Identification of Novel in Vivo Phosphorylation Sites in High Mobility Group N1 Protein from the MCF-7 Human Breast Cancer Cells

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ABSTRACT: High mobility group N1 (HMGN1) protein is a member of nonhistone chromosomal proteins that binds more strongly with nucleosomes than with DNA. Here we report the identification of the sites of in vivo phosphorylation of HMGN1 isolated from the MCF-7 human breast cancer cells. Our results showed that four serine residues, i.e., Ser6, Ser85, Ser88, and Ser98, can be phosphorylated in this protein. To our knowledge, this is the first demonstration that each of the three serine residues in the acidic C-terminal region of human HMGN1 can be phosphorylated. The additional negative charge resulting from the phosphorylation of the C-terminal serine residues is expected to modulate the interaction between HMGN1 and other proteins, which may enhance transcription and facilitate other cellular functions. In addition, the phosphorylation of HMGN1 at Ser85, which precedes Pro86, might play an important role in cellular signaling.

The high mobility group (HMG) proteins are a class of nonhistone chromosomal proteins in higher eukaryotic cells. These proteins are known as architectural transcription factors because of their ability to bind and modify specific structures in DNA or chromatin, and their ability to facilitate and enhance chromatin functions and DNA-dependent activities (*1*−*5*). The HMG proteins are grouped into three distinct families: HMGA family (previously designated as HMG-I/Y, HMGI-C), the HMGB1/B2 family (formerly HMG1/2), and the HMGN1/N2 family (previously HMG14/17) (*1*, *6*).

HMGN1 and N2 are the only nuclear proteins that specifically recognize the 146-bp nucleosomal core particle and they have a higher affinity for nucleosomes than for DNA (*2*, *7*). In addition, the association between HMGN1/N2 and nucleosome core particle is independent of DNA sequence (*8*). The binding of HMGN proteins to nucleosomes unfolds chromatin fiber, enhances the accessibility of the nucleosomal DNA, and facilitates transcription and replication from chromatin templates (*5*). In addition, HMGN1 protein enhances the rate of repair of UV-light damaged DNA in chromatin (*9*).

Posttranslational modifications of the HMGN1 protein, including phosphorylation (*10*−*12*), acetylation (*13*), and glycosylation (*14*), are thought to play important roles in the regulation of chromatin structure and the cellular response to changing environmental stimuli. In this regard, phosphorylation of HMGN1 may influence its binding affinity to DNA or nucleosome-associated proteins, and may affect their localization and function in chromatin (*11*, *15*, *16*). Mitotic phosphorylation of HMGN1 has been shown to prevent its binding to chromatin (*17*), promote its interaction with 14.3.3 proteins, and inhibit its reentry into the newly formed nucleus in late telophase (*18*).

Previous studies with different protein kinases in vitro have shown that several serine residues in HMGN1 protein can be phosphorylated. In this respect, Ser6 and Ser24 are the major and minor sites of phosphorylation catalyzed by cyclic nucleotide-dependent protein kinases, i.e., cAMP-dependent protein kinase (A-kinase) and cGMP-dependent protein kinase (G-kinase) (*19*−*21*). HMGN1 derived from calf thymus can be phosphorylated in vitro by Ca^{2+}-phospholipid-dependent protein kinase (protein kinase C); Ser20 and Ser24 were proposed to be potential major and minor phosphorylation sites (*11*). Moreover, protein serine/threonine kinase 2 (protein kinase CK2), formerly known as casein kinase II, can catalyze the phosphorylation of bovine HMGN1, generating high (Ser89) and low affinity sites (Ser99), which correspond to Ser88 and Ser98 in human HMGN1, respectively (*22*).

Some of the above in vitro phosphorylation sites in HMGN1 were also observed to be phosphorylated in vivo. HMGN1 from mitogen-stimulated mouse cells was observed to be phosphorylated at Ser6 and Ser19, which correspond to Ser6 and Ser20 in human HMGN1, respectively (*23*−*28*). HMGN1 protein from HeLa cells was also found to be phosphorylated at a single identical site as that phosphorylated by protein kinase CK2 in vitro (*29*). The site of phosphorylation, however, was not determined. By using mass spectrometry, Louie et al. (*24*) studied the in vivo phosphorylation of HMGN1/N2 from human K562 cells treated with a phosphatase inhibitor, 12-*O*-tetradecanoylaphorbol 13-acetate (TPA) or okadaic acid (OA), and, by using coupled HPLC-tandem mass spectrometry (LC-MS/MS), they showed that Ser20 and Ser24 in HMGN1 were major and minor phosphorylation sites, respectively, and a third phosphorylation site in HMGN1 was located at either Ser6 or Ser7 (*24*).

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[1] Abbreviations: HMG, high mobility group; NBD, nucleosome binding domain; MS/MS, tandem mass spectrometry; TFA, trifluoroacetic acid; PPIase, peptidyl prolyl isomerase.

