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During Apoptosis of Tumor Cells HMGA1a Protein Undergoes Methylation: Identification of the Modification Site by Mass Spectrometry†

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ABSTRACT: Programmed cell death is characterized by posttranslational modifications of a limited and specific set of nuclear proteins. We demonstrate that during apoptosis of different types of tumor cells there is a monomethylation of the nuclear protein HMGA1a that is associated to its previously described hyperphosphorylation/dephosphorylation process. HMGA1a methylation is strictly related to the execution of programmed cell death and is a massive event that involves large amounts of the protein. In some tumor cells, HMGA1a protein is already methylated to an extent that depends on cell type. The degree of methylation in any case definitely increases during apoptosis. In the studied cell systems (human leukaemia, human prostate tumor, and rat thyroid transformed cells) among the low-molecular-mass HMG proteins, only HMGA1a was found to be methylated. A tryptic digestion map of HPLC-purified HMGA1a protein showed that methylation occurs at arginine 25 in the consensus $G_{24}R_{25}G_{26}$ that belongs to one of the DNA-binding AT-hooks of the protein. An increase of HMGA1a methylation could be related to heterochromatin and chromatin remodeling of apoptotic cells.

Once the first versions of the human genome sequence were obtained, functional genomics constituted a subsequent logical development, linking the products of gene expression (i.e., proteins) with their functional roles. Proteomics deals with both the cellular change of expression patterns in different circumstances or under different stimuli and the definition of protein interactions or complexes able to perform a specific function. Posttranslational modifications of proteins are strongly involved in the latter aspect since they not only modulate secondary and tertiary protein structure but also protein−protein interactions in multiproteic complexes. Indeed, posttranslational modifications increase the number of functional proteins as compared to the number of corresponding genes. Phosphorylation is probably the most frequent protein modification, but other modifications, such as acetylation and glycosylation, also strongly participate in the modulation of protein function (*1*).

Here we report a study concerning the methylation of HMGA1a, a protein that belongs to the HMGA[1] family of nuclear proteins (*2*−*4*). This family has three members, HMGA1a, HMGA1b (deriving from the same gene by alternative splicing), and HMGA2 (deriving from a different gene), previously known as HMGI, HMGY, and HMGI-C, respectively (*5*). They are small and abundant nuclear polypeptides of about 11 kDa that bind to DNA through highly cationic regions called AT-hooks since they bind at the minor groove of AT-rich DNA stretches (*6*, *7*). Each of the HMGA proteins contains three AT-hooks that, however, do not interact with DNA with the same affinity, the second AT-hook (located approximately in the middle of the protein molecule) being the main interacting region (*8*). Another peculiarity of HMGA proteins is a stretch of about 20 amino acid residues at the C-terminus, which has a highly negative charge because of the presence of several glutamic acid residues (*2*). Posttranslational modifications of HMGA proteins such as phosphorylation and acetylation have been widely reported and related to cellular events such as cell cycle or assembly of multiproteic complexes involved in gene expression (*9*−*12*).

In the oncologic field, a special interest is devoted to HMGA proteins since there is evidence that neoplastic transformation is related both to their overexpression in neoplastic cell lines or tumors and to recurrent chromosomal

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[1] Abbreviations: HMGA, high mobility group A; HMGA1a, high mobility group A1a (previously named HMGI); HMGA1b, high mobility group A1b (previously named HMGY); HMGA2, high mobility group A2 (previously named HMGI-C); HMGN1, high mobility group N1 (previously named HMG14); HMGN2, high mobility group N2 (previously named HMG17); PCA, perchloric acid; HPLC, high-pressure liquid chromatography; LC−MS, liquid chromatography−mass spectrometry; cds, coding sequence; SAM, *S*-adenosilmethionine; AdOx, adenosine dialdehyde; DAPI, 4,6-diamidino-2-phenylindole; P, phosphate group; M, methyl group; HDACs, histone deacetylases; HATs, histone acetyltransferases.

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aberrations of their genes found in benign neoplasms (*13*−*22*). The elevated expression of HMGA proteins in cancer cells, while they are not present or only barely detectable in normal differentiated cells, suggested their use in diagnostic pathology (*18*−*20*, *23*−*25*), and looking farther, as drug targets for a rational therapeutic intervention in cancer (*26*−*28*).

In a previous paper, we investigated changes in the degree of phosphorylation of HMGA1a during apoptosis of leukaemic cells (*29*). We found that this protein, constitutively phosphorylated in proliferating cells, undergoes a hyperphosphorylation at the early stages of apoptosis. It is known that hyperphosphorylation leads to the detachment of HMGA proteins from DNA (*2*) that, during apoptosis, is linked to both DNA digestion and nuclear scaffold modification. A dephosphorylation process then initiates, which is linked to the remodeling of chromatin that forms the condensed structure in the apoptotic bodies. In a successive study we also reported that, during apoptosis of leukaemic cells, another posttranslational modification (i.e., methylation) accompanied the change of the degree of phosphorylation of HMGA1a protein. Indeed, a large fraction of this protein becomes monomethylated during apoptosis in parallel to the dephosphorylation process (*30*). Here we extend the study to other tumor cells (human or rat) to ascertain if methylation of HMGA1a protein is a characteristic common to all tumor cells during apoptosis and not restricted to leukaemic ones. Moreover, we investigated other low-molecular-mass HMG proteins as regards their posttranslational modifications during apoptosis. Finally, we carried out a detailed LC−MS study of tryptic digested HMGA1a protein that identified arginine 25, comprised in the first AT-hook of HMGA1a protein, as the site of monomethylation.

EXPERIMENTAL PROCEDURES

Cell Cultures and Treatments. Cultures and apoptotic treatments of leukaemic cells were carried out as previously reported (*29*). Human prostate cancer cell lines DU 145 and PC-3 (*31*, *32*) were grown in RPMI 1640 medium (Hyclone) containing 10% fetal bovine serum, 100 U/L penicillin, 100 μg/L streptomycin, and 2 mM glutamine. Apoptosis was induced in the same medium in cultures at 60% confluence with 0.6 μM camptothecin (DU 145 cells) or 10 μM lovastatin (PC-3) for 48 or 72 h, respectively. Cells were recovered by scraping, washed three times with PBS, and treated for protein or DNA analysis. FRTL5 KiMol rat cells were grown as previously described (*14*), and apoptosis was induced by UVC irradiation at 50 J/m^2.

Protein Extraction, Electrophoretic Analysis, and DNA Fragmentation Assay. Protein extraction, electrophoretic analysis, and DNA fragmentation assay were carried out as previously described (*29*).

Methyltransferase Activity in U937 Cells. Methyltransferase activity in U937 cells was assessed essentially according to ref *33*. Briefly, AdOx treated cells were sonicated in 10 mM phosphate buffer pH 7.2, 150 mM NaCl, 1 mM EDTA, and surnatant recovered by centrifugation at 35 000*g*. Total protein content of the extract, having putative methyltransferase activity, was quantified by Bradford, and a volume containing 30 μg of total protein was treated with 4 μCi of [^{3}H]-5-adenosilmethionine (^{3}H-SAM) together with 6 μg of recombinant HMGA1a protein. After 1 h incubation, the reaction was stopped by SDS and directly loaded into the electrophoretic gel. Methylation of HMGA1a protein was detected by autoradiography using Hyperfilm ECL-Amersham.

Methylation of Endogenous HMGA1a Protein in U937 Cells. U937 cells (1.5−2.0 × 10^6 cells/mL) were incubated for 30 min with 65 μCi of [^{3}H]-SAM (5 μCi/mL), then cells were treated with 68 μM etoposide for 25 h (*29*). HMG proteins were extracted from U937 cells by PCA; the obtained PCA solution was treated with an excess of loading buffer solution and directly loaded on the SDS−electrophoretic gel. Methylated proteins were detected by autoradiography.

Western and Northern Blot Analyses. Western blotting for recognition of HMGA1a and HMGA2 proteins was carried out as previously described (*34*, *35*), and recombinant proteins were used as reference (*7*). mRNA analysis was carried out as previously described (*36*).

