Chromosomal protein HMGN1 enhances the acetylation of lysine 14 in histone H3

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The acetylation levels of lysine residues in nucleosomes, which are determined by the opposing activities of histone acetyltransferases (HATs) and deacetylases, play an important role in regulating chromatin-related processes, including transcription. We report that HMGN1, a nucleosomal binding protein that reduces the compaction of the chromatin fiber, increases the levels of acetylation of K14 in H3. The levels of H3K14ac in *Hmgn1*^{−/−} cells are lower than in *Hmgn1*^{+/+} cells. Induced expression of wild-type HMGN1, but not of a mutant that does not bind to chromatin, in *Hmgn1*^{−/−} cells elevates the levels of H3K14ac. *In vivo*, HMGN1 elevates the levels of H3K14ac by enhancing the action of HAT. *In vitro*, HMGN1 enhances the ability of PCAF to acetylate nucleosomal, but not free, H3. Thus, HMGN1 modulates the levels of H3K14ac by binding to chromatin. We suggest that HMGN1, and perhaps similar architectural proteins, modulates the levels of acetylation in chromatin by altering the equilibrium generated by the opposing enzymatic activities that continuously modify and de-modify the histone tails in nucleosomes.

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Introduction

The chromatin fiber is a dynamic structure that is continuously modified by nuclear factors that remodel the structure of the nucleosome and chemically alter the nucleosomal histones and DNA. Numerous studies link these nucleosomal modifications to the orderly progression of the cell cycle and

differentiation, and to the regulation of nuclear processes that occur in the context of chromatin, including transcription (Strahl and Allis, 2000; Jenuwein and Allis, 2001; Berger, 2002). The reversible acetylation of lysine residues in the amino termini of the core histones was the first to be linked to transcriptional activation, and is one of the most extensively studied chromatin modifications (Hebbes *et al*, 1994; Grunstein, 1997; Kuo and Allis, 1998; Litt *et al*, 2001). Histone acetyltransferases (HATs) and deacetylases (HDACs) continuously add and remove acetyl groups to specific lysine residues in the termini of the core histones and generate a dynamic steady-state level of acetylation (Kadosh and Struhl, 1997; Kuo and Allis, 1998). This dynamic state is perturbed and the acetylation levels of specific lysines at distinct chromatin sites are changed by external and internal cellular events that change the cellular transcription profile. Biochemical and genetic experiments established that HATs and HDACs are coregulators of transcription, replication, and repair, and demonstrated that the levels of acetylation of specific residues in the histone tails correlate with distinct transcriptional states (Kuo and Allis, 1998; Litt *et al*, 2001). For example, in the chicken beta globin locus transcriptional activation correlates with acetylation of specific lysines in the amino termini of H3 and H4, and transcriptional inhibition is associated with their deacetylation (Litt *et al*, 2001). These and numerous other studies established that acetylation plays an important role in regulating chromatin-related events. The acetylated lysines may change the local folding of the chromatin fiber or nucleosome mobility (Cosgrove *et al*, 2004) or serve as recognition markers for the binding of transcriptional regulators to their specific targets (Cheung *et al*, 2000a; Strahl and Allis, 2000; Park *et al*, 2002).

Given that the acetylation levels of the histone tails reflect the equilibrium generated by the opposing activities of HAT and HDAC, it is possible that factors that affect the ability of these enzyme complexes to reach their nucleosomal targets would ultimately affect the overall pattern of acetylation of the histone tails. Thus, the acetylation of histone tails could be modulated by structural proteins that affect the compaction of the chromatin fiber and alter the local accessibility to nucleosomes such as the linker histone H1 (Juan *et al*, 1997; Sera and Wolffe, 1998; Ragab and Travers, 2003) and HMGN nonhistones (Ding *et al*, 1997). Indeed, it has already been demonstrated that both H1 and HMGN proteins affect chromatin access and remodeling. The interaction of histone H1 with nucleosomes, which stabilizes the compact 30-nm higher-order chromatin structure, inhibits the activity of ATP-dependent nucleosome-remodeling enzymes (Horn *et al*, 2002), the PCAF-mediated histone acetylation (Herrera *et al*, 2000), the mitotic phosphorylation of H3 (Van Hooser *et al*, 1998), and the ability of nucleases to access the DNA (Whitlock and Simpson, 1976; Ragab and Travers, 2003). The inhibition of nucleases and PCAF is caused by steric changes at the level of the single nucleosome, while the inhibition

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of ATP-dependent remodeling may be linked to changes in the higher-order chromatin structure.