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In the present investigation, we employed LC-MS/MS and examined the sites of in vivo phosphorylation of HMGN1 protein isolated from the MCF-7 human breast cancer cell line that is not treated with a phosphatase inhibitor. We determined that four serine residues in HMGN1 can be phosphorylated and, among them, three are located in the acidic C-terminal region of HMGN1, and their phosphorylation has not been reported previously.

EXPERIMENTAL PROCEDURES

Cell Culture. The human breast mammary epithelial cell line MCF-7 (ATCC, Manassas, VA) was cultured in minimum essential medium supplemented with 10% fetal bovine serum (ATCC, Manassas, VA), 0.01 mg/mL bovine insulin (Sigma-Aldrich, St. Louis, MO), 100 IU/mL penicillin, and 100 μg/mL streptomycin (ATCC, Manassas, VA).

Protein Extraction and Purification. The HMG proteins were extracted with 5% perchloric acid (PCA) as previously described (*3*). PCA-soluble proteins were purified on a Surveyor HPLC system (ThermoFinnigan, San Jose, CA) by using a 4.6 × 250 mm C4 column (Varian, Walnut Creek, CA). The flow rate was 1.0 mL/min, and a 75-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.

To estimate the amount of HMGN1 protein, 90-μg Type II-A histone (Sigma-Aldrich, St. Louis, MO) was dissolved in 0.1% aqueous solution of TFA and separated under a similar condition as mentioned above by HPLC. The amount of HMGN1 protein was calculated by using the areas of the peaks for the HMGN1 protein and Type II-A histone.

Alkaline Phosphatase Treatment. The dephosphorylation reaction was carried out in a 4-μL 50 mM NH_4HCO_3 solution containing approximately 0.2 μg of HMGN1 protein and 1 unit of calf intestinal alkaline phosphatase (CIP, New England Biolabs, Beverly, MA) at 37 °C for 30 min. The sample was then dried and subjected to MALDI-TOF MS analysis.

Trypsin Digestion. Sequencing-grade modified trypsin was purchased from Roche Diagnostics (Basel, Switzerland). The digestion was carried out in a 50-μL 100 mM NH_4HCO_3 (pH 8.0) solution containing approximately 0.2 μg of HMGN1 protein, and the trypsin-to-substrate ratio was approximately 1 to 50 at 37 °C for 9 or 24 h. The reaction was quenched by adding 1 μL of TFA.

Mass Spectrometry. MALDI-TOF MS experiment was performed in linear mode on a DE-STR instrument equipped with a nitrogen laser (PE Biosystems, Foster City, CA). Each spectrum was calibrated in the default mode in which 0.1% accuracy was typically obtained. 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).

On-line LC-MS/MS was employed for peptide sequencing, and a 0.32 × 50 mm C18 capillary column (300 Å in pore size, 5 μm in particle size, Micro-Tech Scientific, Vista, CA) was used. The Surveyor HPLC system was employed for the LC-MS/MS experiment, and a homemade precolumn splitter was used. The flow rate was 4−6 μL/min after splitting, and a 63-min gradient of 2−65% CH_3CN in 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 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 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-MS/MS experiments were also carried out to monitor the fragmentation of one or a few preselected precursor ions.

RESULTS

1. In Vivo Phosphorylation of HMGN1 Protein in MCF-7 Cells. HMG proteins extracted from the MCF-7 human breast cancer cells were purified by HPLC and the LC fractions were analyzed by MALDI-TOF MS (Figure 1A,B). The HMGN1 protein eluted at 20% acetonitrile among other proteins that are soluble in PCA. As shown in Figure 1B, HMGN1 protein can exist in four forms. The one with *m*/*z* 10536 agrees with the predicted mass of human HMGN1 protein without modification (calculated *m*/*z* 10529 for the $[M + H]^+$ ion), and the other three are attributed to mono- (*m*/*z* 10615; calculated *m*/*z* 10609 for the $[M + H]^+$ ion), di- (*m*/*z* 10695; calculated *m*/*z* 10689), and tri-phosphorylated (*m*/*z* 10770; calculated *m*/*z* 10769) HMGN1. To verify that the modification is due to phosphorylation, the above isolated HMGN1 protein was treated with alkaline phosphatase and analyzed again by MALDI-TOF MS. It turns out that, after phosphatase treatment, only unmodified HMGN1 protein (*m*/*z* 10535) and its oxidized form (the shoulder peak on the right) were observed (Figure 1C), supporting that the HMGN1 protein is phosphorylated.

2. Identification of Phosphorylated Peptides. To locate the phosphorylation sites, we digested the purified HMGN1 protein with trypsin and analyzed the digestion mixture by LC-MS/MS. TurboSequest (ThermoFinnigan, San Jose, CA) search was performed with the obtained product-ion spectra and the Swiss-Prot database was used. Eleven unmodified peptides and four phosphorylated peptides from HMGN1 were identified. The sequence coverage of tryptic peptides identified by LC-MS/MS for HMGN1 was 62% (Figure 2).

