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Interplay between in vitro acetylation and phosphorylation of tailless HMGB1 protein

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ABSTRACT

The postsynthetic acetylation of HMGB1 protein and its truncated form affects significantly its properties as "architectural" factor – recognition of bent DNA and bending of short DNA fragments. We created mutants at the target sites (lysines 2 and 81) in the tailless HMGB1 modified by the histone acetyltransferase CBP. The results show that there is no preferential site for the enzymatic activity of CBP and both lysine moieties are modified independently. Our findings for the first time demonstrate the link between the acetylation and phosphorylation of HMGB1ΔC in vitro. The PKC phosphorylation prior to acetylation inhibits the CBP activity 40–60% for the truncated form and its mutants. The effect of the CBP acetylation on the phosphorylation level turns out to be much more prominent. In the case of HMGB1ΔC modified at Lys 2 and Lys 81 prior to PKC treatment background phosphorylation is detected. If only one of the lysines is modified the inhibitory effect decreases.

The high mobility group box proteins -1 and -2 (HMGB1 and HMGB2, formerly HMG1 and HMG2, see [1]) are nonhistone chromosomal proteins with still unknown functions despite of their abundance and ubiquity and long-standing intensive studies. The ability of these proteins to bend DNA and to bind preferentially to distorted DNA structures [2–4] assigns them the role of "architectural" factors promoting the assembly of nucleoprotein complexes [5]. These peculiar properties of HMGB1 and 2 were shown to be modulated by the acidic C-terminal domain of the molecule [6–8] as well as by some postsynthetic modifications of the proteins such as phosphorylation [9,10] and acetylation [7,11,12]. The inhibitory effect of HMGB1 protein in DNA repair and replication was reduced in the presence of its acetylated form and completely abolished when the assay was performed with the truncated protein [8,13], which confirmed the essential regulatory role of the acetylation and the C tail. Regarding acetylation of HMGB1, although known for about three decades [14], studies on the properties of the acetylated protein started much later. In vivo monoacetylated protein modified at Lys 2 isolated from butyrate-treated cells showed enhanced binding affinity to distorted DNA structures [11] together with a loss of its ability to bend DNA. The in vivo modified protein acquired the property to stimulate joining of short DNA fragments via their ends [12]. The truncated HMGB1 lacking the acidic tail and acetylated at Lys 2 also enhanced the end-joining of DNA fragments. Upon additional acetylation by the histone acetyltransferase CBP (CREB-binding protein) which acted only on the truncated form, an acetyl group was introduced at Lys 81 which restored the bending properties of the protein [7]. Thus the acetylation pattern of the protein appeared to serve as a fine modulator of its "architectural" abilities.

In this study we created recombinant full length and truncated HMGB1 proteins lacking the acidic tail bearing mutations at the acetylation sites – Lys 2 or/and Lys 81. Our aim was to investigate the influence of the different lysine moieties on the CBP acetylation level in vitro as well as the interdependence of in vitro Protein Kinase C (PKC) phosphorylation and acetylation of the truncated HMGB1 protein. The results provided the first information concerning the relation between the two modifications.

Materials and methods

Polymerase chain reaction (PCR). To prepare recombinant HMGB1 protein and HMGB1 lacking its acidic tail (HMGB1ΔC), cDNA encoding full-length rat HMGB1 (lib.N 961, RZPD) was amplified by PCR using primers introducing EcoR1 and XhoI cloning sites: forward 5′-TGCACTGGAATTCATGGGCAAAGGAGATCC-3′ and reverse 5′-CAGTGCACTCGAGTTATTCATCATCATCATCTTC-3′ for the full length protein, and forward 5′-TGCACTGGAATTC ATGGGC AAAGGAGATCC-3′ and reverse 5′-CTTCTTTTTCTTGCTTTTTTCAG CCTTG-3′ for the truncated form. For the mutated recombinant tailless proteins the following primers were used bearing the appropriate mutations: for HMGB1ΔCK2/A2 straight: 5′-TGCACTG GAATTCATGGGCGCAGGAGATCC-3′ and reverse 5′-CAGTGCACTCG AGCTTCTTTTTCTTGCTTTTTTCAGCC-3′ and for HMGB1 ΔCK81/A81 several successive PCRs were carried out. First the DNA coding