HMGN are the only nonhistones known to bind specifically to the 147 base pair nucleosome core particle (CP), the building block of the chromatin fiber. They bind to the nucleosome cores in a highly specific manner, forming unique complexes that yield distinct DNA footprints (Sandeén *et al*, 1980; Alfonso *et al*, 1994). Crosslinking experiments established that the C-terminal region of HMGN1, a major member of the HMGN protein family, is located near the amino-terminal tail of H3 and that the amino-terminal portion of HMGN1 is near H2B (Trieschmann *et al*, 1998). Additional contacts between HMGNs and histones have also been identified (Brawley and Martinson, 1992). The binding of HMGN to nucleosome reduces the compaction of the chromatin fiber and promotes overall accessibility to nucleosomes, but also alters the accessibility of unique sites in the nucleosomes to which they are bound. Thus, HMGNs enhance the rate of transcriptional initiation (Paranjape *et al*, 1995), transcriptional elongation (Ding *et al*, 1997), and the rate of repair of UV-damaged DNA in chromatin (Birger *et al*, 2003), but reduce the rate at which DNaseI digests (Sandeén *et al*, 1980) and hydroxyl radicals cleave (Alfonso *et al*, 1994) nucleosomes. Enhancement of transcription and repair can be attributed to chromatin decompaction, while reduction to DNaseI accessibility is most likely a result of steric changes in the nucleosomes. These steric changes, and the proximity of HMGN to the histone tails, could also affect the pattern of histone post-translational modifications. Indeed, recent studies demonstrated that HMGN1 modulates the levels of phosphorylation of H3S10, and suggested that these proteins also affect the levels of additional histone modifications (Lim *et al*, 2004b).

Here we report that HMGN1 enhances the level of acetylation of Lys 14 in the tail of H3. We find that, in *Hmgn1*^{-/-} cells, loss of HMGN1 protein lowers the steady-state levels of H3K14ac and re-expression of HMGN1 protein elevates the level of this modification. HMGN1 modulates the levels of H3K14ac by binding to chromatin, since an HMGN1 point mutant that does not bind to nucleosomes does not affect this acetylation, and HMGN1 enhances the modification of nucleosomal, but not free, H3. By chromatin immunoprecipitation (ChIP), we find that in a subset of immediate early genes the levels of H3K14ac and phosphoacetylated H3 are elevated in *Hmgn1*^{+/+} cells, but not in *Hmgn1*^{-/-} cells. Kinetic analysis of cells exposed to HDAC inhibitors indicates that HMGN1 protein enhances the levels of H3K14ac in chromatin by stimulating the activity of HATs.

Our studies establish the principle that the equilibrium between the activities that continuously modify and demodify histone tails, such as of HATs and HDACs, can be perturbed by structural proteins such as HMGN1. By shifting this equilibrium, the nucleosome-binding proteins are part of the molecular mechanism that establishes the level of acetylation of specific lysine residues in histone tails, including H3K14ac. This modification has been linked to transcriptional activation in both yeast and vertebrate cells. Thus, HMGN, and perhaps other structural proteins, may affect the cellular transcription profile not only by modifying the higher-order chromatin structure but also by inducing local steric changes that alter the levels of specific modifications in nucleosomes.

Results

Reduced levels of H3K14ac in *Hmgn1*^{-/-} cells

Western analysis of histones extracted from both primary and immortalized *Hmgn1*^{-/-} and *Hmgn1*^{+/+} mouse embryonic fibroblasts (MEFs), grown to the same density under identical conditions, revealed that loss of HMGN1 lowered significantly (30–40%) the steady-state levels of H3K14ac (Figure 1A). To verify that the decreased levels of H3K14ac are indeed due to loss of HMGN1 protein, we examined the levels of this modification in *Hmgn1*^{-/-} MEFs which were stably transformed with vectors expressing either wild-type HMGN1 or an S20,24E HMGN1 mutant, both under the control of the inducible tetracycline response element (TRE) promoter. Exposure of these cells to doxycycline (Dox) induces the expression of either wild-type or mutant S20,24E HMGN1 protein; the latter enters the nucleus, but does not bind to nucleosomes (Prymakowska-Bosak *et al*, 2001). The steady-state levels of H3K14ac in *Hmgn1*^{-/-} cells grown in the presence of Dox and expressing wild-type HMGN1 protein were 2.5 higher than in the cells grown in the absence of Dox, an indication that the levels of this modification are indeed linked to HMGN1 expression. In contrast, expression of the S20,24E HMGN1 mutant did not alter the H3K14ac levels (Figure 1B). Our finding that expression of wild type but not of mutant protein elevates the levels of H3K14ac indicates that the interaction of HMGN1 protein with chromatin enhances the levels of this modification.

HMGN1 enhances acetylation of H3 by HAT

The steady-state level of H3K14ac reflects the equilibrium between the ability of HATs and HDACs to modify and demodify H3K14 in chromatin. The interaction of HMGN1 with chromatin could either promote the ability of HATs to access and modify this residue, or inhibit the ability of HDACs to deacetylate H3K14ac. To distinguish between these two possibilities, we examined the kinetics of H3K14 acetylation in *Hmgn1*^{-/-} and *Hmgn1*^{+/+} cells grown in the presence of trichostatin A (TSA). TSA inhibits HDAC activity; therefore, in its presence the acetylation kinetics reflect the activity of HAT alone, rather than the net result of the equilibrium of opposing activities of HATs and HDACs. Thus, it can be predicted that if HMGN1 enhances HAT activity, then the presence of TSA will increase the difference between the two cell types, while if HMGN1 inhibits HDAC activity, then TSA treatment will minimize, or even eliminate the differences between the two cell types.

