Differential in Vivo Modifications of the HMGI(Y) Nonhistone Chromatin Proteins Modulate Nucleosome and DNA Interactions†

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ABSTRACT: The HMGI(Y) family of "high mobility group" nonhistone proteins are architectural transcription factors whose overexpression is highly correlated with both cancerous transformation and increased malignancy and metastatic potential of tumors in vivo. Here we report on the types of posttranslational modifications found in vivo on the HMG-I and HMG-Y proteins isolated from two human breast epithelial cell lines, MCF-7 and MCF-7/PKC-α, that represent different stages of neoplastic progression. The MCF-7 cell line exhibits many characteristics of normal breast epithelial cells and does not form tumors when injected into nude mice, whereas the MCF-7/PKC-α cell line, a derivative of MCF-7 that expresses a transgene coding for the enzyme protein kinase C-α (PKC-α), is both malignant and highly metastatic. Using MALDI mass spectrometry, we show that the HMG-Y protein is more highly modified than the HMG-I protein in both the MCF-7 and the MCF-7/PKC-α cells. Significantly, the HMG-Y protein isolated from the highly metastatic MCF-7/PKC-α cells possesses a unique constellation of phosphorylations, methylations, and acetylations not found on the HMG-I protein isolated from either the MCF-7 or MCF-7/PKC-α cells. We further demonstrate that some of the same amino acid residues phosphorylated on recombinant HMGI(Y) proteins by purified PKC in vitro are also phosphorylated on the HMG-I(Y) proteins isolated from MCF-7/PKC-α cells, suggesting that PKC phosphorylates these proteins in vivo. Quantitative substrate binding analyses indicate that the biochemical modifications present on the HMG-I and HMG-Y proteins differentially influence the ability of these proteins to interact with both A·T-rich DNA substrates and nucleosome core particles in vitro, suggesting a similar modulation of such binding affinities in vivo. To our knowledge, this is the first demonstration of differences in the types of in vivo biochemical modifications found on the HMG-I and HMG-Y proteins in cells and also the first experimental evidence suggesting a possible linkage between such posttranslational modifications and the neoplastic potential of cells.

The mammalian "high mobility group" proteins HMG-I,[1] HMG-Y, and HMGI-C are founding members of the HMGI-(Y) family of nonhistone chromatin proteins that have been implicated in both positive and negative regulation of gene transcription in vivo (reviewed in ref *1*). The HMG-I (11.5 kDa) and HMG-Y (10.4 kDa) proteins are produced by translation of alternatively spliced transcripts from a single gene (*2*−*4*), while the related HMGI-C protein (12 kDa) is translated from an unspliced transcript coded for by a different gene (*5*). Members of the HMGI(Y) family are known as "architectural" transcription factors because of their ability to regulate gene activity through the recognition and alteration of the structure of DNA and chromatin substrates (*1*). The HMGI(Y) proteins not only bind with high specificity to the narrow minor groove of A·T-rich, random sequence B-form DNA (*6*, *7*) but also have the ability to bind with high affinity to bent or distorted DNA structures such as synthetic four-way junction DNAs (*8*), supercoiled plasmid substrates (*9*), and nucleosome core particles (*10*, *11*). The ability of the HMGI(Y) proteins to bind to a variety of DNA substrates is a consequence of the high degree of intrinsic flexibility of the proteins, particularly within their three "A·T-hook" DNA-binding motifs (*12*, *13*). In addition to binding DNA, HMGI(Y) proteins are postulated to regulate gene transcription in vivo by participating in the formation of stereospecific nucleoprotein "enhanceosomes" through protein−protein interactions with a number of specific transcription factors including NF-κB, ATF-2, Elf-1, AP-1, NFAT, and others (*1*, *14*−*21*) (unpublished data).

In most normal nondividing cells and tissues, the levels of expression of HMGI(Y) mRNAs and proteins are very low or undetectable but are rapidly induced in response to

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[1] Abbreviations: DAG, diacylglycerol; HMGI(Y), high mobility group I and Y proteins; A·T-hook, the highly conserved DNA-binding domain of the HMGI(Y) family of proteins; HMG-I^{MCF7} and HMG-Y^{MCF7}, the HMG-I and HMG-Y proteins, respectively, from MCF-7 cells; HMG-I$^{7\alpha}$ and HMG-Y$^{7\alpha}$, the HMG-I and HMG-Y proteins, respectively, from MCF-7/PKC-α cells; BLT, DNA from the 3′ untranslated tail region of the bovine interleukin-2 cDNA; CIP, calf intestinal alkaline phosphatase; EMSA, electrophoretic mobility shift analysis; MALDI-MS, matrix assisted laser desorption ionization mass spectrometry; PKC, protein kinase C; RP-HPLC, reverse phase high performance liquid chromatography; SDS, sodium dodecyl sulfate; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; *m*/*z*, mass-to-charge; rh, recombinant human.

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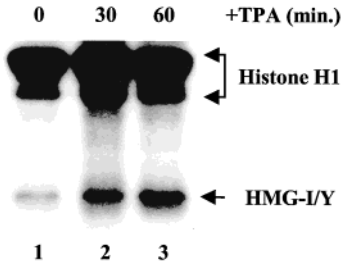

FIGURE 1: Rapid in vivo phosphorylation of the HMGI(Y) proteins in cells treated with TPA. Human breast epithelial cell line Hs578T was in vivo radiolabeled with inorganic [^{32}P] phosphate for the final 15 min of either 0, 30, or 60 min treatment with the phorbol ester TPA (5 ng/mL), as described in Materials and Methods. Lanes: (1) unstimulated cells labeled for 15 min (i.e., a zero time control); (2) cells exposed to TPA for 30 min; and (3) cells exposed to TPA for 1 h. On this 15% SDS gel, the HMG-I and HMG-Y proteins co-migrate and are together designated as HMG-I/Y. The two major species of phosphorylated histone H1 protein are also indicated.

certain growth stimulatory factors (*1*, *2*, *22*−*26*). In contrast, in neoplastically transformed cells, as well as in embryonic cells that have not yet undergone overt differentiation, the constitutive level of HMGI(Y) gene products is often exceptionally high with increasing concentrations being correlated with increasing degrees of metastatic potential (recently reviewed in ref *27*). Furthermore, translocation of A·T-hook DNA-binding motifs to form chimeric cancer-related hybrid proteins appears to be among the most common chromosomal translocations occurring in human tumors (*28*). Nonetheless, the precise in vivo cellular role of either the overexpression of the HMGI(Y) proteins or the chromosomal translocation of their A·T-hook motifs in contributing to processes such as oncogenic transformation, increased tumor metastatic potential, and overt neoplastic malignancy is poorly understood.

The HMG-I and HMG-Y proteins are nearly identical in amino acid sequences [the only difference between the two proteins being an internal deletion of either 11 or 12 residues in the latter (*3*)] and have very similar DNA-binding properties in vitro (unpublished observations). Therefore, HMG-I and HMG-Y have been widely considered biologically interchangeable. Intuitively, however, a total in vivo functional similarity between the two proteins seems unlikely and has not been conclusively demonstrated. An indication that HMG-I and HMG-Y may have distinct cellular functions comes from a recent study that compared the levels of the two proteins in response to treatment with tumor-promoting compounds such as 12-*O*-tetradecanoylphorbol acetate (TPA) (*29*). Utilizing a cell line that is transformed in response to TPA treatment, Cmarik et al. suggested that differential induction of the HMG-Y protein isoform, relative to HMG-I, may be particularly important in the tumor promoter-induced neoplastic transformation process (*29*). Phorbol esters such as TPA function in part by directly activating the Ca^{2+}/phospholipid-dependent enzyme protein kinase C (PKC), which initiates an intracellular signaling cascade that rapidly results in the in vivo phosphorylation of numerous proteins (*30*), including HMGI(Y) (see Figure 1), and culminates in the transcriptional activation of a constellation of cellular genes (*31*) including the HMGI(Y) gene itself (*4*, *24*). To elucidate whether the PKC enzyme plays a role in differentially modulating the functional activity of the HMG-I and HMG-Y proteins, we have used matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) to analyze the sites of phosphorylation on both proteins using purified PKC enzyme in vitro.

We compared the results of these in vitro phosphorylation studies with similar MALDI-MS analyses performed on HMG-I and HMG-Y proteins isolated from MCF-7/PKC-α cells, a human breast epithelial cell line neoplastically transformed by a transgene coding for the α-isoform of the PKC enzyme (i.e., PKC-α) (*32*). In addition, we compared the types of modifications on the HMG-I and HMG-Y proteins isolated from the MCF-7/PKC-α cell line to those same proteins isolated from the nontumorigenic parental MCF-7 cell line. Analysis of the HMGI(Y) proteins isolated from a "matched set" of nontumorigenic and highly malignant cells derived from a common clonal origin allowed us to meaningfully investigate the types of in vivo posttranslational modifications occurring on the HMGI(Y) proteins at different stages of cellular transformation (*33*). Our findings indicate not only that the HMG-I and HMG-Y proteins are differentially modified in cells exhibiting different neoplastic phenotypes but also suggest that variations in the constellations of such in vivo biochemical modifications may be associated with the cancerous transformation of cells by the PKC-α oncogene.

MATERIALS AND METHODS

Cell Lines, Culture Methods, and In Vivo Labeling Procedures. The human breast mammary epithelial cell lines MCF-7 and Hs578T were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and cultured according to the supplier's instructions in Minimal Essential Medium (GibcoBRL, Grand Island, NY), supplemented with sodium bicarbonate, 2 mM L-glutamine, 10% fetal bovine serum, and 100 μg/mL each of sodium penicillin-G and streptomycin sulfate (Sigma Co., St. Louis, MO). The MCF-7/PKC-α cell line was a generous gift of Dr. Kirk Ways, East Carolina University School of Medicine, Greenville, NC, and was maintained according to published protocols (*32*). For in vivo pulse radiolabeling of HMGI(Y) proteins, exponentially growing cells were labeled with inorganic [^{32}P] phosphate (DuPont Co., Wilmington, DW) at a concentration of 100 μCi/mL of medium for the final 15 min of either 0, 30, or 60 min treatment with 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA; Sigma Co.) at a final concentration of 5 ng/mL. Following ^{32}P-labeling, cells were washed with phosphate buffered saline, the acid-soluble proteins were extracted from the cells and separated by SDS−PAGE, and the radiolabeled proteins were detected by autoradiography as previously described (*34*, *35*). Subsequently, the SDS gel was stained with Coomassie brilliant blue, and the HMGI-(Y) proteins were identified by comparison to recombinant HMGI(Y) proteins (data not shown).

