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

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ABSTRACT: High-mobility group (HMG) A1a and A1b proteins are among a family of HMGA proteins that bind to the minor groove of AT-rich regions of DNA. Here we employed tandem mass spectrometry and determined without ambiguity the sites of phosphorylation and the nature of methylation of HMGA1 proteins that were isolated from the PC-3 human prostate cancer cells. We showed by LC−MS/MS that Ser101 and Ser102 were completely phosphorylated in HMGA1a protein, whereas only a portion of the protein was phosphorylated at Ser98. We also found that the HMGA1b protein was phosphorylated at the corresponding sites, that is, Ser90, Ser91 and Ser87. In addition, Arg25, which is within the first DNA-binding AT-hook domain of HMGA1a, was both mono- and dimethylated. Moreover, both symmetric and asymmetric dimethylations were observed. The closely related HMGA1b protein, however, was not methylated. The unambiguous identification of the sites of phosphorylation and the nature of methylation facilitates the future examination of the biological implications of the HMGA1 proteins.

The mammalian high-mobility group A (HMGA) proteins, including HMGA1a, HMGA1b (previously designated as HMGI and HMGY, respectively), and HMGA2, are members of one subfamily of nonhistone chromosomal HMG[1] proteins (*1*). HMGA1a and HMGA1b proteins result from the translation of splicing variants of a single gene (*HMGA1*) and differ only in that HMGA1b protein has an 11-residue internal deletion (*2*, *3*). These proteins contain three highly conserved "AT-hook" domains that preferentially bind to the minor groove of AT-rich DNA stretches (*4*−*6*). In addition to DNA and nucleosome binding, HMGA1 proteins are postulated to be architectural transcription factors that regulate gene expression in vivo and function in a wide variety of cellular processes including cell growth, differentiation, chromatin remodeling, apoptosis, and neoplastic transformation (*7*−*9*).

HMGA1 proteins are among the nuclear proteins that are highly posttranslationally modified (*10*); phosphorylation, methylation, acetylation, and ADP-ribosylation of HMGA1 have been observed (*11*−*17*). In this context, protein kinase CK2, formerly known as casein kinase 2 (CK2), can catalyze the constitutive phosphorylations of Ser98, Ser101, and Ser102 in HMGA1a proteins in vitro and in vivo (*18*−*20*). The function of this constitutive phosphorylation is not clearly understood, though its indirect effect on altering the proteins' DNA binding property has been demonstrated in the past (*10*, *11*).

The HMGA1 proteins are also substrates for cdc2 kinase and protein kinase C. Thr52 and Thr77 in HMGA1a have been shown to be phosphorylated by the former kinase in vitro (*21*). In addition, the two threonines were found to be phosphorylated in vivo in a cell-cycle-dependent manner (*21*, *22*). In HMGA1a, Thr52 and Thr77 are on the N-termini of the second and third "AT hook" domains, respectively (*8*), and the phosphorylation of these two amino acids diminishes significantly (∼20-fold) the binding affinity of the proteins to AT-rich DNA (*21*, *22*). Furthermore, Thr20, Ser43, and Ser63 in HMGA1a were major sites of phosphorylation catalyzed by protein kinase C-α (PKCα) (*13*). The PKC-catalyzed phosphorylation of HMGA1a again reduces the protein−DNA binding affinity (*23*).

In addition to phosphorylation, methylation of HMGA1 proteins has also been reported recently (*12*, *13*, *24*, *25*). In this regard, matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS) results suggested that the HMGA1b protein isolated from human breast cancer cell lines, MCF-7 and MCF-7/PKC-α, might bear several sites of methylations, but the modified amino acid residues were not explicitly determined (*13*). In addition, Giancotti and co-workers (*12*, *24*) showed by LC−MS that Arg25 in HMGA1a is monomethylated and the methylation is correlated with the execution of apoptosis in cancer cells. More recently, Edberg et al. (*25*) reported the dimethylation of arginine and lysine residues in HMGA1a in cultured human breast cancer cells with different metastatic potentials; Arg25, however, was not shown to be methylated.

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[1] Abbreviations: HMG, high-mobility group; PTM, posttranslational modification; CK2, protein kinase CK2 (or casein kinase 2); CIP, calf intestinal alkaline phosphatase; PRMT, protein arginine methyltransferase; CARM, coactivator-associated arginine methyltransferase; MMA, N^G-monomethylarginine; aDMA, asymmetric N^G,N^G-dimethylarginine; sDMA, symmetric N^G,N'^G-dimethylarginine; ESI, electrospray ionization; MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; MS/MS, tandem mass spectrometry; TFA, trifluoroacetic acid; PCA, perchloric acid.

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To understand the biological implications of posttranslational modifications (PTMs) of HMGA1 proteins, it is important to investigate rigorously the sites and types of these modifications. In the present study, we examined the sites of PTMs of HMGA1a and HMGA1b proteins by using tandem mass spectrometry, that is, LC-ESI−MS/MS and MALDI-MS/MS. Our results confirmed the sites of constitutive phosphorylation of the C-terminus of HMGA1a and HMGA1b proteins as well as the site of monomethylation of the former protein. Moreover, we demonstrated for the first time that Arg25 in HMGA1a can be both symmetrically and asymmetrically dimethylated.

EXPERIMENTAL PROCEDURES

Cell Culture. The PC-3 human prostate cancer cells (ATCC, Manassas, VA) were cultured in F-12K medium (ATCC, Manassas, VA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and 100 IU/mL of penicillin.

