Histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation

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Peripheral blood neutrophils form highly decondensed chromatin structures, termed neutrophil extracellular traps (NETs), that have been implicated in innate immune response to bacterial infection. Neutrophils express high levels of peptidylarginine deiminase 4 (PAD4), which catalyzes histone citrullination. However, whether PAD4 or histone citrullination plays a role in chromatin structure in neutrophils is unclear. In this study, we show that the hypercitrullination of histones by PAD4 mediates chromatin decondensation. Histone hypercitrullination is detected on highly decondensed chromatin in HL-60 granulocytes and blood neutrophils. The inhibition of PAD4 decreases histone hypercitrullination and the formation of NET-like structures, whereas PAD4 treatment of HL-60 cells facilitates these processes. The loss of heterochromatin and multilobular nuclear structures is detected in HL-60 granulocytes after PAD4 activation. Importantly, citrullination of biochemically defined avian nucleosome arrays inhibits their compaction by the linker histone H5 to form higher order chromatin structures. Together, these results suggest that histone hypercitrullination has important functions in chromatin decondensation in granulocytes/neutrophils.

Introduction

In eukaryotic cells, 147 bp of DNA is tightly packed around a core histone octamer (including two of each of the histones H3, H2B, H2A, and H4) to form a nucleosome core particle (Kornberg and Lorch, 1999; Richmond and Davey, 2003). The formation of higher order chromatin structures is further mediated by linker DNA and linker histones (Schalch et al., 2005; Brown and Schuck, 2006; Fan and Roberts, 2006; Kan et al., 2007). In particular, the binding of linker histones to the 11-nm poly-nucleosomal fiber plays an important role in chromatin compaction to form a 30-nm fiber. However, the dynamic regulation of chromatin higher order structures during transcription and other nuclear events remains largely unclear.

Posttranslational modification of histone proteins such as acetylation, phosphorylation, and methylation regulates various chromatin functions, including transcription, DNA damage repair, and chromatin condensation/decondensation (Shilatifard, 2006; Kouzarides, 2007; Li et al., 2007). To explain the biological significance of histone modifications in chromatin biology, a “histone code” hypothesis has been proposed, which predicts that covalent histone modifications working singularly or in combination regulate histone structure and multiple nuclear functions (Strahl and Allis, 2000; Turner, 2000; Jenuwein and Allis, 2001).

Peptidylarginine deiminases (PADs) are a family of enzymes previously known to convert protein arginine (Arg) to citrulline (Citrulline), a nonconventional amino acid in proteins (Vossenaar et al., 2003). PAD4 (also called PADI4/PADV) was first identified in human HL-60 leukemia cells upon differentiation.
In response to various stimuli, including pathogen infection and inflammatory response, neutrophils form a highly decondensed chromatin structure, termed neutrophil extracellular traps (NETs), as an innate immune response (Brinkmann et al., 2004; Beiter et al., 2006; Buchanan et al., 2006; Fuchs et al., 2007). However, whether any histone modification plays a role during this change of chromatin higher order structure is largely unknown. In this study, we show that in addition to its gene regulatory role, PAD4-catalyzed histone hypercitrullination appears to play a critical role in chromatin decondensation in granulocytes/neutrophils. Our results support a model in which global histone hypercitrullination regulates the unfolding of chromatin structures during NET formation.

Along the granulocyte lineage (Nakashima et al., 1999) and is highly expressed in peripheral blood neutrophils (Nakashima et al., 2002; Su et al., 2004). PAD4 mainly localizes to the nucleus and targets histones H3, H2A, and H4 for citrullination (Hagiwara et al., 2002; Nakashima et al., 2002; Cuthbert et al., 2004; Wang et al., 2004). We have previously shown that PAD4 also converts histone methylarginine residues to Cit by a novel reaction termed demethylimination, and this activity of PAD4 counteracts the functions of histone Arg methylation at estrogen target genes (Wang et al., 2004). Recently, we have reported that PAD4 functions as a corepressor of p53 to regulate histone Arg modification and gene expression (Li et al., 2008; Yao et al., 2008). These studies support a view that promoter-specific histone citrullination is involved in gene regulation.