Alkaline Phosphatase and Trypsin Treatments. HPLC-purified HMGA1a samples (about 10 μg) from apoptotic cells were treated for 15 h at 37 °C in 50 μL of 5 mM Tris/HCl, 10 mM NaCl, 1 mM $MgCl_2$, 0.1 mM DTT, pH 7.9, containing 10 units of calf intestinal alkaline phosphatase (New England Biolabs). At the end of the reaction, protein sample was directly injected into the HPLC apparatus interfaced with the mass spectrometer. Trypsin treatment for peptide sequence analysis was carried out as previously reported (*29*).

HPLC and LC−MS Measurements. Reverse-phase HPLC chromatography of perchloric acid (PCA) extracted proteins from both control and apoptotic cells was carried out with a Perkin-Elmer Life Sciences apparatus as previously reported (*29*). The HPLC apparatus was interfaced with a mass spectrometer (PE SCIEX, API 1), and reconstructed mass spectra were obtained as previously reported. Mass values are reported as Da ± 1.

Rat HMGA1a/1b Protein Sequences. A reverse transcriptase-PCR was carried out using total RNA from rat Dunning AT-1 cell line (*37*) that expresses high levels of HMGA1 proteins, especially the HMGA1b isoform (our unpublished data). The sequence of the antisense primer for the reverse transcriptase reaction (5′-CCAAGGCTCGAGCGGCACTGCAATGGTCA-3′) was designed on the basis of a sequence from a partial rat *Hmga1* cDNA clone, kindly provided by Dr. D. Hunter. The cDNA synthesized at 42 °C for 1 h with the M-MLV reverse transcriptase (Promega) was then amplified by PCR using the Taq DNA polymerase (Promega) with 35 cycles (annealing temperature: 50 °C) according to standard protocols. The sequence of the sense primer (5′-CACTCTTGAATTCCCCACACATAGAGAAGCAG-3′) was obtained by carrying out a similarity search with the mouse *Hmga1* cDNA against rat genome database using the BLAST program. A rat genomic sequence (gb AC095263) with high similarity with the mouse *Hmga1* sequence was found and shown, in comparison with the mouse and human *HMGA1* genes, to contain a partial sequence of the rat *Hmga1* gene. The 326 bp PCR product was then cloned into the pcDNA3 vector (Invitrogen) and sequenced, obtaining the complete cds of HMGA1b mRNA (gb AF511040). Sequences of the HMGA1a mRNA and of the consequent protein were deduced considering the

HMGA1b cds, and the sequence of the alternative spliced exon was obtained from the partial rat *Hmga1* gene clone sequence. The complete sequence of the HMGA1a protein from Rattus norvegicus has been registered in GenBank by other authors (accession no. AF 507966). This sequence shows few differences from our sequence that we validated by mass spectrometry as reported in this paper.

RESULTS

Increase of Methylation of HMGA1a Protein during Apoptosis of Tumor Cells. We have previously reported that in leukaemic cells (HL60, U937, K562, and NB4) hyperphosphorylation/dephosphorylation of HMGA1a protein is observable during apoptosis induced by different procedures (*29*). Hyperphosphorylation is an early event and is followed by dephosphorylation, which can remove most of the phosphate groups from the protein. We suggested that hyperphosphorylation could be related to the detachment of the protein from DNA to facilitate its digestion by nucleases, whereas dephosphorylation could be linked to the formation of condensed structures of the apoptotic bodies (*29*). Hyperphosphorylation and dephosphorylation partially overlap depending both on cell type and on the procedure used to induce apoptosis. Therefore, the mass spectrum of HMGA1a protein obtained from an apoptotic cell population shows peaks from both hyperphosphorylated and dephosphorylated forms. HMGA1a is constitutively present in tumor cells as the diphosphorylated and triphosphorylated forms (*16*, *29*, *38*, *39*), and at the early stages of apoptosis there is an increase of the amount of the triphosphorylated form in comparison to the diphosphorylated one (*30*). At the same time, other more highly phosphorylated forms, with four or five phosphate groups per molecule, can appear (*29*). In the same leukaemic cells, we observed that the hyperphosphorylation/dephosphorylation process is accompanied by another posttranslational modification (i.e., monomethylation of the HMGA1a molecule), which parallels protein dephosphorylation rather than hyperphosphorylation (*30*).

To verify if HMGA1a methylation during apoptosis is restricted only to leukaemic cells or could be regarded as a general characteristic of tumor cells induced to undergo apoptosis, we examined protein samples extracted from three other tumor cell lines: DU 145, PC-3 (human prostate cancer) (*31*, *32*), and FRTL5 KiMol (virus transformed rat thyroid cells) (*14*). By Western and Northern analyses we ascertained that DU 145 and PC-3 cell lines express the two spliced forms HMGA1a and HMGA1b (data not shown). Moreover, we noted that PC-3 cells also express the HMGA2 protein, however, in a very small amount that did not allow its clear detection by LC−MS. Previous work demonstrated high levels of expression of the three HMGA proteins in FRTL5 KiMol cells (*14*), whereas leukaemic cells express only HMGA1a and HMGA1b (*29*). Therefore, we carried out the study of HMGA2 protein using sample extracted from FRTL5 KiMol cells. Table 1 summarizes the expression of the HMGA proteins in the studied cell lines. DU 145, PC-3, and FRTL5 KiMol cells were induced to undergo apoptosis that was assessed by both DNA fragmentation analysis and fluorescence microscopic observation of DAPI stained nuclei (data not shown). Protein samples extracted from both control and apoptotic cells were LC−MS analyzed; results obtained from HMGA1a and HMGA2 proteins have been reported in Figure 1A−H. Protein samples from HL60 leukaemic cells (Figure 1A,B), used as reference (*29*), show an increase of methylation of HMGA1a protein from 22 to 46% following apoptosis induction. In control DU 145 cells (Figure 1C), HMGA1a protein is detectable as the diphosphorylated (2P: 11 746.0 ± 1 Da) and triphosphorylated (3P: 11 826.3 ± 1 Da) forms, which are also present as monomethylated species (2P/M: 11 760.5 ± 1 Da; 3P/M: 11 840.0 ± 1 Da, respectively). The theoretical mass value of HMGA1a was calculated from the amino acid sequence (*2*) assuming acetylation at the N-terminal serine and adding the mass of two or three phosphate groups; the resulting values were 11 746.8 Da for HMGA1a/2P and 11 826.8 Da for HMGA1a/3P. Following apoptosis induction (Figure 1D), protein dephosphorylation takes place as suggested by the appearance of the unphosphorylated form (0P). At the same time, a massive methylation occurs such that methylated peaks (0P/M, 2P/M, and 3P/M) predominate on the peaks of the unmethylated forms (0P, 2P, and 3P): the total methylated protein in apoptotic cells is more than twice that present in control cells (68 vs 32%). A very similar behavior can be observed for HMGA1a samples obtained from control and apoptotic PC-3 cells (Figure 1E,F, respectively) in which methylation reaches 57% of the total amount of HMGA1a. Mass spectra obtained using protein samples from rat FRTL5 KiMol cells (Figure 1G,H) were more complex than those obtained with protein extracted from the other cell lines shown in Figure 1 because in our reversed-phase chromatographic analysis, HMGA1a and HMGA2 coelute. Therefore, mass spectra of Figure 1G,H show peaks deriving from both proteins, and for the sake of clarity, peaks deriving from HMGA2 protein are empty while those deriving from HMGA1a are filled. Since interpretation of mass data requires a theoretical mass value obtained from the amino acid sequence, we cloned HMGA1 cDNA from rat (see Experimental Procedures). The amino acid sequence thus obtained is reported in Figure 2 together with the sequences of human HMGA1a, human HMGA1b (*2*), and rat HMGA2 (*40*). The rat homologue is also composed of 106 amino acid residues (*2*), and comparison with the human sequence evidenced only four substitutions (human → rat): $S_4 \rightarrow V_4$; $P_{33} \rightarrow S_{33}$; $A_{68} \rightarrow T_{68}$; and $T_{74} \rightarrow V_{74}$. According to this sequence and assuming N-terminal acetylation, the theoretical rat HMGA1a mass was 11 616.9 Da. HMGA1a data of FRTL5 KiMol cells are qualitatively consistent with preceding data from the other cell lines as regards both dephosphorylation during apoptosis and increase of methylation. In UV irradiated cells, dephosphorylated HMGA1a/0P and HMGA1a/1P are clearly visible in comparison with FRTL5 KiMol control cells. At the same time, in apoptotic cells, there is an increase of the methylated HMGA1a protein of 1.4 times with respect to control cells. Using the rat HMGA2 amino acid sequence (*40*) and assuming N-terminal acety-

Table 1: Expression of HMGA Proteins in Different Tumor Cells[a]

cells	HMGA1a	HMGA1b	HMGA2
HL60-U937	+	+	−
DU 145	+	+	−
PC-3	+	+	+/−
FRTL5 KiMol	+	+	+

[a] +, high level of expression; −, no expression; and +/−, low level of expression.