Protein Extraction and Purification. The HMG proteins were extracted by using 5% perchloric acid (PCA) as described previously (*7*, *26*). The PCA-soluble proteins were separated on a Surveyor HPLC system (ThermoFinnigan, San Jose, CA) by using a 4.6 mm × 250 mm C4 column (Varian, Walnut Creek, CA). The flow rate was 1.0 mL/min, and a 50-min gradient of 5−30% CH_3CN in 0.1% aqueous solution of trifluoroacetic acid (TFA) was employed. The chromatogram was obtained by absorbance detection at 220 nm. Fractions containing HMGA1 proteins with different states of modifications were collected, and the individual fractions were subjected to MALDI-MS analysis.

To estimate the amounts of HMGA1 proteins, 90 μg of type II-A histone (Sigma-Aldrich, St. Louis, MO) was dissolved in a 0.1% aqueous solution of TFA and separated by HPLC as described previously (*26*). The amounts of HMGA1 proteins were calculated by using areas of the peaks for the HMGA1 proteins and type II-A histone.

Alkaline Phosphatase Treatment. The dephosphorylation reaction was carried out in a 50-mM NH_4HCO_3 solution containing approximately 0.7 μg of HMGA1 proteins and 1 unit of calf intestinal alkaline phosphatase (CIP, New England Biolabs, Beverly, MA), and the mixture was incubated at 37 °C for 30 min. The reaction mixture was then dried and subjected to MALDI-TOF MS analysis.

Trypsin Digestion. Sequencing-grade modified trypsin was purchased from Roche Applied Science (Indianapolis, IN). Approximately 2 or 0.2 μg of HMGA1 protein was incubated with trypsin at an enzyme-to-substrate ratio of 1:20 in a 100-mM NH_4HCO_3 solution, and the digestion was continued at 37 °C for 3 or 7 h.

Mass Spectrometry. MALDI-TOF MS experiment was performed on a DE-STR instrument equipped with a nitrogen laser (Applied Biosystems, Foster City, CA). The analyses were carried out in linear and reflectron modes for proteins and peptides, respectively. The mass accuracies with default calibration were approximately 1000 and 100 ppm in linear and reflectron modes, respectively. Purified protein samples 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).

MALDI-tandem mass spectrometry (MS/MS) measurements were performed on a QSTAR XL quadrupole/time-of-flight mass spectrometer equipped with an o-MALDI ion source (Applied Biosystems). The mass accuracy in MS/MS mode was approximately 10−30 ppm with external calibration.

On-line LC-electrospray ionization (ESI)−MS/MS was employed for peptide sequencing, and a 0.30 mm × 150 mm C18 capillary column (300 Å in pore size, 5 μm in particle size, Micro-Tech Scientific, Vista, CA) was used. The flow rate was 4−6 μL/min, which was obtained from a 120 μL/min pump flow by using a homemade precolumn splitter, and a 63-min gradient of 2−65% CH_3CN in a 0.6% aqueous solution of acetic acid was employed. The effluent from the HPLC column was directed to an LCQ Deca XP ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA).

Mass calibration for the ion-trap mass spectrometer was carried out by using caffeine, a tetrapeptide MRFA, and Ultramark 1621, which 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 most abundant protonated ions observed in MS mode for collisional activation. The mass width for precursor ion isolation was 3 *m*/*z* units, and the collision gas was helium. To achieve better signal-to-noise ratio in MS/MS, LC-ESI−MS/MS experiments were also carried out for monitoring the fragmentation of one or a few preselected precursor ions.

RESULTS

1. HMGA1a Protein Is Both Phosphorylated and Methylated in PC-3 Cells. We isolated the HMG proteins from the PC-3 cell lysate by acid extraction and separated the subfamilies of the HMG proteins by reversed-phase HPLC. The HMGA1a protein eluted at approximately 20% acetonitrile among other proteins that are soluble in 5% PCA (Figure 1A). The two fractions with retention times of 30 and 31 min (Figure 1A) contain the HMGA1a protein (the MALDI-MS results of the HMGA1a protein from these two fractions are shown in Figure 1B,D). In this respect, the HMGA1a protein from the 30-min fraction appears to be diphosphorylated (2P, *m*/*z* 11 750, calculated *m*/*z* 11 748 for the $[M + H]^+$ ion) and triphosphorylated (3P, *m*/*z* 11 828, calculated *m*/*z* 11 827, Figure 1B). In addition, the HMGA1a protein seems to be also monomethylated (2P/1M, *m*/*z* 11 765, calculated *m*/*z* 11 762; 3P/1M, *m*/*z* 11 844, calculated *m*/*z* 11 842, Figure 1B) and dimethylated (2P/2M, *m*/*z* 11 780, calculated *m*/*z* 11 776; 3P/2M, *m*/*z* 11 858, calculated *m*/*z* 11 856, Figure 1D). The calculated *m*/*z* of HMGA1a was based on the amino acid sequence assuming that the N-terminus of the protein is acetylated (*11*−*13*). It is worth mentioning that the unphosphorylated form of the HMGA1a protein was not detected.

