Structure of a CENP-A–histone H4 heterodimer in complex with chaperone HJURP

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In higher eukaryotes, the centromere is epigenetically specified by the histone H3 variant Centromere Protein-A (CENP-A). Deposition of CENP-A to the centromere requires histone chaperone HJURP (Holliday junction recognition protein). The crystal structure of an HJURP–CENP-A–histone H4 complex shows that HJURP binds a CENP-A–H4 heterodimer. The C-terminal β-sheet domain of HJURP caps the DNA-binding region of the histone heterodimer, preventing it from spontaneous association with DNA. Our analysis also revealed a novel site in CENP-A that distinguishes it from histone H3 in its ability to bind HJURP. These findings provide key information for specific recognition of CENP-A and mechanistic insights into the process of centromeric chromatin assembly.

Results and Discussion

Overall structure

Human HJURP CBD was cocrystallized with CENP-A and histone H4. The 2.6 Å cocrystal structure shows that HJURP CBD forms a complex with a heterodimer of CENP-A and histone H4 [Fig. 1A]. The ordered portion of HJURP CBD consists of amino acids 14–74, and the structure is composed of a long helix (αA) and a three-stranded anti-parallel β sheet, connected by a 15-residue loop [L1] [Fig. 1A]. The structure of the CENP-A and histone H4 heterodimer, as expected, is similar to the ones in the CENP-A–H4 tetramer [Sekulic et al. 2010]. Helix αA of HJURP packs against the central helix [α2] of the histone fold of CENP-A in an anti-parallel manner, with the hydrophobic side of the amphipathic αA facing CENP-A [Fig. 1B]. Polar interactions are found between Glu 96 of CENP-A and three HJURP residues [Arg 32, Lys 39, and Tyr 40], between Glu 107 of CENP-A and Arg 28 of HJURP, and between His 104 and Asp 108 of CENP-A and Ser 25 of HJURP [Fig. 1B]. Side chain interactions with CENP-A, involving amino acids from L1 and the β-sheet domain of HJURP, are mostly hydrophobic [Fig. 1B]. HJURP also makes direct contacts with α3 of histone H4, primarily via hydrophobic and van der Waals interactions through HJURP residues located in the region encompassing the αA–L1 junction [Fig. 1A]. Extensive interactions between HJURP and the CENP-A–H4 heterodimer bury a pairwise surface area of 2459 Å², which is consistent with their stable association at even 1 M salt concentration during the purification process [see the Materials and Methods].

HJURP binds a heterodimer of CENP-A and histone H4

CENP-A and histone H4 normally exist as an equimolar tetramer. The crystal structure of the CENP-A–H4 tetramer shows that two CENP-A–H4 heterodimers dimerize...
through amino acids located on helix $\alpha_3$ and the C-terminal portion of $\alpha_2$, mainly via hydrophobic interactions (Sekulic et al. 2010). Structural superposition shows that the CENP-A–H4 heterodimer superimposes with the ones from the tetrameric structure with a 0.96 Å root-mean-squared deviation (RMSD) of C$_{\alpha}$ positions. The main differences between the two are that (1) the complete C terminus of CENP-A is visible in the current structure, while the last six residues were disordered in the tetrameric structure; (2) an N-terminal segment of L1 (amino acids 77–85) has significantly different main chain and side chain conformations (the largest deviation of the C$_{\alpha}$ position occurs on Asp 83 and is 4.7 Å apart); (3) four additional residues at the C-terminal end of histone H4 became ordered in the current structure; and (4) the C-terminal half of $\alpha_2$ of histone H4 is bent $\sim 10^\circ$ toward $\alpha_1$ of H3 (Fig. 2).

