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The ankyrin repeat domain of Huntingtin interacting protein 14 contains a surface aromatic cage, a potential site for methyl-lysine binding

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Introduction

Huntingtin interacting protein 14 (HIP14), a membrane-bound palmitoyl transferase, palmitoylates a number of neuronal proteins (including Huntingtin) and affects the trafficking, stability, aggregation, and/or functional activity of substrate proteins. HIP14 contains an N-terminal ankyrin repeat domain that may function in its substrate recognition. Sequence analysis suggests that the HIP14 ankyrin repeats share approximately 50% identity with the ankyrin repeats of G9a and G9a-like protein (GLP) histone lysine methyltransferases. The crystal structure of the HIP14 ankyrin repeats reveals a surface aromatic cage, formed by two tryptophans, one tyrosine, and one methionine. The all-hydrophobic cage resembles the tri-methylated lysine binding pocket of the plant homeodomain (PHD) of human BPTF (bromodomain and PHD domain transcription factor) 1.

HIP14, a Huntingtin interacting protein 2, is a 633-residue protein, including 7-8 ankyrin repeats in the N-terminal region followed by five predicted transmembrane helices. The protein contains a signature DHHC palmitoyl transferases motif located close to the predicted fourth transmembrane helix 3. Importantly, the ankyrin repeats (a protein-protein interaction domain that may function in substrate recognition) and the DHHC sequence (the hypothetical active site) are both predicted to reside on the cytoplasmic face of the lipid bilayer 4, presumably allowing the substrate recognition and the active site to interact with the same substrate. Posttranslational palmitoylation involves the attachment of the saturated C16 fatty acid palmitate to specific cysteines via a thioester linkage 5-7. HIP14

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palmitoylates Huntingtin at cysteine 214 8. Other substrates of HIP14-mediated palmitoylation include SNAP-25 (synaptosome associated protein 25 kDa), PSD-95 (postsynaptic density 95 kDa), GAD-65 (glutamate decarboxylase 65 kDa), and Synaptotagmin I 9.

Ankyrin repeats are known to mediate protein-protein interactions 10. Recently we showed that the ankyrin repeat domains of G9a and GLP, two euchromatin associated histone lysine methyltransferases, bind N-terminal histone H3 peptides containing mono- or di-methylated lysine 9 (H3K9me1, me2) via a partial aromatic cage with three tryptophans and one acidic residue 11. Besides the ankyrin repeats of G9a and GLP, other protein domains including the Chromodomain 12, plant homeodomain 1·13·15, and Tudor domain 16, also recognize methylated lysines (Review 17). The common mode of methyl-lysine interactions is via a surface aromatic cage consisting of 2-4 aromatic residues. These aromatic cages are highly selective for methyllysine. In a study of Chromodomain-methyllysine interaction, binding was driven primarily by cation- π interactions (i.e, a methyllysine carries a positive charge), and secondarily by the packing of methyl group(s) against the aromatic ring(s) of the cage, with the hydrophobic effect contributing the least to binding 18. This eliminates the possibility of such cages generically binding hydrophobic residues, and suggests that other methyllysine binding ankyrin repeats could be identified by the presence of a surface aromatic cage.

Materials and Methods

The gene fragment encoding the N-terminal 51-288 residues of human HIP14 (we termed it the HIP14ANK) was amplified from ATCC Clone 5266097 and cloned into the NdeI and XhoI restriction sites of a modified pET28b vector containing 6xHis tag followed by SUMO tag, yielding pXC650.

The hexahistidine-SUMO-HIP14ANK fusion protein was expressed in *E. coli* BL21 (DE3) Gold C⁺ cells (Stratagene) via autoinduction at 37°C 19. The fusion protein was isolated on a 5 ml nickel-charged HiTrap Chelating HP column (GE Healthcare). The imidazole elution from the nickel-column was cleaved with His-tagged Ulp1 protease 20 overnight while in dialysis against buffer without imidazole at 4°C, leaving two amino acids (HisMet) in front of the HIP14 ankyrin repeat domain. The digested mixture was passed through a nickel-column to remove the His-SUMO tag and the protease. The protein was further purified on a Superdex-75 Prep column (GE HealthCare) and stored in the buffer containing 20 mM Tris pH 8.0, 250 mM NaCl, 5% glycerol, and 5 mM dithiothreitol. The selenomethionine-labeled HIP14ANK protein was produced by a methionine auxotrophic *E. coli* strain (B834) grown in the PASM-5052 medium in the presence of 92% selenomethionine (125 mg/L) and 8% methionine (10 mg/L) 19. The growth, induction, and purification were performed following the same protocol as was used for the native protein.

HIP14ANK protein was concentrated to approximately 30 mg/ml. Crystals were obtained by sitting drop with mother liquor containing 100 mM (NH₄)₂SO₄, 25% polyethylene glycol (PEG) 3350, 0.1M Bis-Tris, pH 5.5 at 16°C. Selenomethionine-labeled protein crystals were grown in 200 mM (NH₄)₂SO₄, 30% PEG5KMME, 0.2M MES, pH 5.5. All crystals were cryoprotected by soaking in mother liquor supplemented with 35% xylitol.