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amino acid sequences from 1 to 86 was amplified with fore primer bearing EcoR1 restriction site (5′-TGCACTGGAATTCATGGGCAAAG GAGATCC-3′) and a reverse one bearing a mutation at Lys 81 (5′-CTTTTTTGTTTCCCCTGCGGGAGGGATATAGGT-3′). In a second PCR a DNA fragment coding amino acids from 74 to 186 was synthesized by a fore primer introducing a mutation at Lys 81 (5′-ACCTATATCCCTCCCGCAGGGGAAACAAAAAAG-3′) and a reverse one with a Xho1 restriction sequence (5′-CAGTGCACTCGAGCTTCTTTTTCTT GCTTTTTTCAGCC-3′). The double mutant HMGB1ΔCK2/A2K81/A81 was created as above except that for the amplification of the first fragment (1–86 a.a.) a fore primer bearing a mutation at Lys 2 and EcoR1 restriction site was used (5′-TGCACTGGAATT CATGGGCGCAGGAGATCC-3′). The PCR was carried out in a 50 μl final volume using QB-96 thermocycler (LKB). The reaction mixture contained: 0.125 mM $MgCl_2$, 0.25 mM dNTP, 2 μM of the appropriate primers, 50 ng matrix DNA, 2 U Taq polymerase (Pharmacia) and 5 μl 10× PCR buffer. The purity of all DNA preparations was checked by agarose gel electrophoresis and the accuracy of the obtained constructs was proved by sequencing analysis on ABI PRISM 3100 Genetic Analyzer.

Expression of recombinant proteins. The PCR products were treated with the restriction enzymes EcoR1 and XhoI and cloned in an expression vector pET28a+ and expressed in modified *Escherichia coli* BL21 Poly Lys S. His-tagged protein samples were purified on a HIS-Select HF Nickel Affinity gel (Sigma). Before His-tag removal the proteins were loaded on the Nickel Affinity gel equilibrated with 20 mM Tris–HCl, pH 7.9 containing 60 mM imidazole, 50 mM NaCl and the slurry was washed with thrombin cleavage buffer (20 mM Tris–HCl, pH 8.4, 150 mM NaCl, 2.5 mM $CaCl_2$) supplemented with 0.5 U of thrombin (human plasma, Calbiochem) per milligram recombinant protein and incubated for 4 h. at room temperature. After brief centrifugation the supernatant containing the His-tag depleted protein was collected and used for further experiments. The recombinant histone acetyltransferase CBP was prepared as in [7]. The in vivo acetylated and non modified native HMGB1 proteins were isolated from ascites Guerin tumor cells as in [11]. The purity of all protein preparations was checked by polyacrylamide gel electrophoresis containing sodium dodecyl sulfate (SDS–PAGE).

Successive in vitro acetylation and phosphorylation of truncated HMGB1 protein and its mutated forms. The acetylation by CBP was done as in [7]. The protein samples were incubated at 30 °C for 60 min. in 30 μl buffer containing 50 mM Tris–HCl (pH 8), 10% glycerol, 1 mM DTT, 1 mM PMSF, 10 mM sodium butyrate, 1 mM acetyl CoA (AppliChem) and 60 pmol of each protein. The reaction was started by adding recombinant acetyltransferase CBP. The mock acetylation was carried out with a heat inactivated enzyme. The probes were precipitated with 20% final concentration of three chloroacetic acid (TCA) at −20 °C for 16 h., spin at 15,000 rpm, the pellet was washed several times with cold acetone and dissolved in 5 μl TE (pH 8). The phosphorylation reaction was performed with 0.5 U Protein Kinase Cα (human, recombinant, *Spodoptera frugiperda,* Calbiochem) using Protein kinase C Biotrak enzyme assay system (Amersham, Biosciences) adding 10 nmols [γ^{32}-P] ATP (3000 Ci/mmol) and incubated at 30 °C for 60 min. The samples were analyzed by SDS–PAGE, stained with Coumassie R250 (Sigma), the gel was dried and exposed to KODAK XAR-5 film and quantified by Gel Pro analyzer.