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was not increased at the decondensed chromatin fibers (Fig. 1, I and J), suggesting that the increased staining of histone citrullination is unlikely to be the result of an increase in antibody accessibility. To further test the increase in histone citrullination, Western blot experiments were performed. After calcium ionophore treatment, both H4Cit3 and histone H3 citrullination (the α-H3Cit antibody is generated against a Cit2-, Cit8-, and Cit17-containing H3 peptide) were significantly increased (Fig. 1 K).

Induction of hypercitrullination and global chromatin decondensation by TNF-α in peripheral blood neutrophils

Blood neutrophils are known to form highly decondensed chromatin structures, termed NETs, after bacterial infection (Brinkmann et al., 2004; Fuchs et al., 2007). To analyze whether histone hypercitrullination and chromatin decondensation occur in blood neutrophils, we treated neutrophils with the pro-inflammatory cytokine TNF-α. Before TNF-α treatment, very low levels of H4Cit3 antibody staining were observed in neutrophils (Fig. 2, A–C). After TNF-α treatment for 15 min, ~10% of neutrophils (>300 cells counted from three independent experiments) showed a dramatic increase in histone citrullination (Fig. 2, D–F), suggesting that PAD4 is activated after TNF-α treatment in a subset of blood neutrophils. A close examination

Results and discussion

Histone hypercitrullination and chromatin decondensation in HL-60 granulocytes

In response to DMSO treatment, HL-60 cells differentiate along the granulocyte lineage and express higher levels of PAD4. Previously, we reported that histone H3Arg2, -8, and -17 residues were citrullinated at 0, 27.3, and 6.5% in HL-60 granulocytes after calcium ionophore treatment (Wang et al., 2004). However, the role of histone hypercitrullination remains unclear. In this study, we found that within 15 min after calcium ionophore treatment, a subset of HL-60–derived granulocytes appear to rupture and release long stretches of extensively decondensed chromatin into the extracellular space, forming weblike chromatin structures (Fig. 1 and Fig. S1, A–C, available at http://www.jcb.org/cgi/content/full/jcb.200806072/DC1). In accordance with our previous finding that PAD4 targets histone methylarginine residues for citrullination (Wang et al., 2004), histone H4Arg3 methylation was decreased (Fig. 1 B) and histone H4Cit3 was increased (Fig. 1, E and H) at regions of highly decondensed chromatin, suggesting that chromatin decondensation is associated with PAD activation. The acetylation of histone H4K16 has been correlated with chromatin decondensation (Robinson et al., 2008). In contrast to H4Cit3, this modification was not increased at the decondensed chromatin fibers (Fig. 1, I and J), suggesting that the increased staining of histone citrullination is unlikely to be the result of an increase in antibody accessibility. To further test the increase in histone citrullination, Western blot experiments were performed. After calcium ionophore treatment, both H4Cit3 and histone H3 citrullination (the α-H3Cit antibody is generated against a Cit2-, Cit8-, and Cit17-containing H3 peptide) were significantly increased (Fig. 1 K).
of the decondensed chromatin with NET morphology found that high levels of histone citrullination are associated with decondensed chromatin (Fig. 2, G–L). While we were preparing this manuscript, a study showed that various cytokines and extracellular signals such as TNF, lipopolysaccharide, and H₂O₂ induced histone citrullination and chromatin decondensation in...
neutrophils (Neeli et al., 2008). Collectively, these experiments relate histone hypercitrullination to chromatin decondensation during NET formation.

