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Binding of the CHD4 PHD2 finger to histone H3 is modulated by covalent modifications

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Abstract

CHD4 (chromodomain helicase DNA-binding protein 4) ATPase is a major subunit of the repressive NuRD (nucleosome remodelling and deacetylase) complex, which is involved in transcriptional regulation and development. CHD4 contains two PHD (plant homeodomain) fingers of unknown function. Here we show that the second PHD finger (PHD2) of CHD4 recognizes the N-terminus of histone H3 and that this interaction is facilitated by acetylation or methylation of Lys⁹ (H3K9ac and H3K9me respectively) but is inhibited by methylation of Lys⁴ (H3K4me) or acetylation of Ala¹ (H3A1ac). An 18 μ M binding affinity toward unmodified H3 rises to 0.6 μ M for H3K9ac and to 0.9 μ M for H3K9me3, whereas it drops to 2.0 mM for H3K4me3, as measured by tryptophan fluorescence and NMR. A peptide library screen further shows that phosphorylation of Thr³, Thr⁶ or Ser¹⁰ abolishes this interaction. A model of the PHD2–H3 complex, generated using a combination of NMR, data-driven docking and mutagenesis data, reveals an elongated site on the PHD2 surface where the H3 peptide is bound. Together our findings suggest that the PHD2 finger plays a role in targeting of the CHD4/NuRD complex to chromatin.

Keywords

chromodomain helicase DNA-binding protein 4 (CHD4); histone; methylation; plant homeodomain (PHD)

INTRODUCTION

CHD4 (chromodomain helicase DNA-binding protein 4), also known as Mi2 β , is involved in a variety of fundamental cellular processes including chromatin remodelling, transcriptional repression, and DNA repair and recombination. CHD4 belongs to the class II subfamily of

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AUTHOR CONTRIBUTION

Catherine A. Musselman performed most of the experiments and analysed the data. Robyn E. Mansfield, Foteini Davrazou, Ann H. Kwan, Heather O'Leary and Joel P. Mackay assisted with NMR experiments and mutagenesis and analysed data. Adam L. Garske, Samuel S. Oliver and John M. Denu designed, performed and wrote about the combinatorial on-bead screening assay. Tatiana G. Kutateladze and Catherine A. Musselman wrote the manuscript.

CHD ATPases and is a major subunit of the repressive NuRD (nucleosome remodelling and deacetylase) complex, which is required for regulating transcription and developmental signalling [1-4]. Along with CHD4, the NuRD complex contains MBD2/3, RbAp46/48, MTA1/2/3 and p66 components and HDAC1/2 (histone deacetylase 1/2) (Figure 1a). Coupling of the helicase and HDAC functions makes NuRD unique among chromatin remodelling complexes. Although the purpose of the dual enzymatic activity is unclear, it may be essential for the efficient formation of heterochromatin with densely packed hypoacetylated nucleosomes and the rapid shut-off of gene expression [5,6]. NuRD is broadly distributed throughout the body and is critical for the regulation of numerous genes, however the mechanism by which this complex associates with nucleosomes remains undefined.

CHD4 is a ~ 200 kDa protein, conserved throughout the animal and plant kingdoms. It contains tandem PHD (plant homeodomain) fingers, two chromodomains and a C-terminal ATPase module (Figure 1b). The ~400-residue SNF2-like helicase/ATPase domain provides the energy necessary for histone displacement and sliding during nucleosome remodelling [7]. Unlike other chromodomains that bind methylated histone marks, such as H3K9me3 and H3K27me3 [8-11], the CHD4 chromodomains do not recognize histone tails and instead show a unique DNA binding activity [12]. The function of the PHD fingers of CHD4 is unknown.

The PHD finger is a ~ 60-residue module characterized by a conserved C₄HC₃ sequence that co-ordinates two zinc ions in a cross-brace arrangement. The PHD finger was discovered over a decade ago and has since been found in a wide variety of eukaryotic, mostly nuclear, proteins. Recently, a subset of PHD fingers, including those of BPTF (bromodomain PHD finger transcription factor) and ING2 (inhibitor of growth family, member 2), were shown to recognize H3K4me3 (histone H3 trimethylated at Lys⁴) [13-16], whereas the PHD modules of BHC80 and AIRE (autoimmune regulator) were found to bind H3K4me0 (unmodified H3) [17-19]. However, the residues essential for binding to either H3K4me3 or H3K4me0 are not conserved in the majority of the PHD sequences. This raises the possibility of the existence of distinct subsets of PHD fingers that exhibit selectivity toward other histone modifications and/or are capable of recognizing non-histone ligands. Indeed, recent reports have suggested that PHD domains of ICBP90 and Lys⁴-specific demethylases SMCX and Lid2 may play a role in associating with the H3K9me3 mark [20-22].