To determine whether HMGN1 enhances HAT or inhibits HDAC, we added TSA to *Hmgn1*^{-/-} and *Hmgn1*^{+/+} MEFs grown to 70% confluency and used specific antibodies to determine the levels of the modification various times after the addition of the HDAC inhibitor. Western analysis of the kinetics of H3K14ac accumulation in TSA-treated cells indicates clearly that HMGN1 increases acetylation by enhancing the activity of HATs (Figure 2A).

We excluded the possibility that HMGN1 affects the overall levels of HAT activity in the cells by analyzing the acetyltransferase activity of immunoprecipitates prepared from *Hmgn1*^{-/-} and *Hmgn1*^{+/+} cells with antibodies to either PCAF or p300. These analyses did not reveal differences between the cells in the overall acetylation activity of either PCAF, which is the main H3K14 acetyltransferase, or p300, which has a wider range of acetylation activity (Figure 2B and C).

Figure 1 HMGN1 stimulates the acetylation of H3K14. (A) Western blotting of histones extracted from growing *Hmg1*^{+/+} or *Hmg1*^{-/-} MEFs, resolved by SDS-PAGE, and developed with either anti-H3 (α -H3) or anti-H3K14ac. The relative signal intensities of H3K14ac, normalized to the intensities of total H3, shown in the bar graphs below the Westerns indicate lower levels of H3K14ac in the *Hmg1*^{-/-} cells. (B) Expression of wild type, but not mutant, HMGN1 protein elevates the levels of H3K14ac. Western analysis of extracts from *Hmg1*^{-/-} cells, stably transfected with inducible plasmids expressing either HMGN1 or S20,24E HMGN1 mutant protein, which does not bind chromatin, under the control of the Tet promoter. Dox-induced expression of HMGN1, but not of S20,24E HMGN1 (verified by Western with anti-HMGN1 antibodies) raises the levels of H3K14ac. The relative signal intensities of H3K14ac, normalized to the intensities of total H3, are shown in the bar graphs below the Westerns.

HMGN1 enhances the acetylation of nucleosomal, but not free, H3

PCAF is an efficient H3K14 acetyltransferase in higher eukaryotes, although P300 was reported to also acetylate this residue (Schiltz *et al*, 1999). The acetyltransferase activity resides in a distinct region of the protein known as the HAT domain. In most cells, PCAF associates with several other proteins, including p300, and forms an enzymatically active multiprotein complex (Ogryzko *et al*, 1998). To gain insights into the mechanism whereby HMGN1 enhances H3K14 acetylation, we incubated purified nucleosome CPs with increasing amounts of HMGN1 and tested the relative levels of ¹⁴C incorporation from ¹⁴C-AcCoA into H3, by either the recombinant HAT domain of PCAF or by a PCAF multiprotein complex isolated from HeLa cells (Ogryzko *et al*, 1998). The levels of acetylation were determined by quantitative analysis of protein gels and their corresponding autoradiographs.

Mobility shift assays indicated that HMGN1 bound to the CP and generated complexes containing two HMGN1 per CP (CP+2HMGN1 in Figure 3A, third panel). The autoradiographs indicate a dose- (Figure 3A) and time-dependent (Figure 3D) HMGN-mediated enhancement of H3K14 acetylation by recombinant HAT-PCAF domain, and by the PCAF complex (Figure 3C), that correlated with the binding of HMGN1 to CPs (Figure 4D). The acetylation activity of the PCAF complex was totally inhibited by the PCAF-specific inhibitor H3-CoA-20 (Lau *et al*, 2000), an indication that the complex did not contain additional acetylases, such as p300 (Figure 3E and F). Western analysis verified that rHAT-PCAF acetylated H3K14 (Figure 3B). We conclude that HMGN1 enhances the PCAF-mediated acetylation of nucleosomal H3.

HMGN proteins bind specifically to chromatin, but not to DNA or histones. Therefore, to verify further that histone H3 acetylation is stimulated by HMGN1 only in nucleosomal context, and the stimulation is indeed specific, we repeated these experiments using free, rather than nucleosomal, histone H3 as a substrate. The rHAT-PCAF domain acetylates free H3 very efficiently. The acetylation of free H3 by the rHAT-PCAF domain was not affected by HMGN1 (Figure 4C). The plots of specific activity of H3 acetylation as a function of the HMGN1/H3 input ratio (Figure 4D) indicate clearly that while HMGN1 stimulates the acetylation of nucleosomal histone H3 by both rHAT-PCAF and by PCAF complex, it does not stimulate the acetylation of free, non-nucleosome-bound H3. Thus, HMGN1 enhances the PCAF-mediated H3 acetylation only in the context of chromatin.

