Minireview

Nuclear phosphoproteins HMGA and their relationship with chromatin structure and cancer

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Abstract **The structural characteristics of the three nuclear phosphoproteins of the high mobility group A family are outlined and related to their participation in chromatin structure alteration in many biological processes such as gene expression, neoplastic transformation, differentiation, and apoptosis. The elevated expression of these proteins in tumor cells and their post-translational modifications, such as phosphorylation, acetylation and methylation, are discussed and suggested as suitable targets for cancer chemotherapy.**

Keywords: High mobility group A proteins; Methylation; Phosphorylation; Post-translational modification; Chromatin; Epigenetic process; Cancer

1. Introduction: HMGA, nuclear proteins highly expressed following neoplastic transformation

Since the early identification of high mobility group A (HMGA) proteins in rat thyroid cells transformed by retroviruses, we realized that they were strongly related to cancer because their level of expression was much higher in the transformed cells compared to normal cells in which they resulted practically absent [1,2]. We described a group of three proteins (named C, D, and E) identified in neoplastic cells by electrophoretic analyses. These proteins showed a high electrophoretic mobility both in SDS and acetic acid/urea PAGE, similar to that reported by Johns and Goodwin for a group of nuclear proteins characteristic of all mammalian cells and named HMG [3]. This group comprised the HMG1, HMG2, HMG14, and HMG17 proteins. At the same time, Goodwin and co-workers [4] identified two new proteins in the nuclei of rat thymus and of fibroblasts transformed with avian sarcoma virus. These proteins were named I and I′ because of their resemblance to two proteins named Y and I, previously found by Lund et al. [5] in HeLa cells. In the same period, A. Varshavsky's group [6] studied the binding to the DNA of a mammalian protein called α subsequently found to be an HMG protein. When sequence information became available [7,8], this allowed the restriction of the new HMG proteins to a set of only three polypeptides that were almost universally reported as HMGI (previously I, D, α), HMGY (previously Y, E), and HMGI-C (previously I′, C). Recently [9], researchers in this field decided to rationalize the nomenclature of all HMG proteins as previously done for histone nomenclature. Therefore, the three proteins, subject of this mini-review, are grouped together in the HMGA family that comprises HMGA1a, HMGA1b, and HMGA2 (previously HMGI, HMGY, and HMGI-C, respectively).

2. HMGA structure and interaction properties

HMGA1a, HMGA1b and HMGA2 are polypeptides of about one hundred amino acid residues characterized by a modular sequence organization as shown in Fig. 1. Two different genes are responsible for their expression: *HMGA1* and *HMGA2*: the first gene produces both HMGA1a and HMGA1b by alternative splicing, the second gene HMGA2 [7,8]. These proteins have three highly positively charged regions (highlighted in yellow in Fig. 1A) called AT-hooks, since they bind the minor groove of AT-rich DNA stretches. On the contrary, the C-terminus has completely different feature since it contains a high percentage of negatively charged acidic residues (highlighted in red in Fig. 1A). As shown in Fig. 1B, the three AT-hooks are differently spaced along the protein molecules resulting in an interactive modular system constituted by a set of three proteins able to establish interactions with differently spaced AT-rich DNA regions. The HMGA proteins show an unusual capability to bind other nuclear

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Abbreviations: ERCC1, excision repair cross-complementing group 1; HIV-1, immunodeficiency virus type 1; HMGA, high mobility group A proteins; HPLC, high performance liquid chromatography; HPV 18, human papilloma virus 18; IFN-β, interferon-β; IL-2Rα, interleukin-2 receptor-α; IR, insulin receptor; LC-MS, liquid chromatography-mass spectrometry; MARs/SARs, matrix/scaffold associated regions; PTMs, post-translational modifications; SRF, serum response factor; TF, transcription factor

