

Histone variant H2ABbd confers lower stability to the nucleosome

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Abstract

The histone H2ABbd is a novel histone variant of H2A with a totally unknown function. We have investigated the behavior of the H2ABbd nucleosomes. Nucleosomes were reconstituted with recombinant histone H2ABbd and the changes in their conformations at different salt concentrations were studied by analytical centrifugation. The data are in agreement with H2ABbd being less tightly bound compared to conventional H2A in the nucleosome. In addition, stable cell lines expressing either GFP-H2A or GFP-H2ABbd were established and the mobility of both fusions was measured by FRAP. We show that GFP-H2ABbd exchanges much more rapidly than GFP-H2A within the nucleosome. The reported data are compatible with a lower stability of the variant H2ABbd nucleosome compared to the conventional H2A particle.

Introduction

Within the eukaryotic nucleus DNA is packaged into chromatin. The repetitive subunit of chromatin, the nucleosome, consists of an octamer of conventional core histones (two of each H2A, H2B, H3 and H4) around which two superhelical turns of DNA of ~80 bp each are wrapped (van Holde, 1988). In addition, eukaryotes express histone variants which exhibit a distinct primary sequence and variable identity to their conventional counterparts (Tsanev et al., 1993; van Holde, 1988). As a whole the functions of the histone variants such as H2A.Z, H2AX, and macroH2A are unknown, but they have been implicated in several vital cellular processes. H2A.Z is essential for the survival of mouse and *Drosophila* (Clarkson et al., 1999; Faast et al., 2001; van Daal and Elgin, 1992). This protein is involved in both transcriptional activation (Larochelle and Gaudreau, 2003; Santisteban et al., 2000; Stargell et al., 1993; Suto et al., 2000; White and Gorovsky, 1988) and gene silencing (Dhillon and Kamakaka, 2000; Meneghini et al., 2003). H2AX is phosphorylated at its carboxyl terminus immediately following double strand DNA breakage (Rogakou et al., 1999; Rogakou et al., 1998). This histone variant appears to prevent aberrant repair of DNA breakage and is implicated in the dosage-dependent suppression of genomic instability and tumors in mice (Bassing et al., 2003). Phosphorylation is essential for the function of H2AX, since these effects are abolished in mutants where the conserved serine phosphorylation sites are substituted for alanine or glutamic acid residues (Celeste et al., 2003). MacroH2A, a histone variant with an unusual primary structure, is believed to play an important role in chromosome X inactivation in mammals (Costanzi and Pehrson, 1998; Mermoud et al., 1999) and might be involved in transcriptional repression of autosomes (Perche et al., 2000). Indeed, macroH2A interfered with both transcription factor binding and SWI/SNF nucleosome remodeling (Angelov et al., 2003).

Histone variants are used by the cell to build nucleosomes with specialized architectures with dedicated functions (Ausio and Abbott, 2002). The crystal structure of the H2A.Z nucleosome particle revealed localized changes which may destabilize (H2A.Z-H2B) dimer and (H3-H4)₂ tetramer interactions (Suto et al., 2000). These subtle changes may explain the distinct physicochemical properties of H2A.Z arrays and their specific interactions with nuclear proteins (Abbott et al., 2001; Fan et al., 2002; Suto et al., 2000). In another study, the nucleosomes containing macroH2A exhibited altered structure in the vicinity of the dyad axis which was associated with the inability of SWI/SNF to remodel these particles (Angelov et al., 2003).

Recently, a novel histone variant H2A-Bbd (Barr body deficient), with an identity of 48% to H2A was identified (Chadwick and Willard, 2001). Interestingly, H2ABbd is shorter than the conventional H2A, it contains a row of six arginines at this N-terminus and lacks the non-structured C-terminus typical the other histones from the H2A family. The primary sequence of the histone fold domain of H2ABbd differed significantly from this to H2A (Chadwick and Willard, 2001). This variant is excluded from the inactive X-chromosome and its distribution overlaps with regions of histone H4 acetylation in the nucleus (Chadwick and Willard, 2001). The function of this protein is totally unknown.