**PAD4 activity is important for mediating chromatin decondensation and NET-like chromatin structure formation**

To test whether PAD4 activity is important for chromatin decondensation, we treated HL-60 granulocytes with Cl-amidine, a newly synthesized PAD4 inhibitor (Luo et al., 2006), before calcium ionophore treatment. Double staining of HL-60 granulocytes with the H4Cit3 antibody and the DNA dye Hoechst was performed to analyze histone citrullination and the formation of NET-like chromatin structure. Without calcium ionophore treatment, each cell had only weak H4Cit3 staining and did not display NET-likechromatin structure (Fig. 3A). After calcium ionophore treatment, ~36% of cells were strongly stained with the H4Cit3 antibody (Fig. 3 B). Notably, >8% of cells formed NET-like chromatin structures (Fig. 3 B, arrows). In contrast, Cl-amidine treatment for 15 min before calcium ionophore treatment decreased the number of cells showing positive H4Cit3 staining (Fig. 3 C). Moreover, NET-like chromatin structure was not observed in cells treated with Cl-amidine before calcium ionophore treatment (Fig. 3 C). The effect of PAD4 inhibition by Cl-amidine on histone H3 and H4 citrullination after calcium ionophore treatment was validated by Western blotting (Fig. 3 D, compare lane 2 with lane 3). These results indicate that PAD activity is important for histone citrullination and chromatin decondensation in HL-60 granulocytes after calcium ionophore treatment.

To test whether PAD4 activity directly induces chromatin decondensation, we analyzed chromatin decondensation in undifferentiated HL-60 cells expressing a low level of endogenous PAD4 (Fig. S1 D). First, HL-60 cells were permeabilized by 0.1% Triton X-100 in PBS solution before treatment with wild-type GST-PAD4 fusion protein or a catalytically inactive GST-PAD4C645S mutant in the PAD assay buffer containing a physiological concentration of NaCl. The addition of the wild-type PAD4 but not the inactive PAD4C645S mutant reproducibly caused pronounced chromatin decondensation (Fig. 3, compare E and F with G). Furthermore, Western blot analyses showed a dramatic increase of histone H3 and H4 citrullination in PAD4-treated cells but not in cells treated with the PAD4C645S mutant (Fig. 3 H). Collectively, we conclude that histone citrullination mediates extensive chromatin decondensation in HL-60 cells permeabilized with Triton X-100.

Micrococcal nuclease (MNsase) digests chromatin at the linker DNA region, leading to the release of solubilized poly- and mononucleosome-sized fragments, which is often used as an indication of the accessibility of linker DNA as well as chromatin compaction. The effects of PAD4 treatment on chromatin decondensation prompted us to test whether PAD4 treatment of HL-60 cells alters the accessibility of linker DNA to MNase. After treatment of HL-60 cells with PAD4 or the PAD4C645S mutant, MNase was added to digest chromatin for the time points indicated (Fig. 3 I). MNase digestion generated mononucleosomes more rapidly in cells treated with PAD4 compared with cells treated with the PAD4C645S mutant (Fig. 3 I, compare lane 7 with lane 8). These results indicate that linker DNA is more accessible to MNase after PAD4 treatment. Furthermore, because the size of the nucleosome repeat was not changed as a result of the PAD4 treatment, it appears that the primary nucleosomal structure is not affected by histone citrullination.

**PAD4 activity is required for bacteria-mediated chromatin decondensation**

To analyze whether PAD4 activity is important for NET formation under more physiologically relevant conditions, we treated HL-60 cells, DMSO-differentiated HL-60 cells, or DMSO-differentiated HL-60 cells pretreated with Cl-amidine with IL-8 and bacteria *Shigella flexneri*, which was shown to induce NET formation in peripheral blood neutrophils (Brinkmann et al., 2004). Histone H3 citrullination was rarely detected in undifferentiated HL-60 cells treated with IL-8 and bacteria *S. flexneri* for 3 h (Fig. 4, A and D). Cells occasionally observed with positive staining may reflect HL-60 cell spontaneous differentiation and PAD4 expression (Fig. S1 D). In contrast, after differentiation along the granulocytic lineage to increase PAD4 expression (Fig. S1 D) and IL-8 and bacteria treatment, 9.8 ± 0.7% (n = 4) of cells became histone H3 citrullination positive with a concomitant increase of NET formation in 3.2 ± 0.1% (n = 4) of cells after calcium ionophore treatment was validated by Western blotting. These results indicate that linker DNA is more accessible to MNase after PAD4 treatment. Furthermore, pretreatment of differentiated HL-60 cells with Cl-amidine inhibited histone citrullination and the formation of NETs (Fig. 4, C and D). The percentages of cells with positive staining of H3 citrullination and/or chromatin decondensation in four independent experiments were counted and are shown in a bar graph (Fig. 4 D). Although a histone H3 citrullination–positive cell may have a round nucleus or a nucleus with decondensed chromatin forming NETs, each nucleus with decondensed chromatin was stained with the histone H3 citrullination antibody. To quantify the amount of chromatin forming NETs, we performed MNase digestion using previously described methods (Fuchs et al., 2007) with slight modifications. Compared with the parental HL-60 cells, we consistently observed an increase in MNase digestion and nucleosomal DNA ladder formation in DMSO-differentiated HL-60 cells after IL-8 and bacteria treatment for 15 h (Fig. 4 E, lanes 4 and 5). Furthermore, MNase digestion was decreased when DMSO-differentiated HL-60 cells were pretreated with the PAD4 inhibitor, Cl-amidine (Fig. 4 E, lanes 5 and 6). Together, the aforementioned immunostaining and MNase analyses indicate that PAD4 activity is required for histone citrullination and NET formation.