Successive in vitro phosphorylation and acetylation of truncated HMGB1 protein and its mutated forms. Recombinant truncated HMGB1 protein and its mutant forms (60 pmol) were phosphorylated with 0.5 U Protein Kinase Cα (human, recombinant, *S. frugiperda,* Calbiochem) using Protein kinase C Biotrak enzyme assay system (Amersham, Biosciences) at 30 °C for 30 min. in a final volume of 25 μl, precipitated with TCA (20% final concentration) at −20 °C for 16 h, spin at 15,000 rpm, the pellet was washed several times with cold acetone and dissolved in 5 μl TE (pH 8). The mock phosphorylation was carried out with heat inactivated enzyme. The acetylation reaction with CBP was performed as above only that 100 pmol of [^{14}C] acetyl CoA (Amersham Biosciences) was used. The samples were run on 18% polyacrylamide gel containing SDS, stained with Coumassie R250 (Sigma), soaked in Amplifier (Amersham Biosciences), dried and exposed to KODAK XAR-5 film and quantified by Gel Pro analyzer.

Results

Mutagenesis at the postsynthetic acetylation sites of the nonhistone protein HMGB1 lacking its C terminus

By site directed mutagenesis we generated three mutant tailless HMGB1 proteins schematically presented at Fig. 1. The protein preparations bear mutation at the sites of acetylation proved to be modified by the histone acetyltransferase CBP [7]. The Lys 2 and/or Lys 81 were replaced by alanine.

All mutant proteins were further tested for the level of CBP acetylation compared to the recombinant HMGB1ΔC and how the different acetylated lysine(s) influence the phosphorylation by PKC and vice versa.

In vitro acetylation of HMGB1ΔC and its mutants by CBP

The ability of the histone acetyltransferase CBP to acetylate specifically the truncated HMGB1 lacking the C tail and the respective mutants was estimated by the incorporation of [^{14}C] acetyl CoA. Upon completion of the enzymatic reaction the modified protein samples were separated on a polyacrylamide gel containing SDS (Fig. 2 B, upper part) and the respective autoradiograph presented (Fig. 2 B, bottom part). The histogram corresponded to quantification relied on gel-based assay (Fig. 2 A). The highest amount of label was incorporated in the recombinant HMGB1ΔC accepted relatively as 100% acetylated sample. The truncated HMGB1 pro-

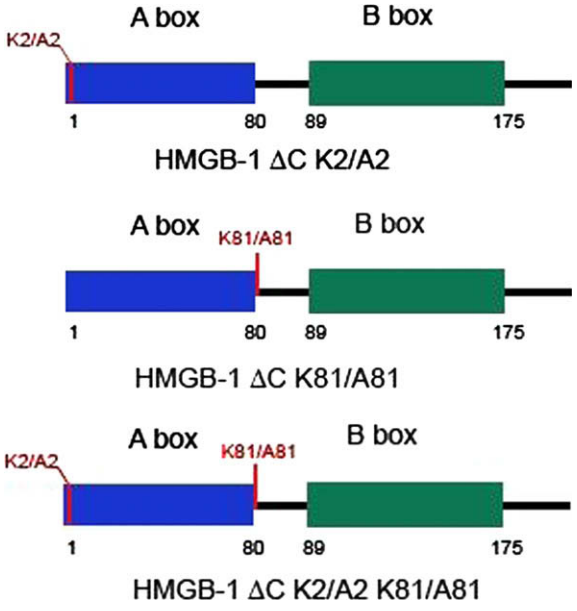

Fig. 1. Schematic presentation of the HMGB1 protein mutants lacking the C terminus. The constructs for the recombinant truncated HMGB1 protein (HMGB1ΔC) and the respective mutants were created by PCR using appropriate primers (see Material and methods). The lysine moieties acetylated by CBP were substituted to alanine and marked in red: Lys 2 (HMGB1ΔCK2/A2), Lys 81 (HMGB1ΔCK81/A81) and both Lys 2 and Lys 81 (HMGB1ΔCK2/A2K81/A81).

