HP1 Binds Specifically to Lys^{26}-methylated Histone H1.4, whereas Simultaneous Ser^{27} Phosphorylation Blocks HP1 Binding*\[5\]

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Histone lysine methylation can have positive or negative effects on transcription, depending on the precise methylation site. According to the “histone code” hypothesis these methylation marks can be read by proteins that bind them specifically and then regulate downstream events. Hetero-chromatin protein 1 (HP1), an essential component of heterochromatin, binds specifically to methylated Lys^{9} of histone H3 (K9/H3). The linker histone H1.4 is methylated on Lys^{26} (K26/H1.4), but the role of this methylation in downstream events remains unknown. Here we identify HP1 as a protein specifically recognizing and binding to methylated K26/\textit{H1.4}. We demonstrate that the Chromo domain of HP1 is mediating this binding and that phosphorylation of Ser^{27} on H1.4 (S27/\textit{H1.4}) prevents HP1 from binding. We suggest that methylation of K26/\textit{H1.4} could have a role in tethering HP1 to chromatin and that this could also explain how HP1 is targeted to those regions of chromatin where it does not colocalize with methylated K9/H3. Our results provide the first experimental evidence for a “phospho switch” model in which neighboring phosphorylation reverts the effect of histone lysine methylation.

In eukaryotic cells the DNA is packaged into chromatin. The building block of chromatin is the nucleosomal core particle containing a histone octamer (two each of the core histones H2A, H2B, H3, and H4) around which 147 bp of DNA are wrapped (1). Linker histone H1 binds to the DNA between the nucleosomal core particles and stabilizes higher order chromatin structure (2).

The N-terminal tails of the core histones protrude from the nucleosomal surface and are subject to multiple covalent modifications including methylation, phosphorylation, and acetylation. These modifications have the potential to regulate chromatin architecture and thereby can affect all aspects of DNA processing. According to the so called “histone code” hypothesis these modifications could be read by proteins that bind to specific modifications and then can regulate downstream events (3, 4).

Methylation of lysines has positive as well as negative effects on transcription, depending on the methylation site. The methylation of lysine 9 of histone H3 (K9/H3) and lysine 27 of histone H3 (K27/H3) has generally been implicated in transcriptional repression. Methylated K9/H3 is specifically recognized and bound by Heterochromatin protein 1 (HP1)\[3\] (5–8). HP1 has a role in heterochromatin organization, maintenance, and in gene repression. In mammals three HP1 isoforms HP1α, HP1β(M31), and HP1γ(M32) have been identified. The HP1 proteins are very similar in their amino acid sequence, they contain a conserved N-terminal Chromo domain (CD), a more variable hinge region and a conserved C-terminal Chromoshadow domain (CSD). This modular organization of HP1 allows several proteins to bind simultaneously to HP1. The CD binds specifically to methylated K9/H3; the hinge region can bind to RNA, DNA, and chromatin, whereas the CSD is involved in self-association and interacts, e.g. with the DNA methyltransferases Dnmt1 and 3a, and the lysine methyltransferase Suz39. Since Suz39 can methylate K9/H3 and HP1 binds to methylated K9/H3, the propagation of heterochromatin could be ensured by a “self-sustaining loop” (for a review, see Ref. 9 and references therein). However, the methylation of K9/H3 is necessary but not sufficient for targeting of HP1 (10), and it has been suggested that other distinct, yet unidentified, HP1 interaction sites could be involved in HP1 targeting (9).

In mammalian cells four histone H1 variants are present in all somatic cells (H1.2 to H1.5), and a fifth (H1.1) is restricted to thymus, testis, and spleen and possibly lymphocytic and neuronal cells (11). Histone H1 is also covalently modified. Multiple phosphorylation sites have been mapped (12). It has been suggested that H1 phosphorylation can promote chromatin decondensation, enabling access to chromatin (13). Histone H1.4 is di-methylated at Lys^{26} (me2K26/H1.4), which is next to a phosphorylation site at Ser^{27} (12). K26/H1.4 is located within the flexible N-terminal domain of H1 just preceding the globular domain. K27/H3 and K26/H1.4 are methylated by a multisubunit methyltransferase complex PRC2 (Polycomb repressive complex 2) whose catalytic subunit is the polycomb protein Ezh2. The specificity depends on the isoform of the EED protein present in the PRC complex (14). The methylation of K26/H1.4 is important for the transcriptional repression by Ezh2 (14). The extension of the so called “histone code” to include linker histones has been recently suggested but not yet demonstrated, and the function of specific methylation in H1.4 is largely unknown (14).