Protein Isolation, Purification, and Detection. Native HMG-I and HMG-Y proteins were acid-extracted with 5% perchloric acid from actively proliferating human mammary epithelial cell cultures as previously described (*35*). Acid-soluble proteins from MCF-7 and MCF-7/PKC-α cells were further purified and separated on a C-18 Vydac reverse phase (RP) HPLC column. A 30% linear gradient of 0.1% trifluoroacetic acid (TFA) in H_2O to 0.1% TFA in acetonitrile

over 75 min was employed to purify the HMG-I and HMG-Y proteins. Recombinant full-length human HMG-I and HMG-Y proteins were prepared and chromatographically purified as described by Reeves and Nissen (*35*). In the present study, the HMG-Y clone 11D was used, which, as a result of alternative splicing of a single mRNA precursor, has a 12-amino acid deletion relative to the HMG-I protein (*3*). Electrophoretic separations of proteins, Western blot analyses, and detection of HMGI(Y) proteins using polyclonal rabbit antiserum raised against recombinant human HMG-I protein followed published protocols (*35*).

In Vitro Phosphorylation by Protein Kinase C. Recombinant human (rh) HMG-I and HMG-Y proteins were phosphorylated in vitro using PKC at 30 °C in a 50-μL reaction volume. Buffer conditions consisted of 2 mM ATP (cold), 20 mM HEPES, pH 7.4, 1 mM DTT, 4 mM $MgCl_2$, 1 mM $CaCl_2$, 200 μg/mL phosphatidylserine (PS), 10 μg/mL diacylglycerol (DAG), and rat brain PKC-α isoform (Calbiochem Inc., CA). Two separate reaction conditions, which differed in the length of reaction time and the ratio of enzyme to substrate, were used to investigate the relative PKC phosphorylation affinity of different residues on the HMGI(Y) proteins. *PKC phosphorylation reaction 1* ("saturating" conditions that should phosphorylate both low and high affinity sites): 0.27 nmol of HMGI(Y) was phosphorylated using a total of 9 pmol of PKC, for an enzyme to substrate molar ratio of 1 to 30, over 9 h (4.4 pmol of PKC was added at time zero; 2.3 pmol of PKC was subsequently added at 3 and 6 h). In the text, unless otherwise noted, references to "PKC phosphorylation" are referring to PKC phosphorylation reaction 1. *PKC phosphorylation reaction 2* ("nonsaturating" phosphorylation reaction conditions that should phosphorylate primarily high affinity sites): 1.0 nmol of HMGI(Y) was phosphorylated using a total of 1.8 pmol of PKC, for an enzyme to substrate molar ratio of 1 to 555, over 6 h (0.9 pmol of PKC was added at 0 and 3 h). Resulting phosphopeptides were purified on a C-18 Vydac RP-HPLC column, lyophilized, reconstituted in water, and appropriate fractions were grouped if necessary.

Dephosphorylation of the HMG-I and HMG-Y Proteins from MCF-7 and MCF-7/PKC-α Cells. The native full-length HMG-I and HMG-Y proteins isolated from both cell lines were exposed to five units of calf intestinal alkaline phosphatase (CIP) (Boehringer Mannheim) at 37 °C for 7 h in 1× buffer supplied by the manufacturer (*36*). The CIP enzyme was precipitated from the HMGI(Y) proteins using a standard trichloroacetic acid precipitation procedure (*35*). Mass spectrometry confirmed the absence of CIP in the acid-purified HMGI(Y) proteins (data not shown).

Tryptic Digests. Tryptic reactions were carried out in 100 mM NH_4HCO_3, pH 8.0, and 1 mM $CaCl_2$ with a trypsin-to-substrate ratio of 1 to 33 (w/w). Trypsin was purchased from Promega (Madison, WI). The length of tryptic digestion, 12 h at room temperature, was empirically determined to yield optimal partial digestion of the substrate proteins. Partial tryptic digestion was necessary since complete digestion of the proteins would result in very small peptide fragments that would be difficult to analyze. Reactions were quenched with 1 μL of trifluoroacetic acid (TFA). Tryptic samples were purified using either a C-18 Vydac RP-HPLC column or a G-25 spin column to remove salt prior to mass spectrometric analysis. All CIP dephosphorylation reactions were conducted prior to tryptic digestion of the proteins.

MALDI Mass Spectrometry. MALDI-MS was performed in linear mode using a PerSeptive Biosystems (Framingham, MA) Voyager DE-RP instrument according to published protocols (*36*−*39*). In addition, all samples were analyzed in positive ion mode using a standard 337-nm nitrogen laser. Two hundred and fifty laser flashes per sample were averaged into a single spectrum, and each spectrum was calibrated either externally and/or internally using mass standards purchased from PerSeptive Biosystems. A difference of less than 0.1% between the observed and the calculated mass values was the criteria used to identify the peptides/proteins (*38*). In addition, mass values were highly reproducible for all tryptic digests in which multiple spectra were generated (on average approximately ±0.5 Da). The samples were dissolved in a 1 to 1 acetonitrile to water and 0.25% TFA solution saturated with either α-cyano-4-hydroxycinnamic acid (for tryptic samples) or 3,5-dimethoxy-4-hydroxy-cinnamic acid (for full-length proteins). Both matrixes were purchased from Sigma Chemical Co. (St. Louis, MO).

Electrophoretic Mobility Shift Assays (EMSAs). Micrococcal nuclease trimmed chicken erythrocyte nucleosome core particles containing ~146 bp of DNA were prepared as previously described (*10*). The double-stranded, A·T-rich, B-form DNA substrate used in the EMSAs was the well-characterized 300 bp 3′ untranslated tail region of the bovine interleukin-2 gene (BLT) and was prepared and used as described previously (*40*, *41*). EMSA reactions for both BLT DNA and nucleosome core particles were carried out as described in Banks et al. (*42*) with slight modifications. For this work, the reactions contained 0.5 nM BLT DNA with a protein binding buffer concentration of 0.5× (5 mM Tris/HCl, pH 7.8, 14 mM NaCl, 25 μg BSA, 0.5 mM EDTA, 0.5 mM DTT, and 0.15 μg of dG−dC).

RESULTS

Phorbol Ester Treatment of Cells Results in Rapid In Vivo Phosphorylation of the HMGI(Y) Proteins. Within half an hour of treatment of exponentially growing human mammary epithelial cells with TPA, the endogenous HMGI(Y) proteins become highly phosphorylated (Figure 1). The major cellular effects of short-term treatment of mammalian cells with tumor-promoting phorbol esters, such as TPA, is to activate enzyme activity in signaling pathways such as the PKC and Ras/MAPK cascades (*31*, *43*). The effect on PKC is due to the structure of TPA, which is similar to the PKC activator molecule diacylglycerol (DAG); therefore, TPA can substitute for DAG and directly activate PKC both in vitro and in vivo (*44*, *45*). As shown in Figure 1, unstimulated cells contain only a low basal level of endogenously phosphorylated HMGI(Y) proteins (lane 1). However, following TPA treatment for 30 min (with radiolabeling occurring during the last 15 min of exposure), the level of phosphorylated HMGI(Y) increases more than 10-fold (lane 2) and remains at this continuously high level for up to 1 h of TPA treatment (lane 3). Interestingly, in the same cells, the level of phosphorylated histone H1 increases only slightly after 30 min of TPA treatment and returns to the basal level of modification after 1 h of phorbol ester exposure (compare lanes 1 and 3). These results indicate that the HMGI(Y)

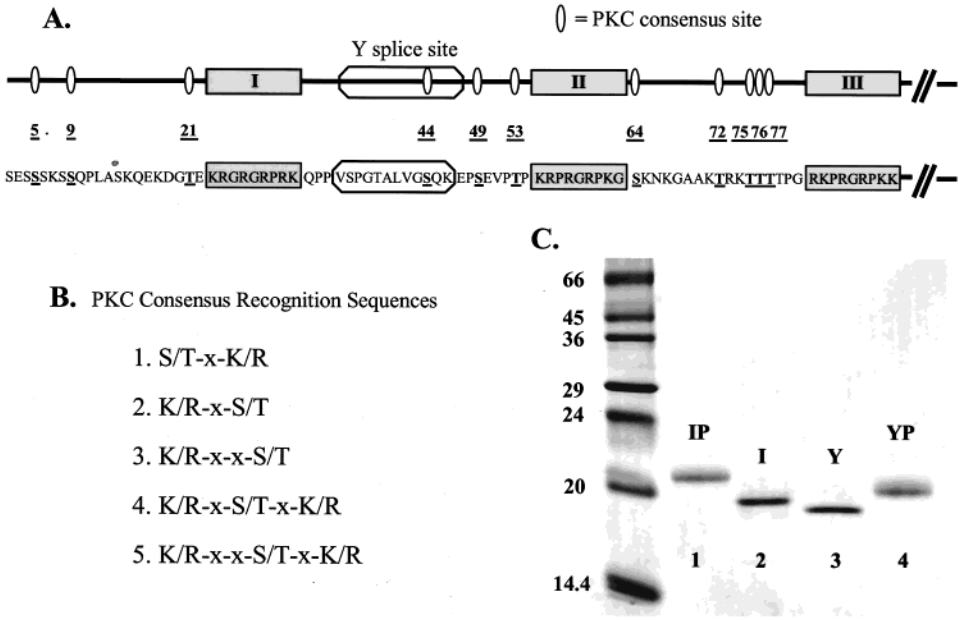

FIGURE 2: Protein kinase C phosphorylates the rhHMG-I and rhHMG-Y proteins in vitro. Panel A illustrates both the amino acid sequence (from Ser2 to Lys89) and a schematic representation of the HMGI(Y) proteins. Residues indicated by the "Y-splice site" are not present in HMG-Y, relative to HMG-I, and represent the only difference between the two proteins. Shaded rectangles correspond to the A·T-hook DNA-binding motifs. On the basis of the PKC consensus recognition sequences, as shown in panel B with "x" equal to any amino acid (*31*), 11 potential PKC phosphorylation sites are present in the HMGI(Y) proteins, which are indicated by open ovals and the corresponding amino acid sequence residue in panel A. No PKC consensus sequences are found in the C-terminal region of the HMGI(Y) proteins. Panel C illustrates an 18% SDS gel: lane 1, PKC phosphorylated rhHMG-I (IP); lane 2, unmodified rhHMG-I (I); lane 3, unmodified rhHMG-Y (Y); and lane 4, PKC phosphorylated rhHMG-Y (YP). PKC phosphorylation reaction 1 was used for the proteins shown in panel C. Protein marker is the Dalton Mark VII from Sigma, and the mass values are shown in kilodalton.

proteins are direct downstream targets of the intracellular PKC signaling pathway and strongly suggest that these proteins are in vivo substrates of the PKC enzymes.

