Phosphorylation and subcellular redistribution of high mobility group proteins 14 and 17, analyzed by mass spectrometry

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Abstract

High mobility group (HMG) proteins 14 and 17 are nonhistone nuclear proteins that have been implicated in control of transcription and chromatin structure. To examine the posttranslational modifications of HMG-14 and -17 *in vivo*, HMG proteins were prepared from nuclear vs. cytosolic fractions of human K562 cells treated with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) or okadaic acid (OA) and examined by electrospray mass spectrometry. Analysis of full-length masses demonstrated mono-, di-, and triphosphorylation of HMG-14 and mono- and diphosphorylation of HMG-17 from OA treated cells, whereas HMG-14 and -17 from TPA treated cells were monophosphorylated. Peptide mass and sequence analysis showed major and minor phosphorylation sites, respectively, at Ser24 and Ser28 in HMG-17, and Ser20 and Ser24 in HMG-14. These sites were found in the consensus sequence RRSARLSAK, within the nucleosomal binding domain of each protein. A third phosphorylation site in HMG-14 was located at either Ser6 or Ser7. Interestingly, the proportion of HMG-14 and -17 found in cytosolic pools increased significantly after 1 h of treatment compared to control cells and showed preferential phosphorylation compared with proteins from nuclear fractions. These results suggest that phosphorylation of HMG-14 and -17 interferes with nuclear localization mechanisms in a manner favoring release from nuclei.

Keywords: high mobility group protein 14; high mobility group protein 17; mass spectrometry; phosphorylation; translocation

High mobility group (HMG) proteins are evolutionarily conserved, abundant nonhistone nuclear proteins in eukaryotes (reviewed by Bustin et al., 1990; Bustin & Reeves, 1996). As a group, these proteins have the characteristics of being small (<30 kDa) and acid soluble under conditions where most proteins are precipitated. HMG-14/17 are representative of one family within this group (Bustin et al., 1995). Two molecules of HMG-14 or -17 bind to two sites on the nucleosome core particle (Mardian et al., 1980; Sandeen et al., 1980; Alfonso et al., 1994; Postnikov et al., 1995), through an evolutionarily conserved basic region called the nu-

cleosomal binding site (Crippa et al., 1992). They have been proposed to inhibit DNA unraveling by bridging entry and exit DNA on the surface of nucleosomal cores (Bustin et al., 1995). They are also found associated with genes being actively transcribed, and their incorporation into chromatin increases transcription *in vitro* (Weisbrod et al., 1980; Einck & Bustin, 1983; Dorbic & Wittig, 1987). Thus, HMG-14 and -17 may also serve as architectural or remodeling proteins that enhance transcription or replication by opening nucleosome/DNA complexes, and facilitating interactions between chromatin templates and components of transcription or replication machineries (Landsman & Bustin, 1991; Ding et al., 1994, 1997; Tremethick, 1994; Paranjape et al., 1995; Trieschmann et al., 1995; Vestner et al., 1998).

HMG-14 and -17 are subject to post-translational modifications that could conceivably alter the interaction of HMG proteins with the nucleosomal core DNA. In particular, phosphorylation of HMG-14 and -17 lowers DNA binding affinity *in vitro* (Palvimo & Maenpaa, 1988; Lund & Berg, 1991; Spaulding et al., 1991). Other studies have shown that HMG-14 and -17 are differentially phosphorylated during the cell cycle (Bhorjee, 1981; Lund et al., 1987).

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Abbreviations: CID, collision-induced dissociation; HMG, high mobility group; HPLC, high-pressure liquid chromatography; LC/MS, reversed-phase HPLC coupled with electrospray ionization mass spectrometry; MS/MS, tandem mass spectrometry; NLS, nuclear localization sequence; OA, okadaic acid; PCA, perchloric acid; TFA, trifluoroacetic acid; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

and are also phosphorylated in log phase cells, suggesting that phosphorylation is also growth associated (Saffer & Glazer, 1982). Previous studies have so far identified Ser6 and Ser19 as *in vivo* sites of phosphorylation on mouse HMG-14 (Walton et al., 1984; Cooper & Spaulding, 1985; Spaulding et al., 1991; Barratt et al., 1994), which correspond to Ser6 and Ser20 in human HMG-14. *In vivo* sites on HMG-17 have not been reported, although Ser24 is an *in vitro* site for Ca^{+2} -calmodulin dependent protein kinases (Ramachandran et al., 1984).

In this study, mass spectrometric analysis was conducted on HMG-14 and -17 isolated from okadaic acid (OA) and 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-treated cells to investigate the cellular distribution of HMG 14/17 and the role of phosphorylation in the distribution. Novel sites of phosphorylation in addition to sites previously identified were found within the consensus nucleosomal binding regions of both proteins. Importantly, HMG-14 and -17 were modified by multiple phosphorylation only in cytosolic fractions of OA- and TPA-treated cells. Thus, our results suggest a novel function for HMG 14/17 phosphorylation, in regulating the cellular distributions of these proteins in response to cell signaling.