Structural superposition shows that HJURP prevents tetramer formation through two means: (1) HJURP blocks the self-association region of CENP-A located at the C-terminal portion of $\alpha_2$, which is involved in interaction with helix $\alpha_3$ of another CENP-A molecule in the tetramer; and (2) the C-terminal end of histone H4 became ordered in the current structure, and the C-terminal half of $\alpha_2$ of histone H4 is bent $\sim 10^\circ$ toward $\alpha_1$ of H3 (Fig. 2). Structural superposition shows that HJURP prevents tetramer formation through two means: (1) HJURP blocks the self-association region of CENP-A located at the C-terminal portion of $\alpha_2$, which is involved in interaction with helix $\alpha_3$ of another CENP-A molecule in the tetramer; and (2) the C-terminal end of histone H4 became ordered in the current structure, and the C-terminal half of $\alpha_2$ of histone H4 is bent $\sim 10^\circ$ toward $\alpha_1$ of H3 (Fig. 2).

HJURP prevents spontaneous association of DNA

In addition to its role in keeping the CENP-A–H4 complex in a dimeric form, HJURP also appears to compete with DNA for binding to the histone complex. As seen in Figure 1B, the convex side of the CENP-A–H4 heterodimer is enriched with positively charged residues. The positively charged area coincides with the path of nucleosomal DNA when the CENP-A–H4 heterodimer is superimposed with a nucleosomal H3–H4 complex (Fig. 4A; Luger et al. 1997). It is immediately clear that the $\beta$-sheet domain and the L1 loop of HJURP block the wrapping of DNA (Fig. 4A).
Specific recognition of CENP-A by HJURP

A critical question concerning CENP-A is what features distinguish it from histone H3. It was shown previously that the CATD of CENP-A serves as a cis-acting element that confers the CENP-A specificity of centromere targeting and nucleosome assembly (Black et al. 2004). In particular, a chimera protein of histone H3 carrying the CATD of CENP-A (H3\textsuperscript{CATD}) was able to bind HJURP in vitro and in vivo (Foltz et al. 2009; Shuaib et al. 2010). Interestingly, among the 22 amino acids of CATD that are different between CENP-A and histone H3, only two residues in loop-1 [Asn 85 and Ala 88] and two residues in α2 [Gln 89 and His 104] of CENP-A make direct interactions with HJURP. The two loop-1 residues are unlikely to be key factors for CENP-A's ability to interact with HJURP, as their histone H3 counterparts (Arg 83 and Ser 86, respectively) can interact with HJURP equally well or better, judging from the number of hydrogen bonds between histone H3 and HJURP residues in the model with a superimposed histone H3–H4 heterodimer. Gln 89 of CENP-A interacts with Phe 44 of HJURP via hydrophobic and van der Waals interactions [Fig. 5A]. The corresponding residue in histone H3, Ser 87, is unable to make such interactions. His 104, which corresponds to Gly 102 in histone H3, stacks with the phenyl ring of Phe 29 and makes one hydrogen bond with Ser 25 of HJURP (Fig. 5A). The extra interactions provided by Gln 89 and His 104 make the binding of CENP-A to HJURP better than that of histone H3, but our analysis did not find any histone H3 residues in loop-1 and helix α2 that will prevent histone H3 from binding to HJURP.

