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NMR assignments and histone specificity of the ING2 PHD finger

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Abstract

The ING2 plant homeodomain (PHD) finger is recruited to the nucleosome through specific binding to histone H3 trimethylated at lysine 4 (H3K4me3). Here, we describe backbone and side chain assignments of the ING2 PHD finger, analyze its binding to the unmodified and modified histone and p53 peptides, and map the histone H3 and H3K4me3 binding sites based on chemical shift perturbation analysis.

Keywords

NMR; histone; chromatin; PHD finger; resonance assignment

Introduction

Histone proteins form the nucleosome core around which DNA is tightly wrapped. The canonical histones contain a conserved globular domain and unstructured but evolutionally conserved tails. The histone tails play a central role in the regulation of gene expression. They undergo a variety of reversible posttranslational modifications, including acetylation and ubiquitination at Lys residues, methylation at Lys and Arg residues, and phosphorylation at Ser and Thr residues, and serve as docking sites for effector proteins. Several single site modifications in the four amino-terminal histone tails give rise to numerous combinations of the so-called ‘histone codes’ responsible for the propagation of signals leading to chromatin remodeling.[1-4] The histone codes are typically ‘written’ or ‘erased’ by histone modifying enzymes, and a few protein domains have been recently identified to specifically recognize or ‘read’ the precise tail modifications. The latter includes bromodomain, which recognizes acetylated Lys residues,[5,6] and methyl-Lys-binding chromodomain,[7,8] tudor,[9] the WD40 repeat,[10,11] malignant brain tumor (MBT),[12,13] the ankyrin repeat,[14] and the plant homeodomain (PHD) finger.[15-18] Depending on the modifications, binding of the ‘reader’ can either facilitate the gene expression or condense the chromatin. A wide variety of epigenetic marks were identified in the last few years, yet the number of histone ‘readers’ remains small.

The zinc binding PHD finger is a recent addition to the family of epigenetic ‘readers’. It was identified in 1993,[19] and has since been found in over 100 human proteins (SMART data base). The seven conserved cysteine and one histidine (C₄HC₃) residues of the PHD finger

coordinate two zinc ions in a cross-brace topology. Many PHD fingers are found in nuclear proteins, transcription factors and regulators of chromatin structure,[20] such as inhibitor of growth (ING) family of tumor suppressors. The second member of this family (ING2) is implicated in a number of critical cellular processes, including regulation of gene expression, cell proliferation and apoptosis.[21-23] ING2 contains a SAP 30-interacting domain (SAID) at the amino-terminus and a carboxy-terminal PHD finger. While the SAID domain of ING2 associates with the SAP30 subunit of the Sin3a/HDAC1/2 repressive complex,[24,25] the PHD finger binds methylated histone tails.[16,26] Consequently, ING2 functions as a tethering protein that stabilizes the chromatin-modifying enzymatic complex at the nucleosome. Furthermore, ING2 and its homolog ING1 are functionally linked to and physically interact with p53 tumor suppressor, and their PHD fingers are essential for p53-mediated apoptosis and acetylation of p53.[22,27,28] Here, we describe backbone and side chain assignments of the ING2 PHD finger, analyze its binding to the unmodified and modified histone tails and p53 methylated at lysine 372 (p53K372me) peptides, and map the histone-binding sites on the basis of chemical shift perturbation analysis.