Characterizing the thermodynamic stability of variant nucleosomes is an important approach for understanding their inherent function(s). Significantly, this analysis has only been performed on H2A.Z complexes *in vitro* (Abbott et al., 2001; Fan et al., 2002). Indeed, no other *in vitro* or *in vivo* studies have been documented for other variant nucleosome assemblies. In this work we present both the *in vitro* and *in vivo* stability of H2A-Bbd nucleosomes as determined by analytical ultracentrifugation and FRAP (Fluorescence Recovery After Photobleaching).

Results and Discussion

H2A-Bbd is able to replace H2A within the nucleosome

The low identity of H2A-Bbd primary structure to conventional H2A suggests that the incorporation of this histone variant into a histone octamer may likely result in a non-canonical nucleosome with a distinct structure and stability. To test this we first cloned, expressed and purified to homogeneity H2A-Bbd (Figure 1, lane 2). Next H2A-Bbd, together with a native H2B, H3, and H4 complement (Figure 1, lanes 3 and 4) were used to reconstitute nucleosomes onto a 146 bp random sequence DNA fragment isolated from chicken erythrocyte core particles (Figure 1 B). The electrophoretic mobility shift assay (EMSA) demonstrates that H2A-Bbd is efficiently incorporated into the nucleosome core particles (Figure 1C, lanes 3-5), a result in agreement with the available data (Chadwick and Willard, 2001). Indeed, at an appropriate histone:DNA ratio no free nucleosomal DNA was detected, thus demonstrating that complete reconstitution was achieved (Figure 1, lanes 4 and 5).

Hydrodynamic studies reveal the low stability of H2A-Bbd particles

Changes in the conformation of nucleosomes as a result of variations of the ionic strength are very informative for determining the nucleosome stability in solution (Angelov et al., 2001; Ausio et al., 1984; Greulich et al., 1985). Within the studied range of salt concentrations (≤ 0.6 M NaCl) the histone N-termini remain still bound to DNA (Angelov et al., 2001; Ausio et al., 1984; Greulich et al., 1985). Such nucleosome conformational transitions may be physiologically relevant since they mimic the nucleosome alterations resulting from the interactions with transcription factors, chromatin remodeling complexes or RNA polymerases (Aalfs and Kingston, 2000; Abbott et al., 2001). With this in mind, we

have characterized the sedimentation behavior of H2A-Bbd nucleosome particles at NaCl concentrations between 0 and 600 mM and compared it to purified core particles from chicken erythrocytes (Figure 2). At low salt concentrations (<50 mM NaCl) both types of particles exhibit similar sedimentation coefficients. Upon raising the ionic strength this parameter decreases for both particles, but the decrease of the $s_{20,w}$ of the H2A-Bbd nucleosomes is clearly more pronounced (Figure 2). For example, at 0.2 M NaCl the $s_{20,w}$ of the H2A-Bbd nucleosome is 9.7S compared to 11S for that of conventional nucleosomes. This distinct drop in the sedimentation coefficient for H2A-Bbd particles is strongly suggestive of a more open structure, which exhibits a higher frictional coefficient compared to the conventional H2A particle. Thus, the increasing ionic strength of the solvent has a differential effect on the conformation of the two particles and suggests a lower inherent stability of the H2A-Bbd particle.

GFP-H2A-Bbd exhibits a higher mobility *in vivo* than GFP-H2A

The lower stability of H2A-Bbd nucleosomes reflects a potential perturbation of nucleosomal DNA and (or) histone octamer interactions. Recent experiments described core histone exchanges *in vivo*, confirming a dynamic organization of the nucleosome (Kimura and Cook, 2001). If the structural lability of the reconstituted variant nucleosomes observed *in vitro* is maintained *in vivo*, one would expect a more dynamic exchange of H2A-Bbd compared to H2A.

