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Deciphering how the chromatin factor RCC1 recognizes the nucleosome: the importance of individuals in the scientific discovery process

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Abstract

The nucleosome repeating unit of chromatin is the target of chromatin enzymes and factors which regulate gene activity in a eukaryotic cell. How the nucleosome is recognized by chromatin enzymes and factors is poorly understood even though such interaction is fundamental to gene regulation and chromatin biology. My laboratory recently determined the structural basis for how the RCC1 (regulator of chromosome condensation) chromatin factor binds to the nucleosome, including the first atomic crystal structure of a chromatin protein complexed with the nucleosome core particle. I describe here how we developed and investigated structural models for RCC1 binding to the nucleosome using biochemical methods, and how we crystallized the 300 kDa complex of RCC1 with the nucleosome core particle. This article highlights the contributions made by key laboratory members and explains our thinking and rationale during the discovery process.

Keywords

molecular recognition; chromatin; nucleosome; X-ray crystallography

If DNA is the blueprint of life, there must be mechanisms to read that blueprint. We now possess a reasonable understanding of how proteins can bind to and read the information contained in naked, double-stranded DNA. In eukaryotes, the packaging of DNA into chromatin by wrapping around histone and other chromosomal proteins offers additional mechanisms to regulate gene expression. Over the past 15 years, we have witnessed the discovery of hundreds of chromatin enzymes and factors that function on the fundamental repeating unit of chromatin, the nucleosome. However, despite the vast and still growing body of information regarding the genetic and biochemical characteristics of such chromatin enzymes and factors, we possess only a rudimentary understanding of how these enzymes and factors recognize and interact with the nucleosome. The crystal structure of the nucleosome core particle in 1997 [1] was a seminal event because it provided a structural framework for understanding chromatin processes and for interpreting genetic results. However, until recently, we lacked an atomic structure of a chromatin protein bound to the nucleosome despite the clear relevance to gene regulation and chromatin biology research. This article will describe how we characterized the RCC1-nucleosome complex through biochemical and structural methods, resulting in the first atomic structure of a chromatin protein bound to the nucleosome [2].

When I was first shown the crystal structure of the nucleosome core particle from coworkers in the Richmond laboratory in 1997, I was struck by how much of the surface of the molecule, both protein and DNA, was available for binding. It seemed obvious that nature would utilize such surfaces for molecular recognition of the nucleosome by chromatin enzymes and factors, but it was not known how such recognition occurred. Key questions include whether recognition would occur primarily through protein or DNA components, whether particular regions of the nucleosome would constitute hotspots for binding and whether different chromatin enzymes and factors would utilize recurring themes to bind to the nucleosome.

My laboratory started studying chromatin enzymes by investigating the yeast SAGA and NuA4 histone acetyltransferase complexes. Together with our collaborators, Patrick Grant and Jacques Cote, we identified minimal HAT subcomplexes that retained the ability to specifically act on nucleosomes [3–6]. Most of this work in my laboratory was carried out by a brilliant technician, Will Selleck. It was Will who convinced me in 2002 that we should try to crystallize the Piccolo NuA4 subcomplex complexed with the nucleosome. Will essentially single-handedly prepared recombinant nucleosome core particles in the quantity and quality needed for crystallization studies. Although we have not yet succeeded to crystallize the Piccolo NuA4-nucleosome complex, Will's work laid the foundation for us to tackle how the nucleosome is recognized by other chromatin enzymes and factors.

Biochemical studies of RCC1-nucleosome interactions

Our investigations of how RCC1 (regulator of chromosome condensation) interacts with the nucleosome were precipitated by a talk given by Rebecca Heald at the 2007 Pew Scholar Reunion meeting. Rebecca described how mitotic spindle formation and transport of macromolecules between the cytoplasm and the nucleus are controlled by the small GTPase protein, Ran. The key signal is a concentration gradient of Ran in its GTP bound state set up around the chromosomes [7,8]. RCC1 plays a central role in the formation of the RanGTP concentration gradient by binding directly to the nucleosome repeating unit of chromatin and by recruiting and activating Ran's nucleotide exchange activity. Given our interest in how the nucleosome is recognized by chromatin factors, RCC1 was an attractive target for us to pursue biochemical and structural studies. Todd Stukenberg, who was also present at the meeting, encouraged our initial efforts through helpful discussions and by providing us the clone for *Xenopus* RCC1.

