In Vitro Acetylation of HMGB-1 and -2 Proteins by CBP: the Role of the Acidic Tail†

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ABSTRACT: Histone acetyltransferases CBP, PCAF, and Tip60 have been tested for their ability to in vitro acetylate HMGB-1 and -2 proteins and their truncated forms lacking the C-terminal tail. It was found that these proteins were substrates for CBP only. Analyses of modified proteins by electrophoresis, amino acid sequencing, and mass spectrometry showed that full-length HMGB-1 and -2 were monoacetylated at Lys2. Removal of the C terminus resulted in (i) an increased incorporation of radiolabeled acetate within the proteins to a level close to that observed with histones H3/H4 and (ii) creation of a novel target site at Lys81. Acetylated and nonmodified HMGB-1 and -2 protein lacking the acidic tail were compared relative to their binding affinity to distorted DNA and the ability to bend linear DNA. Both proteins showed similar affinities to cisplatin-damaged DNA; the acetylated protein, however, was 3-fold more effective in inducing ligase-mediated circularization of a 111-bp DNA fragment. The alterations in the acetylation pattern of HMGB-1 and -2 upon removal of the C-terminal tail are regarded as a means by which the acidic domain modulates some properties of these proteins.

High-mobility group (HMG)[1] proteins 1 and 2 (recently renamed HMGB-1 and -2, see ref *1*) are the most thoroughly studied subgroup of HMG chromosomal proteins, the functions of which are still obscure. Their property to bend DNA and to bind preferentially to distorted DNA structures has led to the view that these non-sequence-specific DNA binding proteins act primarily as architectural elements, promoting the assembly of nucleoprotein complexes (*2*, *3*) and facilitating nucleosome disruption and remodeling (*4*). Surprisingly, work over the past few years demonstrated that these proteins also have functions outside the cell, related to inflammation and tumor growth and metastasis (*5*). The extracellular protein is regarded as a signal that communicates the death of the cell to its neighbors (*5*). The problems about the functions of HMGB-1 and -2 are further extended by the existence of postsynthetic modifications. Phosphorylation of some counterparts of these proteins from insects (*6*, *7*) and from higher plants (*8*) has been found to be essential for their proper folding and DNA binding specificity. Another modification of HMGB proteins is the reversible postsynthetic acetylation, demonstrated so far in vertebrate proteins only (*9*). Although this modification has been known for 25 years, the properties of the acetylated protein were studied very recently. We demonstrated that in vivo acetylation of HMGB-1 at lysine 2 significantly enhanced its affinity to distorted DNA structures (*10*). Acetylation in the presence of butyrate was generally attributed to histone acetyltransferase (HAT) activity (*9*). The discovery in the mid 1990s that this activity is an intrinsic property of some transcriptional adaptors led to the identification of many nuclear HATs, some of them acting also on non-histone proteins (*11*−*13*), including HMG-14 and -17 (*14*, *15*). Thus, our next aim was to study some known HATs for their ability to acetylate HMGB-1 and -2 in vitro, to map the target lysines, and to analyze the consequences for their DNA binding properties. Meanwhile, by testing a large panel of proteins as substrates for recombinant CBP, HMGB-1 was reported among the few that showed a positive result (*16*), but neither have the target lysines been mapped nor the properties of the modified protein studied. Recently, CBP was reported to acetylate the HMG-box containing architectural factor UBF both in vitro and in vivo (*17*). This paper presents our data on in vitro acetylation of rat HMGB-1 and -2 with CBP, the role of the C-terminal domain on the pattern of acetylation as well as some properties of the modified truncated protein.

EXPERIMENTAL PROCEDURES

Preparation of HMGB-1 and -2. Nuclei from Guerin ascites tumor cells were used to isolate the two proteins by a non-denaturing salt extraction procedure (*18*). Removal of

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[1] Abbreviations: CBP, CREB-binding protein; HAT, histone acetyltransferase; HMG, high-mobility group; HMGB-1 and -2, high-mobility group box-containing proteins 1 and 2; PAGE, polyacrylamide gel electrophoresis; PCAF, p300/CBP-associated factor; 0.5×TBE, 0.045M Tris−borate, 1 mM EDTA; Tip60, Tat-interactive protein, 60 kDa; trHMGB-1 and -2, truncated HMGB-1 and -2 proteins, lacking the C-terminal tail.