Among the four phosphopeptides, three are monophosphorylated (P1*, P2*, and P3*), and one is diphosphorylated (P2**, Table 1). Phosphopeptides P1*, P2*/P2**, and P3* correspond to residues 4−17 (KVSSAEGAAKEEPK), 82−97 (TEESPASDEAGEKEAK), and 82−99 (TEESPASDEAGEKEAKSD), respectively. The sites of phosphorylation were established by MS/MS.

3. Serine6 Is Phosphorylated in Monophosphorylated Peptide P1, KVS$_6$SAEGAAKEEPK.* This peptide (residues 4−17 in Figure 2) is within the N-terminal region of the HMGN1 protein. We observed the $[M + 2H]^{2+}$ ions of both the unmodified peptide P1 (*m*/*z* 716.2) and the monophosphorylated P1* (*m*/*z* 756.3). The product-ion spectra of the ions of *m*/*z* 716.2 and 756.3 support our sequence assignment, and the latter spectrum shows the site of phosphorylation in

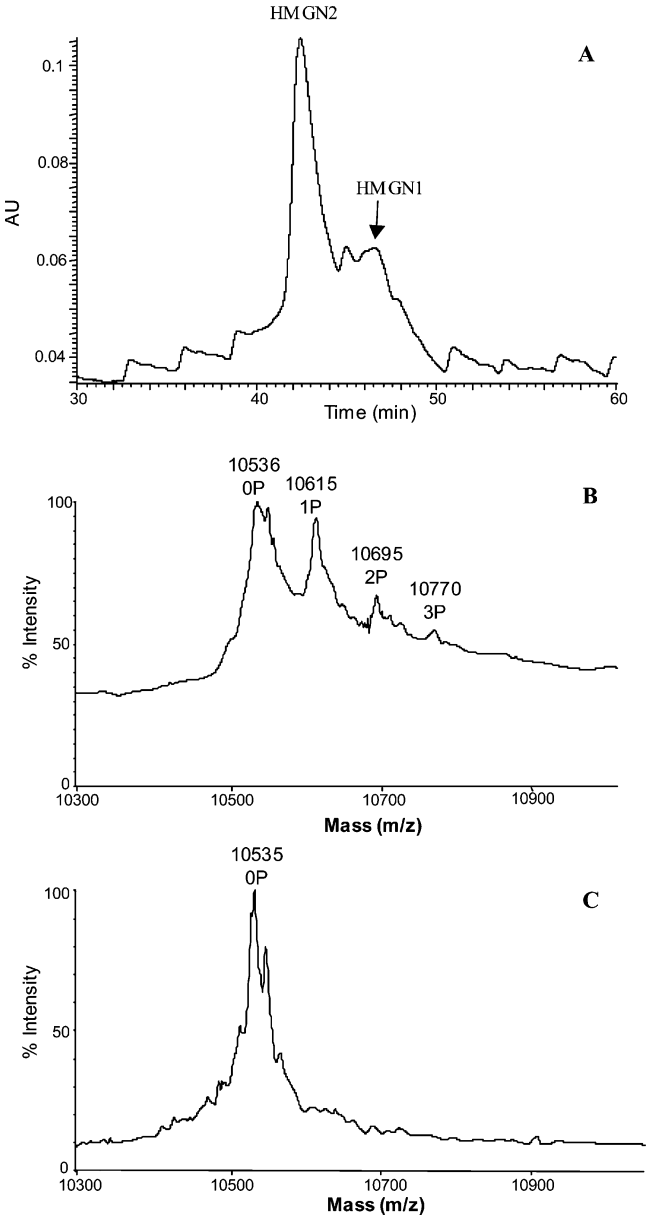

FIGURE 1: HPLC elution trace and MALDI MS measurement show the phosphorylation of HMGN1 protein in MCF-7 cells. (A) PCA-soluble proteins were purified with RP-HPLC, and the effluents were monitored by absorbance at 220 nm. The arrow indicates the HPLC fraction that was used for subsequent MALDI-MS analysis. (B) MALDI mass spectrum of unmodified and phosphorylated HMGN1 protein. (C) MALDI mass spectrum of the above HMGN1 protein after treatment with alkaline phosphatase.

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1                11               21               31
PKRKV**S**SAEG AAKEEPKRRS ARLSAKPPAK VEAKPKKAAA
41               51               61               71
KDKSSDKKVQ TKGKRGAKGK QAEVANQETK EDLPAENGET
81               91
KTEE**S**PA**S**DE AGEKEAK**S**D
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FIGURE 2: Summary of sequence coverage for HMGN1 from the peptides identified by LC-MS/MS analysis of the trypsin digestion mixture. The phosphorylated residues are shown in bold. The covered residues are indicated by underlines. The human HMGN1 sequence was obtained from SWISSPROT and ref 1.