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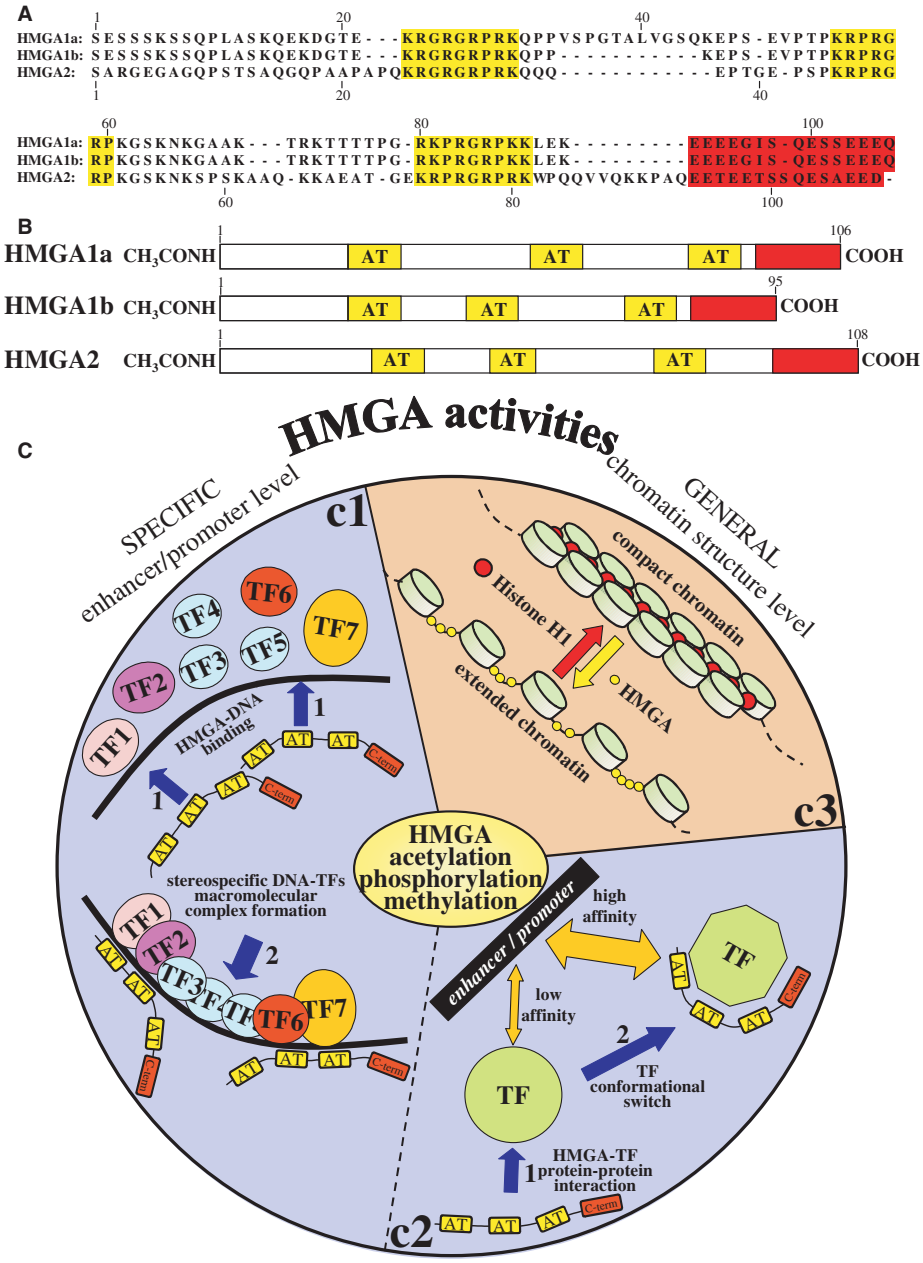


Fig. 1. Primary structure and some proposed functions of human HMGA proteins in the nucleus. (A) Amino acid residues sequences of HMGA1a, HMGA1b, and HMGA2 proteins. AT-hooks (AT) are evidenced in yellow, the C-terminus in red. Note the only difference between HMGA1a and HMGA1b due to the stretch of 11 amino acid residues following the first AT-hook. (B) Scheme of the sequences of HMGA proteins pointing out to the differently spaced AT-hooks (in yellow) and C-terminal ends (in red) along the three protein molecules. (C) Scheme illustrating some of the most important activities of HMGA proteins. c1: An HMGA protein directly binds to the DNA (1) modifying its conformation and consequently facilitating the binding of a group of other TFs (2). HMGA proteins interact with both DNA and TFs generating a multiproteic stereospecific complex bound to DNA [18]. c2: An HMGA protein interacts with a TF (1) that has low affinity for DNA, modifying its conformation (2) and allowing its binding to DNA with high affinity [15,19]. c3: De-repression of transcription by displacement of histone H1 by HMGA proteins [20].

proteins, as reflected by the growing number of HMGA molecular partners [10]. Neither secondary nor tertiary structure has been determined for HMGA proteins free-in-solution, but this does not exclude that in vitro and in vivo interactions could result in locally specific non-canonical structures following the binding with DNA and/or other proteins. Indeed, the core element of AT-hooks is indicated as a motif that assumes a defined structure able to induce structural changes on DNA such as bending, straightening or unwinding [10,11]. Moreover, although different regions have been identified in HMGA proteins as sites of interaction with other nuclear factors, the one most frequently involved in protein–protein interaction comprises the second AT-hook and the amino acid residues between the second and the third AT-hook [12–17].