In Vitro Phosphorylation of the Recombinant Human HMGI(Y) (rhHMG-I and rhHMG-Y) Proteins by PKC. To further investigate whether the HMGI(Y) proteins are substrates for the PKC enzymes, as suggested from the in vivo results above, phosphorylation experiments were performed in vitro using isolated recombinant HMGI(Y) proteins and purified PKC enzymes. Examination of the amino acid sequence for the HMGI(Y) proteins revealed a large number of potential PKC consensus phosphorylation sites (Figure 2A), based on previously published PKC consensus recognition sequences (Figure 2B) (*31*). Interestingly, comparison of the HMG-I and HMG-Y isoforms revealed differences in the number of potential PKC consensus recognition sequences between the two proteins. For instance, as a result of the removal of 12 amino acid residues at the "Y splice site" (Figure 2A), two potential PKC phosphorylation sites, Ser44 and Ser49, are removed in HMG-Y relative to HMG-I. Ser44 is centered in the Y-splice site region and therefore is not present in the HMG-Y protein, and Lys46 is removed with the splice site peptide, thus no longer making Ser49 a consensus PKC phosphorylation site. On the basis of these computer predictions, we chose to investigate whether the HMG-I and HMG-Y proteins are indeed differentially phosphorylated by PKC both in vitro and in vivo.

Recombinant human HMGI(Y) proteins (rhHMG-I and rhHMG-Y) were phosphorylated using cold ATP and the PKC-α isoform enzyme under 'saturating" reactions conditions empirically defined to modify both low and high affinity sites on the proteins (see *PKC phosphorylation reaction 1* in Materials and Methods; unless noted, all in vitro phosphorylations referred to in the text were performed under these conditions). As shown in Figure 2C, under these saturating conditions, both rhHMG-I and rhHMG-Y were extensively phosphorylated by PKC as evidenced by the retarded mobility of the modified proteins on the 18% SDS−PAGE gel relative to the mobilities of the unmodified recombinant proteins (compare lanes 1 vs 2 for rhHMG-I and lanes 3 vs 4 for rhHMG-Y). The molecular mass, based on amino acid composition (minus the N-terminal methionine residue), of the unmodified rhHMG-I protein is 11.5 kDa while that of the rhHMG-Y protein is 10.4 kDa (*1*, *4*, *46*). Nevertheless, as shown in Figure 2C, the unmodified rhHMG-I and rhHMG-Y proteins (lanes 2 and 3) migrate with an apparent molecular mass of ∼18−20 kDa on this gel, an anomaly resulting from the unusually large number of both positively and negatively charged residues in these proteins (*46*).

MALDI-MS Analysis of In Vitro PKC Phosphorylated rhHMG-I and rhHMG-Y Proteins. As shown in Figure 3, the mass-to-charge ratios (*m*/*z*) observed for unmodified rhHMG-Y (*m*/*z* 10 421.5) and rhHMG-I (*m*/*z* 11 549.1) proteins agree with the molecular mass predicted by amino acid sequence, with the first methionine residue removed (HMG-I: 11 544 Da; HMG-Y: 10 420 Da). One phosphate group increases the mass of a protein by 80 Da, and, as illustrated in Figure 3, rhHMG-Y has up to six detectable phosphates (*m*/*z* 10 902.7) while rhHMG-I has up to seven detectable phosphates (*m*/*z* 12 105.1). Therefore, under identical reaction conditions, PKC does appear to differentially phosphorylate the rhHMG-I and rhHMG-Y proteins in vitro.

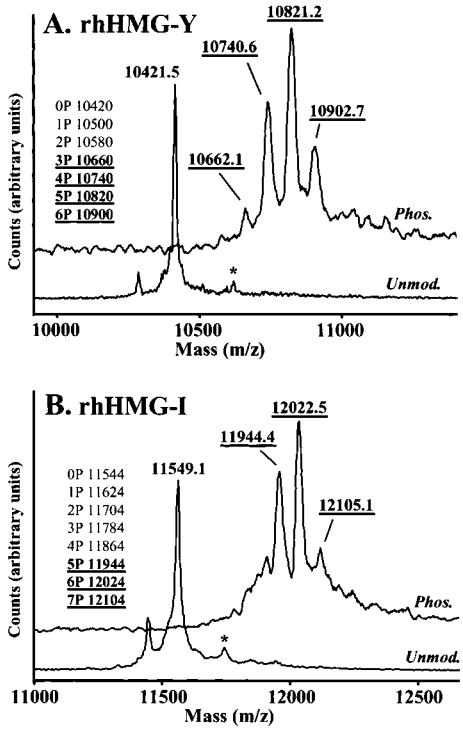

FIGURE 3: MALDI mass spectra of unmodified and in vitro PKC phosphorylated rhHMG-I and rhHMG-Y proteins. The observed mass-to-charge (*m*/*z*) for unmodified (Unmod.) recombinant human (rh) HMG-Y (panel A) and Unmod. rhHMG-I (panel B) closely match the theoretical mass values determined from amino acid sequence, 10 420 Da for HMG-Y and 11 544 Da for HMG-I, minus the initial methionine residue. The theoretical phosphorylated masses, staring at zero phosphates (0P) and increasing by 80 Da per phosphate group, are shown for comparison to the in vitro PKC phosphorylated protein spectra (Phos.) for HMG-Y (panel A) and HMG-I (panel B). A matrix adduct is a common artifact representing one parent ion plus one matrix molecule (3,5-dimethoxy-4-hydroxy-cinnamic acid, *m*/*z* 208) and is identified in the spectra by an asterisk symbol (*) [technical data, PerSeptive Biosystems (Framingham, MA)]. The low intensity peak just before the parent ion in both Unmod. HMG-I and HMG-Y mass spectra is possibly attributed to a degraded form of both proteins lacking the C-terminal glutamine residue.

Several likely explanations exist for the inability of PKC to phosphorylate all potential PKC recognition sequences on the rhHMGI(Y) proteins (see Figure 3). These include the possibility that (i) not all of the computer predicted PKC recognition sites are bona fide phosphorylation sites in the intact proteins; (ii) some of the PKC recognition sites are of such low affinity that they are not phosphorylated by the enzyme under the conditions employed; and (iii) steric hindrance in the proteins may prevent certain sites from being modified in vitro. In any event, experimental results in vitro, such as the multiple phosphoprotein peaks observed in Figure 3, suggest that, even under the saturating enzyme reaction conditions, populations of the rhHMG-I and rhHMG-Y proteins phosphorylated by PKC exhibit a certain degree of heterogeneity.

Tryptic Digestion of PKC Phosphorylated rhHMG-I and rhHMG-Y: Identification of Phosphorylation Sites. MALDI-MS spectra for the tryptic digests of both the unmodified and the PKC phosphorylated rhHMG-I and rhHMG-Y proteins are shown in Figure 4. Table 1 lists the corresponding mass-to-charge ratios of the protonated ion fragments (Obs. MH^+) observed for both rhHMG-Y and rhHMG-I. All

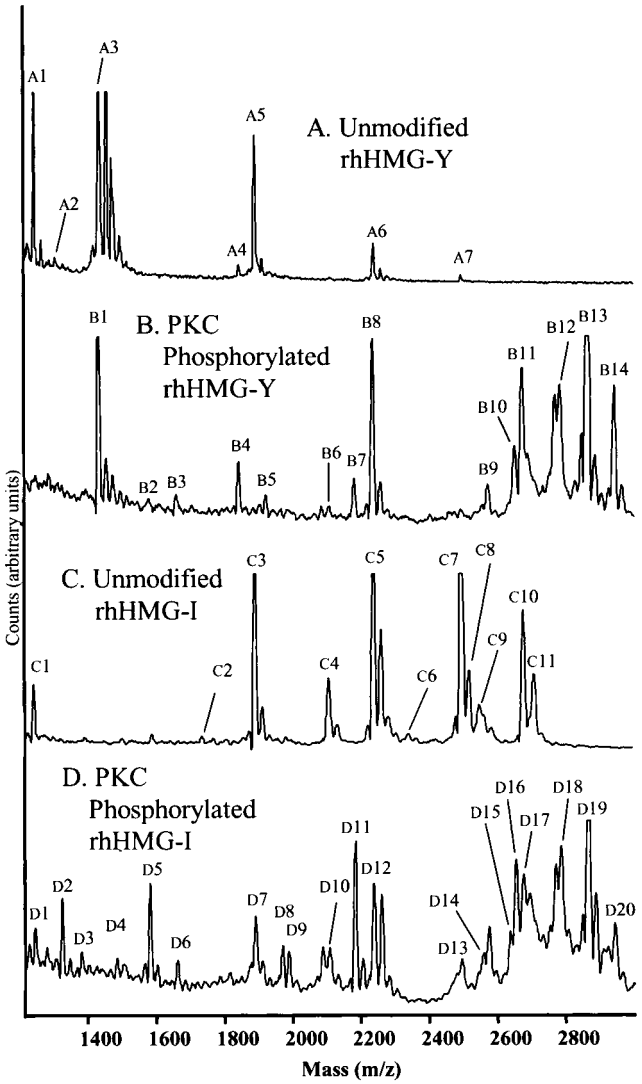

FIGURE 4: MALDI mass spectra of trypsinized unmodified and in vitro PKC phosphorylated rhHMG-I and rhHMG-Y proteins. Tryptic digestion patterns of unmodified rhHMG-Y (panel A) and rhHMG-I (panel C) are illustrated in comparison to in vitro PKC phosphorylated rhHMG-Y (panel B) and rhHMG-I (panel D). See Table 1 for the corresponding mass-to-charge values of the labeled ion peaks. Unlabeled ion peaks are either sodium adducts (*m*/*z* +23) or trypsin self-digestion peptide fragments (unpublished observations).

of the PKC consensus recognition sequences on the rhHMG-I and rhHMG-Y proteins can be found in one or more of the peptide fragments from tryptic spectra analyzed. Therefore, despite the possibility that some of the negatively charged phosphopeptide ions could be suppressed in positive ion mode (*38*, *39*), multiple overlapping peptide fragments resulting from partial tryptic digestion of the proteins allows for accurate determination of all of the phosphorylated residues (*36*). Utilizing a peptide mapping technique described by Kussmann et al. (*36*), we defined the sites of phosphorylation based on the PKC phosphorylated rhHMG-I and rhHMG-Y tryptic ion fragments (summarized in Figure 5).

