Acetylation and Phosphorylation of High-Mobility Group A1 Proteins in PC-3 Human Tumor Cells†

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ABSTRACT: In this paper, we examined the posttranslational modifications (PTMs) of high-mobility group A1 (HMGA1) proteins in PC-3 human prostate cancer cells that are either treated or not treated with a histone deacetylase inhibitor, sodium butyrate. We found that, from a reversed-phase C_4 column, the HMGA1a protein eluted in two different fractions with distinct forms of PTMs: Ser98, Ser101, and Ser102 were phosphorylated and Arg25 was methylated for both fractions; only the minor fraction, however, is hyperphosphorylated where Ser35, Thr52, and Thr77 were also phosphorylated. In addition, Lys14 was acetylated in the major but not the minor HMGA1a fraction isolated from the PC-3 cells that were not treated with butyrate. Likewise, HMGA1b, which is a splicing variant of HMGA1a, was acetylated on Lys14 and phosphorylated on the corresponding residues, i.e., Thr41, Thr66, Ser87, Ser90, and Ser91. The acetylation and phosphorylation of the HMGA1a and HMGA1b proteins may affect their interactions with other protein factors, which in turn may modulate the binding of HMGA1 proteins to DNA and regulate gene expression. In addition, the specifically posttranslationally modified HMGA1 proteins may serve as molecular biomarkers for cancer diagnosis and prognosis.

The chromosomal high-mobility group A (HMGA)[1] proteins, including HMGA1a, HMGA1b (previously known as HMGI and HMGY, respectively), and HMGA2, constitute one subfamily of non-histone HMG proteins (Figure 1 gives the sequences for the HMGA1a and HMGA1b proteins) (*1*). HMGA1a and HMGA1b proteins are translated from the splicing variants of a single gene (*HMGA1*) and differ in that the HMGA1b protein has an 11-residue internal deletion (*2*, *3*). HMGA1a and HMGA1b bear three highly conserved "AT-hook" domains that preferentially bind to the minor groove of AT-rich DNA (*4*−*6*). HMGA1 proteins are considered as architectural transcription factors that function in a number of cellular processes including cell growth and differentiation, chromatin remodeling, apoptosis, and neoplastic transformation (*7*−*9*).

HMGA1 proteins are among the nuclear proteins that are highly posttranslationally modified, and many types of posttranslational modifications (PTMs) have been documented (*10*−*20*). In this context, the HMGA1a protein is a substrate for several protein kinases including cyclin-dependent kinase 1 (cdk1, previously known as cdc2 kinase) (*21*, *22*), protein kinase C (PKC), and protein kinase CK2. Thr52 and Thr77 in human HMGA1a were shown to be phosphorylated by cdk1 in vitro (*21*, *22*), and the phosphorylation of these two residues in HMGA1a was found to be cell cycle-dependent (*21*, *22*). The two threonines are close to the N-terminal sides of the second and third AT-hook domains, respectively (*8*), and the phosphorylation at these two sites reduces greatly the binding affinity of the protein toward AT-rich DNA (*21*−*23*). The phosphorylation of the closely related HMGA1b by cdk1, however, has not been reported. In addition, PKCα was shown to catalyze the phosphorylation of Thr20, Ser43, and Ser63 in HMGA1a (*24*). The PKC-mediated phosphorylation of HMGA1a also decreases the binding affinity between the protein and DNA (*24*). Moreover, protein kinase CK2 can induce the phosphorylations of C-terminal serine residues in HMGA1a in vitro, and Ser98, Ser101, and Ser102 in HMGA1a protein were found to be phosphorylated in vivo (*18*, *25*−*27*). The corresponding serine residues in HMGA1b were also found to be phosphorylated (*18*, *26*). Furthermore, HMGA1a protein was hyperphosphorylated during the early stage of apoptosis, and it underwent dephosphorylation while apoptosis proceeded to its late stage (*11*). The sites of modification for the hyperphosphorylated protein, however, were not determined.

In addition to phosphorylation, methylation of HMGA1a protein was also reported (*12*, *13*, *28*, *29*). In this regard, Giancotti and co-workers (*12*, *28*) showed by LC-MS that Arg25 in HMGA1a is monomethylated and the methylation is correlated with apoptosis in cancer cells. We found recently by LC-tandem mass spectrometry (MS/MS) and matrix-assisted laser desorption/ionization (MALDI) MS/MS that Arg25 can be both mono- and dimethylated in PC-3 human prostate cancer cells, and both forms of dimethylarginine, that is, asymmetric N^G,N^G-dimethylarginine and symmetric N^G,N'^G-dimethylarginine, were present (*18*). Moreover, it was shown that protein arginine methyltransferase 6 might be involved in the methylation of HMGA1a (*19*, *20*).

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[1] Abbreviations: PTMs, posttranslational modifications; MS, mass spectrometry; MS/MS, tandem mass spectrometry; ESI, electrospray ionization; MALDI, matrix-assisted laser desorption/ionization; HMG, high-mobility group; TFA, trifluoroacetic acid; CK2, protein kinase CK2; PKC, protein kinase C; cdk, cyclin-dependent kinase.

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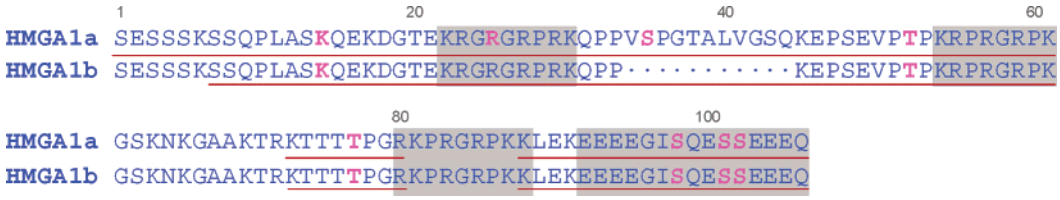

FIGURE 1: Sequence alignments of HMGA1a and HMGA1b. The AT-hook domains and the C-terminal acidic region are highlighted by gray boxes, and the residues that are posttranslationally modified are shown in bold pink letters. The amino acid residues in those peptide sequences covered from the LC-MS/MS and MALDI-MS/MS analysis are underlined.

Other than phosphorylation and methylation, previous studies also revealed that the HMGA1a protein can be acetylated (*15*, *29*−*31*). Enhanceosome is a high-order nucleosome complex formed in response to virus infection (*32*), and the acetylation of Lys64 (Figure 1, the sequence does not include the first methionine residue) in HMGA1a destabilizes the enhanceosome and results in the transcriptional shutoff of interferon-β gene (*30*). The same type of modification of Lys70, however, strengthens the enhanceosome assembly and protects it from premature disruption (*15*). We, however, failed to identify any acetylated peptides from the tryptic digestion mixture of HMGA1a protein isolated from PC-3 cells (*18*).