Several previous studies influenced the direction of our initial investigations. The crystal structure of RCC1 by Alfred Wittinghofer and his colleagues showed that RCC1 was a seven-bladed β -propeller, and that RCC1 used one its two β -propeller faces to bind to Ran [9,10]. We also considered biochemical studies from Iain Machara, David Allis and their colleagues who had shown that RCC1 binds to nucleosome via the histone H2A/H2B dimer unit [11], as well as structural studies from the laboratories of Karolin Luger and Kenneth Kaye which defined an acidic patch on the histone dimer as the binding site for a viral peptide called LANA [12]. Given that both RCC1 and LANA interact with the nucleosome via surfaces contributed by the histone dimer, we asked if RCC1 and LANA shared binding sites on the nucleosome. Jiehuan Huang, a talented graduate student in my laboratory, developed a competition pulldown assay to address this question. His results demonstrated that RCC1 and LANA compete for binding to the nucleosome core particle, presumably because they share overlapping binding sites on the nucleosome [13].

With these results in hand, I set out to develop a structural model for how RCC1 could bind to the nucleosomes. Key ideas that went into the model included (a) the β -propeller structure of RCC1 [10], (b) the fact that β -propeller proteins often interact with other proteins through

loops between the β -strands that make up the repeating propeller structural element [14–16], (c) the expectation that RCC1 would use its non-Ran binding β -propeller face to bind to the nucleosome, (d) Jiehua's localization of RCC1's binding site near or around the acidic patch on the nucleosome, and (e) my observation that the non-Ran binding face of RCC1 contained a groove that I thought could accommodate the C-terminal α -helix of H2B prominently exposed on the nucleosome surface and very close to the acidic patch that LANA interacts with. The model that I developed (Fig. 1a) seemed consistent with the available data, and provided a reasonably large interaction surface between RCC1 and the nucleosome appropriate for their nanomolar binding affinity [17].

The task of testing my model was undertaken by Joe England, a bright undergraduate student in the laboratory. We designed point mutations in the surface of RCC1 that I thought interacted with the nucleosome, and Joe tested these mutations using a pulldown assay. The results were disappointing: none of the 7 mutations covering 9 residues affected binding of RCC1 to nucleosome core particles. I was concerned that Joe might be observing nonspecific interactions in his pulldown assay or that that we needed to test more residues in the putative binding surface of RCC1. For his part, Joe was a little tired of the consistent negative results he obtained testing my model, and decided to develop his own structural model for how RCC1 interacts with the nucleosome. His model placed a stronger emphasis on the competitive binding of RCC1 and LANA to the nucleosome. Joe noted that the LANA peptide employed a critical Arg residue to interact with the histone dimer acidic patch [12] and wondered if RCC1 might not do the same. He examined the crystal structure of RCC1 for exposed basic residues and aided by another undergraduate, Andrew Fleischman, modeled RCC1 binding to the nucleosome. I was frankly not enthusiastic about the model Joe presented to me about a week later (Fig. 1b). I did not like the basis for the model (assumption that RCC1 would bind to the histone dimer acidic patch essentially the same way as the LANA peptide) and I did not like the model's limited contact area between RCC1 and the nucleosome. The model seemed too arbitrary to me. Upon retiring to my office, I decided to examine the implications of Joe's model on Ran binding since Ran should be able to interact with nucleosome bound RCC1. To my surprise, Ran fit neatly in the space between RCC1 and the nucleosome in Joe's model. This suggested an elegant explanation for two observations that I could not account for with my model. Firstly, Macara and Allis had shown that the presence of nucleosomes modestly increased RCC1's ability to activate Ran's nucleotide exchange activity [11]. In my model, the nucleosome and Ran's nucleotide binding site reside on opposite sides of RCC1 and one would have to invoke unprecedented conformational changes within the RCC1 β -propeller to explain this result. In contrast, Joe's model juxtaposed the Ran's nucleotide binding site with RCC1 and the nucleosome providing a simple structural explanation for the how the presence of the nucleosome could affect Ran's binding of nucleotides. Secondly, Peter Becker and Iain Mattaj had observed direct interactions between Ran and the nucleosome [17]. This was simply not possible in my model, but it was easily explained in Joe's model. To his credit, Joe had also noted that his model could account for these unexplained observations although I did not realize this until later.