3. Multiple functions of HMGA proteins

HMGA proteins, short and flexible molecules containing different interacting domains, are able to wedge into many

different compartments assembling or modulating macromolecular complexes that are involved in a variety of biological processes. Indeed, many reports illustrate how HMGA proteins participate in processes such as the regulation of gene expression, virus integration and expression, embryogenesis and differentiation, and neoplastic transformation. Fig. 1C shows a diagram in which some of the proposed functions of HMGA in the nucleus are highlighted.

3.1. HMGA proteins in the regulation of gene expression

HMGA proteins have been shown to participate in the regulation of many genes, but one of the best-studied mechanisms of gene regulation in which they are involved is that of the interferon-β (IFN-β) gene. The activation of the IFN-β expression is due to a multifactor complex that assembles in the nucleosome-free enhancer region of the gene, formed by the factors NF-κB, IRF, ATF2/cJun, and the HMGA1a protein [18,21]. HMGA1a plays a double function in this context: (i) induces allosteric changes in the DNA thus increasing the affinity of the transcription factors (TFs) for their binding sites and (ii) establishes protein–protein interactions with the same factors. This new structure, called enhanceosome, is responsible for the modification and the remodeling of a nucleosome that masks the TATA-box; consequently, transcription can start. This remodeling is triggered by the recruitment from the enhanceosome of GCN5/PCAF that acetylates the nucleosome and also HMGA1a at K64, the latter modification resulting in the stabilization of the enhanceosome. Later, another acetyl transferase called CBP modifies HMGA1a at K70 destabilizing the enhanceosome and, consequently, repressing transcription.

The recruitment on the promoter/enhancer element of chromatin of remodeling factors by gene-specific macromolecular complexes is thought to be a widespread mechanism for gene activation. It is therefore important to underline that the HMGA participate, acting in a manner very similar to that reported for the IFN-β gene, in the regulation of a large set of genes two examples of which are the interleukin-2 receptor-α (IL-2Rα) and the insulin receptor (IR) genes [22,23].

HMGA can influence gene transcription also through direct protein–protein interactions with TFs by inducing changes in their DNA binding affinities. The enhancement of the serum-response factor (SRF) transcriptional activity by HMGA1a is an example of this mechanism [15].

Moreover, given their high abundance in cancer cells, the HMGA have the ability to alter chromatin structure. Indeed, they have been shown to be important elements associated with MARs. MARs/SARs (matrix/scaffold associated regions) are specific segments of genomic DNA that have high affinity for the nuclear matrix and that are enriched in AT sequences. These sequences anchor chromatin to the nuclear scaffold and organize topologically independent DNA domains which have functional roles both in DNA replication and transcription [24]. It has been demonstrated that HMGA proteins displace Histone H1 from MARs, thus participating in chromatin transcriptional activation [20].

3.2. HMGA proteins in virus integration and expression

Further striking importance of the role of HMGA proteins has been evidenced in both virus integration and viral genome expression in host cells [25–28]. Indeed, it has been demonstrated that the pre-integration complex by which immunodeficiency virus type 1 (HIV-1) becomes a part of the host genome of the infected cells contains as an essential component the HMGA1a protein, which is supplied by the host cell. Moreover, the same protein cooperates with the hSWI/SNF complex at the HIV-1 promoter in order to modify chromatin structure and activate the transcription of the viral genes. Similar studies have been also carried out for human papilloma virus type 18 (HPV 18) in which it has been demonstrated that the core of the enhanceosome that stimulates transcription is formed by the hetero-dimers JunB/Fra2 and HMGA1a.