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the acidic C-terminal domain was performed by mild digestion with trypsin (Sigma, TPCK-treated, see ref *19*). Preparative isolation of the acidic C-terminal peptide was carried out as described (*20*). The purity of the final preparations of HMGB-1 and -2 and their truncated forms lacking the acidic tail (trHMGB-1 and -2) were controlled by SDS−polyacrylamide gel electrophoresis. Calf thymus histones H3 and H4 were purified from calf thymus nuclei (*21*).

Expression and Purification of Recombinant HATs. Expression constructs of the His-tagged HAT domain of human PCAF in pET28 and full-length CBP and Tip60 in pGEX2T vectors were expressed in modified *Escherichia coli* BL21 Poly Lys S cells. The cultures were grown at 37 °C to OD_{600} = 0.5, cooled to 20 °C, and induced to expression with 0.5 mM IPTG. After 15 h, the cells were collected by centrifugation and suspended in binding buffer (10 mM Tris-HCl, pH 7.5, 100 mM KCl, 10% glycerol, 0.5 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride) and the complete proteinase inhibitor mix (Boehringer, Mannheim). The cells were disrupted by sonication until OD_{600} reached 20% of its original value and centrifuged at 18000*g* at 4 °C for 30 min, and the supernatant was loaded on Ni-Sepharose or Glutathion-Sepharose columns for His and GST constructs, respectively. The columns were washed with 5 volumes of binding buffer and eluted with either a gradient of imidazole (0.1−0.5 M, 2 column volumes) or 10 mM glutathione.

HAT Assay. Filter binding assays were performed in 20 μL of reaction mixture as reported (*22*) using 70 pmol of [^{3}H]acetyl-CoA (3.5 mCi/mmol, Amersham Life Science Inc.); protein substrates (histone H3/H4, HMGB-1, HMGB-2, trHMGB-1, trHMGB-2, and lysozyme) were added to a concentration of 0.033 μg/mL. After incubation at 30 °C for 40 min, the reaction mixture was spotted onto a Whatman P-81 phosphocellulose filter, and following four washes with sodium carbonate buffer, pH 9.2, the filters were dried and counted in a liquid scintillation counter. For electrophoretic analysis the assay was done as above, except that 870 pmol of [^{14}C]acetyl CoA (570 mCi/mmol, Amersham Life Science Inc.) was used per reaction. The substrate concentrations varied in the range of 0.033−0.165 mg/mL. The reactions were stopped by the addition of an equal volume of gel sample buffer (100 mM Tris-HCl, pH 7.0, 0.2 M dithiothreitol, 2% SDS, 20% glycerol, and 0.1% bromophenol blue) and the proteins resolved by 15% SDS−PAGE at 1.5 V/cm. The gels were stained with Coomassie blue R250, dried, and autoradiographed. Electrophoresis in Triton−urea polyacrylamide gel was carried out as described (*23*) and, when necessary, the gels were scanned using a Gel-Pro analyzer.

Proteolytic Digestion with the Endopeptidase GluC (V8). Digestion with V8 (sequencing grade, Boehringer, Mannheim) was carried out at 37 °C for 5 min in a phosphate buffer, pH 7.8, at a protein/enzyme ratio (w/w) of 50:1 (*24*). The reaction was stopped by adding sample buffer for SDS−PAGE, and the proteins were analyzed by 18% SDS−PAGE.

