THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 14, Issue of April 6, pp. 11354–11361, 2001
Printed in U.S.A.

A Link between Apoptosis and Degree of Phosphorylation of High Mobility Group A1a Protein in Leukemic Cells*

Received for publication, October 18, 2000, and in revised form, December 22, 2000
Published, JBC Papers in Press, January 5, 2001, DOI 10.1074/jbc.M009521200

Francesca Diana‡§, Riccardo Sgarra‡§, Guidalberto Manfioletti‡, Alessandra Rustighi‡, Dalisa Poletto¶, Maria Teresa Sciortino‖, Antonio Mastino‖, and Vincenzo Giancotti‡**

From the ‡Dipartimento di Biochimica, Biofisica e Chimica delle Macromolecole, Università di Trieste, 34127 Trieste, the ¶Centro di Riferimento Oncologico, Aviano (PN), 33081 Pordenone, and the ‖Dipartimento di Scienze Microbiologiche, Genetiche e Molecolari, Università di Messina, 98100 Messina, Italy

Nuclear phosphoprotein HMGA1a, high mobility group A1a, (previously HMGI) has been investigated during apoptosis. A change in the degree of phosphorylation of HMGA1a has been observed during apoptosis induced in four leukemic cell lines (HL60, K562, NB4, and U937) by drugs (etoposide, camptothecin) or herpes simplex virus type-1. Both hyper-phosphorylation and de-phosphorylation of HMGA1a have been ascertained by liquid chromatography-mass spectrometry. Hyper-phosphorylation (at least five phosphate groups/HMGA1a molecule) occurs at the early apoptotic stages and is probably related to HMGA1a displacement from DNA and chromatin release from the nuclear scaffold. De-phosphorylation (one phosphate or no phosphate groups/HMGA1a molecule) accompanies the later formation of highly condensed chromatin in the apoptotic bodies. We report for the first time a direct link between the degree of phosphorylation of HMGA1a protein and apoptosis according to a process that involves the entire amount of HMGA1a present in the cells and, consequently, whole chromatin. At the same time we report that variously phosphorylated forms of HMGA1a protein are also mono-methylated.

Among nonhistone nuclear proteins of mammalian cells, a family of three proteins called HMGA1a, HMGA1b, and HMGA2 (previously termed HMGI, HMGY, and HMGI-C, respectively)[1] have aroused great interest in many laboratories over the last few years, due to the variety of biological processes in which they are involved (Refs. 1–4 and references therein).

HMGA1a and HMGA1b are very similar, differing by only 11 amino acid residues, because they are the splicing products of the same gene (5), whereas HMGA2 is the product of another gene (6). These proteins are composed of about a hundred residues, and all contain three characteristic short basic regions, called AT-*hooks*, that interact with AT-*rich* stretches of DNA in the minor groove (7, 8).

A property that is characteristic not only of the three proteins under discussion, but also of other HMG proteins, is a C-terminal domain having a very high content of acidic residues (1, 9). In HMGA1a, HMGA1b, and HMGA2 the acidic C-terminal domain is constitutively phosphorylated *in vivo* by CK2 (10–12), but additional sites for cell-cycle-dependent phosphorylation by other kinases have also been reported (13–15).

High levels of HMGA1a, HMGA1b, and HMGA2 proteins have been found in embryonic cell lines or tissues as well as in neoplastic cell lines and tumors, but they are absent or expressed at very low levels in normal cells (1, 12, 16–23). These findings support the hypothesis that the three proteins could be linked to growth, differentiation, and neoplastic transformation.

Given the importance of HMGA1a, HMGA1b, and HMGA2 proteins in chromatin dynamics, we addressed the question of chromatin assembly/disassembly by studying their post-translational modifications during apoptosis, a process where profound changes in chromatin structure occur. Apoptosis has been induced in four leukemic cell lines (HL60, K562, NB4, and U937), and the study has been focused on the HMGA1a protein, that is the prevalently spliced form in this type of cells. For the first time we demonstrate that the HMGA1a protein is subject to both hyper-phosphorylation and de-phosphorylation during apoptosis, the two events being time-dependent. We show that a new and fully de-phosphorylated form of HMGA1a is the main form present in the highly condensed chromatin of apoptotic bodies, whereas hyper-phosphorylation is characteristic of early stages of apoptosis and is related to the onset of DNA fragmentation and nuclear envelope degradation. We also report for the first time that HMGA1a is mono-methylated during apoptosis and that this modification is independent of the degree of phosphorylation.

EXPERIMENTAL PROCEDURES

Cell Cultures and Treatments—The four cell lines HL60, K562, NB4, and U937 were cultured in RPMI 1640 medium containing 10% fetal bovine serum at 37 °C. Induction of apoptosis was carried out by three

* This work was supported by grants from Associazione Italiana per la Ricerca sul Cancro, Milano, Italy, by Ministero della Ricerca Scientifica e Tecnologica, Roma, Italy (Grants 9706274625 and 9806279300), by ASI (Grants ARS-99-47 and I/R/129/00), and by the Università di Trieste, Italy. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Both authors contributed equally to this work.

** To whom correspondence should be addressed: Università degli Studi di Trieste, Dipartimento di Biochimica, Biofisica e Chimica delle Macromolecole, Via Licio Giorgieri, 1-34127 Trieste, Italy. Tel.: 39-040-6763676; fax: 39-040-6763694; E-mail: giancot@bbcm.univ.trieste.it.

[1] The nomenclature of the high mobility group (HMG) proteins has been recently revised (see the Chromosomal Proteins Nomenclature on the Web). In this report we use the new nomenclature, but we report the old one in parenthesis. The abbreviations used are: HMGA1a, high mobility group A1a (HMGI); HMGA1b, high mobility group A1b (HMGY); HMGA2, high mobility group A2 (HMGI-C); HMGN1, high mobility group N1 (HMG 14); HMGN2, high mobility group N2 (HMG 17); CK2, casein kinase 2; Z-VAD-fmk, *n*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; PCA, perchloric acid; LC-MS, liquid chromatography-mass spectrometry; TIC, total ion counts; p34-Cdc2, p34-cycle-dependent cyclin 2 kinase; Cdk2, cyclin-dependent kinase 2; MARs, matrix attachment regions; SARs, scaffold attached regions; BURs, base-unpairing regions; kbp, kilobase pair(s); HSV-1, herpes simplex virus type-1; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; HPLC, high pressure liquid chromatography.

different procedures. All cell lines were treated with etoposide (VP-16, Calbiochem), 68 μM, for different times to have a considerable amount of apoptotic cells: 4.5 h (HL60, NB4), 24 h (U937), and 48 h (K562). HL60 cells were also treated with 0.1 μg/ml camptothecin (Calbiochem) for 4.5 h following synchronization with 1 μg/ml aphidicolin (Calbiochem) for 15 h (24). U937 cells were also induced to apoptosis by herpes simplex virus type-1 (HSV-1) (25).

The percentage of apoptotic cells was evaluated by double fluorescence emission of cells treated with both propidium iodide (PharMingen) and annexin-V fluorescein isothiocyanate-conjugated (PharMingen) with a FACScan from Becton Dickinson (Franklin Lakes, NJ).

The time course experiment on HL60 cells was carried out with a procedure similar to that of Morana *et al.* (26).

