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AFM imaging of protein movements: Histone H2A–H2B release during nucleosome remodeling

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Abstract **Being able to follow assembly/disassembly reactions of biomolecular complexes directly at the single molecule level would be very useful. Here, we use an AFM technique that can simultaneously obtain topographic images and identify the locations of a specific type of protein within those images to monitor the histone H2A component of nucleosomes acted on by human Swi–Snf, an ATP-dependent nucleosome remodeling complex. Activation of remodeling results in significant H2A release from nucleosomes, based on recognition imaging and nucleosome height changes, and changes in the recognition patterns of H2A associated directly with hSwi–Snf complexes.**

Keywords: Chromatin; Nucleosome; Remodeling; Swi–Snf; Histones; SPM; AFM

1. Introduction

The ability to be able to follow directly, at the single molecule level, the fates of individual proteins during the dynamic assembly/disassembly processes that biological complexes often undergo would be very useful. Electron microscopy requires fixed or frozen substrates, necessitating comparisons of two different sample preparations in order to monitor changes. The atomic force microscope (AFM) can provide before and after comparisons of the same single molecules, imaged in solution cf. [1,2], but until recently could not identify specific types of proteins in compositionally complex samples. Stroh et al. [3] presented a solution to the specificity problem in AFM, by scanning with a tip tethered to an antibody against a protein of interest. This approach generates a traditional topographic image simultaneously and in exact spatial registration with a recognition image that locates the sites of antigen-antibody binding events, and thus the locations of specific types of individual proteins, within the topographic image. Recognition can be both efficient and specific.

Here, we use this technique to monitor histone H2A changes on mouse mammary tumor virus (MMTV) promoter nucleosomal arrays after activation of the human Swi–Snf (hSwi–Snf) ATP-dependent nucleosome remodeling complex. hSwi–Snf plays a crucial role in relieving nucleosome repression during MMTV transcription activation in vivo [4–6], a common role for these complexes in many genomic processes [7–10]. Nucleosome remodeling is known to involve many types of DNA alterations (cf. [1,2]) but it was believed that the histone octamer remained intact during remodeling. However, recent biochemical evidence [11,12] suggests that under certain (but not all [13]) conditions, H2A–H2B dimers are removed from the nucleosome during remodeling. Nucleosome height decreases during remodeling also suggested H2A loss [2]. Here, we use recognition imaging with AFM tips containing an anti-H2A antibody to test directly for H2A loss during nucleosome remodeling. This technique should be applicable to the study of other processes in which proteins are assembled, disassembled or change locations vis-a-vis a complex(es).

2. Methods

2.1. In situ AFM imaging

Reconstituted MMTV promoter nucleosomal arrays were incubated with hSwi–Snf, deposited onto glutaraldehyde-treated mica and imaged as previously described [1,2]. Subsaturated arrays must be used in these studies, see Ref. [14]. All imaging was done in solution using magnetic mode excitation of the cantilever (1,2).

2.2. Recognition imaging

Antibodies to histones H2A, H2B and BRG1 were purchased from Upstate (NY) and Abcam (UK), tethered to AFM tips and samples scanned as described [2,3]. Images were median filtered with 2 nm radius and analyzed with custom software that identified the recognition spots as local dips in intensity. The threshold for recognition signal identification was set by comparing the intensity distribution in areas free of recognition signals (but adjacent to the spot being analyzed) with that in areas that contained recognition spots. Recognition events were clearly evident as a new low intensity feature in the distribution, and their location was marked on the corresponding topographic image.

2.3. ELISA assays

The component to be tested was incubated with the antibody, rinsed and sandwich- assayed with anti-rabbit alkaline phosphatase following standard protocols [15]. Absorbance at 406 nm was measured as a function of time after addition of PNPP. Numbers in Table 1 are the

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Table 1
Testing antibody specificity with specific histone and chromatin preparations

	ELISA				Recognition imaging					
	H3/H4 tetramer	H2A–H2B dimer	HeLa core histones	MMTV chromatin	H2A histone	H2B histone	H2A–H2B dimer	H3/H4 tetramer chromatin	MMTV chromatin	hSwi–Snf
Anti-H2A	4.1	18.6	27.6	50.8	46%	12%	NT	6%	99%	7%
Anti-H2B	15.1	20.1	32.9	NT	NT	NT	NT	76%	94%	71%

ELISA data are normalized absorbances at 406 nm (see Section 2). Recognition imaging data are the percentage of topographic features that are coincident with a recognition signal. The specific components tested in the recognition imaging assays were deposited and imaged just as for the chromatin+hSwi–Snf samples analyzed in this work (see Section 2). "NT" means not tested. Note the lack of target specificity of the anti-H2B antibodies. This is also observed with other antibodies (data not shown).

ratio of the slope of the absorbance vs. time for the various anti-histone treated samples to the slope for blanks treated with anti-rabbit alkaline phosphatase alone.