On the basis of analysis of tryptic spectra of proteins phosphorylated in vitro by PKC under saturating conditions, the rhHMG-Y protein has seven modified residues (aa 5, 9, 21, 64, and 75−77), while the rhHMG-I protein has eight

Table 1: Peptide Mapping of Trypsinized Unmodified and PKC Phosphorylated rhHMG-Y and rhHMG-I Proteins Defines Sites of Phosphorylation[a]

pept[b]	obs MH^{+}[d]	calc MH^{+}[e]	residues[f,i]	PKC sites[g]	mod[h]
A1	1241.8	1242.7	74−84		
A2	1305.0	1305.7	32−34|47−55		
A3/B1	1433.9/1433.7	1433.6	31−34|47−55		
B2	1579.3	1579.7	72−84	75, 76, 77	1P
B3	1660.6	1659.7	72−84	75, 76, 77	2P
A4/B4	1844.4/1843.6	1843.1	31−34|47−58		
A5	1888.8	1889.0	8−24		
B5	1922.4	1921.0	74−88	75, 76, 77	3P
B6	2108.8	2109.1	90−107		
B7	2183.2	2182.3	8−26	21 (9)	1P
A6/B8	2238.1/2237.1	2237.3	89−107		
A7	2494.9	2494.2	2−24		
B9	2575.7	2574.7	2−24	21 (5, 9)	1P
B10	2654.4	2654.7	2−24	21 and 5 or 9	2P
B11	2679.2	2679.1	24−34|47−58		
B12	2787.9	2787.9	2−26	21 (5, 9)	1P
B13	2868.4	2867.9	2−26	21 and 5 or 9	2P
B14	2948.1	2947.9	2−26	21 and 5 and 9	3P
B15	3100.7	3100.5	27−34|47−65	64 (53)	1P

pept[c]	obs MH^{+}[d]	calc MH^{+}[e]	residues[f]	PKC sites[g]	mod[h]
C1/D1	1242.8/1243.7	1242.7	74−84		
D2	1323.0	1322.7	74−84	75, 76, 77	1P
D3	1379.1	1378.7	59−71	64	1P
D4	1483.2	1482.6	74−84	75, 76, 77	3P
D5	1579.7	1579.8	72−84	75, 76, 77	1P
D6	1659.6	1659.8	72−84	75, 76, 77	2P
C2	1733.7	1732.9	8−23		
C3/D7	1888.8/1889.8	1889.0	8−24		
D8	1969.2	1968.9	8−24	21 (9)	1P
D9	1987.9	1987.0	68−84	75, 76, 77 (72)	2P
C4/D10	2108.4/2108.3	2108.9	90−107		
D11	2182.3	2182.1	8−26	21 (9)	1P
C5/D12	2237.8/2237.6	2237.0	89−107		
C6	2345.1	2345.3	47−67		
C7/D13	2495.8/2493.5	2494.2	2−24		
C8/D14	2557.3/2559.7	2558.4	31−55		
C9	2567.1	2568.4	8−30		
D15	2639.3	2638.3	31−55	44 (49, 53)	1P
D16	2655.4	2654.2	2−24	21 and 5 or 9	2P
C10/D17	2676.7/2676.9	2675.3	85−107		
C11	2708.2	2707.4	2−26		
D18	2788.1	2787.3	2−26	21 (5, 9)	1P
D19	2867.8	2867.3	2−26	21 and 5 or 9	2P
D20	2946.4	2947.3	2−26	21 and 5 and 9	3P

[a] rhHMG-Y and rhHMG-I proteins were phosphorylated using PKC phosphorylation reaction 1 (see Materials and Methods). [b] The peptide number corresponds to the labeled ion peaks observed in Figure 4, panels A and B for HMG-Y. [c] The peptide number corresponds to the labeled ion peaks observed in Figure 4, panels C and D for HMG-I. [d] Observed *m*/*z* values of the protonated ions from Figure 4. [e] Calculated monoisotopic mass. [f] Amino acid residues of the corresponding peptide fragment. [g] Potential PKC consensus recognition sequences within each phosphopeptide fragment. [h] Number of phosphate groups observed from tryptic analysis. [i] To avoid confusion, the numbering of the residues in HMG-Y are the same as in HMG-I, with the splice site (which removes residues 35−46) indicated by the symbol "|". When a phosphopeptide fragment contains more PKC consensus sites than observed phosphate groups, those sites in parentheses are reasoned not to be phosphorylated based on observations from all tryptic spectra analyzed (data not shown).

phosphorylated residues (aa 5, 9, 21, 44, 64, and 75−77) (see Figure 5). Interestingly, the total number of phosphate groups defined from the MALDI mass spectra for the full-length PKC phosphorylated rhHMG-Y (six phosphates) and rhHMG-I (seven phosphates) proteins (Figure 3) differed from the number identified by the tryptic analysis: rhHMG-Y (seven phosphorylated residues); rhHMG-I (eight phosphorylated residues). This discrepancy in phosphorylation status is likely attributed to nonstoichiometric phosphorylation, even under saturating conditions, of all of the low-affinity PKC consensus sequences by the PKC enzyme in vitro.

Nonsaturating PKC phosphorylation conditions were employed to characterize the relative affinity of the PKC phosphorylation sites in the rhHMG-I and rhHMG-Y proteins. Under the nonsaturating PKC reaction conditions (see *PKC phosphorylation reaction 2* in Materials and Methods), it was reasoned that high affinity sites would be preferentially phosphorylated over low affinity sites. The results from the tryptic analysis of the rhHMG-I and rhHMG-Y proteins phosphorylated under both saturating and nonsaturating conditions are summarized in Figure 5. Importantly, note that Ser44 is a high affinity PKC site in the rhHMG-I protein and that this site is not present in the rhHMG-Y protein. In addition, even under the supposed saturating PKC phosphorylation reaction conditions used in Figures 3 and 4 (PKC phosphorylation reaction 1), Ser5 and Ser9 as well as Thr75, 76, and 77 are not stoichiometrically modified (see peptides D18 through D20 and D2 in Table 1). Nevertheless, in all tryptic spectra analyzed, no other residues besides those shown in Figure 5 were observed to be phosphorylated on the rhHMG-I and rhHMG-Y proteins, which suggests that all sites of PKC phosphorylation have been defined under the conditions employed.

In some instances, ion fragments observed from the tryptic spectra of the in vitro PKC phosphorylated rhHMG-I and rhHMG-Y proteins contain fewer phosphate groups than the total number of PKC consensus recognition sequences. In such cases, those residues considered not to be phosphorylated are indicated by parenthesis in Table 1 and are done so based on patterns observed throughout all tryptic spectra analyzed for the in vitro PKC phosphorylated proteins (data not shown). However, due to limitations in peptide mapping using MALDI mass spectrometry, precisely identifying residues in these peptide fragments that are phosphorylated can only be accomplished by tandem mass spectrometry (MS/MS).

PKC Phosphorylation of the rhHMG-I and rhHMG-Y Proteins In Vitro Disrupts High Affinity A·T-Rich B-Form DNA Binding. Electrophoretic mobility shift assays (EMSAs) were employed to assess the effect of PKC phosphorylation on the DNA-binding properties of the rhHMG-I and rhHMG-Y proteins. In Figure 6, panels A and B, both unmodified rhHMG-I and rhHMG-Y proteins exhibit a high affinity for the A·T-rich DNA substrate, BLT, used in these EMSAs. BLT is an acronym for the 3′ UTR of the bovine IL-2 cDNA and is an excellent, and extensively studied, substrate for HMGI(Y) because it contains numerous A·T-rich binding sites (*40*−*42*). In contrast to the unmodified proteins, the PKC phosphorylated rhHMG-I and rhHMG-Y proteins (PKC phosphorylation reaction 1) exhibit a significant reduction in DNA-binding affinity (Figure 6, panels C and D). Interestingly, despite the high level of phosphorylation defined in Figures 3−5 for rhHMG-I and rhHMG-Y, the phosphorylated proteins are still able to form some specific protein−DNA complexes with the naked DNA substrate (Figure 6, panels C and D). A similar decrease in binding affinity was observed for the phosphorylated rhHMG-I and

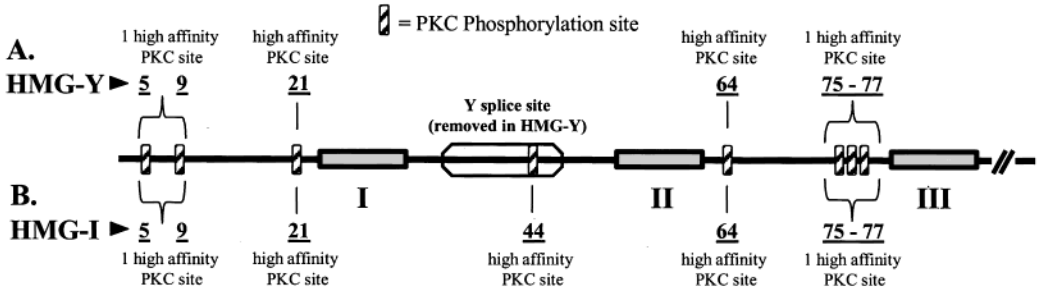

FIGURE 5: Summary of the sites of in vitro PKC phosphorylation. PKC phosphorylation sites for both the rhHMG-Y (panel A) and the rhHMG-I (panel B) proteins are summarized using a protein schematic. High affinity sites were defined using nonsaturating phosphorylation reaction conditions (see Materials and Methods and text for explanation). No sites of phosphorylation were observed on the C-terminal region past A·T-hook III. Note: for simplicity, we have chosen to designate one site between Thr75, 76, and 77 and one site between Ser5 and Ser9 as high affinity since an average of only one phosphate group is observed for each of the two groups of residues under nonsaturating PKC phosphorylation conditions.

