Mutations to the histone H3 αN region selectively alter the outcome of ATP-dependent nucleosome-remodelling reactions

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ABSTRACT

Mutational analysis of the histone H3 N-terminal region has shown it to play an important role both in chromatin function in vivo and nucleosome dynamics in vitro. Here we use a library of mutations in the H3 N-terminal region to investigate the contribution of this region to the action of the ATP-dependent remodelling enzymes Chd1, RSC and SWI/SNF. All of the enzymes were affected differently by the mutations with Chd1 being affected the least and RSC being most sensitive. In addition to affecting the rate of remodelling by RSC, some mutations prevented RSC from moving nucleosomes to locations in which DNA was unravelled. These observations illustrate that the mechanisms by which different ATP-dependent remodelling enzymes act are sensitive to different features of nucleosome structure. They also show how alterations to histones can affect the products generated as a result of ATP-dependent remodelling reactions.

INTRODUCTION

Chromatin presents an obvious impediment to nuclear processes that require access to DNA. To counter this obstruction the eukaryotic cell has evolved mechanisms that alter chromatin structure and/or dynamics. An assortment of enzymes have been found to participate in gene regulation that act to post-translationally modify histone proteins (1). Several of these modifications, including phosphorylation, acetylation and methylation have been shown at least in some cases to create epitopes that are specifically recognized by chromatin-binding proteins (2). However, it is also possible that a subset of chromatin modifications act to directly alter the biophysical properties of chromatin (3–5). A second class of enzyme acting to manipulate chromatin structure during gene regulation non-covalently reconfigures chromatin in energy-consuming reactions (6). These ATP-dependent remodelling enzymes share homology with the superfamily 2 group of helicase related proteins (7) and have been referred to as Snf2 family proteins as they share additional homology within the catalytic subunit of the SWI/SNF complex (8,9). There is likely to be interplay between these two classes of enzyme as many ATP-dependent remodelling enzymes are associated with protein motifs capable of recognizing histone modifications.

To investigate this interplay we previously investigated the effects of histone truncation on ATP-dependent remodelling (10). This pointed to an important role for the histone H3 tail in nucleosome dynamics. A peptide ligation approach was used to show that H3 K14 acetylation acted to direct recruitment of RSC complexes to chromatin (11). Consistent with this H3 K14 acetylation is important for RSC function (12). In addition to this specific effect on recruitment of RSC, point mutations in histone H3 were found to have a range of distinct effects on the dynamic properties of nucleosomes (10). In particular, it was found that mutation of the histone H3 αN helix could alter different aspects of the dynamic properties of nucleosomes such as inherent mobility, site exposure and histone dimer stability (10).

Here we take advantage of this designer library of mutant nucleosomes to investigate how the dynamic properties of nucleosomes influence the action of ATP-dependent remodelling enzymes. We find that modifications affecting different aspects of the nucleosome have specific effects on different remodelling enzymes, and that these can act to alter either the rate of remodelling or the products generated.

MATERIALS AND METHODS

Chromatin assembly

Xenopus laevis histones were bacterially expressed, purified and assembled into tetramers and octamers, as appropriate, as described (13). Site-directed mutagenesis was performed using the Stratagene QuikChange Kit.

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Nucleosomes were assembled by salt dialysis using an equimolar ratio of octamer and purified PCR-amplified DNA fragments (14). The nomenclature used to define DNA fragments is aBe, with a and c are numbers that describe the upstream and downstream bp extensions, respectively. B is the nucleosome positioning sequence source, with A representing the MMTV nucleosome A (15). Fluorescently labelled oligos were from Eurogentec (Belgium) and unlabelled oligos from the Oligonucleotide Synthesis Laboratory (University of Dundee, UK). The oligo sequences to amplify the 54A18 fragment are 5'-TGTAATAGCTTATGTGACCA and 5'-TACATCTAATGCTTATGTGACCA; for the 54A0 fragment 5'-TACATCTAATGCTTATGTGACCA and 5'-ATACAAAACCTGTCGCCAG. The PCR was purified by ion exchange chromatography using a 1.8-ml SOURCE 15Q (GE Healthcare) column.

Purification of chromatin-remodelling enzymes

Yeast strains TAP tagged for RSC (16) and SWI/SNF (17) were purified as described previously (11).

