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NMR Structure Note: PHD Domain from Human SHPRH

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Abstract

SHPRH (SNF2, histone linker, PHD, RING, helicase) is a SWI2/SNF2-family ATP-dependent chromatin remodeling factor, and one of E3 ubiquitin ligases responsible for Ubc13-Mms2-dependent K63 poly-ubiquitination of PCNA (proliferating cell nuclear antigen) that promotes error-free DNA damage tolerance in eukaryotes. In contrast to its functional homologues, *S. cerevisiae* Rad5 and human HLTF (helicase like transcription factor), SHPRH contains a PHD (plant homeodomain) finger embedded in the ‘minor’ insert region of the core helicase-like domain. PHD fingers are often found in proteins involved in chromatin remodeling and transcription regulation, and are generally considered as ‘readers’ of methylation state of histone tails, primarily the lysine 4 (K4) residue of histone H3 (H3K4). Here we report the solution NMR structure of the SHPRH PHD domain and investigate whether this domain is capable of recognizing H3K4 modifications. The domain adopts a canonical PHD-finger fold with a central two-stranded anti-parallel β -sheet flanked on both sides by the two interleaved zinc-binding sites. Despite the presence of a subset of aromatic residues characteristic for PHD-fingers that preferentially bind methylated H3K4, NMR titration experiments reveal that SHPRH PHD does not specifically interact with the H3-derived peptides irrespective of K4 methylation. This result suggests that the SHPRH PHD domain might have evolved a different function other than recognizing histone modifications.

Keywords

DNA damage tolerance; template switching; translesion synthesis; ubiquitination; PCNA; HLTF; Rad5; Mms2-Ubc13

Biological Context

SHPRH (SNF2, histone linker, PHD, RING, helicase) is a 193 kDa member of SWI2/SNF2 family of ATP-dependent chromatin remodeling enzymes (Flaus et al., 2006), containing the C₃HC₄ (Cys3-His-Cys4) RING-finger characteristic of E3 ubiquitin ligases. The human SHPRH gene maps to the chromosomal region 6q24 whose allelic loss or deletions are observed in many types of cancer, leading to the hypothesis that this region may encode putative tumor suppressor gene(s) (Sood et al., 2003). Consistent with this notion, SHPRH

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has been subsequently shown to play a key role in the error-free branch of DNA damage tolerance - a mechanism by which cells can bypass replication blocks caused by sites of DNA damage (lesions) and/or fill postreplicational lesion-containing single-stranded DNA gaps (Chang and Cimprich, 2009; Unk et al., 2010).

SHPRH, and the closely-related protein HLTF (helicase like transcription factor), are functional homologues of *S. cerevisiae* Rad5, acting as ubiquitin ligases in Ubc13-Mms2-dependent poly-ubiquitination of PCNA (proliferating cell nuclear antigen) (Motegi et al., 2008; Motegi et al., 2006; Unk et al., 2006). Human PCNA is a ring-shaped DNA-sliding clamp that provides a binding platform for various proteins involved in DNA replication and damage response (Moldovan et al., 2007). PCNA ubiquitination at replication forks that are stalled by DNA damage regulates switching between normal DNA replication and the two distinct modes of DNA damage tolerance (Chang and Cimprich, 2009; Unk et al., 2010; Waters et al., 2009). Thus, PCNA mono-ubiquitination at K164 by the Rad6 (E2)/Rad18 (E3) pair triggers the exchange between replicative (pol δ and pol ϵ) and low-fidelity translesion synthesis (TLS) DNA polymerases (Rev1, pol η , pol ι , pol κ and pol ζ) that constitute the *error-prone* branch of DNA damage tolerance responsible for the majority of mutagenesis in eukaryotic cells (Waters et al., 2009). Subsequent extension of K63-linked poly-ubiquitin chain by Ubc13-Mms2 (E2)/Rad5, SHPRH or HLTF (E3) promotes *error-free* DNA lesion bypass occurring *via* the template switching (TS) mechanism, where the newly synthesized strand of the sister duplex is used as a template for DNA synthesis across the lesion (Chang and Cimprich, 2009; Unk et al., 2010).