Apoptosis inhibition was carried out by treating HL60 cells with 10 or 25 μM Z-VAD-fmk (Calbiochem) in culture medium for 1 h, after which etoposide 136 μM was added and cells were left to incubate for 30 min. Following etoposide incubation, cells were washed and left in culture medium containing Z-VAD-fmk for another 2 h.

Protein Extraction, Electrophoretic Analysis, and Western Blotting—Total HMG proteins and histone H1 were selectively extracted by treating cells with 5% perchloric acid (PCA) (w/v) as previously reported (17, 18). Electrophoretic analysis was carried out on 15% polyacrylamide gels in acetic acid/urea or in SDS/Tris/Tricine buffer as described previously (12). For Western blot analysis, a rabbit polyclonal antibody raised against an N-terminal peptide of HMGA1a protein was used (23).

DNA Fragmentation Assay—DNA from control or apoptotic cells was extracted essentially according to Zhu and Wang (27). DNA fragments were analyzed on 2% agarose gel in Tris-acetate-EDTA buffer and visualized with ethidium bromide under UV light.

HPLC and LC-MS Measurements—Reverse-phase HPLC chromatography of PCA extracts was carried out with a PerkinElmer Life Sciences apparatus (series 200 LC pump and 785A UV-visible detector) using a Vydac Protein C4 column (2.1 × 150 mm). Protein was eluted by a water/acetonitrile gradient, and the chromatogram was obtained by absorbance detection at 220 nm. At the same time, an aliquot of the eluted solution was directly injected into an interfaced mass spectrometer (PE SCIEX, API 1), which gave an equivalent chromatogram by counting total ions (TIC) that reach the mass detector (28). Search for ion composition was carried out on each TIC peak, and a succession of m/z^+ values was obtained for every protein form contained in that peak. The identification of all successions of m/z^+ allowed to obtain reconstructed mass spectra (±1 Da) that gave the molecular composition of the analyzed peak. The same procedure was employed for the analysis of tryptic fragments, the only difference being that a Waters Delta-Pak C18 column (2 × 150 mm) was used. LC-MS then was used to identify both different forms of the whole protein molecule and different forms of tryptic fragments.

Trypsin Treatment—HPLC-purified HMGA1a (15 μg) from both K562 control and apoptotic cells was treated for 15 h at 37 °C in 50 μl of Tris/HCl, 100 mM, pH 8.5, containing 0.75 μg of sequencing grade trypsin (Roche Diagnostics), and the reaction was stopped by freezing. Thawed tryptic samples were directly injected into the HPLC apparatus interfaced with the mass spectrometer, at room temperature.

RESULTS

An Electrophoretic Retarded Band of HMGA1a Protein Appears in Leukemic Cells during Apoptosis—Protein extracts from four leukemic cell lines (K562, HL60, NB4, and U937) have been analyzed by acetic acid/urea electrophoresis, which showed, in the extract from apoptotic cells, a double band at the migration position of the HMGA1a protein, in comparison with the single band obtained from control cells (Fig. 1*A*). To verify if the retarded band could be due to any new apoptotic form(s) of HMGA1a, the regions comprising HMGN2 and HMGA1a of the acid/urea first dimension (Fig. 1*A*) were analyzed by SDS two-dimensional analysis. The pattern obtained was blotted for Western identification using a polyclonal antibody raised against an N-terminal peptide common to both HMGA1a and HMGA1b (5, 23) (Fig. 1*B*). From the result reported in Fig. 1*B*, it is evident that the antibody identified both *normal* HMGA1a and HMGA1b proteins as well as the retarded band, which can consequently be considered as one or more new forms of HMGA1a appearing during apoptosis.

Both Hyper-phosphorylation and De-phosphorylation of

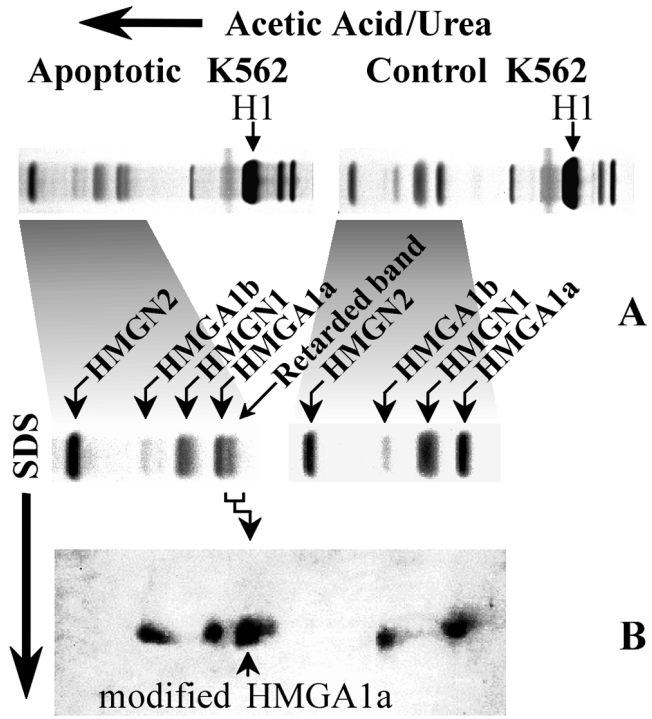

FIG. 1. **Electrophoretic and Western analyses of PCA-extracted proteins from K562 cells show new form(s) of HMGA1a protein during apoptosis.** *A*, first-dimension electrophoretic analysis in acetic acid/urea 15% polyacrylamide gels of PCA-extracted protein samples from control and etoposide-induced apoptotic K562 cells (Coomassie Blue staining). *B*, the regions comprising HMGN2 and HMGA1a proteins in the first dimension acetic acid/urea were analyzed in a second SDS dimension, then blotted for Western analysis. Protein identification was carried out using a polyclonal antibody raised against an N-terminal peptide common to both HMGA1a and HMGA1b and proteins detected by the ECL + Plus procedure (Amersham Pharmacia Biotech). Before carrying out the second SDS electrophoretic analysis, to prevent protein mixing by diffusion in the course of the run, HMGA1a and the retarded band have been separately excised and a piece of blank gel interposed between them.

