Distinct Domains in High Mobility Group N Variants Modulate Specific Chromatin Modifications*

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We have demonstrated that levels of specific modification in histone H3 are modulated by members of the nucleosome-binding high mobility group N (HMGN) protein family in a variant-specific manner. HMGN1 (but not HMGN2) inhibits the phosphorylation of both H3S10 and H3S28, whereas HMGN2 enhances H3K14 acetylation more robustly than HMGN1. Two HMGN domains are necessary for modulating chromatin modifications, a non-modification-specific domain necessary for chromatin binding and a modification-specific domain localized in the C terminus of the HMGNs. Thus, chromatin-binding structural proteins such as HMGNs affect the levels of specific chromatin modifications and therefore may play a role in epigenetic regulation.

Post-translational modifications of core histones in chromatin are important molecular markers of epigenetic regulation and part of the mechanisms that regulate the orderly progression of DNA-related nuclear events, such as transcription, recombination, replication, and repair (1–3). The levels and patterns of histone modification in chromatin are not fixed; they are in a constant state of flux and are the result of an equilibrium established by the activities of enzymes that continuously add, and those that continuously remove, chemical groups from histones. Among the best-studied examples are the changes in the levels of acetylation and phosphorylation in the tail of histone H3. These and studies with histone H1 (10, 11) suggest that the levels of chromatin modifications are determined, not only by the opposing activities of kinases and phosphatases that access and modify specific residues in the tails of the nucleosomal histones (6, 7).

In recent studies (8, 9) with cells derived from Hmgn1−/− mice and in vitro analysis of the kinetics of histone modification in HMGN1−/− nucleosome complexes reveal that HMGN1 affects the levels of phosphorylation of both S10 and S28 and the levels of acetylation of K14 in H3. These and studies with histone H1 (10, 11) suggest that the levels of chromatin modifications are determined, not only by the opposing activities of the enzymes that continuously modify and demodify histones, but also by architectural chromatin-binding proteins that alter the ability of these enzymes to access and modify their nucleosomal targets. Significantly, the interaction of HMGN1 with nucleosomes reduced the levels of phosphorylation but enhanced the levels of acetylation, suggesting specificity in the effects of HMGN1 on chromatin modifications.

HMGN is a family of proteins that binds specifically to the 147-bp nucleosome core particle, i.e. the building block of chromatin, and alters the structure and activity of the chromatin fiber. The major members of this protein family, HMGN1 and HMGN2, are present in most vertebrate cells, have very similar structures, bind to nucleosomes with similar affinities, and produce identical footprints on nucleosomal DNA (12, 13). Yet, both in vivo and in vitro, they bind to nucleosomes specifically and form HMGN-specific homodimeric complexes containing either two molecules of HMGN1 or two molecules of HMGN2; heterodimeric nucleosome complexes containing one molecule of HMGN1 and one of HMGN2 are not formed (14). Because HMGN1 modulates the levels of histone modifications only when bound to nucleosomes, it is possible that the HMGN-specific nucleosome binding modes will lead to specific effects on chromatin modifications.

Here we tested whether modification in the tail of H3 is modulated in an HMGN-specific manner. We found that HMGN1 and HMGN2 affect differently the MSK1-mediated phosphorylation of H3S10 and H3S28 and the PCAF-mediated acetylation of H3K14. Using a battery of HMGN domain swap mutants, we identified the protein domains regulating the levels of these histone modifications. These findings demonstrate that structural proteins that interact with chromatin without any obvious specificity for the DNA sequence may have specific effects on the levels of chromatin modifications. As such, HMGN and similar chromatin-binding structural proteins may play a role in epigenetic regulatory mechanisms.

EXPERIMENTAL PROCEDURES

Preparation of Nucleosomes and Proteins and Construction of Domain Swap cDNAs—Nucleosome core particles were prepared from chicken red blood cells (15). Wild-type and mutant HMGN proteins were expressed in and purified from Escherichia coli cells as described previously (16). The cDNAs, which encode domain swap mutants of HMGN proteins (see Fig. 2), were created by overlap extension PCR (17). Additional details can be found in the supplemental material.

Biochemical Assays—For electrophoretic mobility shift assays and calculation of dissociation constants, core particles were incubated with the various concentrations of HMGNs in 2× Tris borate-EDTA, electrophoresed on 5% native polyacrylamide gels, and analyzed as described previously (18). Phosphorylation and histone acetyltransferase assay were performed as described previously (8, 9). Additional details can be found in the supplemental material.