Kinetic analysis at varying concentrations of either nucleosomes or acetyl-CoA, the two substrates of the reaction, indicates that HMGN1 affects both the apparent $V_{\max}$ and the apparent K_m of the reaction (Figure 4A and B). In both types of experiments, addition of HMGN1 increased both the apparent $V_{\max}$ and the apparent K_m . At constant HMGN to nucleosome levels, but varying acetyl-CoA concentrations, addition of HMGN1 increased the apparent $V_{\max}$ from 0.025 to 0.043 and the apparent K_m from 0.0023 to 0.004 (Figure 4B). Similarly, in Figure 4A, addition of HMGN1 increases the apparent $V_{\max}$ from 0.05 to 0.2, and the $K_{m\text{app}}$ from 0.033 to 0.2. Given that the nucleosomes are heterogeneous and the reaction involves more than one substrate, it is not clear whether the parameters obtained from the Michaelis-Menten equation can be used to interpret the effect of HMGN1 on the HAT assay with nucleosomes. However, at

Figure 2 HMGN1 elevates the levels of H3K14ac by stimulating HAT activity. **(A)** Kinetics of H3K14 acetylation in *Hmgn1*^{-/-} and *Hmgn1*^{+/+} cells grown in the presence of the HDAC inhibitor TSA. *Hmgn1*^{+/+} and *Hmgn1*^{-/-} fibroblasts were grown in the presence of TSA for various times. Western analyses of the histones extracted from these cells are shown on the right. The graph depicts the relative signal intensities of H3K14ac normalized to the intensities of total H3. Non-TSA-treated *Hmgn1*^{+/+} (NT in the graph) cells are used as controls. The broken lines extrapolate the initial slopes of the curves. **(B)** Similar levels of HAT activities in *Hmgn1*^{-/-} and *Hmgn1*^{+/+} cells. Cell extracts were immunoprecipitated with antibodies to either PCAF or p300, and the immunoprecipitates incubated with histone H3 and [¹⁴C]acetyl-CoA. The reaction mixtures were fractionated on 15% SDS-PAGE and the gels autoradiographed. Control reaction of H3 incubated with [¹⁴C]acetyl-CoA and with either purified p300, purified PCAF HAT domain, no enzymes, or immunoprecipitated with normal, non-immune antibody (N-IgG) are included. **(C)** The relative signal intensities of ¹⁴C-H3, normalized to the intensities of total H3, are shown in the bar graphs below.

all kinetic points, HMGN1 stimulated the acetylation of H3. Our studies with free histones (Figure 4C) indicate that HMGN1 do not act directly on the free, non-chromatin-bound enzyme. Therefore, the simplest interpretation is that the binding of HMGN1 to nucleosomes induces changes in the nucleosome itself, which ultimately enhance the ability of the enzyme to modify the tail of H3.

The major site of interaction between HMGN1 and nucleosomes is a positively charged, 34-amino-acid sequence

known as the nucleosome-binding domain (NBD). A double point mutant, S20,24E-HMGN1, in which two serines contained in this domain were changed to glutamic acid, does not bind to nucleosomes (Prymakowska-Bosak *et al*, 2001) and does not enhance acetylation (Supplementary Figure S1, D). Truncation mutants lacking either the C-terminal 26 amino acids (HMGN1- Δ C26, Supplementary Figure S1, C) or the N-terminal 11 amino acids (Δ N11-HMGN1, Supplementary Figure S1, B), which do bind to nucleosomes

Figure 3 HMGN1 enhances the acetylation of nucleosomal H3K14. (A) HMGN1 enhances the activity of the recombinant HAT domain of PCAF (r PCAF-HAT). Coomassie blue-stained 15% polyacrylamide SDS-containing gels, and corresponding autoradiograms (^{14}C), of reaction mixtures containing equal amounts of chicken erythrocyte CPs, reconstituted with increasing amounts of HMGN1, and incubated with [^{14}C]acetyl-CoA and r PCAF-HAT. Note the direct correlation between HMGN1 input and acetylation. The EtBr-stained panel depicts mobility shift assays of the reaction mixtures, on native 4% polyacrylamide gels in 2 $\times$ TBE. Note that, under these reaction conditions, HMGN1 bound cooperatively to CPs and formed a complex (CP + 2HMGN1). (B) Western analysis of reaction mixtures performed as in (A) indicates that HMGN1 enhances the acetylation of H3K14. (C) HMGN1 stimulates the activity of PCAF complex. Reaction performed as in (A). (D) HMGN1 enhances the rPCAF-mediated acetylation of H3. Time course of the reaction of nucleosomes incubated either without HMGN1, or with a four-fold molar excess of HMGN1. (E) PCAF is the only active HAT in the complex. PCAF complex incubated with 0–250 μM concentration of H3-CoA-20, a specific inhibitor on PCAF. Note that the inhibitor abolished the acetylation of H3 in either the presence or absence of HMGN1. (F) Quantification of the radioactivity incorporated into H3 at various concentrations of inhibitor.

Figure 4 Effect of HMGN1 on PCAF-mediated H3 acetylation (A). Lineweaver-Burk plot showing the effect of HMGN1 on PCAF-mediated acetylation of nucleosome cores at a fixed concentration of [14 C]acetyl-CoA (100 μ M) and various concentrations of nucleosomes (0.006–1 μ M) in either the presence or absence of HMGN1. For each point, the concentration of HMGN1 was adjusted to maintain a constant HMGN1:core nucleosome molar ratio. (B) Lineweaver-Burk plot showing the effect of HMGN1 on r-PCAF-mediated acetylation of H3 in nucleosome cores. HAT assays were carried out with a fixed concentration of nucleosomes (1 μ M) and increasing concentrations of [14 C]acetyl-CoA, in either the presence (4 μ M) or absence of HMGN1. (C) Coomassie blue-stained 15% polyacrylamide SDS-containing gels and corresponding Westerns of reaction mixtures containing equal amounts of histone H3 incubated with increasing amounts of HMGN1, followed by incubation with [14 C]acetyl-CoA and recombinant HAT domain of PCAF. Note that HMGN1 does not stimulate acetylation. (D) Binding HMGN1 to CP enhances H3 acetylation by either PCAF HAT domain (●) or PCAF complex (■). HMGN1 does not enhance the acetylation of free histone H3 (▲). The relative acetylation levels were determined by quantifying the data in panels A and C in Figure 3 and panel C in Figure 4.

with an affinity similar to that of the intact protein (Trieschmann *et al*, 1995), do not stimulate H3K14 acetylation (Supplementary Figure S1). Thus, stimulation of acetylation involves the interaction of the full-length HMGN1 protein with nucleosome cores.