Experimental

Subcloning, expression and purification of the ING2 PHD finger

The mouse ING2 PHD finger (gi: 31 542 996; aa 201–281) subcloned into a pGEX-6P vector (Amersham) was expressed in *Escherichia coli* BL21(DE3) pLysS (Stratagene). The unlabeled, ^{15}N -labeled and $^{15}\text{N}/^{13}\text{C}$ -labeled proteins were produced by growing the culture in zinc chloride-enriched (40 μM) Luria-Bertani or minimal media supplemented with $^{15}\text{NH}_4\text{Cl}$ and $^{13}\text{C}_6$ -glucose (Cambridge Isotope). Bacteria were harvested by centrifugation after induction with isopropyl-1-thio- β -D-galactopyranoside (0.1 mM) at 22 °C for 16 h and lysed by French Press. The glutathione S-transferase (GST)-fusion proteins were purified on a glutathione Sepharose 4B column (Amersham). The GST tag was cleaved with PreScission protease (Amersham). The proteins were concentrated in Millipore concentrators (Millipore), purified by AKTA FPLC using a Superdex-75 column and concentrated into d_{11} -tris buffer containing 150-mM NaCl, 10-mM d_{10} -dithiothreitol and 1-mM NaN_3 in 7% $^2\text{H}_2\text{O}/\text{H}_2\text{O}$, pH 6.5.

NMR spectroscopy and sequence specific assignments

NMR experiments were performed at 298 K on Varian INOVA 600 and 500 MHz spectrometers using pulse field gradients to suppress artifacts and eliminate water signal. The PHD finger construct contained residues 211–262 of ING2 and additional unstructured 10 amino-terminal and 19 carboxy-terminal amino acids which together with the GPLGSPEF sequence derived from the vector were not included in the assignment statistics. There are four Pro residues in the PHD finger sequence. The NMR samples contained 1–2 mM unlabeled or uniformly ^{15}N - and $^{15}\text{N}/^{13}\text{C}$ -labeled ING2 PHD. The ^1H , ^{15}N heteronuclear single quantum coherence (HSQC), ^{15}N -edited NOESY–HSQC,[29,30] HSQC–NOESY–HSQC,[31] TOCSY–HSQC,[32] ^{13}C -edited NOESY–HMQC,[33] HCCH–TOCSY,[34] HNCACB,[35] CBCA(CO)NH,[36] HNCO,[36] C(CO)NH[37] and H(CCO)NH[37] were recorded and analyzed for sequential and spin system assignments and to collect NOEs. $^3J_{\text{HNH}\alpha}$ coupling constants were measured from HNHA[38] and HMQC- J .[39] The NMR data were processed with nmrPipe[40] and analyzed with Pipp,[41] nmrDraw and in-house software programs on Sun and Silicon Graphics workstations.

NMR titrations of histone and p53 peptides

The ^1H , ^{15}N HSQC spectra of 0.2–0.26-mM uniformly ^{15}N -labeled ING2 PHD finger were collected using 1024 t_1 increments of 2048 data points, 96 number of increments and spectral widths of 7510 and 1367 Hz in the ^1H and ^{15}N dimensions, respectively. The binding was characterized by monitoring chemical shift changes in ^1H , ^{15}N HSQC spectra of the PHD finger

as histone peptides (up to 8 mM) or p53K372me3 and p53K372me1 peptides (up to 5 mM) were added stepwise. The dissociation constants (K_D s) were determined by a nonlinear least-squares analysis using the program Xmgr and the equation: $\Delta\delta = \Delta\delta_{\max}((([L] + [P] + K_D) - \sqrt{([L] + [P] + K_D)^2 - 4 \times [P] \times [L] }) / (2 \times [P]))$, where $[L]$ is concentration of the peptide, $[P]$ is concentration of PHD, $\Delta\delta$ is observed chemical shift change, and $\Delta\delta_{\max}$ is the difference in chemical shifts of the free and ligand-bound protein. The synthetic histone peptides: NH₂-ARTKQTARKSTG (H3), NH₂-ARTKme3QTARKSTG (H3K4me3), NH₂-SGRGKGGKGLGKGGAKRHRKVLRL (H4), Ac-SHLKSKme3KGQST (p53K372me3) and Ac-SHLKSKme1KGQST (p53K372me1) were synthesized at the Biophysics Core Facility of the University of Colorado Denver School of Medicine. The GST-fusion yeast histone H2A (residues 1–35) and H2B (residues 1–35) constructs (a gift of M. Grunstein) were expressed in *E. coli* and purified on a glutathione Sepharose 4B column using a standard procedure. Following the removal of a GST tag with Thrombin, the peptides were purified by FPLC, the buffer was exchanged into water, and after lyophilization the peptides were obtained as a white powder.