To get insight into the functional role of linker histone modifications, we chose to study the recently described K26/H1.4 di-methylation. The identification of proteins that are able to bind specifically to methylated K26/H1.4 is essential to understand how this modification might be read. We found that HP1(s) binds specifically via its CD to methylated Lys^{26} of histone H1 and that simultaneous phosphorylation of neigh-
FIGURE 1. HP1 binds to methylated K26/H1.4. A, the sequences of Histone H3 aa 5–11 and histone H1.4 aa 22–28. B, unmodified (un), meK26/H1.4 (meK26), me9/K9/H3 (meK9, lane 3), meK64/H3 (meK64, lane 4), or meK20/H4 (meK20, lane 5) peptides were immobilized onto Sulfolink-Sepharose via a C-terminal cysteine residue and used as affinity columns. Recombinant GST-HP1α, GST-HP1β, or GST-HP1γ was assayed for binding. Bound proteins were visualized by Coomassie staining. Lane 6 represents 20% of the input. C, binding to meK26/H1.4 peptide as affinity column was performed as described for B but in the presence of free meK26/H1.4 (lane 1) or unmodified H1.4 peptides (lane 2) as competitors. Lane 4 represents 20% of input for HP1α and HP1β and 40% for HP1γ. D, binding of HP1γ CD to meK26/H1.4 peptide was performed as described for C, but 1, 3, 10, or 20 μg of free meK26/H1.4 (lanes 3–6) or meK9/H3 peptides (lanes 7–10) were used as competitors. Lane 1 represents 20% of input. E, Western blot of meK26/H1.4 affinity column-purified 38B9 nuclear extract with HP1 subtype-specific antibodies. F, permeabilized HeLa cell nuclei were challenged with no peptide (lane 2), unmodified H1 (lane 1), or meK26/H1.4 peptides (lane 3). After incubation the supernatants were assayed by Western blot and probed with an antibody specific for HP1α and β.
Binding of HP1 to Methylated H1

90 min on a wheel at 4°C and washed three times in IPH-E before resolution on a 15% SDS-PAGE. Bound proteins were visualized by Coomassie staining or Western blotting as indicated. For competition experiments HP1 was preincubated with free competitor peptides, and the binding was performed in presence of the competitor.

Preparation of Nuclei and Peptide Challenge—HeLa nuclei were purified, permeabilized, diluted into PBS, and incubated with and without peptide for 2 h on ice as described (6). The supernatants were analyzed by Western blot for the presence of HP1.

Far-Western-type Overlay Assays and Immunocytochemistry—As described previously (19) mouse NIH 3T3 cells were fixed for 10 min with 4% paraformaldehyde in PBS and permeabilized with PBS, 0.6% Triton X-100 for 10 min. Coverslips were incubated overnight with 0.5 μg/ml recombinant GST or GST-HP1α protein in PBS, 5% bovine serum albumin, 0.2% Tween 20 either in the absence or in the presence of 100-fold molar excess of histone peptides. Next we performed primary and secondary antibody stainings with an anti-GST antibody (Sigma) and Alexa Fluor 488 anti-rabbit IgG (Jackson Immunoresearch) before counterstaining of nuclei acid with DAPI and image acquisition with a Leica SP2 UV confocal microscope (Leica Microsystems).

RESULTS

HP1 Binds Specifically to Methylated K26/H1.4—To identify proteins specifically binding to Lys26-methylated H1.4 we followed a candidate approach. The CD of HP1 binds specifically to methylated K9/H3 (meK9/H3) (5–8). Based on the sequence conservation of the regions surrounding K9/H3 and K26/H1.4 (Fig. 1A) we wanted to know whether HP1 can also bind to methylated K26/H1.4. We coupled H1.4 peptides methylated or unmethylated at Lys26 onto Sepharose beads and used them as affinity columns. As shown in Fig. 1B, recombinantly expressed mouse HP1α, HP1β, and HP1γ bind, as expected, to meK9/H3 peptides (lane 3) but also to meK26/H1.4 peptides (lane 2). No binding was observed to unmethylated H1.4 peptides (lane 1) or to meK64/H3 or meK20/H4 peptides (lanes 4 and 5). This binding to meK26/H1.4 was specific and reproducible using two different H1.4 peptides (corresponding to amino acids 20–33 and 23–32) (data not shown).