Results

Post-translational modifications on HMG-14 and -17 by full-length mass analysis

HMG proteins were purified from K562 cells treated for 1 h with 1 μM OA or 100 nM TPA, and analyzed by LC/MS. HMG-14 and -17 eluted at 8 and 10% acetonitrile, respectively, among other PCA soluble proteins (Fig. 1A). Each protein was observed as a co-eluting series of charge forms with varying m/z values, which were summed over several scans to produce ion spectra, illustrated by MH_8^{+8} to MH_{16}^{+16} for HMG-17 (Fig. 1B). Average masses of HMG-17 and -14 isolated from untreated cells, calculated from the charge forms with greatest intensity, were $9,262.0 \pm 0.7$ Da and $10,527.8 \pm 0.7$ Da, respectively, which agree with calculated masses from translated cDNA sequences (9,261.5 Da, human HMG-17; 10,527.7 Da, human HMG-14; Landsman et al., 1986a, 1986b).

To determine the relative amount of each form, scans containing HMG-14 or -17 were summed and deconvoluted to show the protein masses (Fig. 2A,B). The areas under peaks provide semi-quantitative estimates of different forms of each protein, assuming

Fig. 1. LC/MS analysis of HMG-14 and -17 proteins. HMG proteins were prepared from nuclear fractions of untreated K562 cells and analyzed by LC/MS, as described in Materials and methods. **A:** Mass spectral data were collected as proteins eluted from a capillary POROS R120 HPLC column and converted digitally to gray scale. The m/z values are plotted against elution time, measured by mass spectral scan number and minutes. Each set of discrete bands eluting together represents an ion series of a single protein. Arrows indicate mass spectral data for HMG-17 and -14 proteins. **B:** Mass spectrum of unmodified HMG-17 protein from nuclear fractions of untreated cells, derived by summing scans 140–150 from Figure 1A. Peaks represent individual charged forms. Similar results were obtained for HMG-14, by summing scans 158–163 (not shown). **C:** Mass spectrum of unmodified and modified HMG-17 prepared from the cytosolic fraction of OA-treated cells. Inset shows an expansion of scans 800–900, indicating discrete ions with m/z values corresponding to 0P, 1P, and 2P.

Fig. 2. Effects of OA and TPA treatment on HMG-17 and -14. K562 cells were treated with 1 μ M okadaic acid (OA) or 100 nM TPA for 1 h, and HMG proteins were prepared from nuclear and cytosolic fractions, as described in Materials and methods. **A:** Deconvoluted mass spectra derived from corresponding m/z series are shown for HMG-17 from nuclear and cytosolic fractions of control (CON), OA, or TPA-treated cells. The average molecular mass is indicated for peaks of unmodified and modified proteins. **B:** The deconvoluted mass spectra of HMG-14 from nuclear or cytosolic fractions of control (CON), OA, or TPA-treated cells.

equivalent ionization efficiency and HPLC recovery in each case. Minor peaks with masses of $9,341.3 \pm 1.9$ Da and $10,608.0 \pm 1.3$ Da, respectively, for HMG-17 and -14, reflecting mass increments of 80 Da were observed in the cytosolic fractions of control cells (Fig. 2A,B; CON cytosolic). Thus, under unstimulated conditions, HMG-14 and -17 are largely unmodified, with low-level phosphorylation.

HMG-17 and -14 from cells treated with OA or TPA showed significant increases in phosphorylation. For example, Figure 1C shows the charge envelope of HMG-17 from the cytosolic fraction of OA-treated cells, which, when deconvoluted, revealed increases in peaks with masses of 9,341.3 and 9,421.5 Da (Fig. 2A; cytosolic OA), indicating monophosphorylation and diphosphorylation of this protein. In response to TPA, a monophosphorylated form was apparent (Fig. 2A; cytosolic TPA). Similarly, HMG-14 from cytosolic fractions of OA-treated cells showed protein mass increases of 80, 160, and 240 Da (10,608, 10,688, and 10,768 Da; Fig. 2B; cytosolic OA), which correlate to mono-, di-, and triphosphorylated protein. Following TPA treatment, mono- and diphosphorylated forms were observed for HMG-14 (Fig. 2B; cytosolic TPA).

HMG-14 and -17 in nuclear vs. cytosolic pools

Interestingly, subcellular fractionation of extracts prior to HMG purification showed that the phosphorylated forms were recovered predominantly in cytosolic fractions of OA- or TPA-treated cells (Fig. 2A,B). Masses of HMG-14 and -17 in nuclear fractions were identical to controls, indicating a strong bias against post-translational modification in nuclear fractions.

To quantify the enhanced phosphorylation of proteins in the cytoplasmic fractions, three separate experiments involving different sample preparations and LC/MS analyses were performed, and the areas under each peak following deconvolution of spectra were measured and averaged. Figure 3 shows the results of this analysis, reporting each form of HMG-14 or -17 as a percentage of the total in cytosolic + nuclear fractions. In untreated cells, cytosolic fractions contained 13.9% of total HMG-17, which included $13.1 \pm 2.1\%$ unphosphorylated and $0.8 \pm 0.5\%$ monophosphorylated forms (Fig. 3A,B, left panels). OA treatment elevated the cytosolic amount of monophosphorylated HMG-17 to $18.5 \pm 2.3\%$ and diphosphorylated protein to $3.0 \pm 0.8\%$ (Fig. 3A, right panel). Thus, the HMG-17 in OA-treated cytosolic fractions amounted to 34% of total protein, increasing by 2.4-fold compared to controls. TPA treatment resulted in an increase of monophosphorylated HMG-17 in cytosolic fractions to $10.2 \pm 1\%$, and decrease in unphosphorylated form from $12.2 \pm 2\%$ to $7.4 \pm 0.2\%$ (Fig. 3B), so that overall, HMG-17 in the cytosolic fractions increased by 5% in TPA-treated cells compared to controls. In contrast, phosphorylated HMG-17 was negligible in nuclear fractions of control or TPA-treated cells and was no greater than $1.7 \pm 2\%$ in nuclear fractions of OA-treated cells. Thus, HMG-17 protein increased in cytosolic fractions, in a manner that correlated with OA- or TPA-stimulated phosphorylation.