P1* (Figure 3A,B, observed fragment ions for P1* are summarized in Figure 3C). The most abundant fragment ion (m/z 707.1) in the product-ion spectrum of the $[M + 2H]^{2+}$ ion of the phosphorylated peptide is due to the neutral loss of an H_3PO_4 (98 Da) from the parent ion (ΔP1 in Figure 3B. We use "Δ" to designate the elimination of an H_3PO_4 moiety throughout this paper). This is in accord with the finding that facile elimination of H_3PO_4 is commonly observed in the fragmentation of phosphorylated peptides (*30*). Further loss of a water molecule from ΔP1 was also found (m/z 698.1).

In this peptide, Ser6 and Ser7 are the two potential phosphorylation sites. The interpretation of the product-ion spectrum is somehow complicated because of the presence of fragment ions containing either a phosphorylated or a dehydrated serine, and the latter forms from the elimination of a neutral H_3PO_4. In this regard, several b_n ions, e.g., b_6, b_9, b_{10}, b_{11}, b_{12}, were observed in both forms (Figure 3B). Although the $*b_3$ ion (m/z 395.2) is of low abundance, the Δb_3 ion (m/z 297.2) and its complementary y_{11} ion (m/z 1116.6) are clearly observed (Figure 3B), demonstrating that Ser6 is phosphorylated. The ion of m/z 1098.5 can be assigned as either the $[y_{11} - H_2O]$ for the peptide with Ser6 being phosphorylated or the Δy_{11} ion for the peptide with Ser7 being phosphorylated. The presence of this ion prevents us from excluding the possibility that a portion of the peptide contains a phosphorylated Ser7. The relative abundances of the y_{11} ion and the ion of m/z 1098.5, however, indicate that phosphorylation of Ser7 is a minor component, i.e., at most 25%, if it is there. This result is also consistent with many previous studies, which show that Ser6 is phosphorylated in HMGN1 (*19*−*21*, *24*).

4. Serine85 and Serine88 Are Phosphorylation Sites in Phosphopeptides P2 and P2**, $TEES_{85}PAS_{88}DEAGEKEAK$.* We detected three different forms of a C-terminal peptide P2 (residues 82−97), i.e., unmodified (P2), monophosphorylated (P2*), and di-phosphorylated (P2**). Product-ion spectra of the $[M + 2H]^{2+}$ ions (m/z 839.6, 879.5, and 919.5) of the three different forms of P2 are shown in Figure 4A−C, and most fragment ions observed in the product-ion spectra of the $[M + 2H]^{2+}$ ions of P2* and P2** are summarized in Figure 4D,E.

Similar to phosphopeptide P1*, the neutral loss of H_3PO_4 can occur readily upon collisional activation of the $[M + 2H]^{2+}$ ions of P2* and P2**, which gives rise to fragment ions of m/z 830.6 ($-1H_3PO_4$) in Figure 4B as well as m/z 870.5 ($-1H_3PO_4$) and 821.5 ($-2H_3PO_4$) in Figure 4C. In addition, further loss of a water molecule was observed, which gives fragment ions of m/z 821.6 for P2* and m/z 812.8 for P2**.

In P2, three amino acid residues can be phosphorylated, i.e., Thr82, Ser85, and Ser88. In Figure 4B, we observed $*y_{13}^{2+}$, Δy_{13}^{2+}, Δy_{13}, Δy_{14}^{2+}, Δy_{14}, and $[*y_{14} - H_2O]^{2+}$ ions, but we did not detect any unmodified y_{13} or y_{14} ion for P2*, demonstrating without ambiguity that Thr82 is not phosphorylated in this peptide. The phosphorylation, therefore, must occur at either Ser85 or Ser88. The product-ion spectrum of the $[M + 2H]^{2+}$ ion indicates that the monophosphorylated P2* is heterogeneous, i.e., phosphorylation can occur at either Ser85 or Ser88. In this context, y_3, y_4, y_6, y_7, y_8, and y_9 ions give the same m/z values as those observed in the product-ion spectrum of the unmodified P2 (Figure 4A,B), whereas y_{10} and y_{12} ions can bear either an unmodified serine (m/z 1063.5 for y_{10} and m/z 1231.6 for

Table 1: Phosphopeptides Detected in LC-MS/MS Analysis of the Tryptic Digestion Mixture of HMGN1

peptide name	predicted mass (Da)[a]	observed ions (*m*/*z*)[b]	observed ions (*m*/*z*)[b]	residues[d]	modification[c]
P1	1430.6	716.2 (2+)	1430.4	4−17	
P1*	1510.6	756.3 (2+)	1510.6	4−17	1P
P2	1677.7	839.6 (2+)	1677.2	82−97	
P2*	1757.7	879.5 (2+)	1757.0	82−97	1P
P2**	1837.7	919.5 (2+)	1837.0	82−97	2P
P3	1879.9			82−99	
P3*	1959.9	980.6 (2+)	1959.2	82−99	1P

[a] Mass calculated from amino acid sequence of human HMGN1 using average masses. [b] Charges on ions observed by LC-MS/MS are indicated in parentheses. [c] Peptide average mass calculated from observed ions. [d] Amino acid residues of the corresponding peptide fragment. [e] Number of phosphate groups present in the peptide.