To verify the specificity of HP1 for meK26/H1.4 we performed pull-down experiments in the presence of free competitor peptides. Fig. 1C shows that meK26/H1.4 (lane 1) but not unmethylated H1.4 peptides (lane 2) disrupt the binding of HP1 to meK26/H1.4. Additionally we performed cross-competition experiments using the H1.4 and the H3 peptides. These experiments show that the meK26/H1.4 peptide competes better for the binding of the HP1β CD to meK26/H1.4 than the meK9/H3 peptide (Fig. 1D) and vice versa (data not shown). Taken together this data demonstrates that all subtypes of HP1 (HP1α, HP1β, HP1γ) bind directly and specifically to methylated K26/H1.4.

We next asked whether endogenous HP1 also binds to methylated K26/H1.4. As shown by Western blot with HP1 subtype-specific antibodies (Fig. 1E), all three isoforms of HP1 can be affinity purified from 38B9 cell nuclear extract with meK26/H1.4 (lane 2) but not with unmethylated (lane 3) H1.4 peptides. We obtained the same result using human nuclear extract suggesting that this interaction is conserved (data not shown).

We then investigated whether methylated H1.4 has a role in tethering HP1 to chromatin. To address this we prepared nuclei from HeLa cells, permeabilized them, and challenged them with methylated or unmethylated H1.4 peptides to elute HP1 from chromatin. After pelleting the chromatin fraction we probed the supernatant for the presence of HP1. Fig. 1F shows that challenging nuclei with excess of meK26/H1.4 peptide increases the proportion of HP1 in the soluble fraction (compare lanes 2 and 3), whereas challenging with the unmethylated H1.4 peptides did not result in a significant increase of HP1 in the soluble fraction (lane 1). Therefore a meK26/H1.4 peptide can displace HP1 from native chromatin, confirming the specific binding of HP1 to methylated Lys26.

HP1 Binding to meK26 is Mediated by the CD—To map the domain of HP1 that interacts with meK26/H1.4 we used HP1 deletion constructs. Fig. 2A shows that the HP1α CD binds specifically to meK26/H1.4 peptides (lane 2, top panel). The HP1α hinge region and the HP1α CSD are not binding. Full-length HP1α only binds to the meK26/H1.4 peptides (lane 2, bottom panel). Additionally we analyzed the binding of the three HP1 CD His fusions to K26/H1.4. As shown in Fig. 2B, the CD

FIGURE 2. The chrom domain of HP1 binds to methylated K26/H1.4. A, bacterially expressed and purified GST-HP1α proteins encompassing the CD domain (aa 1–66), the hinge region (aa 67–119), the CSD domain (aa 117–191), and full-length HP1 were tested for binding to columns as described in Fig. 1. Lane 3, Inp. represents 20% of the input. Bound proteins were visualized with GST-specific antibody. B, His fusions of HP1α, HP1β, or HP1γ-CD were tested for binding to unmethylated (lane 2) or meK26/H1.4 (lane 3) peptides. Bound proteins were visualized with His-specific antibody. C, HP1β with a point mutation in the CD, K41W42 to AA, and HP1 wild type (wt) were assayed for binding to unmethylated (lane 1) or meK26/H1.4 (lane 2) peptides. Bound proteins were visualized by Coomassie staining.
of HP1α, HP1β, and HP1γ bind all to meK26/H1.4 but not to unmethylated peptides (compare lanes 1 and 2).

To examine whether an intact CD is required for meK26/H1.4 binding, we tested mutations in the CD of HP1 for binding. The K41W42 to AA mutation affects central residues in the CD (8). This mutation abolishing the binding of HP1 to meK26/H1.4 peptides (Fig. 2, lane 2), testifying for the importance of an intact CD for the binding to methylated H1.4.