To confirm the presence of phosphorylation and to visualize other form(s) of PTMs better, especially methylation, we treated the isolated HMGA1a protein with alkaline phosphatase and subjected the resulting products to MALDI-MS analysis. The MALDI-MS result showed that such treatment results in N-acetylated HMGA1a protein (*m*/*z* 11 590, calculated *m*/*z* 11 588) and its monomethylated form (*m*/*z* 11 603, calculated *m*/*z* 11 602) for the 30-min fraction

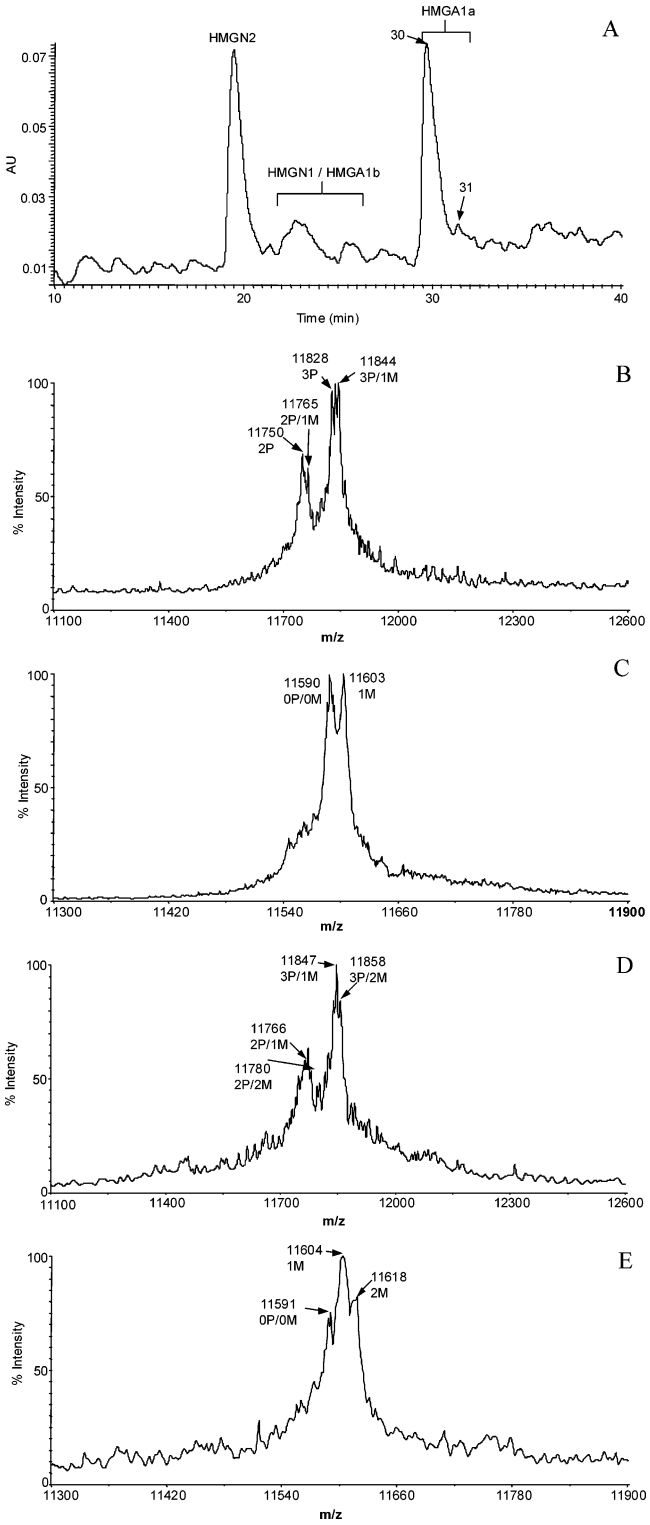


FIGURE 1: HPLC trace and MALDI-TOF MS measurements show the phosphorylation and methylation of HMGA1a protein in PC-3 cells. (A) HPLC trace for the separation of the PCA-soluble proteins. The effluents were monitored by absorbance at 220 nm. The arrows indicate the HPLC fractions that were used for subsequent MALDI-MS analysis. (B, D) MALDI-TOF mass spectra of HMGA1a protein from the 30- and 31-min fractions. (C, E) MALDI-TOF mass spectra of HMGA1a protein from the 30- and 31-min fractions that have been treated with alkaline phosphatase (CIP).

(Figure 1C). In addition, both monomethylated (*m*/*z* 11 604, calculated *m*/*z* 11 602) and dimethylated (*m*/*z* 11 618, calculated *m*/*z* 11 616, Figure 1E) forms of HMGA1a were more evident after the alkaline phosphatase treatment. These results not only confirmed that the HMGA1a protein is phosphorylated but also highlighted that the protein can be mono- and dimethylated in PC-3 cells.

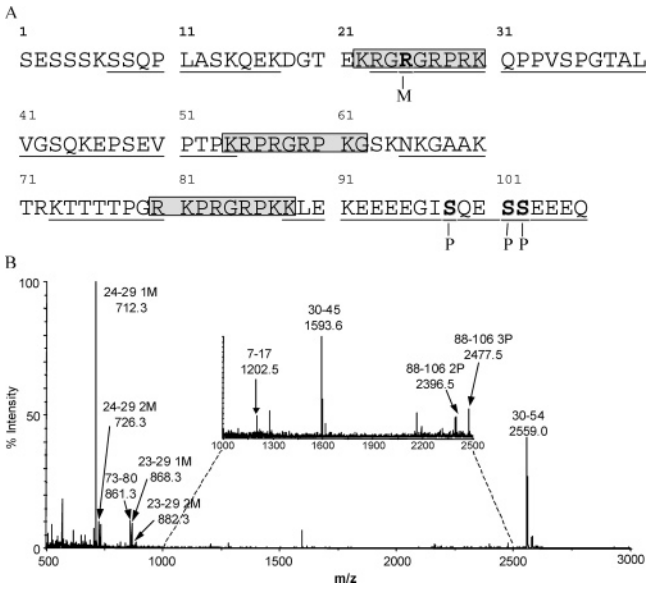


FIGURE 2: (A) Summary of sequence coverage for HMGA1a protein from the peptides identified by LC-ESI−MS/MS analysis of the tryptic digest. The covered residues are underlined. "P" and "M" underneath a vertical bar represent the phosphorylation and methylation sites, respectively. The shaded rectangular boxes illustrate the three AT-hook DNA-binding domains. The human HMGA1a sequence was obtained from Swiss-Prot. (B) MALDI-TOF mass spectrum of the tryptic digestion mixture of HMGA1a protein isolated from the PC-3 cells. Sequence assignments for tryptic fragments are shown above the *m*/*z* values of the ions; "P" and "M" represent phosphorylation and methylation, respectively.