A pGEX-6p plasmid containing the Saccharomyces cerevisiae CHD1 gene made by Daniel Ryan was transformed into Rosette 2 cells (Novagen). 61 of cells were grown in 2YT media (1.6% bactotryptone, 1.0% yeast extract, 0.5% NaCl, pH 7.5) to an A600 of ~0.6, induced with 0.4 mM IPTG and expressed at 25°C overnight. All subsequent steps were performed at 4°C. The cells were pelleted in a Beckman JS 4.2 rotor at 4000 r.p.m. for 25 min, washed with 1× PBS and re-suspended in 120 ml of lysis buffer [20 mM Tris pH 7.5, 350 mM NaCl, 0.1% (v/v) Tween, 0.05% (v/v) 2-mercaptoethanol, 2 mM E-64, 1 mM AEBSF, 1 mM pepstatin, 2.6 mM apro tinin], before freezing at –80°C. The thawed cells were homogenized with a 30-cm² dounce, followed by sonication with a Branson S450 digital sonicator at 30% intensity using a 10-min cycle of 20 s ON, 30 s OFF and centrifugation in a Beckman JA-25.50 rotor at 31 000 g for 15 min. The supernatant was added to 300 µl TALON metal affinity resin (Clontech Laboratories Incorporated), followed by rotation for 2 h, before centrifugation in a Heraeus Megafuge 1.0 centrifuge (Thermo Fischer Scientific, USA) and pre-running at 300 V for 3 h with continuous pump recirculation of 0.2 × TBE buffer between the upper and lower compartments at 4°C. Gels were run at 300 V for 3.5 h and imaged using a Phosphoimager FLA-5100 (Fujifilm, Japan). Gel band intensities were quantitated using AIDA software (Raytest, Germany). The proportion of nucleosomes repositioned was calculated as the intensity of the sum of all detectable products over the sum of the major initial position plus products. In many assembly reactions a proportion of nucleosomes are assembled at a minor assembly location marked as the P position (e.g. *in Figure 2A*) (18).

Including this band in the calculations was found not to significantly affect the determination of initial rates. In some histone mutants the proportion of nucleosomes assembled at the P position was altered, and some additional less abundant sites of deposition were also observed. However, again inclusion nucleosomes assembled at these locations was not found to significantly affect the comparison of initial rates. As a result, in the analysis presented here nucleosomes deposited at locations other than the major NuclA locations are not in included in quantitation. For some mutations a small proportion of nucleosomes were observed to undergo thermal repositioning in control reactions lacking remodelling enzyme. However, this was generally detected after long incubations and the proportion of nucleosomes repositioned at early time points was low enough not to affect the calculation of initial rates. The reproducibility of the assay when carried out in this manner was found to be high (Supplementary Figure 1).

In this way the proportion of nucleosomes repositioned is calculated independently of the total signal detectable in each lane. The initial rate was calculated as previously described (11). Each initial rate was repeated at least
three times using chromatin prepared in separate assembly reactions.

High-resolution nucleosome mapping

Nucleosomes for high-resolution site-directed mapping (H3 C110A, H4 S47C) were prepared as previously described (18). Each 10 μl reaction contained 4 pmol mapping nucleosome, 50 mM Tris pH 7.5, 50 mM KCl, 1 mM MgCl₂ and the RSC quantities specified in Figure 5. The samples were incubated at 30°C for 2 h, before termination by the addition of 500 ng of HindIII-digested bacteriophage lambda competitor DNA (Promega) and incubation on ice. One microlitre of 33 nM and 333-nM ammonium ferrous sulphate was added to the no RSC control and RSC repositioned samples, respectively and incubated on ice for 10 min. One microlitre of the reaction was removed and resolved on a 5% native polyacrylamide gel. To the remaining sample, 5 μl of 19.2-mM ascorbic acid and 0.05% (v/v) hydrogen peroxide was added to each sample, followed by incubation on ice for 90 min. The reactions were terminated by phenol:chloroform:isoamyl alcohol (25:24:1) extraction and ethanol precipitation. Each sample was re-suspended in 5 μl of mapping loading buffer (5 mM EDTA, 0.1% bromophenol blue in formaldehyde), heated at 90°C for 3 min and loaded onto a sequencing gel [8% acrylamide:bis acrylamide (49:1 ratio), 8 M urea, 1 x TBE, 0.1% APS and 0.1% TEMED]. The gels were pre-run using 1 L of boiling 1 x TBE buffer until the temperature probe reached 50°C. The gel was loaded immediately and run at 50°C temperature, for 1.5–2 h. Fluorescent DNA markers for the 54A18 DNA fragment were created using a CycleReader Auto DNA Sequencing kit (Fermentas, Canada). The dideoxyribonucleotide sequencing products migrate 1 bp slower relative to the mapping cleavage products, as determined by examining the previously characterised +70 cleavage site for nucleosomes assembled on the 54A18 DNA fragments (19).