Peptide microarray

Peptide microarray experiments were performed as described previously.[42] Briefly, biotinylated histone peptides were printed in six replicates onto a streptavidin-coated slide (ArrayIt) using a VersArray Compact Microarrayer (Bio-Rad). After a short blocking incubation with biotin (Sigma), the slides were incubated with GST-fusion ING2 PHD in peptide binding buffer (50-mM Tris-HCl, pH 7.5, 150-mM NaCl, 0.1% Nonidet P-40, 20% fetal bovine serum) overnight at 4 °C with gentle agitation. After washing with the same buffer, slides were probed first with anti-GST antibody and then fluorescein-conjugated secondary antibody and visualized with a GenePix 4000 scanner (Molecular Devices).

Results and Discussion

Sequence specific resonance assignments of the ING2 PHD finger

To examine the interactions involving the PHD finger of ING2, the ¹H, ¹³C and ¹⁵N resonances of this domain were assigned. The ¹H, ¹⁵N HSQC spectrum of the PHD finger is shown in Fig. 1. Initial amino acid spin system and sequential assignments were made from ¹⁵N-edited NOESY–HSQC ($\tau_{\text{mix}} = 50$ and 135 ms), HSQC–NOESY–HSQC ($\tau_{\text{mix}} = 150$ ms) and TOCSY–HSQC (DIPSII). The triple resonance HNCACB, CBCA(CO)NH, HNCO, C(CO)NH and H(CCO)NH spectra of uniformly ¹⁵N/¹³C-labeled PHD domain were recorded and analyzed to complete the assignments. Quantitative ³J_{HNH α} coupling constants were estimated from HNHA and HMQC-*J* spectra (data not shown). Complete backbone assignments and near complete side chain assignments were made for all the residues of ING2 PHD, with the exception of Y255 (Table 1). The ¹H and ¹⁵N resonances of 47 of 48 detectable backbone amides of the PHD finger (residues 211–262), the NH₂ side chains of three Asn and two Gln and ¹³C resonances of 45 of 48 detectable backbone carbonyl groups were assigned. The ¹H and ¹³C resonance assignments of the side chains are 93 and 76% complete, respectively.

Significant chemical shift changes in the ING2 PHD finger are induced by histone H3

To determine whether the unmodified histone tails can be recognized by the ING2 PHD finger, ¹H, ¹⁵N HSQC spectra of the ¹⁵N-labeled protein were collected as the peptides corresponding to the amino-terminal regions of H3, H4, H2A and H2B were titrated in. The histograms in Fig. 2(a) show normalized chemical shift changes observed in backbone amides of the PHD finger upon addition of the peptides. Of the four peptides tested, only unmodified H3 caused considerable resonance perturbations in the PHD finger, whereas the H4, H2A and H2B peptides induced very small changes. The ligand-free and the bound states of the PHD finger were in the fast exchange regime on the NMR time scale, allowing us to determine

binding affinities. The K_D values were found to be $\sim 1\text{--}2\text{ mM}$ for H3, H4 and H2A peptides (Fig. 2(b) and (c)). The H2B interaction appeared to be weaker, with the lower affinity limit of 10 mM .

The ING2 PHD finger exhibits specificity for H3K4me but does not recognize other histone modifications or p53K372me

The amino-terminal histone H3 tail undergoes posttranslational modifications including methylation at Lys4 and Lys9, the best understood epigenetic marks. As shown in Fig. 2(a) and (d), binding of histone H3 trimethylated at Lys9 (H3K9me3) induced chemical shift changes in the ING2 PHD finger almost identical to those observed upon addition of unmodified H3. Furthermore, the K9me3 group did not alter binding affinity of the PHD finger for the histone tail (Fig. 2(b)), indicating that Lys9 is located beyond the interacting sequence. In contrast, the interaction with H3K4me3 was found to be much stronger (Fig. 2(b)). Analysis of the chemical shift perturbations revealed that in addition to the two clusters of residues (Y223/E225/M226/C229 and K249/G252/K253/W254) that were perturbed due to binding of unmodified H3, two other clusters of residues (Y215/C216/V221/S222 and E237/W238/H240) exhibited significant chemical shift changes in the presence of K4me3, suggesting that the latter two are affected by the coordination of the trimethylammonium group of lysine (Fig. 2(d)).