It is clear that the CATD of CENP-A is important for HJURP binding, but it seems insufficient for distinguishing CENP-A from histone H3. We searched for CENP-A residues outside of the CATD region that may be important for differentiating the abilities of CENP-A and histone H3 to bind HJURP. We found that Ser 68 on helix α1 of CENP-A is situated in a shallow hydrophobic pocket formed by Val 50, Met 52, Leu 55, and Trp 66 [Fig. 5A]. The corresponding residue in histone H3 is Gln 68, which has a bigger size and is difficult to fit into the hydrophobic pocket without making steric clashes with the HJURP residues. We predicted that a Ser 68 to Gln mutation would result in a weak binding of CENP-A to HJURP, while changing Ser 68 to a leucine, which is hydrophobic and has a smaller side chain than glutamine, should be benign to HJURP binding. Indeed, GST pull-down experiments confirmed our prediction [Fig. 5B]. To further test the significance of the newly discovered CENP-A site for HJURP binding, we mutated the corresponding residue in histone H3, Gln 68, to a serine and tested its binding to HJURP. Indeed, GST pull-down experiments show that Q68S gained the ability to bind HJURP, and robust binding of H3\textsuperscript{CATD} to HJURP was also detected in a parallel experiment [Fig. 5C]. Thus, we conclude that Ser 68 of CENP-A is an important determinant for
HJURP. Surprisingly, analyses of the HJURP-CENP-A–H4 chimera protein revealed that the CATD plays a critical role for HJURP recognition, as previous studies have shown that the CATD provides major binding affinities for HJURP, and the Ser 68 site serves as a principal determinant of CENP-A specificity. In this model, the histone H3 region corresponding to the CATD of CENP-A can interact with HJURP, perhaps suboptimally, but Gln 68 pushes away HJURP, the latter force wins and there is no binding. In H3CATD, the artificially introduced CATD can overcome the energy barrier caused by the unfavorable contact of Gln 68, resulting in the binding of HJURP. Less intuitive in this model is how the CATD overpowers Gln 68 in H3CATD, as the S68Q mutant of CENP-A with a native CATD loses the ability to bind HJURP. It should be pointed out that structural change may play an important role in the resolution of this puzzle, as it is evident from the deuterium exchange experiments that a non-CATD region in helix α1 of H3CATD has a different conformation from the corresponding region in histone H3 or CENP-A (Black et al. 2004). It is also evident from structural comparisons that the α1–L1 region is the most variable region among the structures of histone H3–H4 and CENP-A–H4 complexes. Thus, it is possible that introduction of a CATD into H3 resulted in an environment that remedied the adverse effect of Gln 68. A cocrystal structure of HJURP CBD in complex with the H3CATD–H4 complex should clarify the role of Gln 68 in H3CATD and, together with in vivo experiments, will provide further tests of the “yin-yang” model of centromere targeting proposed here.

The discovery that HJURP binds a heterodimeric form of the CENP-A–H4 complex also has profound implications in understanding the molecular mechanism of assembly of CENP-A-containing nucleosomes. The precise model of CENP-A-containing nucleosomes is still a matter of debate (Henikoff and Furuyama 2010; Black and Cleveland 2011). A closely relevant point here is whether a centromeric nucleosome contains a CENP-A–H4 heterodimer or heterotetramer (Dimitriadis et al. 2010; Sekulic et al. 2010). The structural result cannot distinguish whether a CENP-A nucleosome is octameric or a hemisome. However, it does point out that, if CENP-A nucleosomes were octameric, additional processes or regulations would be required to ensure that the CENP-A-containing nucleosomes are predominantly homotypic, as heterotypic nucleosomes with both CENP-A and histone H3 are a minority species (Foltz et al. 2006).

In summary, our structural and biochemical analyses of the HJURP CBD–CENP-A–histone H4 complex have provided novel insights into the specificity of CENP-A recognition by HJURP, and advanced our understanding of the histone chaperone activities of HJURP in preventing the formation of a CEN-P-A–histone H4 tetramer and modulating the DNA-binding activity of the CENP-A–histone H4 complex. The results of this study should facilitate in-depth analyses of the molecular mechanism of centromeric chromatin assembly.

Materials and methods

Protein expression, purification, and crystallization

The CBD encompassing the first 80 amino acids of human HJURP was expressed as a polyhistidine- and sumo-tagged fusion protein from a pET28a-smt3-HJURP [1- to 80-amino-acid] plasmid. Human CENP-A and...
histone H4 were coexpressed using a pCDFDUET (CENPA + H4) plasmid. To purify the complex HJURP-CBD with CENP-A and histone H4, Escherichia coli cells overexpressing HJURP-CBD and CENP-A-H4 were mixed and lysed together in a buffer containing 20 mM Tris-HCl (pH 8.0), 1 M NaCl, and 1 mM PMSF. The trimeric complex was first purified using a HisTrap FF column (GE Healthcare), followed by cleavage of the polyhistidine-sumo tag with the sumo protease for 2 h at room temperature. The his-tagged sumo was removed by passing the protein sample through the HisTrap FF column one more time. The protein complex was further purified on a Superdex200 sizing column (GE Healthcare). The eluted fractions were analyzed by SDS-PAGE, and highly purified fractions were pooled and concentrated to ~8 mg/mL for crystallization. The HJURP-CENP-A-H4 complex was crystallized by hanging-drop vapor diffusion at 16°C, and the crystals used for data collection grew in a condition with 2 M ammonium sulfate.