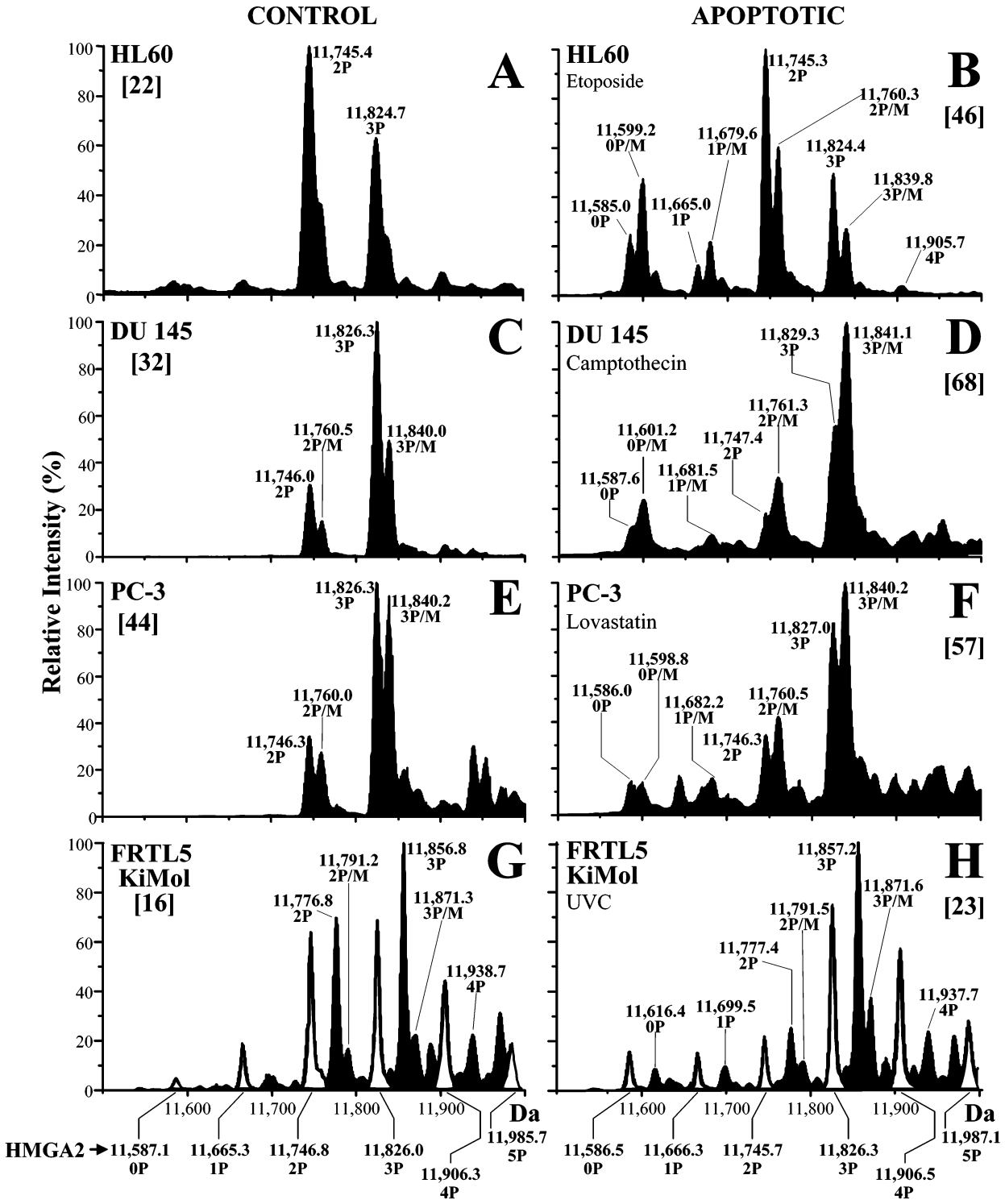

FIGURE 1: Methylation of HMGA1a protein in four tumor cell lines induced to undergo apoptosis. HL60, DU 145, PC-3, and FRTL5 KiMol cell lines were induced to undergo apoptosis by etoposide, camptothecin, lovastatin, and UVC, respectively. Protein mixtures were extracted by PCA, analyzed by LC−MS, and the obtained data reported as reconstructed spectra (Da). Panels A, C, E, and G: protein samples from control cells. Panels B, D, F, and H: protein samples from apoptotic cells. 0P, 1P, 2P, 3P, and 4P indicate the number of phosphate groups (P) borne by HMGA1a protein while /M indicates monomethylation. In panels G and H, peaks of HMGA2 protein are empty to better distinguish them from HMGA1a peaks (filled). The percentage (±2) of total HMGA1a methylated protein found in each sample is shown in square brackets.

lation, we obtained a theoretical mass value of 11 586.8 Da that allowed us to identify up to five different phosphorylated forms of this protein (1P, 2P, 3P, 4P, and 5P) together with the unphosphorylated form (0P) that increases in UV irradiated cells. We point out that while HMGA2 behaves as HMGA1a regarding phosphorylation, interestingly, no methylated forms have been detected either in control or in UV irradiated FRTL5 KiMol cells. All the experimental mass values of peaks in Figure 1 are summarized in Table 2. Since in previous studies (*29*, *30*), using time course experiments, we demonstrated by microscopic observation of stained nuclei, DNA fragmentation analysis, and mass spectrometry that chromatin condensation and dephosphorylation/methylation of HMGA1a protein are related events, we can

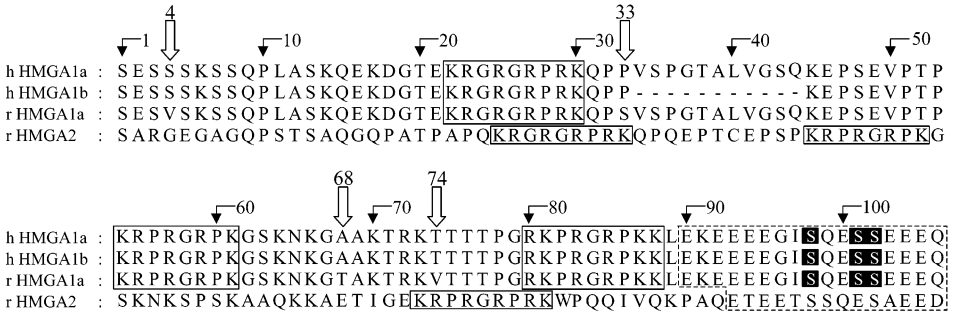

FIGURE 2: Human (h) and rat (r) amino acid sequences of studied HMGA proteins. DNA binding AT-hooks are included in solid boxes, while the 11 amino acid residues that differentiate HMGA1b from HMGA1a are shown as a dotted line (- - -). Acidic C-termini are included in a dotted box in which the constitutively phosphorylated serines are evidenced. The four human → rat substitutions (i.e., 4, 33, 68, and 74) are also indicated.