Mapping of the Acetylated Sites in HMG-1 and -2 and Their Truncated Forms Lacking the C-Terminal Domain. Proteins were acetylated by CBP with non-radioactive acetyl-CoA (*22*) and separated by 15% SDS−PAGE; after staining with Coomassie blue, the appropriate bands were excised from the gel. In-gel digestion with trypsin was performed as described (*25*) with slight modifications. The slices were cut into small pieces, transferred into 0.25 mL polyethylene vials, and washed twice (15 and 30 min) with 150 μL of 10 mM NH_4HCO_3, pH 8.9. The supernatant was discarded, and the gel pieces were shrunk by dehydration in 150 μL of 50% acetonitrile−10 mM NH_4HCO_3. This step was repeated, and after removal of the liquid, the probe was dried in a vacuum centrifuge. The gel pieces were swollen in a buffer containing 10 mM NH_4HCO_3 and 0.05 μg/μL of trypsin (Roche, sequencing grade) and incubated for 20 min at 4 °C. The supernatant was removed and replaced with 20 μL of the same buffer without trypsin. Digestion continued overnight at 37 °C. Peptides were extracted successively with 50 μL of 10 mM NH_4HCO_3 and 50 μL of acetonitrile (37 °C, 10 min for each extraction). After collection of the supernatant, 100 μL of a solution of formic acid (10%), acetonitrile (20%), and 2-propanol (20%) was added to the gel pieces; following incubation for 10 min at 37 °C, the supernatant was added to the first one, dried to ∼5 μL, and stored at −20 °C. Peptides from the protein digests were separated using capillary HPLC connected on-line to a mass spectrometer. The solvent delivery system consisted of a Rheos 2000 pump connected with an ERC 3215α degasser (Flux Instruments, Karlskoga, Sweden) running at a flow of 80 μL/min, which was reduced to a column flow of ∼500 nL/min using a precolumn tee split. A fused silica microcapillary column (100 μm i.d. × 365 μm o.d.) was pulled with a model P-2000 laser puller (Sutter Instrument Co., Novato, CA) and packed with 7 cm/5 μm reverse-phase C_{18} material. The gradient (solvent A, 0.1% formic acid; solvent B, 0.1% formic acid in 85% acetonitrile) started at 0% B. The concentration of solvent B was maintained at 0% for 5 min and then increased linearly from 0 to 60% for 40 min and from 60 to 100% for 10 min. Mass spectral analysis of peptides was performed using an LCQ ion trap instrument (ThermoFinnigan, San Jose, CA) equipped with a nanospray interface. The nanospray voltage was set at 1.6 kV, and the heated capillary was held at 1700 °C. MS/MS spectra were searched against the Swiss−Prot database using SEQUEST (LCQ BioWorks, ThermoFinnigan).

Amino Acid Sequence Analysis. Sequencing was performed on an Applied Biosystems Inc. (ABI) model 492 Procise protein sequenator. Samples were sequenced from a Biobrene-treated glass fiber filter and, typically, 12 amino acid residues were determined.

Ligase-Mediated Circularization Assay. The DNA probe used was a 111-bp fragment obtained by *Bam*H1 and *Pvu*II digestion of pUC19. The probe was labeled at the protruding 5′ termini with [α-^{32}P]dCTP and a Klenow enzyme (Promega). The assay generally followed a procedure described elsewhere (*26*). After termination of ligation, some reaction mixtures were treated with exonuclease III (Boehringer, Mannheim) at 37 °C for 30 min with 30 units per 10 μL. All samples were made 1% in SDS, 4% in sucrose, and 0.25% in bromphenol blue, digested with protease K (1 μg, 37 °C, 1 h), run on 5% polyacrylamide gel (29:1 acrylamide/*N*,*N*′-methylenbisacrylamide) in 0.5×TBE buffer (0.045 M Tris−borate and 1 mM EDTA, pH) at 10 V/cm, vacuum-dried, and autoradiographed. Generated circles were identified in the gel by their resistance to exonuclease III digestion. The band density of the monomer DNA circles was quantified after scanning with a Gel Pro analyzer.

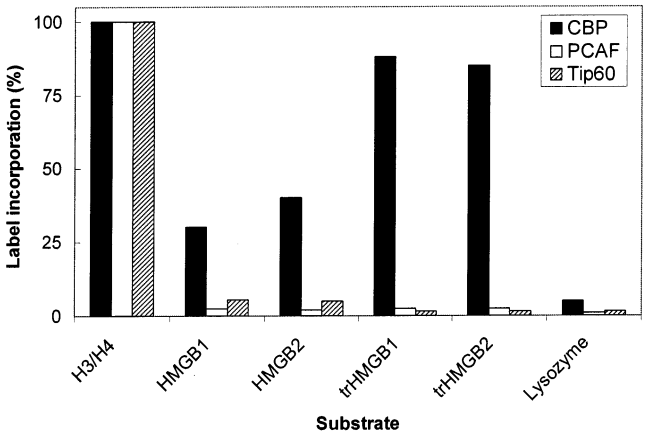


FIGURE 1: In vitro acetylation of HMGB-1 and -2 proteins and their truncated forms lacking the C-terminal domain (trHMGB-1 and -2) by CBP, PCAF, and Tip60. Data are from a filter-binding assay using [^{3}H]acetyl-CoA.