The Arg217 residue that Joe had surmised would be critical for RCC1 to bind to the nucleosome resides in a loop region on the edge of the β -propeller structure. We decided to call this region the switchback loop because its in-and-out structure reminded me of a switchback hiking trail. Joe engineered mutations at different position in the RCC1 switchback loop and to our delight (or perhaps relief), he found that the side chain of Arg217 was required for RCC1 to bind to the nucleosome. Additional experiments demonstrated that the RCC1 switchback loop is necessary but not sufficient for nucleosome binding and that a second Arg residue in the switchback loop is also required for RCC1 to bind nucleosomes [13].

Crystallization of the RCC1-nucleosome complex

While Joe was performing his RCC1-nucleosome binding studies, I was trying to crystallize the RCC1-nucleosome complex. Although I had managed to express and purify *Xenopus* RCC1, that protein aggregated at high concentrations under the conditions that I tested. I decided to switch to using human RCC1 since the human protein had been crystallized [10] and was therefore likely to be unaggregated at the high concentrations used for protein crystallization. The first key experiment after producing human RCC1 was to examine whether it formed a well-behaved complex with the nucleosome. This is where all the effort we had expended into trying to crystallize the Piccolo NuA4-nucleosome complex started to pay off. I used procedures developed by Will Selleck and me to reconstitute human RCC1 with recombinant nucleosome core particles, and to assay for formation of the complex using size exclusion chromatography. The results showed a new peak for the RCC1-nucleosome complex well resolved from the nucleosome core particle on its own. This was surprising given the molecular weight of the nucleosome core particle of 204 kDa and the expected molecular weight of the RCC1-nucleosome complex of 290 kDa (2 copies of 43 kDa RCC1 + 1 nucleosome core particle at 204 kDa). Baseline separation of molecules in size exclusion chromatography is usually only possible if the molecules differ by at least a factor of two in size. This result suggested to us that the RCC1-nucleosome complex was not globular, i.e. that it was significantly elongated.

The first crystallization trials of the human RCC1-nucleosome complex I set up in April 2007 produced clustered needle crystals under multiple pH and salt conditions. Optimization of crystallization conditions based on these initial crystals resulted in larger but still clustered needle crystals. One disturbing feature of the crystals was the imperfect growth which produced hollow needles or tubes (Fig 2a). Nevertheless, the crystals were large enough to be washed and their contents analyzed by SDS-PAGE gel. The result that the washed crystals contained both RCC1 and histone protein components was our first confirmation that we had crystallized a complex of RCC1 and the nucleosome. However, when analyzed in an X-ray beam, these crystals displayed only smeary diffraction to about 20 Å, clearly unsuitable for diffraction studies.

To improve the crystals, I first tried varying crystallization conditions but to little avail. Influenced by the crystal packing of nucleosomes in nucleosome core particle crystals, I next modified different aspects of the recombinant nucleosome core particles used to form RCC1-nucleosome complexes in my crystallization trials. Two technicians in the laboratory, Kristen Wiley and Dylan Schlaich, were instrumental in the production of recombinant nucleosome core particles with different DNA sequences or lengths, and truncations in individual histone tails. Interestingly, crystals with similar morphology to the original RCC1-nucleosome crystals were obtained despite making these changes. I was particularly surprised that I could increase the length of nucleosomal DNA by 5 or 10 bp without apparent effect on RCC1-nucleosome crystallization since the precise DNA length is critical for nucleosomes to crystallize on their own. Having essentially exhausted my options for altering the nucleosome, I turned next to the other component of the complex, RCC1. Human RCC1 is composed of a well defined β -propeller structural unit that I did not wish to disrupt and an N-terminal extension. To this point, I had employed human RCC1 lacking its first 20 amino acids because this region was believed to be dispensable for binding to the nucleosome, but reintroducing these 20 N-terminal residues did not affect RCC1-nucleosome crystallization. In summary, I obtained similar clustered needle crystal whether changes were made in the nucleosome core particle or in the human RCC1 construct used.