Selenium anomalous diffraction data were collected at peak (0.9794 Å) and inflection (0.9796 Å) wavelengths at the SBC-CAT 19-ID beamline at the Advanced Light Source at Argonne National Laboratory on an ADSC Quantum 315 detector. SOLVE 21 found 21 selenium sites, which gave a clear, traceable density with many recognizable side chains.

Model building with O 22 and refinement with CNS 23 was then continued using a native data set.

In addition, GST tagged HIP14ANK (pXC648) and GST tagged HP1 β chromodomain were purified and used in the binding of protein domains to peptide SPOT arrays 24.

Results and Discussion

Ankyrin repeats exist in a large number of intracellular and extracellular eukaryotic proteins 25-27. To investigate whether a subset of ankyrin repeats could function in the recognition of methylated lysines as exemplified by G9a and GLP 11, we searched protein database for sequence homologues of G9a and GLP. We placed particular emphasis on the conservation of aromatic residues corresponding to the cage residues of G9a and GLP, and their appropriate placement on the beta-turn and first alpha helix of neighboring repeats. Table 1 summarizes the protein sequence alignment of ankyrin repeat containing proteins with the potential to bind methyllysine. These proteins have conserved spacing of aromatic, hydrophobic or acidic residues, like in G9a and GLP. The ankyrin repeat domain of HIP14 is one of these candidates.

We generated a hexahistidine-SUMO (small ubiquitin-like modifier) tagged construct 28 containing human HIP14 residues 51-288 (pXC650). The SUMO fusion was cleaved off by Ulp1 protease 20. The protein crystallized in the space group P6₁22 and the structure was determined to the resolution of 1.99 Å (Table 2). The crystallographic asymmetric unit contains three molecules, A, B, and C. The three molecules are highly similar, except at the N- and C-termini (Fig. 1A).

The crystallized HIP14 fragment contains seven ankyrin repeats. Each repeat has a helix-turn-helix- β -turn structure. The helices stack against each other and the β -turns project out at right angles. The sequence conservation between HIP14 and GLP indicates the invariant residues lie between ankyrin repeats 2 and 7 (Fig. 1B and Table 3). Structural superimposition of HIP14 and GLP ankyrin repeats positions the methylated lysine in a surface cage formed by M191, W196, and Y198 of ANK5 and W231 of ANK6 (Fig. 1C). In the GLP structure, an acidic residue interacts with the lone proton of the N_ϵ atom of the dimethylated lysine whose methyl moieties point toward the aromatic residues 11. Surveying structurally characterized cage-like methyl-lysine binding pockets revealed a glutamate or aspartate residue in (or near) the binding pocket having an important role in selecting for lower (mono- or di-) versus higher (tri-) methylation states of lysine 17. An all-hydrophobic cage in HIP14 suggests that it would bind a tri-methylated lysine.

The first 17 amino acids of Huntingtin before the polyglutamine track contains a third lysine, K15, followed by a serine, resembling the histone H3 lysine 9 and serine 10. Other substrates of HIP14, SNAP-25, PSD-95, GAD-65, and Synaptotagmin I, all contain sequences resembling histone H3 lysine 4 (H3K4) and H3K9 (supplementary Table 1). Known histone lysine methyltransferases, such as SET7/9 (acting on H3K4) and G9a (acting on H3K9), are able to methylate non-histone targets with consensus sequence as short as two-to-three residues 24-29-32. Thus, we investigated the potential interaction of the HIP14 ankyrin-repeat domain with modified histone peptides arrays 24. We employed two different peptide arrays including in total >1000 peptides containing >80 known modifications of the H3, H4, H2A and H2B tails in various combinations, but no specific binding to the membranes was detected (not shown). In comparison, as a positive control HP1 β shows a strong H3K9 specific signal with the same arrays (Supplementary Figure 1). In addition, there was no detectable binding to the Huntingtin peptides (Supplementary Table 2). We speculate that the methylation of Huntingtin may crosstalk with other modifications,

because, in addition to palmitoylation, Huntingtin can be posttranslationally modified either by sumoylation or by ubiquitylation on its N-terminal lysine residues K6 and K9 33. The crosstalk between ubiquitylation and methylation of neighboring lysine residues has been observed in regulation of the protein stability of estrogen receptor α 32. It remains to be seen whether Huntingtin is methylated and whether the methylated Huntingtin is recognized by the ankyrin repeats of HIP14.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Accession Numbers

Atomic coordinates and structure factors are available in the Protein Data Bank (<http://www.rcsb.org/>) at 3EU9 (PDB ID code).

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Figure 1. Structure of HIP14 ankyrin repeats

(A) Superimposition of molecule A (green) and C (magenta), two of the three molecules in the crystallographic asymmetric unit. Molecule A contains an intact N-terminus, but the last seven residues (282-288) are disordered; while the first eight (51-58) and the last four residues (285-288) are disordered in molecule C. In molecule B (not shown), the first two (51-52) and the last nine residues (280-288) are disordered.