Beyond their function as E3 ubiquitin ligases, Rad5 and HLTF act as ATP-dependent double-stranded DNA translocases capable of replication fork reversal (Chang and Cimprich, 2009; Unk et al., 2010). In the process of fork reversal the Y-shaped DNA structure with homologous arms is converted into a four-way Holliday junction ('chicken-foot' intermediate) where one of the arms consists of the two newly synthesized daughter DNA strands. The formation of such an intermediate allows switching from the lesion-containing to the undamaged DNA template, facilitating error-free bypass replication across sites of DNA damage (Chang and Cimprich, 2009; Unk et al., 2010). It is likely that, similar to Rad5 and HLTF, SHPRH also possess a fork reversal activity, however this function remains to be demonstrated.

Rad5, SHPRH and HLTF share similar architectures that are characteristic of SWI2/SNF2 family members, including seven short conserved helicase motifs I, Ia, II, III, IV, V and VI (Flaus et al., 2006) spread over the length of each protein separated by insert regions of up to several hundred amino acids long (Figure 1A). Such a discontinuous arrangement for a long time obscured identification of the domain structure for this protein family. The recently-determined spatial structures of SWI2/SNF2 family members, such as zebrafish Rad54 (Thoma et al., 2005), revealed that the helicase motifs define a core helicase-like domain, despite their long sequence separation, and consists of two recA-like domains and several other structural elements (Flaus et al., 2006) (Figure 1B). Embedded in the helicase-like domain are so-called 'minor' and 'major' insert regions that may harbor additional accessory domains (Flaus et al., 2006). Thus, the 'major' insert region of Rad5, SHPRH and HLTF contains the embedded C₃HC₄ RING-fingers, while the 'minor' insert region of SHPRH includes the H1.5 (linker histone H1 and H5) domain and the C₄HC₃ (Cys4-His-Cys3) PHD (plant homeodomain) finger (Figure 1B) (Chang and Cimprich, 2009; Flaus et al., 2006; Unk et al., 2010). Additionally, the N-terminal parts of Rad5 and HLTF preceding the helicase-like domain contain ~120 amino-acid region known as the HIRAN domain, which is not found in SHPRH (Chang and Cimprich, 2009; Unk et al., 2010) (Figure 1B).

Consistent with their roles as E3 ubiquitin ligases responsible for PCNA poly-ubiquitination, human SHPRH and HLTF interact with PCNA, Ubc13-Mms2 (their E2), and Rad18 (E3 transferring the first ubiquitin moiety) (Motegi et al., 2008; Motegi et al., 2006; Unk et al., 2006). SHPRH and HLTF were also shown to bind one another (Motegi et al., 2008). Recent report suggests that SHPRH, but not HLTF, can interact with TLS DNA polymerases pol η and pol κ , and thereby participate in error-prone DNA lesion bypass (Lin et al., 2011). At this time, however, protein domains that mediate these interactions remain unknown. It is likely that beyond ubiquitin ligase and DNA translocase activities (Chang and Cimprich, 2009; Unk et al., 2010) SHPRH and HLTF might have other functions, and that the list of their interaction partners will be extended as these functions are identified.

Here we report the solution NMR structure of the PHD-finger embedded in the ‘minor’ insert region of human SHPRH (Figure 1B), which, along with the adjacent H1.5 domain, are not found in its homologue HLTF. PHD zinc fingers are small modules of approximately 50 to 80 residues stabilized by the two zinc atoms anchored by 8 zinc-coordinating residues, C₄HC₃ (Cys4-His-Cys3), found in proteins involved in chromatin remodeling and transcription regulation (Bienz, 2006; Sanchez and Zhou, 2011). PHD domains are generally considered as epigenome readers that recognize lysine and arginine methylation of the histone H3 tail, primarily H3K4 (three- and di-methylated, K4me3 and K4me2, vs. non-modified, K4me0) (Sanchez and Zhou, 2011). Therefore, we have also investigated SHPRH PHD interaction with the peptides derived from the histone H3 tail with different methylation states of K4 residue. Surprisingly, no specific binding has been observed, suggesting that SHPRH PHD may belong to a small group of PHD-fingers that recognize other histone modifications, or bind other yet unknown molecular targets.