Modulation of H3 modifications during immediate-early gene induction

Induction of immediate-early gene expression by various stress agents such as the protein synthesis inhibitor anisomycin coincides with increased levels of histone post-translational modifications, including elevated levels of H3S10 phosphorylation (Barratt *et al*, 1994; Cheung *et al*, 2000b) and H3K14 acetylation (Thomson *et al*, 2001). A small fraction of the nucleosomes are 'phosphoacetylated', that is they are dimodified and are both acetylated at K14 and phosphorylated at S10 (Barratt *et al*, 1994; Cheung *et al*, 2000b). We recently reported that loss of HMGN1 protein affected the kinetics of phosphorylation of a subset of the IE-response genes (Lim *et al*, 2004b). The effect of HMGN1 on the H3S10 phosphorylation kinetics could be detected by comparative Western analysis of total histones extracted from anisomycin-treated *Hmgn1*^{-/-} and *Hmgn1*^{+/+} cells.

In contrast, Western analysis with antibodies to H3K14ac did not detect differences in the kinetics of acetylation of total histones extracted from anisomycin-treated *Hmgn1*^{-/-} and *Hmgn1*^{+/+} cells. The global levels of H3K14ac did not change during the entire course of anisomycin exposure, and were always lower in *Hmgn1*^{-/-} cells (Figure 5B). Most likely, changes in the H3K14ac levels in the IE genes cannot be detected against the large background of pre-existing acetylation. Nevertheless, these results re-emphasize the difference between *Hmgn1*^{-/-} and *Hmgn1*^{+/+} cells and suggest that the levels of H3K14ac are different throughout the cellular chromatin rather than in only a few genes of these cells.

Since HMGN1 reduces the rate of H3S10 phosphorylation (Lim *et al*, 2004b) but enhances H3K14 acetylation (Figures 1 and 2), we examined whether HMGN1 affects the levels of phosphoacetylation, that is, the doubly modified H3 containing both phosphorylated S10 and acetylated K14. Western analysis of histones extracted from *Hmgn1*^{-/-} and *Hmgn1*^{+/+} cells, grown to the same density under identical conditions, revealed that loss of HMGN1 reduced the steady-state levels of phosphoacetylated H3 by approximately 35% (Figure 5A). Conceivably, the reduced levels of dimodified H3 could be due to lower levels of either H3S10p or H3K14ac. Since in *Hmgn1*^{-/-} cells loss of HMGN1 enhances the levels of H3S10p (Lim *et al*, 2004b), the reduced levels of H3-phosphoacetylation are due to decreased acetylation, supporting our conclusion that loss of HMGN1 protein reduces the acetylation of H3K14.

In serum-starved quiescent *Hmgn1*^{+/+} cells, the levels of phosphoacetylated H3 are similar to those of *Hmgn1*^{-/-} cells (Figure 5A–C). Anisomycin stimulation induces a rapid and transient phosphorylation of HMGN1, which weakens the interaction of the protein with chromatin. Considering that *Hmgn1*^{-/-} cells lack HMGN1, and anisomycin stimulation reduces the binding of HMGN1 to nucleosomes, it can be expected that HMGN1 protein would not have a significant effect on the kinetics of H3 phosphoacetylation during IE induction. Indeed, kinetic analysis revealed that H3 was

Figure 5 HMGN1 does not affect global acetylation and phosphoacetylation of histone H3 during IE gene induction. (A) HMGN1 enhances the levels of H3 phosphoacetylation in growing but not quiescent mouse fibroblasts. (B) Western analysis of the histone modifications analyzed with the antibodies indicated on the left during the course of anisomycin treatment of *Hmgn1*^{+/+} and *Hmgn1*^{-/-} cells. (C) Plots depicting the time course of phosphoacetylation and phosphorylation of H3 in *Hmgn1*^{+/+} or *Hmgn1*^{-/-} cells during anisomycin treatment.

dimodified at the same rate in both *Hmgn1*^{-/-} and *Hmgn1*^{+/+} cells (Figure 5C). While HMGN1 does not affect the global phosphoacetylation (Figure 5C, left), it does affect the phosphorylation (Figure 5C, right), a finding that we previously studied in detail (Lim *et al*, 2004b). Thus, HMGN1 enhances acetylation, decreases the levels of phosphorylation, and does not affect the levels of phosphoacetylation. These findings demonstrate the multifaceted nature of the effect of HMGN1 on the pattern of post-translational modification in the histone tails.