HMGA1a Takes Place in Leukemic Cells during Apoptosis—To understand the nature of the HMGA1a form(s) generated during apoptosis, the same extracts of Fig. 1 have been separated by HPLC, and the eluted protein was detected by both UV absorption (Fig. 2, *A* and *B*) and LC-MS total ion count (Fig. 2, *C* and *D*). LC-MS molecular masses of HMGA1 components have been determined (Fig. 2, *E* and *F*): control K562 cells contain two forms of HMGA1a protein having masses of 11,745.9 and 11,826.0 Da, respectively. The predicted mass of human HMGA1a protein without modifications (*i.e.* 106 amino acid residues, no initial methionine) is 11,544.8 Da, which becomes 11,586.8 Da if N-terminal acetylation at the first serine is assumed, as found in preceding studies. It is possible to infer this also from both the mass spectrometric data recently reported by Reeves and coworkers (11, 19, 29) and by tryptic digestion experiments as reported below. Because one phosphate group increases the mass of a protein by 80 Da, the two forms of HMGA1a protein found in control K562 can be considered as the di-phosphorylated (found, 11,745.9 Da; calculated, 11,746.8 Da) and the tri-phosphorylated (found, 11,826.0 Da; calculated, 11,826.8 Da) modifications. This result is in agreement with preceding data that reported *in vitro* and *in vivo* phosphorylation of the three last serines at the C-terminal of both HMGA1a and HMGA1b proteins (*i.e.* serines 98, 101, and 102) as consensus sites for CK2 (10–12). The extract from K562 apoptotic cells contains, in addition to the di-phosphorylated and tri-phosphorylated forms present in the control cells,

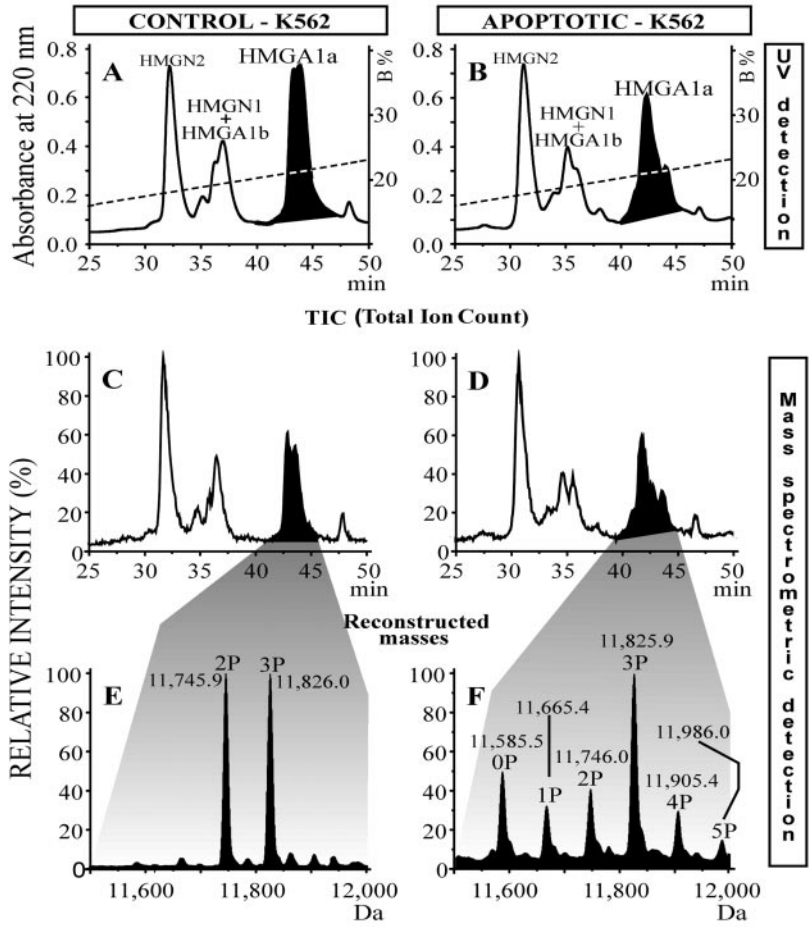

FIG. 2. **HPLC elution profiles and reconstructed masses of PCA-extracted proteins from K562 cells show changes in the degree of phosphorylation of HMGA1a protein during apoptosis.** Reverse phase HPLC elution profiles of PCA extracted proteins from control (*A*, *C*) and etoposide-induced apoptotic (*B*, *D*) K562 cells detected as absorbance at 220 nm (*A*, *B*) or as total ion count (*C*, *D*). *Dotted lines* in *A* and *B* refer to the percentage of acetonitrile (solvent B) in the gradient used for the HPLC elution. Proteins contained in each peak are labeled in *A* and *B*. Composition of HMGA1a peaks from control (*E*) and apoptotic (*F*) K562 cells was obtained by mass spectrometry, and results are reported as reconstructed masses (*Da*). In *E* and *F* the identified mass values (*Da*) are reported; from these values the number of phosphate groups (*OP*, *1P*, *2P*, *3P*, *4P*, and *5P*) borne by HMGA1a protein were deduced.

other forms whose molecular masses are consistent with hyper-phosphorylated forms (four phosphate groups, 11,905.4 Da and five phosphate groups, 11,986.0 Da) and with de-phosphorylated forms (one phosphate group, 11,665.4 Da and zero phosphate group, 11,585.5 Da). Assuming that LC-MS peak intensity is approximately proportional to the amount of protein, it is possible to estimate that the sample from K562 control cells of Fig. 2*E* contains about 50% of di-phosphorylated HMGA1a protein and about 50% of tri-phosphorylated, whereas in the apoptotic sample (Fig. 2*F*) the main form is tri-phosphorylated, all other forms being present at lower percentage.

LC-MS data shown in Fig. 3*A* indicate that in all four analyzed cell lines the main forms of HMGA1a are those that are di-phosphorylated and tri-phosphorylated. The same data from apoptotic cells (Fig. 3*B*) show both hyper-phosphorylated and de-phosphorylated forms. However, inspection of the data indicates that the degree of phosphorylation is not only cell type-dependent but also related to the percentage of apoptotic cells present in the analyzed cell sample. In fact, di-phosphorylated and tri-phosphorylated forms of HMGA1a are the main molecules in both HL60 and NB4 cells, which contain 36% of apoptotic cells, whereas in K562, having 77% of apoptotic cells, the tri-phosphorylated molecule is the main form accompanied by a considerable amount of de-phosphorylated forms, and in U937, containing 97% of apoptotic cells, the main form of HMGA1a is without phosphate. Thus the data of Fig. 3 allow us to conclude that, in the control cell lines studied, HMGA1a is mainly present as the di- and tri-phosphorylated forms. During apoptosis induced by etoposide, two different processes take place, one hyper-phosphorylates the di- and tri-phosphorylated forms, the other de-phosphorylates all phosphorylated molecules. Very similar results were obtained for camptothecin-induced HL60 cells (data not shown). Fig. 3 also shows that, on the right side of most of the variously phosphorylated peaks, there is a shoulder that in U937 apoptotic cells resolves into a definite peak having a constant increase of about 14 Da. It seems likely that this increase is due to methylation concomitant with the phosphorylation/de-phosphorylation processes. Methylation of HMGA1b protein (the other spliced protein) has also been reported by Reeves and coworkers (29) in MCF-7 cells in which up to four methyl groups have been identified by tryptic peptide mapping. No methylation has, however, been reported previously for HMGA1a that on the contrary results mono-methylated in the cell lines of this study.

To obtain further confirmation that the identified forms of HMGA1a protein are due to phosphorylation, HPLC-purified protein samples from both control and apoptotic K562 cells were alkaline phosphatase-treated. This removed phosphate groups from all phosphorylated forms of HMGA1a and gave only the unphosphorylated, N-terminally acetylated molecule, having molecular masses of 11,586.2 Da (control) and 11,585.9 Da (apoptotic) (data not shown).