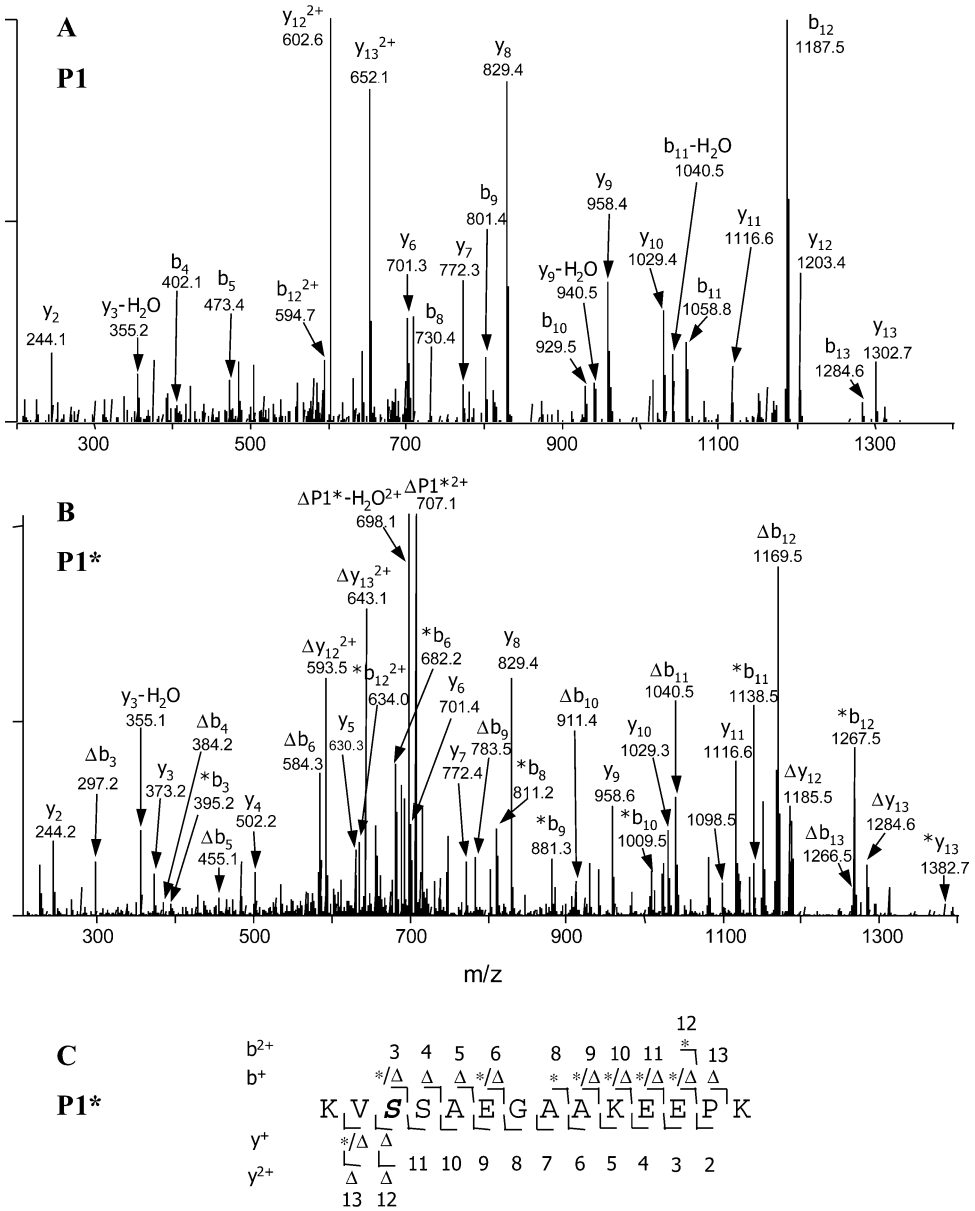

FIGURE 3: Product-ion spectra of the $[M + 2H]^{2+}$ ions of unmodified P1 (A) and monophosphorylated P1* (B). An asterisk (*) indicates that an ion bears a phosphate group, and neutral loss of an H_3PO_4 is represented by a triangle (△). The symbol */△ indicates that both forms were observed. (C) Summary of the observed b^+, b^{2+} ions (⌉) and y^+, y^{2+} ions (⌊) in the P1* sequence. The phosphorylated residue is indicated as a bold italic letter.

y_{12}) or a phosphoserine (*m*/*z* 1143.2 for $*y_{10}$ and *m*/*z* 1311.5 for $*y_{12}$). Moreover, both b_4 (*m*/*z* 447.1) and Δb_4 (*m*/*z* 429.0) were observed. These results demonstrate that some portion of the peptide P2* has a phosphorylated Ser85, whereas the other portion has a phosphorylated Ser88.