Methods and Results

Protein expression and purification

The gene encoding PHD domain from human SHPRH (residues 652–716) was sub-cloned into PGEX-4T3 vector using SalI and NotI restriction sites. The recombinant protein was overexpressed in *E. Coli* BL21(DE3) cells transformed with SHPRH PHD plasmid. Cell culture was inoculated overnight at 37°C in 50 ml LB medium and then transferred to 1L M9 medium with ¹⁵NH₄Cl and ¹³C-glucose used as sole nitrogen and carbon sources, respectively. Cells were grown at 37°C to OD₆₀₀ of 1.0–1.2, at which point 0.05 mM ZnCl₂ was added to the medium. After an additional 1h growth to OD₆₀₀ of ~1.4 protein expression was induced by 1 mM IPTG overnight at 20 °C. Cells were harvested by centrifugation, resuspended in PBS buffer (Na₂HPO₄ 10 mM pH 7.5, KH₂PO₄ 1.8 mM, NaCl 140 mM) and lysed by sonication. Soluble fraction was collected, loaded onto GST-column and incubated for 30 minutes, followed by protein elution with 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM reduced glutathione. GST-tag was cleaved with thrombin at room temperature overnight, followed by protein purification on a HiLoad Superdex 75 column (GE Healthcare). The final protein sample used for NMR structure determination contained 0.7 mM ¹⁵N/¹³C labeled protein, 50 mM HEPES, 150 mM NaCl, 0.05 mM ZnCl₂, 1 mM DTT, pH 7.0. The same protocol was used to express and purify ¹⁵N labeled SHPRH PHD domain for the H3 histone binding experiments (see below) with the exception that cells were grown in M9 medium containing ¹²C-glucose.

NMR resonance assignment and protein structure calculation

NMR spectra for the backbone and side-chain resonance assignment and spatial structure determination of the SHPRH PHD domain were acquired at 20 °C on 11.7 and 18.8 T Agilent VNMRs spectrometers (500 and 800 MHz ¹H frequencies) equipped with cold probes. The resonance assignments were obtained from 2D ¹H-¹⁵N HSQC, ¹H-¹³C HSQC

and 3D HNCO, HNCACB, CBCA(CO)NH, HBHA(CO)NH, HC(C)H-TOCSY, (H)CCH-TOCSY, ^{15}N - and ^{13}C -edited NOESY-HSQC (150 ms mixing time) spectra (Sattler et al., 1999). The spectra were processed with NMRPipe (Delaglio et al., 1995) and analyzed in CARA software. Nearly complete (98%) set of the assigned backbone and side-chain chemical shifts was deposited to BioMagResBank (BMRB accession number 19229). The annotated assigned ^1H - ^{15}N HSQC spectrum of the SHPRH PHD is shown in Figure 2.

It was previously shown that PHD finger is a zinc-dependent fold and that zinc removal from the purified domain by addition of the excess EDTA results in the loss of the domain structure (Pascual et al., 2000). To ascertain that the SHPRH PHD domain in our samples is bound to zinc we have additionally recorded ^1H - ^{15}N HSQC spectrum of 0.2 mM PHD domain in the presence of 10 mM EDTA (Figure 2, inset). Addition of EDTA resulted in significant precipitation of the SHPRH PHD sample. The remaining protein displays NMR spectrum characteristic of disordered protein (Figure 2, inset), suggesting that apo-form of the SHPRH PHD domain is unstable in solution.