X-ray data collection and structure determination
Diffraction data were collected at beamline BL17U of Shanghai Synchrotron Radiation Facility (SSRF) using a Quantum 315r detector (ADSC). A cryoprotectant with a mixture of crystallization well solution and 15% glycerol was used for data collection at liquid nitrogen temperature. A 2.6° resolution cryoprotectant was produced in

Molecular weight determination by MALS
The molecular masses in solution were determined by MALS using the DAWN HELEOSTM II 18-angle static light-scattering system [Wyatt Technology], connected to an Agilent HPLC, hooked up with a WTC SEC column (Wyatt Technology). The system was first equilibrated using a buffer containing 20 mM Tris-HCl (pH 8.0), 1 M NaCl, and 1 mM DTT for 12 h. The equilibrated system was then calibrated with BSA at a concentration of 3 mg/mL. The HJURP-CENP-A-H4 complex was prepared as described before, and the CENP-A-H4 tetramer was reconstituted and purified according to a published protocol (Tanaka et al. 2004). Protein samples at three different concentrations ([HJURP-CENP-A-H4]: 1 mg/mL, 2 mg/mL, and 4 mg/mL; CENP-A–H4: 1 mg/mL, 2 mg/mL, and 5 mg/mL) were subjected to MALS analyses at a flow rate of 0.5 mL/min at 16°C. The molecular mass was calculated using ASTRAS.3.4.14 software [Wyatt Technology].

GST pull-down experiments
CENP-A mutations were generated in the pCDFDUET (CENPA + H4) plasmid by PCR. Wild-type and mutant CENP-A complexes were purified on a HisTrap FF column (GE Healthcare). GST-tagged HJURP CBD was produced in E. coli using a pGEX-6P-1 vector. Histone H3–H4 complexes were reconstituted using bacterially expressed human histone H3, H3(Q68S), or H3(S68P), and H4 using an established protocol (Luger et al. 1999). In vitro binding assays were performed as described previously (Foltz et al. 2009). Briefly, a portion of GST or GST-HJURP CBD-bound glutathione-sepharose beads (~5.0 μg of protein each) were incubated with recombinant wild-type or mutant CENP-A–H4 complexes in the binding buffer (100 mM NaH2PO4 at pH 7.4, 300 mM NaCl, 0.025% NP-40, 10% glycerol, 1 mM DTT) for 30 min at room temperature. GST pull-down experiments of histone H3 complexes were carried out similarly, with the exception that 0.05% of NP-40 was used. Protein-bound glutathione resins were washed six times with the binding buffer, and the bound samples were analyzed by Coomassie blue-stained SDS-PAGE on a 6%–25% polyacrylamide gradient gel.

DNA binding
A biotin-labeled 208-bp α-satellite DNA fragment (0.2 μg) was incubated with 0.5 μg of reconstituted wild-type CENP-A–H4 complex in the
absence or presence of increasing amounts of GST-HJURP CBD or GST for 1 h at room temperature in the binding buffer containing 10 mM HEPS-KOH (pH 7.6), 0.1 mM EDTA, and 100 mM NaCl. DNA binding was analyzed by 5% native PAGE in 0.5× TBE, transferred, cross-linked onto a Hybond-N nylon membrane, and detected with a streptavidin-alkaline phosphatase conjugate and CDP-Star chemiluminescent reagent [Roche Applied Science] as described in Li et al. (2003).

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