2. Ser98, Ser101, and Ser102 Can Be Phosphorylated in HMGA1a Protein. To establish unambiguously the sites of phosphorylation and methylation, we digested the HMGA1 proteins with trypsin and subjected the resulting peptide mixture to MALDI-MS (MS/MS) and LC-ESI−MS/MS analyses. The LC−MS/MS results were further searched against the Swiss-Prot database by using software package TurboSEQUEST (ThermoFinnigan, San Jose, CA). The sequence coverage for HMGA1a protein was 72% based on LC-ESI−MS/MS and database search results (Figure 2A).

Now we begin our discussion on the sites of phosphorylation in HMGA1a. MALDI-MS of the tryptic digestion mixture showed both di- and triphosphorylated forms of a peptide segment containing amino acid residues 88−106, that is, ions of *m*/*z* 2396.5 and 2477.5 (Figure 2B). This result is in agreement with the fact that the protein can adopt two or three sites of phosphorylation, suggesting that the phosphorylation occurs exclusively in this peptide segment. This conclusion is further supported by the fact that we did not observe the unphosphorylated form of this peptide segment (residues 88−106) (Figure 2B).

The LC-ESI−MS/MS results allow us to establish the sites of phosphorylation of the HMGA1a protein. The product-ion spectrum of the $[M + 2H]^{2+}$ ion (ion of *m*/*z* 1198.8) of the diphosphorylated form of the peptide KLEKEEEEGISQESSEEEQ (residues 88−106) showed that Ser101 and Ser102 are phosphorylated, whereas Ser98 is unmodified (Figure 3A, and most fragment ions are summarized in Figure

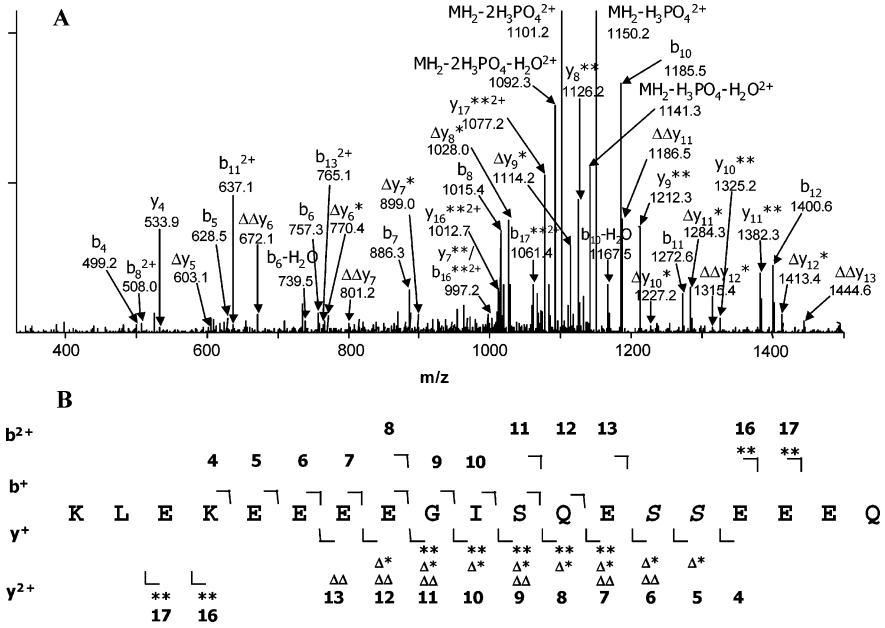


FIGURE 3: Ser101 and Ser102 are the modification sites in the diphosphorylated HMGA1a protein. (A) Product-ion spectrum of the $[M + 2H]^{2+}$ ion (*m*/*z* 1198.8) of diphosphorylated peptide 88−106 (KLEKEEEEGISQE***S*****S***EEEQ) obtained from an LC-ESI−MS/MS experiment. An asterisk (*) indicates that an ion bears a phosphate group, and neutral loss of an H_3PO_4 is represented by a triangle (Δ). The symbol */Δ indicates that both forms were observed. (B) A summary of the observed b^+, b^{2+} ions (+) and y^+, y^{2+} ions (+) for the peptide bearing residues 88−106. The phosphorylated residues are highlighted by a bold italic letter.

3B). In this regard, we found that the *m*/*z* values of the b_{11}, b_{12} and b_{13}^{2+} ions were the same as the calculated *m*/*z* values of the corresponding b ions for the unmodified peptide. This, together with the failure in detecting the respective phosphoserine-bearing b_{11}, b_{12}, and b_{13} ions, demonstrates conclusively that Ser98 is not modified in the diphosphorylated peptide.

In this peptide segment, Ser98, Ser101, and Ser102 are the only residues that can possibly be phosphorylated. The above results, therefore, also illustrate that both Ser101 and Ser102 are phosphorylated, which is further supported by the presence of y_7** and y_8** ions in the product-ion spectrum (the symbol "*" represents the presence of a phosphorylated residue).

There are only three residues that can be phosphorylated in the peptide segment with residues 88−106. Therefore, all three serines (Ser98, Ser101, and Ser102) are phosphorylated in the triphosphorylated peptide, which is supported by ESI−MS/MS results (Figure S1, Supporting Information).