Table 2: Mass Spectrometric Values (Da) of the Variously Modified Forms of Human (HL60, DU 145, and PC-3) or Rat (FRTL5 KiMol) HMGA1a Protein and of Rat HMGA2 (FRTL5 KiMol) Protein[a]

	HUMAN						RAT	
	HL60		DU 145		PC 3		FRTL5 KiMol	
	C	A	C	A	C	A	C	A
HMGA1a /0P		11 585.0		11 587.6		11 586.0		11 616.4
HMGA1a/0P/M		**11 599.2**		**11 601.2**		**11 598.8**		
HMGA1a/1P		11 665.0						11 699.5
HMGA1a/1P/M		**11 679.6**		**11 681.5**		**11 682.2**		
HMGA1a/2P	11 745.4	11 745.3	11 746.0	11 747.4	11 746.3	11 746.3	11 776.8	11 777.4
HMGA1a/2P/M		**11 760.3**	**11 760.5**	**11 761.3**	**11 760.0**	**11 760.5**	**11 791.2**	**11 791.5**
HMGA1a/3P	11 824.7	11 824.4	11 826.3	11 829.3	11 826.3	11 827.0	11 856.8	11 857.2
HMGA1a/3P/M		**11 839.8**	**11 840.0**	**11 841.1**	**11 840.2**	**11 840.2**	**11 871.3**	**11 871.6**
HMGA1a/4P		11 905.7					11 938.7	11 937.7
HMGA2/0P							11 587.1	11 586.5
HMGA2/1P							11 665.3	11 666.3
HMGA2/2P							11 746.8	11 745.7
HMGA2/3P							11 826.0	11 826.3
HMGA2/4P							11 906.3	11 906.5
HMGA2/5P							11 985.7	11 987.1

[a] Protein theoretical mass values obtained from amino acid sequence and assuming N-terminal acetylation of human HMGA1a, rat HMGA1a, and rat HMGA2 were 11 586.8, 11 616.9, and 11 586.8 Da, respectively. Mass values of methylated forms are shown in bold. C, control cells; A, apoptotic cells.

conclude that the data from Figure 1 complete the view of HMGA1a participation in chromatin remodeling during apoptosis.

Alkaline Phosphatase Treatment Highlights Methylation of HMGA1a Protein. To better underline that HMGA1a protein undergoes two different posttranslational modifications during apoptosis (i.e., phosphorylation and methylation), HMGA1a purified samples obtained from apoptotic U937, HL60, DU 145, and PC-3 cells were alkaline phosphatase treated (Figure 3). Removal of the phosphate groups from the variously modified forms (i.e., phosphorylated but unmethylated (1P, 2P, 3P, and 4P) or phosphorylated and monomethylated (1P/M, 2P/M, and 3P/M)) provided simpler mass spectra containing two main peaks (0P and 0P/M) and highlights methylation as the other main HMGA1a posttranslational modification during apoptosis. We point out that monomethylation (+14 Da) is the predominant form and that higher degrees of methylation are negligible if at all detectable.

Among Low-Molecular-Mass HMG Proteins Only HMGA1a Is Methylated during Apoptosis of Tumor Cells. Mammalian cells express many low-molecular-mass HMG proteins including HMGN1, HMGN2, and HMGA1a, HMGA1b, HMGA2, the three HMGA proteins being expressed only in cancer cells (*4*, *15*−*17*). Figure 4 shows mass spectra of protein samples obtained from control U937 cells (panels A, D, and G) or U937 cells induced to undergo apoptosis by camptothecin (panels B, E, and H) or etoposide (panels C, F, and I). No evident change in the degree of phosphorylation or methylation was found for HMGN1/N2 proteins during apoptosis of U937 cells (Figure 4A−F). Similar results were obtained for the other cell lines used in this work (data not shown). The behavior of HMGA1b, the other spliced protein of the *HMGA1* gene, deserves a longer discussion. With regard to the hyperphosphorylation/dephosphorylation process during apoptosis, HMGA1b behaves in a manner similar to the HMGA1a protein in all studied systems, although the intensity of changes is different (Figure 4D−F). HMGA1b, always detectable as the diphosphorylated and triphosphorylated forms in control cells (as the HMGA1a protein), was never found in the methylated form either in control or in apoptotic cells (Figure 4D−F). It is worthwhile to mention that HMGA1a protein was instead found in the methylated form already in some proliferating control cells such as DU 145, PC-3, and FRTL5 KiMol (as shown in Figure 1); however, following apoptosis a substantial increase of methylation is observed. Further demonstration that only HMGA1a is methylated derived from in vitro and in vivo labeling experiments. First, we demonstrated the presence of methyltransferase activity toward HMGA1a in cells under

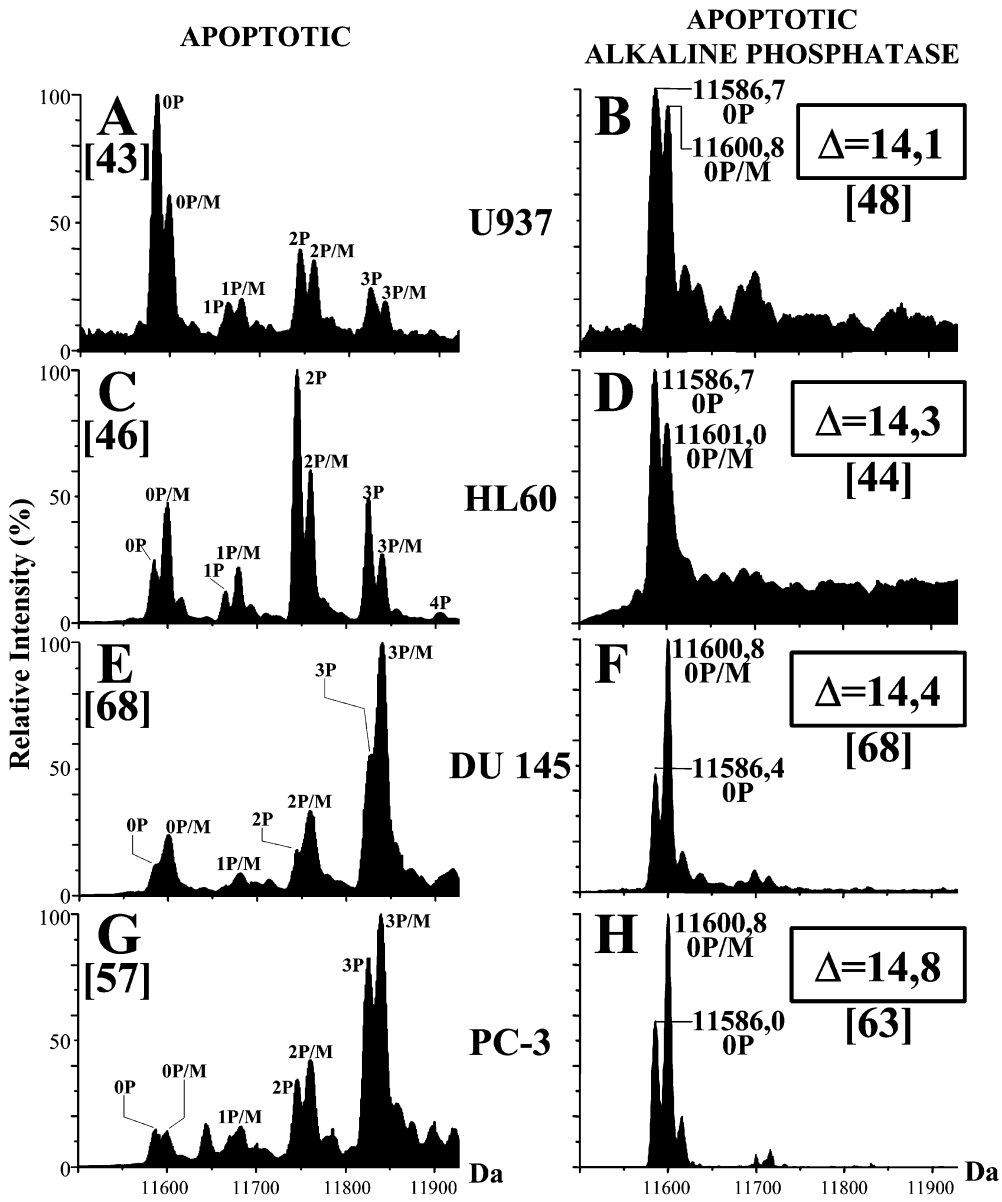

FIGURE 3: Mass spectrometry of extracted proteins from apoptotic cells, alkaline phosphatase treated to eliminate all phosphate groups, allows to evidence monomethylation of HMGA1a protein. HPLC-purified HMGA1a protein samples from apoptotic cells (panels A, C, E, and G) were treated with alkaline phosphatase (panels B, D, F, and H). 0P, 1P, 2P, 3P, and 4P indicate the number of phosphate groups (P) borne by HMGA1a protein while /M indicates monomethylation. The increase of mass (about 14 Da) because of methylation is shown in panels B, D, F, and H. Percentage (±2) of total HMGA1a methylated protein found in each sample is shown in square brackets.

investigation. To this end, HMGA1a recombinant protein was incubated with a U937 protein extract in the presence of [^{3}H]-SAM as methyl group donor. Results in Figure 5A show that the protein extract from U937 cells (lane 2) is able to methylate recombinant HMGA1a protein (lane 3). Subsequently, U937 cells were incubated with [^{3}H]-SAM and etoposide treated to induce apoptosis. PCA extracted proteins were analyzed by SDS−gel electrophoresis (Figure 5B). Because of the low amount of HMGA1a protein (in comparison with the other co-extracted proteins), its migration position in the electrophoretic patterns (lanes 2 and 3) was assessed by using HPLC-purified HMGA1a protein from both control and apoptotic U937 cells as reference (lanes 1 and 4, respectively). The autoradiography shown in Figure 5B indicates that HMGA1a protein is methylated during apoptosis (lane 3), whereas it is not in control cells (lane 2).