Electrophoretic Mobility Shift Assay. The analysis was carried out as described previously (*27*) using a ^{32}P-labeled 40-bp oligonucleotide, site-specifically platinated at a single GpG inserted in a restriction site for *Bam*H1 endonuclease (*8*).

RESULTS

CBP Acetylates HMGB-1 and -2 Proteins and Their Truncated Forms Lacking the C-Terminal Domain. Three known HATs were tested for their ability to acetylate HMGB-1 and -2 proteins and their truncated forms lacking the acidic tail (trHMGB-1,-2): the His-tagged HAT domain of human PCAF and full-length CBP and Tip60. Highly purified histones H3/H4 from calf thymus and lysozyme were included in the assays as positive and negative controls, respectively. The reaction mixtures contained equivalent amounts of PCAF, CBP, and Tip60 as normalized by filter binding assay using histone H3/H4 as substrates. The ability of these HATs to specifically acetylate the above proteins was tested by the enzyme-dependent incorporation of radioactive acetate. Radiolabeling was analyzed by both filter binding assay with reaction mixtures containing [^{3}H]acetyl-CoA (Figure 1) and SDS−PAGE of proteins incubated in the presence of [^{14}C]acetyl-CoA (Figure 2). The results summarized in Figure 1 show that among the different HATs used, only CBP specifically acetylates HMGB-1 and -2 and their truncated forms. As expected, the highest amount of the radiolabel was incorporated in histones H3/H4; surprisingly, the radioactivity of trHMG-1 and -2 is lower but close to that of H3/H4. As for the full-length proteins, they incorporated less acetate than truncated molecules, HMGB-2 being a slightly better substrate than HMGB-1. The lack of radioactivity on the filter with lysozyme, a protein with a high lysine content, together with the lack of radioactivity in the proteins incubated with [^{3}H]acetyl-CoA in the absence of enzyme (not shown) favors the conclusion that CBP specifically acetylates HMGB-1 and -2 and their truncated forms. Another inference from the filter binding experiment is that removal of the C-terminal tail sharply increases the acetylation. The two conclusions were further justified when the proteins from the HAT assay were resolved on SDS−polyacrylamide gels and visualized with Coomassie blue and with autoradiography (Figure 2). As seen, much higher

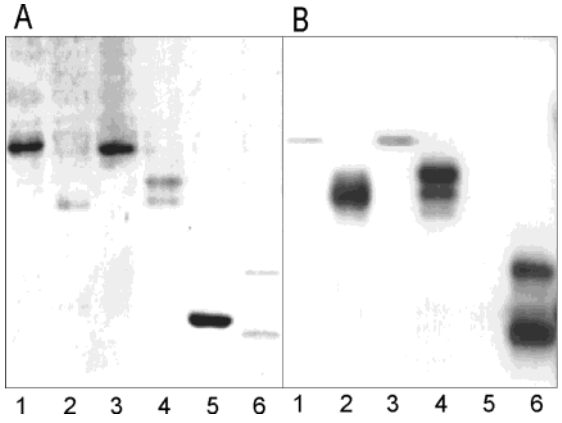


FIGURE 2: Specific acetylation of HMGB-1 and -2 and trHMGB-1 and -2 proteins by CBP as demonstrated by SDS−PAGE: (A) protein gel stained with Coomassie blue; (B) corresponding autoradiograph, representing the incorporation of [^{14}C]acetate. Substrates used are HMGB-1 (lane 1), trHMGB-1 (lane 2), HMGB-2 (lane 3), trHMGB-2 (lane 4), lysozyme (lane 5), and histone H3/H4 (lane 6).