Here, in this paper, we treated the cultured PC-3 human prostate cancer cells with a histone deacetylase inhibitor, and we found that Lys14 in HMGA1a and HMGA1b can be acetylated. In addition, we determined the sites of phosphorylation of the hyperphosphorylated HMGA1a, and we found that the corresponding residues in HMGA1b are also phosphorylated.

MATERIALS AND METHODS

Cell Culture. The PC-3 human prostate cancer cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA). Under control conditions, the cells were cultured in F-12 media (ATCC) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and 5% CO_2 at 37 °C. For histone deacetylase (HDAC) treatment, the PC-3 cells were cultured in the same way as the control except that the cells were treated with 7 mM sodium butyrate for overnight before harvesting for protein extraction.

Protein Extraction and Purification. The HMG proteins were extracted with 5% perchloric acid (PCA) as previously described (*7*, *18*, *33*). PCA-soluble proteins were purified on an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA) by using a 1.5 × 250 mm C4 column (Varian, Walnut Creek, CA). The flow rate was 200 μL/min, and a 75 min gradient of 5−30% CH_3CN in 0.1% aqueous solution of trifluoroacetic acid (TFA) was employed. The chromatograms were recorded by absorbance detection at 220 nm. The HMG proteins were collected in four fractions according to absorbance peaks (Figure 2), and these fractions were dried in a Speedvac concentrator.

Enzymatic Digestion. The above dried HMG protein fractions were redissolved in 50 mM ammonium bicarbonate and digested by modified sequencing grade trypsin (Roche, Indianapolis, IN) at an enzyme:substrate ratio of 1:50 at 37 °C for overnight. The enzymatic reaction was terminated by adding glacial acetic acid (5 μL). The tryptic digestion mixture was dried and stored at −20 °C prior to mass spectrometric analysis.

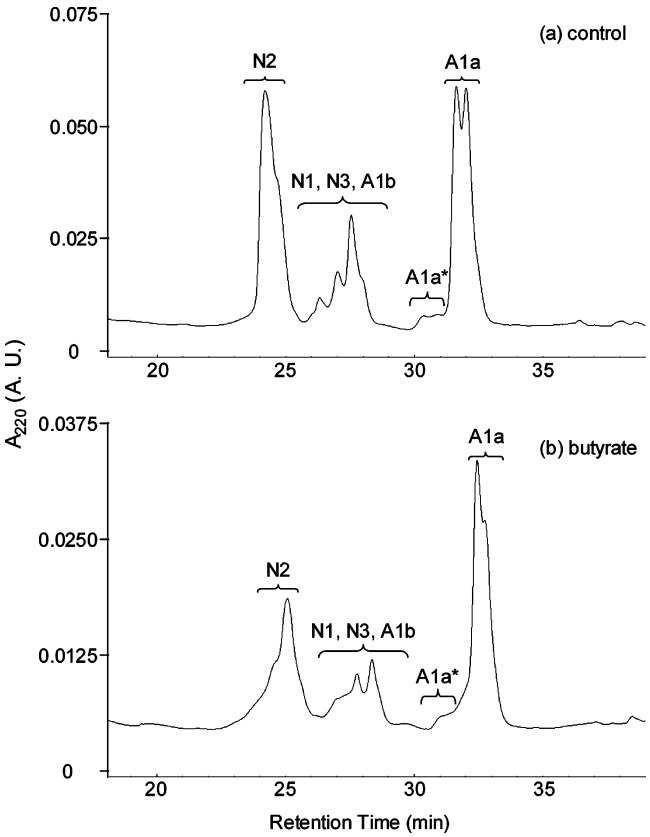

FIGURE 2: HPLC traces for the separation of HMG proteins isolated from sodium butyrate-treated (a) and untreated PC-3 human prostate cancer cells (b).

Mass Spectrometry and Database Search. Two mass spectrometric techniques were employed for peptide sequencing, i.e., matrix-assisted laser desorption/ionization−tandem mass spectrometry (MALDI-MS/MS) on a QSTAR XL quadrupole/time-of-flight mass spectrometer equipped with an o-MALDI ion source (Applied Biosystems, Foster City, CA) and LC-electrospray ionization-MS/MS (LC-ESI-MS/MS) on an LCQ Deca XP ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA). On the basis of our previous studies on HMGA1a (*18*), we noticed that the methylated peptide segment GRGRPR (residues 24−29) of HMGA1a can be readily detected by MALDI-MS, but it is somewhat difficult to be detected by LC-MS/MS. This might be attributed to the formation of multiply charged ions of this peptide segment, whose low *m*/*z* value makes it difficult to be detected because of background noises in the low *m*/*z* region of ESI-MS. On the other hand, MALDI-MS/MS is not very capable in fragmenting ions formed from large peptides because MALDI usually produces singly charged ions. Thus, these two mass spectrometric techniques were

Table 1: Summary of Posttranslational Modifications of HMGA1 Proteins Isolated from Control and Butyrate-Treated PC-3 Cells[a]

peptide sequence	HMG type	PTM site(s)	residues
SSQPLAS***K***$_{Ac}$QEK	A1a	Lys14	7−17
TTT***T***$_p$PGR	A1a	Thr77	74−80
KTTT***T***$_p$PGR	A1a	Thr77	73−80
KQPPVSPGTALVGSQKEPSEVP***T***$_p$PK	A1a	Thr52	30−54
KQPPV***S***$_p$PGTALVGSQK	A1a	Ser35	30−45
KQPPV***S***$_p$PGTALVGSQKEPSEVP***T***$_p$PK	A1a	Ser35, Thr52	30−54
KLEKEEEEGISQE***S***$_p$S$_p$EEEQ	A1a	Ser101, Ser102	88−106
KLEKEEEEGI***S***$_p$QE***S***$_p$***S***$_p$EEEQ	A1a	Ser98, Ser101, Ser102	88−106
SSQPLAS***K***$_{Ac}$QEK	A1b	Lys14	7−17
KQPPKEPSEVP***T***$_p$PK	A1b	Thr41	30−43
KTTT***T***$_p$PGR	A1b	Thr66	62−69
KLEKEEEEGISQE***S***$_p$***S***$_p$EEEQ	A1b	Ser90, Ser91	77−95
KLEKEEEEGI***S***$_p$QE***S***$_p$***S***$_p$EEEQ	A1b	Ser87, Ser90, Ser91	77−95

[a] Phosphorylations at Ser98, Ser101, and Ser102 in HMGA1a as well as Ser87, Ser90, and Ser91 in HMGA1b were identified in our previous studies (*18*).

chosen because they are complementary in protein identification and in the studies of PTMs of proteins.

