Sumoylated Human Histone H4 Prevents Chromatin Compaction by Inhibiting Long-range Internucleosomal Interactions*

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Background: Human histone H4 is post-translationally modified at Lys-12 by the small ubiquitin-like modifier protein (SUMO-3).

Results: Chemical sumoylation at H4 Lys-12 revealed the inhibition of chromatin compaction and oligomerization by SUMO-3.

Conclusion: Sumoylation changes chromatin structure by inhibiting long-range internucleosomal interactions and decreasing the affinity between adjacent nucleosomes.

Significance: Learning how sumoylation changes the structure of chromatin suggests that it may mediate gene repression without chromatin compaction.

The structure of eukaryotic chromatin directly influences gene function, and is regulated by chemical modifications of the core histone proteins. Modification of the human histone H4 N-terminal tail region by the small ubiquitin-like modifier protein, SUMO-3, is associated with transcription repression. However, the direct effect of sumoylation on chromatin structure and function remains unknown. Therefore, we employed a disulfide-directed strategy to generate H4 homogenously and site-specifically sumoylated at Lys-12 (suH412). Chromatin compaction and oligomerization assays with nucleosomal arrays containing suH412 established that SUMO-3 inhibits array folding and higher order oligomerization, which underlie chromatin fiber formation. Moreover, the effect of sumoylation differed from that of acetylation, and could be recapitulated with the structurally similar protein ubiquitin. Mechanistic studies at the level of single nucleosomes revealed that, unlike acetylation, the effect of SUMO-3 arises from the attenuation of long-range internucleosomal interactions more than from the destabilization of a compacted dinucleosome state. Altogether, our results present the first insight on the direct structural effects of histone H4 sumoylation and reveal a novel mechanism by which SUMO-3 inhibits chromatin compaction.

Histones are the principal protein component of eukaryotic chromatin and constitute an important class of nuclear proteins that are extensively post-translationally modified during the cell cycle (1). The wide range of histone post-translational modifications (PTMs)5 includes phosphorylation, methylation, acetylation, ubiquitylation, and modification by the small ubiquitin-like modifier (SUMO) (2). Studies of the chromatin localization and cellular functions of histone PTMs have identified their critical roles in DNA-templated processes such as transcription, repair, replication, and segregation (3, 4). Not surprisingly, the dysregulation of histone PTMs has drastic effects, resulting in a range of human diseases including cancers of the blood (5) and brain (6), ataxias (7), and muscular dystrophy (8). Given their essential roles in normal eukaryotic development and disease initiation, it is crucial to understand how specific histone modifications influence the structure and function of chromatin.

Histone sumoylation (modification by the SUMO-1 or 2/3 family of proteins) occurs in parasites (9), plants (10), yeast (11, 12), and humans (13, 14). Similar to the range of histone targets of ubiquitin (Ub), SUMO is conjugated to all four core histones (11), the linker histone H1 (15), and histone variants H2A.Z (12) and H2A.X (14). Among the core histones, H4 is the primary target for modification by SUMO-1, and SUMO-3 in human 293T cells and B-lymphocytes (13). The site of sumoylation in H4 was initially localized to 26 amino acids in the flexible N-terminal, or tail, region (13) and more recently Lys-12 in H4 was identified as the site of modification by SUMO-3 in HeLa cells (16). H4 sumoylation was associated with gene repression through genetic studies in yeast (11) and HeLa cells (13), but in the absence of sumoylated histone-specific antibodies these studies could not ascribe a direct mecha-

5 The abbreviations used are: PTM, post-translational modification; SUMO, small ubiquitin-like modifier protein; suH, sumoylated histone; ESI, electrospray ionization; Ub, ubiquitin; MN, mononucleosome; NRL, nucleosome repeat length; GdmHCl, guanidinium hydrochloride; uH, ubiquitylated histone.

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**FIGURE 1. Histone H4 tail-mediated internucleosomal interactions.** A, structure of mononucleosomes showing interaction of the H4 tail region in one nucleosome with the H2A-H2B interface in an adjacent symmetry-related mononucleosome. B, surface electrostatic potential rendering of two adjacent mononucleosomes showing the acidic patch (red) and basic H4 tail (blue). Structures were rendered from Protein Data Bank code 1AOI with PyMol software.

nistic role to this modification. Thus, despite its discovery over a decade ago and its implication in gene regulation, the direct effects of histone sumoylation on chromatin structure and function are unknown.

One common mechanism by which histone PTMs influence gene function is by directly modulating the degree of chromatin compaction and higher order oligomerization (17–20). The primary driving force for chromatin compaction and oligomerization, in the absence of additional protein factors, is an electrostatic interaction between the basic H4 tail and an exposed acidic patch at the H2A-H2B interface on an adjacent nucleosome (Fig. 1) (21, 22). Structural transitions in chromatin may arise from in cis mechanisms wherein a PTM directly modulates the degree of interaction between two nucleosomes, such as acetylation at Lys-16 in the H4 tail (H4 K16ac) (17) or ubiquitylation at Lys-120 in H2B (uH2B) (18). Alternatively, PTMs may act in trans to recruit chromatin binding and remodeling proteins that modulate the structure of chromatin, such as trimethylation at H3 Lys-9, which recruits the heterochromatin-associated protein 1 (23, 24).