Consistent with the above observation with the monophosphorylated P2*, the product-ion spectrum of the $[M + 2H]^{2+}$ ion of P2** shows that both Ser85 and Ser88 are phosphorylated (Figure 4C). In the product-ion spectrum, we observed Δy_{10} (*m*/*z* 1045.9), $*y_{10}$ (*m*/*z* 1143.4), Δy_{12} (*m*/*z*

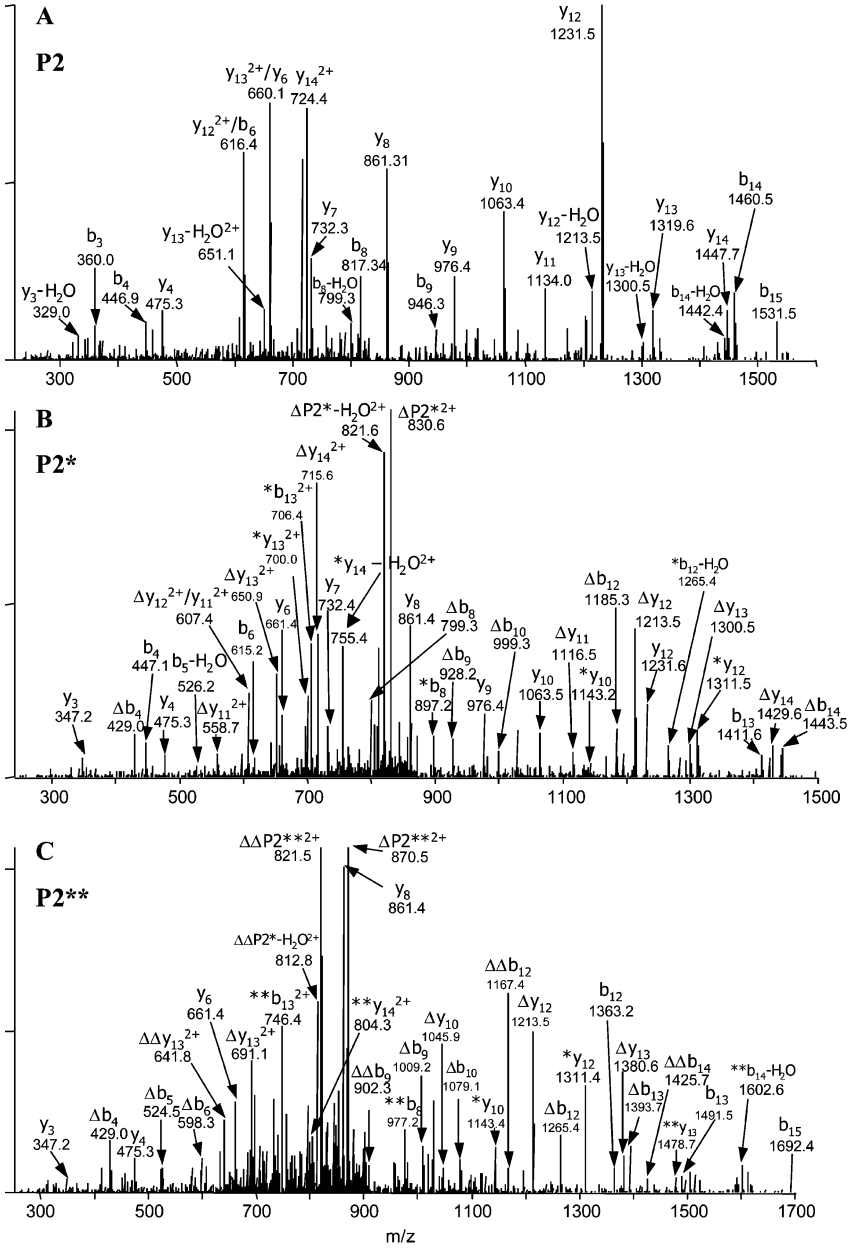

FIGURE 4: Product-ion spectra of the $[M + 2H]^{2+}$ ions of unmodified P2 (residue 82−97) (A), monophosphorylated P2* (B), and diphosphorylated P2** (C). An asterisk (*) indicates that an ion carries a phosphate group, and neutral loss of H_3PO_4 is represented by a triangle (△). */△ indicates that both forms were observed. "**" and "△△" represent ions that contain two phosphate groups and with the elimination of two H_3PO_4 molecules, respectively. (D, E) Summary of the observed b^+, b^{2+} ions (⌋) and y^+, y^{2+} ions (⌊) in the P2* and P2** sequences. The phosphorylated residues are indicated as a bold italic letter. U/Δ in panel D indicates that both unmodified and dehydrated ions are observed.