Similar effects were observed with HMG-14. In cytosolic fractions of control cells, the monophosphorylated form of this protein was $1.0 \pm 0.9\%$ of the total protein (Fig. 3C,D, left panels). In response to OA, mono-, di-, and triphosphorylated forms of HMG-14 increased in the cytosolic fraction to $12.6 \pm 2.0\%$, $23.0 \pm 7.6\%$,

Fig. 3. Quantitation of HMG-17 and -14 proteins. Deconvoluted mass spectral peaks of unphosphorylated vs. phosphorylated proteins were integrated and averaged over three separate experiments, shown with standard deviations. **A,B:** HMG-17 prepared from nuclear and cytosolic fractions of (A) OA- or (B) TPA-treated cells. **C,D:** HMG-14 from (C) OA- or (D) TPA-treated cells. The sum of intensities for all forms of each protein was unchanged between samples (data not shown).

and $3.8 \pm 1.4\%$ of the total protein (Fig. 3C). Monophosphorylated HMG-14 increased somewhat in nuclear fractions (from $3.4 \pm 2.7\%$ in controls, to $18.0 \pm 7.8\%$ in OA-treated cells). However, the effect of OA on the cytoplasmic pool was greater, with the amount of HMG-14 in the cytoplasmic pool increasing from 8 to 42%, corresponding to a fivefold increase in response to OA. In TPA-treated cells, enhanced mono- and diphosphorylated HMG-14 ($8.7 \pm 0.9\%$ and $1.8 \pm 0.3\%$, respectively) and decreased unphosphorylated protein ($7.0 \pm 5.0\%$ in controls to $3.4 \pm 1.6\%$ in TPA-treated) were observed in cytosolic fractions (Fig. 3D). No significant changes in monophosphorylated HMG-14 were observed in nuclear fractions of control vs. TPA-treated cells (3–4% monophosphorylated; Fig. 3D). Overall, the amount of HMG-14 in cytosolic fractions increased from 7 to 14% in response to TPA. As with HMG-17, shifts in HMG-14 localization to cytoplasmic pools correlated with phosphorylation of protein.

Negative controls for this behavior were provided by other HMG proteins. In particular, proteins with masses of $10,751.2 \pm 2.7$ and $11,746.8 \pm 1.5$ Da were observed by LC/MS of PCA soluble proteins (data not shown), respectively, eluting 0.6 and 3.3 min following the elution of HMG-14. Previous work has shown that mouse HMG-Y is acetylated at Ser2 following removal of Met1 and exists in a diphosphorylated form (Ferranti et al., 1992). Thus, the protein with observed mass of 10,751 Da was tentatively assigned to human HMG-Y based on its calculated mass of 10,749.7 Da for a demethionylated, N-acetylated, and diphosphorylated form. The protein with observed mass 11,747 Da was tentatively assigned to HMG-I, based on its calculated mass of 11,746.8 for a form with the same modifications. Although these assignments were not confirmed by sequencing, the delayed elution of these proteins established their dissimilarity from HMG-17 or -14. Like HMG-14/17, phosphorylation of HMG-I/Y in response to OA or TPA treatment was evident by mass increases of 80, 160, and 240 Da. Phosphorylation of HMG-I/Y has been characterized by oth-

ers (Lund et al., 1987; Ferranti et al., 1992; Siino et al., 1995; Schwanbeck & Wisniewski, 1997); therefore, these proteins were not studied further. However, we observed that levels of HMG-I/Y in nuclear vs. cytosolic pools were unchanged compared to control cells (control vs. OA-treated cells: 94 vs. 94% nuclear for HMG-I; 97 vs. 96% nuclear for HMG-Y). This argues that the cytosolic enrichment of HMG-14 and -17 in response to cell stimulation was not caused by breakdown of the nuclear envelope and is more likely due to subcellular relocalization.

Identification and quantitation of phosphorylated peptides

To verify protein assignments and locate phosphorylation sites, HMG-17 and -14 from nuclear or cytosolic fractions of control vs. treated cells were partially purified by HPLC, digested with endoproteinase Lys-C or Asp-N, and analyzed by LC/MS. Observed peptide masses were compared with those predicted from HMG-17 and -14, and the peptides recovered are indicated in Figure 4. Assignment of the 9,262 Da protein as HMG-17 was confirmed based on three major peptides from Lys-C digestion (K7, K8/9, and K20) and three major Asp-N peptides (D1, D3, and D4; Table 1), each of which appeared as more than one charge species. Similarly, the assignment of HMG-14 was verified by identification of four Lys-C peptides (K5/6, K18, K19, and K20) and three large Asp-N peptides (D1, D3, and D4; Table 2), which also appeared with confirming charge species. Smaller, more hydrophilic peptides were not observed, as expected from the nature of the C18 resin used for analysis. The observed sequence coverage from Lys-C and Asp-N digestions was 66% for HMG-17 and 91% for HMG-14 (Fig. 4A,B), and was the same in preparations from control vs. treated cells. All phosphorylatable residues were included among the peptides from HMG-17, and all but three serines (Ser44, Ser45, Ser98) were included among the peptides from HMG-14.