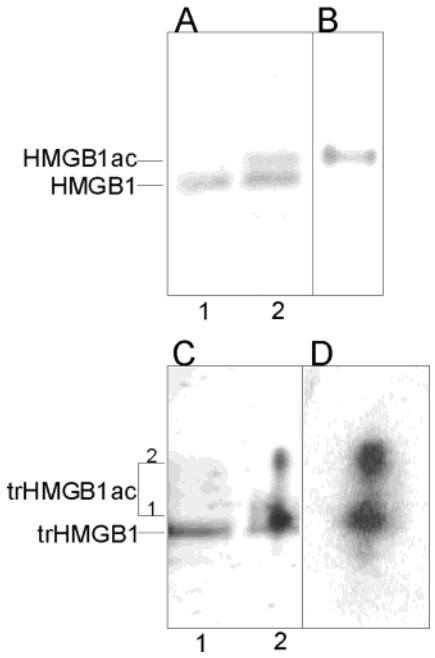


FIGURE 3: Electrophoresis in 18% polyacrylamide gel containing Triton−urea−acetic acid: (A) Coomassie blue-stained protein gel of nonmodified (lane 1) and CBP-acetylated full-length HMGB-1 (lane 2); (B) corresponding autoradiograph, showing the incorporation of [^{14}C]acetate; (C) Coomassie-stained protein gel of nonmodified (lane 1) and CBP-acetylated trHMGB-1 (lane 2); (D) corresponding autoradiograph.

amounts of HMGB-1 and -2 proteins as compared to those of trHMGB-1 and -2 incorporated much less radioactivity (compare lines 1 with 2 and lines 3 with 4 in Figure 2A,B).

Acetylated HMGB-1 and -2 and trHMGB-1 and -2 were separated on Triton−acetic acid−urea polyacrylamide gels. This system was successfully used for the separation of acetylated species of core histones (*23*) and, very recently, for the identification of monoacetylated HMGB-1 isolated from butyrate-treated cells (*10*). Coomassie blue staining of acetylated HMGB-1 (Figure 3A, lane 2) revealed two bands: one that comigrated with the parental molecules (Figure 3A, lane 1) and one with retarded migration, which

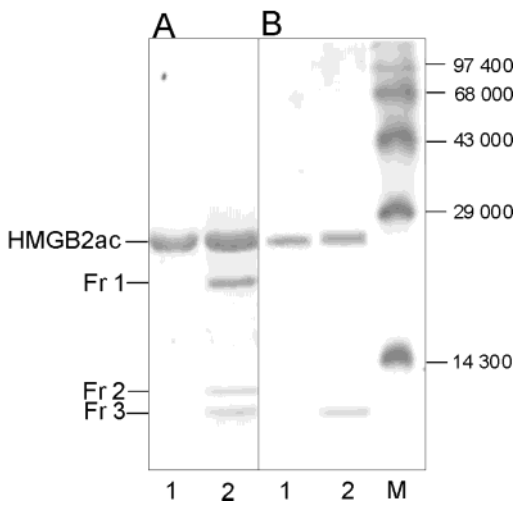


FIGURE 4: GluC−protease digestion pattern of CBP-acetylated full-length HMGB-2 as revealed by 18% SDS−PAGE: (A) Coomassie blue-stained protein gel of control, nondigested (lane 1) and protease-digested protein (lane 2) [digestion peptides obtained are marked Fr1 (m.m. 18 kDa), Fr2 (m.m. 13 kDa), and Fr3 (m.m. 11.5 kDa)]; (B) corresponding autoradiographs, showing the distribution of incorporated [^{14}C]acetate among digestion products. M, radiolabeled molecular mass marker proteins (Da).