Mechanistic studies of the precise roles for histone PTMs in dictating chromatin structure are typically limited by the inhomogeneity of modified histones obtained from biological sources (1). Furthermore, many of the enzymes that install site-specific histone modifications remain unknown, which precludes enzymatic generation of uniformly modified histones in vitro. Therefore, semisynthetic strategies that yield uniformly and site-specifically modified histones have provided valuable insight regarding the structural effects on chromatin of methylation (25), acetylated (17), and ubiquitylated histones (18, 26).

We now report the application of a disulfide-directed protein modification strategy (27) to generate human histone H4 site-specifically modified by SUMO-3 at Lys-12 (referred to as suH4ss). Our semisynthesis of suH4ss permitted the unprecedented biophysical analysis of homogenously site-specifically sumoylated mononucleosomes (MNs) and nucleosomal arrays at physiological Mg²⁺ concentrations. In addition to ensemble sedimentation coefficient measurements that probed the effect of SUMO-3 on chromatin compaction (28), a single-molecule Försters resonance energy transfer (FRET)-based technique was adopted to measure the effect of SUMO-3 on internucleosomal interactions (20).

Corroborative results from these studies indicated that suH4ss inhibits intra- and inter-array interactions that underlie chromatin compaction and oligomerization, respectively. Furthermore, the effect of SUMO-3 was recapitulated by Ub, suggesting a role for the steric bulk of the protein in the observed effects. Importantly, the mode of action of SUMO-3 differs from acetylation, and is predominantly mediated by the inhibition of long-range internucleosomal interactions. Thus, our results offer the first mechanistic insights on how SUMO-3 alters the structure of chromatin in cis to form an open euchromatin-like structure, and set the stage for future biochemical studies of the in trans effects of sumoylated H4.

**EXPERIMENTAL PROCEDURES**

Purification of Wild-type Histones—Gene sequences encoding wild-type human histone isoforms H2A 2-A, H2B 1-K, H3.2, and H4 G were cloned in a pET3a vector. BL21(DE3) cells transformed with the histone expression plasmids were grown in 6 liters of YT medium (16 g/liters of tryptone, 10 g/liters of yeast extract, 5 g/liters of NaCl) at 37 °C until A600 ~0.7. Protein expression was induced by the addition of 0.3 mM isopropyl β-D-thiogalactopyranoside followed by growing for an additional 3 h at 37 °C. Cells were harvested by centrifugation at 7,000 × g, lysed by sonication, and then centrifuged at 20,000 × g. Insoluble histones were recovered from inclusion bodies by dissolving them in 6 M guanidinium hydrochloride (GdmHCl), 10 mM Tris, pH 7.5, and purified by size exclusion chromatography on a Superdex 200 column. Histones were further purified by RP-HPLC and characterized by electrospray ionization-mass spectrometry (ESI-MS).

Synthesis of suH4ss—The histone H4 K12C mutant was generated by site-directed mutagenesis, using the forward primer, 5′-GGTAAAAGGGTGTTAACGCTGGTTGCGGTTGCGCTAAACGTCACCCTAAA-3′ and reverse primer, 5′-TTTTACGTTGACGTTTAGCACCACCGCACCACGACCTTTACCACCTTACC-3′. Due to the low yields of H4 K12C from the insoluble lysate pellet, it was purified as a His₆-TEV-H4K12C fusion protein and pure H4 K12C was obtained after
cleavage of the His$_6$ tag with tobacco etch virus (TEV) protease. Purified H4 K12C, 1, was dissolved in a 3:1 (v/v) acetic acid: water mixture and reacted with an excess of 2,2’-dithiobis(5-nitro-3-pyridyldisulfide) for 2 h at 25 °C. The H4 K12C-Npys asymmetric disulfide, 2, was subsequently purified by RP-HPLC and characterized by ESI-MS.

A C47S mutant form of human SUMO-3 was generated by site-directed mutagenesis using the forward primer, 5’-AGCAAGCTGATGAGGCTCTGCCTTGCAGCTTGCT-3’ and reverse primer, 5’-CATTGACAAGGCCCTGGCCTCAGATGGGCTTCATGCAGCTGCT-3’, cloned in-frame with a C-terminal Mycobacterium xenopi GyrA intein and chitin binding domain in the pTXB1 vector (27). The resultant fusion protein was overexpressed in E. coli BL21(DE3) cells by growing them to an A$_{600}$ of 0.5 at 37 °C followed by the addition of 0.3 mM isopropyl β-D-thiogalactopyranoside at 25 °C. The cells were grown for an additional 4 h at 25 °C, harvested by centrifugation at 7,000 × g, and lysed by sonication in a lysis buffer containing 20 mM Tris, pH 7.5, 200 mM NaN$_3$, and 1 mM β-mercaptoethanol. The lysate supernatant obtained after centrifugation at 20,000 × g was applied to chitin beads and washed extensively. Intein-mediated cleavage of SUMO-3 C47S in the presence of the small molecule thiol cysteamine afforded the SUMO-3 C47S-cysteamine adduct, 3. The modified SUMO was eluted from the column, purified by RP-HPLC, and characterized by ESI-MS.