Similar to the HMGA1a protein, the HMGA1b protein extracted from PC-3 cells can be either di- or triphosphorylated. In addition, we determined by LC-ESI−MS/MS that Ser90 and Ser91 are completely phosphorylated, whereas Ser87 is not phosphorylated in the di-phosphorylated peptide (product-ion spectra are shown in Figures S2 and S3, Supporting Information). Considering that there is a deletion of the 11 amino acids in the HMGA1b protein, the Ser87, Ser90, and Ser91 in HMGA1b correspond to Ser98, Ser101, and Ser102 in HMGA1a protein. The above findings are consistent with previous studies for the HMGA1a protein isolated from human placenta, in which the sites of phosphorylation were established by amino acid sequencing of tryptic fragments (*18*), and for the HMGA1b protein purified from Lewis lung carcinoma, where the sites of phosphorylations were determined via fast-atom bombardment (FAB)−MS analyses of proteolytic fragments (*19*).

3. Mono- and Dimethylation of Arg25 in HMGA1a Protein. In addition to the phosphorylated peptides, MALDI-MS analysis of the tryptic digestion mixture of the HMGA1a protein also showed a mono- and dimethylated peptide segment containing amino acid residues 24−29 (GRGRPR, *m*/*z* 712.3 and 726.3) (Figure 2B).

To identify the site of monomethylation, we first acquired the product-ion spectrum of the ion of *m*/*z* 712.3 in an LC−MS/MS experiment. The resulting MS/MS (Figure 4) showed the formation of not only y and b ions but also product ions initiated from losses of neutral fragments, including eliminations of NH_3 (17 Da), H_2O (18 Da), methylamine (CH_3NH_2, 31 Da), carbodiimide [$C(NH)_2$, 42 Da], and monomethylcarbodiimide ($CH_3N{=}C{=}NH$, 56 Da). The losses of CH_3NH_2 and $CH_3N{=}C{=}NH$ support that this peptide bears a monomethylarginine (MMA) (Scheme 1). The eliminations of these two neutral fragments have been previously observed for G−MMA−GLSLSR (*27*). Furthermore, characteristic fragment ions allow us to determine that only Arg25 is methylated. In this respect, we found that the y_3 and y_4 ions have the same *m*/*z* values as those calculated for the unmodified peptide (Figure 4), suggesting that neither Arg27 nor Arg29 is methylated. Moreover, we observed $b_4^{\#}$ (*m*/*z* 441.2), $b_5^{\#} + H_2O$ (*m*/*z* 556.3) and product ions resulting from neutral losses from these fragments, for example, $b_4^{\#} - C(NH)_2$ (*m*/*z* 399.2), $b_5^{\#} + H_2O - NH_3$ (*m*/*z* 539.3), $b_5^{\#} + H_2O - CH_3NH_2$ (*m*/*z* 525.2), $b_5^{\#} + H_2O - C(NH)_2$ (*m*/*z* 514.2), and $b_5^{\#} + H_2O - CH_3N{=}C{=}NH$ (*m*/*z* 500.2) ions (Figure 4, "#" designates those ions carrying a monomethylarginine, that is, a molecular mass increase of 14 Da). The above results, therefore, demonstrate that Arg25 is monomethylated. It is worth mentioning that the formation of $[b + H_2O]$ ion from the loss of the C-terminal amino acid residue has been reported previously (*28*). Moreover, MALDI-MS/MS (Figure S4) of the ion of *m*/*z* 712.3 allowed us to draw the same conclusion. In this context, we also observed y_1,

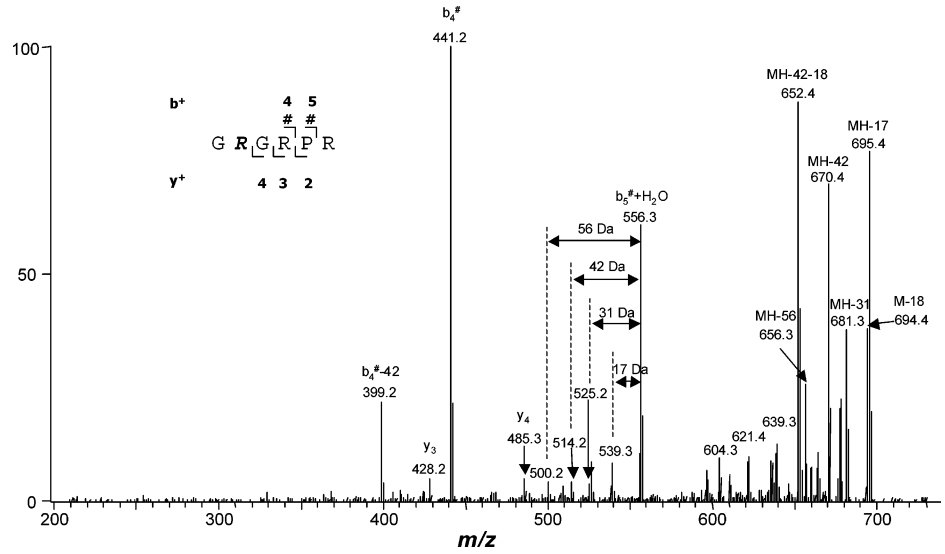
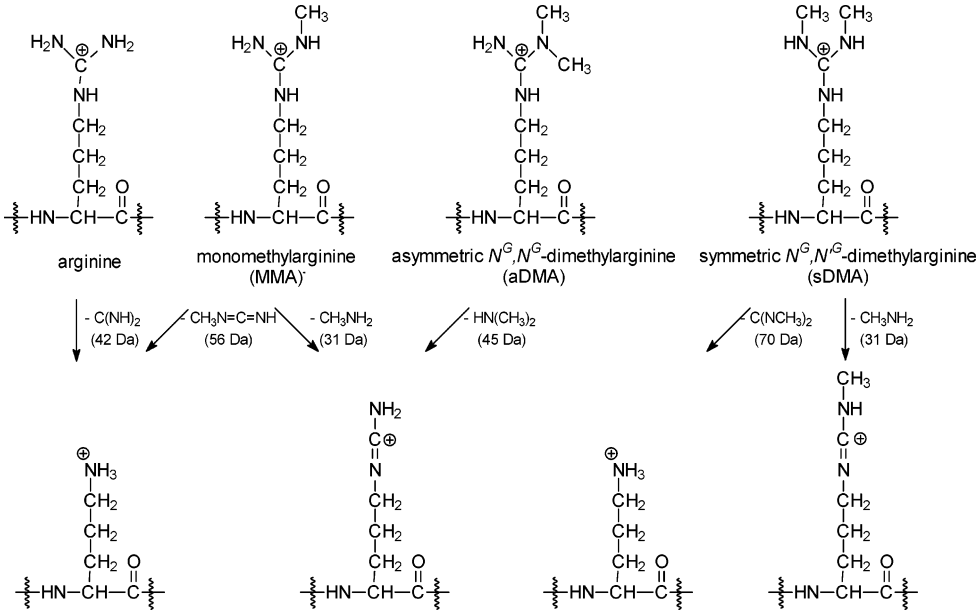