Fig. 4. Summary of peptide coverages for HMG-17 and -14. Amino acid sequences indicating the peptide coverages for endoproteinase Lys-C and Asp-N peptides for (A) HMG-17 and (B) HMG-14. Serine phosphorylation sites, Ser24 and Ser28 in HMG-17, and Ser20 and Ser24 in HMG-14, are indicated by outlined letters.

Peptides identified for the phosphorylated HMG-17 and -14 in cytosolic preparations of OA- and TPA-treated cells were the same, except that peptides with masses 80 or 160 Da larger than predicted were observed. For HMG-17 from cytosolic fractions of OA-treated cells, digests contained mono- and di-phosphorylated forms of Lys-C peptide K7, which contains two phosphorylatable residues, Ser24 and Ser28 (residues 18–30; predicted 0P mass = 1,513.6 Da, observed 1P and 2P masses = 1,593.5 and 1,673.4 Da). This was confirmed by the appearance of mono- and di-phosphorylated Asp-N peptide D4, which also contains Ser24 and Ser28

(predicted 0P mass = 4,513.4 Da, observed 1P, 2P masses = 4,593.7, 4,672.9 Da). The only other phosphorylatable residue in HMG-17 is Thr76, which is present in peptide D5/6. Phosphorylated forms of D5/6 were not observed, indicating that Ser24 and Ser28 are the primary sites of phosphorylation in OA-treated cells. Similarly, in HMG-17 preparations from cytosolic fractions of TPA-treated cells, K7 and D4 were mostly monophosphorylated and diphosphorylated to a low amount, and D5/6 was unphosphorylated. This indicates that TPA and OA treatment lead to phosphorylation of the same sites, accounting for the phosphorylation observed in the intact protein (Fig. 2A).

Digests of HMG-14 from cytosolic fractions of OA-treated cells contained mono- and diphosphorylated forms of Lys-C peptide K5/6 (predicted 0P mass = 1,436.9 Da, observed 1P and 2P masses = 1,517.6 and 1,598.0 Da). This peptide corresponds to residues 18–29, containing two phosphorylatable residues, Ser20 and Ser24 (Fig. 4B). Interestingly, K5/6 in HMG-14 contains a sequence identical to the site of phosphorylation in K7 of HMG-17 (RRSARLSAK), located within the N-terminal region of each protein. An overlapping Asp-N peptide D1 was found in mono-, di-, and triphosphorylated forms (predicted 0P mass = 4,354.1 Da; observed 1P, 2P, 3P masses = 4,433.9, 4,514.3 Da, 4,594.3 Da). This peptide contains Ser6 and Ser7, in addition to Ser20 and Ser24 (Fig. 4B). Therefore, based on the observed stoichiometry of D1, the most likely site for the third phosphorylation is Ser6 or Ser7. The mono-, di-, and triphosphorylated peptides accounted for all the phosphate observed in the protein (see below); therefore, serines 44, 45, and 98, which were not observable in the analysis of HMG-14, are probably not phosphorylated. Only monophosphorylated forms of K5/6 or D1 were observed in TPA-treated cells, as well as in nuclear fractions of cells treated with either stimulant.

The distributions of phosphorylated forms of K7 and D4 in HMG-17 and K5/6 and D1 in HMG-14 were next examined in OA- or TPA-treated cells. Although peptides are subject to variability in ionization, relative yields can often be estimated when their chemical structures are nearly identical, as when comparing modified vs. unmodified forms (Resing et al., 1995a, 1995b). Figure 5 shows the relative recovery of peptides, determined by in-

Table 1. Peptide ions observed by LC/MS in HMG-17 digests^a

Peptide name ^b	Predicted mass (Da) ^c	Observed ions (Da/e) ^d	Observed mass (Da) ^e
K7	1,513.6	757.6(+2); 505.2(+3); 379.4(+4)	1,513.1
K8/9	959.5	960.7(+1); 481.1(+2); 320.9(+3)	960.0
K20	1,214.5	1,216.4(+1); 608.3(+2)	1,215.0
D4	4,513.4	1,129.9(+4); 903.5(+5); 753.1(+6); 646.0(+7); 565.5(+8); 502.6(+9); 452.6(+10)	4,514.3
D5	1,271.5	1,272.7(+1); 636.8(+2)	1,271.7
D5/6	1,687.7	845.0(+2); 563.9(+3)	1,688.4

^aHMG-17 prepared from nuclear fractions of control K562 cells. Smaller hydrophilic peptides were not observed by LC/MS and are not shown.

^bK and D denote peptides from Lys-C and Asp-N digests, respectively. Incomplete products are indicated by adjacent peptides separated by a slash.

^cMass calculated from amino acid sequence of human HMG-17, using monoisotopic masses for peptides less than 1,500 Da and average masses for peptides larger than 1,500 Da.

^dCharges on ions observed by LC/MS are indicated in parentheses.

^eAverage mass of observed ions.