(B) Superimposition of HIP14 ankyrin repeats (molecule C) and GLP ankyrin repeats (blue; PDB 3B95). Sequence conservation between the two proteins is scattered throughout of ANK2 to ANK7 (Table 3). It is possible that either protein could have an additional repeat either extended in the N-terminus (GLP) or in the C-terminus (HIP14). The histone H3 peptide bound with GLP is shown in stick model (yellow) with blue for nitrogen and red for oxygen atoms.

(C) Close-up view of the cages (magenta for HIP14 and blue for GLP) formed by the residues of ANK4 and ANK5. The di-methylated lysine (yellow) is inserted into the partial hydrophobic cage of GLP. A tri-methylated lysine could be inserted into the all-hydrophobic cage of HIP14.

Table 1

Sequence alignment of ankyrin repeat domains with the potential to bind methyllysine

Protein	Accession	Length	Repeat	Score	Identity	Positives	Gaps	Conserved
ANKRD1A	U01401	100	1	100	100	100	0	100
ANKRD1B	U01402	100	1	100	100	100	0	100
ANKRD1C	U01403	100	1	100	100	100	0	100
ANKRD1D	U01404	100	1	100	100	100	0	100
ANKRD1E	U01405	100	1	100	100	100	0	100
ANKRD1F	U01406	100	1	100	100	100	0	100
ANKRD1G	U01407	100	1	100	100	100	0	100
ANKRD1H	U01408	100	1	100	100	100	0	100
ANKRD1I	U01409	100	1	100	100	100	0	100
ANKRD1J	U01410	100	1	100	100	100	0	100
ANKRD1K	U01411	100	1	100	100	100	0	100
ANKRD1L	U01412	100	1	100	100	100	0	100
ANKRD1M	U01413	100	1	100	100	100	0	100
ANKRD1N	U01414	100	1	100	100	100	0	100
ANKRD1O	U01415	100	1	100	100	100	0	100
ANKRD1P	U01416	100	1	100	100	100	0	100
ANKRD1Q	U01417	100	1	100	100	100	0	100
ANKRD1R	U01418	100	1	100	100	100	0	100
ANKRD1S	U01419	100	1	100	100	100	0	100
ANKRD1T	U01420	100	1	100	100	100	0	100
ANKRD1U	U01421	100	1	100	100	100	0	100
ANKRD1V	U01422	100	1	100	100	100	0	100
ANKRD1W	U01423	100	1	100	100	100	0	100
ANKRD1X	U01424	100	1	100	100	100	0	100
ANKRD1Y	U01425	100	1	100	100	100	0	100
ANKRD1Z	U01426	100	1	100	100	100	0	100

Table 2

Statistics of X-ray data reduction, phasing, and structure refinement

Crystal Number	09 (Native)	03 (Se)	03 (Se)
Data collection			
Space group	P6 ₁ 22		
Cell constants a=b (Å) c (Å) =	106.66 255.53	107.29 256.43	106.81 256.01
Beamline (APS)	SER-CAT	SBC-CAT	
Wavelength (Å)	1.0	0.9796	0.9794
Resolution (Å) *	35.0-1.99 (2.05-1.99)	50.0-2.50 (2.59-2.50)	50.0-2.50 (2.59-2.50)
R _{sym} or R _{merge} *	0.078 (0.404)	0.152 (0.780)	0.180 (0.839)
I/σI	14.0	5.8	5.3
Completeness (%) *	99.1 (99.9)	98.3 (99.3)	99.9 (100)
Redundancy	11.0	12.0	21.6
Measured Reflections	656,010	367,177	664,119
Unique Reflections	59,687	30,731	30,889
Mean FOM (2.5Å), Solve	0.59		
Refinement			
Resolution (Å) *	34.27-1.99 (2.06-1.99)		
Number of reflections *	58,266 (4,980)		
R _{work} /R _{free} *	0.222/0.251 (0.269/0.289)		
No. of atoms			
Protein	5212 (three molecules per asymmetric unit)		
Sulfate ions	7		
Glycerol	2		
Water	344		
B-factors (Å ²)	40.1		
R.m.s. deviations			
Bond Lengths (Å)	0.005		
Bond Angles (°)	1.1		
Dihedral (°)	20.3		
Improper (°)	0.75		
Estimated coordinate error			
From Sigmaa (Å)	0.15		

* Highest resolution shell is shown in parenthesis.

Table 3

Sequence alignment of HIP14 (NP_056151.2) and GLP (Q9H9B1) ankyrin repeats. White-on-black residues (59) are invariant between the two sequences examined , while gray-background positions (22) are conserved (T and S; I, V, L and M; D and E; Q and N; R and K). Cage residues are highlighted in red letters. Grey-letter residues, either in the N-terminus (GLP) or in the C-terminus (HIP14), are not included in the structure. Vertical lines between the blocks indicate conserved hydrophobic residues important for the anti-parallel packing of two helices (in cyan) of each repeat. HIP14 P207 of ANK5 is in a cis-conformation