RESULTS

Histone H3 mutations have specific effects on nucleosome repositioning by Chd1 and RSC

A competitive repositioning assay was used to compare the initial rates at which mutated and wild-type nucleosomes are moved by RSC and Chd1 (10,11). This involves mixing equimolar amounts of wild-type and mutated nucleosomes assembled on cy3 and cy5 labelled DNA fragments, ATP and the appropriate amount of remodelling enzyme at 30°C, before reactions were stopped by the addition of excess competitor DNA. The reaction products were analysed on native polyacrylamide gels and the extent to which the wild-type and mutant nucleosomes are repositioned established through the use of selective filters. As Chd1 repositionings mononucleosomes most efficiently from positions close to DNA ends to more centrally located positions (18), we used a DNA fragment that positions nucleosomes such that 54 bp is located on one side and none on the other (54A0). Native gel electrophoresis can be used to track the loss of nucleosomes form their initial location and accumulation at destinations (Figure 1A). Quantitation enables the proportion of nucleosomes moved to be tracked over a time course (Figure 1B). Approximately three-quarters of the histone mutations tested caused relatively minor changes of 25% or less to the initial rate (Figure 1C). The H3 H39A mutation caused the greatest effect on Chd1 repositioning with a 1.9-fold ± 0.1 (mean ± standard error of the mean) increase to the initial rate relative to wild-type controls (Figure 1A and B). These results suggest that Chd1 is not greatly affected by mutations in this region of H3.

To determine whether the above effects were a property shared among all chromatin-remodelling enzymes we tested the ability of the RSC complex to reposition the H3 mutated nucleosomes. RSC and related SWI/SNF
Histone H3 mutations differentially affect RSC repositioning. 

Collectively, these results show that single alanine mutations within the histone H3 N-terminal region cause different effects on the repositioning capabilities of the Chd1- and RSC-remodelling enzymes.

Table 1. Effects of histone mutations on repositioning by RSC and Chd1

| Mutation | Initial rate of RSC repositioning$^a$ | Initial rate of Chd1 repositioning$^a$ |
|----------|--------------------------------------|--------------------------------------|
| H3 P38A  | 1.3 ± 0.1                            | 1.0 ± 0.05                           |
| H3 H9A   | 1.4 ± 0.05                           | 1.9 ± 0.1                            |
| H3 R40A  | 1.3 ± 0.1                            | 1.1 ± 0.02                           |
| H3 Y41A  | 1.5 ± 0.2                            | 0.8 ± 0.05                           |
| H3 R42A  | 2.2 ± 0.3                            | 1.4 ± 0.1                            |
| H3 P43A  | 1.1 ± 0.05                           | 0.6 ± 0.05                           |
| H3 G44A  | 1.7 ± 0.1                            | 0.9 ± 0.01                           |
| H3 T45A  | 1.6 ± 0.1                            | 1.2 ± 0.1                            |
| H3 V46A  | 0.9 ± 0.05                           | 0.9 ± 0.05                           |
| H3 L48A  | 1.1 ± 0.05                           | 0.8 ± 0.05                           |
| H3 R49A  | 2.1 ± 0.1                            | 1.2 ± 0.05                           |
| H3 E50A  | 1.2 ± 0.1                            | 0.9 ± 0.05                           |
| H3 I51A  | 0.6 ± 0.1                            | 0.8 ± 0.05                           |
| H3 R52A  | 1.2 ± 0.1                            | 0.9 ± 0.1                            |
| H3 R53A  | 1.1 ± 0.1                            | 0.8 ± 0.1                            |
| H3 Y54A  | 1.3 ± 0.2                            | 0.9 ± 0.05                           |
| H3 Q55A  | 0.7 ± 0.1                            | 1.1 ± 0.05                           |
| H3 K56A  | 1.2 ± 0.1                            | 0.9 ± 0.05                           |
| H3 S57A  | 1.4 ± 0.2                            | 0.8 ± 0.05                           |
| H3 K56Q  | 1.3 ± 0.1                            | ND                                   |

$^a$Each value represents the mean ± SEM. ND, not determined.

1.7- and 1.4-fold decreases to the initial rate of RSC repositioning, but had far smaller effects on Chd1 action.

In principle, these differences to the products observed could reflect either a slower rate of remodelling and the accumulation of intermediates or a genuine difference in the products generated. To investigate this further, reactions were performed with higher concentrations of RSC over extended time courses. We observed that wild-type nucleosomes were initially converted to band 2, but at completion equivalent amounts of band 2 and band 3 accumulated (Figure 4).