Table 2. Peptide ions observed by LC/MS in HMG-14 digests^a

Peptide name	Predicted mass (Da)	Observed ions (Da/e)	Observed mass (Da)
K5/6	1,436.9	719.8(+2); 480.2(+3); 360.5(+4); 288.5(+5)	1,437.7
K18	1,116.5	1,117.6(+1); 559.5(+2)	1,116.8
K19	1,201.5	1,202.6(+1); 601.9(+2)	1,201.7
K20	1,348.6	1,349.8(+1); 675.5(+2)	1,348.9
D1	4,354.1	871.7(+5); 726.7(+6); 623.2(+7); 545.6(+8); 484.9(+9); 436.6(+10)	4,354.8
D3	2,857.2	1429.7(+2); 953.6(+3); 715.1(+4); 572.9(+5)	2,857.8
D4	1,774.8	1,775.9(+1); 888.7(+2)	1,775.2

^aHMG-14 prepared from nuclear fractions of control K562 cells. Masses and other nomenclature are reported as in Table 1.

tegrating ion currents and summing over all charged forms of each peptide. To test whether results from different samples could be compared, intensities of different forms of each peptide were normalized against the sum of all observable peptides in each LC/MS analysis. For example, various forms of K7 were normalized against the sum of five peptides in Lys-C digests of HMG-17 (0P-K7, 1P-K7, 2P-K7, K8/9, and K20). Likewise, D4 peptides were normalized against the sum of five peptides from Asp-N digestions (0P-D4, 1P-D4, 2P-D4, D5, D5/6). Recoveries of peptides K8/9, K20, D5, and D5/6 showed minimal variation between samples from control vs. treated cells, in nuclear or cytosolic pools (Fig. 5A,B), validating the comparison of K7 or D4 phosphopeptide levels between different samples. The same conclusion was reached with HMG-14, based on the low variation between peptides K18, K19, and K20, and D3 and D4 (Fig. 5C,D).

Levels of monophosphorylated K7 were less than 1% in nuclear fractions of untreated, OA-, or TPA-treated cells. In contrast, cyto-

solic fractions showed increases in monophosphorylated and diphosphorylated peptides, respectively, to 21 and 6% of total peptides in OA-treated cells, and 26 and 1% in TPA-treated cells (Fig. 5A). The increases were similar to those observed with D4, where monophosphorylated D4 was 11% of total in nuclear fractions, but mono- and diphosphorylated forms were, respectively, 46 and 10% of the total in cytosolic fractions of OA-treated cells. In TPA-treated cells monophosphorylated D4 was 4% of total peptides in nuclear fractions, but mono- and diphosphorylated forms were, respectively, 37 and 2% of the total in cytosolic fractions (Fig. 5B). Thus, the phosphopeptide distributions of K7 and D4 agree well with and account for the distribution of cytosolic HMG-17 protein into different phosphorylated forms (Fig. 3A).

Similar conclusions were made for HMG-14. Monophosphorylated K5/6 was absent in untreated cells and increased to 12% of the total in nuclear fractions of OA-treated cells and to 9% in that of TPA-treated cells. However, cytosolic fractions showed

Fig. 5. Peptide quantitation in nuclear vs. cytoplasmic fractions. Mass spectral data were collected during on-line LC/MS analysis of HPLC elutes and were scanned for predicted ion masses of (A,C) endoproteinase Lys-C or (B,D) Asp-N peptides, in (A,B) HMG-17 and (C,D) HMG-14 (see Fig. 4 for peptide nomenclature). Signal intensities for the peptides observed in nuclear (NUC) or cytosolic (CYTO) fractions of control, OA-, or TPA-treated cells were measured and reported as percent of total signal intensities. Non-, mono-, and diphosphorylated peptides are indicated by 0P, 1P, and 2P.

increased mono- and diphosphorylated forms to 27 and 9%, respectively, of total peptides in OA-treated cells, and 29% monophosphorylated K5/6 in TPA-treated cells (Fig. 5C). Similarly, phosphorylated D1 was absent in untreated cells, increasing to 14% monophosphorylated in nuclear fractions of OA-treated cells, whereas mono-, di-, and triphosphorylated D1 in cytosolic fractions increased to 13, 18, and 5%, respectively, of total peptide in OA-treated cells, and monophosphorylated D1 increased to 29% in TPA-treated cells (Fig. 5D). The data support the model for phosphorylation of major sites at Ser20 and Ser24, and a minor site at Ser6 or 7.

Identification of phosphorylation sites by LC/MS/MS

Phosphorylation sites were confirmed by LC/MS/MS sequencing of Lys-C peptides from cytosolic fractions of control, OA-, and TPA-treated cells. MS/MS spectra of the doubly charged K7 ion from HMG-17 showed loss of the two N-terminal residues and little or no cleavage at other sites. MS/MS spectra of the triply charged K7 ion cleaved readily at the same sites, but produced a more complex spectrum, most likely due to the presence of several doubly charged ions and potential rearrangements into ions with masses that could not be simply accounted for from the sequence (Fig. 6A–C). Some cleavage at the C-terminus was observed, yielding y_1 and low intensity y_3 , y_5 , and y_6 ions. An ion with mass 732.5 was observed, which most likely corresponds to the doubly charged dehydrated parent ion; although it is equal to the predicted mass of y_7 , it is also present in spectra of phosphorylated K7, where y_7 should be shifted by +80 Da. Observed fragment ions are summarized above the spectrum.