FIGURE 4: Product-ion spectrum of the ESI-produced $[M + H]^+$ ion (*m*/*z* 712.3) of monomethylated peptide 24−29 (G***R***#GRPR) shows that Arg25 is monomethylated in HMGA1a protein. Neutral losses are indicated in the spectrum. The ions bearing a monomethylarginine are labeled with a "#". The neutral losses of 17-, 31-, 42-, and 56-Da fragments correspond to the losses of an NH_3, CH_3NH_2, $C(NH)_2$, and $CH_3N{=}C{=}NH$, respectively (see discussion in text and Scheme 1). The methylated residue is indicated as a bold italic letter.

Scheme 1: Characteristic Neutral Losses from the Side Chains of Unmodified Arginine, Monomethylarginine (MMA), Asymmetric N^G,N^G-Dimethylarginine (aDMA), and Symmetric N^G, N'^G-Dimethylarginine (sDMA) (*27*, *29*)



y_2, and $b_2^{\#}$ ions in the latter spectrum, which provides additional evidence supporting the monomethylation of Arg25.

The nature of dimethylation in GRGRPR (residues 24−29) was also determined by a tandem mass spectrometry experiment. First of all, MALDI-MS/MS results showed that the two isomeric forms of dimethylarginine (DMA), that is, asymmetric N^G,N^G-dimethylarginine (aDMA) and symmetric N^G,N'^G-dimethylarginine (sDMA), are both present in this dimethylated peptide containing amino acids 24−29. In this respect, it has been demonstrated in the past that asymmetric and symmetric dimethylarginines can be differentiated by MS/MS based on characteristic side chain neutral losses (*27*, *29*). As shown in Scheme 1, the side chain of aDMA can cleave and result in the neutral loss of a dimethylamine [$NH(CH_3)_2$, 45 Da] (*27*, *29*). The side chain of sDMA, on the other hand, can fragment to lead to the losses of both a methylamine (CH_3NH_2, 31 Da) and a dimethylcarbodiimide [$C(NCH_3)_2$, 70 Da] (*27*, *29*). These specific fragmentations can be utilized as "reporter neutral losses" in the identification of aDMA and sDMA (*27*, *29*).

The product-ion spectrum of the ion of *m*/*z* 726.3 (Figure 5) showed the above "reporter" neutral losses from the molecular ion ($[M + H]^+$ ion), that is, the losses of methylamine (31 Da, *m*/*z* 695.4), dimethylamine (45 Da, *m*/*z* 681.4), and dimethylcarbodiimide (70 Da, *m*/*z* 656.4). Moreover, neutral losses of those fragments from $b_5^{\#\#} + H_2O$, $b_4^{\#\#}$, and $b_2^{\#\#}$ ions were also found. These observations, therefore, support again the presence of both aDMA and sDMA in this peptide. Because the neutral loss of a methylamine (31 Da) can also arise from the cleavage of the side chain of a monomethylarginine (vide supra), it is possible that there are two MMAs in this peptide. Such possibility, however, can be excluded from the absence of