For MALDI analysis, purified protein samples or tryptic digestion products were dissolved in an aqueous solution of 0.1% TFA, and the sample aliquots were mixed with an equal volume of matrix solution, which was a saturated solution of α-cyano-4-hydroxycinnamic acid in a solvent mixture of CH_3CN, H_2O, and TFA (50/50/0.1 v/v).

On-line LC-MS/MS was employed for the identification of PTMs of HMGA proteins, and a 0.32 × 150 mm C18 capillary column (300 Å in pore size, 5 μm in particle size; Micro-Tech Scientific, Vista, CA) was used. A Surveyor HPLC system (ThermoFinnigan) was employed for the LC-MS/MS experiment, and a homemade precolumn splitter was used. The flow rate was approximately 6 μL/min after splitting, and the percentage of acetonitrile in 0.6% aqueous solution of acetic acid changed from 2% to 65% in 65 min. The effluent from the HPLC column was directed to the ion-trap mass spectrometer. Mass calibration was carried out by using caffeine, a tetrapeptide MRFA, and Ultramark 1621 that were supplied by the instrument vendor. The spray voltage was 4.0 kV, and the capillary temperature was maintained at 225 °C. MS/MS was done in data-dependent scan mode by selecting the first and second most abundant ions observed in MS mode for collisional activation. The mass width for precursor ion isolation was 2.5 *m*/*z* units, and the collision gas was helium. To achieve better signal-to-noise ratio in MS/MS and more sensitive identification of PTMs, LC-MS/MS experiments were also carried out to monitor the fragmentation of one or a few preselected precursor ions.

The LC-MS/MS data were subjected to SEQUEST (Thermo Electron, Waltham, MA) search with the SWISSPROT protein database. Multiple forms of PTMs were considered in the search: phosphorylation of serine and threonine; acetylation as well as mono-, di-, and trimethylation of lysine; mono- and dimethylation of arginine.

RESULTS

Acetylation of Lys14 in HMGA1a. Intrigued by the previous observations that Lys64 and Lys70 in HMGA1a can be acetylated and the acetylation of these two lysines can result in distinct outcomes on the enhanceosome complex (*15*, *30*), we decided to ask the question of whether these and other lysine residues can be acetylated in HMGA1a isolated from PC-3 prostate cancer cells. In the viewpoint that we were not able to identify any acetylated peptide from this protein isolated from PC-3 cells in our previous study (*18*), we decided to treat the PC-3 cells with sodium butyrate, an inhibitor for histone deacetylases (*34*), and examined the PTMs of HMGA1 proteins isolated from the butyrate-treated cells. For comparison, we further characterized the PTMs of HMGA1 proteins isolated from the control PC-3 cells. It turned out that many sites of PTMs are present in HMGA1 proteins (Figure 1; Table 1 lists the peptides that carry the posttranslationally modified residues).

Here we start our discussion with the acetylation of Lys14. This acetylation was first found for HMGA1a isolated from butyrate-treated PC-3 cells. In this respect, SEQUEST search of the LC-MS/MS results acquired in the data-dependent scan mode facilitated us to discover that Lys14 in SSQPLASK_{Ac}QEK (residues 7−17, and "K_{Ac}" represents an acetylated lysine) was acetylated. To obtain a tandem mass spectrum with an improved signal-to-noise ratio, we selectively monitored the fragmentation of the $[M + 2H]^{2+}$ ion (*m*/*z* 622.8) of the acetylated lysine-bearing peptide in another LC-MS/MS experiment.

The resulting tandem mass spectrum allows us to assign the site of modification without ambiguity (Figure 3). In particular, we observed a mass difference of 170 Da, which corresponds to the mass of an acetylated lysine, between y_3 and y_4 ions, and the same mass difference was found for b_7 and b_8 ions, supporting that Lys14 is acetylated. The observed *m*/*z* values of other y and b ions are also consistent with the acetylation of Lys14 (Figure 3). In addition, we failed to observe a y_3 ion whose *m*/*z* value is in line with the acetylation of Lys17, demonstrating that the acetylation occurs exclusively on Lys14.

It is worth noting that the acetylation of lysine shared the same increase in nominal mass (i.e., 42 Da) as the trimethylation of the same residue. Previous studies showed that tandem mass spectra can allow for the differentiation of these two forms of PTMs (*35*). In this regard, upon collisional activation in the mass spectrometer, the acetylated lysine-carrying peptide usually gives a characteristic immonium ion of *m*/*z* 126, whereas the trimethylated peptide can often have the diagnostic neutral loss of a 59 Da fragment, i.e., $(CH_3)_3N$, either from the parent ion or from the backbone cleavage ions (*35*). Although the ion-trap mass spectrometer does not allow us to observe low *m*/*z* fragment ions (*36*) including