Only monophosphorylated K7 was sequenced by MS/MS, because the modification sites on diphosphorylated K7 were unambiguous. Despite the complexity of the spectra, the data were sufficient to clearly establish that peptides observed with $M = 1,593.0$ Da were indeed identical to monophosphorylated K7. Furthermore, peptides derived from OA- and TPA-treated cells showed identical spectra (Fig. 6B,C), indicating phosphorylation at identical sites. Although weak, recovery of y_3 (305.5 Da), Δy_4 (400.2 Da), and y_5 (574.0 Da) occur in about the same yield, in spectra of unphosphorylated and phosphorylated forms of K7. Phosphorylation at Ser28 would eliminate this ion; therefore, the monophosphorylated peptide must be primarily modified at Ser24. This is confirmed by an increased yield of y_6 (645.1 Da) and Δy_7 (714.5 Da) in phosphorylated K7, which reflect cleavages next to Ser24. Enhanced cleavages are often observed adjacent to phosphorylated residues, along with loss of H_3PO_4 to produce dehydrated ions (Resing et al., 1995a, 1995b).

In the case of HMG-14, MS/MS spectra of monophosphorylated K5/6 were identical between OA- and TPA-treated cells (Fig. 6D–F, summarized above the spectra). In this case, several b ions were observed corresponding to changes of +80 Da, as well as neutral loss of phosphate (Fig. 6E,F). No fragmentation occurred in the vicinity of Ser20 or Ser24 that allowed distinction of phosphorylation at either site. The y_8 ion (811.2 Da) was observed in all spectra, suggesting that the major phosphorylation site occurs at Ser20, but the behavior of this ion was equivocal, because the chemistry of fragmentation strongly favored cleavage at the C-terminus. In analogy to K7, which contains the identical primary sequence surrounding the sites of phosphorylation, the major phosphorylation site most likely occurs at Ser20.

Discussion

Our study identifies physiological sites for phosphorylation on HMG-14 and -17 using electrospray mass spectrometry. Previous work has shown *in vivo* phosphorylation at Ser6 and Ser19 on mouse HMG-14 (Ser6 and Ser20 in human HMG-14) (Walton et al., 1984; Cooper & Spaulding, 1985; Spaulding et al., 1991; Barratt et al., 1994). In contrast, no *in vivo* sites have been reported to date on HMG-17. Our work confirms previous studies by demonstrating phosphorylation at homologous residues Ser6 and Ser20 on human HMG-14, and further demonstrates an additional phosphorylation site at Ser24. We also report new physiological modifications of HMG-17 at Ser24 and Ser28, which appear in a region of identical sequence to that surrounding Ser20 and Ser 24 in HMG-14 (RRSARLSAK), suggesting coordinate regulation of HMG-14 and -17 phosphorylation. A novel and important finding in our study is that the phosphorylated forms of HMG-14 and -17 are preferentially found in cytosolic pools, suggesting a role for phosphorylation in regulating the subcellular localization of these proteins.

Analysis of full length protein masses demonstrates triphosphorylated and diphosphorylated forms of HMG-14 and -17, respectively, indicating that all sites are significantly occupied in intact cells. The ability to observe phosphorylation at high stoichiometry was facilitated by the suppression of intracellular phosphatase activity with OA. In contrast, TPA treatment resulted in phosphorylation of only two sites in HMG-14 and one site in HMG-17. This indicates that phosphorylation sites in HMG-14 and -17 are subject to rapid turnover, most likely via type 1 and 2 classes of serine/threonine phosphatases.

Protein kinases that phosphorylate HMG-14/17 at the major sites have been implicated from previous *in vitro* studies. Protein kinase C and a similar calcium/phospholipid-dependent kinase have been reported to phosphorylate both proteins *in vitro* (Ramachandran et al., 1984; Palvimo et al., 1987), where the phosphorylation of HMG-17 occurs predominantly at Ser24 and to a lesser degree at Ser28. We, therefore, hypothesize that phosphorylation within the sequence RRSARLSAK in response to TPA occurs via protein kinase C. Although TPA also indirectly stimulates MAP kinases, we have observed no effect of the MKK inhibitor, PD98059, on HMG-14/17 phosphorylation (data not shown). Phosphorylation of HMG-14 at Ser6 by cAMP- or cGMP-dependent kinases has also been reported (Walton et al., 1982; Palvimo et al., 1983). Thus, other kinases may contribute to phosphorylation at Ser6 in response to OA. Ser88 and Ser98 on HMG-14 are also phosphorylated by casein kinase II *in vitro* (Walton & Gill, 1983; Walton et al., 1985). We did not observe phosphorylation at these sites, although residues modified with low stoichiometry may not be detected by our methods.