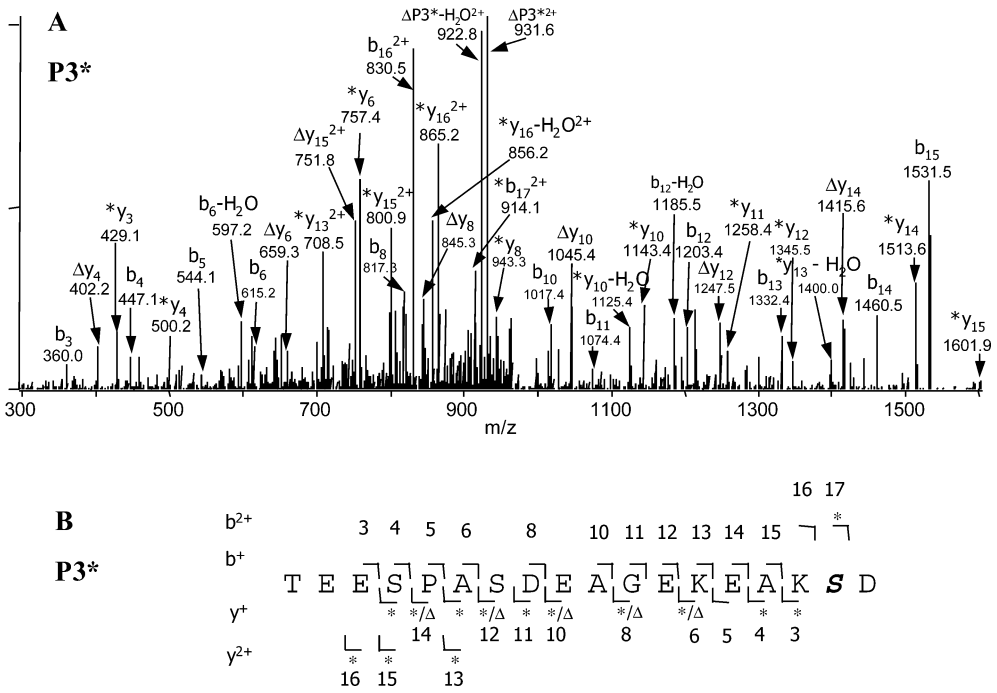

FIGURE 5: (A) Product-ion spectrum of the $[M + 2H]^{2+}$ ion of monophosphorylated P3*. An asterisk (*) indicates that the ion contains a phosphate group, and neutral loss of H_3PO_4 is represented by a triangle (△). The symbol */△ indicates that both forms were observed. (B) Summary of the observed b^+, b^{2+} ions (⌉) and y^+, y^{2+} ions (⌊) in the P3* sequence. The phosphorylated residue is indicated as a bold italic letter.

1213.5), and $*y_{12}$ (*m*/*z* 1311.4) ions, which carry either a dehydrated or phosphorylated serine, whereas the corresponding fragments bearing an unmodified serine were not detectable. These results demonstrate that Ser88 is phosphorylated. In addition, we observed the $**y_{13}$ and $**y_{14}^{2+}$ ions. These two ions carry two phosphoserine residues, which furnishes strong evidence supporting that Ser85 is also phosphorylated.

Although Ser89 in bovine HMGN1, which is homologous to Ser88 in human HMGN1, has been shown to be phosphorylated by protein kinase CK2 in vitro (*22*), this is the first demonstration that Ser85 and Ser88 in HMGN1 can be phosphorylated in human cells.

5. Serine98 Is the Phosphorylation Site in Monophosphorylated Peptide P3, TEESPASDEAGEKEAKS$_{98}$D.* Monophosphorylated peptide P3* (residue 82−99, TEESPASDEAGEKEAKSD) was observed in the tryptic digests of HMGN1 protein, whereas the unphosphorylated peptide P3 was not detected. The absence of the unphosphorylated P3 is very likely due to that the amide bond between Lys97 and the unphosphorylated Ser98 is susceptible to proteolysis by trypsin. The negative charge introduced by the phosphorylation of Ser98, however, may inhibit the cleavage of the amide bond between Lys97 and Ser98, thereby giving rise to phosphopeptide P3*. This appears to be supported by the fact that the Lys94−Glu95 peptide bond was not cleaved in P2, P2*, P2**, and P3*, where Lys94 is surrounded by two acidic amino acids.

The product-ion spectrum of the $[M + 2H]^{2+}$ ion of P3* supports that Ser98 is phosphorylated (Figure 5A, and observed fragment ions of P3* are summarized in Figure 5B). The most abundant product ion ($\Delta P3^{2+}$, *m*/*z* 931.6) is again due to the neutral loss of an H_3PO_4 moiety from the parent ion. The further loss of a water molecule from the $\Delta P3^{2+}$ ion also occurs and it gives an ion of *m*/*z* 922.8. In addition, many y_n ions bearing one phosphorylated amino acid (*y) were observed, i.e., $*y_3$ (*m*/*z* 429.1), $*y_4$ (*m*/*z* 500.2), $*y_6$ (*m*/*z* 757.4), $*y_8$ (*m*/*z* 943.3), etc. We, however, did not observe any corresponding y_n ions that do not carry a phosphorylated amino acid. These results demonstrate that Ser98 is the only amino acid residue that is phosphorylated in P3*. In addition, all observed b_n ions except the $*b_{17}^{2+}$ ion (*m*/*z* 914.1) have the same *m*/*z* values as those predicted from the unmodified peptide, which again supports that Ser98 is phosphorylated. It is interesting to note that neither Ser85 nor Ser88 is phosphorylated in P3*, showing again that the sites of phosphorylation in HMGN1 are heterogeneous. This result might also implicate that the phosphorylation of Ser98 inhibits the phosphorylation of the other two serines on the C-terminus. Further experiments need to be carried out to determine whether the latter argument is valid.