In contrast, the H3 I51A and Q55A mutations caused a similar distribution...
to wild-type. Class 2 mutations, such as H3 K56A, displayed increased accumulation of band 1. Class 3 mutations, such as H3 R40A, were under-represented for band 2 and class 4 mutations, such as H3 I51A, showed the most marked differences to wild-type controls with the majority of nucleosomes only converted to band 1 (Figure 4).

**H3 I51A prevents RSC from unravelling nucleosomes**

In order to characterize the products generated following remodelling of the different histone mutations, we employed site-directed hydroxyl radical mapping (22). Briefly, this involves the incorporation of cysteaminyl EDTA at H4 S47C, a residue that is close to the DNA at the nucleosome dyad axis. Ferrous ions, ascorbic acid and hydrogen peroxide are added to produce a localized Fenton reaction at the EDTA derivative. The resulting hydroxyl radicals cleave strongly at a single proximal DNA site and more weakly at 7 and 8 bp away on each DNA strand. This characteristic cleavage pattern can be used to determine the position of the nucleosomal dyad axis at base-pair resolution (22).

Site-directed mapping showed increasing amounts of RSC progressively remove control nucleosomes (H4 S47C) away from the +70 position. Nucleosomes appear at new locations up to 111 bp along the longer arm (Figure 5, lanes 3, 7–9). This involves moving the nucleosome to locations in which DNA is removed from one side of the histone octamer as has been described previously (19–21). These results suggest that for wild-type nucleosomes the faster migrating products on native PAGE gels correspond to nucleosomes being repositioned by RSC to a series of locations on what was originally the longer linker DNA arm (Figure 5).

In contrast, repositioning of H3 I51A nucleosomes (a Class 4 mutation), resulted in accumulation at only a subset of the locations observed for wild-type nucleosomes. The major positions observed are at +27, +26 and +22, which correspond to the edge of the nucleosome being positioned at 11, 10 and 6 bp from the end of the DNA fragment (Figure 5 lanes 5, 11–13). Although wild-type nucleosomes were moved to a series of positions beyond this in which from 2 to 57 bp of DNA are removed from the edge of the nucleosome, these locations are all

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**Figure 3.** Histone H3 mutations alter banding patterns following remodelling with RSC. When nucleosomes that have been remodelled by RSC (0.44 fmol) are separated on a native polyacrylamide gel three faster migrating species can be detected. These are labelled 1, 2 and 3. Nucleosomes H3 G44A and T45A were predominantly relocated to band 1 after a 64-min incubation at 30°C, in contrast wild-type nucleosomes were relocated equally to bands 1 and 2. Repositioning of mutations H3 K56A and Y41A produced a similar banding pattern relative to wild-type. Note band 3 represents a doublet in some gels. WT, wild-type.
under-represented for I51A nucleosomes. The single predominant band on native PAGE gels observed following remodelling of H3 I51A nucleosomes corresponds to the co-migration of nucleosomes at a subset of locations in which DNA remains fully wrapped.

Comparison of remodelling by RSC and SWI/SNF

The SWI/SNF complex has a similar subunit composition to RSC. The catalytic subunits Snf2 and Sth1 share several sequence motifs as do other members of the complexes (23). Biochemical characterization of the activities of these complexes has also suggested they function similarly (19,24,25). In order to investigate whether interactions with histone H3 affect the action of these enzymes similarly, the products of remodelling reactions driven by SWI/SNF were resolved by native PAGE and assigned to one of four classes dependent on the distribution pattern of the repositioned products (bands 1, 2 and 3). Wild-type nucleosomes represent class 1, H3 K56A nucleosomes class 2, H3 R40A nucleosomes class 3 and H3 I51A nucleosomes class 4. The band intensities of the RSC remodelled nucleosomes are plotted in the graph to the right of each respective native polyacrylamide gel. Band 0, main initial nucleosome position. Asterisk denotes P position.

DISCUSSION

The ability to manipulate the coding sequence of histone genes together with the expression and assembly of these proteins in vitro provides an opportunity to monitor the direct effects of these mutations on chromatin dynamics. We have identified differences in the sensitivities of chromatin-remodelling enzymes to mutations affecting different aspects of nucleosome structure. This indicates there are differences in the underlying mechanisms by which even closely related enzymes act.

