A Quantitative Study on the In Vitro and In Vivo Acetylation of High Mobility Group A1 Proteins

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High mobility group (HMG) A1 proteins are subject to a number of post-translational modifications, which may regulate their function in gene transcription and other cellular processes. We examined, by using mass spectrometry, the acetylation of HMGA1a and HMGA1b proteins induced by histone acetyltransferases p300 and PCAF in vitro and in PC-3 human prostate cancer cells in vivo. It turned out that five lysine residues in HMGA1a, i.e., Lys-14, Lys-64, Lys-66, Lys-70, and Lys-73, could be acetylated by both p300 and PCAF. We further quantified the level of acetylation by analyzing, with LC-MS/MS, the proteolytic peptides of the in vitro or in vivo acetylated HMGA1 proteins where the unmodified lysine residues were chemically derivatized with a perdeuterated acetyl group. Quantification results revealed that p300 and PCAF exhibited different site preferences for the acetylation; the preference of p300 acetylation followed the order of Lys-64~Lys-70 > Lys-66 > Lys-14~Lys73, whereas the selectivity of PCAF acetylation followed the sequence of Lys-70~Lys-73 > Lys-64~Lys-66 > Lys-14. HMGA1b was acetylated in a very similar fashion as HMGA1a. We also demonstrated that C-terminal phosphorylation of HMGA1 proteins did not affect the in vitro acetylation of the two proteins by either p300 or PCAF. Moreover, we examined the acetylation of lysine residues in HMGA1a and HMGA1b isolated from PC-3 human prostate cancer cells. Our results showed that all the above five lysine residues were also acetylated in vivo, with Lys-64, Lys-66 and Lys-70 in HMGA1a exhibiting higher levels of acetylation than Lys-14 and Lys-73. (J Am Soc Mass Spectrom 2007, 18, 1569–1578) © 2007 American Society for Mass Spectrometry

High mobility group (HMG) proteins, comprising three families of structurally unrelated proteins including HMGA, HMGB, and HMGN, are nonhistone chromosomal proteins that are thought to play important roles in the assembly of chromatin and in the regulation of transcription in higher eukaryotic cells [1]. HMGA1 proteins contain three independent DNA-binding regions, called AT-hook motifs, which bind to the minor groove of AT-rich DNA sequences both in vitro and in vivo [1–3]. HMGA1a and HMGA1b, formerly known as HMG-I and HMG-Y, respectively, are translated from the splicing variants of a single gene (HMGA1) and they are identical in sequence except for an 11-amino acid internal deletion in HMGA1b (Figure 1) [4, 5]. Both HMGA1a and HMGA1b are recognized as architectural transcription factors that up- or down-regulate the transcriptional activity of a number of human genes [6, 7]. Despite the structural similarities between HMGA1a and HMGA1b, these two proteins may have distinct biological functions [8–10].

Among the highly modified nuclear proteins, HMGA1 proteins adopt a number of post-translational modifications (PTMs) including acetylation, methylation, and phosphorylation. Phosphorylation of HMGA1 proteins was first detected by Lund et al. [11] in Ehrlich ascites cells. Further research revealed that HMGA1 proteins were phosphorylated by several kinases such as cyclin-dependent kinase 1 (previously known as cdc2 kinase) [12, 13], protein kinase C (PKC) [14], and protein kinase CK2 [15]. These phosphorylations attenuated the DNA binding affinities of mammalian HMGA1a [14, 16, 17] and HMGA1b [17, 18].

In addition to phosphorylation, methylation was also detected in HMGA1 proteins [8, 19–23]. Dimethylation of both arginine and lysine residues in HMGA1a was reported and it might be correlated with the metastatic potential and apoptosis of cells [19–21]. Recently, we reported that Arg-25 in HMGA1a, but not in HMGA1b, was both mono- and dimethylated in PC-3 human prostate cancer cells, with the dimethylation being in either symmetric or asymmetric form [24]. Importantly, some of the above phosphorylation and methylation were also found in HMGA1a and HMGA1b proteins isolated from human breast cancer tissues [25].

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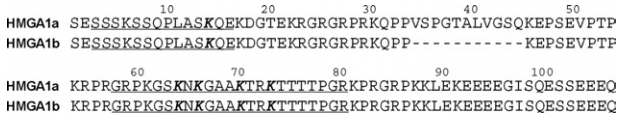

Figure 1. The sequences of HMGA1a and HMGA1b. The acetylated lysine residues identified in this study were highlighted in bold, and peptides containing these lysine residues were underlined.