Loss of HMGN1 affects the anisomycin response of a subset of the IE genes we examined. For most of these genes, the transcription in *Hmgn1*^{-/-} cells was higher than in *Hmgn1*^{+/+} cells, indicating that loss of HMGN1 enhanced IE gene expression. We linked the elevated expression of these IE genes in the *Hmgn1*^{-/-} cells to an enhanced level of phosphorylation in H3S10 (Lim *et al*, 2004b). A notable exception was *junD*, whose expression in *Hmgn1*^{-/-} cells was actually lower than in *Hmgn1*^{+/+} cells, suggesting that the presence of HMGN1 enhances its expression. Real-time RT-PCR analysis of RNA extracted from anisomycin-treated *Hmgn1*^{+/+} and *Hmgn1*^{-/-} cells verified that HMGN1 enhances the stress-induced expression of *junD*, but not of *c-jun*, a gene whose expression is the same in both cell types (Figure 6A). ChIP analysis indicated that anisomycin expo-

sure elevated the levels of H3K14ac in both the promoter and transcribed portion of *junD* in *Hmgn1*^{+/+} cells to a higher degree than in *Hmgn1*^{-/-} cells (Figure 6B). In the promoter, and to some extent also in the transcribed portion of *junD*, HMGN1 also enhances the levels of anisomycin-induced phosphoacetylation of H3. The expression of *c-jun* is not affected by HMGN1 (Lim *et al*, 2004b; Figure 6A), perhaps because its anisomycin-induced expression is not tightly linked to either acetylation or phosphoacetylation (Soloaga *et al*, 2003). Indeed, throughout the course of anisomycin treatment, the levels of modification of *c-jun* did not differ between *Hmgn1*^{-/-} and *Hmgn1*^{+/+} cells. Our findings that HMGN1 affects the acetylation and induction of *junD* but not *c-jun*, together with our previous observation of the link between HMGN1, H3S10 phosphorylation, and IE gene induction, suggest that HMGN1 affects the levels of histone modifications in only a subset of the anisomycin-induced IE genes.

Discussion

Our main finding is that the nucleosome-binding protein HMGN1 enhances the levels of H3K14ac in chromatin, a modification that has been linked to transcriptional activation and gene expression. The results presented in the manuscript

Figure 6 Loss of HMGN1 alters the expression and modification levels of *junD* but not *c-jun*. (A) Real-time RT-PCR analysis of *junD* and *c-jun* expression during anisomycin treatment of *Hmgn1*^{+/+} or *Hmgn1*^{-/-} cells. (B) HMGN1 enhances post-translational modification levels in *junD*, but not in *c-jun*. The relative enrichment of *junD* and *c-jun* sequences in the chromatin of anisomycin-treated *Hmgn1*^{+/+} or *Hmgn1*^{-/-} cells is shown, which were immunoprecipitated with antibodies to either phosphoacetylated-H3 or H3K14ac. The immunoprecipitates were analyzed by quantitative real-time PCR with primers specific to the regions indicated in the schematic diagram of the gene. All time points refer to duration of anisomycin exposure.

provide experimental evidence that the levels of acetylation in chromatin are modulated not only by the continuous action of HATs and HDACs but also by structural chromatin-binding proteins such as HMGN1.

Several types of experiments indicate that the levels of H3K14ac are linked to the interaction of HMGN1 with nucleosomes: first, the levels of H3K14ac in growing *Hmgn1*^{-/-} cells are lower than in growing *Hmgn1*^{+/+} cells. Second, expression of HMGN1 in *Hmgn1*^{-/-} cells elevates the levels of H3K14ac. Third, wild type, but not the S20,24 HMGN1 mutant protein, which does not bind to nucleosomes, elevates the levels of H3K14ac. Fourth, HMGN1 enhances the acetylation of nucleosomal, but not free, histone H3. We also found that the acetyltransferase activity of PCAF and p300 in immunoprecipitates from *Hmgn1*^{-/-} cells was indistinguishable from that present in immunoprecipitates from *Hmgn1*^{+/+} cells (Figure 2B and C), and that inhibition of HDAC activity increased the levels of H3K14ac in *Hmgn1*^{+/+} cells to a larger extent than in *Hmgn1*^{-/-} cells (Figure 2). Although

some effect on HDAC cannot be absolutely excluded, all the results are consistent with the conclusion that HMGN1 enhances the ability of HAT to modify H3K14.

Several different mechanisms could account for the HMGN1 effects on the levels of H3K14ac. By competing with H1 for chromatin-binding sites, or by changing the entry-exit angle of the nucleosomal DNA, HMGN1 reduces the compaction of the higher-order chromatin structure (Bustin, 2001; Catez *et al*, 2004). Therefore, one possibility is that the HMGN1-induced changes in the compaction of the higher-order chromatin structure preferentially enhance the ability of HATs, as compared to HDACs, to reach their specific target. The second possibility involves steric changes operative at the level of the single nucleosome. This possibility is supported by our finding that the binding of HMGN1 to isolated nucleosome CPs stimulates the acetylation of H3 by both the recombinant HAT domain of PCAF and by the multiprotein PCAF complex, the physiologically relevant form of the enzyme. The kinetic data, indicating that