3.3. HMGA proteins in embryogenesis and differentiation

In normal cells the expression of HMGA proteins is restricted to embryogenesis, it decreases with organogenesis and in normal adult cells is very low or almost absent. In particular, both genes are expressed at high levels in the entire embryo until 8.5 dpc [29,30]. At later stages, the expression pattern becomes more restricted; in particular, HMGA1 expression is confined to specific body organs of ectodermal, mesodermal and endodermal origin, while HMGA2 expression is restricted to mesenchimal tissues. A role for both factors in development has been demonstrated. Pivotal studies carried out by Chada's group demonstrated that mice showing a *pygmy* phenotype carry a disrupted *Hmga2* gene and are characterized by a large reduction of fat tissue [30]. The same group confirmed the role of HMGA2 in adipogenesis, demonstrating that the deficiency of the *Hmga2* gene in mice results in resistance to obesity induced by diet [31]. The phenotype of *Hmga1* knockout mice has not been reported possibly because the more general expression of this factor could severely impair development. Indeed, suppression of HMGA1 expression impairs differentiation of pre-adipocytic cells [32], loss of *Hmga1* gene function affects lymphohematopoietic differentiation [33] and *Hmga1* is required for normal sperm development [34].

3.4. HMGA proteins in neoplastic transformation

After embryogenesis, HMGA are re-expressed at high levels in transformed cells and in tumors. This elevated expression, detected in a variety of tumors having different origins [35–38], prompted us and other laboratories to suggest HMGA1a and HMGA1b as diagnostic markers of neoplastic transformation/progression. Indeed, it has been well established by immunohistochemistry [39–48] that many human neoplasias, including thyroid, prostatic, cervical, colorectal, pancreatic and ovarian carcinoma, show a strong increase of HMGA1a and HMGA1b proteins [40–46,48]. The first evidence of a direct role played by these factors in tumorigenesis came from transfection in normal rat thyroid cells of an antisense construct for HMGA2 that prevented retrovirally induced neoplastic transformation [49]. The increased expression of HMGA proteins was later shown to promote tumor progression in different cell lines. Overexpression of the three HMGA proteins led to transformation with anchorage-independent growth in Rat 1a fibroblasts and human lymphoid CB33 cells and similar results have been obtained by overexpressing the HMGA1a in the human breast epithelial cell line MCF-7 [50–53]. Transgenic mice overexpressing HMGA proteins confirmed the role of these proteins in tumorigenesis also in vivo [54,55].

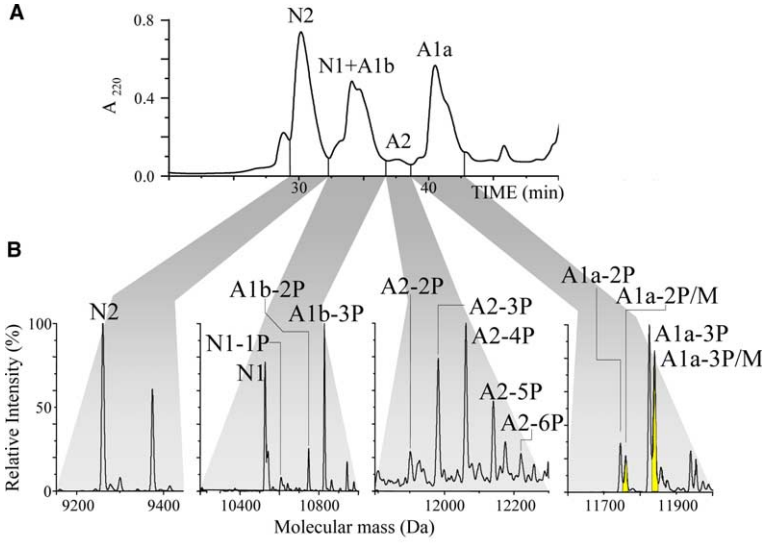
In addition of overexpression, HMGA disregulation as a result of specific chromosomal rearrangements has also been reported in a variety of common benign tumors. Structural alterations for both *HMGA* genes have been reported, but rearrangements of the *HMGA2* gene at 12q15 are particularly frequent especially in lipomas and leiomyomas, making this gene probably the most commonly rearranged one in human neoplasms. The pivotal role of *HMGA2* rearrangements in the process of lipomagenesis has been previously reviewed [56].