Phosphorylation of Ser27 Prevents HP1 Binding—According to the “binary switch” model a phosphorylation next to a lysine methylation could reverse the effect of the methylation (15). In the case of H1.4, a Lys26-methylated and Ser27-phosphorylated form has been described (12). This prompted us to investigate how the phosphorylation of Ser27 influences the binding of HP1 to meK26/H1.4. Fig. 3A shows that recombinant HP1α, HP1β, and HP1γ no longer bind to meK26/H1.4 peptide when it is simultaneously phosphorylated on Ser27 (compare lanes 2 and 1). To test whether the absence of binding was indeed due to the phosphorylation of Ser27 we used λ-phosphatase to remove the phospho group from the meK26/phosphoS27/H1.4 peptide. Fig. 3B shows that dephosphorylation of the peptide restores the binding (compare lane 4 with lanes 2 and 3) confirming that Ser27 phosphorylation blocks HP1 binding.

We next asked whether competition with H1.4 peptides affects the localization of HP1 in NIH 3T3 cells performing overlay assays as previously described (19). Fixed and permeabilized NIH 3T3 cells were incubated with recombinant GST–HP1α fusion protein in the presence of different peptide competitors. Fig. 3C, panels 2 and 10, show a specific nuclear localization of GST–HP1α (compared with control GST, panels 1 and 9), similar to the predominant localization of endogenous HP1α to foci of pericentromeric heterochromatin (Ref. 9 and data not shown). The localization of HP1 did not change when unmethylated H1.4 peptide was added as competitor (panels 3 and 11). However, addition of the meK26/H1.4 peptide results in displacement of GST–HP1 from the pericentromeric heterochromatin foci and a clear reduction of the nuclear staining (panels 4 and 12) as it is observed with the meK9/H3 peptide competitor (panels 7 and 15). Addition of the meK26/phosphoS27/H1.4 peptide fails to compete for GST–HP1α binding and does not affect the HP1α localization (panels 5 and 13). Use of phosphatase treated meK26/phosphoS27/H1.4 peptide restores the competition capacity of the peptide (compare panels 6 and 14 with panels 4 and 12 and 5 and 13). Note that as described previously (19) the meK9/phosphoS10/H3 peptide causes HP1 displacement from pericentromeric heterochromatin foci, suggesting that our approach with the short peptides is relevant to the native histone molecules (panels 8 and 16).

HP1 Binds to Native Methylated H1.4—We then used purified native H1.4 from human HL60 cells to assess HP1 binding. Fig. 4A shows that HP1 full-length (lane 1) and HP1 CD (lane 2) bind native H1.4. No binding is observed with the HP1 CSD (lane 6). Strikingly, only a meK26/H1.4 peptide competitor disrupts this binding (lane 4), but not an unmethylated H1.4 peptide (lane 3), indicating that the Lys26 methylation of native H1.4 is important for the binding. Interestingly, a meK26/phosphoS27/H1.4 peptide cannot compete for this binding (lane 5).

To verify the importance of the Lys26 methylation for the binding to HP1 we asked whether HP1 binds preferentially to methylated H1.4. To this end we raised a meK26/H1.4-specific antibody (see supplemental Fig. 1). We then tested whether using an HP1β affinity column results in an enrichment of Lys26-methylated H1.4. Using the meK26/H1.4-spe-

FIGURE 3. Phosphorylation of S27/H1.4 prevents HP1 binding. A, meK26/H1.4 (lane 2) or meK26/phosphoS27/H1.4 (me/P, lane 1) peptides were used as affinity columns to assess binding of recombinant HP1. Bound HP1α, HP1β, or HP1γ was visualized by Coomassie staining. Lane 3 represents 20% of the input. B, H1.4 peptide columns were used to test binding of recombinant HP1 except that the meK26/phosphoS27/H1.4 peptide was dephosphorylated by incubation with λ-phosphatase (lane 4), and binding efficiency was compared with the untreated peptide (lane 3). Lane 1 represents 20% of the input. C, fixed and permeabilized NIH 3T3 cells were incubated with recombinant GST (panels 1 and 9) or GST–HP1α (panels 2–8 and 10–16), and peptide competition experiments were performed by incubating cells with the following peptides: unmodified K26/H1.4 (panels 3 and 11), meK26/H1.4 (panels 4 and 12), meK26/phosphoS27/H1.4 (panels 5 and 13), meK9/H3 (panels 7 and 15) meK9/phosphoS10/H3 (panels 8 and 16), and dephosphorylated meK26/phosphoS27/H1.4 peptide (lanes 6 and 14). Foci observed upon staining with anti-GST antibody (panels 2–8) correspond to the previously described HP1 localization to pericentromeric heterochromatin (compare with DAPI staining, panels 10–16). All cells were analyzed in parallel using the same confocal parameters.
Binding of HP1 to Methylated H1