The kinetics and interactions of a protein with its partners in living cells can be studied through expression of a Green Fluorescent Protein (GFP) fusion construct. This chimera is visualized by GFP autofluorescence. Irradiation with the laser light results in a photobleached area with an observable loss of fluorescence. The subsequent fluorescence recovery kinetics of GFP-fusion proteins can be measured by FRAP. Such kinetic experiments directly reflect

the mobility of GFP-fusion proteins, which in turn, provide strong insights into the interaction of the protein with its partners *in vivo* (Lippincott-Schwartz et al., 2001).

To measure the mobility of H2A and H2A-Bbd we first established stable cell lines expressing GFP fused to either conventional H2A or to H2A-Bbd. In these cell lines the GFP-histone fusions were assembled into nucleosomes (Figure 3). This was demonstrated as follows. Nuclei were prepared from the stable cell lines and were digested with micrococcal nuclease. Nucleosomes were isolated by centrifugation through a sucrose gradient containing 0.6 M NaCl (Mutskov et al., 1998). At this NaCl concentration, nucleosomes are depleted of linker histones and non-histone proteins, i.e. only the histone octamers properly organized into nucleosomes were isolated (Figure 3A and B). The presence of GFP-fusions within this preparation was demonstrated by using antibodies against GFP. Indeed, western blot analysis clearly shows that GFP-H2A-Bbd and GFP-H2A are both successfully incorporated in nucleosomes isolated from stable cell lines (Figures 3 C). Importantly, the amount of expressed GFP-H2A was much lower (less than 10%) compared to this of the conventional H2A (Figure 3D), while this of GFP-H2ABbd was even smaller (compare the signals for GFP-H2A and for GFP-H2ABbd relative to these of H2A, Figures 3D and 3E). Thus, the expression of the GFP-fusions led to an insignificant increase of the total amount of H2A-type histones and no free GFP-histone fusions should be present. If this was correct, treatment of the cells with increasing NaCl concentrations up to 0.6 M should not release GFP-histone fusions from the cells and the intensity of the GFP signals should remained unchanged (Kimura and Cook, 2001). And this was the case for both cell lines expressing the respective GFP-histone fusions (Figure 3 F and G). We conclude that no detectable amount of free GFP-H2A or GFP-H2ABbd is present in the stable cell lines. This validates the use of FRAP technique to measure the mobility of the GFP-histone fusions.

For the FRAP experiments, a small nuclear area was photobleached in single cells of both GFP-H2A and GFP-H2A-Bbd cell lines, and images were collected at concurrent intervals (Figure 4A). Clearly, the recovery kinetics differed in both samples (Figure 4 A and B). Conventional GFP-H2A displayed slower recovery, since even 30 minutes after photobleaching the fluorescence reaches only ~ 40% of its initial value (Figure 4 A and B). This result agrees with the available data (Kimura and Cook, 2001). In contrast, the photobleaching recovery for the GFP-H2A-Bbd fusion was fast, since after 10-15 minutes an essentially complete recovery was observed (Figure 4 A and B). Therefore, GFP-H2ABbd exchanges quicker than GFP-H2A within the nucleosome, an observation in agreement with the *in vitro* biophysical analysis that detected possible perturbations in the H2A-Bbd nucleosome structure.

In conclusion, our data show that the variant H2A-Bbd nucleosome exhibits lower stability *in vitro* compared to the conventional nucleosome particle. Importantly, the FRAP experiments demonstrate that *in vivo* H2ABbd is more rapidly exchanged than conventional H2A histone. In addition, the available data indicate that this histone variant is tightly associated with acetylated euchromatic regions of the genome and possibly with active genes (Chadwick and Willard, 2001). This suggests that H2ABbd nucleosomes might be inherently specialized to enhance localized nucleosome disruption and facilitate transcription.