We observed that remodelling by the enzyme Chd1, was affected very little in terms of either the products generated or the rates at which nucleosomes were repositioned (Figure 7B; Table 1). Although the initial rate of
remodelling by RSC was affected to a greater extent than Chd1 the magnitude of the effects remained low, with the maximum effect being 2.2-fold (Figure 7E). This indicates that both enzymes were able to engage and reposition nucleosomes efficiently. Consistent with this the binding of RSC to nucleosomes bearing the H3 E50A, I51A and Q55A mutations was equivalent to wild-type nucleosomes (Supplementary Figure 2). The limited effects of these mutations on the rate of nucleosome sliding by RSC and Chd1 contrasts with the more substantial effects these mutations were observed to have on spontaneous thermal nucleosome repositioning (compare Figure 7B, E and F). This lack of correlation is likely to reflect significant differences in the mechanisms for thermal and ATP-dependent nucleosome repositioning.

In contrast to the limited effects on the initial rates of remodelling, the outcome of RSC remodelling reactions was affected dramatically with many mutations altering the spectrum of products generated (Figure 7C). A related but less dramatic effect was observed with the SWI/SNF complex (Figure 7D). While RSC repositions wild-type nucleosomes to a series of locations in which the nucleosome dyad is so close to the edge of the DNA fragment that up to 57 bp of DNA are removed from one edge of the nucleosome, these locations were under-represented when H3 I51A mutant nucleosomes were used as a

Figure 5. RSC redistributes H3 I51A nucleosomes to a subset of locations. The base-pair locations of RSC repositioned nucleosomes on the 54A18 fragment were determined by site-directed hydroxyl radical mapping. At top, sequencing gels. M, guanine marker of the 54A18 fragment, lane 1. No RSC controls, lanes 2, 4, 6 and 10. Repositioning of nucleosomes at 30°C for 128 min using 10 fmol RSC, lanes 7, 11; 83 fmol RSC, lanes 8, 12; and 660 fmol RSC, lanes 3, 5, 9 and 13. The cleavage products and an illustration of nucleosome locations (black ovals initial locations, clear ovals RSC redistributed locations) on the 54A18 DNA fragment are shown alongside the sequencing gels. At bottom, the corresponding native polyacrylamide gel. * (caret sign), non-specific bands. WT, wild-type mapping nucleosomes.
substrate (Figure 5). This means that the mechanics of moving nucleosomes to locations in which DNA is unravelled differ from those involved in repositioning within the confines of a DNA fragment.

It is notable that there is a good correlation between residues that have strong effects on thermally driven nucleosome positioning and the inability of RSC to move nucleosomes into positions where DNA is unravelled (compare Figure 7C and F). At first this appears to be counterintuitive as nucleosomes that are inherently more mobile cannot be repositioned so effectively by RSC. However, it is also true that the H3 E50A, I51A and Q55A mutations reduce the stability of histone octamers (10; data not shown). One model for RSC action involves the RSC complex drawing DNA over the surface of nucleosomes while remaining bound to the histone octamer in a fixed orientation (26). The reduction to octamer stability resulting from the H3 E50A, I51A and Q55A mutations might result in the octamer rather than the DNA being distorted as a result of ATP hydrolysis. Furthermore, as RSC starts to move nucleosomes to locations in which DNA is unravelled from their surface, the overall loss of histone contacts will require the ATPase motor within the RSC complex to exert greater forces. Indeed, it has been estimated that a force of 3 pN will be required to dissociate each helical contact (27). The increased forces that are applied during DNA unravelling might drive a reconfiguration of the histone octamer that prevents any further repositioning. This could explain why RSC is able to move nucleosomes to positions where histone DNA contacts are retained, but not to locations where DNA is unravelled. In this case the reason why Chd1 is not affected in the same way could be simply that the repositioning of nucleosomes to more central locations does not involve unravelling DNA to the same extent.

Having observed that mutations to the H3 zN region of the nucleosome can affect the outcome of remodelling reactions, it will be of interest to investigate whether histone modifications exert similar effects. Our own preliminary observations made using mutations designed to mimic a selection of modifications known to occur in this region of the nucleosome revealed only subtle effects on the action of ATP-dependent remodelling enzymes (data not shown). It is possible that the use of amino acid substitutions is not a sufficiently accurate mimic for post-translational modifications and hopefully the development of new technologies will allow the effect of histone modifications within the globular core of nucleosomes to be tested directly (28). Nonetheless, our observations illustrate a principle by which alterations to the structure of nucleosomes can selectively affect the outcome of remodelling reactions. It is of special note that the unravelling of DNA is affected as this might be anticipated to affect the repertoire of different means by which post-translational modification of histones can potentially exert effects on chromatin dynamics (3).

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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