HMGA1a Methylation Site Is at Arginine 25 Comprised in a GRG Consensus. HPLC-purified HMGA1a samples from HL-60, DU 145, and PC-3 apoptotic cells were fully dephosphorylated by alkaline phosphatase and further purified by HPLC (data not shown). LC−MS of tryptic digests of this HMGA1a protein provided the tryptic maps reported in Figure 6. Since trypsin treatment digested both the methylated and the unmethylated protein, some fragments were detected in both methylated and unmethylated forms (i.e., differing by about 14 Da). Among all fragments whose position within the HMGA1a sequence was mapped, we detected only four monomethylated peptides (i.e., peptides 1−29, 7−27, 24−29, and 24−54). These peptides are shown in Figure 6, and their overlapping allowed us to identify arginine 25 as the methylated amino acid residue included in the sequence $G_{24}R_{25}G_{26}$, which belongs to the first AT-hook of the HMGA1a protein. Moreover, peptides 26−29, 26−54, and 26−57 were always found only in the unmethylated form and allowed us to rule out R_{27} and R_{29} as putative sites of modification. The fact that mass values

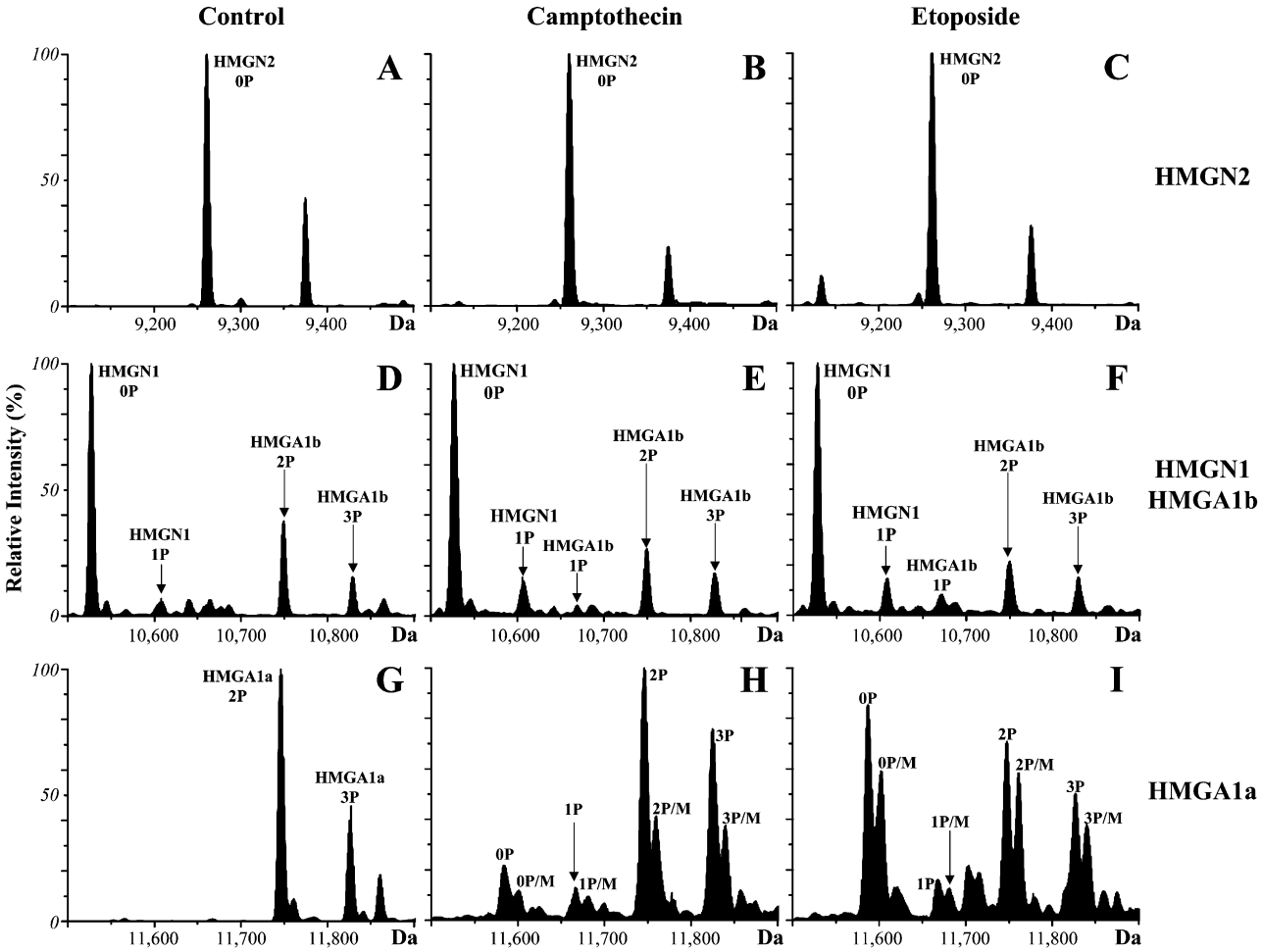

FIGURE 4: Mass spectrometry demonstrates that among low-molecular-mass HMG proteins, only HMGA1a protein undergoes methylation during apoptosis. Panels A, D, and G: Mass spectrum in the range of 9000−12 000 Da of protein sample from control U937 cells. Panels B, E, and H: Mass spectrum in the range of 9000−12 000 Da of protein sample from U937 cells induced to under go apoptosis by camptothecin. Panels C, F, and I: Mass spectrum in the range of 9000−12 000 Da of protein sample from U937 cells induced to undergo apoptosis by etoposide.

obtained for the entire HMGA1a molecule indicated monomethylation (+14 Da) leads us to conclude that R_{25} is the only methylated residue.

DISCUSSION

An increasing interest is at the moment devoted to protein methylation, for which two types of N-modification have been described: methylation of the guanidino side chain of arginine or methylation of the ϵ-amino group of lysine. The latter modification is better known because of its identification at the N-terminal tails of histones within processes that regulate chromatin structure and gene silencing (*41*−*45*). A site-specific domain involved in the methylation of the lysine (called SET) has been identified in many methyltransferases, and crystal structures of this domain have been recently reported (*46*−*48*). Methylation of arginine is also under investigation particularly regarding histones (*49*−*51*). In this paper, we report data on methylation of HMG proteins that are histone partners in chromatin structure organization and show that (1) HMGA1a protein is heavily monomethylated during apoptosis of different tumor cells, while the other low-molecular-mass HMG proteins are unmethylated; (2) this type of modification could be considered as a general characteristic of all tumor cells induced to undergo apoptosis; (3) methylation is independent of the apoptosis inducing procedure since chemical (etoposide, camptothecin, lovastatin), viral (HSV-1), or physical (UVC) agents cause the same effects; and (4) monomethylation is at arginine 25 present in the consensus GRG sequence in the first DNA binding AT-hook.