Structure calculation for the SHPRH PHD domain was performed in CYANA software (Guntert, 2003) using (i) the backbone ϕ and ψ angle restraints obtained from chemical shifts with TALOS+ program (Shen et al., 2009), (ii) ^1H - ^1H distance restraints based on correlations observed in NOESY spectra, and (iii) hydrogen bond restraints added on the basis of NOE analysis. NOE cross-peaks were peak-peaked manually and assigned automatically in CYANA (Guntert, 2003). Additional restraints for distances between zinc-ions and atoms of zinc-coordinating residues were introduced to enforce tetrahedral geometries of the two zinc centers based on zinc center geometries observed in high-resolution crystal structures (Alberts et al., 1998). A total of 100 structures of the domain were generated, followed by refinement of lowest-energy 20 models in CNS software (Brunger et al., 1998) using constrained molecular dynamic simulations in explicit solvent. Atomic coordinates of the final SHPRH PHD structural ensemble were deposited in the Protein Data Bank (PDB entry 2M85)

Spatial structure of SHPRH PHD domain

Table 1 summarizes NMR-based restraints used for the SHPRH PHD structure determination and structure refinement statistics. The ensemble of 20 least-energy structures of the domain shown in Figure 3A exhibits mean pairwise RMSD of 1.2 Å calculated for heavy atoms of residues 657–712, displays minimal violations from idealized molecular geometry, and agrees well with the input experimental data (Table 1). The overall backbone fold for the least-energy model of SHPRH PHD domain is illustrated in Figure 3B. The domain adopts a canonical PHD-finger fold with a central two-stranded anti-parallel β -sheet (residues 674–677 and 682–685) flanked on both sides by the two interleaved zinc binding sites formed by residues C661, C663, H685, C688 (ZnI site) and C677, C680, C703, C706 (ZnII site) (Figure 3). The β -sheet region harbors two pairs of zinc-coordinating residues: one in the end of strand β 1 and in a short β 1- β 2 connector loop (C677, C680 from ZnII site) and the other in the end of and immediately after strand β 2 (H685, C688 from ZnI site). Loop 1 between the first pair of zinc-coordinating residues from ZnI site (C661, C663) and strand β 1 lacks regular secondary structure, while loop 2 connecting strand β 2 and the last pair of zinc-coordinating residues from ZnII site (C703, C706) includes two short helices, α 1 (residues 686–688) and α 2 (residues 693–697). Additionally, the C-terminal part of the domain forms α -helix α 3 spanning the residues 704–710.

Figure 3C shows sequence alignment of the SHPRH PHD domain and the two groups of PHD fingers: one that recognizes K4-methylated tail of histone H3, H3K4me3 or H3K4me2, over non-modified H3K4me0 (red), and another that preferentially binds H3K4me0 (blue) (Sanchez and Zhou, 2011). Structure analysis of various PHD-finger complexes with H3-

derived peptides revealed amino-acid residues responsible for H3K4 recognition (Sanchez and Zhou, 2011), marked by red and blue in Figure 3C. Thus, recognition of the tri- or di-methyl ammonium group of H3K4 occurs *via* an aromatic cage formed by 2 to 4 residues with aromatic and hydrophobic side-chains (Figure 3C, red). Among the cage-forming residues, the highly conserved tryptophan in strand $\beta 2$ is present in all PHD fingers that read H3K4me3 and H3K4me2 modifications. Additional cage-forming aromatic residues are located in the loop 1 and the beginning of strand $\beta 1$, or in the N-terminal part preceding the first two zinc-coordinating residues of ZnI site (Figure 3C, red). In contrast, recognition of non-modified H3K4me0 does not require an aromatic cage, and occurs *via* hydrophobic and acidic amino-acids occupying the same positions as cage-forming residues in PHD domains that preferentially bind methylated H3K4 (Figure 3C, blue).