RESULTS

To test for HMGN specificity in the modulation of H3 modification levels, we measured the ability of the H3 kinase MSK1 and the acetyltransferase PCAF to modify nucleosomal H3 in the presence of various amounts of either HMGN1 or HMGN2. MSK1 phosphorylates H3S10

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2 The abbreviations used are: HMGN, high mobility group N; CHUD, chromatin unfolding domain; PCAF, p300/CBP-associated factor; NBD, nucleosome binding domain; MSK1, mitogen- and stress-activated kinase; NLS, nuclear localization signal.
and H3S28 (19, 20), whereas PCAF acetylates mainly H3K14 (21). We have already demonstrated (8, 9) that HMGN1 enhances the PCAF-mediated acetylation of H3K14 and inhibits the MSK1-mediated phosphorylation of H3S10 and H3S28 only in the context of chromatin; the modification levels of free histones are not affected by HMGN1. We have also reported that MSK1 phosphorylates two conserved serines in the HMGN proteins (8) and that PCAF acetylates lysine 2 in HMGN2 but not in HMGN1 (11). These modifications can also be seen in Fig. 1.

Surprisingly, although HMGN1 inhibits histone H3 phosphorylation by MSK1 (Fig. 1A, lanes 3–6 and Fig. 1B, open circles), the closely related homologue HMGN2, which binds to the nucleosome core particle with the same affinity as HMGN1 (Fig. 2), does not inhibit the phosphorylation of histone H3 by MSK1 (Fig. 1, A and B). Western blots indicate that HMGN1 inhibits the phosphorylation of both H3S10 and H3S28, the two MSK1 targets of histone H3, whereas HMGN2 does not effect the phosphorylation of either (Figs. 1C and Fig. 3). The inhibition of the phosphorylation of nucleosomal H3 by HMGN1 is dependent on its nucleosome binding; HMGN1 mutants that do not bind to chromatin do not inhibit this phosphorylation (8). Because HMGN1 and HMGN2 bind to nucleosomes with similar affinity (Fig. 2A, right column), these results indicate a HMGN variant-specific effect on histone H3 phosphorylation.

HMGN variant-specific effects were also observed by analysis of the PCAF-mediated acetylation of nucleosomal H3K14. Thus, although HMGN1 enhances the acetylation of histone H3 <2-fold, (Fig. 1D; E, open circles), HMGN2 has a significantly stronger effect and enhances the acetylation levels almost 3.5-fold (Fig. 1D, lanes 7–10; E, closed triangles). These results reveal HMGN-type specificity in the modulation

![Figure 1. Differential effect of HMGN1 and HMGN2 on histone phosphorylation and acetylation. A, HMGN1 (but not HMGN2) inhibits the MSK1-mediated phosphorylation of H3. Shown are autoradiograms (upper panel) and corresponding Coomassie Blue (CBB) gels. B, quantification results of the autoradiogram in A. C, Western analysis demonstrates that HMGN1 (but not HMGN2) inhibits the phosphorylation of both H3S10 and H3S28. D, HMGN2 enhances the PCAF-mediated acetylation of H3 more efficiently than HMGN1. The upper panel shows the autoradiogram of the lower panel stained by CBB. E, quantification results of the autoradiogram in D. Open circles and closed triangles represent results of HMGN1 and HMGN2, respectively. Error bars in B and E show S.D. calculated from three independent experiments.](10183f1a.png)
FIGURE 2. Generation and characterization of HMGN domain swap mutants. A, schematic structure of HMGN1, HMGN2, and the swap mutants used in this study. HMGNs contain the four domains: (13) NLS1 (residues 1–12 of HMGN1 or 1–16 of HMGN2) and NLS2 (residues 42–73 of HMGN1 or 47–67 of HMGN2), which contain nuclear localization signals and NBD (residues 13–41 of HMGN1 or 17–46 of HMGN2) and CHUD (residues 74–99 of HMGN1 or 68–89 of HMGN2), which facilitate chromatin unfolding. The $K_d$ values of the mutants for the binding to nucleosomes is shown on the right. B, Coomassie Blue-stained gel of the purified swap domain mutant proteins. C, mobility shift assay of nucleosome cores (CP) with the various swap mutants. Nucleosome core particles were incubated with HMGN proteins and then separated on 5% native PAGE in 2X Tris borate-EDTA. In this cooperative binding condition, two molecules of HMGN bound to one molecule of nucleosome core particle, and the complex between HMGN and the nucleosome formed a specific mobility shift indicated as $CP + 2HMGNs$. 

HMGN Variant-specific Effects on Histone Modifications
of both the phosphorylation and acetylation of histone H3 in nucleosomes.