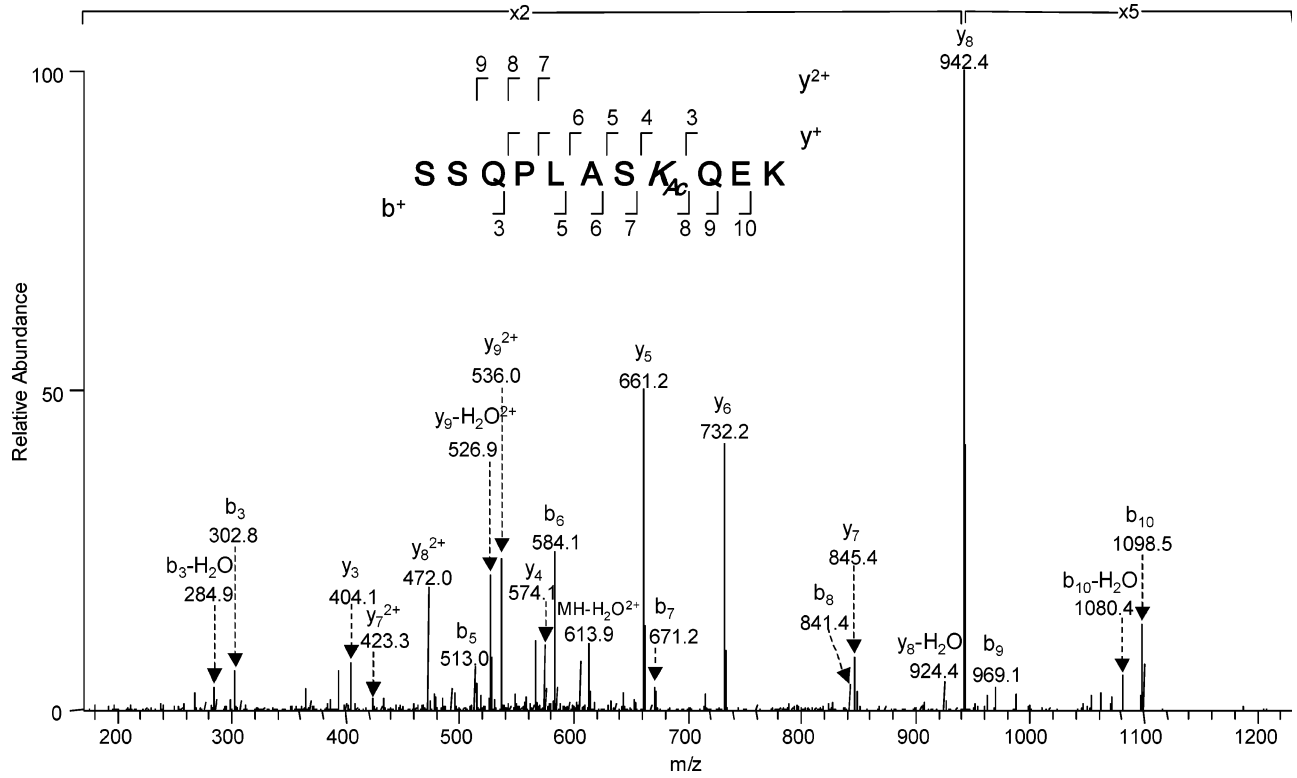

FIGURE 3: Acetylation of Lys14 in HMGA1a peptide SSQPALSK_{Ac}QEK (residues 7−17). Shown is the product ion spectrum of the $[M + 2H]^{2+}$ ion (*m*/*z* 622.9) of the tryptic peptide obtained from the major HMGA1a fraction isolated from sodium butyrate-treated PC-3 cells. The y and b ions are calculated on the basis of the Lys14-acetylated peptide segment. To better view low-abundance fragment ions, some spectral regions are amplified by 2- and 5-fold as indicated by ×2 and ×5, respectively.

the immonium ion of the acetylated lysine, the failure in finding the neutral loss fragment (−59 Da) supports that the modification is acetylation, not trimethylation. In addition, the increased abundance of the modified peptide upon HDAC inhibitor treatment suggests that Lys14 is acetylated, but not trimethylated.

Similar data-dependent scan analysis of the tryptic digestion products of HMGA1a fractions isolated from the control PC-3 cells, however, did not allow us to identify the same acetylated peptide segment; the failure in detecting this peptide might be attributed to the low level of acetylation of Lys14. On the other hand, while we analyzed the tryptic digestion mixture of the corresponding HMGA1a fraction by monitoring specifically the fragmentation of the ion of *m*/*z* 622.9, which corresponds to the doubly protonated ion of SSQPLASK_{Ac}QEK, we were able to obtain a tandem mass spectrum supporting the acetylation of Lys14 in this peptide (Figure S1). In this context, we consider that the percentage of the acetylation of Lys14 should be positively correlated with the ratio of the relative abundance of the doubly charged ion corresponding to the acetylated peptide (SSPQLASK_{Ac}QEK) over the sum of relative abundances of ions for both acetylated and unacetylated peptides carrying Lys14, i.e., SSPQLASK_{Ac}QEK, SSPQLASKQEK, and SSPQLASK. (The first two peptides contain a trypsin miscleavage site.) From this analysis, we may estimate that the level of Lys14 acetylation was approximately four times higher in butyrate-treated than in control samples (Figure S2). In this context, it is worth noting that the ionization efficiencies for the three peptides are expected to be different; therefore, the absolute percentages of acetylation are expected to be different from the peak area ratios shown in Figure S2.

We also monitored the fragmentation of the ion of *m*/*z* 622.8 for the tryptic digestion mixture of the minor HMGA1a fraction and the HMGA1b fraction. It turned out that we were not able to find a product ion spectrum corresponding to the acetylated peptide segment in the minor HMGA1a fraction obtained from control cells, though the minor HMGA1a fraction from the butyrate-treated cells also carries this modification. In addition, we detected the respective acetylated peptide from the tryptic digestion mixture of the fraction containing HMGA1b (data not shown).

It is worth noting that the acetylation of Lys14 was also observed previously for HMGA1a protein isolated from other cell lines (*29*, *31*). The acetylation of Lys14 in HMGA1b was, however, observed here for the first time.

Phosphorylation of HMGA1a and HMGA1b. Other than the acetylation of Lys14 and the phosphorylation of C-terminal serines (*18*), we also found that Ser35, Thr52, and Thr77 can be phosphorylated in HMGA1a protein isolated from the PC-3 human prostate cancer cells. In this respect, the phosphorylation of these three residues occurs in the HMGA1a fraction eluting earlier than the major HMGA1a fraction (labeled as HMGA1a* in Figure 2). MALDI-MS analysis of the minor HMGA1a fraction showed that the protein carries up to six phosphate groups (Figure S3). In addition, the phosphorylation of these three residues is not dependent on whether the PC-3 cells have been treated with butyrate. Thr52 and Thr77 were shown previously by peptide sequencing to be phosphorylated (*21*, *22*). We confirmed their phosphorylation in HMGA1a isolated from both control and butyrate-treated PC-3 cells. (The product ion spectra for the Thr52- and Thr77-bearing phosphopeptides from the control cells are shown in Figures S4 and S5.) In addition,