The HMGA1a protein sequence has 11 arginines, 10 of which are contained in the AT-hook segments that are the interacting sites with DNA (*2*, *3*, *8*). Arginine methylation frequently occurs at sites in which arginine flanks a glycine residue (*49*, *50*). In the HMGA1a protein, among the 11 arginines, only arginine 25, which is contained in a GRG consensus (Figure 2), was found to be methylated even if the other five arginines flank a glicine residue. This is similar to what was found for histone H4, which is monomethylated at arginine 3 present in a similar GRG consensus, although H4 shows up to 14 arginines, one of which (arginine 8) belongs to the RGG sequence that together with the GAR sequence is a well-known recognition site for arginine methyltransferases (*49*, *50*). It has been shown that the enzyme PRMT1 is the major H4-specific methyltransferase in human cells for arginine 3, but other in vivo substrates for PRMT1 are as yet unknown (*50*, *51*). It is likely that this enzyme could be also involved in HMGA1a monomethylation. Interestingly, some but not all H4 molecules resulted methylated at arginine 3 (*50*), and similarly, we

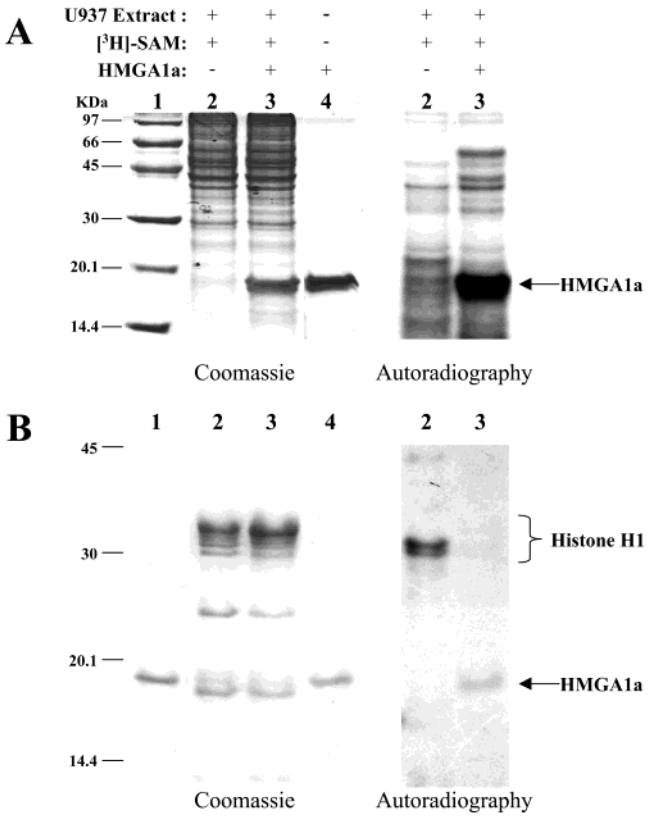

FIGURE 5: [^{3}H]-labeled methylation demonstrates that the mass increase (about 14 Da) found for HMGA1a protein is actually because of monomethylation. (A) SDS-electrophoretic analysis of AdOx treated U937 cell total protein extract incubated with [^{3}H]-SAM (lane 2) or with [^{3}H]-SAM and recombinant HMGA1a protein (lane 3); lanes 1 and 4 show molecular weight protein markers and recombinant HMGA1a protein, respectively. (B) SDS-electrophoretic analysis of PCA protein extracts from control or apoptotic U937 cells both treated with [^{3}H]-SAM (lanes 2 and 3, respectively); HPLC-purified HMGA1a protein from control or apoptotic U397 cells (lanes 1 and 4, respectively) were used as reference.

found a high degree of methylation of HMGA1a, but a protein fraction remains unmethylated even if cells are fully apoptotic. Moreover, in our cell systems, the isoform HMGA1b resulted unmethylated, although it has exactly the same sequence at the site of methylation as HMGA1a (see Figure 2). This means that methylation of HMGA1a protein during apoptosis is not a response to the general disorganization of chromatin structure that could allow the modification of all unsecured protein. Methylation of HMGA1a protein (and likely histone H4) could thus interest only some nuclear compartments and not the entire chromatin. However, it has also been reported that HMGA1b protein is methylated in MCF-7 cells (*52*).

Inhibition experiment using adenosine dialdehyde demonstrated that methylation of HMGA1a protein is not one of the molecular events that cause apoptosis but rather could be related to the cell death induced chromatin remodeling (*30*). In this structural reorganization there is also its concurrent dephosphorylation. Taken together, these results suggest that the execution of cell death requires not only a chain of events to be programmed, but also a programmed chromatin condensation following DNA degradation that could be more or less marked in a cell type dependent manner. In other words, it seems likely that the condensed chromatin of apoptotic cells is not formed by a random crowding of proteins onto the digested DNA but rather that there is the self-assembly of a new chromatin that needs the concurrent activation of both methyltransferases and phosphatases that modify not only HMGA1a protein but likely also other chromatin associated proteins as well. In this respect, we carried out an experiment in which HMGA1a protein was extracted from U937 cells induced to undergo necrosis and LC−MS analyzed; mass spectra showed no evidence of methylation of HMGA1a (data not shown).

What could the specific molecular role of methylated arginine be in the dephosphorylated HMGA1a molecule? While it seems likely that the dephosphorylation process increases the affinity of the protein for DNA because of the loss of the negative charges of the phosphate groups, thus promoting chromatin packaging, it is more difficult to suggest a mechanistic model for the interaction of the methylated arginine of the HMGA1a protein with DNA. This is for more than one reason. Methylation of arginine residues (as well as lysine residues) does not alter their positive charge; therefore, the effect of methylation as regards the DNA binding, at least in terms of electrostatic interaction, could be of moderate importance. Actually, in band-shift experiments that we carried out using AT-rich oligonucleotides and both methylated and unmethylated HMGA1a protein (unphosphorylated), we did not find important differences in DNA binding affinities (data not shown). Furthermore, it is known that the contribution of the first AT-hook to the overall DNA affinity of HMGA1a protein is rather low, the main contribution coming from the second AT-hook (*53*). It thus follows that the function of HMGA1a methylation is more likely related to protein−protein interaction mechanisms, in which the introduction of the extra hydrophobic methyl group and loss of H-bonding from NH could change the quaternary organization of the interacting proteins. An interplay between different modifications such as phosphorylation, acetylation, and methylation is observed at histone tails, and more generally it has been suggested that "the presence of a given modification may dictate or prevent the presence of a second modification elsewhere" (*42*, *44*). Cross-talk between methylation and phosphorylation has been reported for heterochromatin protein 1 (HP1), which is able to bind histone H3 when the latter is methylated at lysine 9 by SUV39 enzyme. This methylation negatively affects phosphorylation of serine 10 of histone H3 and allows binding of HP1 (*54*, *55*). Therefore, we think that the coupled processes of methylation/dephosphorylation that we have found for HMGA1a protein could be located in the general context of combination of three modifications (i.e., phosphorylation, acetylation, and methylation) that manage the formation of protein complexes in regulating chromatin remodeling and gene activity (*41*, *56*).

Methylation of HMGA1a protein is already present in many tumor proliferating cells and increases during apoptosis. This is particularly true for proliferating human tumor prostate and rat FRTL-5 KiMol cell lines (see Figure 1), but small amounts of methylated HMGA1a protein can also be detected in proliferating leukaemic cells such as U937 and HL-60 cells (*29*). It has been suggested that methylation of nuclear proteins (histones, in particular) could represent a way to regulate chromatin structure and function in a manner analogous to acetylation (*57*). Because up to now protein methylation as been reported to result in an irreversible