In the present study, we demonstrate that the second PHD finger of CHD4 is a reader of the histone H3 N-terminal tail. We use a combination of tryptophan fluorescence and NMR binding data, chemical shift perturbation analysis, immunoprecipitation data, peptide library screening, mutagenesis and data-driven docking to infer the molecular mechanism for H3 recognition. Our findings offer new insight into the role of the PHD2 finger in the CHD4/NuRD complex, suggesting that it plays a part in targeting the complex to chromatin.

MATERIALS AND METHODS

Protein purification

The wild-type CHD4 PHD2 finger and its mutants were expressed in *Escherichia coli* BL21 (DE3) pLysS cells grown in LB (Luria-Bertani) broth or ¹⁵NH₄Cl-supplemented minimal media. Bacteria were harvested by centrifugation after induction with IPTG (0.3–1.0 mM) and lysed by sonication. The unlabelled and ¹⁵N-labelled GST (glutathione transferase)-fusion proteins were purified on glutathione-Sepharose 4B beads (Amersham Biosciences). The GST tag was either cleaved with Prescission protease, or left for the purposes of Western blot analysis, in which case the GST-fusion protein was eluted off the glutathione-Sepharose beads using 0.05 M reduced L-glutathione (Sigma Aldrich). The proteins were concentrated into 20 mM Tris (or d₁₀-Tris), pH 6.8–7.5, in the presence of 150 mM NaCl, 10 mM dithiothreitol and 1 mM NaN₃.

PCR mutagenesis

Point mutants were prepared using the Stratagene QuikChange XL site-directed mutagenesis kit according to the manufacturer's instructions.

Western blot analysis

GST-fusion CHD4 PHD fingers were incubated with C-terminal biotinylated peptides (Upstate Biotechnology) corresponding to the unmodified H3 (residues 1–21) and singly modified H3K4me1/2/3 (residues 1–21), H3K9me1/2/3 (histone H3 mono-/di-/tri-methylated at Lys⁹; residues 1–21), H3K9ac (histone H3 acetylated at Lys⁹; residues 1–21), H3K27me1/2/3 (histone H3 mono-/di-/tri-methylated at Lys²⁷; residues 21–44) and H3K36me1/2/3 (histone H3 mono-/di-/tri-methylated at Lys³⁶; residues 21–44) histone tails in the presence of streptavidin–Sepharose beads (GE Healthcare) in binding buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl and 0.05 % Nonidet P-40. The beads were collected via centrifugation and washed five times with the peptide binding buffer. Bound protein was detected by Western blot using anti-GST HRP (horseradish peroxidase)-conjugate monoclonal antibodies (GE Healthcare). Negative controls using GST-fusion proteins in the absence of the peptides were run in parallel to ensure that the proteins did not bind to the streptavidin beads.

NMR spectroscopy

Chemical shift perturbation experiments were carried out using uniformly ¹⁵N-labelled wild-type or mutant PHD2. ¹H, ¹⁵NHSQC (heteronuclear single quantum coherence) spectra were recorded in the presence of increasing concentrations of 12-mer histone tail peptides (synthesized by the Peptide Core Facility, University of Colorado Denver, CO, U.S.A.) or 20-mer H3K9ac (Anaspec). NMR experiments were carried out on a 500 MHz Varian INOVA spectrometer in the on-site NMR Core Facility. *K_d* values were calculated by a nonlinear least-squares analysis in Kaleidagraph using the equation:

$$\Delta\delta = \Delta\delta_{\max} \left\{ ([L] + [P] + K_d) - \sqrt{([L] + [P] + K_d)^2 - 4[P][L]} \right\} / 2[P]$$

where [L] is concentration of the peptide, [P] is concentration of the protein, $\Delta\delta$ is the observed chemical shift change, and $\Delta\delta_{\max}$ is the normalized chemical shift change at saturation. Normalized chemical shift changes [23] were calculated using the equation:

$$\Delta\delta = \sqrt{(\Delta\delta_H)^2 + (\Delta\delta_N/5)^2}$$

where $\Delta\delta$ is the change in chemical shift in parts per million (ppm). NMR assignments of CHD4 PHD2 were taken from [24] (BMRB 5555).