HMGN1 increases both the apparent $V_{\max}$ and the apparent K_m , also seem to support the notion that the effects are due to steric changes in nucleosomes. The increase in $V_{\max}$ is not due to the direct effects of HMGN1 on the enzyme, since HMGN1 does not enhance the acetylation of non-nucleosomal H3. The increase in K_m suggests that HMGN1 does not improve the binding of the enzyme to the nucleosomal substrate. We therefore suggest that the binding of HMGN1 to nucleosomes induces steric changes in the nucleosomes, and perhaps even in the histone tail itself, which ultimately increase the ability of the enzyme to reach and acetylate K14 in H3. The 'steric' model also provides an explanation for the opposite effects of HMGN1 on phosphorylation of H3S10 (inhibition) and acetylation of K14 (enhancement). The HMGN1-induced steric changes in nucleosomes or in histone tails do not affect all histone modifications equally; each type of modification needs to be considered separately.

Indeed, our previous studies (Lim *et al*, 2004b) show additional effects of HMGN1 on the post-translational modification of histones. Thus, it is very likely that HMGN1 affects the ability of additional remodeling enzymes to modify the histone tails. We also found that the close homolog HMGN2 enhances the PCAF-mediated rate of acetylation of H3K14 in isolated nucleosomes (Supplementary Figure S2). Conceivably, all members of the HMGN family may affect the rate of histone modifications.

Previous studies with a preparation of preactivated full-length flag-tagged recombinant PCAF suggested that HMGN1 inhibited the incorporation of radioactivity from [14 C]acetyl-CoA into nucleosomal H3 (Herrera *et al*, 1999). Since subsequent studies suggested that the native PCAF could be copurified with HDAC activity (Yamagoe *et al*, 2003), and that there was contaminating kinase activity in the preparation (unpublished), those studies need to be re-evaluated. Our current studies with cells in tissue culture, with the recombinant PCAF-HAT domain that lacks the HDAC-binding site, and with the PCAF complex, together with the western analyses that identified the acetylated lysine in H3, lead us to conclude that HMGN1 enhances the rate of PCAF-mediated acetylation of K14 in nucleosomal H3.

In living cells, the interaction of HMGN1 with chromatin is dynamic (Catez *et al*, 2003) and the protein competes with other members of the HMGN family, and with histone H1 variants for nucleosome-binding sites (Catez *et al*, 2004; Bustin *et al*, 2005). This dynamic competition may also be part of the molecular mechanism whereby HMGN1 affects the ability of chromatin-remodeling factors to modify specific sites in the histone tails. Thus, HMGN may affect histone post-translational modifications at three distinct levels: (1) at the level of the higher-order chromatin structure it modifies the overall access to nucleosomes, (2) at the level of the single nucleosome it changes the levels to which specific residues in the histone tails are modified, and (3) competition with other chromatin-binding proteins leads to structural changes that could either enhance or reduce the levels of histone modifications. The multiple mechanisms whereby HMGN1 affects the activity of histone modifiers could explain the complex pattern of differences in histone modifications between *Hmgn1*^{-/-} and *Hmgn1*^{+/+} cells. Loss of HMGN1 protein leads not only to decreased levels of H3K14ac but also to increased levels of phospho-H3S10, and several additional changes in modifications of H3 and other core histones. In

some cases the differences may be functionally compensatory as may be the case for IE genes, only a few of which are affected by the loss of HMGN1. The induction of these genes is associated with increased phosphorylation of H3S10, acetylation of H3K14, and in some cases with dimodified phospho-S10/acetyl-K14 form. We have not detected any differences between *Hmgn1*^{-/-} and *Hmgn1*^{+/+} cells in the kinetics of phosphoacetylation during IE gene induction, perhaps because HMGN1 has opposite effects on these modifications: it enhances the levels of H3K14ac, but inhibits the levels of H3S10p (Lim *et al*, 2004b). In several systems there seems to be a link between the tandem phosphorylation of H3S10 and acetylation of H3K14, since it has been demonstrated that PCAF and its yeast homolog GCN5 acetylate K14 in H3 molecules in which S10 is phosphorylated more efficiently than in nonphosphorylated H3 (Cheung *et al*, 2000b; Berger, 2002). This tandem phosphoacetylation, however, only occurs in a small fraction of the modified H3 (Barratt *et al*, 1994; Cheung *et al*, 2000b) and the effects modulated by HMGN1 are pertinent to the bulk of H3S10 phosphorylation and H3K14 acetylation.

We suggest that HMGN1, and similar nucleosome-binding proteins, modulate the ability of nucleosome-modifying factors to access and modify their targets. Loss of HMGN1 has a pleiotropic effect leading to numerous changes in histone modifications, some of which are compensatory, thereby ensuring cell survival. However, *Hmgn1*^{-/-} cells are significantly more sensitive to various stresses, including UV irradiation (Birger *et al*, 2003), X-ray irradiation, and heat shock (Birger, unpublished). It is tempting to speculate that the hypersensitivity of *Hmgn1*^{-/-} cells to stress is related to an altered pattern of post-translational modifications in histone tails.