The differences between HMGN1 and HMGN2 in modulating histone modification is unexpected, because the structure and biochemical properties of these two proteins are very similar (12, 22). To further verify that the two proteins indeed have distinct effects on histone modifications, we examined whether their ability to modulate histone modification levels localizes to specific HMGN protein domains. All HMGNs contain four major domains (12); two domains marked as NLS1 and NLS2 (Fig. 2A) contain nuclear localization signals, a nucleosome binding domain (Fig. 2A, NBD), and a chromatin unfolding domain (Fig. 2A, CHUD). To identify the region of HMGN1 that is responsible for the inhibition of histone H3 phosphorylation or enhancement of H3 acetylation, we generated a series of domain swap mutants between HMGN1 and HMGN2 (Fig. 2A). All of the swap mutants were expressed in bacteria and purified to homogeneity (Fig. 2B). Electrophoretic mobility shift assays (Fig. 2C) and Kd calculations (Fig. 2A) indicate that all of the swap mutants bound to nucleosomes with affinities similar to those of the native proteins.

To test whether a specific domain in HMGN1 is mainly responsible for the inhibition of the MSK1-dependent phosphorylation of histone H3, we first tested the inhibitory activity of the swap mutants HMGN2-N1NBD, in which the NBD of HMGN2 (residues 17–46 of HMGN2) is replaced by the NBD of HMGN1 (residues 13–41 of HMGN1), and of HMGN2-N1C, in which the entire HMGN2 C terminus was replaced by domains NLS2 and CHUD of HMGN1. We found that HMGN2-N1NBD does not inhibit the phosphorylation of either H3S10 or H3S28, whereas HMGN2-N1C inhibits the phosphorylation of both H3S10 and H3S28 to the same degree as native HMGN1 (Fig. 3A, lanes 1–10). Thus, the inhibitory activity localizes to the C-terminal portion of

FIGURE 3. Identification of the HMGN domains affecting post-translational modification in H3. A, the NLS2 domain of HMGN1 inhibits the MSK1-mediated phosphorylation of H3S10 and H3S28. Phosphorylation assays containing purified nucleosome cores and the protein indicated on the top of each lane (molar ratio of CP to protein, either 1 or 3) were fractionated on native gels and analyzed by Western blot, with the antibodies indicated on the left. Western blots with anti-H3 indicate equal loading and transfer of the protein from the gel to the membrane. B, the CHUD of HMGN2 is the region responsible for the strong enhancement of the acetylation of histone H3 by PCAF. The acetylation reaction of the nucleosome core particle by PCAF was performed as described for A, except that the PCAF-mediated incorporation of 14C was measured. The relative acetylation levels, calculated from the autoradiogram, are indicated below the lanes of the upper panel. Coomassie blue (CBB)-stained gel indicates equal loadings. C, scheme depicting the HMGN domains involved in the modulation of post-translational modifications in the tail of histone H3.
HMGN1. The swap mutant HMGN2-N1L52, in which the NLS2 region of HMGN1 replaces that of HMGN2, inhibits the phosphorylation of both H3S10 and H3S28, whereas the swap mutant HMGN2-N1CHUD, which contains only the CHUD region of HMGN1, does not (Fig. 3A, lanes 1–15). Thus, the MSK1-inhibitory activity localizes to the NLS2 domain of HMGN1. Indeed, the deletion mutant HMGN1-Cdel52, which lacks the CHUD domain but contains the NLS2 domain, did inhibit the phosphorylation of both H3S10 and H3S28, whereas the deletion mutant HMGN1-Cdel55, which lacks 55 amino acid residues from the C terminus of HMGN1 and does not contain the NLS2 domain, did not (Fig. 3A, lanes 16–20). Taken together, the results indicate that the specific inhibitory action of HMGN1 on the phosphorylation of S10 and S28 in the N terminus of H3 resides in the NLS2 region of the protein.

The swap mutants described above were also used to identify the HMGN2 region involved in the enhancement of the PCAF-mediated H3 acetylation. We found that HMGN2-N1NBD enhances the H3 acetylation to the same extent as native HMGN2, whereas HMGN2-N1C enhances acetylation significantly less, to the same degree as native HMGN1 (Fig. 3B, lanes 1–10). Thus, the C-terminal domain of HMGN2 contains the acetylation-enhancing activity. HMGN2-N1L52, which contains the CHUD domain of HMGN2, enhances acetylation, whereas HMGN2-N1CHUD, which contains the NLS2 of HMGN2 and the CHUD of HMGN1, did not enhance acetylation (Fig. 3B, Lanes 11–16). Thus, the CHUD domain of HMGN2 is the main HMGN domain specifically involved in enhancing the PCAF-mediated H3 acetylation; the effect of HMGN1 is significantly smaller.

**Discussion**

Our major finding is that the levels of specific post-translational modifications in the N terminus of histone H3 are differentially regulated by two closely related proteins, HMGN1 and HMGN2. HMGN modulate chromatin modifications only when bound to chromatin; however, the modulating activity of the proteins localize to specific domains that are distinct from the domain necessary for chromatin binding. The HMGN specificity in modulating distinct modifications in the tail of H3 establishes the principle that structural chromatin-binding proteins modulate the levels of chromatin modifications and therefore may play a role in epigenetic regulatory mechanisms.