To find out if the ING2 PHD finger is capable of associating with the less-common histone modifications, it was tested by *in vitro* peptide microarrays (Fig. 3(a)). The biotinylated peptides corresponding to methylated, acetylated and phosphorylated histones H2A, H2B, H3 and H4 were incubated with GST-fusion ING2 PHD. Subsequently the peptide-bound protein was detected using anti-GST antibody and fluorescein-conjugated secondary antibody. Whereas H3K4me3 peptide was distinctly recognized by the PHD finger, no binding was observed to other modified histone peptides, pointing to the high selectivity of the ING2 PHD finger.

We next examined whether the ING2 PHD finger interacts with p53K372me peptides that share sequence similarity with H3K4me (Fig. 3(b) and (c), and data not shown). It has recently been shown that p53 can be methylated at K372 by Set9 methyltransferase,[44] and that the PHD finger of ING1/2 is required for p53 activity.[21,22,28] Addition of the p53K372me3 or p53K372me1 peptides resulted in small chemical shift changes in the ING2 (and homologous ING1) PHD finger, however the binding was at least tenfold weaker than the interaction with unmodified H3.

Identification of the H3 and H3K4me3 binding sites by chemical shift perturbation analysis

The most perturbed (due to the interaction with H3 and H3K4me3) residues were mapped onto the surface of the ING2 PHD finger[16] (Fig. 4(a) and (b)). The Y223/E225/M226/C229 and K249/G252/K253/W254 clusters, affected by unmodified H3, define well the extensive site where the first six amino-terminal residues of H3 are bound (Fig. 4(a)). The additional two clusters of residues that are perturbed by H3K4me3 form a hydrophobic/aromatic cage around the trimethylammonium group of Lys4 (Fig. 4(b)). The aromatic side chains of the W238 and Y215 residues, positioned perpendicular to each other and orthogonal to the protein surface, play a central role in restraining the K4me3 group through cation- π contacts. The chemical shifts of $H^{\epsilon 1}$ and $N^{\epsilon 1}$ in the indole ring of W238 are particularly sensitive to the presence of the trimethylammonium moiety and are significantly deshielded in the H3K4me3 complex but not when associated with H3K9me3 (Fig. 4(c)). Notably, the coordination of methyl-lysine by an aromatic cage has also been reported for other histone-binding modules, including chromodomains and MBT,[12,13,45-50] indicating that the mechanism of this 'histone code' recognition is strongly conserved.

The aromatic moiety of W238 separates two grooves where the extended side chains of Arg2 and Lys4me3 are located (Fig. 4(b)). The grooves are connected by a narrow channel, and flanked by W238 and K249, in which Thr3 of the H3K4me3 peptide fits precisely. Thus, the specificity of ING2 PHD may be attributed at least in part to the small size of this connecting channel. The PHD finger binds to H3K4me3, containing a small residue preceding Lys4, but discriminates against H3K9me3, H3K27me3 or H4K20me3 that have a bulky residue (Arg) in this position. Although p53K372me3 peptide contains a small residue (Ser371) preceding methylated lysine, this sequence is not recognized by the PHD finger either. Most likely, the Lys370 and Leu369 residues of p53 that replace Arg2 and Ala1, respectively, in the H3K4me3 sequence (Fig. 3(b)) are incapable of making multiple hydrogen bonds involving the guanidino moiety of Arg2 and the primary amino group of Ala1. Furthermore, the methyl group of Ala1 lies in the small hydrophobic cavity, formed by W254 and I227, which would exclude the large Leu369 residue of p53.