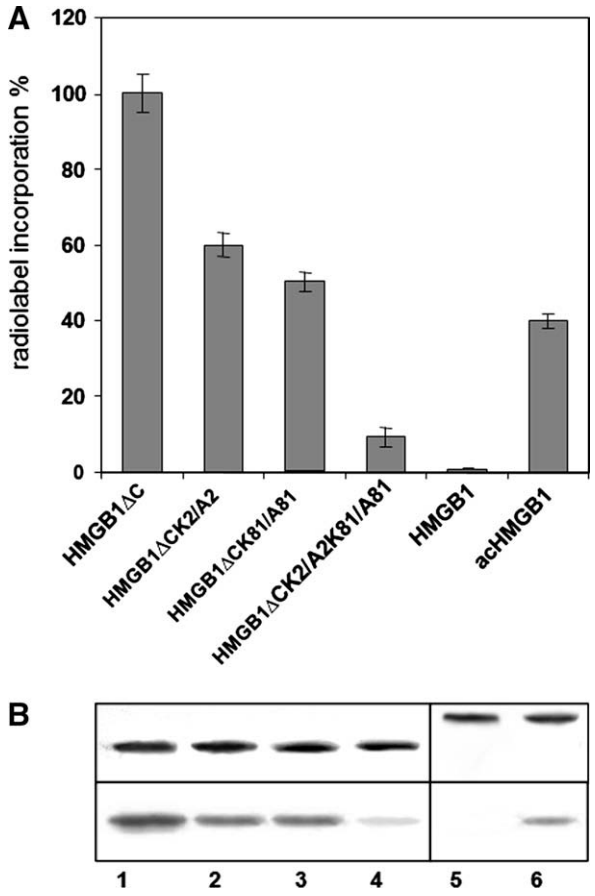

Fig. 2. The acetylation level of the truncated HMGB1 protein and its mutant forms. The protein samples are subjected to acetylation by CBP using [^{14}C] acetyl CoA, run on a 18% polyacrylamide gel containing SDS and stained with Coumassie (B, upper part), dried and exposed to KODAK XAR-5 film and the corresponding autoradiograph presented (B, bottom part). Lanes 1–6; truncated HMGB1 protein (HMGB1ΔC), truncated HMGB1 protein mutated at Lys 2 (HMGB1ΔCK2/A2), truncated HMGB1 protein mutated at Lys 81 (HMGB1ΔCK81/A81), truncated HMGB1 protein mutated at Lys 2 and Lys 81 (HMGB1ΔCK2/A2K81/A81), full length HMGB1 protein isolated from Guerin ascite tumor cells (HMGB1), and in vivo acetylated HMGB1 protein isolated from Guerin ascite tumor cells in the presence of butyrate (acHMGB1), respectively. The data from gel-based quantification assay are shown on (A).