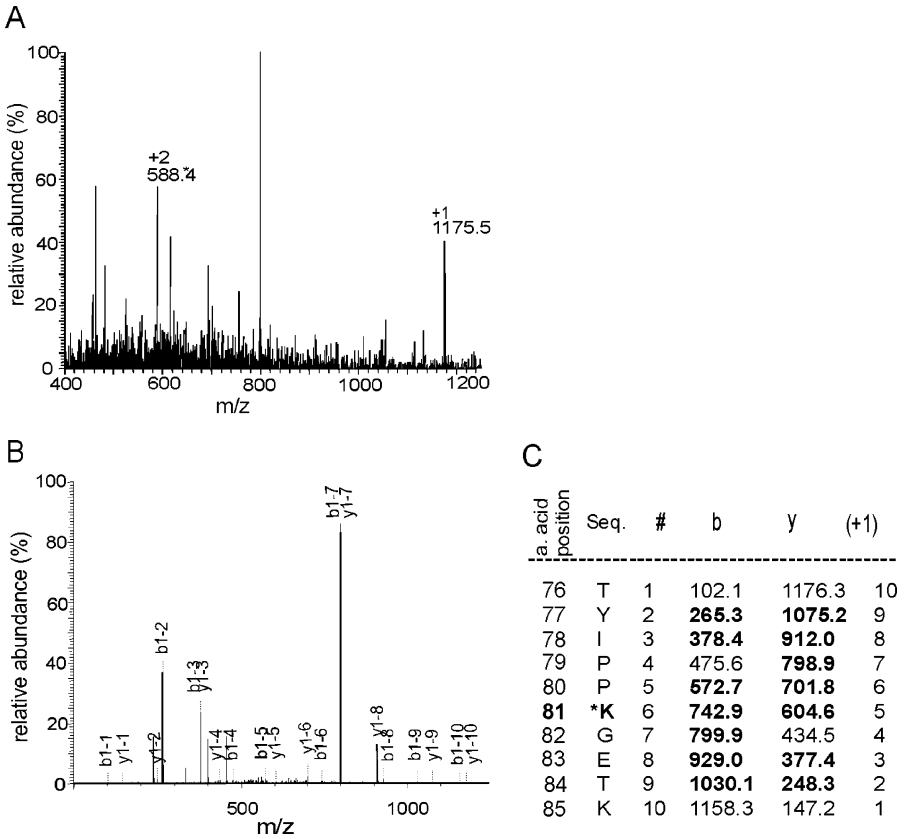
we attribute to acetylated molecules by analogy with the pattern of in vivo acetylation (*10*). The autoradiograph confirmed the existence of a single band of a monoacetylated protein (Figure 3B, lane 2). Acetylated trHMGB-1, however, appeared as two bands, both with retarded migration relative to the nonmodified molecules (Figure 3C). The same pattern was observed on the autoradiograph (Figure 3D). Similar results were obtained with both HMGB-2 and trHMGB-2 (not shown). These data suggest the existence of two acetylated forms of the truncated protein: mono- and diacetylated. If so, it should have been modified at two lysine residues. Mapping the sites of acetylation by both mass spectrometry and amino acid sequencing confirmed this assumption (see below).

Monoacetylation of full-length HMGB-2 is predicted by the experiments with controlled digestion of the modified protein with the protease V8 (Glu-C). An early time-course digestion study has demonstrated (*24*) that treatment of HMGB-1 and -2 with the protease results in a restricted pattern of three peptides, consisting of amino acids 1−73 (m.m. 11 500), 74−185 (m.m. 13 000), and 74−213 (m.m. 18 000). Glu-C digestion profiles of HMGB-2 as revealed by Coomassie blue staining and autoradiography are shown on Figure 4. Three digestion fragments are seen on the protein gel (panel B, lane 2) and only one on the autoradiograph: the band of the peptide with m.m. 11 500, representing amino acids 1−73. These data are confirmed by mass spectrum and amino acid sequence analyses (see below).

HMGB-1 and -2 Proteins Are Acetylated at Lys2; Removal of the C-Terminal Tail Creates an Additional Acetylation Site at Lys81. An early work on in vivo acetylation of HMGB-1 protein showed two acetylation sites: a major one at Lys2 and a minor site at Lys11 (*9*). Our recent study with acetylated HMGB-1 purified from butyrated cells confirmed the modification of Lys2 (*10*). To identify the acetylated lysines upon in vitro acetylation with CBP of full-length and truncated HMGB-1 and -2, the modified proteins were subjected to mass spectrum and sequence analyses. After prior purification of the proteins by running in SDS gels, the bands of interest were excised and in-gel-digested with trypsin. Mass spectrum analysis of trypsin-generated peptide containing the modification revealed an acetylation of lysine 81 in the truncated form (Figure 5) but not in the full-length protein (see the above experiments with Glu-C digested HMGB-2). It turned out, however, that short peptides from the N-terminal region have not been efficiently extracted. For this reason, amino acid sequencing was carried out with proteins, purified by reverse phase chromatography. The analysis was performed for the first 12 amino acids and showed an acetylation of Lys2 in both full-length and truncated HMGB-1 and -2. Concerning the acetylation of Lys81 in the truncated protein but not in the full-length molecule, a plausible explanation of this finding is an exposure of the residue to the enzyme due to tail removal. An alternative explanation, however, cannot be excluded: the free acidic tail interacts with CBP, and the complex formed impedes the enzyme action, the effect being much more pronounced toward Lys81 than toward Lys2. If so, the acetylation of Lys81 upon tail removal should not reflect changes in the HMGB-1 and -2 molecules, leading to an increased accessibility to CBP. To check this, the C-terminal peptide was cut from HMGB-1, and after purification, increasing amounts of the peptide were incubated with CBP under conditions of the HAT assay. Truncated HMGB-1 was then acetylated with CBP alone and with CBP, preincubated with the acidic tail. The two assays showed similar incorporations of [^{3}H]acetate (not shown), thus ruling out the second possibility.