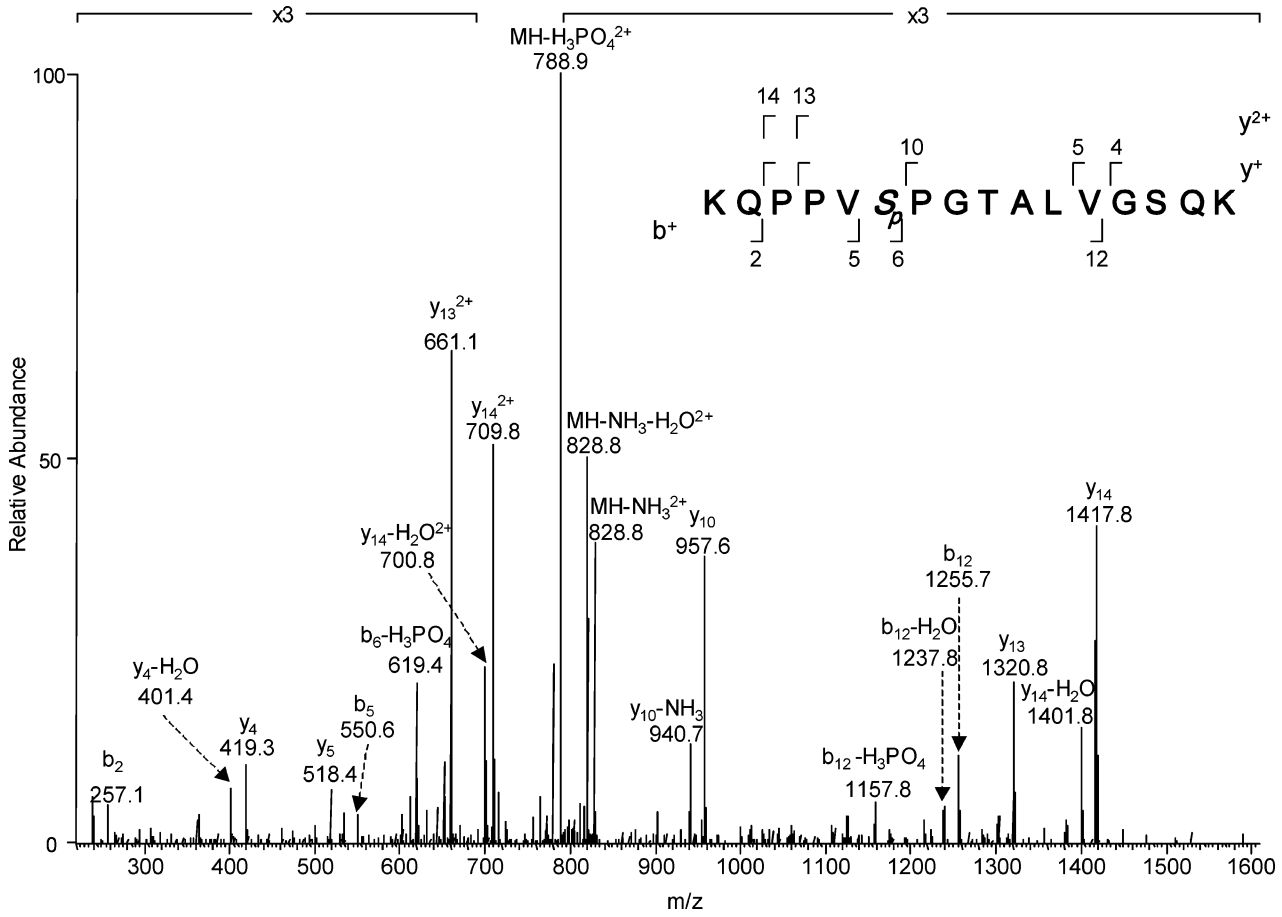

FIGURE 4: Phosphorylation of Ser35 in KQPPVS_pPGTALVGSQK (residues 30−45). Shown is the product ion spectrum of the $[M + 2H]^{2+}$ ion (*m*/*z* 837.4) of the tryptic peptide from the minor HMGA1a protein fraction isolated from control PC-3 cells. The spectrum was obtained by LC-MS/MS in data-dependent scan mode. To better view low-abundance fragment ions, some spectral regions are amplified by 3-fold as indicated by ×3 in the figure.

we identified Ser35 as a new phosphorylation site in HMGA1a protein.

The phosphorylated Ser35-bearing peptide, i.e., KQPPVS_p-PGTALVGSQK (S_p represents a phosphorylated serine), was identified by LC-MS/MS analysis carried out in data-dependent scan mode. In the product ion spectrum of the $[M + 2H]^{2+}$ ion (*m*/*z* 837.4) of the tryptic peptide KQPPVS_p-PGTALVGSQK (Figure 4, the labeled y and b ions are based on the calculated *m*/*z* values for Ser35-phosphorylated peptide), we observed the formation of a $[b_6 - H_3PO_4]$ ion (*m*/*z* 619.2), which supports the phosphorylation of Ser35. In addition, the observed y_{10} ion exhibits the same *m*/*z* value as that calculated for the unmodified peptide. This result, together with the failure in detecting a y_{10} ion with a *m*/*z* value being consistent with the phosphorylation of either Thr38 or Ser43 (Figure 4), demonstrates that Ser35 is the only residue being phosphorylated in this peptide; i.e., neither Thr38 nor Ser43 is modified.

The phosphorylation of Ser35 was also confirmed by the observation of a diphosphorylated peptide, KQPPVS_pPG-TALVGSQKEPSEVPT_pPK, where both Ser35 and Thr52 are phosphorylated (data not shown). In this respect, it is worth noting that Ser35, Thr52, and Thr77 all precede a proline residue, suggesting that their phosphorylation might be catalyzed by the same kinase. The major HMGA1a protein fraction is, however, mostly di- or triphosphorylated, where the three C-terminal serine residues (i.e., Ser98, Ser101, and Ser102) are phosphorylated. The corresponding residues in HMGA1b, namely, Thr41, Thr66, Ser87, Ser90, and Ser91, were also found to be phosphorylated (Table 1; the product ion spectrum of the $[M + 2H]^{2+}$ ion of the Thr41-bearing peptide is shown in Figure S6). Taken together, among all of the phosphorylation sites that we identified in this paper, the phosphorylation of Ser35 in HGMA1a as well as Thr41 and Thr66 in HMGA1b were reported here for the first time.

In addition to acetylation and phosphorylation, we also examined the methylation of HMGA1a protein from control and butyrate-treated PC-3 cells by MALDI-MS and MS/MS. Consistent with our previous findings with the control PC-3 cells (*18*), Arg25 was also found to be methylated for HMGA1a protein isolated from butyrate-treated PC-3 cells (data not shown).