In conclusion, our data demonstrate that chemical shift mapping can be used for defining both, the interacting interface of the histone H3 tail and the precise binding pocket for the K4me3 epigenetic mark. The small size of the Thr3-binding channel and a network of precise hydrophobic and hydrogen-bonding contacts involving Arg2 and Ala1 likely account for the high specificity of the ING2 PHD finger toward H3K4me3.

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Figure 2.

Identification of the binding partner of the ING2 PHD finger: (a) and (d) the histograms show normalized ^1H , ^{15}N chemical shift changes in backbone amides of the PHD finger upon addition of indicated peptides. The normalized[43] chemical shift change was calculated using equation $[(\Delta\delta\text{H})^2 + (\Delta\delta\text{N}/5)^2]^{0.5}$, where δ is the chemical shift in parts per million (ppm). The protein : peptide ratio is 1 : 5. (b) The binding affinities of the ING2 PHD finger determined by chemical shift perturbation analysis in the ^1H , ^{15}N HSQC spectra. (The values with * symbol are taken from Peña *et al.*, 2006). (c) The binding curves used to determine the K_D values based on the analysis of ^{15}N chemical shift changes in ^1H , ^{15}N HSQC spectra of the ING2 PHD finger.

Figure 3.

The histone specificity of the ING2 PHD finger. (a) Peptide microarrays containing the indicated histone peptides were probed with GST-ING2 PHD or GST. Red spots indicate binding; me, methylation; ac, acetylation; ph, phosphorylation; s, symmetric; a, asymmetric. (b) Sequence alignments of the indicated peptides. (c) A region of superimposed ^1H , ^{15}N HSQC spectra of the ING2 PHD finger (0.2 mM) collected during titration with up to 5 mM p53K372me1.

Figure 4.

The histone-binding sites of the ING2 PHD finger. Residues that exhibit significant chemical shift changes upon addition of H3 (a) and H3K4me3 (b) peptides are colored on the ING2 PHD finger surface. The bound H3K4me3 peptide is shown as a ball-and-stick model with C, O and N atoms colored green, red and blue, respectively. (c) Corresponding regions of superimposed ^1H , ^{15}N HSQC spectra of the ING2 PHD finger (0.2 mM) display chemical shift changes in the $\text{NH}^{\epsilon 1}$ group of W238 during titration with up to 1 mM H3K4me3 (left), H3K4me1 (middle) and H3K9me3 (right).

Table 1
¹H, ¹³C and ¹⁵N resonance assignments of the ING2 PHD finger at 25 °C and pH 6.6

Residue	Chemical shift (ppm)													
	N	NH	αH	βH	γH	δH	εH	αC	βC	γC	δC	CO	δNy	εN
Asn211	117.44	8.53	4.67	2.7;2.8		6.92;7.79		53.19	39.13			174.91	114.39	
Glu212	122.82	7.79	4.52	1.98	2.33			54.47	30.22					
Pro213			4.27	1.97;2.06	1.54	3.66		62.94	32.22	27.65	51.22	174.62		
Thr214	109.21	7.51	4.11	3.65	0.96			59.49	70.65	22.1		173.05		
Tyr215	118.47	8.26	5.05	1.98;3.1		7.11	6.84	57.2	43.67			174.09		
Cys216	115.85	7.1	4.7	2.37;3.66				59.32	30.28			175.33		
Leu217	126.88	10.07	4.39	1.87;2.02	2.18	1.13		57.08	42.77	25.81	23.78	178.11		
Cys218	114.23	7.65	4.66	3.16;3.31				58	31.23			175.93		
Asn219	121.17	7.85	4.51	2.76;3.09		6.82;7.51		54.81	37.91			173.57	113.64	
Gln220	116.54	8.04	4.99	2.56	2.47		6.84;7.49	54.27	33.1	34.31		176.10		113.12
Val221	113.56	8.15	4.51	2.56	1.00			61.58	33.14	18;21.69		176.19		
Ser222	115.58	8.89	4.04	3.64				59.49	63.37			173.99		
Tyr223	120.14	7.37	4.59	2.91;3.14		6.92	6.44	57.99	39.41			174.62		
Gly224	109.2	8.62	3.73;3.83					45.69				173.18		
Glu225	122.35	8.22	3.96	1.74	1.87			56.62	28.43			176.04		
Met226	123.9	8.26	5.5	2.1	2.18		1.59	53.08	34.73	31.69		175.08		
Ile227	121.89	9.65	4.75	1.29	1.16;0.44	−0.02		58.17	42.5	15.17;28.92	15.17	172.08		
Gly228	116.42	8.32	2.56;4.75					43.4				174.90		
Cys229	125.83	8.3	4.54	3.12				60.16	32.77			176.82		
Asp230	127.08	8.91	4.66	1.63;2.45				57.06	40.67			176.64		
Asn231	121.84	8.97	4.99	3.06;4.04		7.11;9.55		53.08	38.18			176.40	117.13	
Glu232	131.43	9.53	4.14	2.1	2.38			59.06	29.21	36.54		177.03		
Gln233	114.93	7.28	4.46	1.79	2.35		6.82;7.59	54.47	28.22	34.4		174.73		114.75
Cys234	126.35	7.6	4.12	2.83				59.17	31.25					
Pro235			4.54	2.35	2.08;2.35			63.94	32.46	26.8		176.61		
Ile236	124.16	8.58	4.13	1.71	1.05;1.48	0.33		63.08	40.95	18.75;28.78	13.65	177.28		
Glu237	120.29	9.61	3.75	2.44	1.71;1.94			63.01	29.96	36.91		176.09		
Trp238	119.98	7.77	5.34	2.42;2.6	arom:	6.92;7.11;7.43	9.91	55.99	33.32			174.15		128.6