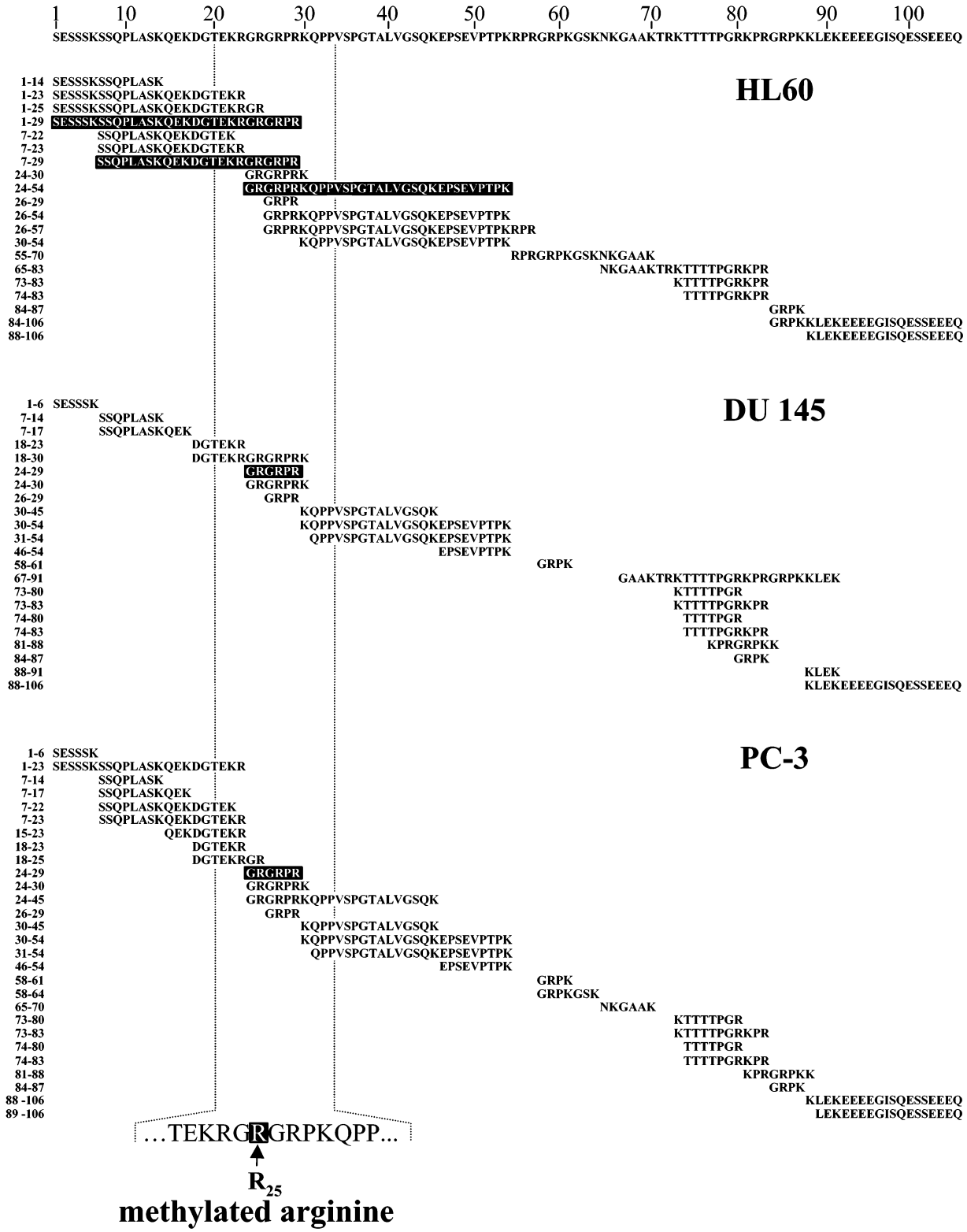

FIGURE 6: Tryptic digestion map of methylated HMGA1a protein shows that arginine 25 is the modified site. Identified fragments by mass spectrometry of trypsin digested HPLC-purified and alkaline phosphatase treated HMGA1a protein from HL60, DU 145, and PC-3 apoptotic cells. Identified methylated fragments 1−29, 7−29, 24−29, and 24−54 are evidenced.

modification that persists over the lifetime of a protein (*57*), it is conceivable to find methylation in the condensed apoptotic chromatin when the commitment of the cell toward apoptosis is irreversible. Similarly, methylation of chromatin structural proteins in tumor cells could participate in the formation of nuclear compartments containing heterochromatin or in the inactivation of genes positioned adjacent to heterochromatin domains to lock a portion of DNA (*58*−*60*). Condensed chromatin could allow cancer cells to evade the apoptotic program to which they are initially committed to and enable them to survive by repressing the expression of pro-apoptotic factors (*28*, *61*, *62*). It has been suggested that a repressive chromatin conformation could be generated by cooperation of methyltransferases (that methylate histone

H3) and histone deacetylases (HDACs) in opposition to histone acetyltransferases (HATs) that, on the contrary, unfold chromatin by acetylation of histone amino-terminal tails and generate a template for transcription (*63*−*65*). Methylation of HMGA1a protein could participate to this methylation program of nuclear proteins mediating the repositioning and permanent silencing of genes (*66*).

In conclusion, we here report that methylation of HMGA1a protein is a relevant posttranslational modification to be considered together with phosphorylation and acetylation of other nuclear proteins in studies concerning both cell proliferation and apoptosis. We underline that in this paper we describe a posttranslational modification that interests a protein that is abundant in the nucleus and consequently could induce structural alteration of large portions of the chromatin. In this context, HMGA1a methylation could be an additional structural contribution to the so-called histone code that regulates the gene-reading machinery (*67*).