Fluorescence spectroscopy

A Fluoromax-3 spectrofluorometer was used to carry out tryptophan fluorescence measurements on PHD2. Spectra were recorded at 25°C on samples containing 0.5 μM PHD2 and progressively increasing concentrations of histone peptides. Samples were excited at 295 nm and emission spectra were recorded between 305 and 405 nm with a 0.5 nm step size and a 1 s integration time, averaged over three scans. *K_d* values were determined by a nonlinear least-squares analysis in Kaleidagraph using the equation:

$$\Delta I = \Delta I_{\max} \left\{ ([L] + [P] + K_d) - \sqrt{([L] + [P] + K_d)^2 - 4[P][L]} \right\} / 2[P]$$

where [L] is the concentration of the peptide, [P] is the concentration of the protein, ΔI is the observed change of signal intensity, and $\Delta I_{\max}$ is the difference in signal intensity of the free and fully bound states of the CHD4 PHD finger. The K_d values were averaged over three or more separate experiments, with error calculated as the standard deviation between the runs.

Combinatorial on-bead screening assay

A 5000-member PTM (post-translational modification)-randomized combinatorial peptide library based on the first 10 residues of the histone H3 N-terminus was developed as described in (A.L. Garske, S.S. Oliver and J.M. Denu, unpublished work and [25]). The library was incubated first with the GST-tagged version of CHD4 PHD2, second with a GST-specific primary antibody, third with a biotinylated secondary antibody, and finally with streptavidin-conjugated alkaline phosphatase, catalysing the turnover of BCIP (5-bromo-4-chloroindol-3-yl phosphate), which results in formation of a turquoise precipitate on beads bearing sequences that bind to the target protein. The bead colour intensity is proportional to affinity of the interaction [25]. Peptides from individual beads were cleaved with cyanogen bromide and analysed by MALDI-TOF (matrix-assisted laser-desorption ionization-time-of-flight) MS. PTM patterns were determined from the resulting mass ladders. Discrimination factors were obtained by dividing the frequency of each modification observed in the intensely blue beads by the frequency of each corresponding modification from a random group of 100 library members. Discrimination factors represent the likelihood of observing a particular modification in a protein screening experiment relative to random chance.

HADDOCK docking calculations

Modelling of the PHD2-H3 complex was performed using the flexible docking program HADDOCK (www.haddock.org). The protein co-ordinates were taken from the structure of the ligand-free CHD4 PHD2 finger (1MM2) [24]. Co-ordinates for the unmodified H3 (residues 1–10) ligand were taken from the structure of the BHC80-H3 complex (2PUY) [17]. The modified peptides, H3K9me3 and H3K9ac, were generated using the Insight II molecular modelling system. NMR chemical shift perturbation data were used to restrain the calculation. Specifically, residues Glu⁴⁴⁷, Gly⁴⁵⁵, Gly⁴⁵⁶, Leu⁴⁵⁸, Cys⁴⁶⁰, Thr⁴⁶³, Ser⁴⁶⁷, His⁴⁶⁹ and Trp⁴⁸⁵ in PHD2 were identified as active residues, and residues Gly⁴⁸³ and Glu⁴⁸⁴ were identified as passive residues. For the peptides, residues 1–9 were identified as active residues and residue 10 was identified as passive. The four lowest energy structures from the top cluster were analysed for each run. The ensemble of the four best structures for PHD2-H3 and the plot of the HADDOCK score versus rmsd (root-mean-squared deviation) for the top cluster of all three calculations are shown in Supplementary Figure S1 (at <http://www.BiochemJ.org/bj/423/bj4230179add.htm>).

RESULTS AND DISCUSSION

CHD4 PHD2 recognizes histone H3

The function of the CHD4 PHD2 finger as a histone H3-binding module was initially identified by pull-down experiments (Figure 1c). GST-tagged PHD2 was first incubated with biotinylated histone peptides corresponding to unmodified H3 (residues 1–21) and singly modified H3K4me1/2/3 (residues 1–21), H3K9me1/2/3 (residues 1–21), H3K9ac (residues 1–21), H3K27me1/2/3 (residues 21–44) and H3K36me1/2/3 (residues 21–44), and then with streptavidin-Sepharose beads. After collecting the beads by centrifugation, the histone peptide-

bound protein was detected by Western blot analysis. As shown in Figure 1(c), the PHD2 finger was able to recognize unmodified H3, H3K9me1/2/3 and H3K9ac. Binding to the H3K4me1/2/3 peptides was significantly weaker, gradually diminishing with accumulation of each additional methyl group at Lys⁴. Little to no interaction was observed with methylated H3K27 and H3K36 peptides. Together, these results suggested that the CHD4 PHD2 finger binds to a region within the first 21 residues of the H3 tail and that methylation at Lys⁴ disrupts this interaction.