**Loss of heterochromatin and lobular nuclear morphology in HL-60 granulocytes after calcium ionophore treatment**

In electron microscopy analyses, HL-60 granulocytes display a multilobular nuclear structure with distinct heterochromatin underlying the nuclear envelope (Fig. 4, F and G, left). To test whether these characteristic nuclear structures are affected by PAD4 activation, we analyzed nuclear morphology in HL-60 granulocytes by electron microscopy. Without calcium ionophore...
To test whether these nuclear structure changes are related to histone citrullination, we performed immunofluorescence analyses. Before calcium treatment, HL-60 granulocyte nuclei showed a clear multilobular structure (Fig. 4 H, left; arrows denote two nuclei). Furthermore, a rimlike structure of heterochromatin treatment, each HL-60 granulocyte has a rim of electron-dense heterochromatin underlying the nuclear envelope (Fig. 4 F and G, left). In contrast, heterochromatic regions appear more diffuse and electron lucent in ~35% of cells after calcium ionophore treatment for 15 min (Fig. 4, F and G, right).

To test whether these nuclear structure changes are related to histone citrullination, we performed immunofluorescence analyses. Before calcium treatment, HL-60 granulocyte nuclei showed a clear multilobular structure (Fig. 4 H, left; arrows denote two nuclei). Furthermore, a rimlike structure of heterochromatin
underneath the nuclear envelope and weak H4Cit3 staining were detected (Fig. 4 H). In contrast, after calcium ionophore treatment, nuclei became round and lost the multilobular nuclear structure with a concomitant increase in H4Cit3 staining (Fig. 4 H, right), suggesting that the activation of PAD4 and subsequent histone citrullination caused dramatic changes in HL-60 granulocytes’ nuclear structure.

**Histone citrullination inhibits nucleosome array folding by linker histone H5**

To analyze whether histone citrullination affects the nucleosome compaction, we analyzed the density of the 207 × 12 nucleosome core particle array (Springhetti et al., 2003), which contains 12 nucleosome core particles assembled with core histones H3, H2B, H2A, and H4 on a defined DNA template, treated with GST-PAD4 or the GST-PAD4C645S mutant. The ability of PAD4 to citrullinate nucleosomal histones was confirmed by Western blotting (unpublished data). Consistent with the maintenance of the primary nucleosome structure (Fig. 3 I), there was no difference between the density of the 207 × 12 nucleosome core particle array treated with PAD4 or the PAD4C645S mutant (Fig. 5 A). This result suggests that under current experimental conditions, citrullination of histones by PAD4 did not generate detectable conformation changes in the nucleosomal core particle arrays.

