3–8 with a mass shift of 374 was observed. These results suggest that the reactivity of the e-amino group of Lys4 is much higher than the activity of other residues in the amino-terminal and core domains.

**DISCUSSION**

Human histone H3, which is a small protein consisting of 135 amino acid residues, is very basic due to the presence of 13 lysine and 17 arginine residues in the molecule. The amino-terminal tail domain of histone H3 is sensitive to acetylation changes the electrostatic environment of the histone and loosens folding, which results in unstable chromatin structures and increased transcription (22). Although hyperacetylation has caused apoptosis in cancer cells (23), such acetylations are reversible by the action of histone deacetylase and subsequent methylation (20). Histone methyl transferase, which is present in the nucleus (24), acts on lysine residues deacetylated by histone deacetylase (20), and methylation of lysine 4 relates to activation of transcription (25).
Fig. 3. MALDI mass spectrum of the reaction mixture resulting from the incubation of equimolar amounts of deoxycholyl adenylate and human histone H3. A, before immunoaffinity extraction; B, after immunoaffinity extraction. MS conditions: instrument, Voyager DE-STR (reflector mode); matrix, α-cyano-4-hydroxycinnamic acid; accelerating voltage, 25 kV; grid voltage, 18 kV; guide wire voltage, 50 V. The asterisk indicates the peak derived from enzymatic digestion of the immobilized IgG fraction.
Secondary bile acids such as deoxycholic and lithocholic acids, are more cytotoxic than the primary bile acids, cholic and chenodeoxycholic acids, which are synthesized in hepatocytes. Low doses of primary bile acids increase the number of aberrant crypt foci in the colon (29); conversely, higher concentrations of deoxycholic acid induce apoptosis in colon cancer cells (30). Nair et al. (31) have found a tissue-bound form of lithocholate with a free form in human liver, which can be hydrolytically released using clostridial cholanylaminooic acid hydrolase. They have demonstrated a specific conjugation of the α-amino group of a lysine residue to the carboxyl group of a bile acid and investigated the relationship to promotion of carcinogenesis (32). In addition, they have found tissue-bound lithocholate in various commercially available histones (33). Secondary bile acids are better substrates for phase II metabolism, including amino acid, sulfenic acid, and glucuronic acid conjugation. As mentioned above, acyl adenylate is an active intermediate in the metabolic reaction that converts carboxylic acid to the corresponding thioester. Rat liver bile acid CoA ligase has been cloned, and it shows affinity for deoxycholate binding (34). Furthermore, acyl-CoA synthetase, also known as ACS-5, which can produce bile acid acyl adenylates as intermediates, has been purified from rat intestinal epithelial cells and proliferating preadipocytes (35). In addition, a more recent publication (36) has reported the expression of ACS-5 in human epithelial tumors of the small intestine. These findings suggest the possible synthesis of bile acid acyl adenylate in epithelial cells. The formation reaction of acyl adenylate is due to an ATP-dependent ping-pong mechanism (37), and acyl adenylate usually does not become soluble in cells. However, when the amount of absorbed bile acids is greater than the amount of CoA, which is required for the transformation from acid adenylate to CoA thioester, it is possible that active acyl adenylates will be released into the periplasm and react with α-amino groups in protein molecules. The addition of butyrate, a short chain fatty acid, inhibits histone deacetylase and results in subsequent histone hyperacetylation, induction of p21WAF overexpression, and apoptotic death in colon cancer cells (23, 38, 39). Fabrizi et al. (40) have reported the adduction of phosgene, the major active metabolite of chloroform, to lysine residues of human histone H2B in their investigation of the mechanism of chloroform mutagenicity. The modification of the α-amino group of lysine residues in the histone H3 amino-terminal domain observed herein may inhibit histone hyperacetylation and result in the promotion of carcinogenesis.

In conclusion, we have demonstrated that bile acid acyl adenylate can react with α-amino groups in the histone H3 amino-terminal tail domain under nonenzymatic conditions. These data suggest that the α-amino groups of lysine residues in the amino-terminal domain of histone H3 display higher reactivity than the α-amino groups of lysine residues in the core domain and, in particular, that the α-amino group of Lys4 may have the highest reactivity of any lysine residue in histone H3. This novel modification, bile acid adduct formation on the amino-terminal tail domain of histone H3 through bile acid acyl adenylate as an active intermediate, may also relate to the action of secondary bile acids as colon tumor promoters.

5.1, v/v/v); declustering potential, 60.0 V; collision energy, 25.0–35.0 eV; collision gas, 3 units of N2. The peaks marked with an asterisk indicate the possible fragment ions at m/z 471 and 503 in B and m/z 445 and 503 in C, which are produced from the DCA bound at amino-terminal lysine residue fragments.
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FIG. 5. Protein modeling of recombinant human histone H3 constructed by SWISS-MODEL.

TABLE II

The list of b-ion and y-ion series observed in the product ion mass spectra of peptide fragments bound DCA

| Ion       | Measured | Ion       | Measured | Ion       | Measured | Ion       | Measured |
|-----------|----------|-----------|----------|-----------|----------|-----------|----------|
| b2        | 604.3130 | b1        | 129.0993 | b1        | 129.0943 | (b2H2O)   | 228.1718 |
| b2-H2O    | 588.4217 | b2        | 216.1218 | y1        | 175.1062 | (b1H2O)   | 385.1582 |
| b2-H2O    | 568.3850 | b3        | 317.1658 | y2        | 246.1393 | (b1H2O)   | 449.2042 |
| b2-H2O    | 714.4270 | b4        | 374.1531 | y2        | 317.1706 | b1         | 175.1062 |
| y1        | 175.1239 | y1        | 175.1239 | y3        | 819.4849 | b2         | 409.2129 |
| y2        | 214.1262 | y2        | 214.1262 | y6        | 214.1262 | y1         | 175.1239 |
| y3        | 343.1735 | y3        | 343.1735 | y6        | 343.1735 | y1         | 175.1239 |
| y4        | 845.0333 | y4        | 845.0333 | y5        | 845.0333 | y1         | 175.1239 |
| y5        | 959.6134 | y5        | 959.6134 | y5        | 959.6134 | y1         | 175.1239 |
| y6        | 941.5656 | y6        | 941.5656 | y6        | 941.5656 | y1         | 175.1239 |
| y7        | 1232.7107 | y7       | 1232.7107 | y7        | 1232.7107 | y1         | 175.1239 |
| y8        | 1175.5277 | y8       | 1175.5277 | y8        | 1175.5277 | y1         | 175.1239 |
| y9        | 660.3055 | y9        | 660.3055 | y9        | 660.3055 | y1         | 175.1239 |
| y10       | 1315.8544 | y10      | 1315.8544 | y10      | 1315.8544 | y1         | 175.1239 |
| y11       | 1297.9136 | y11      | 1297.9136 | y11      | 1297.9136 | y1         | 175.1239 |

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