The SHPRH PHD domain contains tryptophan residue, W683, at the position highly conserved in PHD fingers that recognize tri- or di-methylated H3K4, as well as two aromatic residues, Y657 and F659, N-terminal to the first pair of zinc-coordinating cysteins of ZnI site, C661 and C663 (Figures 3C, first line). The presence of these spatially close aromatic amino-acids that could potentially form an aromatic cage (Figures 3A,B, yellow) might prove consistent with the ability of the domain to recognize K4-methylated tail of H3 histone.

SHPRH PHD domain is not a reader of H3K4 methylation state

To test the hypothesis that SHPRH PHD finger may bind and preferentially recognize histone H3 methylated at K4 we have performed a series of NMR titration experiments. In these experiments 25 mM solutions of custom synthesized (GenScript) 11 residue peptides derived from the H3 tail, ARTKQTARKST, non-modified, di- or tri- methylated at K4 were gradually added to ~0.2 mM samples of ^{15}N labeled SHPRH PHD domain. The titrations were continued until protein to peptide ratio of 1:28 (H3K4me0 and H3K4me2 peptides) or 1:36 (H3K4me3 peptide) and were monitored by recording 11.7 T ^1H - ^{15}N HSQC spectra at each titration step.

Surprisingly, no chemical shift changes consistent with specific binding were observed upon SHPRH PHD titrations with either of the H3-derived peptides irrespective of the K4 methylation state. Furthermore, no changes in the backbone $^1\text{H}^{\text{N}}$ - ^{15}N and side-chain $^1\text{H}^{\text{e1}}$ - $^{15}\text{N}^{\text{e1}}$ chemical shifts were observed for W683, whereas the indole ring of a conserved tryptophan at this position in PHD fingers that bind tri- and di-methylated H3K4 is involved in cation- π and van der Waals interactions with the K4 methyl ammonium group crucial for stabilization of the complex (Sanchez and Zhou, 2011). It should be noted that in all titration experiments we have noticed small chemical shift changes for the backbone amide of H705 at large excess of the added peptide (data not shown). Similar changes were observed in ^1H - ^{15}N HSQC spectra of SHPRH PHD upon small variations in the sample pH. NMR titration experiments clearly suggest that, despite the presence of a conserved tryptophan and two more aromatic residues that could potentially form an aromatic cage, the SHPRH PHD finger *is not* capable of specific binding H3 histone tail irrespective of the methylation state of K4 residue. One possible explanation for the lack of H3K4 binding by the SHPRH PHD domain is the presence of positively charged R674 in the beginning of $\beta 1$ -strand (marked by star in Figure 3C) in a conserved position occupied by a hydrophobic residue in PHD domains that recognize H3K4 modifications.

Discussion and Conclusions

In this work we have determined NMR structure of the PHD domain from the ‘minor’ insert region of human SHPRH (Figures 1,3), an E3 ubiquitin ligase responsible for poly-ubiquitination of DNA-sliding clamp PCNA and a tentative ATP-dependent DNA

translocase that plays an important role in error-free branch of DNA damage tolerance (see Biological Context). PHD fingers have been reported as universal readers of methylation state of H3 histone tail (Sanchez and Zhou, 2011). Most of the domains described so far preferentially bind tri- or di-methylated H3K4, H3K4me3 and H3Kme2, while a smaller group recognizes non-modified H3K4me0 (Figure 3C). Several PHD fingers were also identified that discriminate between methylation states of H3R2, H3R2me0 vs H3R2me2 (Sanchez and Zhou, 2011). PHD domains are also known to interact with proteins other than histones (Bienz, 2006), and some PHD domains such as the second PHD finger from AIRE do not recognize any histone H3 modifications (Gaetani et al., 2012). Here we have shown with the use of NMR titration experiments that SHPRH PHD domain does not bind either K4-methylated or unmodified histone H3 tail, contrary to what might be anticipated from its amino-acid composition (Figure 3C). Thus, SHPRH PHD domain may have evolved another function distinct from reading histone H3 methylation state, or might be involved in recognition of a different histone modification.