4. Modified forms of HMGA proteins

HMGA proteins are subjected to a variety of post-translational modifications (PTMs) that modulate their multi-interacting property with both DNA and proteins [57–67]. Radioactive labeling and liquid chromatography/mass spectrometry (LC/MS) are techniques of choice for the identification of protein post-translational modified forms. We used LC/MS to study changes of HMGA PTMs in different cell lines subjected to different stimuli. Protein samples containing all families of HMG proteins have been obtained by extraction of tumor cells with perchloric acid [2,35] and then analyzed by reverse-phase chromatography in order to separate HMGA proteins from the other two HMG families, i.e., HMGB (formerly HMG1/2) and HMGN (formerly HMG14/17) [3,9,68], still present in neoplastic cells. A typical chromatographic profile of the five low molecular mass HMG proteins (HMGA1a, HMGA1b, HMGA2, HMGN1, and HMGN2) is shown in Fig. 2A. Mass spectrometric analysis allows the identification of all the modified forms as shown in Fig. 2B, in which it is possible to note that more than one species co-elute in the same chromatographic peak. As shown in Fig. 2C, experimental masses can be compared with theoretical ones deduced from the amino acid sequences of Fig. 1A. HMGA proteins are among the most highly post-translationally modified nuclear proteins. Fig. 3 summarizes PTM sites of the HMGA1a protein so far identified by our and other laboratories [18,58–67].

4.1. Phosphorylation

Nearly all of HMGA1a and HMGA1b proteins are constitutively phosphorylated by casein kinase 2 (CK2) at the two or three serine residues of the C-terminal end (see Fig. 1A), however, the function of this constitutive phosphorylation is not clearly understood [58–67]. Experimental evidence obtained on the HMGA2 protein suggests that the acidic tail could have a role in the transcriptional regulation of target genes by regulating the DNA-binding affinity rather than specificity [69,70]. Phosphorylation of the serines located at the C-terminus could therefore represent an additional modulation of HMGA DNA-binding properties. Moreover, other



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	Molecular mass (Da)			Molecular mass (Da)	
	theoretical	experimental		theoretical	experimental
HMGN2	9261.5	9261.2	**HMGA1b 2P**	10749.7	10749.1
HMGN1	10527.7	10528.3	**HMGA1b 3P**	10829.7	10829.0
HMGN1 1P	10607.7	10608.7	**HMGA2 2P**	11902.8	11901.0
HMGA1a 2P	11746.8	11746.0	**HMGA2 3P**	11982.8	11982.5
HMGA1a 2P/M	11760.8	11760.0	**HMGA2 4P**	12062.8	12063.1
HMGA1a 3P	11826.8	11826.0	**HMGA2 5P**	12142.8	12142.1
HMGA1a 3P/M	11840.8	11840.2	**HMGA2 6P**	12222.8	12222.9

Fig. 2. LC-MS analysis of a protein sample obtained by 5% perchloric acid extraction from the prostate tumor cell line PC-3. (A) Reverse-phase HPLC profile obtained by a water/acetonitrile gradient using a C18 column. (B) Reconstructed mass spectra obtained by LC-MS. P, phosphate group; M, methyl group (peaks highlighted in yellow). (C) Comparison of experimental mass values of detected HMG proteins and theoretical mass values calculated according to the amino acid residue sequence.

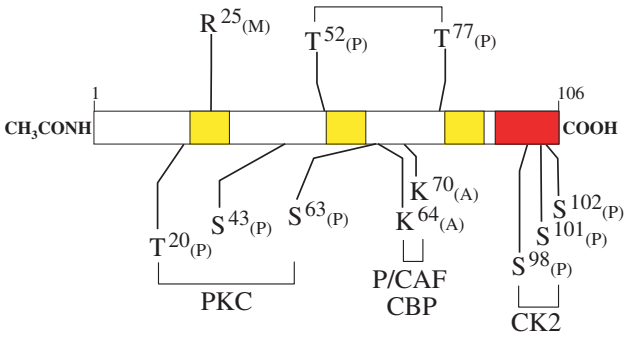

Fig. 3. Schematic description of post-translational modifications found for human HMGA1a protein. AT, AT-hook; P, phosphorylation site; A, acetylation site; M, Methylation site; PKC, protein kinase C; P/CAF and CBP, acetyl transferases; CK2, casein kinase 2; cdc2, cdc2 kinase. Apex numbers at the amino acid symbols indicate the position of the modified residue along the protein sequence.