In considering the molecular mechanisms whereby HMGN proteins affect the acetylation and phosphorylation of H3, we first note that the effects require direct interaction of the proteins with nucleosomes. We have already demonstrated that HMGN point mutants that do not bind to nucleosomes do not affect the levels of H3 modification either in vitro or in vivo (8, 9). Likewise, we have demonstrated that HMGN1 does not affect the modification of free, non-nucleosomal histones. One possible explanation for the inhibitory effects of HMGN1 on MSK1-mediated phosphorylation is that the binding of the entire molecule to the nucleosomes hinders the ability of the kinase to reach its target. This possibility is not compatible with the domain swap mutants. First, neither the mutant HMGN2-N1NBD nor the mutant HMGN1-Cdel55, both of which contain the HMGN1 nucleosome binding domain, inhibit phosphorylation of H3, which is proof that the binding itself is not the main cause for inhibition. Second, although HMGN1 and HMGN2 generate very similar nucleosomal DNA footprints, only HMGN1 inhibits phosphorylation, additional proof that the inhibition is not simply due to the binding of the protein to chromatin. Finally, the studies with additional mutants localizes the inhibitory effect to the NLS2 domain of HMGN1, indicating that only a specific region of the protein is responsible for specifically inhibiting the ability of MSK1 to phosphorylate nucleosomal H3.

Significantly, the NLS2 domain is not a general, nonspecific inhibitory domain. It does not affect the PCAF-mediated acetylation of H3K14, located only four amino acids away from H3S10. The net charge of the NLS2 domain of HMGN1 is similar to that of HMGN2; however, the amino acid sequence is significantly different. When inserted into HMGN2 protein, this sequence inhibits phosphorylation. Thus, the sequence of the NLS2 region of HMGN1 functions as an MSK1 inhibitory domain. These considerations are also relevant to understanding the main HMGN2 determinants that enhance the PCAF-mediated acetylation of H3K14. The difference in the amino acid sequence of the CHUD domains of HMGN1 and HMGN2 may be the underlying reason for their different acetylation-promoting activities. Our findings that the NLS2 domain of HMGN1 inhibits phosphorylation and that the CHUD domain of HMGN2 enhances the PCAF-mediated acetylation of H3 are strong evidence that HMGN proteins affect post-translational modifications at the local, nucleosome levels. Thus, the effect of HMGNs is not simply due to either the steric hindrance that inhibits the ability of a modifying enzyme to reach its target nor to their known “chromatin unfolding” activity that may enhance the ability of modifiers to reach their target.

Taken together, these results indicate that two domains of HMGN are involved in regulating chromatin modifications. First, the NBD domain is necessary for chromatin interaction; modifications in this domain that abolish chromatin binding also abolish the ability of the proteins to modulate chromatin modification. With respect to the modification affected, the NBD domain acts as a nonspecific module in that it will affect all chromatin modifications that are mediated by HMGNs. The second domain is located in the C-terminal region of the proteins. Within this region, we have identified subdomains that either up-regulate or down-regulate the levels of specific modifications. Thus, the domains in the C terminus can be considered as modification-specific domains. We suggest that, once the HMGN protein is bound to nucleosomes, domains in the C terminus interact with the tails of histone and induce steric changes that alter the ability of modifying enzymes to modify their targets. This possibility is supported by the observation that these proteins form HMGN variant-specific complexes with nucleosomes. We have already suggested that, upon binding, HMGNs induce allosteric transitions in the nucleosomes, which favor the formation of homodimeric HMGN complexes (14). HMGNs affect, not only the phosphorylation of H3S10 and H3S28 and the acetylation of H3K14, but also H3K4 methylation, H3K9 acetylation, H3K9 methylation (8), and modifications in H2A (3). Thus, the interaction of HMGN with chromatin may affect the levels of several types of modifications in the tail of the core histones.

Our findings that HMGN1 and HMGN2 have differential effects on the phosphorylation and the acetylation of histone H3 are the first observations describing functional differences between HMGN1 and HMGN2 proteins. Together with our previous findings (8, 9), they reinforce and expand the notion that HMGN proteins are part of the mechanisms that regulate the dynamics of histone modifications on chromatin. In living cells, HMGN functions within a network of chromatin-binding proteins, such as H1, HMGB, and HMG1, which compete for nucleosomal binding sites (23). As demonstrated for H1 (10, 11), it is likely that other structural chromatin-binding proteins will also affect the levels of histone modifications by mechanisms similar to those described here. Therefore, we suggest that the histone modification pattern in chromatin is determined, not only by the equilibrium between the enzymatic activities that modify and demodify the histones, but also by HMGNs.

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3 Y. V. Postnikov and M. Bustin, unpublished data.
but also by the dynamic and competitive binding of structural chromatin proteins.

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