Tryptic Digestion of HMGA1a from both Control and Apoptotic K562 Cells: Identification of Phosphorylated Peptides—Tryptic fragments of HPLC-purified HMGA1a from both control and apoptotic K562 cells were analyzed by LC-MS spectrometry. 26 different peptides, spanning the entire HMGA1a sequence, have been identified and are reported in Fig. 4*A*. For each of the identified peptides, a search for the phosphorylated forms was carried out in both control and apoptotic samples, and the following conclusions were reached: (i) in both control and apoptotic cells N-terminal fragments were acetylated; (ii) in control K562 cells the main phosphorylated fragment was the 88- to 106-animo acid peptide present as the

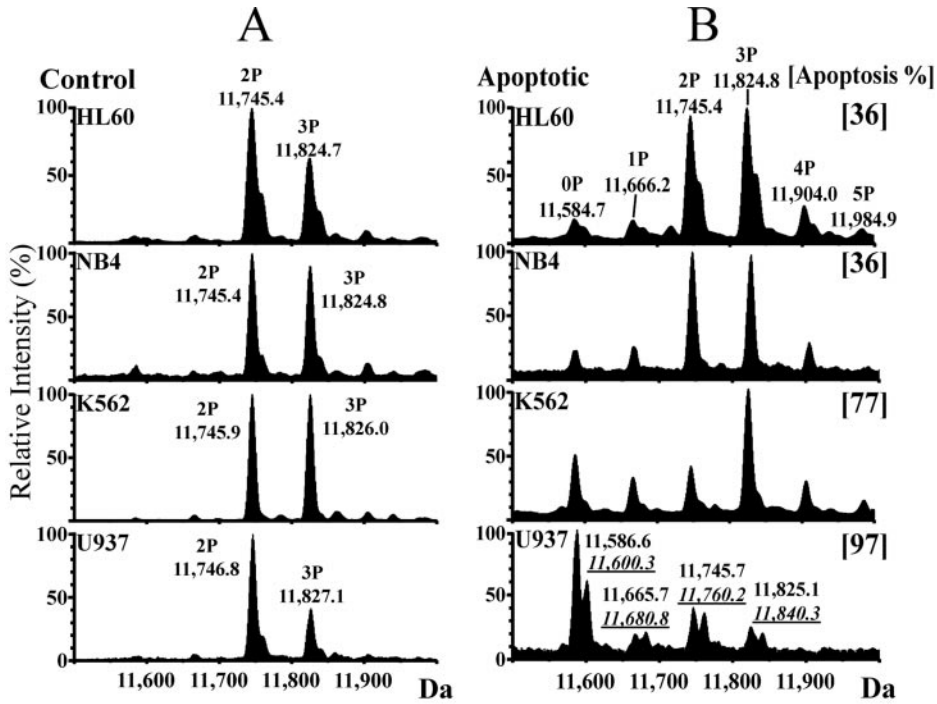

FIG. 3. **Both hyper-phosphorylated and de-phosphorylated forms of HMGA1a protein are present in different apoptotic leukemic cell lines.** Proteins from both control (*A*) and etoposide-induced apoptotic (*B*) leukemic cell lines (HL60, NB4, K562, and U937) were PCA-extracted and LC-MS-analyzed. Reconstructed masses were obtained for the HMGA1a peak of each extract. *A*, mass values (*Da*) of bi-phosphorylated (*2P*) and tri-phosphorylated (*3P*) forms are shown. These forms resulted as constitutive modifications of HMGA1a protein in the four studied cell lines. *B*, reconstructed mass values (*Da*) are reported for each sample together with the deduced number of phosphate groups (from *OP* up to *5P*). The percentage of apoptotic cells was obtained by FACScan analysis and shown in *square brackets*. *Underlined* mass values refer to phosphorylated HMGA1a forms that are also methylated.

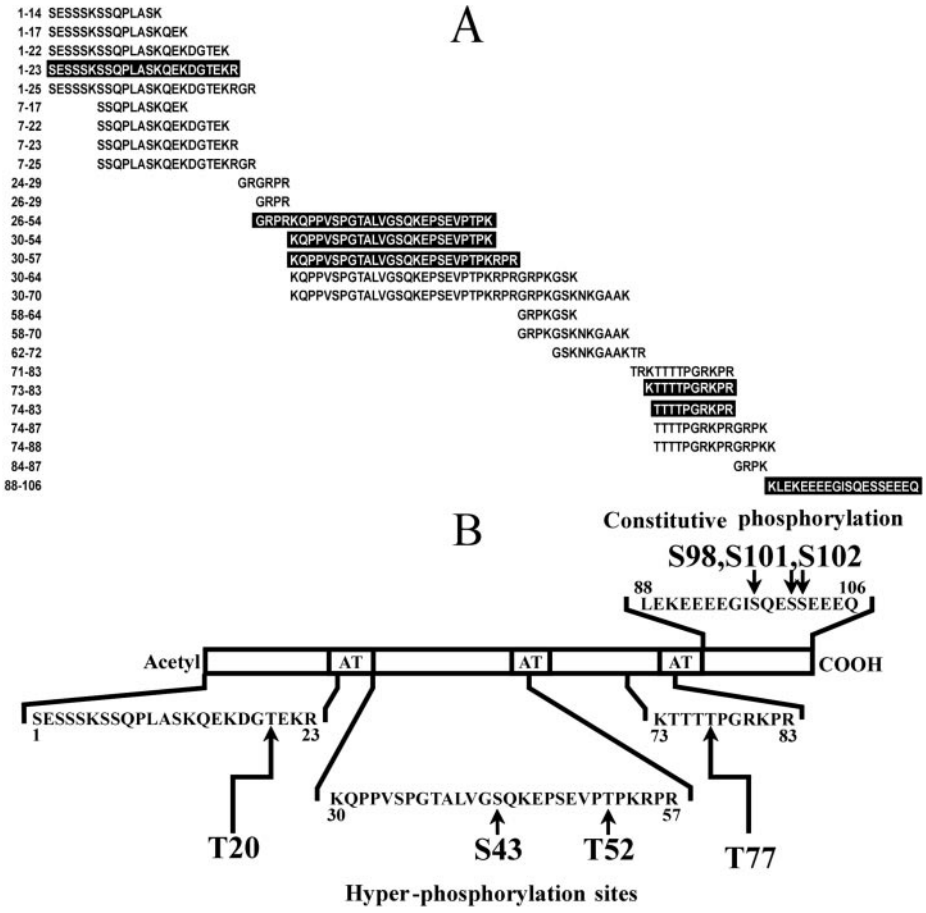

FIG. 4. **Constitutive and hyper-phosphorylation suggested sites of HMGA1a protein in leukemic cells.** *A*, tryptic fragments of HMGA1a protein identified by LC-MS of HPLC-purified protein samples from both control and apoptotic K562 cells. Identified phosphorylated fragments (1–23, 25–54, 30–54, 30–57, 73–83, 74–83, 88–106) are evidenced. *B*, the results of LC-MS analyses on whole molecule and tryptic fragments from both control and etoposide-induced apoptotic K562 cells are summarized. The sequences of the identified phosphorylated peptides are shown, and suggested modified residues are indicated. *AT*, AT-*hooks*.

di- or tri-phosphorylated forms; (iii) in apoptotic K562 cells the 88- to 106-amino acid peptide was mainly unphosphorylated; one phosphate group has been detected in fragments 1–23, 73–83, and 74–83; mono- and di-phosphorylated forms have been identified for the fragments 26–54, 30–54, and 30–57. This means that the additional phosphates in the hyper-phosphorylated forms are located inside the protein molecule and that the de-phosphorylation process starts by removing the phosphate groups from the C-terminal side.