Other than phosphorylation and methylation, acetylation of HMGA1 proteins was also detected [8, 9, 19, 26–28]. In this respect, Lys-64 and Lys-70 in HMGA1a protein can be acetylated by CBP (CREB-binding protein) and PCAF (p300/CBP-associated factor), respectively [26, 27]. In addition, the acetylation at these two sites conferred distinct biological outcomes; the acetylation of Lys-64 destabilized the enhanceosome, whereas the acetylation of Lys-70 potentiated transcription of interferon-β gene by stabilizing the enhanceosome and preventing the acetylation of HMGA1a by CBP [26, 27]. Moreover, multiple acetylation sites in HMGA1 proteins from MCF-7 human breast cancer cells have been suggested, though the assignment of these modifications to specific lysine residues was not made [8]. Very recently, we reported that Lys-14 in HMGA1a and HMGA1b was acetylated in PC-3 cells. We, however, were not able to identify any acetylated peptides containing Lys-64 and/or Lys-70 from the tryptic digestion mixture of HMGA1a [28].

Mass spectrometry is commonly used for the identification of PTMs in proteins [29, 30]. However, PTMs often alter the ionization efficiency of the peptide being modified and, as a consequence, the observed relative abundances of ions corresponding to the modified and unmodified forms of a peptide may not reflect accurately the level of modification. This problem can be resolved by using stable isotope-labeled proteins or peptides, which are chemically identical to the naturally occurring proteins or peptides [31–33]. Incorporation of non-radioactive isotopes into peptides or proteins of interest can be achieved either in cell culture [34, 35] or after the extraction of peptides/proteins from cells or tissues [36, 37]. In this regard, quantitative analysis of phosphorylation [38, 39], glycosylation [40, 41], and acetylation [42] has been reported.

Herein, we adopted the quantification method introduced by Smith et al. [42], which was developed to quantify the levels of acetylation at individual lysine residues in the N-terminal tail of histone H4, and examined systematically the sites and levels of acetylation of HMGA1 proteins induced by histone acetyltransferases p300 and PCAF in vitro. Because HMGA1 proteins isolated from cancer cells showed simultaneous acetylation and phosphorylation [28, 43], we further assessed how C-terminal constitutive phosphorylation affects the acetylation of the two proteins. Moreover, we investigated the acetylation of lysine residues in HMGA1a and HMGA1b isolated from PC-3 human prostate cancer cells.

Experimental

Purification of HMGA1 Proteins

Full-length recombinant human HMGA1a and HMGA1b proteins were overexpressed in *E. coli* BL21 DE3 pLysS cells (Invitrogen, Carlsbad, CA) followed by extraction with 5% perchloric acid (PCA) as reported previously [44, 45]. Recombinant HMGA1 proteins were further purified on an Agilent 1100 system (Agilent Technologies, Palo Alto, CA) by using a 4.6 × 250 mm C4 column (Grace Vydac, Hesperia, CA). The flow rate was 1.0 mL/min, and a 60-min gradient of 5% to 50% CH_3CN in 0.1% aqueous solution of trifluoroacetic acid (TFA) was employed. The purified proteins were then quantified by Bradford protein assay (Bio-Rad, Hercules, CA).

The HMGA1 proteins were also isolated from PC-3 human prostate cancer cells following previously described procedures [24]. Briefly, the PC-3 cells were cultured in F-12 media (ATCC, Manassas, VA) supplemented with 10% fetal bovine serum (Invitrogen) and 5% CO_2 at 37 °C. Cells were harvested and homogenized by sonication in the lysis buffer followed by PCA extraction. The HMGA1 proteins were then isolated from the PCA-soluble fractions by using HPLC with a 4.6 × 250 mm C4 column (Varian, Walnut Creek, CA).

In Vitro Phosphorylation of HMGA1 Proteins by Protein Kinase CK2

Recombinant HMGA1a or HMGA1b (15 μg) was incubated with 600 U of protein kinase CK2 (New England BioLabs, Beverly, MA) and 200 μM ATP at 37 °C for 1 h in a 150-μL reaction buffer supplied by the vendor. The phosphorylated HMGA1 proteins were then isolated from the reaction mixture by using HPLC on the Agilent 1100 system with a 2.0 × 250 mm C4 column (Phenomenex, Torrance, CA). The flow rate was 200 μL/min, and a 40-min gradient of 5% to 40% CH_3CN in 0.1% aqueous solution of TFA was employed. The chromatogram was obtained by absorbance detection at 220 nm. Fractions containing phosphorylated HMGA1a or HMGA1b were collected and subjected to MALDI-MS analysis to confirm the phosphorylated products.

In Vitro Acetylation of HMGA1 Proteins by p300 and PCAF

Recombinant and CK2-phosphorylated HMGA1 proteins were acetylated by the HAT (histone acetyltransferase) domain of p300 (Upstate, Temecula, CA) or PCAF (Upstate) at enzyme-to-substrate molar ratios of 1:20 and 1:30, respectively. The acetylation reactions were carried out in the presence of 1.5 nmol acetyl CoA in a HAT buffer (50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 1.0 mM dithiothreitol, and 10% glycerol) at 30 °C for 1 h [46, 47].