PHD fingers are structurally similar to RING domains characteristic of E3 ubiquitin ligases, which poses an intriguing possibility that PHD fingers might also function as components of E3 ligases. The PHD domain dependent ubiquitin ligase activity has indeed been reported for several nuclear proteins (Dul and Walworth, 2007). Furthermore, PHD domain from the KAP1 co-repressor binds Ubc9 (SUMO E2 conjugating enzyme) and acts as E3 ligase for sumoylation of the adjacent bromodomain (Ivanov et al., 2007). Although E3 ubiquitin ligase activity of SHPRH is dependent on its RING domain that is embedded in the 'major' insert region (Motegi et al., 2006) (Figure 1), it is conceivable that PHD finger might play an accessory role in Ubc13-Mms2-SHPRH ubiquitin-conjugation cascade. Other potential functions of SHPRH PHD might involve mediating interactions with various proteins of DNA damage tolerance pathways such as TLS DNA polymerases and/or Rad18 (E3 ligase responsible for PCNA mono-ubiquitination) (Lin et al., 2011; Motegi et al., 2008; Motegi et al., 2006). These and other possibilities need to be systematically addressed in future studies aimed at establishing functional role and identifying interaction partners of SHPRH PHD domain.

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Figure 1.

(A) Conserved motifs in SWI2/SNF2 family of chromatin remodeling enzymes: seven original helicase motifs I, Ia, II, III, IV, V and VI and additional short conserved blocks labeled Q to N (Flaus et al., 2006). (B) Structural sub-domains of a core helicase-like domain of SWI2/SNF2 family enzymes, including two recA-like domains separated by the two helical protrusions and a linker domain flanked by the N-terminal Q-motif and the C-terminal brace domain (Flaus et al., 2006). Additional modular domains found in the 'major' and 'minor' insert regions and the N-terminal parts of *S. cerevisiae* Rad5, human HLTF and SHPRH are indicated in the bottom of the plot.

Figure 2. ^1H - ^{15}N HSQC spectrum of the SHPRH PHD domain (residues 652–716) acquired at 11.7 T magnetic field, 20 °C with peaks annotated with the backbone amide resonance assignments. The spectrum of 0.2 mM ^{15}N labeled domain in the presence of 10 mM EDTA is shown as inset.

Figure 3. (A) Stereo-view of the NMR-derived ensemble of 20 least-energy structures, and (B) the backbone conformation for the least-energy model of the SHPRH PHD domain (residues 652–716). Zinc-ions and side-chains of zinc-coordinating residues are shown in green, while the cluster of spatially closed aromatic residues Y657, F659 and W683 is shown in yellow. (C) Sequence alignment of the SHPRH PHD domain with PHD fingers that preferentially bind methylated H3K4me3 and H3K4me2 (red), or non-modified H3K4me0 (blue) histone H3 tail (Sanchez and Zhou, 2011). Residues involved in recognition of H3K4 modifications are highlighted by red and blue colors, respectively.

Table 1

NMR-based restraints used for the SHPRH PHD domain structure calculation and structure refinement statistics.

NMR distance and dihedral restraints	PHD (652–716)
Distance restraints	
Total NOE	1343
Intra-residue	824
Inter-residue	519
Sequential ($ i - j = 1$)	824
Medium range ($ i - j < 4$)	200
Long range ($ i - j > 5$)	319
Protein-zinc restraints	46
Hydrogen bonds	14
Total dihedral angle restrains (ϕ, ψ)	87
Deviations from idealized geometry	
Bond lengths ($\text{\AA}$)	0.02
Bond angles ($^\circ$)	1.3
Ramachandran plot	
Residues in most favored regions (%)	75.1
Residues in additional allowed regions (%)	23.7
Residues in generously allowed regions (%)	0.3
Residues in disallowed region (%)	0.9
Mean pairwise rmsd ($\text{\AA}$)	residues 657–712
Heavy	1.18 ± 0.14
Backbone	0.5 ± 0.11