Recognition of the shorter H3 peptides was characterized by NMR titration experiments. ¹H, ¹⁵N HSQC spectra of the uniformly ¹⁵N-labelled PHD2 finger were recorded as unmodified H3, H3K4me3, H3K9me3, H3K9ac, H3K27me2, H3K36me3 or H4K20me3 peptides were gradually added to the NMR samples (Figure 1d). Substantial chemical-shift changes in the PHD2 finger were detected during titration with either unmodified H3, H3K9me3 or H3K9ac. These interactions were in the intermediate-to-fast exchange regime on the NMR time scale, characterized by the progressive shifting and broadening of amide resonances, indicative of strong binding.

Addition of H3K4me3 induced small chemical shift changes in the CHD4 PHD2 spectrum, and the observed fast exchange regime pointed to a weak association with this peptide (Figure 1d). A lack of significant resonance perturbations upon titrating in H3K27me2, H3K36me3 or H4K20me3 indicated that the PHD2 finger does not recognize these epigenetic marks (Figure 1d). The NMR data were in agreement with pull-down experiments, corroborating the fact that methylated Lys⁴ inhibits the interaction of CHD4 PHD2 with histone H3.

Selectivity of the CHD4 PHD2 finger for histone modifications

To determine whether other post-translational histone modifications influence the interaction with H3, the CHD4 PHD2 finger was tested in an on-bead combinatorial H3 tail library assay (Figure 2). Construction of the library involved a split-pool approach, generating a library with 5000-member diversity [25]. Using the ten most N-terminal residues of histone H3, the library encompassed the possible modification states for 7 residues within the peptide. Sites of modification included positions 2 (R, Rme1, Rme2s, Rme2a, Cit), 3 (T, Tph), 4 (K, Kme1, Kme2, Kme3, Kac), 6 (T, Tph), 8 (R, Rme1, Rme2s, Rme2a, Cit), 9 (K, Kme1, Kme2, Kme3, Kac) and 10 (S, Sph). We screened the library with GST-tagged CHD4 PHD2, employing a colorimetric assay to identify beads that bound the protein with high affinity. To determine the preferred modification state at different positions, 32 beads that indicated positive binding were selected and the PTM state was revealed by MALDI MS. Positional discrimination factors for each site were determined by dividing the observed frequency of the positive beads by the frequency determined among a random set of 100 beads.

The screening results substantiated the strong preference of CHD4 PHD2 for unmodified Lys⁴ (discrimination factor of 7.1) compared with methylated or acetylated Lys⁴ (Figure 2). A significant preference for K9me2 and K9ac (discrimination factors of 2.4 and 1.5, respectively) over unmodified Lys⁹ (discrimination factor of 0.6) was also observed. Phosphorylation at Thr³, Thr⁶ or Ser¹⁰ was not tolerated at all (discrimination factors of 2.5, 1.9 and 2.6 respectively for the unphosphorylated threonine and serine residues); not a single phosphorylation mark was identified at any of these positions in the positive hits. In contrast, no significant preferences were observed for Arg² and Arg⁸ with regard to either citrullination or mono- or di-methylation (see Supplementary Figure S2 at <http://www.BiochemJ.org/bj/423/bj4230179add.htm>). Collectively, the on-bead combinatorial library data points to the high selectivity of the CHD4 PHD2 finger, which shows clear preference for unmodified Thr³, Lys⁴, Thr⁶ and Ser¹⁰, and favours modified Lys⁹ (methylated or acetylated) over the unmodified residue.