On the basis of literature data (10–14, 29, 30), we have drawn the scheme shown in Fig. 4*B*, where all the putative sites for hyper-phosphorylation of the HMGA1a protein are shown together with the constitutive C-terminal modified serines. Data summarized in Fig. 4*B* indicate that it could be

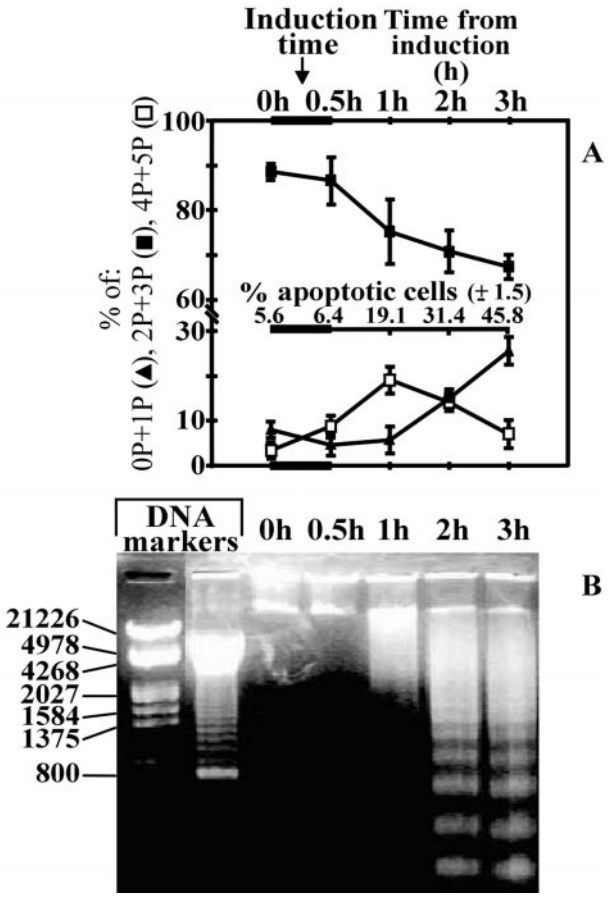

FIG. 5. **Time course of etoposide-induced HL60 cells: Hyper-phosphorylation of HMGA1a protein precedes its de-phosphorylation and is related to initial DNA fragmentation, whereas de-phosphorylation accompanies formation of inter-nucleosomal fragments.** *A*, HL60 cells were treated with 136 μM etoposide for 30 min and after washing left to proceed toward apoptosis quantified by FACScan (%).The degree of phosphorylation of HMGA1a protein was evaluated at the beginning of the experiment (*Control*, 0 h), after 30 min of etoposide treatment, and after 1, 2, and 3 h from washing. Phosphorylated forms are reported as follows: 0P + 1P (▲); 2P + 3P (■); 4P + 5P (□). All reported values are the results of three independent experiments. *B*, DNA fragmentation in the time course of apoptosis of HL60 cells was analyzed on 2% agarose gel and visualized with ethidium bromide.

possible to find HMGA1a molecules bearing up to seven phosphate groups at a time. LC-MS data shown in Fig. 3*B* clearly indicate up to five phosphate groups; however, very low amounts of HMGA1a having six or seven phosphate groups have been detected (data not shown). In any case, it must be pointed out that phosphorylation and de-phosphorylation processes could partially overlap.

Hyper-phosphorylation of HMGA1a Precedes its De-phosphorylation during Apoptosis—From preceding data, results show that the hyper-phosphorylation of HMGA1a and its de-phosphorylation should be related to different events that take place at different times during apoptosis and concern different regions of the molecule. To clarify this point, we have carried out a time course at the beginning of apoptotic induction. To this end, HL60 cells have been induced with 136 μM etoposide for 30 min, then the drug was removed and washed cells were left to proceed toward apoptosis far up to 3 h. Proteins were PCA extracted and LC-MS analyzed at four different times (after 30-min treatment and 1, 2, and 3 h from washing). In Fig. 5*A*, the result of the time course is reported grouping together de-phosphorylated HMGA1a forms (*0P + 1P*), di- and tri-phosphorylated forms (*2P + 3P*), and hyper-phosphorylated forms (*4P + 5P*). The time course shows that hyper-phosphorylation of HMGA1a starts as soon as etoposide treatment begins, reaching a maximum 1 h after drug removal when the forms having one or no phosphate groups begin to appear. Phosphate groups introduced at an early stage of the apoptotic process are added to the two or three phosphate groups already present at the C-terminal of the molecule and produce the 4P and 5P (or more phosphorylated) modifications. De-phosphorylation starts with the removal of the *constitutive* phosphate groups, *i.e.* C-terminal phosphates (serines 98, 101, and 102) probably when phosphorylation inside the HMGA1a molecule is still active. This is the reason why di- and tri-phosphorylated forms can have a *constitutive* origin (*i.e.* control cells or cells at very early stages of the process) or an apoptotic origin, deriving from the removal of *constitutive* phosphate groups from molecules bearing four or five (or more) phosphates (*i.e.* hyper-phosphorylated forms). Further support for the hypothesis that hyper-phosphorylation of HMGA1a protein should be related to the early events of the apoptotic process derives from the electrophoretic analysis of the DNA reported in Fig. 5*B* where the maximum level of HMGA1a phosphorylation observable after 1 h does correspond to high molecular weight digested DNA, whereas the subsequent de-phosphorylation process correlates with the formation of low molecular weight DNA fragments.

Caspase Inhibition Evidences HMGA1a Hyper-phosphorylation as an Early Event of Apoptosis—It is well known that topoisomerase I and II poisons such as etoposide and camptothecin cause apoptosis through cell cycle block and activation of a group of cysteine proteases called caspases (31–35). Inhibition of caspase activity slows down or stops the advance of the apoptotic process and could allow one to obtain information on its very early events. Therefore, we used the caspase inhibitor Z-VAD-fmk (35–38) on HL60 etoposide-treated cells and both DNA fragmentation and HMGA1a phosphorylation were analyzed. To understand to which nuclear morphologic change phosphorylation of HMGA1a protein could be related during apoptosis, we carried out microscopic observation of aliquots of the same cells stained with 4,6-diamidino-2-phenylindole. Fig. 6*A* shows that using 25 μM Z-VAD-fmk (*lane 2*) DNA fragmentation is almost blocked, and small differences in the chromatin status are observable comparing representative cells from the control to the 25 μM Z-VAD-fmk-treated cells (Fig. 6*B*, *1* and *2*). Moreover, mass data (Fig. 6*C*, *2*) show that hyper-phosphorylated forms (*4P* and *5P*) have been produced. If a lower Z-VAD-fmk concentration is used (10 μM, Fig. 6*A*, *lane 3*), an increased digestion of DNA is observable, but it is lower, however, than the substantial nucleosomal cleavage reached after 2 h of etoposide treatment in the absence of Z-VAD-fmk (Fig. 6*A*, *lane 4*). Consistently, an increased level of hyper-phosphorylation has been detected in this protein sample: note that the *4P peak* in *mass spectrum 3* (Fig. 6*C*) has the same intensity as the *2P peak*. At this stage, in which a clear beginning of DNA fragmentation is seen (Fig. 6*A*, *lane 3*), chromatin starts to condense showing a typical alteration of nuclear organization (Fig. 6*B*, *3*) but not yet forming well defined apoptotic bodies as those observed after reported 2 h treatment of cells without Z-VAD-fmk (Fig. 6*B*, *4*). In this last sample both hyper-phosphorylation and de-phosphorylation are observable (Fig. 6*C*, *4*), because only about 30% of cells are definitively apoptotic (see Fig. 5*A*), while the remaining cells are still running through the preceding steps of the apoptotic process. It is then obvious that protein mixtures extracted from such a composite system would contain both hyper-phosphorylated forms (*i.e.* early stages of apoptosis) and de-phosphorylated forms (*i.e.* late stage of apoptosis). In conclusion, we think that inhibition experiments using Z-VAD-fmk clearly show that hyper-phos-