**FIGURE 4. HP1 binds to methylated native H1.4.** A, HP1-3-His (HP1 full-length (HP1fl)) (lane 1), HP1 CD (lanes 2–5), and HP1 CSD (lane 6) fusions were coupled to beads and then used as affinity columns to assess the binding of human native H1.4 purified from HL60 cells (that do not express H1.1). Unmodified K26/H1.4 (lane 3), meK26/H1.4 (lane 4), or meK26/ phosphoS27/H1.4 (lane 5) peptide was used as competitors. Bound H1.4 was visualized with antibody 2376 (see "Material and Methods"). Lane 7 represents 30% of the input. B, Coomassie staining of the fusion proteins used described for A. C, HP13 full-length (HP1fl) was used as affinity column (as described for A) to assay for an enrichment of methylated H1.4 relative to total H1.4. To detect bound H1.4 we used antibody 2376 (top panel), to detect methylated H1.4 we used a meK26/H1.4-specific antibody (bottom panel) (see "Material and Methods" and supplemental Fig. 1). There is a significant enrichment of Lys26-methylated H1.4 bound to HP1 compared with total H1.4 (compare lanes 1 and 2, top and *bottom panels*) bound to HP1. We quantified the bands with the Quantify one 4.2.3 software (Bio-Rad) and normalized the signals of bound protein relative to input. The enrichment of methylated H1.4 relative to total H1.4 was estimated to 2.5-fold (see histogram).

A specific antibody we detected a significant enrichment of Lys26-methylated H1.4 bound to HP1 compared with total H1.4 bound to HP1 (Fig. 4C, compare lanes 1 and 2, top and *bottom panels*). With the help of quantification software we determined an ~2.5-fold enrichment of methylated H1.4 bound to HP1 (Fig. 4C, *histogram*).

Taken together these data suggest that binding of HP1 to methylated K26/H1.4 is important for tethering HP1 to chromatin. Moreover simultaneous phosphorylation of Ser27 prevents binding of HP1 to H1.

**DISCUSSION**

**HP1 Binds to Methylated K26/H1.4**—In this study we show that HP1 binds in vitro specifically to H1.4 peptides and native H1.4 methylated on Lys26 and that this binding is specifically competed with meK26/H1.4 peptides (Figs. 1 and 4). Moreover, we have mapped the interaction site to the CD of HP1 and demonstrated that the CD needs to be intact for HP1 to bind to meK26/H1.4 (Figs. 2 and 4). Importantly the phosphorylation of Ser27 prevents the binding of HP1 to methylated H1.4 (Fig. 3).

Despite the sequence similarity around K26/H1.4 and K9/H3 our competition studies suggest that the binding to meK26/H1.4 is specific (Fig. 1D). The fact that we detect binding of purified human H1.4 to HP1 and an enrichment of methylated H1.4 (Fig. 4) strongly supports the relevance of our finding.

Losson and colleagues (20) showed that HP1 interacts with H1. They demonstrated by far-Western analysis that the hinge region of HP1α binds to H1. This binding might provide an additional contact and might have a role in stabilizing the interaction between HP1 and H1. We observe a specific interaction of the HP1 CD with human H1.4. In our experiments the HP1 CD can also bind to purified human H1 containing nucleosomes (data not shown). Additionally, we can compete HP1 in vitro from chromatin (Fig. 1F) and change the nuclear localization of HP1 (Fig. 3C) by competition with methylated H1.4 peptides. Differences to Nielsen et al. (20) might be due to different experimental conditions and approaches and the use of different H1 sources. We used purified human H1.4 and human-based H1.4 peptides in our experiments, while Nielsen et al. (20) used calf thymus H1. There is so far no evidence that calf H1 can be methylated at Lys26. Moreover among different species, the region surrounding Lys26, which may be important for the binding of HP1 varies. All our results point strongly toward an interaction of the HP1 CD with methylated human H1.4.