CBP-Induced Acetylation of HMGB-1 and -2 Proteins Lacking the C-Terminal Tail Increases Their Ability To Bend Linear DNA but Has No Effect on the Binding Affinity to Distorted DNA Structures. The fundamental properties of HMG box-containing proteins are to bind preferentially to bent DNA and to induce bending in linear DNA. Experiments were designed to see how in vitro acetylation by CBP influences these propeties. The DNA-binding affinity of the acetylated proteins was assayed with site-specifically platinated 40-bp synthetic oligonucleotide by using mobility shift analysis, whereas their ability to bend DNA was analyzed by a DNA circularization assay. The truncated HMGB-1 and -2 proteins, both acetylated and unmodified, demonstrated comparable binding affinities to that of cisplatinated DNA (not shown). Acetylation of trHMGB-1 and -2, however, enhanced three times the DNA bending ability as judged by the ligase-mediated circularization of a 111-bp fragment (Figure 6, compare lanes 2 and 4 and lanes 3 and 5).

The effect of in vitro acetylation on the binding to platinated oligonucleotide was investigated also with full-length HMGB-1 and -2. The affinity of the acetylated molecules was 1.5−2 times higher than that observed with the control parental protein (not shown). In a previous work, the same experiments were performed with in vivo acetylated HMGB-1 isolated from butyrated cells. The modified protein, also monoacetylated at Lys2, showed an at least 6-fold higher affinity as compared with the nonmodified protein (*10*). The observed differences in the binding affinities of the two acetylated proteins we atrribute to the fact that the experiments with in vivo acetylated HMGB-1 were carried out with



a. acid position	Seq.	#	b	y	(+1)
76	T	1	102.1	1176.3	10
77	Y	2	**265.3**	**1075.2**	9
78	I	3	**378.4**	**912.0**	8
79	P	4	475.6	**798.9**	7
80	P	5	**572.7**	**701.8**	6
81	***K**	6	**742.9**	**604.6**	5
82	G	7	**799.9**	434.5	4
83	E	8	**929.0**	**377.4**	3
84	T	9	**1030.1**	**248.3**	2
85	K	10	1158.3	147.2	1

FIGURE 5: Mass spectrum identification of the acetylated lysine in trHMGB-1: (A) mass analysis of the peptide (generated upon tryptic in-gel digestion of trHMGB-1) containing the modified lysine [calculated mass = 1174.6; peak sequenced by MS/MS is labeled (*)]; (B) MS/MS spectrum of the double-charged peptide ion at *m*/*z* 588.4. Assignment of fragment masses to b- and y-ions was done following identification of the peptides by SEQUEST. The deduced sequence is shown in panel C. *K = acetylated lysine.

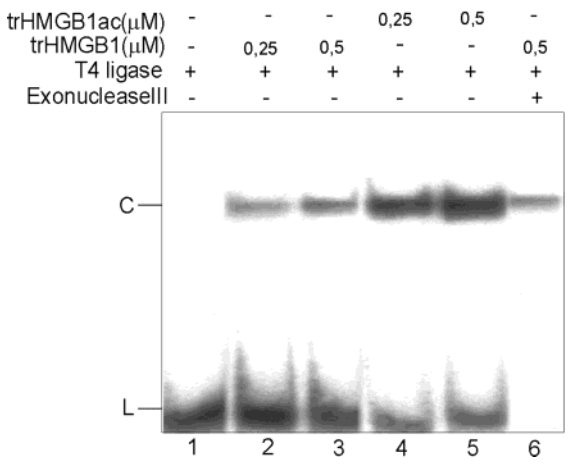


FIGURE 6: DNA circularization assay in the presence of trHMGB-1: effect of acetylation by CBP. DNA has been fractionated by 5% PAGE. L, C, linear and circular forms, respectively, of a ^{32}P-labeled 111-bp fragment obtained by *Bam*H1 and *Pvu*II digestion of pUC19. Compare lanes 2 and 4 and lanes 3 and 5.

highly purified protein, containing negligible amounts of unmodified protein (*10*), whereas CBP-treated HMGB-1 contained ~30% acetylated molecules as determined from the pattern of acetylation, shown in Figure 3A, lane 2.