Residue	Chemical shift (ppm)													
	N	NH	α H	β H	γ H	δ H	ε H	α C	β C	γ C	δ C	CO	δ Ny	ε N
Phe239	117.06	9.15	4.81	2.81;3.22		7.67	7.23	56.29	42.76			176.03		
His240	123.2	9.43	4.97	3.63;3.95				58.05	30.92			178.02		
Phe241	124.41	8.48	3.95	3.1;3.33		6.68	6.88	60.84	37.78			178.82		
Ser242	110.07	8.96	4.34	4.07				61.46	62.34			178.20		
Cys243	121.95	7.39	4.31	3.32				62.54	30.6			176.86		
Val244	107.6	7.39	4.76	2.7	0.85;0.98			59.93	31.25	18.13;22.91		174.30		
Ser245	112.34	7.74	4.13	4.03				59.06	61.54			173.74		
Leu246	119.28	7.95	4.55	1.24	0.84	0.49;-0.26		53.50	44.30			177.01		
Thr247	112.28	8.39	4.31	4.12	0.96			62.18	69.56			173.32		
Tyr248	123.06	8.77	4.72	2.74;3.08		7.04	6.84	55.71	40.31			173.49		
Lys249	123.31	8.77	4.08	1.87	1.39	1.55								
Pro250			4.42	1.91;2.29	1.69			62.83	32.48	27.27	51.03	176.30		
Lys251	121.14	8.55	4.36	1.79	1.49	1.75		56.18	33.07	25.15	29.24	177.36		
Gly252	110.88	8.23	3.98					44.49	31.40			173.29		
Lys253	120.49	8.12	4.3	1.78	1.38	1.58	3.03	55.95	33.97	25.3	28.77	175.93		
Trp254	123.84	9.33	4.51	3.17	arom:6.36;	6.80;7.00;7.55	9.77	58.79						127.17
Cys256	128.78	9.16												
Pro257				2.47	1.73	3.83		65.32	32.64	28.29	50.68	177.43		
Lys258	117.06	8.73	4.26	1.92;2.09	1.49	1.65		57.47	32.26	24.82	28.32	177.93		
Cys259	122.4	8.09	4.01	2.62;2.91				61.72	32.22			171.45		
Arg260	114.83	8.08	4.00	2.06	1.55			55.69	29.21			174.71		
Gly261	108.66	8.54	3.21;4.08					43.97				175.79		
Asp262	125.5	8.39	4.24	2.38;2.71										