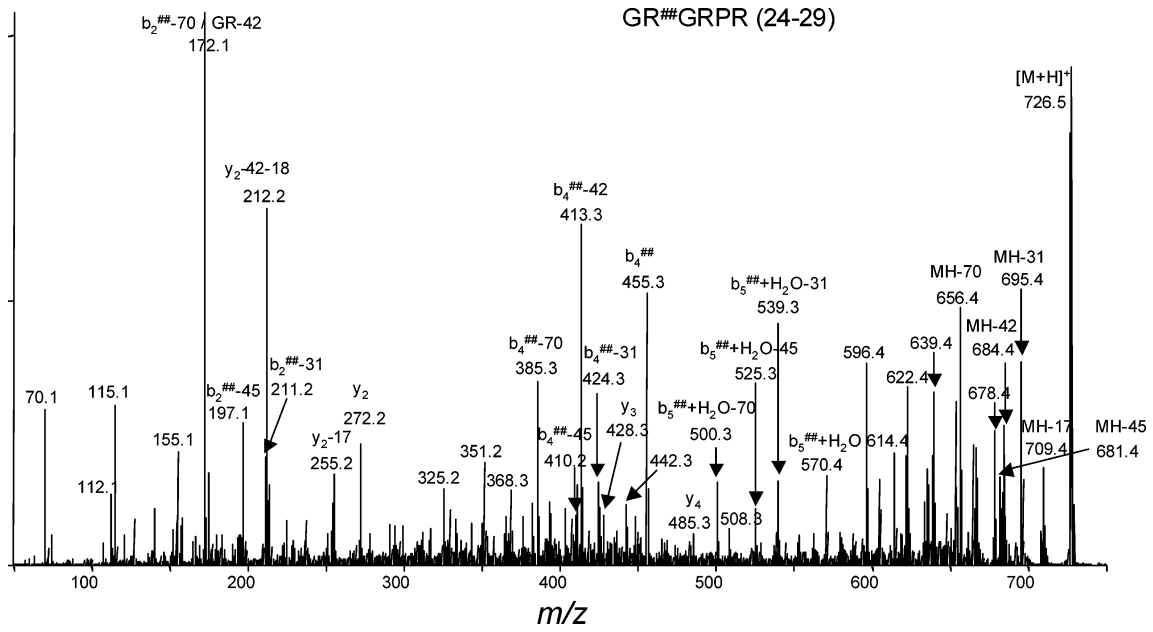


FIGURE 5: Product-ion spectrum of the MALDI-produced $[M + H]^+$ ion (*m/z* 726.4) of dimethylated peptide G***R*****##**GRPR (24−29). The symbol "##" designates fragment ions bearing a dimethylarginine. The neutral losses of 17-, 31-, 42-, 45-, and 70-Da fragments correspond to the losses of an NH_3, CH_3NH_2, $C(NH)_2$, $(CH_3)_2NH$, and $C(NCH_3)_2$, respectively (see discussion in text and Scheme 1). The methylated residue is indicated as a bold italic letter.

the neutral loss of monomethylcarbodiimide (56 Da), which is a characteristic cleavage from monomethylated arginine (vide supra, Scheme 1) from either the precursor ion or the b/b + H_2O ions. For instance, the peaks corresponding to [M − 56] (calculated *m/z* 670.4), [$b_5^{\#\#}$ + H_2O − 56] (*m/z* 514.3), and [$b_4^{\#\#}$ − 56] (*m/z* 399.3) ions are all below the noise level (Figure 5). Therefore, we can conclude that this peptide does not contain two MMAs.

The site of dimethylation can be determined from fragment ions emanating from backbone cleavages. In this context, we observed unmodified y_2 (*m/z* 272.2), y_3 (*m/z* 428.3), and y_4 ions (*m/z* 485.3, Figure 5), suggesting that neither Arg27 nor Arg29 is methylated. Along this line, we observed $b_2^{\#\#}$ − CH_3NH_2 (*m/z* 211.2), $b_2^{\#\#}$ − $HN(CH_3)_2$ (*m/z* 197.1), and possibly $b_2^{\#\#}$ − $C(NCH_3)_2$ (*m/z* 172.1, this ion can also be assigned as an internal fragment ion of GR − 42) ions, though $b_2^{\#\#}$ ion is undetectable. In addition, we found $b_4^{\#\#}$ (*m/z* 455.3), $b_4^{\#\#}$ − CH_3NH_2 (*m/z* 424.3), $b_4^{\#\#}$ − $C(NH)_2$ (*m/z* 413.3), $b_4^{\#\#}$ − $C(NCH_3)_2$ (*m/z* 385.3), $b_5^{\#\#}$ + H_2O (*m/z* 570.4), $b_5^{\#\#}$ + H_2O − CH_3NH_2 (*m/z* 539.3), $b_5^{\#\#}$ + H_2O − $NH(CH_3)_2$ (*m/z* 525.3), and $b_5^{\#\#}$ + H_2O − $C(NCH_3)_2$ ions (*m/z* 500.3). These results strongly support that the Arg25 is dimethylated.

To examine whether the sites of dimethylation of this peptide are heterogeneous, we searched for ions which might suggest the dimethylation of other arginine residue(s). In this regard, we did not observe any ions supporting the dimethylation of Arg27 or Arg29. For instance, neither $y_2^{\#\#}$ (*m/z* 300.2) nor $y_4^{\#\#}$ (*m/z* 513.3) ion is detectable in Figure 5. Thus, the dimethylation occurs exclusively on Arg25. It is important to note that neither monomethylation nor dimethylation was observed in HMGA1b protein isolated from PC-3 cells (data not shown), which is consistent with previous studies (*12*).

DISCUSSION

The mammalian HMGA1 proteins have been suggested to be central "hubs" of nuclear function because of their intimate involvement in a wide variety of biological processes in vivo (*8*). The overexpression of HMGA1 proteins is among the most consistent feature observed in a variety of cancers with elevated concentrations being correlated with increasing malignancy (*7*, *8*). In addition, various types of in vivo posttranslational modifications (PTMs) have been observed and demonstrated to influence markedly their ability to interact with DNA substrates, other proteins, and chromatin (*7*). Moreover, both the transcription of the *HMGA1* gene and the biochemical modifications of HMGA1 proteins are downstream targets of numerous signal transduction pathways, which make them exquisitely responsive to various environmental stimuli (*8*).