DISCUSSION

The "bottom-up" strategy for the mass spectrometric identification of the sites of PTMs in proteins is usually achieved by analyzing the proteolytic digestion products with LC-MS/MS in data-dependent scan mode (*37*). In this mode of analysis, the software can allow for the automatic selection of one or a few ions observed in MS for fragmentation based on the relative abundances of the ions, which gives tandem mass spectra for peptide sequencing and PTM identification. When the level of modification is low, it is, however, difficult

for the ions corresponding to modified peptides to be chosen for fragmentation in this mode of analysis. Here we treated PC-3 cells with butyrate, which is an HDAC inhibitor. The resulting enhanced level of acetylation of proteins facilitates the identification of sites of modification in proteins by LC-MS/MS in data-dependent scan mode. After the modification sites are identified, we analyzed the tryptic digestion mixture of the HMGA1a protein isolated from untreated cells by LC-MS/MS, where we monitored selectively the fragmentation of ions corresponding to the acetylated peptides. This latter approach facilitates the fragmentation of ions of low abundance, and the resulting product ion spectra permit the identification of low-level acetylation.

This approach enabled us to determine that Lys14 is acetylated in HMGA1a protein, whereas this information could not be gained by data-dependent LC-MS/MS analysis of the corresponding tryptic digestion mixture of HMGA1a protein isolated from untreated cells. HDAC inhibitor treatment has been used previously to facilitate the identification of low levels of acetylation in other proteins (*38*, *39*). We reason that the comparison of the acetylation of proteins isolated from HDAC inhibitor-treated cells with those obtained from untreated cells may allow one to reveal the dynamics of acetylation and deacetylation of specific residues in proteins. In this respect, monitoring the fragmentation of specifically acetylated peptide segments, which was employed in this study, is important for the identification of low-abundance acetylation in proteins isolated from untreated cells.

HMGA1a is among the most abundant phosphoproteins in the nucleus. The phosphorylations of Thr52, Thr77, Ser98, Ser101, and Ser102 were previously known (*18*, *21*, *22*). In this respect, Thr52 and Thr77 were reported to be phosphorylated by cdk1 in vitro, and the phosphorylation of these two residues occurs in a cell cycle-dependent manner (*21*, *22*). The two threonines are close to the N-terminal sides of the second and third AT-hook regions (*9*), and the phosphorylation at these sites decreases significantly the binding affinity of HMGA1a to AT-rich DNA (*21*, *22*). The new phosphorylation site that we identified here, that is, Ser35, is close to the first AT-hook, and we reason that the phosphorylation of this site may also affect the binding of the protein to AT-rich DNA. Moreover, Ser35, Thr52, and Thr77 in HMGA1a and the corresponding Thr41 and Thr66 in HMGA1b all precede a proline residue (Figure 1), suggesting that the phosphorylation of these three residues might be catalyzed by the same kinase, e.g., cdk1. HMGA1a protein was previously shown to be hyperphosphorylated in early-stage apoptotic cells (*11*, *12*, *28*). Therefore, we speculate that the minor hyperphosphorylated HMGA1a fraction might be due to the presence of a small amount of apoptotic cells in the PC-3 cell culture. This argument appears to be consistent with the observations showing that a small fraction of HL-60 cells undergoes apoptosis even without drug induction (*11*) and that the activation of cdk1 may be linked with apoptosis (*40*, *41*).

The phosphorylation of Ser35, Thr52, and Thr77 in HMGA1a and the corresponding Thr41 and Thr66 in HMGA1b may play a special role in the biological function of these two proteins because reversible phosphorylation of proteins on serine or threonine preceding a proline (Ser/Thr-Pro) is a major cellular signaling mechanism (*42*). In this respect, peptidyl prolyl isomerase (PPIase) Pin1 (*43*) isomerizes specifically the phosphorylated Ser/Thr−Pro bonds in certain proteins (*42*). The protein conformational changes caused by the isomerization affect profoundly the protein's catalytic activity, dephosphorylation, and interaction with other proteins, as well as subcellular localization (*42*). Although no study has shown that the above phosphorylated residues in HMGA1a or HMGA1b are substrates for Pin1, a recent study demonstrated that Pin1 is overexpressed in a variety of cancer tissues and cell lines (*44*, *45*). In addition, the stable expression of Pin1-specific small interfering RNA constructs in PC-3 and LNCaP cells significantly reduced cellular proliferation, colony formation, migration, and invasion (*46*). Moreover, Pin1 was also suggested to be a prognostic marker for prostate cancer (*47*). Thus, the phosphorylation of those Ser or Thr residues on the N-terminal side of proline in HMGA1a and HMGA1b may facilitate the interaction of these two HMG proteins with Pin1 thereby affecting the downstream signaling pathways. Moreover, we speculate that the phosphorylations of Ser35, Thr52, and Thr77 in HMGA1a as well as Thr41 and Thr66 in HMGA1a might also be correlated with cancer progression. Thus, these phosphorylations may serve as biomarkers for the diagnosis and prognosis of cancers.

The biological implications of acetylation of Lys14 are not very clear, though we suspect that this acetylation may affect the interaction of HMGA1 proteins with other protein factors. In this context, it is worth noting that the acetylation of the Lys64 in an HMGA1a peptide PKRPRGRPKG-SKNKGAA (residues 53−69, Figure 1) was shown to have little effect on the binding of the peptide to a duplex DNA carrying a central AT-rich region (*48*). Thus, the repression of the human interferon-β gene, which follows the acetylation of Lys64 in HMGA1a (*15*, *30*), was suggested not to be a direct result of the abrogation of DNA binding (*48*). In the viewpoint that Lys14 is in close proximity to the first AT-hook domain of HMGA1a, we suspect that the acetylation of this residue may affect the association of HMGA1a with other proteins, which, in turn, may modulate the binding of HMGA1a protein to DNA.

It is of interest to note that the acetylation of Lys14 was either not identified, for the control PC-3 cells, or at markedly decreased level, for the butyrate-treated cells, in the hyperphosphorylated minor HMGA1a fraction, whereas this acetylation occurs at a detectable level for the major HMGA1a protein isolated from the control PC-3 cells. On the other hand, the phosphorylation of Ser35, Thr52, and Thr77 was observed for the minor, but not the major HMGA1a fraction. The three C-terminal serine residues were, however, phosphorylated in a similar fashion in the two fractions of the HMGA1a proteins. That modification at one site affects that of another is well-known for histone proteins (*49*, *50*). Our observations here, together with the previous findings that the acetylations of Lys64 and Lys70 have distinct functions on enhanceosome activity (*15*), suggest that the PTMs of HMGA1a protein may be highly regulated, which, in turn, may control the expression of a large number of target genes. In this regard, the unambiguous identification of the acetylation of Lys14 in HMGA1a and the determination of the sites of phosphorylation in the heavily phosphorylated HMGA1a protein set the stage for the future examination of the biological implications of these modifications.