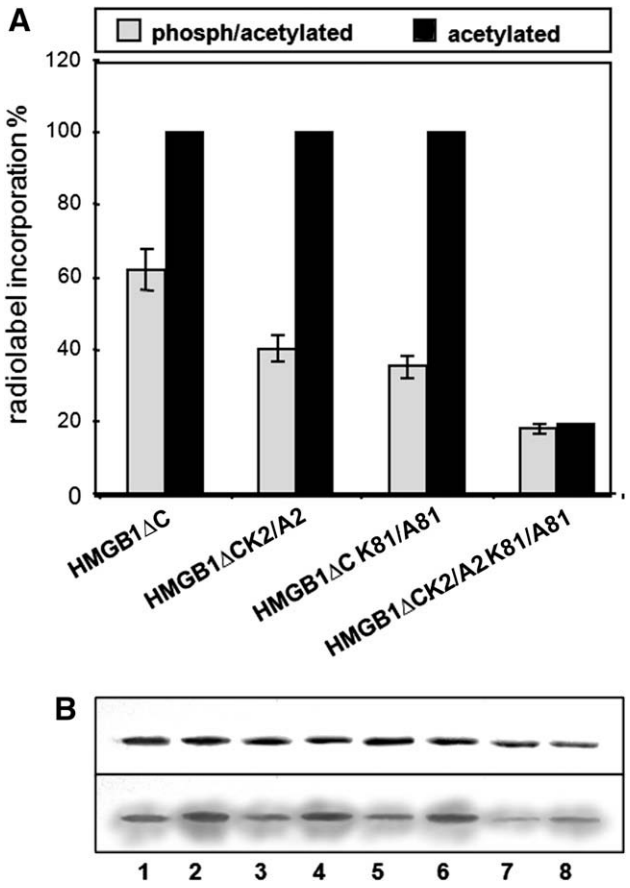

Fig. 3. The effect of PKC phosphorylation on the CBP acetylation level of the truncated HMGB1 protein and its mutant forms. The protein samples are subjected to PKC phosphorylation using cold ATP prior to CBP acetylation with [^{14}C] acetyl CoA, run on a 18% polyacrylamide gel containing SDS and stained with Coumassie (B, upper part), dried and exposed to KODAK XAR-5 film and the corresponding autoradiograph presented (B, bottom part). Lanes 1, 3, 5, and 7 represent truncated HMGB1 protein (HMGB1ΔC), truncated HMGB1 protein mutated at Lys 2 (HMGB1ΔCK2/A2), truncated HMGB1 protein mutated at Lys 81 (HMGB1ΔCK81/A81), and truncated HMGB1 protein mutated at Lys 2 and Lys 81 (HMGB1ΔCK2/A2K81/A81) PKC phosphorylated followed by acetylation with CBP, respectively. Lanes 2, 4, 6, and 8 represent truncated HMGB1 protein (HMGB1ΔC), truncated HMGB1 protein mutated at Lys 2 (HMGB1ΔCK2/A2), truncated HMGB1 protein mutated at Lys 81 (HMGB1ΔCK81/A81), and truncated HMGB1 protein mutated at Lys 2 and Lys 81 (HMGB1ΔCK2/A2K81/A81) acetylated by CBP, respectively. The data from quantification gel-based assay are shown on (A).

tein preparations bearing mutations at the sites of CBP acetylation (Lys 2 or Lys 81) were modified at similar extent – 40–50% less than the non mutant tailless protein while the double mutant HMGB1ΔCK2/A2K81/A81 showed background level of label incorporation.

As previously shown [7] the enzyme CBP effectively acetylated HMGB1 protein only when its acidic C terminus was removed. One probable explanation is the fact that the long C-tail forms a flexible structure that shields some residues within and between the HMG boxes [15]. In some cases the in vivo acetylated at Lys 2 HMGB1 protein mimics the properties of the tailless one [8,13] which motivated us to use the in vivo acetylated HMGB1 as a substrate for the histone acetyltransferase CBP. The label incorporation level was compared to this of non modified native HMGB1 (Fig. 2 B, compare lane 5 with lane 6). It is clearly seen that the presence of an acetyl group at position 2 in the full length HMGB1 protein resulted in a higher acetylation level in comparison with the parental protein shown to be marginally acetylated by the same enzyme [7]. The recombinant HMGB1 sample exhibited no label incorporation at all (data not shown).

Interplay of in vitro acetylation and phosphorylation of HMGB1ΔC and its mutants

Resent studies on the histone code provided a set of evidences how a modification of one residue of the histone protein(s) can influence that of another [16]. We investigated the link between two enzymatic reactions: acetylation by CBP and phosphorylation by the lipid dependant serine/threonine kinase PKC. This approach was applied for the recombinant HMGB1 lacking the C tail (HMGB1ΔC) as for the mutated proteins HMGB1ΔCK2/A2, HMGB1ΔCK81/A81 and HMGB1ΔCK2/A2K81/A81 in order to discriminate the role of the two acetylated lysine moieties at position 2 and 81.

In the first set of experiments the protein preparations were PKC phosphorylated using cold ATP as a precursor in the reaction mixture and subsequently acetylated by CBP. The level of acetylation was estimated by the incorporation of [^{14}C] acetyl CoA compared to the same protein preparations not phosphorylated in advance. The samples were run on a 18% polyacrylamide gel con-

taining SDS (Fig. 3 B, upper part) and the autoradiograph was presented in Fig. 3 B, bottom part. The results from the quantification gel-based assay were summarized in Fig. 3 A. The label incorporation by CBP of the non phosphorylated protein samples was considered 100% except for the double mutant HMGB1ΔCK2/A2K81/A81. It is clearly demonstrated that the presence of phosphate(s) group in the molecule of HMGB1ΔCK2/A2 and HMGB1ΔCK81/A81 mutant proteins inhibited similarly the modification of both Lys 81 and Lys 2 approximately 2.5 times (Fig. 3 B, compare lanes 3 and 4, 5 and 6, respectively). The inhibitory effect of phosphorylation on the enzyme activity of CBP was slightly decreased when a non-mutant truncated HMGB1 protein was used as a substrate where the two lysines were accessible for acetylation (Fig. 3 B, compare lanes 1 and 2). As expected the double mutant HMGB1ΔCK2/A2K81/A81 incorporated negligible amount of [^{14}C] acetyl CoA nevertheless it was phosphorylated or not (Fig. 3 B, lanes 7 and 8). In order to estimate the eventual effect of PKC itself on the CBP activity we carried out mock phosphorylation of the protein samples with heat inactivated PKC prior to acetylation. In all cases the incorporation of [^{14}C] acetyl CoA was not affected (data not shown).