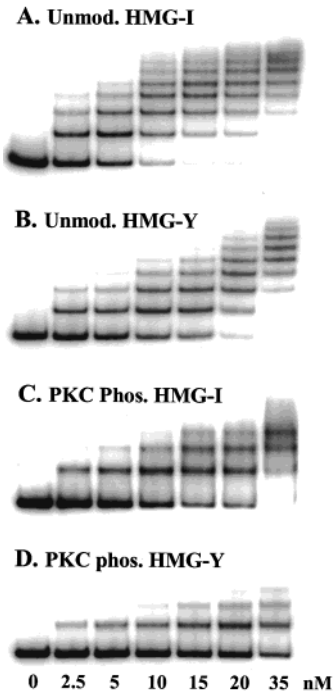

FIGURE 6: Phosphorylation of the rhHMG-I and rhHMG-Y proteins in vitro by PKC reduces A·T-rich DNA-binding affinity. Electrophoretic mobility shift assay (EMSA) was performed using the 300 bp 3′ untranslated tail region of the bovine Interleukin-2 cDNA (BLT). Unmodified rhHMG-I (panel A) and rhHMG-Y proteins (panel B) are compared to the in vitro PKC phosphorylated rhHMG-I (panel C) and rhHMG-Y (panel D) proteins to assess the relative binding affinity of the phosphorylated proteins. Each gel has identical concentrations of protein ranging from 0 to 35 nM as seen in panel D.

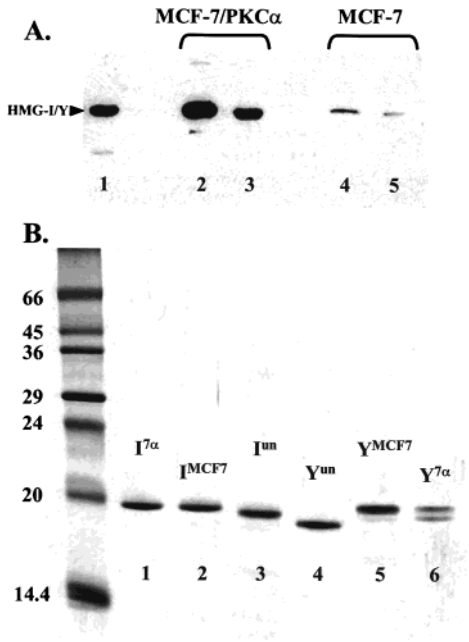

FIGURE 7: (Panel A) HMGI(Y) proteins are expressed at a low level in nonmetastatic MCF-7 cells but at a high level in metastatic MCF-7/PKC-α cells. Total acid-soluble cellular proteins were isolated from MCF-7 and MCF-7/PKC-α cells, separated by 15% SDS−PAGE, transferred onto nitrocellulose membrane, and analyzed by Western blot with a specific polyclonal antibody against the human HMG-I protein. Lanes: (1) control rhHMG-I protein (1 μg); (2) 12 μg of total protein isolated from MCF-7/PKC-α cells; (3) 6 μg of total protein isolated from MCF-7/PKC-α cells; (4) 12 μg of total protein isolated from MCF-7 cells; and (5) 6 μg of total protein isolated from MCF-7 cells. On this gel, the HMG-I and HMG-Y proteins co-migrated and are designated as HMG-I/Y. (Panel B) HMG-I and HMG-Y proteins from MCF-7 and MCF-7/PKC-α cells are posttranslationally modified. An 18% SDS gel comparing the proteins isolated from MCF-7/PKC-α (HMG-$I^{7\alpha}$, lane 1 and HMG-$Y^{7\alpha}$, lane 6) and MCF-7 (HMG-I^{MCF7}, lane 2 and HMG-Y^{MCF7} lane 5) to unmodified rhHMG-I and rhHMG-Y proteins in lanes 3 and 4, respectively, is shown. The masses for molecular weight marker, Dalton Mark VII, are listed in kilodalton.

rhHMG-Y proteins, in comparison to the unmodified proteins, on isolated nucleosome core particles (data not shown).

HMG-I and HMG-Y are Posttranslationally Modified In Vivo. Although the difference in number of PKC in vitro phosphorylation sites on the rhHMG-I and rhHMG-Y proteins is small, the above experimental results suggested the potential for differential posttranslational modifications of these proteins in vivo. To investigate this possibility, we acid-extracted and purified the HMG-I and HMG-Y proteins from two different cell lines: MCF-7 and MCF-7/PKC-α. As noted in the introduction, these two cell lines were chosen for study on the basis of a marked difference in their tumorigenic potential in nude mice. These studies therefore allowed for investigation of a possible connection between the posttranslational modification of the HMGI(Y) proteins in vivo and different stages of cellular transformation. As shown in the Western blot in Figure 7A, the MCF-7/PKC-α cells, consistent with their highly malignant phenotype, constitutively contain much higher levels (>10-fold) of endogenous HMGI(Y) proteins (lanes 2 and 3) than do the preneoplastic parental MCF-7 cells (lanes 4 and 5). Furthermore, SDS−PAGE analysis (Figure 7B) of the HMG-I and

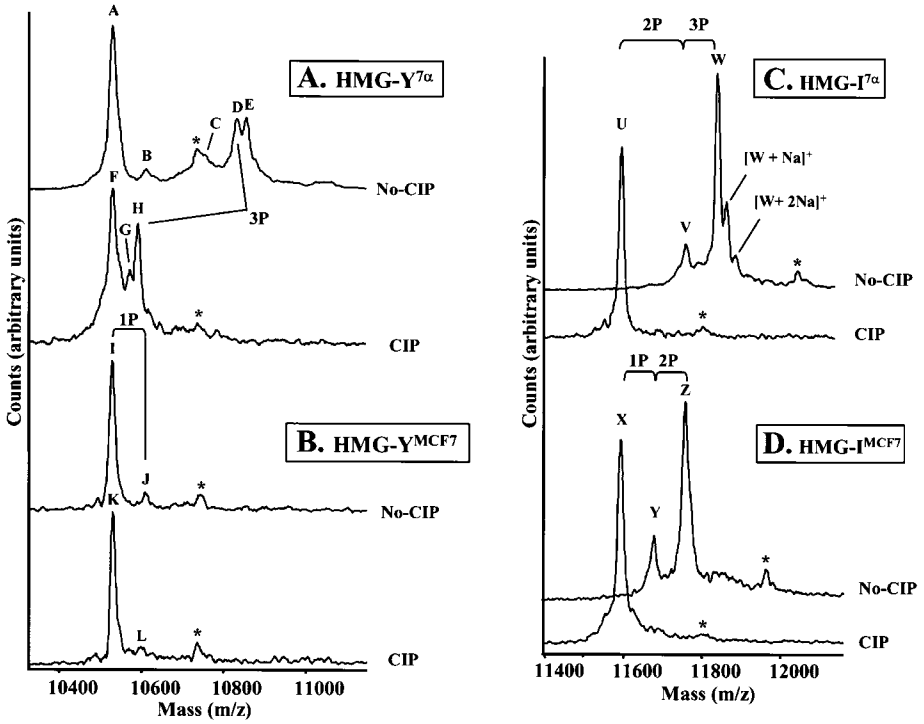

FIGURE 8: MALDI mass spectra of full-length HMG-Y^{MCF7}, HMG-$Y^{7\alpha}$, HMG-I^{MCF7}, and HMG-$I^{7\alpha}$. HMG-I and HMG-Y isolated from MCF-7 and MCF-7PKC-α cells were dephosphorylated using calf intestinal alkaline phosphatase "CIP" and were analyzed by MALDI-MS in comparison to the "no-CIP" (in vivo phosphorylated) proteins. The observed *m/z* values for HMG-$Y^{7\alpha}$, panel A, are A = 10 530.2; B = 10 611.5; C = 10 755.4; D = 10 833.8; E = 10 856.2; F = 10 529.8; G = 10 571.9; and H = 10 591.6, and the observed *m/z* values for HMG-Y^{MCF-7}, panel B, are I = 10 528.5; J = 10 608.6; K = 10 529.1; and L = 10 599.8. The observed *m/z* values for HMG-$I^{7\alpha}$, panel C, are U = 11 593.2; V = 11 753.8; and W = 11 834.0, and the observed *m/z* values for HMG-I^{MCF-7}, panel D, are X = 11 592.6; Y = 11 672.9; and Z = 11 752.9. An asterisk (*) illustrates a matrix adduct and is commonly observed when the sinapinic acid matrix is used (see Figure 3). The sodium adducts (+23 *m/z*) observed in panel C are $[W + Na]^+ = 11\,857.0$; $[W + 2Na]^+ = 11\,880.3$. Note that ion peak L (*m/z* 10 599.8) in the "CIP" spectrum for HMG-Y^{MCF7} in panel B is labeled to illustrate that it is not the same mass as the phosphopeptide ion peak J (*m/z* 10 608.6) in the "no-CIP" HMG-Y^{MCF7} spectrum in panel B. Symbols 1P, 2P, and 3P denote the number of phosphate groups.

HMG-Y proteins isolated from MCF-7 cells (HMG-I^{MCF7} and HMG-Y^{MCF7}, lanes 2 and 5) and from MCF-7/PKC-α cells (HMG-$I^{7\alpha}$ and HMG-$Y^{7\alpha}$, lanes 1 and 6) illustrates that all of these proteins are posttranslationally modified in vivo as evidenced by their retarded mobilities relative to unmodified recombinant proteins (I^{un} and Y^{un}, lanes 3 and 4).

MALDI-MS Analysis of the HMG-I and HMG-Y Proteins from MCF-7 and MCF-7/PKC-α Cells. All four of the native proteins (HMG-I^{MCF7}, HMG-Y^{MCF7}, HMG-$I^{7\alpha}$, and HMG-$Y^{7\alpha}$) isolated from the two cell lines were digested with calf intestinal alkaline phosphatase (CIP) to assess each protein's relative level of in vivo phosphorylation. Comparison of the MALDI-MS spectra (Figure 8) of the "no-CIP" (in vivo phosphorylated) and "CIP" (dephosphorylated) treated proteins, confirmed that all four proteins were posttranslationally phosphorylated in vivo. Although the unmodified mass of each of the four native proteins from the two cell lines is unknown, it appears that the mass is the same as their unmodified recombinant protein counterparts (rhHMG-I: observed *m/z* 11 549.1, Figure 3B; rhHMG-Y: observed *m/z* 10 421.5, Figure 3A). For example, ion peak X (*m/z* 11 593.2) in the CIP dephosphorylated HMG-I^{MCF7} spectra in Figure 8D cannot be the full-length HMG-I protein with the initial methionine residue, a moiety that has a theoretical mass of 11 676 Da.