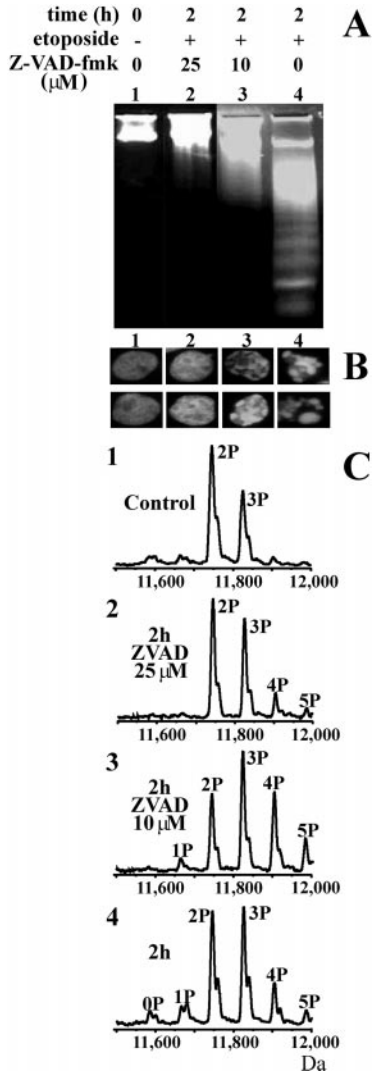

FIG. 6. **Use of the caspase inhibitor Z-VAD-fmk slows down the etoposide-induced apoptosis of HL60 cells and allows observation of hyper-phosphorylation as an early event associated with initial chromatin condensation.** HL60 cells were preincubated with the caspase inhibitor Z-VAD-fmk for 1 h, then 136 μM etoposide was added for 30 min and, after washing, cells were left for other 2 h in the presence of the inhibitor. *1*, HL60 control; *2*, Z-VAD-fmk (25 μM); *3*, Z-VAD-fmk (10 μM); *4*, HL60 apoptotic control sample (*i.e.* 136 μM etoposide, no inhibitor). *A*, DNA fragmentation was determined by electrophoresis on 2% agarose gel, and bands were visualized by ethidium bromide. *B*, under each electrophoretic pattern microscope images of 4,6-diamidino-2-phenylindole-stained representative nuclei are shown. *C*, LC-MS mass data (reconstructed masses) of HMGA1a protein from each cell sample are reported, and the number of phosphate groups is indicated.

phorylation of HMGA1a is an early apoptotic event and, at the same time, demonstrate that the level of phosphorylation of this protein is truly linked to the apoptosis of leukemic cells.

U937 Leukemic Cells Induced to Apoptosis by Herpes Simplex Virus 1 (HSV-1) Show Degrees of HMGA1a Phosphorylation Similar to That Found in Cells Induced by Nonviral Agents—To verify that the degree of phosphorylation of HMGA1a during apoptosis is independent of the agent capable of triggering the signal pathway that leads to apoptosis, we analyzed protein extracts from apoptotic U937 cells induced by herpes simplex virus type-1 (HSV-1) (25). In Fig. 7, LC-MS data of the HMGA1a protein from U937 apoptotic cells are compared with those of control mock cells, and it is possible to see that a massive de-phosphorylation of HMGA1a takes place during apoptosis. Moreover, it is noteworthy that methylation

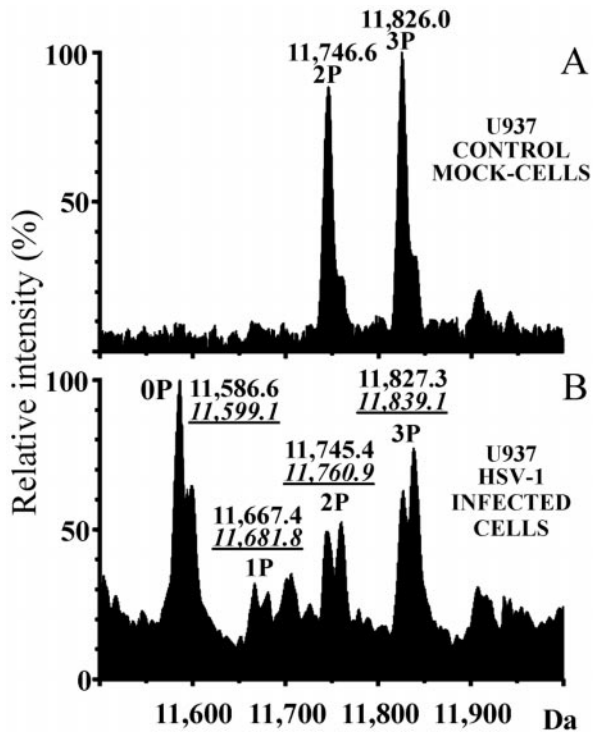

FIG. 7. **LC-MS experiments demonstrate that U937 cells induced to apoptosis by HSV-1 contain de-phosphorylated forms similar to those found in cells induced by nonviral agents.** Proteins were selectively PCA-extracted from both control (*A*) and virus-induced U937 cells (*B*) and LC-MS-analyzed. Reconstructed masses were obtained for the HMGA1a peak of each extract. Mass values (*Da*) of variously phosphorylated forms are shown (*0P*, *1P*, *2P*, *3P*) together with the values (*underlined*) of the mono-methylated species.

of HMGA1a as revealed in drug-induced leukemic cells (Fig. 3*B*) is also observable in virus-induced U937 cells. We conclude that both alteration of phosphorylation and methylation are related to the apoptotic process *per se* rather than to the agent used to induce apoptosis.

DISCUSSION

This report concerns the study of post-translational modifications of HMGA1a protein during apoptosis induced in four leukemic cell lines (HL60, K562, NB4, and U937). These cell lines show constitutive expression of the two proteins HMGA1a and HMGA1b, whereas the HMGA2 has not been detected; HMGA1a is the predominant species as compared with HMGA1b. In fact, from Coomassie Blue-stained electrophoretic patterns we evaluated that the ratio HMGA1a/HMGA1b in both HL60 and K562 cells is about 10:1 (data not shown). The present paper deals only with the most abundant protein (*i.e.* HMGA1a), but a similar behavior was ascertained also for HMGA1b during apoptosis, from both a careful inspection of the Western analysis shown in Fig. 1 and mass data not shown. Electrophoretic patterns have been also used to evaluate the molecular ratio between histone H1 and HMGA1a that indicated a ratio of about 20:1 in control HL60 cells (data not shown). This means that in these cells there is on average one HMGA1a molecule for every 20 nucleosomes, assuming about one histone molecule is bound to the linker DNA of each nucleosome (39).