DISCUSSION

This work presents our experiments on in vitro acetylation of HMGB1 and -2 proteins and communicates data on (i) the identification of both the acetylating enzyme and the target lysines and (ii) the role of the acidic C-terminal domain in modulating the acetylating pattern of these proteins.

The earlier identification of HMGB-1 as a substrate of CBP among many tested proteins (*16*) together with our present observations that HMGB-1 and -2 and their truncated forms are acetylated by CBP and not by PCAF and Tip60 testifies to the specificity of CBP-mediated modification of these proteins in vitro. Their acetylation by CBP within the cell remains to be investigated. The demonstration that in vivo acetylated HMGB-1 is modified at Lys2 (*9*, *10*) and that the same lysine residue is a target site upon in vitro acetylation with CBP (this paper) motivates such a study.

The finding that acetylations of HMGB-1 and -2 in vivo in butyrated cells (*10*) and in vitro by CBP are targeted to a common site (lys2) means that the two acetylation procedures will equally influence the properties of the protein. Indeed, this has been demonstrated for the binding affinity to ciplsatin-damaged DNA. The advantage of the acetylation by CBP is the possibility of raising acetylated molecules of HMGB-1 and -2 lacking the acidic tail. CBP acetylated the truncated protein at two sites: Lys2 and Lys81. The modification had no effect on the binding affinity to cisplatin-damaged DNA but resulted in a 3-fold increase of the DNA bending ability as demonstrated by a ligase-mediated circularization of a linear DNA fragment. One explanation of the latter finding might be the modification of Lys81, located in close proximity to box B, supposed to be responsible for the DNA bending ability of HMGB-1 and -2 (*28*, *29*). Whatever the mechanism of this effect, it is due to tail removal.

The general function of the C-terminal domain of HMGB-1 and -2 is not clear despite its numerous effects on the properties of these proteins. It reduces the DNA bending ability (*30*) as well as the affinity of HMG boxes for both linear DNA (*19*, *30*, *31*) and distorted DNA structures (*27*, *32*, *33*) with little effect on the affinity to DNA minicircles (*30*). This domain has been found to be essential for the formation of stable complexes with other proteins (*34*). The effects on DNA binding affinity were generally attributed to contacts with A and B boxes or to charge repulsion (*3*, *35*) without any ideas about the intimate mechanisms by which tail removal affected the properties of the protein. The data described in this paper suggest a mechanism operating via the acetylation pattern of the protein. We showed that tail removal created an additional target site at Lys81. Moreover, an increase of the overall level of acetylation has also been observed (see Figure 1). Concerning the latter finding, our experiments do not say whether it is due solely to the additional site or if more frequent acetylation at Lys2 is also taking place. The exposure of Lys81 for acetylation upon removal of the C terminus agrees well with the hypothetical tertiary model structure of HMGB-2 (*36*) derived from the HMG box B structure, determined by NMR spectroscopy (*37*, *38*). According to this model, the acidic domain is expected to interact with the linker region between the two boxes (75−88 amino acid residues) or the carboxyl-flanking sequence of box B (*36*). Our data are consistent with the first possibility: removal of the C-terminal tail interrupted its contacts with the region between A and B boxes, thus creating favorable steric conditions for acetylation of Lys81. Thus, it seems reasonable to suggest that the elimination of the highly charged C terminus makes the remaining portion of the molecule more accessible to acetylation by CBP. In this context it is worth remembering that the earliest report on acetylation of HMGB-1 protein claimed an increased susceptibility to deacetylating activity of HMGB-1 lacking this domain (*9*). The enhanced accessibility of truncated proteins to acetylating−deacetylating enzymes, respectively, the alterations in their acetylation patterns, we regard as a mechanism by which the acidic domain may modulate the DNA bending properties of these proteins and, probably, their interactions with other proteins. Moreover, tail removal may influence the pattern of other postsynthetic modifications of HMGB-1 and -2. The question is whether such a mechanism is operating within the cell. The isolation from tissue homogenates of a degradation product of HMGB-1 that lacks the acidic tail (*39*) and the identification of a trypsin-like chromatin-bound protease that cleaves HMGB-1 (*40*) favor the possibility for intracellular elimination of the C terminus and support such a view.

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