The HMGA1 proteins have a very high content of acidic residues in the C-terminal domain, which is a common feature for HMG proteins (*11*). Protein kinase CK2 has been shown to be involved in the constitutive phosphorylations of serine residues in this domain of the HMGA1 proteins in vitro and in vivo, and Ser98, Ser101, and Ser102 in HMGA1a were shown to be phosphorylated (*11*, *13*, *18*−*20*, *30*). In the present work, we employed tandem mass spectrometry, that is, LC-ESI−MS/MS and MALDI-MS/MS, and confirmed that these three serine residues in HMGA1a can be phosphorylated in PC-3 human prostate cancer cells. A recent analysis of 308 sites that are phosphorylated by protein kinase CK2 highlights the importance of acidic amino acids that are at positions $n + 3$ (the most crucial one), $n + 1$, and $n + 2$, where n is the position of phosphorylation (*31*). There are three glutamic acid residues C-terminal to Ser101 and Ser102 (Figure 2A). Therefore, these two serines can both be phosphorylated by CK2 (*18*). Moreover, phosphorylation of Ser98 can occur after Ser101 is phosphorylated (*18*). This may explain why Ser98 is phosphorylated only in a portion of the HMGA1a protein. Similar as what was observed here, we reported recently that all three serine residues in the acidic C-terminal tail of HMGN1 protein can be phosphorylated in MCF-7 human breast cancer cells (*26*),

and some of these sites have been shown to be phosphorylated by CK2 in vitro (*32*). The C-terminal domain of HMGA1 proteins is postulated not to be involved in DNA-binding because of the high content of negatively charged residues in this region. However, the phosphorylation of HMGA1 proteins in the C-terminal region may alter the protein conformation and decrease the protein's DNA binding affinity (*19*, *20*, *30*).

The monomethylation of Arg25 in HMGA1a, but not in HMGA1b, was observed in cultured human cancer cells, and the methylation was suggested to be associated with the hyperphosphorylation/dephosphorylation processes in apoptosis (*12*, *24*). In that study, the site of methylation in HMGA1a was not determined by tandem MS, rather by careful MS measurements of the molecular weights of tryptic peptides with overlapping sequences (*12*). Here we employed LC−MS/MS and MALDI-MS/MS and provided solid evidence confirming that Arg25 is indeed monomethylated in HMGA1a protein. Moreover, we demonstrated for the first time that the same arginine residue is also dimethylated, and both isoforms of dimethylarginine, that is, aDMA and sDMA, are present.

Protein arginine methylation has been implicated in the regulation of transcription (*33*−*35*), signal transduction (*36*−*38*), RNA transport (*39*, *40*), and possibly RNA splicing (*41*). There are four types of protein arginine methyltransferases (PRMTs) (*42*). Type I and type II PRMTs catalyze the formation of aDMA and sDMA, respectively (*42*−*45*). Type III and type IV enzymes, on the other hand, facilitate the formation of only N^G-monomethylarginine (*42*). In addition, both type I and type II enzymes can methylate arginine residues to form MMA, presumably as an intermediate for the formation of DMA (*42*). Among the mammalian PRMTs that have been identified, PRMT1, PRMT2, PRMT3, coactivator-associated arginine methyltransferase 1 (CARM1/PRMT4), and PRMT6 are suggested to be type I methyltransferases (*42*, *43*, *45*), whereas PRMT5 is a type II enzyme (*44*). Substrates for type I PRMTs include Sam68 (*38*), STAT1 (*46*), HnRNP A (*47*), nucleolin (*48*), poly(A)-binding protein II (*49*), and so forth. On the other hand, myelin basic protein (*50*), coilin (*51*), and the spliceosomal proteins SmB/B′, SmD1, SmD3, and Sm-like protein LSm4 (*52*−*54*) are the only substrates for type II enzymes reported to date. The nature of PRMT(s) involved in the dimethylation of Arg25 in HMGA1a remains unclear; the presence of both isoforms of dimethylation, however, indicates that more than one type of methyltransferase enzymes are involved in the methylation process.

Similar to the HMGA1a protein, histone proteins can be methylated on arginine residues. In this respect, histone H4 can be monomethylated at Arg3 by PRMT1 in vitro and in vivo, and the methylation facilitates subsequent acetylation of H4 tails, which plays an important role in transcriptional activation (*34*, *55*). Likewise, Arg17 in histone H3 has been shown to be susceptible to methylation by CARM1 (*35*), and the CARM1-mediated histone H3 methylation is correlated with gene activation and transcription regulation (*33*, *35*).

Although Arg25 in HMGA1a is located in the first DNA-binding domain of this protein, the methylation of this residue may not affect the protein−DNA binding affinity to a considerable extent because the methylation does not change the overall charge on an arginine residue. The introduction of hydrophobic methyl groups to the side chain of an arginine residue, however, increases steric hindrance and removes amino hydrogens that can potentially be involved in hydrogen bonding interaction(s) (*43*). Therefore, the methylation of Arg25 in HMGA1a may alter the conformation of the protein and modulate protein−protein interactions. It is of importance to note that Arg25 of the closely related HMGA1b protein isolated from PC-3 cells was not methylated, which may suggest that the 11 amino acid deletion (amino acids 34−44) somehow impairs the methylation process. Moreover, it indicates that the HMGA1a and HMGA1b proteins may assume distinct biological functions in vivo.

To summarize, our study clearly established the nature of in vivo phosphorylation and methylation of HMGA1 proteins in human PC-3 prostate cancer cells. We found that both Ser101 and Ser102 in HMGA1a are completely phosphorylated, whereas Ser98 is partially phosphorylated. Furthermore, we demonstrated that Arg25 in HMGA1a can be mono- or dimethylated. HMGA1b protein is phosphorylated on the corresponding serine residues in the C-terminus; this protein, however, is not methylated.

SUPPORTING INFORMATION AVAILABLE

LC-ESI−MS/MS results of the triphosphorylated peptide (residues 88−106) in the HMGA1a protein, the diphosphorylated and triphosphorylated peptide (residues 77−95) in the HMGA1b protein, the MALDI-MS/MS of the monomethylated peptide (residues 24−29) of the HMGA1a protein, and the proposed mechanism for neutral losses from arginine, monomethylarginine, and dimethylarginines (in pdf format). This material is available free of charge via the Internet at http://pubs.acs.org.

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