What could the function for this high amount of HMGA1a protein be? It is not conceivable that it is entirely involved in the formation of specific protein entities that regulate transcription of specific genes.

Involvement of HMGA1a protein has been reported not only at promoter regions of specific genes, where a limited amount of

protein appears to be necessary, but also at more global nuclear structures related to higher order chromatin bound to the nuclear matrix and forming distinct nucleoproteic loops. Such structures have been called MARs (matrix attachment regions) or SARs (scaffold attached regions), and it has been shown that they contain specialized AT-*rich* DNA regions with high unwinding aptitude termed BURs (base-unpairing regions) (40–42). BURs specifically bind HMGA1a and HMGA1b proteins (43), although this should involve DNA regions different from those bound to histone H1. In fact, it has been reported that HMGA1a displaces histone H1 from chromatin and nuclease-sensitive chromatin releases HMGA1a, HMGA1b, and HMGA2 proteins but not histone H1 (41, 44, 45). At the same time, in an immunocytochemical study we have demonstrated that topoisomerase IIα and HMGA1a-HMGA1b proteins colocalize in the interphase nucleus of HeLa cells (46). The mutually exclusive localization of histone H1 and HMGA1a protein could account for a different involvement of these two factors in the processes of chromatin condensation/de-condensation, which are in turn related to the phosphorylation of these proteins, both well known substrates for cyclin-dependent kinases p34-Cdc2 and Cdk2 (13, 14, 47). H1 phosphorylation due to these kinases appears to be related to mitosis (48–52) rather than to apoptosis, whereas data from this report indicate a relationship of HMGA1a phosphorylation with apoptosis and preceding data associated HMGA1a phosphorylation by p34-Cdc2 with mitosis (13, 14).

As reported under "Results," in addition to the C-terminal constitutive phosphates due to CK2, HMGA1a protein bears other phosphate groups that could derive from the action of different kinases. The constitutive C-terminal phosphorylation due to CK2 is not directly involved in DNA binding alteration, although an indirect effect could be elicited by affecting protein tertiary structure (53). On the other hand, hyper-phosphorylation of HMGA1a protein could be related to its displacement from DNA; this results in a more open chromatin structure that is a more accessible substrate for nucleases, which produce large DNA fragments at the very early stages of apoptosis, together with lamin degradation. DNA fragmentation may have the release of MARs from nuclear scaffold as a first step followed by chromatin unfolding that allows progressive DNA digestion. Large DNA fragments (20–50 kbp) should thus be related to HMGA1a hyper-phosphorylation and initial chromatin condensation as shown in Fig. 6. Further DNA fragmentation generates DNA ladders that are characteristic of the highly condensed chromatin of apoptotic bodies. This last event is related to de-phosphorylated HMGA1a protein as shown in Fig. 3*B* for U937 cells. It is worthwhile to mention that at neither the initial stage of apoptosis nor the later stage of apoptotic bodies formation is there loss of HMGA1a protein (data not shown). We wish to point out that the phosphorylation/de-phosphorylation process evidenced for HMGA1a involves the total amount of protein present in the cell and, consequently, chromatin as a whole. Very recently, phosphorylation of both histones H2B and H2AX has been reported during apoptosis of HL60 cells induced by etoposide (54, 55). Phosphorylation of these histones, detected by ^{32}P autoradiography, concerns only a fraction of the total protein (about 5–10% in the case of histone H2B) and has been related to the early phase of DNA fragmentation during apoptosis. These results are consistent with our data on hyper-phosphorylation of HMGA1a as an early event of apoptosis in which some histones, HMGA1a and HMGA1b proteins, and other proteins of the nuclear scaffold are substrates of a programmed process of phosphorylation that initiates cell death. However, apoptotic hyper-phosphorylation of HMGA1a is a quantitatively more important event both for the number of new phosphorylation sites (at least four) and the involvement of the entire amount of protein. Moreover, the following massive de-phosphorylation, leading to a completely de-phosphorylated form (here observed for the first time), could be one of the events required for an irreversible chromatin condensation, just as the final committed apoptosis is. The de-phosphorylation of HMGA1a protein parallels another de-phosphorylation process recently described for H1 histone during apoptosis of HL60 cells. In fact, Kratzmeier *et al*. (56) reported that histone H1 sub-types become rapidly de-phosphorylated upon apoptosis induction and interpret this phenomenon as an important event for the process of chromatin condensation and/or chromatin fragmentation. Our data are consistent with the apoptotic de-phosphorylation of histone H1 and demonstrate, for the first time, that phosphorylation/de-phosphorylation of HMGA1a is involved in the apoptotic process and that HMGA1a could be considered as a structural element in the chromatin of leukemic cells. Moreover, a characteristic mono-methylation of HMGA1a has been evidenced that could reserve further information on stress-exposed cells.

We have demonstrated that, at least in the cells studied, alteration in the degree of phosphorylation of HMGA1a is independent of the agent that induces apoptosis, *i.e.* drug or virus. However, it is necessary to recall that HMGA1a protein is not present or present at very low levels in normal cells, whereas its expression is increased in transformed cells. Therefore, the link between alteration of constitutive HMGA1a phosphorylation, chromatin, and apoptosis should refer only to cells characterized by high levels of this protein. If, on one hand, this limits the extension of the observed phenomenon to all cells, on the other hand, it constitutes an interesting difference between normal and neoplastic cells, which could provide a possible way to induce or at least to influence apoptosis only in the latter. This aspect of the question, concerning the differences of nuclear organization and matrix protein composition in cancer and normal cells, is a promising area for application in both cancer diagnosis and prognosis (57).