The next on my list of parameters to manipulate was the RCC1 species. The use of orthologs has a rich history in macromolecular crystallization: the crystallography literature is replete

with examples where appropriate crystals were obtained only when other orthologs were tried [18,19], usually because the surface features of the ortholog permitted appropriate crystal contacts. Switching from human RCC1 to the yeast ortholog, Srm1 proved helpful. Like the RCC1-nucleosome crystals containing human RCC1, RCC1-nucleosome crystal containing yeast RCC1 grew under a wide variety of crystallization conditions. Clustered needles were still present in several crystallization drops, but block crystals grew under some conditions. It was a happy coincidence I obtained these results just before a terrific postdoctoral fellow, Ravindra Makde, joined the lab in February 2008. I turned the RCC1-nucleosome crystallization project to Ravi, and in just one month, he grew beautiful looking single crystals of the complex.

X-ray diffraction studies of these yeast RCC1-nucleosome crystals (Fig. 2b) showed diffraction to about 4.5 Angstroms in one direction, but only to about 7 Angstroms in the other directions, i.e. the crystals were anisotropic (Fig. 3a). Ravi was able to improve diffraction to about 3.5 Angstroms in the best direction by optimizing the post-crystallization soaking conditions, but we were still limited to about 6.5 Angstroms in the other directions. We explored different approaches to further improve the diffraction limits including creating new yeast RCC1 constructs or using RCC1 from other species. When Ravi first examined crystals of the *Drosophila* RCC1-nucleosome complex, he observed isotropic (similar in each direction) diffraction data to about 5 Angstroms. Ravi decided to focus his efforts on the *Drosophila*-nucleosome crystals (Fig. 2c) and he also decided to pursue crystal dehydration to improve the diffraction limits. I was influenced by the large amount of solvent space in the crystal packing we determined for the yeast and *Drosophila* RCC1-nucleosome complexes, and I wanted to see if we could encourage additional crystal contacts using protein domains fused to the RCC1 construct. Ravi's approach proved to be more effective and in April 2009, he produced diffraction pictures taken using our home X-ray generator which showed isotropic diffraction spots to 3.5 Angstroms (Fig. 3b). We were thrilled with this result because it suggested we might be able to produce an atomic structure for the complex. Ravi needed to soak the crystal in a dehydrating solution to produce this diffraction result, a potentially variable process, and so he was careful to show that he could reproduce this result at least once. We had a celebration in the laboratory to commemorate this major breakthrough, and then to our horror, Ravi was not able to reproduce the result again.

For several weeks afterwards, Ravi was only able to observe diffraction to about 5 or 6 Angstroms. During this trying time, we were comforted by the fact that he had been able to reproduce his good result at least once, suggesting that it was not simply a fluke. We reasoned that Ravi's crystal dehydration procedure was critical for improving the diffraction characteristics and that the key parameters of the protocol had not been reproduced in the later experiments. I was particularly concerned whether the crystals had sufficient time to come to equilibrium in the final dehydration soaking solution in that protocol. For about three months between April and July of 2009, Ravi worked tirelessly, through the day and often through the night, to develop a robust crystal dehydration protocol for his crystals. Then in July of 2009, Tom Koerber, Hemant Yennawar, Ravi and I drove to the Advanced Photon Source, an excellent synchrotron source outside of Chicago, Illinois with more than 50 crystals of the RCC1-nucleosome complex pretreated by dehydration and flash cooled in liquid nitrogen. Over the next two days, we screened through these crystals and collected 19 diffraction data sets. We had hoped to improve the diffraction limits to 3.2 Angstroms or better, but we were not prepared for the beautiful diffraction spots produced by a pair of crystals. These crystals diffracted X-rays to about 2.8 Angstroms with low mosaicity (a measure of the order within a crystal, the lower the better) (Fig. 3c). The quality of the diffraction data was a testament to the quality of crystals Ravi had grown, to his crystal

dehydration protocol and the quality of 24-ID-E X-ray beam provided by the Northeastern Collaborative Access Team (NE-CAT).