On the basis of the no-CIP and CIP MALDI-mass spectra, both the HMG-I and the HMG-Y proteins from the MCF-7/PKC-α cell line had a higher level of modification relative to the MCF-7 cell line (see Figure 8). For example, HMG-$Y^{7\alpha}$ (Figure 8A) had an average of three phosphate groups [no-CIP ion peak D (*m/z* 10 833.8) is three phosphate groups away from the CIP ion peak H (*m/z* 10 591.6)] while HMG-Y^{MCF7} (Figure 8B) had an average of one phosphate group [no-CIP ion peak J (*m/z* 10 608.6) is one phosphate group away from the CIP ion peak K (*m/z* 10 529.1)].

In addition to the higher level of phosphorylation observed in the MCF-7/PKC-α cells, other differences in posttranslational modification were also observed between the HMG-I and the HMG-Y proteins isolated from these cell lines. For example, the ion peaks A, F, I, and K observed in both the CIP and no-CIP spectra for the HMG-Y proteins in Figure 8, panels A and B (~*m/z* 10 530) are approximately 110 *m/z* units higher than the unmodified HMG-Y protein (observed rhHMG-Y = *m/z* 10 421 Da, Figure 3A). In contrast, ion peaks U and X in the CIP HMG-$I^{7\alpha}$ and HMG-I^{MCF7} spectra both have a mass that is approximately 44 *m/z* units from the unmodified HMG-I protein (observed rhHMG-I = *m/z* 11 549). As the CIP spectra correspond to the dephosphorylated protein species, differences between the observed mass-to-charge ratios of the CIP proteins and the theoretical mass for unmodified HMG-I and HMG-Y must correspond to posttranslational modifications other than phosphorylation. Therefore, even aside from differences in the levels of phosphorylation, HMG-Y has a higher level of "other types" of posttranslational modifications than does HMG-I isolated from the same two cell lines. As defined by the tryptic

Table 2: Peptide Mapping of Trypsinized HMG-Y$^{7\alpha}$, HMG-Y^{MCF7}, HMG-I$^{7\alpha}$, and HMG-I^{MCF7} Defines Sites of Modification[a]

HMG-Y$^{7\alpha}$						HMG-Y^{MCF-7}					
obs[b] MH$^+$	calc[f] MH$^+$	residues[g,j]	no-CIP[h]	CIP[h]	mod[I]	obs[c] MH$^+$	calc[f] MH$^+$	residues[g,j]	no-CIP[h]	CIP[h]	mod[i]
1114.4	1114.6	75−84	N	Y		1115.0	1114.6	75−84	Y	N	
1243.4	1242.7	74−84	Y	Y		1243.4	1242.7	74−84	Y	Y	
1306.5	1305.7	32−34|47−55	Y	N		1303.4	1303.7	16−26	Y	N	
1585.2	1585.7	19−31	Y	Y	1A + 2M	1464.5	1465.7	2−15	Y	Y	1A
1615.0	1615.9	59−73	Y	Y	4M	1615.9	1615.9	59−73	Y	Y	4M
1743.8	1744.0	59−74	Y	Y	4M	1744.4	1744.0	59−74	Y	Y	4M
1791.9	1790.9	8−23	Y	Y	1A + 1M	1808.3	1809.1	74−89	N	Y	
1890.3	1889.0	8−24	Y	Y		1890.0	1889.0	8−24	Y	N	
1972.3	1971.0	16−31	Y	Y	1A + 2M	1972.1	1971.0	16−31	N	Y	1A + 2M
2236.4	2237.0	89−107	N	Y		2308.3	2309.3	27−34|47−58	Y	N	
2470.0	2469.2	27−34|47−58	Y	N	2P	2494.9	2494.2	2−24	Y	N	
2537.5	2537.2	2−24	Y	Y	1A	2675.8	2675.3	85−107	N	Y	
3178.3	3179.6	27−34|47−65	Y	N	2P	2870.7	2871.6	27−34|47−62	Y	N	1P + 1A
3216.0	3216.4	82−107	Y	N	2P[k]						

HMG-I$^{7\alpha}$						HMG-I^{MCF7}					
obs[d] MH$^+$	calc[f] MH$^+$	residues[g]	no-CIP[h]	CIP[h]	mod[i]	obs[e] MH$^+$	calc[f] MH$^+$	residues[g]	no-CIP[h]	CIP[h]	mod[i]
1013.7	1014.4	16−23	Y	N	1P [21]	1114.1	1114.6	75−84	Y	Y	
1114.4	1114.6	75−84	Y	N		1137.9	1138.7	56−65	N	Y	
1201.9	1202.6	8−18	Y	N		1242.5	1242.7	74−84	Y	Y	
1242.6	1242.7	74−84	Y	Y		1499.8	1499.9	72−84	Y	Y	
1297.5	1298.8	59−71	Y	Y		2102.3	2102.1	8−26	Y	Y	
1378.0	1378.7	59−71	Y	N	1P [64]	2536.9	2537.2	2−24	Y	Y	1A
1556.8	1555.9	59−73	N	Y		2558.8	2558.4	31−55	Y	Y	
1673.6	1673.9	31−46	Y	N	1P	2676.8	2675.3	85−107	Y	N	
1685.1	1684.0	59−74	N	Y		2750.4	2750.4	2−26	Y	Y	1A
1789.4	1788.0	56−71	Y	N	1P [64]	2835.7	2835.2	85−107	Y	N	2P[k]
1889.2	1889.0	8−24	Y	Y		3048.5	3047.6	31−58	Y	N	1P
2103.6	2103.2	47−65	N	Y		3127.2	3127.6	31−58	Y	N	2P
2238.2	2237.0	89−107	N	Y							
2558.8	2558.4	31−55	Y	Y							
2696.7	2697.2	2−24	Y	N	1A + 2P						
2750.8	2750.4	2−26	Y	Y	1A						

[a] MALDI-MS of trypsinized proteins were conducted as in Materials and Methods (data not shown). [b] Observed *m*/*z* values of the protonated ions for the HMG-Y$^{7\alpha}$ proteins are listed. [c] Observed *m*/*z* values of the protonated ions for the HMG-Y^{MCF7} proteins are listed. [d] Observed *m*/*z* values of the protonated ions for the HMG-I$^{7\alpha}$ proteins are listed. [e] Observed *m*/*z* values of the protonated ions for the HMG-I^{MCF7} proteins are listed. [f] Calculated monoisotopic mass. [g] Amino acid residues of the corresponding peptide fragment. [h] Those ion fragments observed in the "CIP" (dephosphorylated) or "no-CIP" (in vivo phosphorylated) tryptic spectra are designated with a "Y", and those ions not observed are designated with an "N". [i] Number and type of modification [A = acetyl (43 amu); M = methyl (15 amu); P = phosphate (80 amu)]. In some instances, specific modified residues can be defined and are so indicated in brackets. [j] To avoid confusion, the numbering of the residues in HMG-Y is the same as in HMG-I, with the splice site (which removes residues 35−46) indicated by the symbol "|". [k] C-terminal phosphorylation likely from casein kinase II at residues 99, 102, or 103 (*52*).

analysis below, these other modifications include acetylation and methylation.

Importantly, the relative heights of the MALDI-MS ion peaks are not quantitative and cannot be directly correlated to the species observed on the SDS gel in Figure 7B. In addition, mass comparisons between the proteins from SDS−PAGE and MALDI-MS are not possible because of the well-documented anomalous mobility that the HMGI(Y) proteins exhibit on protein gels (*1*, *35*).

Tryptic Digestion of the HMG-I and HMG-Y Proteins from MCF-7 and MCF-7/PKC-α Cells: Identification of Sites of In Vivo Modification. MALDI-MS spectra of tryptic fragments from both the CIP and the no-CIP HMG-I and HMG-Y proteins isolated from MCF-7 and MCF-7/PKC-α were analyzed to identify sites and types of posttranslational modification (data not shown). Table 2 lists the observed mass-to-charge ratios for the protonated ion fragments (Obs. MH$^+$) obtained from these tryptic spectra. Those ion fragments that were present in the no-CIP (in vivo phosphorylated) spectra and were missing in the CIP (dephosphorylated) spectra are likely to be phosphorylated. Although one might normally expect to see all of the corresponding dephosphorylated tryptic peptide ion fragments in the CIP-dephosphorylated spectrum, for a variety of technical reasons this is, in fact, seldom the case (*36*). More importantly, the presence of an ion fragment in both the no-CIP and the CIP tryptic spectra was a strong indication that the ion fragment is not phosphorylated and, therefore, must be either an unmodified peptide fragment or a peptide fragment that contains posttranslational modifications other than phosphorylation.

From a careful analysis of the MALDI-MS-derived molecular masses of the tryptic peptides of the HMG-I and HMG-Y proteins isolated from MCF-7 and MCF-7/PKC-α cells, we conclude that while the HMG-I protein is modified by both phosphorylation and acetylation, the HMG-Y protein is additionally modified by methylation in both cell types (see Table 2 and summaries in Figure 10 and Table 3).