Methylation or acetylation at Lys⁹ increases the binding affinity of CHD4 PHD2, whereas methylation at Lys⁴ decreases it

The histone H3-binding affinities of the PHD2 finger were measured using tryptophan fluorescence and NMR. We found that CHD4 PHD2 associates with unmodified H3 with a K_d of 18 μ M (Figure 3). However, this binding was gradually diminished by mono-, di- and tri-methylation of Lys⁴, with the affinities for H3K4me1, H3K4me2 and H3K4me3 dropping to ~300 μ M, 1 mM and 2 mM respectively. In contrast, the affinity of PHD2 was augmented as the number of methyl groups attached to Lys⁹ was increased, ultimately reaching 0.9 μ M for H3K9me3. Likewise, acetylation of Lys⁹ enhanced the binding, yielding a K_d of 0.6 μ M. Overall, the CHD4 PHD2 finger bound ~100-fold stronger to unmodified H3 than to H3K4me3, but 20- and 30-fold stronger to H3K9me3 and H3K9ac than to H3. Consistent with these observations, it has previously been shown that association of the entire CHD4-containing NuRD complex with H3 is disrupted by methylation of Lys⁴ but is tolerated when Lys⁹ is methylated [26].

The H3-binding site of the CHD4 PHD2 finger

The histone-binding site of the PHD2 finger was defined by analysing NMR resonance perturbations in PHD2 caused by the unmodified H3 peptide. Plotting chemical shift changes for each backbone amide allowed us to identify the residues most affected due to interaction with H3 (Figure 4a). The largest perturbations or disappearance of signals were observed for Glu⁴⁴⁷, Gly⁴⁵⁵, Gly⁴⁵⁶, Leu⁴⁵⁸, Cys⁴⁶⁰, Thr⁴⁶³, Ser⁴⁶⁷, His⁴⁶⁹, Gly⁴⁸³ and Trp⁴⁸⁵, suggesting that these residues are directly or indirectly involved in the binding. Mapping these residues onto the surface of the ligand-free CHD4 PHD2 finger [24] revealed a well-defined, elongated site where the histone peptide is bound (Figure 4c).

To establish the contribution of each binding site residue in the PHD2-H3 interaction, Glu⁴⁴⁷, Gly⁴⁵⁵, Gly⁴⁵⁶, Leu⁴⁵⁸, Cys⁴⁶⁰ and Trp⁴⁸⁵ were mutated in turn, and the mutant proteins were tested by NMR. The ¹H, ¹⁵N HSQC experiments showed that substitution of the core residues Gly⁴⁵⁶, Leu⁴⁵⁸ and Trp⁴⁸⁵ with alanine, and Gly⁴⁵⁵ with valine, resulted in collapse of the tertiary structure of the protein, thus pointing to the importance of these residues for the structural stability of PHD2. In contrast, the E447A and C460W mutants remained folded. We note that Glu⁴⁴⁷ and Cys⁴⁶⁰ of CHD4 correspond to Asp⁴⁸⁹ and Met⁵⁰² of BHC80 [17] and to Asp²⁹⁷/Asp²⁹⁹ and Cys³¹⁰/Cys³¹² of human/murine AIRE [18,19]. The conserved aspartate residues of BHC80 and AIRE form a salt bridge with Lys⁴, a hallmark of the K4me0 recognition, whereas the hydrophobic side chains of methionine/cysteine insert between Arg² and Lys⁴ of the peptide [17-19]. Replacement of the aspartate residues with alanine or the hydrophobic residues with a bulky tryptophan in the PHD fingers of BHC80 and AIRE abolish interactions with H3K4me0 [17,27]. Likewise, Glu⁴⁴⁷ and Cys⁴⁶⁰ of CHD4 are required for the interaction with unmodified H3. NMR titrations demonstrate that E447A and C460W mutants bind to the H3 peptide significantly weaker than the wild-type protein (K_d = 0.9 mM and 3.7 mM respectively), suggesting that these residues play critical roles in the binding energetics (Figure 3b).

A model of the PHD2-H3 complex

Employing the NMR data as restraints in the flexible docking algorithm HADDOCK, a model of the PHD2-H3 complex was generated. The modelling revealed that the H3 peptide is bound to PHD2 through a number of hydrogen bonds and salt bridges (Figure 4b). The peptide forms a third antiparallel β -strand, pairing with the first β -strand of PHD2, and is stabilized through backbone interactions between Arg², Lys⁴ and Thr⁶ of H3 and the protein residues Cys⁴⁶⁰, Leu⁴⁵⁸ and either Gly⁴⁵⁵ or Gly⁴⁵⁶. The peptide is further restrained through the co-ordination of the amino group of Ala¹ by Gly⁴⁸³ and Glu⁴⁸⁴, the formation of a salt bridge between

Arg² and Asp⁴⁶², and hydrogen bonding interactions involving Lys⁴, Thr⁶ and Arg⁸ (Figure 4b).