3. Results and discussion

The samples to be imaged were compositionally complex (four core histones, hSwi–Snf and BSA) so it was necessary to establish the extent of antibody cross-reaction with the relevant non-antigens. This was tested by ELISA assays and recognition imaging (Table 1). Clearly, the specificity of these H2A antibodies is appropriate for these experiments; anti-H2A reacts strongly when its antigen (H2A) is present but weakly when it is not, in both assays. Previously, we showed that the recognition reaction between MMTV nucleosomes and these anti-H2A antibodies is abolished by the specific peptide used to generate the antibody, but not by nonspecific peptides or proteins, and these antibodies do not recognize BSA or DNA [3]. Under the solution conditions used here, H2A and H2B occur as obligate dimers [16], thus, results obtained with anti-H2A antibodies are sufficient to describe H2A–H2B dimer changes in these samples.

3.1. Recognition imaging detects histone H2A release during remodeling

To monitor remodeling events, MMTV nucleosomal arrays and the ATP-dependent nucleosome remodeling complex human Swi–Snf (hSwi–Snf) are incubated together in solution, then deposited on GD-APTES mica and imaged [1,2]. The approach used here allows the same image field, and thus the *same* set of individual molecules, to be scanned repetitively [1,2,14], twice *before* ATP addition to determine the rate of technique-induced change (due to the scanning process itself etc.) and then again after in situ addition of ATP, which activates hSwi–Snf, to monitor remodeling changes.

Typical results are shown in Fig. 1. The top two rows show images of a field of molecules scanned twice before ATP addition, scan 1 (i) and scan 2 (ii), and then again after ATP addition, scan 3 (iii). The topographic images are in the top row ("Topo") and the corresponding recognition images are in the second row ("recog"). For both, the images from scans 1 and 2 are virtually identical. Thus, the technique itself does not cause changes. However, the addition of ATP results in a major change (an increase) in the number of H2A recognition events. Some remodeling changes are also observed near hSwi–Snf complexes (see below, also Refs. [1,2]).

In order to quantify the H2A release, we developed a program that superimposes the recognition signals, i.e. the antigen-antibody binding events in the field being scanned, at their correct locations in the corresponding topographic image. The program filters the recognition signals, identifies recognition events based on an analysis of pixel intensities, and then superimposes the filtered signals as colored circles on the topographic image (see Section 2). This electronic approach insures a high level of spatial accuracy in the matching of topography and recognition.

In the leftmost two panels in the third row, we show such superimposed images for scan 2 (ii), pre-ATP, and scan 3 (iii), post-ATP addition. Green dots mark recognition signals that are coincident with the location of nucleosomes in the topographic image while yellow dots mark recognition signals that are not nucleosome-coincident. These results confirm the significant recognition changes after ATP addition. The total number of recognition signals increases and most of the new signals are not coincident with nucleosomes (yellow dots). Thus, activation of hSwi–Snf appears to result in ejection of H2A–H2B dimers from nucleosomes.

Table 2 combines quantitative results from several experiments. "On-nucleosome" signals reflect recognition that is coincident with nucleosomes. "Off-nucleosome" signals reflect recognition that does not lie within a tether-length (see below) of a nucleosome. The frequency of histone change resulting from technique-associated effects, scans 1 vs. 2 (both −ATP), is minor. Most of the recognition signals are on-nucleosome in both scans and the ratios of off:on nucleosome signals differ little. After hSwi–Snf activation (scan 3), the total number of H2A recognition signals increases by over 40% (from 727 to 1025), mainly due to a large increase in off-nucleosome recognition signals (to 615 from 217). As a result, only 40% of the recognition signals are on nucleosomes after ATP addition vs. 60–70% in scans 1 and 2. No H2A release is detected after addition of ATP to a deposited sample of chromatin minus hSwi–Snf (Table 3).

Note that signals up to 12 nm from a nucleosome in the topographic image were considered to be "on-nucleosome". This reflects the expectation that the finite length of the tether between the antibody and the AFM tip will lead to some inherent variability, up to ± the tether length or ~12 nm, in the precise location of the recognition signal relative to the position of a particle in the corresponding topographic image. As a result, histones that are released but happen to fall within 12 nm of a nucleosome will be scored as nucleosome-associated. Thus, the number of on-nucleosome signals in scan 3 (Table 2) may include some released H2A and therefore underestimate the extent of release.

Vicent et al. [11] observed H2A–H2B release from a specific nucleosome in a trinucleosomal MMTV promoter fragment