HMGA proteins are considered as hubs for nuclear functions (*8*). As studies on histone PTMs have led to a wealth of new insights into the mechanisms of transcription, we reason that systematic characterizations of the PTMs of HMGA1 proteins pave the way for a better understanding of the role of these non-histone chromosomal proteins on transcriptional regulation (*8*, *9*), DNA repair (*51*), and cancer development (*8*, *9*).

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SUPPORTING INFORMATION AVAILABLE

Extracted ion chromatograms and ESI-MS/MS of post-translationally modified peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Bustin, M., and Reeves, R. (1996) High-mobility-group chromosomal proteins: architectural components that facilitate chromatin function, *Prog. Nucleic Acid Res. Mol. Biol. 54*, 35−100.
2. Johnson, K. R., Lehn, D. A., Elton, T. S., Barr, P. J., and Reeves, R. (1988) Complete murine cDNA sequence, genomic structure, and tissue expression of the high mobility group protein HMG-I(Y), *J. Biol. Chem. 263*, 18338−18342.
3. Johnson, K. R., Lehn, D. A., and Reeves, R. (1989) Alternative processing of mRNAs encoding mammalian chromosomal high-mobility-group proteins HMG-I and HMG-Y, *Mol. Cell. Biol. 9*, 2114−2123.
4. Strauss, F., and Varshavsky, A. (1984) A protein binds to a satellite DNA repeat at three specific sites that would be brought into mutual proximity by DNA folding in the nucleosome, *Cell 37*, 889−901.
5. Solomon, M. J., Strauss, F., and Varshavsky, A. (1986) A mammalian high mobility group protein recognizes any stretch of six A·T base pairs in duplex DNA, *Proc. Natl. Acad. Sci. U.S.A. 83*, 1276−1280.
6. Reeves, R., Elton, T. S., Nissen, M. S., Lehn, D., and Johnson, K. R. (1987) Posttranscriptional gene regulation and specific binding of the nonhistone protein HMG-I by the 3′ untranslated region of bovine interleukin 2 cDNA, *Proc. Natl. Acad. Sci. U.S.A. 84*, 6531−6535.
7. Reeves, R. (2004) HMGA proteins: isolation, biochemical modifications, and nucleosome interactions, *Methods Enzymol. 375*, 297−322.
8. Reeves, R. (2001) Molecular biology of HMGA proteins: hubs of nuclear function, *Gene 277*, 63−81.
9. Reeves, R., and Beckerbauer, L. (2001) HMGI/Y proteins: flexible regulators of transcription and chromatin structure, *Biochim. Biophys. Acta 1519*, 13−29.
10. Sgarra, R., Rustighi, A., Tessari, M. A., Di Bernardo, J., Altamura, S., Fusco, A., Manfioletti, G., and Giancotti, V. (2004) Nuclear phosphoproteins HMGA and their relationship with chromatin structure and cancer, *FEBS Lett. 574*, 1−8.
11. Diana, F., Sgarra, R., Manfioletti, G., Rustighi, A., Poletto, D., Sciortino, M. T., Mastino, A., and Giancotti, V. (2001) A link between apoptosis and degree of phosphorylation of high mobility group A1a protein in leukemic cells, *J. Biol. Chem. 276*, 11354−11361.
12. Sgarra, R., Diana, F., Bellarosa, C., Dekleva, V., Rustighi, A., Toller, M., Manfioletti, G., and Giancotti, V. (2003) During apoptosis of tumor cells HMGA1a protein undergoes methylation: identification of the modification site by mass spectrometry, *Biochemistry 42*, 3575−3585.
13. Banks, G. C., Li, Y., and Reeves, R. (2000) Differential in vivo modifications of the HMGI(Y) nonhistone chromatin proteins modulate nucleosome and DNA interactions, *Biochemistry 39*, 8333−8346.
14. Struhl, K. (2001) Gene regulation. A paradigm for precision, *Science 293*, 1054−1055.
15. Munshi, N., Agalioti, T., Lomvardas, S., Merika, M., Chen, G., and Thanos, D. (2001) Coordination of a transcriptional switch by HMGI(Y) acetylation, *Science 293*, 1133−1136.
16. Elton, T. S., and Reeves, R. (1986) Purification and postsynthetic modifications of Friend erythroleukemic cell high mobility group protein HMG-I, *Anal. Biochem. 157*, 53−62.
17. Giancotti, V., Pani, B., D'Andrea, P., Berlingieri, M. T., Di Fiore, P. P., Fusco, A., Vecchio, G., Philp, R., Crane-Robinson, C., Nicolas, R. H., Wright, C. A., and Goodwin, G. H. (1987) Elevated levels of a specific class of nuclear phosphoproteins in cells transformed with v-ras and v-mos oncogenes and by cotransfection with c-myc and polyoma middle T genes, *EMBO J. 6*, 1981−1987.
18. Zou, Y., and Wang, Y. (2005) Tandem mass spectrometry for the examination of the posttranslational modifications of high-mobility group A1 proteins: symmetric and asymmetric dimethylation of Arg25 in HMGA1a protein, *Biochemistry 44*, 6293−6301.
19. Sgarra, R., Lee, J., Tessari, M. A., Altamura, S., Spolaore, B., Giancotti, V., Bedford, M. T., and Manfioletti, G. (2006) The AT-hook of the chromatin architectural transcription factor high mobility group A1a is arginine-methylated by protein arginine methyltransferase 6, *J. Biol. Chem. 281*, 3764−3772.
20. Miranda, T. B., Webb, K. J., Edberg, D. D., Reeves, R., and Clarke, S. (2005) Protein arginine methyltransferase 6 specifically methylates the nonhistone chromatin protein HMGA1a, *Biochem. Biophys. Res. Commun. 336*, 831−835.
21. Lund, T., and Laland, S. G. (1990) The metaphase specific phosphorylation of HMG I, *Biochem. Biophys. Res. Commun. 171*, 342−347.
22. Nissen, M. S., Langan, T. A., and Reeves, R. (1991) Phosphorylation by cdc2 kinase modulates DNA binding activity of high mobility group I nonhistone chromatin protein, *J. Biol. Chem. 266*, 19945−19952.
23. Reeves, R., Langan, T. A., and Nissen, M. S. (1991) Phosphorylation of the DNA-binding domain of nonhistone high-mobility group I protein by cdc2 kinase: reduction of binding affinity, *Proc. Natl. Acad. Sci. U.S.A. 88*, 1671−1675.
24. Xiao, D. M., Pak, J. H., Wang, X., Sato, T., Huang, F. L., Chen, H. C., and Huang, K. P. (2000) Phosphorylation of HMG-I by protein kinase C attenuates its binding affinity to the promoter regions of protein kinase C gamma and neurogranin/RC3 genes, *J. Neurochem. 74*, 392−399.
25. Palvimo, J., and Linnala-Kankkunen, A. (1989) Identification of sites on chromosomal protein HMG-I phosphorylated by casein kinase II, *FEBS Lett. 257*, 101−104.
26. Ferranti, P., Malorni, A., Marino, G., Pucci, P., Goodwin, G. H., Manfioletti, G., and Giancotti, V. (1992) Mass spectrometric analysis of the HMGY protein from Lewis lung carcinoma. Identification of phosphorylation sites, *J. Biol. Chem. 267*, 22486−22489.
27. Wang, D. Z., Ray, P., and Boothby, M. (1995) Interleukin 4-inducible phosphorylation of HMG-I(Y) is inhibited by rapamycin, *J. Biol. Chem. 270*, 22924−22932.
28. Sgarra, R., Diana, F., Rustighi, A., Manfioletti, G., and Giancotti, V. (2003) Increase of HMGA1a protein methylation is a distinctive characteristic of leukaemic cells induced to undergo apoptosis, *Cell Death Differ. 10*, 386-389.
29. Edberg, D. D., Bruce, J. E., Siems, W. F., and Reeves, R. (2004) In vivo posttranslational modifications of the high mobility group a1a proteins in breast cancer cells of differing metastatic potential, *Biochemistry 43*, 11500−11515.
30. Munshi, N., Merika, M., Yie, J., Senger, K., Chen, G., and Thanos, D. (1998) Acetylation of HMG I(Y) by CBP turns off IFN beta expression by disrupting the enhanceosome, *Mol. Cell 2*, 457−467.
31. Edberg, D. D., Adkins, J. N., Springer, D. L., and Reeves, R. (2005) Dynamic and differential in vivo modifications of the isoform HMGA1a and HMGA1b chromatin proteins, *J. Biol. Chem. 280*, 8961−8973.
32. Merika, M., and Thanos, D. (2001) Enhanceosomes, *Curr. Opin. Genet. Dev. 11*, 205−208.
33. Zou, Y., Jiang, X., and Wang, Y. (2004) Identification of novel in vivo phosphorylation sites in high mobility group N1 protein from the MCF-7 human breast cancer cells, *Biochemistry 43*, 6322−6329.
34. Zhu, W. G., and Otterson, G. A. (2003) The interaction of histone deacetylase inhibitors and DNA methyltransferase inhibitors in