The significance of the Ala¹ recognition was demonstrated by examining binding of the PHD2 finger to a histone H3 peptide in which the primary amino group of Ala¹ was acetylated (H3A1ac). The K_d value of the PHD2–H3A1ac interaction was found to be ~400 μ M, which is ~20-fold lower than the affinity of PHD2 for unmodified H3 (Figure 3b). These results suggest that the proper co-ordination of unmodified Ala¹ is necessary for the formation of the complex. In addition, the backbone amide cross peaks of Asn⁴⁸², Gly⁴⁸³, Glu⁴⁸⁴ and Trp⁴⁸⁵ exhibited different perturbation patterns upon binding to H3A1ac as compared with unmodified H3, providing further evidence that these residues are involved in the recognition of Ala¹ (see Supplementary Figure S3 at <http://www.BiochemJ.org/bj/423/bj4230179add.htm>).

To define further the role of the N-terminus of H3, a peptide comprising residues Thr³–Ser¹⁰ but lacking Ala¹ and Arg² [H3(3–10)], was tested in NMR titration experiments. Even a 10-fold excess of H3(3–10) induced no changes in the NMR spectra of PHD2, implying that CHD4 PHD2 does not recognize H3 lacking the first two N-terminal residues (Figure 3b). Given that the loss of binding was substantially greater than the reduction in binding to H3A1ac, where Ala¹ is N-terminally blocked, these data suggest that both Ala¹ and Arg² and/or their backbone contacts are required for robust interaction with CHD4 PHD2.

The H3-binding mode of CHD4 PHD2 in the model is similar to that observed in the BHC80-PHD–H3 and AIRE-PHD1–H3 complexes [17–19], indicating that the molecular mechanism of the H3 recognition by this subset of PHD fingers is conserved.

Increasing hydrophobicity of Lys⁹ facilitates the PHD2–H3 interaction

To obtain insight into the role of Lys⁹, we modelled the complexes of PHD2 with H3K9ac and H3K9me3 (Figures 4f and 4g). A superimposition of these complexes with the PHD2–H3 complex showed that the first seven residues of the H3 peptide interacts with PHD2 similarly. However, methylated or acetylated Lys⁹ is positioned more closely to the protein surface in the H3K9ac and H3K9me3 complexes, allowing Arg⁸ to form a salt bridge with Glu⁴⁴⁷.

The position of Arg⁸ and modified Lys⁹ in the complexes was corroborated by NMR data. The majority of chemical shift changes in PHD2 induced by H3K9ac or H3K9me3 were paralleled to those caused by unmodified H3, indicating that the modified and unmodified peptides occupy the same binding pocket. However, the amide cross peaks of Glu⁴⁴⁷/Phe⁴⁴⁸/Cys⁴⁴⁹/Lys⁴⁵⁰/Gly⁴⁵⁵/Gly⁴⁵⁶/Ser⁴⁶⁷ and Asp⁴⁵⁴/Gly⁴⁵⁵/Gly⁴⁵⁶ were shifted to a larger extent in the PHD2–H3K9ac and PHD2–H3K9me3 complexes respectively (Figure 5). The solvent-exposed Glu⁴⁴⁷, Phe⁴⁴⁸, Asp⁴⁵⁴, Gly⁴⁵⁵ and Gly⁴⁵⁶ residues form a contiguous patch on the surface, with Cys⁴⁴⁹ and Ser⁴⁶⁷ lying underneath this patch. Interestingly, in the AIRE PHD–H3K4me0 complex, the Glu²⁹⁸ and Asp³⁰⁴ residues, equivalent to Phe⁴⁴⁸ and Asp⁴⁵⁴ of CHD4, are involved in transient electrostatic interactions with unmodified Lys⁹ of the peptide [19]. Mutations of Glu²⁹⁸ or Asp³⁰⁴ in the AIRE PHD finger cause a 5- to 16-fold decrease in the binding affinity, revealing a role of Lys⁹ in the formation of the complex [19]. Likewise, when Asp⁴⁵⁴ of CHD4 was substituted with valine, binding of the CHD4 PHD2 finger to H3K9me3 was significantly compromised (Figure 3b).