Ravi solved the structure of the RCC1-nucleosome complex by molecular replacement using the Richmond laboratory's high resolution structure of the nucleosome core particle [20,21], and the crystal structure of *Drosophila* RCC1 which was solved by a graduate student in the laboratory, Manoj Saxena with Ravi's guidance. The structure (Fig. 1c) showed that RCC1 uses the switchback loop to contact the histone dimer acidic patch essentially as Joe England had predicted, although Joe did not get the correct orientation of RCC1 with respect to the nucleosome. Joe also did not anticipate the beautiful network of hydrogen bonds linking the switchback loop to the histone dimer surface in the crystal structure [2]. Still, Joe's assumption that RCC1 would mimic the LANA peptide's use of an arginine residue to bind to the histone surface proved to be exactly right. One does wonder if the histone dimer acidic patch, one of the few acidic protein patches on the histone octamer surface, will prove to be a hotspot for other proteins to bind to the nucleosome. Ravi's structure also showed that the RCC1 switchback protein-protein contacts with histones are stabilized by protein-DNA contacts involving an adjacent RCC1 loop and RCC1's N-terminal arm. In summary, RCC1 recognizes the architecture of the nucleosome by interacting with both histone and DNA components.

We learned much about how the nucleosome can be recognized by a chromatin protein by crystallizing the RCC1-nucleosome complex and solving the crystal structure. The final result was very rewarding, but the process by which we reached this goal was equally rewarding and informative. Working with talented individuals like Joe and Ravi was the icing on the cake. We have only taken a small step in understanding how chromatin proteins and enzymes bind to the nucleosome. Nevertheless we are hopeful that our experience will help us and others to tackle crystal structure determination of other chromatin enzyme-nucleosome complexes and in so doing, elucidate structural mechanisms for key genetic processes.

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Fig. 1.

Theoretical models and crystal structure of RCC1-nucleosome core particle complex. The histone H3, H4, H2A, H2B and DNA components of the nucleosome core particle (NCP) are shown in cornflower blue, light green, wheat, pink and light blue respectively, while RCC1 is presented in yellow-orange (central strand region), blue and green (loop regions) and red (switchback loop). (a) Song Tan's model incorrectly uses RCC1's face opposite to its Ran-binding face to bind to the exposed histone surface. (b) Joe England's model employs RCC1's switchback loop and in particular an arginine residue (shown) in that loop to mimic a critical LANA peptide Arg in its binding to the histone dimer. (c) Ravi Makde's crystal structure of the RCC1-nucleosome core particle complex with the corresponding Arg residue shown. Although Joe's model did not anticipate the RCC1-DNA contacts and the orientation of RCC1 with respect to the nucleosome is quite different from the crystal structure, Joe did correctly predict the binding of RCC1's switchback loop and its arginine residue to the histone dimer.

Fig. 2. RCC1-nucleosome complex crystals. (a)–(c) Crystals of the human, yeast or *Drosophila* RCC1 in complex with the nucleosome core particle respectively.

Fig. 3.

Diffraction studies of RCC1-nucleosome complex crystals. (a) Diffraction image of yeast RCC1-nucleosome complex crystal showing anisotropic diffraction, (b) Diffraction image of *Drosophila* RCC1-nucleosome complex crystal showing isotropic diffraction. This image prompted a short-lived celebration in the laboratory, (c) Diffraction image of *Drosophila* RCC1-nucleosome complex crystal taken at Advanced Photon Source (APS) NE-CAT beamline ID-24E showing diffraction to 2.8 Angstroms.