The organization of nucleosome core particles to higher order chromatin structure is mediated by linker histones (Schalch et al., 2005; Brown and Schuck, 2006; Fan and Roberts, 2006; Kan et al., 2007). 207 × 12 nucleosomal core particle arrays sediment at 35 S without linker histone and at 53 S with linker histone added. Next, we used this defined biochemical system to test whether PAD4 treatment affects linker histone–mediated array compaction. After treatment with PAD4 or the PAD4C645S mutant and analytical ultracentrifugation, the peak of the 207 × 12 nucleosomal core particle arrays was recovered, and the linker histone H5 was added to further compact the array. Interestingly, after adding H5, the 207 × 12 nucleosomal core particle array treated by the PAD4C645S mutant and PAD4 formed an ~50-S and ~40-S structure, respectively (Fig. 5 B). The 10-S difference in the density of these arrays indicates that citrullination of core histones in the 207 × 12 array by PAD4 decreased the ability of H5 to compact the array.

Because the physiological substrate of PAD4 in cells is chromatin (i.e., nucleosomes associated with linker histones), we further analyzed whether PAD4 can alter the structure of the 207 × 12 array assembled in the presence of H5. The H5-containing 207 × 12 array formed a 53-S structure after treatment with the PAD4C645S mutant (Fig. 5 C), whereas a 45-S structure was formed after treatment with PAD4 (Fig. 5 C). These results show that PAD4 mediates the formation of a less condensed nucleosome array structure.

In summary, our experiments showed that PAD4 and histone hypercitrullination play a unique role in chromatin decondensation in HL-60 granulocytes and peripheral blood neutrophils. Using the HL-60 granulocytes as a model system, we showed that PAD4 activity and histone hypercitrullination are important for the extensive chromatin decondensation during NET formation. Because the release of PAD4 into extracellular spaces from neutrophils may generate self-antigens that may induce autoimmune diseases, we envision that the activation of PAD4 and NET formation is an event regulated by multiple signals, including the generation of reactive oxygen species (Fuchs et al., 2007).
Many steps are involved in the formation of a high order chromatin structure in an interphase cell nucleus. The first regulatory step to form a condensed high order chromatin structure is mediated by the association of linker histones to the nucleosome arrays (Trojer and Reinberg, 2007). It is very likely that a decrease in linker histone binding to nucleosomes may help to unfold chromatin during NET formation. We propose that histone cistronellation can play a dual role. First, limited cistronellation of histones at specific gene promoters regulates gene activity (Cuthbert et al., 2004; Wang et al., 2004; Balint et al., 2005; Li et al., 2008; Yao et al., 2008). Second, global histone hypercistronellation in granulocytes appears to represent one of the underlying mechanisms by which chromatin decondenses during NET formation in granulocytes/neutrophils.

Materials and methods

HL-60 cell differentiation and GST-PAD4 treatments

HL-60 differentiation with DMSO and treatment with calcium ionophore in Locke’s solution (10 mM Hepes-HCl, pH 7.3, 150 mM NaCl, 5 mM KCl, 2 mM CaCl₂, and 0.1% glucose) were performed essentially as previously described (Wang et al., 2004). To analyze the effects of PAD4 inhibition on histone cistronellation and chromatin decondensation, HL-60 granulocytes were pretreated with Cl-amidine at 200 μM concentration for 15 min at 37°C before calcium ionophore treatment. For Triton X-100 treatment, the medium was removed, and 50 μl PBST (PBS with 0.1% Triton X-100) supplemented with protease inhibitors (1 μg/ml aprotinin and 1 mM PMSF) was added to permeabilize cells for 2 min. After briefly rinsing the cells twice with PBS, 0.5 μg GST-PAD4 or the GST-PAD4C645S mutant in 50 μl of PAD assay buffer (50 mM Hepes, pH 7.6, 5 mM CaCl₂, 5 mM DTT, and 150 mM NaCl, supplemented with 0.1 μg/ml aprotinin) was added to treat the cells for 2 h at 37°C. After the treatment, the cells were fixed with 3.7% paraformaldehyde in PBS containing 1% Triton X-100 and 2% NP-40 and immunostained similarly as other cells. In parallel, cellular proteins were acid extracted to purify histones for Western blotting. Cl-amidine was provided by P.R. Thompson (University of South Carolina, Columbia, SC).