In the second set of experiments the truncated HMGB1 protein and the respective mutants were subjected to acetylation with CBP using cold acetyl CoA followed by radiolabeled PKC phosphorylation with [γ^{32}-P] ATP. The electrophoretic and autoradiograph analysis were done as described above. The label incorporation by PKC of the non acetylated protein samples was considered 100%. The effect of the acetylation on the phosphorylation level is demonstrated on Fig. 4. Introducing an acetyl group either at position 2 or position 81 reduced the PKC modification to 60–40%, respectively (Fig. 4 B, compare lanes 5 and 6, 3 and 4). Surprisingly the simultaneous acetylation at both lysines 2 and 81 practically inhibited almost 100% the phosphorylation of the tailless HMGB1 protein (Fig. 4 B, compare lanes 1 and 2). As for the double mutant where the two acetylable lysines were substituted to alanines the treatment with CBP logically had no effect on the phosphorylation level (Fig. 4 B, compare lanes 7 and 8). A mock acetylation with heat inactivated CBP prior to phosphorylation was carried out to rule out the possibility of any PKC inhibition due to the presence of the acetyltransferase in the reaction mixture. In all tailless HMGB1 protein samples the phosphorylation level remained unchanged (data not shown).

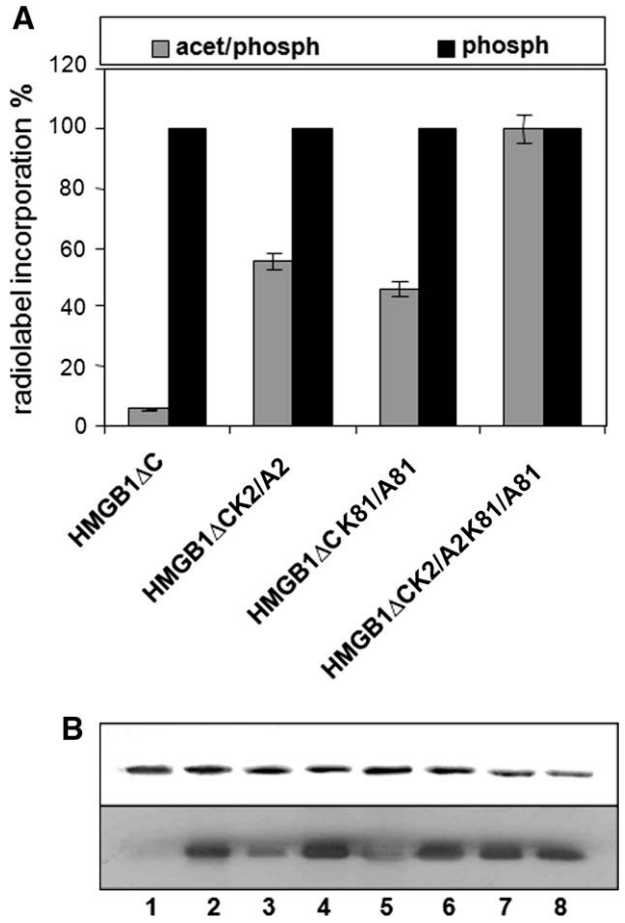

Fig. 4. The effect of CBP acetylation on the PKC phosphorylation level of the truncated HMGB1 protein and its mutant forms. The protein samples were subjected to CBP acetylation with cold acetyl CoA prior to radiolabeled [γ^{32}-P] ATP PKC phosphorylation, run on a 18% polyacrylamide gel containing SDS and stained with Coumassie (B, upper part), dried and exposed to KODAK XAR-5 film and the corresponding autoradiograph presented (B, bottom part). Lanes 1, 3, 5 and 7 represent truncated HMGB1 protein (HMGB1ΔC), truncated HMGB1 protein mutated at Lys 2 (HMGB1ΔCK2/A2), truncated HMGB1 protein mutated at Lys 81 (HMGB1ΔCK81/A81) and truncated HMGB1 protein mutated at Lys 2 and Lys 81 (HMGB1ΔCK2/A2K81/A81) acetylated with CBP prior to PKC phosphorylation, respectively. Lanes 2, 4, 6, and 8 represent truncated HMGB1 protein (HMGB1ΔC), truncated HMGB1 protein mutated at Lys 2 (HMGB1ΔCK2/A2), truncated HMGB1 protein mutated at Lys 81 (HMGB1ΔCK81/A81), and truncated HMGB1 protein mutated at Lys 2 and Lys 81 (HMGB1ΔCK2/A2K81/A81) phosphorylated by PKC, respectively. The data from quantification gel-based assay are shown on (A).

Discussion

We created recombinant truncated HMGB1 protein samples mutated at the sites of acetylation Lys 2 or/and Lys 81 for several reasons: (i) to see if there is a preferential site for the enzymatic activity of CBP, (ii) to investigate the existence of a mutual dependence for the acetylation of the lysine moieties, (iii) to estimate the interdependence of in vitro PKC phosphorylation and CBP acetylation. It is already known that Lys 2 in the HMGB1 molecule is acetylated in vivo [11] and in vitro by CBP as an additional site 81 is exposed and modified by CBP upon removal of the acidic C terminus [7]. Lysine acetylation is a well known histone modification that forms a stable epigenetic mark on chromatin. For example the histone acetyltransferase Gcn5 interacts