Materials and methods

Materials

Antibodies to H3K14ac, H3S10p, and phosphoacetylated H3 and active PCAF HAT domain were from Upstate Biotechnology. Nucleosome CPs, histones, HMGNs, mutant HMGNs, and corresponding antibodies were prepared as described (Bustin, 1989; Lim *et al*, 2004a). The anti-HMGN1 antibodies recognize the S20,24E-HMGN1 mutant.

Cell culture

Mouse *Hmgn1*^{-/-} and *Hmgn1*^{+/+} embryonic fibroblasts (MEFs) and *Hmgn1*^{-/-} stably transformed with inducible vectors expressing either wild-type (clone 622, *Hmgn1*^{-/-}, pTet-HMGN1) or mutant (clone M101, *Hmgn1*^{-/-}, pTet-HMGN1 S20,24E) HMGN1 proteins under the control of the inducible TRE promoter were generated and grown as described (Birger *et al*, 2003). To accumulate an adequate amount of HMGN in nuclei, the cells were treated with Dox for various periods of time. After reaching 90% confluence, the cells were made quiescent by growing in serum-deprived medium (DMEM, 0.1% serum) for 48–72 h prior to the addition of 10 μ g/ml TSA. A minor level of leakiness was detected in clone M101 (Figure 1B, right).

Protein extraction and Western blotting

Approximately 2×10^6 cells were used for protein extraction and 2×10^7 cells were used for ChIP experiments. Cells were scraped with modified 1 $\times$ SDS sample buffer (45 mM Tris-HCl, 1 mM EDTA, 1% SDS, 2 mM DTT, 10% glycerol, 0.01% bromophenol blue, protease inhibitor cocktail, 50 nM okadaic acid, 100 μ M sodium orthovanadate, 10 mM sodium butyrate, 0.5 μ g/ml of TSA). After freezing and thawing, the samples were passed at least 10 times through a 25-gauge needle or sonicated, boiled for 10 min, centrifuged, subjected to SDS-PAGE, stained with Coomassie blue,

and quantified. The amount of protein in each extract was equalized, and the proteins were resolved by SDS-PAGE again. After semidry transfer onto PVDF membranes, the samples were analyzed by Western immunoblotting using ECL Plus kit (Amersham).

In vitro acetylation assay

All assays were performed in HAT buffer (50 mM Tris-HCl, pH 8.0, 10% glycerol (v/v), 1 mM dithiothreitol (DTT), 0.1 mM EDTA, and 5 mM butyric acid). Each 10 μ l reaction contained: nucleosome cores 0.1 mg/ml (or free histone H3 0.025 mg/ml), 0.2 mM [14 C]acetyl-CoA (20 nCi), 100 ng of recombinant PCAF (Upstate Cell Signalling Solutions, Cat #14-309), and various amounts of HMGN1 (added at specific molar ratio to H3 ratios varied from 0.5 to 4, depending on the experiment). The assay was performed at 37°C for 30 min. As the cPCAF is a more potent HAT than rPCAF, the quantity of rPCAF or cPCAF added to each assay was empirically determined as the amount of preparation required to yield nearly equivalent activities on nucleosome CPs. The reactions were stopped by the addition of an equal volume of a SDS-gel sample buffer (100 mM Tris-HCl (pH 6.8), 200 mM DTT, 2% SDS, 0.1% bromophenol blue, 20% glycerol), denatured for 5 min at 95°C, and the proteins were resolved on a 15% SDS-polyacrylamide gel. The gels were stained with Coomassie Blue for estimation of protein quantities, soaked in Enlightening Enhancer solution (Dupont) for 30 min, and vacuum dried. The radioactivity incorporated into the protein bands was visualized by a PhosphorImager (Molecular Dynamics) and quantified with ImageQuant software. Lineweaver-Burk plot showing the effect of HMGN1 on pCAF-mediated acetylation of nucleosome core was derived from HAT assays that were carried out either with a fixed concentration of nucleosomes (1 μ M) or increasing concentrations of [14 C]acetyl-CoA, in either the

presence or absence of HMGN1 (4 μ M), or with a fixed concentration of [14 C]acetyl-CoA (100 μ M) and various concentrations of nucleosomes (0.006–1 μ M) in either the presence or absence of HMGN1. In the latter series, the concentration of HMGN1 was adjusted to maintain a constant molar ratio of 4 HMGN1 to 1 core nucleosome. For inhibition studies, 0–250 μ M H3-CoA-20, a PCAF-specific inhibitor, was added to the PCAF complex (Lau *et al*, 2000).

ChIP and real-time quantitative PCR

ChIP experiments were performed according to the protocol recommended by Upstate as previously described (Birger *et al*, 2003). Each experiment was carried out with at least two different clones, each DNA was analyzed by real-time quantitative PCR at least three times. In brief, crosslinked chromatin (formaldehyde, final concentration 1%, at 37°C for 10 min) was lysed, sonicated to an average DNA length of 600 bp, and subjected to immunoprecipitation with ~8–10 μ g of affinity pure antibodies and 50 μ l of protein A-agarose beads. The immunoprecipitate was released by SDS, treated by proteinase K, phenol-extracted, and ethanol precipitated. Real-time quantitative RT-PCR was performed with an ABI PRISM 7900HT according to the supplier's recommendation using β -actin and β -globin gene primers for normalization.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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