phosphorylation sites have been detected that are related to the cell cycle, such as those at T52 and T77 due to cdc2 kinase, or to signal transduction pathways such as T20, S43, and S63 that are modified by protein kinase C (PKC). Phosphorylation of T52 and T77 of the human HMGA1a protein takes place at G2/M-phase of the cell cycle and causes a strong decrease of the DNA-binding affinity [62,71]. On the other hand, phosphorylation by PKC of T20, S43, and S63 is the result of the treatment of mammary epithelial cells with phorbol esters that activate the Ca^{2+}/phospholipid pathway [61]. Likewise the cdc2 phosphorylation, a significant reduction of DNA-binding affinity has been found for HMGA1a protein modified by PKC. Detailed studies carried out by J.R. Wiśniewski and coworkers elegantly differentiated the aptitude of the three AT-hooks and of the three proteins (see Fig. 1A and B) to bind to the DNA [72,73]. Using the promoter region of the IFN-β gene as a model, these authors demonstrated that the two proteins HMGA1a and HMGA1b engage all three AT-hooks in binding DNA, whilst the HMGA2 protein employs its N-terminal and central AT-hooks. Phosphorylation of HMGA1b by cdc2 kinase impairs the contacts of the first AT-hook with the IFN-β promoter, whereas phosphorylation of HMGA2 by the same kinase affects mainly the binding of the second AT-hook.

4.2. Acetylation

HMGA are costitutively N-terminally acetylated (Fig. 3); the function of K64 and K70 acetylation in the context of IFN-β enhanceosome structure has been already discussed.

4.3. Methylation

Methylation is the most recent HMGA modification that has been taken into consideration [59–61,67]. By mass spectrometry analysis of trypsin-digested HMGA1a, we demonstrated that this protein, at least in the analyzed cells, is mono-methylated at R25 that is located in the middle of the first AT-hook (see Fig. 1A). With regard to this modification, two points are worthwhile to mention: (i) in the analyzed tumor cells, the percentage of mono-methylated HMGA1a protein is very high reaching in some samples up to fifty per cent of total HMGA1a protein and (ii) among HMGA proteins only HMGA1a resulted methylated, at least at the level of sensitivity of the adopted method.

4.4. PTMs conclusive remarks

Results similar to those reported in Fig. 2 have been obtained for many other neoplastic cells and taken together allow us to draw the following conclusions:

- HMGA1a and HMGA1b are usually detectable in tumor cells and, in addition to these proteins, many highly transformed cells express also the HMGA2 protein;
- HMGA proteins are constitutively bi- or tri-phosphorylated at their C-terminus;
- The three HMGA proteins are not equivalently modified by PTMs;
- Many neoplastic cell lines show large amounts of mono-methylated HMGA1a protein that appears to be the only HMGA protein methylated at high level.

5. HMGA proteins and apoptosis

The most extended variation in HMGA PTMs seems to occur during programmed cell death chromatin condensation. We carried out detailed LC/MS studies on the HMGA1a protein of a number of tumor cells induced to undergo apoptosis by various procedures [59,60,67]. At early apoptotic stages, the HMGA1a protein undergoes an increase in the degree of phosphorylation that probably results in its displacements from DNA and, consequently, allows faster DNA digestion because of the more accessible chromatin structure. These new phosphate groups added to those already present at the C-terminal tail result in hyper-phosphorylated forms (up to 5 phosphates/protein molecule). Hyper-phosphorylation is followed by a de-phosphorylation process that, depriving HMGA1a protein of many negative charges, facilitates re-aggregation of DNA and the placement of HMGA into apoptotic bodies. On the contrary, during apoptosis, there is a continuous increase of the degree of HMGA1a methylation at R25 that reaches the highest level when completely de-phosphorylated protein is observed. The function of this modification is currently under investigation.

6. Conclusion and perspectives

Core histones (H2A, H2B, H3, and H4) organize the DNA into fibers that are further compacted by linker histone H1 [74]. The compactness of this structure is variously modulated, depending on the necessities of the cell, by loss or weakness of binding of histone H1, by post-translational modification of N-sides of core histones, and by the interference of other non-histone nuclear proteins. Among these, HMGA proteins play an important role since:

(a) They are present in large amounts in the nucleus (particularly in tumor cells).
(b) They compete with histone H1 to bind to the DNA.
(c) They are capable of forming multiprotein complexes involved in DNA-binding.
(d) They are, to a great degree, subjected to several PTMs.