preferentially with previously acetylated H3 peptides and tetra-acetylated H4 peptides [17]. Our results show that both lysines when modified separately incorporate comparable levels of [^{14}C] acetyl CoA (Fig. 2, lanes 2 and 3) which represents 55–60% from the label incorporation in the non mutant tailless HMGB1 protein. These data might be interpreted on one hand that the enzyme does not demonstrate preference for either Lys 2 or Lys 81 and on the other hand both sites are acetylated independently. As previously mentioned the in vivo acetylated HMGB1 protein exhibited properties closer to the truncated form lacking the C tail [8,13]. One explanation might be that the long C peptide changes its configuration in the molecule upon acetylation and the protein behaves as a truncated one. This hypothesis is supported by the fact that the in vivo acetylated HMGB1 is modified in a higher extent by CBP compared to the full length protein showing no acetylation or a very low background level (Fig. 2, lanes 5 and 6). One may speculate that introducing an acetyl group at Lys 2 alters the position of the C tail and thus diminishes its shielding effect.

The histone code comprises different sets of combination of enzymatic modifications in many cases dependent on each other. For example the phosphorylation of S10 and acetylation of K9 in the molecule of histone H3 prevent K9 methylation [18,19]. Several transcriptionally-associated histone acetyltransferases display strong preferences for H3 phosphorylated at S10 over the unmodified peptide as a substrate [20]. There are no data related to the

interplay of the postsynthetic modifications of HMGB1 protein. Our results for the first time demonstrate the link between the acetylation and phosphorylation of the tailless HMGB1 in vitro. This truncated form of the protein was found in the nucleus since a chromatin bound protease was purified from calf thymus which cleaved specifically the protein removing the C terminus [21]. The PKC phosphorylation prior to acetylation inhibits the CBP activity 40% for the HMGB1ΔC and approximately 60% for the mutants HMGB1ΔCK2/A2 and HMGB1ΔCK81/A81. The double mutant where the two acetylable sites are omitted is logically not affected. The effect of the CBP acetylation on the phosphorylation level turns out to be much more prominent. In the case of HMGB1ΔC modified at Lys 2 and Lys 81 prior to PKC treatment background phosphorylation is detected. Therefore the double acetylated sample is no more a substrate for the serine/threonine kinase. If only one of the lysines is modified the inhibitory effect decreases.

Our findings for the first time demonstrate the interdependence of the postsynthetic acetylation and phosphorylation of HMGB1 lacking the C terminus. In our case the acetylation by CBP has stronger effect on the subsequent phosphorylation than the PKC modification on the following enzymatic activity of the acetyltransferase. The dynamic of postsynthetic modifications and the different combinations of phosphate/acetyl groups in the protein molecule may serve as fine modulation of its functions and also as specific marks for protein/ protein interactions during cellular processes.

Acknowledgments

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