REFERENCES

1. Doyle, H. A., and Mamula, M. J. (2001) *Trends Immunol. 22*, 443−449.
2. Bustin, M., and Reeves, R. (1996) in *Progress in Nucleic Acids Research and Molecular Biology* (Cohn, W. E., and Moldave, K., Eds.) Vol. 84, pp 35−100, New York, Academic Press.
3. Bustin, M. (1999) *Mol. Cell. Biol. 19*, 5237−5246.
4. Jansen, E., Petit, M. M. R., Schoenmakers, E. F. P. M., Ayoubi, T. A. Y., and Van de Ven, W. J. M. (1999) *Gene Ther. Mol. Biol. 3*, 387−395.
5. Bustin, M. (2001) *Trends Biochem. Sci. 26*, 152−153.
6. Huth, J. R., Bewley, C. A., Nissen, M. S., Evans, J. N., Reeves, R., Gronenborn, A. M., and Clore, G. M. (1997) *Nat. Struct. Biol. 4*, 657−665.
7. Mantovani, F., Covaceuszach, S., Rustighi, A., Sgarra, R., Heath, C., Goodwin, G. H., and Manfioletti, G. (1998) *Nucleic Acids Res. 26*, 1433−1439.
8. Reeves, R., and Beckerbauer, L. (2001) *Biochim. Biophys. Acta 1519*, 13−29.
9. Reeves, R., Langan, T. A., and Nissen, M. S., (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 1671−1675.
10. Nissen, M. S., Langan, T. A., and Reeves, R. (1991) *J. Biol. Chem. 266*, 19945−19952.
11. Schwanbeck, R., and Wiśniewski, J. R. (1997) *J. Biol. Chem. 272*, 27476−27483.
12. Munshi, N., Agalioti, T., Lomvardas, S., Merika, M., Chen, G., and Thanos, D. *Science 293*, 1133−1136.
13. Giancotti, V., Berlingieri, M. T., Di Fiore, P. P., Fusco, A., Vecchio, G., and Crane-Robinson, C. (1985) *Cancer Res. 45*, 6051−6057.
14. Giancotti, V., Pani, B., D'Andrea, P., Berlingieri, M. T., Di Fiore, P. P., Fusco, A., Vecchio, G., Philp, R., Crane-Robinson, C., Nicolas, R. H., Wright, C. A., and Goodwin, G. H. (1987) *EMBO J. 6*, 1981−1987.
15. Giancotti, V., Buratti, E., Perissin, L., Zorzet, S., Balmain, A., Portella, G., Fusco, A., and Goodwin, G. H. (1989) *Exp. Cell. Res. 184*, 538−545.
16. Giancotti, V., Bandiera, A., Buratti, E., Fusco, A., Marzari, R., Coles, B., and Goodwin, G. H. (1991) *Eur. J. Biochem. 198*, 211−216.
17. Giancotti, V., Bandiera, A., Ciani, L., Santoro, D., Crane-Robinson, C., Goodwin, G. H., Boiocchi, M., Dolcetti, R., and Casetta, B. (1993) *Eur. J. Biochem. 213*, 825−832.
18. Chiappetta, G., Avantaggiato, V., Visconti, R., Fedele, M., Battista, B., Trapasso, F., Merciai, B. M., Fidanza, V., Giancotti, V., Santoro, M., Simeone, A., and Fusco, A. (1996) *Oncogene 13*, 2439−2446.
19. Chiappetta, G., Tallini, G., De Biasio, M. C., Manfioletti, G., Martinez-Tello, F. J., Pentimalli, F., de Nigris, F., Mastro, A., Botti, G., Fedele, M., Berger, N., Santoro, M., Giancotti, V., and Fusco, A. (1998) *Cancer Res. 58*, 4193−4198.
20. Bandiera, A., Bonifacio, D., Manfioletti, G., Mantovani, F., Rustighi, A., Zanconati, F., Fusco, A., Di Bonito, L., and Giancotti, V. (1998) *Cancer Res. 58*, 426−431.
21. Zhou, X., Benson, K. F., Ashar, H. R., and Chada, K. (1995) *Nature 376*, 771−774.
22. Anand, A., and Chada, K. (2000) *Nat. Genet. 24*, 377−380.
23. Chiappetta, G., Bandiera, A., Berlingieri, M. T., Visconti, R., Manfioletti, G., Battista, S., Martinez-Tello, F. J., Santoro, M., Giancotti, V., and Fusco, A. (1995) *Oncogene 10*, 1307−1313.
24. Fedele, M., Bandiera, A., Chiappetta, G., Battista, S., Viglietto, G., Manfioletti, G., Casamassimi, A., Santoro, M., Giancotti, V., and Fusco, A. (1996) *Cancer Res. 56*, 1896−1901.
25. Abe, N., Watanabe, T., Masaki, T., Mori, T., Sugiyama, M., Uchimura, H., Fujioka, Y., Chiappetta, G., Fusco, A., and Atomi, Y. (2000) *Cancer Res. 60*, 3117−3122.
26. Hughes, J. H., and Cohen, M. B. (1999) *Am. J. Clin. Pathol. 111*, 267−274.
27. Sawyers, C. L. (2002) *Curr. Opin. Genet. Dev. 12*, 111−115.
28. Johnstone, R. W., Ruefli, A. A., and Lowe, S. W. (2002) *Cell 108*, 153−164.
29. Diana, F., Sgarra, R., Manfioletti, G., Rustighi, A., Poletto, D., Sciortino, M. T., Mastino, A., and Giancotti, V. (2001) *J. Biol. Chem. 276*, 11354−11361.
30. Sgarra, R., Diana, F., Rustighi, A., Manfioletti, G., and Giancotti, V. *Cell Death Diff.*, in press.
31. Webber, M. M., Bello, D., and Quader, S. (1996) *Prostate 29*, 386−394.
32. Mitchell, S., Abel, P., Ware, M., Stamp, G., and Lalani, E.-N. (2000) *Br. J. Urol. 85*, 932−940.
33. Najbauer, J., and Aswad, D. (1990) *J. Biol. Chem. 265*, 12717−12721.
34. Patel, U. A., Bandiera, A., Manfioletti, G., Giancotti, V., Khau, K., and Crane-Robinson, C. (1994) *Biochim. Biophys. Res. Commun. 201*, 63−70.
35. Berlingieri, M. T., Manfioletti, G., Santoro, M., Bandiera, A., Visconti, R., Giancotti, V., and Fusco, A. (1995) *Mol. Cell. Biol. 15*, 1545−1553.
36. Manfioletti, G., Giancotti, V., Bandiera, A., Buratti, E., Sautière, P., Cary, P., Crane-Robinson, C., Coles, B., and Goodwin, G. H. (1991) *Nucleic Acids Res. 19*, 6793−6797.
37. Tennant, T. R., Kim, H., Sokoloff, M., and Rinker-Schaeffer, C. W. (2000) *Prostate 43*, 295−302.
38. Palvimo, J., and Linnala-Kankkunen, A. (1989) *FEBS Lett. 257*, 101−104.
39. Ferranti, P., Malorni, A., Marino, G., Pucci, P., Goodwin, G. H., Manfioletti, G., and Giancotti, V. (1992) *J. Biol. Chem. 267*, 22486−22489.
40. Li, D., Lin, H. H., McMahon, M., Ma, H.-Y., and Ann, D. K. (1997) *J. Biol. Chem. 272*, 25062−25070.
41. Rea, S., Eisenhaber, F., O'Carroll, D., Strahl, B. D., Sun, Z.-W., Schmid, M., Opravil, S., Mechtler, K., Ponting, C. P., Allis, C. D., and Jenuwein, T. (2000) *Nature 406*, 593−599.
42. Zhang, Y., and Reinberg, D. (2001) *Genes Dev. 15*, 2343−2360.
43. Jenuwein, T., and Allis, C. D. (2001) *Science 293*, 1074−1080.
44. Kouzarides, T. (2002) *Curr. Opin. Gen. Dev. 12*, 198−209.
45. Lachner, M., and Jenuwein, T. (2002) *Curr. Opin. Cell Biol. 14*, 286−298.
46. Trievel, R. C., Beach, B. M., Dirk, L. M. A., Houtz, R. L., and Hurley, J. H. (2002) *Cell 111*, 91−103.
47. Wilson, J. R., Jing, C., Walker, P. A., Martin, S. R., Howell, S. A., Blackburn, G. M., Gamblin, S. J., and Xiao, B. (2002) *Cell 111*, 105−115.
48. Zhang, X., Tamaru, H., Khan, S. I., Horton, J. R., Keefe, L. J., Selker, E. U., and Cheng, X. (2002) *Cell 111*, 117−127.
49. McBride, A. E., and Silver, P. A. (2001) *Cell 106*, 5−8.
50. Strahl, B. D., Briggs, S. D., Brame, C. J., Caldwell, J. A., Koh, S. S., Ma, H., Cook, R. G., Shabanowitz, J., Hunt, D. F., Stallcup, M. R., and Allis, C. D. (2001) *Curr. Biol. 11*, 996−1000.
51. Wang, H., Huang, Z. Q., Xia, L., Feng, Q., Erdjument-Bromage, H., Strahl, B. D., Briggs, S. D., Allis, C. D., Wong, J., Tempst, P., and Zhang, Y. (2001) *Science 293*, 853−857.
52. Banks, G. C., Li, H., and Reeves, R. (2000) *Biochemistry 39*, 8333−8346.
53. Yie, J., Liang, S., Merika, M., and Thanos, D. (1997) *Mol. Cell. Biol. 17*, 3649−3662.
54. Lachner, M., O'Carroll, D., Rea, S., Mechtler, K., and Jenuwein, T. (2001) *Nature 410*, 116−120.
55. Bannister, A. J., Zegerman, P., Partridge, J. F., Miska, E. A., Thomas, J. O., Allshire, R. C., and Kuzarides, T. (2001) *Nature 292*, 110−113.

56. Stallcup, R. M. (2001) *Oncogene 20*, 3014−3020.
57. Rice, J. C., and Allis, C. D. (2001) *Curr. Opin. Cell. Biol. 13*, 263−273.
58. Brown, K. E., Guest, S. S., Smale, S. T., Hahm, K., Merkenschlager, M., and Fisher, A. G. (1997) *Cell 91*, 845−854.
59. Brown, K. E., Baxter, J., Graf, D., Merkenschlager, M., and Fisher, A. G. (1999) *Mol. Cell 3*, 207−217.
60. Brown, K. E., Amoils, S., Horn, J. M., Buckle, V. J., Higgs, D. R., Merkenschlager, M., and Fisher, A. G. (2001) *Nat. Cell. Biol. 3*, 602−606.
61. Evan, G. I., and Vousden, K. H. (2001) *Nature 411,* 342−348.
62. Hickman, J. A. (2002) *Curr. Opin. Gen. Dev. 12*, 67−72.
63. Harbour, J. W., and Dean, D. C. (2000) *Genes Dev. 14*, 2393−2409.
64. Nielsen, S. J., Schneider, R., Bauer, U. M., Bannister, A. J., Morrison, A., O'Carroll, D., Firestein, R., Cleary, M., Jenuwein, T., and Herrera, R. E. (2001) *Nature 412*, 561−565.
65. Klochendler-Yeivin, A., Muchardt, C., and Yaniv, M. (2002) *Curr. Opin. Gen. Dev. 12*, 73−79.
66. Fisher, A. G., and Merkenschlager, M. (2002) *Curr. Opin. Gen. Dev. 12*, 193−197.
67. Milne, T. A., Briggs, S. D., Brock, H. W., Martin, M. E., Gibbs, D., Allis, C. D., and Hess, J. L. (2002) *Mol. Cell 10*, 1107−1117.

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