Ser99 in bovine HMGN1, which corresponds to Ser98 in human HMGN1, has been shown to be a minor site of phosphorylation catalyzed by protein kinase CK2 in vitro (*22*). To our knowledge, this is the first report that Ser98 can also be phosphorylated in MCF-7 human breast cancer cells in vivo.

DISCUSSION

HMGN1 protein contains three major functional motifs, corresponding to four evolutionarily conserved domains, i.e., bipartite nuclear localization signal (residues 1−4 and 52−56), nucleosome binding domain (NBD, residues 13−41), and chromatin unfolding domain (residues 74−81) (*1*, *5*).

Phosphorylation at serine residues in or close to the NBD has been observed in many previous investigations, including Ser20, Ser24, and Ser6 or Ser7 (*19*−*21*). The addition of a phosphate group to these serines, which neighbor the two basic triplets (Lys2-Arg3-Lys4 and Lys17-Arg18-Arg19),

effectively reduces their positive charge and, consequently, compromises the DNA binding affinities of these regions (*16*). Our study shows that Ser6 is phosphorylated in HMGN1 isolated from the MCF-7 cells. We, however, were not able to detect any peptide containing Ser20, which might be due to extensive trypsin cleavages in the region surrounding Ser20. Therefore, it remains unclear whether Ser20 is phosphorylated in MCF-7 cells. We also identified an unmodified peptide that contains Ser24 (Figure 2); however, we did not observe the corresponding phosphorylated peptide.

There are few reports about the phosphorylation of serine residues in the acidic C-terminus of HMGN1 (*22*, *29*), and the biological implications of the phosphorylation of these sites remain unexplored. In this respect, in vitro studies demonstrated that bovine HMGN1 can be phosphorylated by protein kinase CK2, and the sites of phosphorylation are Ser89 and Ser99, which correspond to Ser88 and Ser98 in human HMGN1, respectively (*22*). The in vivo phosphorylation of HMGN1 protein from HeLa cells was found to occur at a single identical site with the site phosphorylated by protein kinase CK2 in vitro (*29*). The site of phosphorylation, however, was not established in that study. We demonstrate for the first time that each of the three serines in the C-terminal region of HMGN1 can be phosphorylated in vivo. In this context, the acidic C-terminus of other HMG proteins, i.e., HMGA1a and A1b, was previously shown to be phosphorylated as well (*31*−*34*).

The phosphorylation of multiple serine residues in the acidic C-terminus of HMGN1 may have significant biological implications. Trieschmann et al. (*35*) showed that the deletion of 26 C-terminal amino acid residues of HMGN1 reduces drastically the ability of the protein to enhance transcription from chromatin templates, demonstrating that the negatively charged C-terminal region of the protein is important in transcriptional enhancement. The phosphorylation of the serine residues, i.e., Ser85, Ser88, and Ser98, in the acidic C-terminus will introduce more negative charges to the C-terminal region. The HMGN1 is known to decompact the chromatin structure by targeting linker histone H1 and the amino tails of core histones (*5*, *36*). Phosphorylation of HMGN1 at the C-terminus may, therefore, further decompact the chromatin structure and enhance transcription by strengthening its interaction with histone H1 and the N-terminal tails of core histones. In addition, recent studies showed that HMGN1 protein may associate with proteins other than histones to form protein complexes (*37*). A more negative C-terminus imposed by phosphorylation of the serine residues in this region may exert significant effect on the interaction of HMGN1 proteins with other proteins in these complexes.

Among the three C-terminal serines that are phosphorylated, the phosphorylation of Ser85 may play a special role in the biological function of HMGN1 protein because reversible phosphorylation of proteins on serine or threonine preceding a proline (Ser/Thr-Pro) is a major cellular signaling mechanism (*38*). Recently, a novel peptidyl prolyl isomerase (PPIase) Pin1 was discovered (*39*). Pin1 isomerizes specifically the phosphorylated Ser/Thr-Pro bonds in certain proteins (*38*). The protein conformational changes induced by the isomerization have a profound effect on the protein's catalytic activity, dephosphorylation, interaction with other proteins, and subcellular localization (*38*). Although no study has shown that HMGN1 with a phosphorylated Ser85 is a substrate for Pin1, a recent study demonstrated that Pin1 is overexpressed in breast cancer tissues (*40*), which may point to such a possibility.

To conclude, we used LC-MS/MS and determined that Ser6, Ser85, Ser88, and Ser98 can be phosphorylated in HMGN1 isolated from the MCF-7 human breast cancer cells. The identification of novel in vivo phosphorylation sites in the acidic C-terminal tail in HMGN1 should stimulate further studies of the biological implications of these modifications.

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