the treatment of human cancer cells, *Curr. Med. Chem. Anti-cancer Agents 3*, 187−199.
35. Zhang, K., Yau, P. M., Chandrasekhar, B., New, R., Kondrat, R., Imai, B. S., and Bradbury, M. E. (2004) Differentiation between peptides containing acetylated or tri-methylated lysines by mass spectrometry: an application for determining lysine 9 acetylation and methylation of histone H3, *Proteomics 4*, 1−10.
36. March, R. E. (1997) An introduction to quadrupole ion trap mass spectrometry, *J. Mass Spectrom. 32*, 351−369.
37. Mann, M., and Jensen, O. N. (2003) Proteomic analysis of post-translational modifications, *Nat. Biotechnol. 21*, 255−261.
38. Huq, M. D., and Wei, L. N. (2005) Post-translational modification of nuclear co-repressor receptor-interacting protein 140 by acetylation, *Mol. Cell. Proteomics 4*, 975−983.
39. Sarg, B., Helliger, W., Talasz, H., Koutzamani, E., and Lindner, H. H. (2004) Histone H4 hyperacetylation precludes histone H4 lysine 20 trimethylation, *J. Biol. Chem. 279*, 53458−53464.
40. Castedo, M., Perfettini, J. L., Roumier, T., and Kroemer, G. (2002) Cyclin-dependent kinase-1: linking apoptosis to cell cycle and mitotic catastrophe, *Cell Death Differ. 9*, 1287−1293.
41. Borgne, A., and Golsteyn, R. M. (2003) The role of cyclin-dependent kinases in apoptosis, *Prog. Cell Cycle Res. 5*, 453−459.
42. Lu, K. P., Liou, Y. C., and Zhou, X. Z. (2002) Pinning down proline-directed phosphorylation signaling, *Trends Cell Biol. 12*, 164−172.
43. Lu, K. P., Hanes, S. D., and Hunter, T. (1996) A human peptidyl-prolyl isomerase essential for regulation of mitosis, *Nature 380*, 544−547.
44. Wulf, G. M., Ryo, A., Wulf, G. G., Lee, S. W., Niu, T., Petkova, V., and Lu, K. P. (2001) Pin1 is overexpressed in breast cancer and cooperates with Ras signaling in increasing the transcriptional activity of c-Jun towards cyclin D1, *EMBO J. 20*, 3459−3472.
45. Bao, L., Kimzey, A., Sauter, G., Sowadski, J. M., Lu, K. P., and Wang, D. G. (2004) Prevalent overexpression of prolyl isomerase Pin1 in human cancers, *Am. J. Pathol. 164*, 1727−1737.
46. Ryo, A., Uemura, H., Ishiguro, H., Saitoh, T., Yamaguchi, A., Perrem, K., Kubota, Y., Lu, K. P., and Aoki, I. (2005) Stable suppression of tumorigenicity by Pin1-targeted RNA interference in prostate cancer, *Clin. Cancer Res. 11*, 7523−7531.
47. Ayala, G., Wang, D., Wulf, G., Frolov, A., Li, R., Sowadski, J., Wheeler, T. M., Lu, K. P., and Bao, L. (2003) The prolyl isomerase Pin1 is a novel prognostic marker in human prostate cancer, *Cancer Res. 63*, 6244−6251.
48. Dragan, A. I., Liggins, J. R., Crane-Robinson, C., and Privalov, P. L. (2003) The energetics of specific binding of AT-hooks from HMGA1 to target DNA, *J. Mol. Biol. 327*, 393−411.
49. Jenuwein, T., and Allis, C. D. (2001) Translating the histone code, *Science 293*, 1074−1080.
50. Peterson, C. L., and Laniel, M.-A. (2004) Histones and histone modifications, *Curr. Biol. 14*, R546−R551.
51. Adair, J. E., Kwon, Y., Dement, G. A., Smerdon, M. J., and Reeves, R. (2005) Inhibition of nucleotide excision repair by high mobility group protein HMGA1, *J. Biol. Chem. 280*, 32184−32192.

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