As observed for PKC phosphorylation of the rhHMG-I and rhHMG-Y proteins in vitro using purified PKC, there are differences in the relative number of modifications defined from the MALDI mass spectra of the full-length

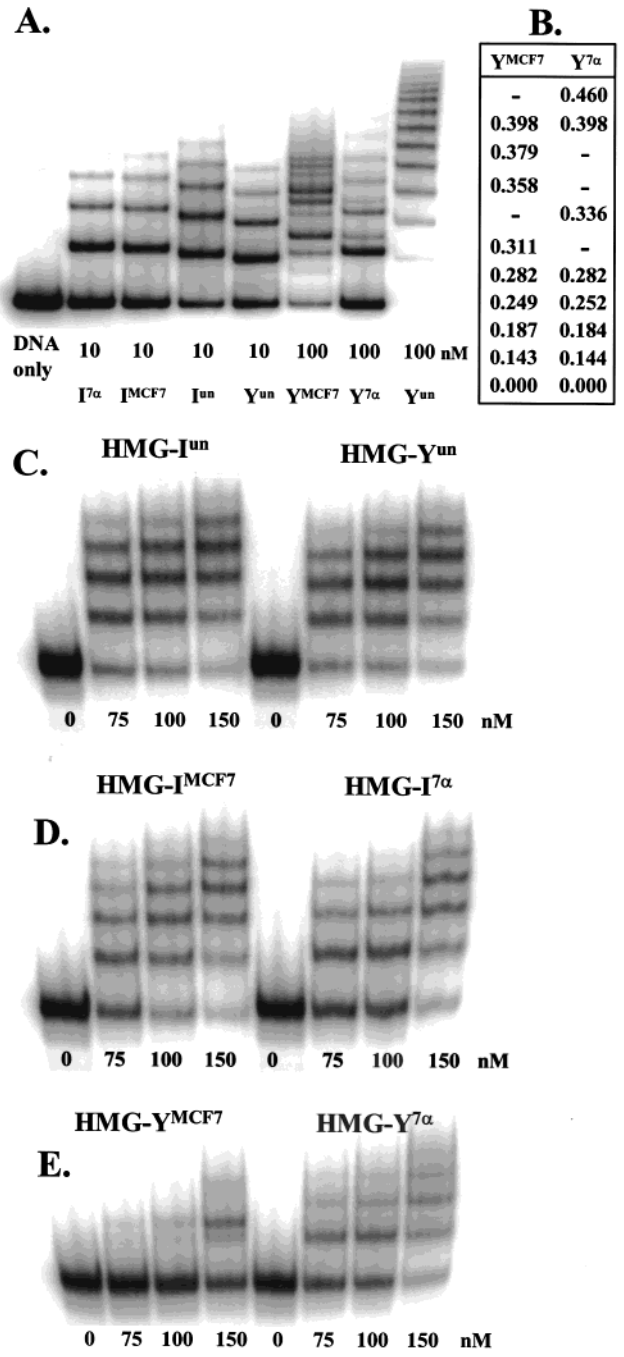

FIGURE 9: HMG-Y, but not HMG-I, from MCF-7 and MCF-7/PKC-α cells exhibit a reduced affinity for A·T-rich DNA and nucleosome core particles. In panel A, BLT DNA was used (see Figure 6 for definition of BLT) for EMSAs of proteins from MCF-7/PKC-α (HMG-$I^{7\alpha}$; HMG-$Y^{7\alpha}$) and MCF-7 (HMG-I^{MCF7}; HMG-Y^{MCF7}) cells. For comparison, unmodified rhHMG-I (I^{un}) and unmodified rhHMG-Y (Y^{un}) are shown. R_f (relative mobility) values for the HMG-Y^{MCF7} and HMG-$Y^{7\alpha}$ proteins binding to BLT DNA from panel A are shown in panel B, with free DNA set to zero, to illustrate the difference in the mobility of the protein−DNA complexes between these two proteins. In panels C through E, EMSAs were preformed using chicken erythrocyte nucleosome core particles: panel C, unmodified recombinant HMG-I and HMG-Y proteins; panel D, HMG-I from MCF-7 and MCF-7/PKC-α cells; and panel E, HMG-Y from MCF-7 and MCF-7/PKC-α cells.

HMG-$I^{7\alpha}$, HMG-I^{MCF7}, HMG-$Y^{7\alpha}$, and HMG-Y^{MCF7} proteins in comparison to that obtained from analysis of tryptic digests. For instance, the tryptic analysis revealed a higher number of phosphate groups than were defined by MALDI-MS analysis of their corresponding full-length proteins (compare Figure 10 and Table 3). Therefore, the HMG-I and HMG-Y proteins isolated from MCF-7 and MCF-7/PKC-α appear to be mixtures of nonstoichiometrically modified proteins, which suggests that there are distinct cellular subpopulations of the HMG-I and HMG-Y proteins with either different types of in vivo modifications and/or different levels of similar modifications in the cells.

HMG-I and HMG-Y Proteins from MCF-7 and MCF-7/PKC-α Cells Display a Differential Affinity for A·T-Rich B-Form DNA and Nucleosome Core Particles. As with the analysis of in vitro PKC phosphorylated rhHMG-I and rhHMG-Y proteins (Figure 6), EMSAs were employed to assess the effect of in vivo modifications on HMGI(Y) DNA-binding properties. Although HMG-$I^{7\alpha}$ and HMG-I^{MCF7} have an average of two and three phosphate groups, respectively (Table 3), both proteins have nearly the same affinity for naked BLT DNA (Figure 9A, lanes $I^{7\alpha}$ and I^{MCF7}), and only a slightly decreased affinity relative to unmodified rhHMG-I (Figure 9A, lane I^{un}). In contrast, we have previously reported that phosphorylation of HMG-I by the enzyme cdc-2 kinase results in a 20-fold reduction in DNA-binding affinity upon phosphorylation at residues Thr53 and Thr78 (*34*, *47*). This marked difference between the effects of cdc2 kinase phosphorylation and the phosphorylation described here suggests that in vivo phosphorylation of HMG-I from MCF-7 and MCF-7/PKC-α may serve to modulate protein−protein interactions to a greater extent than it regulates protein−DNA interactions.

In contrast to the HMG-$I^{7\alpha}$ and HMG-I^{MCF7} proteins, HMG-Y isolated from both the MCF-7 and the MCF-7/PKC-α cells exhibits a significant reduction in DNA-binding affinity relative to unmodified rhHMG-Y (compare Y^{MCF7} and $Y^{7\alpha}$ to 100 nM Y^{un}, Figure 9A). Interestingly, at the same concentrations (100 nM), both Y^{MCF7} and $Y^{7\alpha}$ display a markedly different BLT DNA-binding pattern as compared to the 100nM Y^{un}. In addition, there are differences in the binding pattern between Y^{MCF7} and $Y^{7\alpha}$, as observed by comparing R_f (relative mobility) values in Figure 9B. Clearly, the binding patterns observed for HMG-Y^{MCF7} and HMG-$Y^{7\alpha}$ on BLT DNA are a consequence of these proteins having been adducted in vivo by a combination of three different posttranslational modifications (phosphorylation, methylation, and acetylation). These binding patterns are clearly different from those on BLT DNA observed for the rhHMG-Y protein modified only by phosphorylation (Figure 6D) or for the HMG-I protein modified in vivo by a combination of both phosphorylation and acetylation (cf. $I^{7\alpha}$ and I^{MCF7}, Figure 9A).

EMSAs of the HMG-I and HMG-Y proteins from MCF-7 and MCF-7/PKC-α cells on isolated nucleosome core particles are illustrated in Figure 9, panels D and E in comparison to unmodified rhHMG-I and rhHMG-Y proteins in Figure 9C. Figure 9D indicates that the HMG-I proteins isolated from MCF-7 and MCF-7/PKC-α cells (i.e., HMG-I^{MCF7} and HMG-$I^{7\alpha}$) behave similarly in these EMSAs to the unmodified rhHMG-I. In contrast, Figure 9E illustrates that the HMG-Y proteins isolated from both cell types (i.e., HMG-Y^{MCF7} and HMG-$Y^{7\alpha}$) exhibit a marked reduction in core particle binding affinity relative to the unmodified rhHMG-Y protein, with HMG-$Y^{7\alpha}$ having a somewhat higher

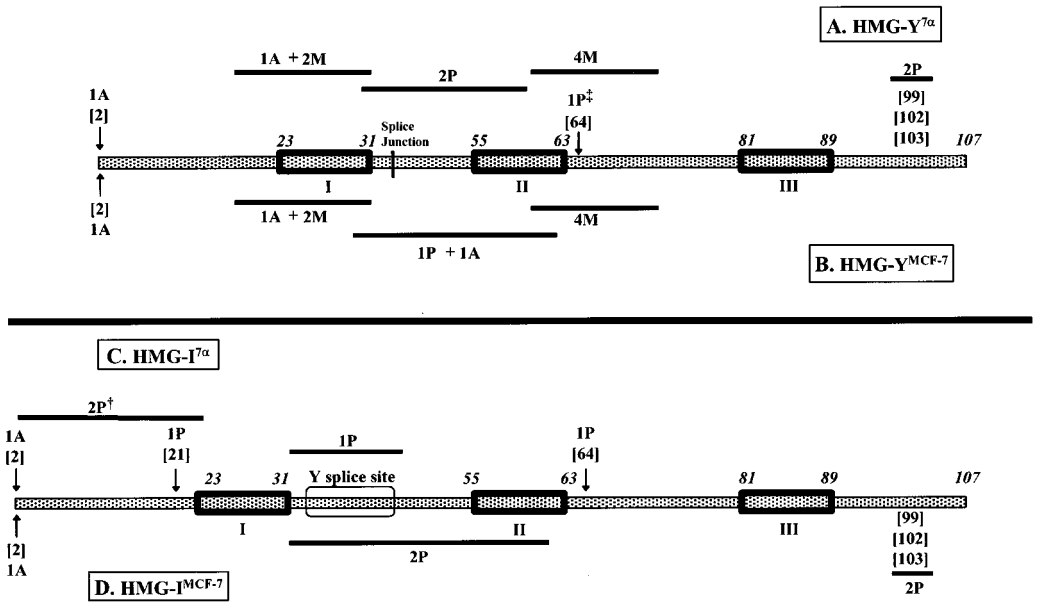

FIGURE 10: Summary of sites of modification for the HMG-I and HMG-Y proteins from MCF-7 and MCF-7/PKC-α cells. Sites of modification were determined using a peptide mapping technique (see Table 2). Because of limitations of the peptide mapping technique, in most cases, only regions of the protein can be defined as being modified (as indicated by a line with the number and type of modification). However, in some instances, specific modified residues can be defined and are so indicated in brackets. Sites of modification are A = acetyl; M = methyl; and P = phosphate. Numbering of the residues in HMG-Y is the same as in HMG-I. Shaded rectangles indicate the regions of the proteins corresponding to the three A·T-hook DNA-binding motifs. (‡) Identification of the phosphate group on Ser64 in panel A is based on the diphosphopeptide tryptic fragment corresponding to residues 27−34|47−65 (Obs. MH^+ = 3178.3). (†) One of the two phosphate groups identified on the tryptic fragment corresponding to residues 2−24 (Obs. MH^+ = 2696.7) is likely on Thr21.