Acid extraction of histones and Western blotting

The acid extraction of histones and Western blotting with H4Cit (1:2,000; Millipore), H3Cit (1:3,000; Abcam), and H3 (1:3,000; Abcam) antibodies were performed essentially as previously described (Wang et al., 2004).

Immunostaining and electron microscopy

After fixation of samples with 3.7% paraformaldehyde in PBS supplemented with 1% Triton X-100 and 2% NP-40, cells were washed with PBS three times for 10 min each. After the third wash, cells were blocked first with 2% BSA and 5% normal goat serum in PBS for at least 30 min at RT. Primary antibodies were diluted in PBS supplemented with 2% BSA and 5% normal goat serum as follows: 1:100 H4Arg3Me (Millipore), 1:500 H4Cit (Millipore), and 1:100 H4K16Ac (Millipore). Cells were stained with the primary antibodies in a humid chamber overnight at 4°C. After staining, cells were washed with PBS three times for 10 min each. Cells were washed with the appropriate secondary antibodies conjugated with Cy3 at RT for 2 h in the dark. After washing three times for 10 min each, cells were counterstained with 1 μg/ml Hoechst and mounted for imaging with a fluorescence microscope (Axioskop 40; Carl Zeiss, Inc.) using an Axioplan 10x NA 0.45 (Carl Zeiss, Inc.) or a Fluor 40x NA 1.30 oil Ph3 objective lens (Carl Zeiss, Inc.). Images were captured using a camera (AxioCam MRm; Carl Zeiss, Inc.) and the Axiovision AC software (Carl Zeiss, Inc.) at RT. Images were further processed and pseudocolored using Photoshop (Adobe) with the appropriate adjustment of levels. Electron microscopy work was performed at the Bio-Imaging Resource Center at the Rockefeller University.

Blood neutrophil isolation and TNF-α treatment

Peripheral neutrophils were used according to the Institutional Review Board–approved protocols of the Well Medical School of Cornell University. Neutrophils were isolated to >95% purity using PolymorphPrep (Axis-Shield PoC AS) and treated with TNF-α as previously described (Han et al., 2005).

MNase digestion of HL-60 cells after permeabilization with Triton X-100

After cells were permeabilized with PBST (0.1% Triton X-100) and treated with GST-PAD4 or the GST-PAD4C645S mutant, cells were collected by spinning at 2,000 rpm for 10 min at 4°C. The cell pellet was resuspended in 250 μl of buffer N2 (10 mM Pipes, pH 6.5, 0.5 mM sodium metabisulfite, 0.5 mM benzamidine-HCl, and 5 mM MgCl₂; freshly supplemented with 0.5 mM DTT and 0.1 mM PMSF). The samples were kept on ice, and 0.5 U RNase A and CaCl₂ was added to 5 μM MNase (the amount of MNase was optimized for each batch). Samples were incubated at 37°C and stopped at 0, 5, 10, and 15 min by increasing the EDTA concentration to 10 mM. Then, 75 μl H2O, 30 μl of 10% SDS, 25 μl of 4 M NaCl, and 200 μl phenol-chloroform were added to the digested samples in a sequential order and mixed by vortex. Samples were centrifuged at 13,000 rpm for 10 min. 1/3 vol of 7.5 M NH₄Ac, pH 7.6, and 1 vol of isopropanol were added in a sequential order. Samples were left at RT for 10 min to precipitate DNA and centrifuged at 13,000 rpm for 10 min. DNA was washed with cold 70% ethanol three times to remove salt, air dried, resuspended in 10 μl H2O, and analyzed in 1.5% DNA agarose gel.