Another key acidic residue of AIRE (Glu²⁹⁸) is replaced with a hydrophobic Phe⁴⁸⁸ in the CHD4 PHD2 sequence. The aromatic ring of Phe⁴⁸⁸ lies orthogonal to the protein surface and may favourably interact with K9ac or form hydrophobic and cation- π contacts with methylated Lys⁹. Methylation of a lysine residue is known to enhance interactions with aromatic residues due to the increased hydrophobic contacts involving the aromatic side chain and both the lipophilic methyl groups and the ϵ CH₂ of lysine [28]. Furthermore, the loss or shielding of the

positive charge in K9ac and K9me3 may prevent repulsion between Lys⁹ and Lys⁴⁵³, a residue adjacent to Phe⁴⁸⁸. These factors could lead to a higher affinity for the CHD4 PHD2–H3K9ac/me interaction compared with the interaction between PHD2 and unmodified H3. In agreement with this, the F448A mutant associates with the H3K9ac and H3K9me3 peptides more weakly than the wild-type protein (Figure 3c), and displays chemical shift changes in ¹H, ¹⁵N HSQC spectra almost identical to those observed for binding of wild-type PHD2 to the unmodified H3 peptide (results not shown). Since both methylation and acetylation of Lys⁹ enhance the affinity, we concluded that increased hydrophobicity of the side chain most likely draws this residue toward the hydrophobic surface of the N-terminal region of the protein, further facilitating the interaction of Arg⁸ with PHD2.

Comparison of the subsets of PHD fingers

Recent studies suggest that the large family of PHD fingers can be separated into subsets based on the specificity of this module toward post-translational histone modifications. At least four subsets have been identified, including PHD fingers that recognize (i) H3K4me3 [BPTF, ING/YNG, Pygopus, RAG2 (recombination-activating gene 2) and TAF3] [13-16,29-37], (ii) unmodified H3 (AIRE, BHC80 and the cysteine-rich domain of DNMT3L) [17-19,38], (iii) H3K9me3 (ICBP90, Lid2 and SMCX) [20-22], and (iv) H3K36me3 (Ecm5 and Nto1) [34]. In addition, the RAG2 PHD finger has been recently shown to bind a double modification, H3R2me2/K4me3 [33]. A comparison of the amino acid sequences reveals that PHD fingers which bind H3K4me3 must contain an aromatic residue preceding the first cysteine, a hydrophobic residue upstream from the third cysteine, and a tryptophan residue preceding the zinc-co-ordinating histidine to form a (semi)aromatic/hydrophobic cage around trimethylated Lys⁴. The PHD fingers that recognize unmodified H3 are characterized by the presence of an acidic patch at the N-terminus (NEDE in AIRE and HEDF in BHC80) and a hydrophobic residue (methionine/cysteine) preceding the third cysteine. In the CHD4 PHD2 finger, the N-terminal patch (HMEF), being significantly more hydrophobic, favours acetylated or methylated Lys⁹ of H3K4me0. The signature motif(s) responsible for the recognition of other modifications remain to be determined.

In summary, we demonstrate that the second PHD finger of CHD4 recognizes the N-terminus of histone H3 and that methylation at Lys⁴ abolishes this interaction, whereas methylation or acetylation of Lys⁹ facilitates it. A model of the complex reveals that the H3 tail occupies an elongated binding site of PHD2 and is held in place through a conserved set of salt bridges and hydrogen bonds. The preference of the CHD4 PHD2 finger for modified Lys⁹ is most likely attributed to the N-terminal sequence, which is more hydrophobic compared with the acidic sequences of other H3K4me0-binding PHD fingers. Our results suggest that CHD4 may be involved in stabilization of the CHD4–NuRD complex at chromatin through binding of the PHD2 finger to the histone H3 tail carrying unmodified, acetylated or methylated Lys⁹. It is intriguing that the same domain is able to bind two distinct histone marks, which are often associated with opposite processes such as gene activation and repression. One possible explanation of these findings is in the multiple and dynamic function of the CHD4–NuRD–HDAC repressive complex, which may require initial association with H3K9ac and later with H3K9me3, promoting both the deacetylase activity of HDAC and methylation of Lys⁹ in the pathway from an active to inactive state of chromatin [26]. Another important question concerns the interplay between the activities of two PHD fingers of CHD4 and two chromodomains of this protein. Future studies are required to establish the relationship and functional significance of multiple interactions of the CHD4 modules with chromatin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