Table 3: Summary of the Levels of Modifications for HMG-I and HMG-Y from MCF-7 and MCF-7/PKC-α Cells

	MCF-7	MCF-7/PKC-α
phosphorylation:		
HMG-I[a]	2	3
HMG-Y[a]	1	3
acetylation:		
HMG-I[b]	1	1
HMG-Y[b]	3	2
methylation:		
HMG-I[b]	0	0
HMG-Y[b]	6	6

[a] The number of phosphate groups for HMG-I and HMG-Y are based on the average determined from MALDI-MS of the full-length proteins in Figure 8. Discrepancies in the number of phosphate groups defined by MALDI-MS analysis of full-length proteins as compared to the tryptic analysis as summarized in Figure 10 reflect the nonstoichiometric modification of the proteins. [b] The number of methyl and acetyl groups is based on the tryptic analysis of the proteins in Table 2 (summarized in Figure 10).

affinity for the nucleosomal substrate than HMG-Y^{MCF7}. This is significant because HMG-$Y^{7\alpha}$ has a higher level of phosphorylation relative to HMG-Y^{MCF7} (see Table 3) and could indicate that specific phosphorylation sites on HMG-$Y^{7\alpha}$ are positioned to interact with the positively charged N-terminal tails from the nucleosome core particles. In addition, note that a somewhat similar binding pattern is observed in the core particle EMSA (Figure 9E) for the two HMG-Y proteins as seen with the BLT DNA substrate in Figure 9A. Therefore, differences in modifications between the two HMG-Y proteins from the MCF-7 and MCF-7/PKC-α cells appear to differentially influence both their A·T-rich DNA and nucleosome core particle binding affinity, and perhaps even specificity, relative to each other and to the HMG-I proteins from the same cells.

DISCUSSION

In Vivo Posttranslational Modifications of the HMGI(Y) Proteins. An extensive body of literature exists concerning posttranslational modifications of the HMGI(Y) proteins and the potential role(s) of these modifications in regulating the function of these proteins in vivo (*34*, *47*−*59*). Members of the HMGI(Y) family are among the most highly phosphorylated proteins in the mammalian cell nucleus (*47*, *60*), and these modifications have been demonstrated to modulate the DNA-binding activity of the proteins in vitro. For example, phosphorylation of both human and mouse HMG-I proteins at specific amino acid residues by cdc2 kinase [previously referred to as histone H1 kinase (*51*)] decreases the in vitro affinity of binding of these proteins to A·T-rich DNA substrates by 20−50-fold. In vivo, cdc2 kinase has also been demonstrated to phosphorylate these same amino acid residues on the human HMG-I protein in a cell cycle-dependent manner (*34*, *47*). In addition, the evolutionarily distant (*54*), but still related, insect HMG-I homologue from the genus *Chironomus* has also been demonstrated to be an in vitro substrate for cdc2 kinase and a number of other kinases including mitogen-activated protein kinase (MAPK) and PKC (*54*). In contrast, and contrary to the results presented here, a previous report has suggested that the mammalian HMGI(Y) proteins are not in vitro substrates for PKC phosphorylation (*61*). The reasons for this apparent discrepancy are presently unknown but reinforce the significance of the present observations that the HMGI(Y) proteins are indeed in vitro and in vivo phosphorylation substrates for this family of kinases. Nevertheless, our current identification of regions of the HMGI(Y) proteins that are modified in vivo (Figure 10 and Table 3) confirm earlier reports that the HMGI(Y) proteins are acetylated, for

example, in murine Friend erythroleukemic cells (*46*), and are phosphorylated by casein kinase II in human placenta cells (see Figure 10, residues 99, 102, and 103) (*52*).

Differential In Vivo Modification of the HMG-I and HMG-Y Proteins. Although mass spectrometric peptide mapping techniques have certain intrinsic limitations that do not always allow for precise identification of the all sites of protein biochemical modification (*36*), it is unambiguously clear from the mass spectra data presented for both the full-length proteins (Figure 8) and their tryptic peptides (Table 2), that the HMG-I and HMG-Y proteins are differentially modified by posttranslational modifications in vivo in the MCF-7 and MCF-7/PKC-α cell lines (summarized in Figure 10). For example, both the HMG-I and the HMG-Y proteins exhibit a higher level of phosphorylation in the highly metastatic MCF-7/PKC-α breast epithelial cell line containing a PKC-α transgene than they do in the nonmetastatic parental MCF-7 cell line (summarized in Table 3). Furthermore, it is also readily apparent that the HMG-Y isoform protein is more heavily modified in both the MCF-7 and the MCF-7/PKC-α cells than is the HMG-I protein in these same cells (Table 3). The major differences between the HMG-I and the HMG-Y proteins are that HMG-Y is both more extensively acetylated and methylated than the HMG-I protein in these cells (Table 3). Nevertheless, precise confirmation of the posttranslational assignments can only be done using tandem mass spectrometric techniques (MS/MS) (*37*, *38*, *62*, *63*).

Differential In Vitro PKC Phosphorylation of the rhHMG-I and rhHMG-Y Proteins. Using MALDI-MS to analyze tryptic digests, rhHMG-I was shown to be phosphorylated in vitro by PKC at eight residues (Ser5, Ser9, Thr21, Ser44, Ser64, Thr75, Thr76, and Thr77), while rhHMG-Y was phosphorylated at seven residues (Ser5, Ser9, Thr21, Ser64, Thr75, Thr76, and Thr77) (summarized in Figure 5). In addition, utilizing nonsaturating PKC enzymatic phosphorylation conditions, the relative affinity of the HMGI(Y) PKC phosphorylation sites were defined (Figure 5). Two of the "high affinity" phosphorylation sites on the rhHMGI(Y) proteins that are efficiently modified by the purified PKC enzyme in vitro (e.g., residues Thr21 and Ser64; Figures 3−5) are also phosphorylated in vivo on the HMG-$I^{7\alpha}$ protein isolated from MCF-7/PKC-α cells that harbor an active PKC-α transgene (*32*). In addition, it is likely that Ser64 is phosphorylated on the HMG-$Y^{7\alpha}$ protein from MCF-7/PKC-α cells as a diphosphopeptide tryptic fragment corresponding to residues 27−65 (Obs. $MH^+ = 3178.3$) was observed (see Figure 10). These results provide strong support for PKC being the modifying enzyme that phosphorylates the Thr21 and Ser64 residues on the HMGI(Y) proteins in vivo (compare Figure 5 to Figure 10).

A recent study by Xiao et al., using various PKC isoforms (α, *β*, *γ*, *δ*, *ϵ*, and *ζ*) to in vitro phosphorylate HMG-I, supports the findings presented in this work (*64*). Using Edman degradation, this group demonstrated that Ser44 and Ser64 are high affinity PKC sites and that Thr75 through Thr78 are also phosphorylated to a lesser extent relative to Ser44 and Ser64 (*64*). Our results are consistent with this previous work but also define other sites of phosphorylation on the rhHMG-I and rhHMG-Y proteins by the PKC-α isoform in vitro (Figure 5).

The MALDI-MS spectra observed for the PKC phosphorylated full-length rhHMG-I and rhHMG-Y proteins (Figure 3) contain multiple mass peaks that suggest that the substrates are not completely saturated by PKC in vitro, even under our saturating reaction conditions (PKC phosphorylation reaction 1). However, we believe that it is not possible to completely saturate the HMGI(Y) proteins by PKC because of the proximity of residues Thr75, 76, and 77. For example, note that Thr75 is centered in the predicted PKC consensus recognition sequence for Thr76 and Thr77 (N-$R_{73}K_{74}\mathbf{T_{75}T_{76}T_{77}}$-$T_{78}P_{79}$-C); (see Figure 2B for consensus PKC phosphorylation sites) therefore, the initial phosphorylation of Thr75 likely blocks subsequent phosphorylation of Thr76 and Thr77 through steric hindrance in the PKC active site. On the other hand, a free PKC consensus residue, N-terminal to a previously phosphorylated residue, could be available for phosphorylation and would suggest that all residues Thr75, 76, and 77 can be phosphorylated if done so in the proper order. Therefore, mono-, di-, and triphosphoprotein species exist in the reaction population as evidenced by the multiple phosphoprotein peaks in Figure 3. Nevertheless, despite incomplete saturation of the substrates, all possible sites of phosphorylation by PKC were defined in vitro (Figure 5)

Spacing is the Key. The present work suggests that the splice site peptide that distinguishes the HMG-I protein from HMG-Y plays a much more important role in differentiating these two proteins in vivo than previously thought. The only difference between the HMG-I and HMG-Y proteins is the absence of the "Y-splice site" amino acids in the HMG-Y isoform (*3*). Removal of the 11−12 amino acid peptide segment in HMG-Y changes the spatial relationship of the A·T-hook DNA binding motifs by shortening the region separating A·T-hooks I and II. Thus, similar modifications may affect substrate interactions of the two proteins differently based on their spatial differences. In addition, removal of the splice peptide from the HMG-Y protein not only removes known sites for posttranslational modifications (e.g., as seen with PKC phosphorylation in vitro, Figure 5A) from this isoform but also creates new peptide sequences at the splice site junction that could potentially be recognized by other modifying enzymes in vivo. Therefore, unique sites of modification may be present that serve to differentiate HMG-Y from HMG-I, in vivo. For example, for HMG-$Y^{7\alpha}$, there are two phosphate groups that are near the splice site junction (see Figure 10) and likely represent the creation of new protein kinase consensus sequences that are different from HMG-I.

Significance of Findings. The present findings are important for a number of reasons. The protein kinase C enzymes are known to play a critical role in cell proliferation, differentiation, neoplastic transformation, and apoptosis (*31*, *65*). Here we demonstrate that, contrary to a previous report (*61*), the mammalian HMGI(Y) proteins are efficient in vitro substrates for phosphorylation by the PKC enzymes and, additionally, provide compelling evidence that these proteins are also in vivo substrates for this group of enzymes. The fact that both the HMG-I and the HMG-Y proteins are more highly phosphorylated in the tumorigenic MCF-7/PKC-α cells than they are in the nonmalignant parental MCF-7 cells (Table 3) provides compelling, but still indirect, support for the hypothesis that the mechanism of malignant transformation by the PKC oncogene involves in vivo phosphorylation

of specific substrates. The present work also demonstrates that, in addition to phosphorylation, both the HMG-I and the HMG-Y proteins are subject to multiple other posttranslational modifications in vivo, with the HMG-Y isoform being much more highly modified than the HMG-I isoform in both preneoplastic and overtly malignant cells. These observations suggest that the HMG-I and HMG-Y proteins may participate in different cellular processes based on their unique constellations of in vivo posttranslational modifications, perhaps being mediated by their differing abilities to bind to nuclear DNA and nucleosomal substrates (Figure 9, panels C−E). And, finally, the very rapid phosphorylation of the HMGI(Y) proteins in cells treated with phorbol esters (Figure 1) indicates that these nuclear proteins are direct downstream nuclear targets of the PKC enzyme in vivo and suggests that activation of the PKC signal transduction pathway may play a significant role in regulating the functional activity of the HMGI(Y) proteins in normal cells in vivo.

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