Methods

Nucleosome reconstitution

By using appropriate primers the H2A-Bbd histone variant cDNA (Chadwick and Willard, 2001) was PCR-amplified from human testis Marathon-ready cDNA library, expressed in *E. coli* and purified to homogeneity (Angelov et al., 2003; Luger et al., 1999). A titration was carried out using SDS-PAGE to ensure that all histones in the final mixture were present in

equimolar amounts. The histone mixture thus obtained was dialyzed overnight against 2.0 M NaCl, 10 mM Tris (pH 7.5), 10 mM β -mercaptoethanol, 0.1 mM EDTA at 4°C and mixed with 146 bp random sequence DNA in the same buffer at a ratio of 1.13 histone: 1.0 DNA (w/w). Nucleosome core particle reconstitution was carried out using salt gradient dialysis (Mutskov et al., 1998). The integrity of the core particles was analyzed by 4% native PAGE and sedimentation velocity in the analytical ultracentrifuge (see below).

Analytical Ultracentrifugation

Reconstituted H2ABbd nucleosomes were dialyzed against buffers of varying ionic strength and subjected to analytical ultracentrifuge analysis as described elsewhere (Ausio et al., 1989). Briefly, Sedimentation velocity runs were performed in a Beckman XL-A ultracentrifuge using an An-55 Al aluminum rotor and double-sector cells with aluminum filled Epon centerpieces. A value of 0.650 cm³/g was used for the partial specific volume of the nucleosome core particle.

Cell Culture, Transfection, immuno-blotting

Human A431 cells were grown on Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum (Biowhittaker, Europe). Cells were transfected with either GFP-H2A or GFP-H2A-Bbd constructs using FuGENE 6 reagent (Roche) according to the manufacturer's protocol. Stable transfected cells were selected by gentamicin (500 μ g/ml). Cell clones were amplified and their positivity was checked by fluorescence.

Nuclei were isolated from stable A431 human cell lines expressing either GFP-H2A or GFP-H2A-Bbd. After appropriate digestion with micrococcal nuclease, linker histone depleted nucleosomes were prepared by using sucrose gradients containing 0.6 M NaCl. Nucleosomal DNA was extracted and analyzed by agarose gels and the protein composition of the particles

was determined by SDS-PAGE electrophoresis. Alternatively, cells were harvested by trypsinization and lysed in Laemmli sample buffer containing 7M urea. GFP-fusions proteins were then detected by immunoblotting with a monoclonal anti-GFP antibody (BD Clontech). Blots were developed by using the ECL plus western blotting detection system (Amersham Bioscience).

Quantification of the GFP-fluorescence of interphase and mitotic cells at different NaCl concentrations.

Cells were arrested at mitosis by treatment with nocodazole (40ng/ml final concentration) for 16 hours. Both mitotic and interphase cells were washed with PBS and after incubation at room temperature for 10 minutes with PBS-T-G buffer (PBS, 0.1% Triton-X100, 50% glycerol) containing NaCl at different concentrations, they were fixed with 4% PAF. The cells used as a control were fixed immediately after the washing with PBS. Fluorescence intensities were quantified by using a home-made software. The intensity of fluorescence of the NaCl treated cells was presented as a ratio (in percentage) to the fluorescence intensity of the control cells.

In vivo experiments: FRAP

Cells were grown on Lab-Tek chambered cover glass (Nalge Nunc International). For imaging cells were maintained at 37°C on a temperature controlled stage while medium was buffered with Hepes (10 mM, pH 7.5). Photobleaching and confocal microscopy were performed on a ZEISS LSM510 Laser Scanning Confocal apparatus using a PlanApochromat 40x water immersion objective. GFP was excited with a 488-nm Argon2 laser (power varying from 0.1 to 1%). FRAP experiments: Outlined regions were bleached by 10 iterations of a full

power laser and subsequently recovery was monitored over about one hour. Bleaching due to the acquisition was corrected and was less than 10% in all experiments.

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Figure legends

Figure 1. The histone variant H2A-Bbd is able to substitute conventional H2A in the nucleosome particles.

(A) 18% PAGE containing SDS of H2A-Bbd and the complement conventional histone proteins. Lane 1: chicken erythrocyte histone marker; Lane 2: recombinant H2A-Bbd monomer; Lane 3: equimolar mixture of H2B, H3 and H4; Lane 4: H2A-Bbd, H2B, H3, H4 mixture used to reconstitute mononucleosomes.