REFERENCES

1. Bustin, M., and Reeves, R. (1996) *Prog. Nucleic Acids Res. Mol. Biol.* **84,** 35–100S. O.
2. Bustin, M. (1999) *Mol. Cell. Biol.* **19,** 5237–5246
3. Jansen, E., Petit, M. M. R., Shoenmakers, E. F. P. M., Torik, A. Y., and Van de Ven, W. J. M. (1999) *Gene Ther. Mol. Biol.* **3,** 387–395
4. Brunetti, A., Manfioletti, G., Chiefari, E., Goldfine, I. D., and Foti, D. (2001) *FASEB J*. **15,** 492–500
5. Johnson, K. R., Lehn, D. A., Elton, T. S., Barr, P. J., and Reeves, R. (1988) *J. Biol. Chem.* **263,** 18338–18342
6. Manfioletti, G., Giancotti, V., Bandiera, A., Buratti, E., Sautière, P., Cary, P., Crane-Robinson, C., and Goodwin, G. H. (1991) *Nucleic Acids Res.* **19,** 6793–6797
7. 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
8. Mantovani, F., Covaceuszach, S., Rustighi, A., Sgarra, R., Heath, C., Goodwin, G. H., and Manfioletti, G. (1998) *Nucleic Acids Res.* **26,** 1433–1439
9. Johns, E. W. (1982) *The HMG Chromosomal Proteins*, Academic Press, New York
10. Palvimo, J., and Linnala-Kankkunen, A. (1989) *FEBS Lett.* **257,** 101–104
11. Ferranti, P., Malorni, A., Marino, G., Pucci, P., Goodwin, G. H., Manfioletti, G., and Giancotti, V. (1992) *J. Biol. Chem.* **267,** 22486–22489
12. Giancotti, V., Bandiera, A., Buratti, E., Fusco, A., Marzari, R., Coles, B., and Goodwin, G. H. (1991) *Eur. J. Biochem.* **198,** 211–216
13. Reeves, R., Langan, T. A., and Nissen, M. S. (1991) *Proc. Natl. Acad. Sci.* U. S. A. **88,** 1671–1675
14. Nissen, M. S., Langan, T. A., and Reeves, R. (1991) *J. Biol. Chem.* **266,** 19945–19952
15. Schwanbeck, R., and Wisniewski, J. R. (1997) *J. Biol. Chem.* **272,** 27476–27483
16. Giancotti, V., Berlingieri, M. T., Di Fiore, P. P., Fusco, A., Vecchio, G., and Crane-Robinson, C. (1985) *Cancer Res.* **45,** 6051–6057
17. 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
18. 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
19. 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
20. 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
21. Zhou, X., and Chada, K. (1998) *Keio J. Med.* **47,** 73–77
22. 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
23. 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
24. Del Bino, G., Lassota, P., and Darzynkiewicz, Z. (1991) *Exp. Cell Res.* **193,** 27–35
25. Mastino, A., Sciortino, M. T., Medici, M. A., Perri, D., Ammendolia, M. G., Grelli, S., Amici, C., Pernice, A., and Guglielmino, S. (1997) *Cell Death Differ.* **4,** 629–638
26. Morana, S. J., Wolf, C. M., Li, J., Reynolds, J. E. Brown, M. K., and Eastman, A. (1996) *J. Biol. Chem.* **271,** 18263–18271
27. Zhu, N., and Wang, Z. (1997) *Anal. Biochem.* **246,** 155–158
28. Wilm, M., and Mann, M. (1996) *Anal. Chem.* **68,** 1–8
29. Banks, G. C., Li, Y., and Reeves, R. (2000) *Biochemistry* **39,** 8333–8346
30. Xiao, D.-M., Pak, J. H., Wang, X., Sato, T., Huang, F. L. Chen, H.-C., and Huang, K.-P. (2000) *J. Neurochem.* **74,** 392–399
31. Froelich-Ammon, S. J, and Osheroff, N. (1995) *J. Biol. Chem.* **270,** 21429–21432
32. Martins, L. M., Mesner, P. W., Kottke, T. J., Basi, G. S., Sinha, S., Tung, J. S., Svingen, P. A., Madden, B. J., Takahashi, A., McCormick, D. J., Earnshaw, W. C., and Kaufman, S. H. (1997) *Blood* **90,** 4283–4296
33. Martins, L. M., Kottke, T. J., Kaufman, S. H., and Earnshaw, W. C. (1998) *Blood* **92,** 3042–3049
34. Widmann, C., Gibson, S., and Johnson, G. L. (1998) *J. Biol. Chem.* **273,** 7141–7147
35. Sun, X.-M., MacFarlane, M., Zhuang, J., Wolf, B. B., Green, D. R., and Cohen, G. M. (1999) *J. Biol. Chem.* **274,** 5053–5060
36. Pronk, G. J., Ramer, K., Amiri, P., and Williams, L. T. (1996) *Science* **271,** 808–810
37. Slee, E. A., Zhu, H., Chow, S. C., MacFarlane, M., Nicholson, D. W., and Cohen, G. M. (1996) *Biochem. J.* **315,** 21–24
38. Shao, R.-G., Cao, C.-X., and Pommier, Y. (1997) *J. Biol. Chem.* **272,** 31321–31325
39. Wolffe, A. (1998) *Chromatin, Structure and Function*, Academic Press, New York
40. Käs, E., and Laemmli, U. *K. (1992) EMBO J.* **11,** 705–716
41. Zhao, K., Käs, E., Gonzales, E., and Laemmli, U. K. (1993) *EMBO J.* **12,** 3237–3247
42. Göhring, F., and Fackelmayer, F. O. (1997) *Biochemistry* **36,** 8276–8283
43. Liu, W.-M., Guerra-Vladusic, F. K., Kurakata, S., Lupu, R., and Kohwi-Shigematsu, T. K. (1999) *Cancer Res.* **59,** 5695–5703
44. Giancotti, V., Bandiera, A., Sindici, C., Perissin, L., and Crane-Robinson, C. (1996) *Biochem. J.* **317,** 865–870
45. Hill, D. A., Pedulla, M. L., and Reeves, R. (1999) *Nucleic Acids Res.* **27,** 2135–2144
46. Martelli, A., Riccio, M., Bareggi, R., Manfioletti, G., Tabellini, G., Baldini, G., Narducci, P., and Giancotti, V. (1998) *J. Histochem. Cytochem.* **46,** 863–864
47. Choi, K. S., Eom, Y. W., Kang, Y., Ha, M. J., Rhee, H., Yoon, J.-W., and Kim, S.-J. (1999) *J. Biol. Chem.* **274,** 31775–31783
48. Shibata, K., Inagaki, M., and Ajiro, K. (1990) *Eur. J. Biochem.* **192,** 87–93
49. Shibata, K., and Ajiro, K. (1993) *J. Biol. Chem.* **268,** 18431–18434
50. Marushige, Y., and Marushige, K. (1995) *Anticancer Res.* **15,** 267–272
51. Yoshida, M., Usui, T., Tsujimura, K., Inagaki, M., Beppu, T., and Horinouki, S. (1997) *Exp. Cell. Res.* **232,** 225–239
52. Hendzel, M. J., Nishioka, W. K., Raymond, Y., Allis, C. D., Bezett-Jones, D. P., and Th'ng, J. P. (1998) *J. Biol. Chem.* **273,** 24470–24478
53. Wang, D.-Z., Ray, P., and Boothby, M. (1995) *J. Biol. Chem.* **270,** 22924–22932
54. Ajiro, K. (2000) *J. Biol. Chem.* **275,** 439–443
55. Rogakou, E. P., Nieves-Neira, W., Boon, C., Pommier, Y., and Bonner, W. M. (2000) *J. Biol. Chem.* **275,** 9390–9395
56. Kratzmeier, M., Albig, W., Hänecke, K., and Doenecke, D. (2000) *J. Biol. Chem.* **275,** 30478–30486
57. Bosman, F. T. (1999) *Virchows Arch.* **435,** 391–399

A Link between Apoptosis and Degree of Phosphorylation of High Mobility Group A1a Protein in Leukemic Cells

Francesca Diana, Riccardo Sgarra, Guidalberto Manfioletti, Alessandra Rustighi, Dalisa Poletto, Maria Teresa Sciortino, Antonio Mastino and Vincenzo Giancotti

J. Biol. Chem. 2001, 276:11354-11361.
doi: 10.1074/jbc.M009521200 originally published online January 5, 2001