AIRE	autoimmune regulator
BPTF	bromodomain PHD finger transcription factor
CHD4	chromodomain helicase DNA-binding protein 4
GST	glutathione transferase
H3	histone H3
H3A1ac	histone H3 acetylated at Ala ¹
H3K9ac	histone H3 acetylated at Lys ⁹
H3K9me1/2/3	histone H3 mono-/di-/tri-methylated at Lys ⁹
H3K4me0	unmodified histone H3
H3K4me1/2/3	histone H3 mono-/di-/tri-methylated at Lys ⁴
H3K27me1/2/3	histone H3 mono-/di-/tri-methylated at Lys ²⁷
H3K36me1/2/3	histone H3 mono-/di-/tri-methylated at Lys ³⁶
HDAC	histone deacetylase
HSQC	heteronuclear single quantum coherence
ING	inhibitor of growth family
MALDI	matrix-assisted laser-desorption ionization
NuRD	nucleosome remodelling and deacetylase
PHD	plant homeodomain
PTM	post-translational modification
RAG2	recombination-activating gene 2

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Figure 1. The CHD4 PHD2 finger binds histone H3

(a) The NuRD complex and its major components. The nucleosomes are shown as blue cylinders. (b) Architecture of CHD4: the N-terminal of PHD fingers, the two adjacent chromodomains and a catalytic ATPase module. (c) Binding of the GST-fusion CHD4 PHD2 finger to biotinylated histone peptides. (d) Superimposed ^1H , ^{15}N HSQC spectra of 0.2 mM PHD2, collected during titrating in the indicated histone peptides. The spectra are colour-coded according to the protein/peptide ratio, the position of low intensity peaks of E447 are marked by an asterisk.

Figure 2. Binding specificity of the CHD4 PHD2 finger was established using positional discrimination factors

The discrimination factor for a single modification at any one position is calculated as a mathematical ratio of frequencies between the results of screening the library against CHD4 PHD2 and a random library sampling (50 sequences). A larger discrimination factor (a larger bar in graph) represents a modification favouring binding. A discrimination factor of 1 denotes no difference between the CHD4 screen and a random bead sampling. χ^2 analysis was performed at each position. For positions with 4 (n-1) degrees of freedom (DF), a χ^2 value greater than 13.28 denotes statistical significance at a 99% confidence level. Positions with 1 degree of freedom (DF) must have a value greater than 6.63 in order to be considered statistically significant at the 99% confidence level. A non-significant result means that no effects were discovered and chance could explain the observed differences at that position. The modification states at positions Thr³ (T, pT), Lys⁴ (K, Kme1, Kme2, Kme3, Kac), Thr⁶ (T, pT), Lys⁹ (K, Kme1, Kme2, Kme3, Kac) and Ser¹⁰ (S, pS) are shown. ac, acetylated; me, methylated; p, phosphorylated. We note that K9me3 is not well represented in the screen. This could be due to the sample size, which was not large enough to resolve all tendencies at this position and because any of the three modifications (acetylation, di- and tri-methylation) results in enhanced binding. Additionally, based on χ^2 analysis, PTM discrimination at Lys⁹ is less pronounced compared with positions 3, 4 and 6.

Figure 3. Histone H3 binding affinities of the CHD4 PHD2 finger

(a) Representative binding curves used to determine the K_d values of the PHD2–H3, PHD2–H3K9me3 and PHD2–H3K9ac interactions by tryptophan fluorescence. (b) The binding affinities of the CHD4 PHD2 finger, wild-type and mutants for indicated peptides, measured by tryptophan fluorescence (^a) and NMR (^b).

Figure 4. Identification of the H3-, H3K9ac- and H3K9me3-binding sites of the CHD4 PHD2 finger and models of the PHD2-H3, PHD2-H3K9ac and PHD2-H3K9me3 complexes

(a, d and e) The histograms show normalized ^1H , ^{15}N chemical shift changes in backbone amides of PHD2 upon addition of the H3 (a), H3K9ac (d) and H3K9me3 (e) peptides. An asterisk indicates the disappearance of the resonances, and P indicates a proline residue. (b, f and g) Models of the PHD2-H3, PHD2-H3K9ac and PHD2-H3K9me3 complexes as obtained using the flexible docking program HADDOCK. Residues of PHD2 and H3 are labelled in black and green, respectively. Hydrogen bonds and salt bridges are shown by red dotted lines. (c) Residues that exhibit significant H3-induced resonance perturbations [more than average plus one quarter (yellow), one half (orange) and one (red) standard deviation] in (a) are mapped onto the surface of the ligand-free CHD4 PHD2 [24] and labelled.

Figure 5. Differences in NMR resonance perturbations observed in the CHD4 PHD2 finger upon binding to H3K9me3 and H3K9ac as compared with unmodified H3

An asterisk indicates the disappearance of the resonances, and P indicates a proline residue.