(B) Native gel electrophoresis of H2A-Bbd containing mononucleosomes. Lane 1: *CfoI* digest of pBR 322 used as a DNA molecular marker; Lane 2: purified chicken erythrocyte mononucleosomes; Lane 3-5: reconstituted mononucleosomes with H2A-Bbd. Lane 3 contains an excess of free DNA; Lane 4, 5 are different loadings of the same sample. Lane 6: 146 bp random sequence nucleosomal DNA used as a reconstitution substrate for core particles. **N** = nucleosome, **D** = 146bp DNA used in reconstitution experiments

Figure 2. Sedimentation profile of nucleosome core particles reconstituted with H2A-Bbd or a full native histone complement under different ionic strength (0-600 mM NaCl). The dotted line is the predicted behavior obtained from native nucleosome core particles (Ausio and van Holde, 1986).

Figure 3. GFP-H2A and GFP-H2A-Bbd histone fusions are assembled into nucleosomes.

(A) Nuclei were isolated from stable A431 human cell lines expressing either GFP-H2A or GFP-H2A-Bbd and nucleosomes prepared as described under methods. 1.5% agarose gel

electrophoresis of DNA isolated from nucleosomes prepared from GFP-H2A (lane 2) or GFP-H2A-Bbd (lane 3) expressing cells. Lane 1, molecular mass DNA markers.

(B) 18% SDS electrophoresis of histones isolated from nucleosomes prepared from GFP-H2A (lane 1) and GFP-H2A-Bbd (lane 2) stable cell lines. The positions of the core histones are designated on the right part of the figure. Note that histones H2A and H2B co-migrate.

(C) Western blot of proteins isolated from GFP-H2A (lane 1) and GFP-H2A-Bbd (lane 2) expressing cell line nucleosomes. An anti-GFP antibody was used for detection of the GFP-fusion histones. The arrow shows the position of the protein marker with molecular mass 45 kD.

(D) GFP-H2A is expressed in trace amount compared to H2A. Western blot of proteins isolated from GFP-H2A expressing cell line. Increasing amounts of total cell extract was loaded on 15% SDS gel. An anti-H2A antibody was used for detection of both H2A and the fusion GFP-H2A proteins. Note the very small amount of the fusion GFP-H2A relative to the amount of histone H2A.

(E) Western blot analysis GFP-H2A and GFP-H2ABbd fusions. Total cell extracts from GFP-H2A (lane 1) or GFP-H2ABbd (lane 2) stable cell lines were loaded on a 15% SDS gel. The blot was cut into two parts. The lower part containing the blotted proteins with molecular masses below 30 kD was revealed by using an anti-H2A antibody to detect histone H2A. The upper part of the blot was revealed by using an anti-GFP antibody. The positions of histone H2A and the GFP-fusions are designated.

(F) The amount of GFP-H2A and GFP-H2ABbd fusions in the stable cell lines is not affected upon treatment with elevated concentrations of NaCl. Interphase or mitotic cells expressing either GFP-H2A or GFP-H2ABbd were treated with buffered solutions containing NaCl at the concentrations indicated and the cells were imaged by using fluorescence microscopy.

(G) Quantification of the data presented on (F). Values represent means and standard deviations for 45 cells. Note that the intensity of the GFP-H2A and GFP-H2ABbd signals did not change upon treatment with NaCl concentrations up to 0.6 M.

Figure 4. FRAP analysis of GFP-H2A and GFP-H2A-Bbd proteins. (A) Cells expressing either GFP-H2A or GFP-H2ABbd were imaged before (pre-bleach) or during recovery after bleaching. Images were recorded at the indicated times post-bleaching. The arrows designate the rectangular bleached area. (B) Quantification of the FRAP data. Values represent means and standard deviations for 11 nuclei from three independent experiments. The scale bar represents 2.5 μm .