IL-8 and S. flexneri treatment of HL-60 cells and MNase digestion to analyze DNA release during NET formation

HL-60 cells were treated with 1.25% DMSO for 3 d for differentiation. For Cl-amidine treatment, differentiated HL-60 cells were preincubated with 200 μM Cl-amidine for 1 h to inhibit PAD4 activity. Then, 3 x 10⁴ cells from each group (HL-60 cells, differentiated HL-60 cells, and differentiated HL-60 cells pretreated with Cl-amidine) were resuspended in Locke’s solution (10 mM Hepes HCl, pH 7.3, 150 mM NaCl, 5 mM KCl, 2 mM CaCl₂, and 0.1% glucose) supplemented with 0.1% PBS and spun down to cover slips by centrifugation at 700 g for 10 min. Cells were incubated with 100 ng/ml IL-8 for 30 min. 3 x 10⁵ exponential-phase S. flexneri were added to cells (MOI = 100) and spun at 200 g for 10 min. Cells were incubated for 3 h in a 5% CO₂, 37°C incubator before immunostaining analyses or for 12 h to increase NET formation before MNase treatment and DNA release. For MNase treatment, after incubation with S. flexneri for 15 h, DTT and Ca²⁺ were added to the sample to a final concentration of 1 mM and 5 mM, respectively. Samples were incubated with 0.5 U MNase for 3 min at RT, and the reaction was stopped by adding 20 mM EDTA. Samples were centrifuged at 200 g for 5 min, and supernatant was collected for DNA extraction. Released DNA was extracted using phenol-chloroform, ethanol precipitated, and analyzed in 1.2% agarose gels.

Nucleosome array reconstitution, PAD4 reaction, and analytical centrifugation

Nucleosome positioning templates containing 12 clone 601 repeats uniformly positioned every 207 bp (207 x 12) were constructed and reconstituted with core histones isolated from chicken erythrocytes as previously described (Nikkitiva et al., 2007). Competitor DNA was obtained from the pUC19 vector and added to the reconstitution mixture at a template DNA to competitor DNA ratio of 2:1. The reconstituted nucleosome core arrays were purified on a sucrose gradient. Purified fractions were collected, dialyzed for 48 h against 10 mM Hepes, pH 7.5, 0.1 mM EDTA, and 5 mM NaCl, and concentrated to A₂₆₀ = 2.0. For linker arrays, linker histone H5 was isolated from chicken erythrocytes and reconstituted with the core arrays at a ratio of one molecule of H5 per nucleosome.

PAD4 was incubated with either core arrays or linker arrays. Reaction mixtures contained 0.1 μg PAD4 per 1 μg histone with 20 mM Tris, pH 7.5, 50 mM CaCl₂, and 2 mM DTT and were incubated for 1 h at 37°C. Control reactions were performed the same way but using the inactive mutant form, PAD4C645S, with either the core or linker arrays. After incubation, EDTA was added to a concentration of 2 mM to stop the reaction. The reaction mixtures were analyzed on 18% SDS-PAGE and by analytical ultracentrifugation. After analytical ultracentrifugation of the PAD4 core arrays, linker histone H5 was added to the centrifuged sample at a ratio of one molecule of H5 per nucleosome and incubated for 1 h at 20°C before repeating the analytical ultracentrifugation.

Sedimentation velocity experiments were conducted to examine nucleosome array compaction using an ultracentrifuge (Optima XL-A; Beckman Coulter). Samples were centrifuged in 150 mM NaCl at 20,000 rpm and 20°C for ~3 h. Data analysis was performed using the continuous c(s) distribution model (Brown and Schuck, 2006) with SEDFIT software (http://www.analyticalultracentrifugation.com).
Online supplemental material

Fig. S1 shows the increase of histone citrullination and chromatin decondensation in HL-60 granulocytes after calcium ionophore treatment and the increase of PAD4 expression in HL-60 cells after DMSO differentiation. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200806072/DC1.

We thank colleagues in the Allis, Coonrod, and Wang laboratories for discussions and suggestions during the course of this study. We thank the BioImaging Center at the Rockefeller University for electron microscopy support. We thank Dr. Paul R. Thompson and Dr. Na Xiong (Pennsylvania State University) for critical reading of the manuscript. This research is supported in part by a Pennsylvania State University startup fund to Y. Wang, an Era of Hope Scholar Award (grant V871XVH-07-1-0372) to S.A. Coonrod, and a National Science Foundation grant (MCB-0615536) to S.A. Grigoryev.

Submitted: 12 June 2008
Accepted: 18 December 2008

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