Normally, HMG-14 and -17 are predominantly nuclear and DNA bound, and our estimates of 15% cytoplasmic distribution for each protein under basal conditions are in reasonable agreement with measurements by others (Kuehl et al., 1984). However, cell stimulation increased cytoplasmic HMG-14 and -17 levels, particularly in response to OA, where redistribution to 42 and 34% cytoplasmic protein was observed, predominantly represented by multiphosphorylated forms. Under our conditions involving only 1 h of treatment, total levels of each protein were unchanged; therefore, it is unlikely that the phosphorylated forms are derived from newly synthesized protein or preferential degradation of unphosphorylated forms. Furthermore, the subcellular distribution of other pre-

Fig. 6. MS/MS of the major phosphorylation sites in HMG-17 and -14. Doubly and triply charged y and b ions, observed with m/z +40 and +20 representing addition of HPO_3 , are indicated by asterisks (*), and neutral loss of H_3PO_4 with m/z -49 and -32.6 are represented by triangles (Δ). For clarity, internal fragment ions are not labeled. HMG-17: MS/MS spectral data were obtained for (A) the unphosphorylated parent ion of K7 from nuclear fractions of control cells ($\text{MH}^{+3} = 505.4$), (B) monophosphorylated K7 from cytosolic fractions of OA-treated cells ($\text{MH}^{+3} = 532.0$), and (C) monophosphorylated K7 from cytosolic fractions of TPA-treated cells ($\text{MH}^{+3} = 531.8$). Dehydrated and neutral loss of parents are represented by $\text{MH}^{+3} = 499.7$ in (A), and $\text{MH}^{+3} = 499.3$ in (B,C). The unlabeled arrow in (A) indicates a weak y_6 ion that was enhanced in fragmentations of the phosphorylated peptide in (B) and (C). HMG-14: MS/MS spectral data were obtained for unphosphorylated K5/6 from (D) control cells ($\text{MH}^{+3} = 479.9$) and for monophosphorylated K5/6 from (E) OA ($\text{MH}^{+3} = 506.6$) and (F) TPA-treated cells ($\text{MH}^{+3} = 506.6$). Dehydrated or neutral loss of parent ions are indicated by (A) $\text{MH}^{+3} = 474.1$ and (B,C) $\text{MH}^{+3} = 474.1$. In OA and TPA spectra (E,F), doubly and triply charged ions of phosphorylated b_{12} were observed with addition of water ($*b_{12} + 18$) $^{+2}$, ($*b_{12} + 18$) $^{+3}$. The arrow in E and F indicate a weak ion of unphosphorylated y_8 , suggesting phosphorylation at Ser20. Sequence results of K7 and K5/6 are displayed above their respective spectra. Singly and doubly charged b ions are displayed above the sequence ($\square$), and singly, doubly and triply charged y ions are below ($\square$). Stippled lines indicate weak ions. Symbols indicate ions shifted in m/z from that predicted by the amino acid sequence by +80, -18, or +62, representing addition of one phosphate ("P"), neutral loss of one H_3PO_4 (" Δ "), or addition of one phosphate plus neutral loss of one H_3PO_4 (" ΔP "), respectively. Phosphorylated residues deduced from sequencing results are indicated by asterisks.

dominantly nuclear proteins such as HMG-I/Y were unchanged, eliminating the possibility that the redistribution is an artefact of nuclear envelope breakdown. Thus, we conclude that the correlation we observe reflects a causal relationship, in which phosphorylation somehow facilitates the redistribution of HMG-14 and -17 toward non-nuclear pools.

Two mechanisms for this process can be envisioned, based on the known behavior of HMG-14/17. One model involves phosphorylation of these proteins in the nucleus, which leads to disruption of chromatin interaction, enabling net export and cytoplasmic translocation, or leakage from nuclei following cell disruption. Consistent with this, the major phosphorylation sequence, RRSARLSAK,

is found within a highly conserved nucleosomal binding domain (residues 17–47; Bustin & Reeves, 1996), and phosphorylation of HMG-14 and -17 has been shown to lower binding affinity to DNA (Palvimo & Maenpää, 1988; Lund & Berg, 1991; Spaulding et al., 1991). Alternatively, phosphorylation of nonchromatin bound HMG-14 or -17 may promote interactions with cytoplasmic anchoring proteins. A second model involves rapid nuclear import and export of HMG proteins, and modulation of binding to importins or exportins by phosphorylation. Recent work has demonstrated nuclear uptake of both HMG-14 and -17 through active nuclear import processes (Hock et al., 1998). This, in part, involves a possible bipartite nuclear localization sequence (NLS) within conserved residues 1–4 and 53–56 (HMG-17) or 52–55 (HMG-14). Conceivably, phosphorylation within RRSARLSAK may facilitate cytoplasmic localization by disrupting NLS interactions with importin- α , or by enhancing interactions with exportins. Further work is needed to investigate and extend these intriguing possibilities.

Materials and methods

Cell culture

Human erythroleukemia K562 cells (American Type Culture Collection) were maintained in spinner cultures at a density of 1×10^6 cells/mL in RPMI medium supplemented with 10% fetal bovine serum (Gibco/BRL, Gaithersburg, Maryland) and 100 μ g/mL streptomycin and penicillin. Prior to treatment, cells were passed into fresh media at a density of $5\text{--}8 \times 10^5$ cells/mL, transferred to 15 cm² plates (50 mL), and incubated at 37 °C for 4 to 6 h. Cells were then treated with 1 μ M OA (LC Laboratories, Woburn, Massachusetts) or 100 nM TPA (LC Laboratories) for 1 h [0.1% (v/v) of stock solution in dimethyl sulfoxide or ethanol, respectively]. Control cells were treated with either dimethyl sulfoxide or ethanol.