HMGA proteins participate locally and temporally to the dynamic modification of the chromatin structure, mainly imposed by the histones, and, therefore, they have been defined "architectural factors" [68,74,75]. Consequently, cells that express high levels of HMGA proteins (such as tumor cells) should have a profound alteration in the chromatin structure when compared with normal cells.

In addition to being a particular "feature" of transformed cells, a number of papers have pointed out to the oncogenic activity of HMGA at least in some selected cell lines [49–56]. For all these reasons, the HMGA proteins could represent a good target for the development of new anticancer strategies. Actually, in order to interfere with their function or block their expression, some papers reported the use of antisense technology in cancer cells to suppress the expression of HMGA proteins that leads to a decrease in cell proliferation or to apoptotic cell death [48,76,77]. Moreover, an already known antitumoral drug named FR900482 [78] has been suggested as cross-linking agent of HMGA1a and HMGA1b to the minor groove of DNA in order to avoid any further use of the protein in the nucleus [79].

Another, not yet explored, way to prevent the action of HMGA proteins could be found in their natively disordered structure that is able to establish multiple interactions. It is conceivable that HMGA proteins could be forced to assume a fixed structure, thereby loosing the original multi-interacting capability. This modification could be obtained by introducing or producing into tumor cells specific compounds able to turn a flexible molecule into a rigid and no further on usable one. A deeper understanding of the HMGA molecular network of protein–protein interaction could provide essential information for the development of such a chemotherapeutic agent. Following this idea, in our laboratory we have already started a proteomic project to search for HMGA molecular partners. The preliminary HMGA molecular network we obtained suggests an involvement of HMGA proteins not only at a transcriptional level but also in other basal nuclear DNA- and RNA-linked processes [manuscript in preparation]. Identifying HMGA molecular partners and subsequently the domains/structures involved in HMGA protein–protein contacts could provide unchallenged opportunities to develop molecules able to selectively perturb the various activities of HMGA proteins. However, it is our opinion that HMGA proteins should be targeted in combination therapies in which there are at least two targets: the first specific and characteristic of a stated tumor (such as an inhibitor of a specific kinase or siRNA against specific mRNAs), the second with a general influence on chromatin (such as histones or HMGA proteins).

In many tumor cells, the HMGA2 protein is overexpressed in addition to the other two proteins. Until now, the reason for this extra-expression is not known but our recent results suggest that HMGA2 is involved in cell-cycle control by regulating the cyclin A gene [17]. Indeed, HMGA2 knockout mice show the *pygmy* phenotype, which is characterized by growth retardation and a drastic reduction of body fat content [30]. Moreover, it was also shown that HMGA2 can negatively affect the expression of DNA-repair gene ERCC1, linking therefore HMGA2 expression with genomic instability [80]. These results suggest that HMGA2 could be related to proliferation rather than neoplastic transformation. However, repression of this protein could be an attractive tool for a combined chemotherapeutic treatment, at least for a limited group of cancers.

The epigenetic modification of chromatin takes into account also the methylation process that should be discussed from a triplicate point of view. (i) DNA methylation at the 5-position of cytosine in the di-nucleotide sequence CpG is a well-known phenomenon and hyper-methylation of DNA promoter regions has been associated with transcriptional silencing of tumor suppressor genes [81,82]. (ii) Histone methylation also seems to be associated with repressive chromatin structure formation that brings DNA into inactive heterochromatin [83]. (iii) The third methylation is that of HMGA1a that reaches high levels in tumor cells [59,60]. It could be possible that also methylated HMGA1a is involved in the silencing program in which both DNA and histone methylation participate. Inhibition of methyltransferases that catalyze the three types of methylation could be a very promising tool in cancer therapy. Studies on both DNA and histone methylation are more advanced compared to those concerning the HMGA proteins. Indeed, clinical trials are already carried out with decitabine (NSC-127716, Dacogen, Supergen) that is a specific DNA methyltransferase inhibitor used in order to reduce the chromatin repression pathway.

In conclusion, the scenario of epigenetic modifications of DNA and chromatin associated proteins is wide and includes many different events that, however, are linked and cooperate in cancer cell chromatin remodeling. Among these factors and modifications there are HMGA proteins, whose function in chromatin structure alteration is at the moment of increasing interest in many laboratories.

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