Lys26 is present in all of the somatic linker histone subtypes but, however, not in more specialized H1 variants. At least H1.2 and H1.4 are methylated at Lys26 (21), but only H1.4 is known to be phosphorylated at the neighboring serine. The regions around Lys26 differ between the variants. Therefore it will be interesting to test the binding of HP1 to other methylated H1 variants besides H1.4.

At a Possible Function for H1 Methylation—The binding of HP1 to methylated K9/H3 is necessary but not sufficient (10) for targeting HP1 to chromatin. In line with this, HP1 and methylated K9/H3 do not completely colocalize (9), which raises the question whether additional HP1 interaction sites are involved in tethering HP1. Binding of HP1 to methylated H1.4 could provide these additional interactions.

So far the only described HMT that can methylate K26/H1 is EzH2. The binding of HP1 to methylated K26/H1.4 would be consistent with the role of H1 methylation by EzH2 in transcriptional repression (14). Recently, an interaction between Su(z)12, a component of PRC2, and HP1 has been described (22). Therefore PRC2 could have a role in recruiting HP1 (and vice versa). This suggests the existence of an extended self-sustaining loop; HP1 can bind to methylated K9/H3 and/or methylated K26/H1.4 and interacts with the methyltransferases EzH2 and SuV39 that methylate K26/H1.4 and K9/H3, respectively. This loop might have an important role in gene repression and heterochromatinization.

H1 binding to chromatin is dynamic; the linker histone does not remain associated with a specific site throughout the cell cycle (23). In a similar way HP1 binding to chromatin seems to be highly dynamic (24). It is tempting to speculate that the dynamic localization of HP1 on chromatin could be, at least partially, a consequence of the dynamic association of H1 with chromatin. It is important to notice that post-translational modifications of H1 can modulate its association with nucleosomes (25). If the methylation of K26/H1.4 might modulate its residence time on a specific nucleosome, the hypothesis of a differential targeting of HP1 to specific sites of chromatin via methylated H1.4 is quite appealing. Testing this and being able to demonstrate how general the role of H1 methylation in targeting of HP1 to specific sites of chromatin awaits the development of methyl-K26/H1.4 specific antibodies that are suitable for immunocytochemistry or immunoprecipitation.

**Simultaneous Phosphorylation and Methylation of H1.4 Prevents HP1 Binding**—The phosphorylation of H1 increases the dynamic exchange of H1 from chromatin (26), and it is involved in gene activation and repression (27). Phosphorylation of H1 has also been correlated with chromatin decondensation (28). Lys26 in H1.4 is within a modification cluster (15); it can be methylated or acetylated, and neighboring Ser27 can be phosphorylated (12). Given that methylation of H1.4 might have a role in tethering HP1 to chromatin and heterochromatinization, then loss of HP1 could be necessary for decondensation. This could be regulated by phosphorylation of Ser27/H1.4 and the resulting displacement of HP1.

In the case of H3, the phosphorylation of S10/H3 does not result in loss of HP1 binding (Fig. 3C and Ref. 29). Thus, it is possible that despite a sequence similarity each phospho-methyl mark behaves differently. Our finding that phosphorylation leads to loss of HP1 binding is in agreement with the binary switch hypothesis postulated by Allis and colleagues (15) and extends it toward linker histone H1. This is also the
first experimental evidence showing that adjacent phosphorylation could reverse the effect of histone lysine methylation.

In conclusion we have shown a new, specific binding of HP1 to methylated Lys26 on histone H1.4, a mechanism that may explain, at least partially, how HP1 could be tethered to chromatin particularly to regions lacking methylated K9/H3. Importantly, simultaneous phosphorylation of the neighboring residue blocks HP1 binding. However, the events that lead to HP1 recruitment to methylated K26/H1.4 in vivo await further investigation.

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