Preparation of HMG proteins

The following procedures were modified from Barratt et al. (1994) and were performed at 4 °C unless otherwise stated. After treatment, cells were harvested by centrifugation ($2,000 \times g$, 5 min), and washed once with 30 mL Hank's buffered saline solution. Cell pellets were resuspended in 1 mL lysis buffer (10 mM HEPES, pH 7.6, 0.25 M sucrose, 15 mM MgCl₂, 10 mM KCl, 1 mM benzamidine, 20 μ g/mL leupeptin, 20 μ g/mL aprotinin, 5 μ g/mL pepstatin, 100 μ M sodium orthovanadate, 1 mM dithiothreitol, 0.5 μ M okadaic acid) and 0.2% Triton X-100. Nuclei were separated from cytosolic fractions by centrifugation ($3,000 \times g$, 3 min), and the cytosolic supernatant was set aside for perchloric acid (PCA) precipitation. Pellets were washed with 1 mL lysis buffer and centrifuged at $3,000 \times g$, 3 min, then resuspended with 100 μ L lysis buffer, layered onto a 1 mL sucrose pad (1 M sucrose in lysis buffer), and centrifuged again. Crude purified nuclei pellets were resuspended in 100 μ L lysis buffer for PCA precipitation. Proteins of nuclear or cytosolic fractions were precipitated with 5% PCA (v/v) on ice for 5 min, followed by centrifugation ($3,000 \times g$, 3 min). The supernatants containing PCA-soluble proteins were acetone precipitated overnight at -20 °C. Final acetone precipitated pellets containing HMG proteins were stored at -80 °C until use.

Prior to MS analyses, protein pellets of nuclear fractions were resuspended in 200 μ L water, and pellets of cytosolic fractions in

100 μ L of 9 M urea, 0.1 M Tris-HCl, pH 8, followed by 100 μ L water. Samples were acidified to a final concentration of 1% (v/v) formic acid before HPLC in purifying individual proteins or in analyzing their masses directly by HPLC-coupled mass spectrometry (LC/MS).

Proteolysis of HMG proteins for mass spectral analysis

HMG proteins were fractionated on a POROS R120 PEEK reversed-phase column (PerSeptive Systems, Framingham, Massachusetts, 2.1 mm $\times$ 3 cm, run on an Applied Biosystems Inc., Foster City, California, HPLC, Model 140B). The column was equilibrated in 0.1% trifluoroacetic acid (TFA) in water, and acidified proteins were eluted at 200 μ L/min with a gradient of 0.6% acetonitrile/min in 0–50% acetonitrile and 0.08% TFA. Protein elution was monitored at 215 nm by ultraviolet absorbance detection (Applied Biosystems Inc. Model 685A detector, 2.4 μ L flow cell), and fractions were collected manually. Acetonitrile/TFA in the fractions was removed by lyophilization, and proteolysis was carried out after solubilization with 10 μ L 8 M urea and 90 μ L of 0.1 M Tris-HCl pH 8, by two consecutive additions of 5% (w/w) endoproteinase Lys-C (Wako Bioproducts, Richmond, Virginia) each reacted for 3 h at 30 °C, or by a 5% (w/w) endoproteinase Asp-N (Boehringer Mannheim, Mannheim, Germany) reaction for 3 h at 37 °C.

Mass spectral analyses

Mass determination of proteins was performed by MS as described (Louie et al., 1996) using a Perkin-Elmer Sciex (Foster City, California) API-III triple quadrupole mass spectrometer equipped with a nebulization-assisted electrospray source and a high-pressure collision cell. Acetone-precipitated PCA-soluble proteins from nuclear or cytosolic fractions were solubilized with water or urea/Tris-HCl as described above, acidified to 1% formic acid, and loaded on fused silica capillary columns (500 μ m $\times$ 20 cm), which were packed with POROS R120 resin (PerSeptive Systems) and equilibrated with 0.1% formic acid in water. Proteins were eluted at 20 μ L/min with a gradient of 0.6% acetonitrile/min in 0–50% acetonitrile and 0.1% formic acid. Peptides generated by Lys-C or Asp-N proteolysis were analyzed in a similar manner except that Columbus C18 resin (Phenomenex, Torrance, California) was used and the gradient was run at 1% acetonitrile/min.

Mass spectra for proteins were obtained by scanning from 350 to 1,800 Da/e with 0.13 Da/e step size and 0.5 ms dwell time. The ionspray needle was set at a potential of 4.8 kV with a nebulizing air flow rate of 0.6 L/min and an orifice voltage of 75 V. Conditions for peptide analysis were identical except that spectra were obtained by scanning from 200 to 1,800 Da/e with 0.15 Da/e step size, and the ionspray needle and orifice voltage were decreased to 4.6 kV and 65 V, respectively. Peptides were quantified by integrating ion currents and summing over charged species.

Peptide sequencing was performed by HPLC elution coupled to tandem mass spectrometry (LC/MS/MS) as described (Louie et al., 1996). Individual ions were selected in the first quadrupole, then accelerated to a kinetic energy of 15 eV and collisionally activated with argon at a thickness of $2.5\text{--}3.2 \times 10^{14}$ atoms/cm³ in the high-pressure collision cell of the second quadrupole. MS/MS data were collected in the third quadrupole by scanning from 50 to 1,600 Da/e with 0.15 Da/e step/size and 1.0 ms dwell time. LC/MS and LC/MS/MS data were analyzed using software pro-

vided by Perkin-Elmer Sciex. Figures were prepared by importing data into Sigmaplot (Jandel Scientific, Austin, Texas), Photoshop (Adobe, San Jose, California), or Canvas (Deneba, South Miami, Florida).

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