Finally, 1 eq of 2 and 2 eq of 3 were dissolved in reaction buffer consisting of 1 M HEPES, 6 mM GdmHCl, pH 7.0, and allowed to react for 1 h at 25 °C with continuous shaking. The resultant disulfide-linked product, suH4$_{ss}$, was purified by RP-HPLC and characterized by ESI-MS.

Synthesis of H4 K12ac and K16ac—Purified H4 K16C (2.2 mg), or H4 K12C (2.2 mg) was dissolved in 200 μl of 6 M GdmHCl, 200 mM sodium acetate, pH 6.0. To this solution was added 15 mM L-glutathione, 50 mM N-vinylacetamide, 100 mM dimethylsulfide, and 50 mM of the azo radical initiator VA-044 (2,2’-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride). The thermally activated thiol–ene click reaction was initiated by incubating the above mixture in the dark at 37 °C for 2 h. The product was purified by RP-HPLC and characterized by ESI-MS.

Octamer Formation—Individual histones were dissolved in an unfolding buffer consisting of 7 M GdmHCl, 20 mM Tris, pH 7.5, at final concentrations of ~4 mg/ml. Equimolar amounts of the four histones were combined and the resultant mixture dialyzed into refolding buffer consisting of 10 mM Tris, 2 M NaCl, 1 mM EDTA, pH 7.5. The self-assembled crude octamers were concentrated and then purified by size exclusion. Fractions containing octamer were identified by 15% SDS-PAGE, combined, and concentrated prior to nucleosome preparation.

MN Formation—Octamers and 147 bp of Widom 601 DNA (1_147_601) were combined in 10 μl of high-salt octamer refolding buffer to a final concentration of 2 μM. After incubation at 37 °C for 15 min, 3.3 μl of dilution buffer 1 (10 mM HEPES, 1 mM EDTA, 0.5 mM PMSF, pH 7.9) was added and the temperature was dropped to 30 °C. Further dilutions with 6.7, 5, 3.6, 4.7, 6.7, 10, 30, and 20 μl of buffer 1 were then undertaken every 15 min. A final dilution with 100 μl of dilution buffer 2 (10 mM Tris, 1 mM EDTA, 0.1% (v/v) Nonidet P-40, 0.5 mM PMSF, 20% (v/v) glycerol, pH 7.5) was carried out and after an additional 15 min at 30 °C, the MNs were concentrated and their composition verified by non-denaturing polyacrylamide gel electrophoresis followed by staining with ethidium bromide.

Array Formation—Histone octamers and the 12_177_601 DNA were combined at concentrations of 2 μM octamers and 2 μM 601 sites in 75 μl of reconstitution buffer (2 M KCl, 0.1 mM EDTA, 10 mM Tris, pH 7.8). In addition, 0.7 mM of a weaker binding 147-bp Nucleosome A (Nuca)-positioning sequence found in the mouse mammary tumor virus long terminal repeat (29) was added to prevent overloading of 12-mer arrays with octamers. Stepwise dialysis was performed at 4 °C against reconstitution buffers containing 1.4 M NaCl, 1.2 M NaCl, 1 M NaCl, 0.8 M NaCl, 0.5 M NaCl, and 10 mM NaCl for 90 min each, followed by a final dialysis step against reconstitution buffer containing 10 mM NaCl. Mouse mammary tumor virus DNA and MNs were removed by selective precipitation of the arrays with MgCl$_2$. After removal of the supernatant, the arrays were resuspended in TEN buffer, and dialyzed against fresh TEN buffer prior to application in biophysical assays.

Saturation of 12_177_601 DNA with octamers was confirmed by digestion of 0.17 pmol of arrays with 10 units of Scal restriction enzyme at room temperature for 12 h, followed by non-denaturing PAGE and staining with ethidium bromide. The presence of a MN band as well as the absence of free DNA and higher running species indicated full array occupancy. Reducing agents in the restriction enzyme buffer led to partial loss of the SUMO moiety in suH4$_{ss}$ arrays resulting in three distinct MN bands. The presence of 12 MNs per array was further confirmed by partial digestion with Micrococcal nuclease. Briefly, 0.17 pmol of arrays were digested with 0.2 units of M. nuclease for 60 s on ice. The reaction was stopped by the addition of 0.2% (w/v) SDS and 20 mM EDTA. The DNA fragments were purified and separated on native PAGE prior to staining with ethidium bromide.

Sedimentation Velocity Measurements—All samples were prepared in 400 μl of measurement buffer (10 mM Tris, pH 7.8, 10 mM NaCl) containing either 0.1 mM EDTA (0 mM Mg$^{2+}$) or 1 mM Mg$^{2+}$. Samples were equilibrated under vacuum for 2 h at 20 °C in an analytical ultracentrifuge (Beckman XL-1) before sedimentation at 12,000 rpm in 12-mm double-sector cells held in an An-50 Ti rotor (8-place). Absorbance was measured at 260 nm in continuous scan mode. Boundary fractions were calculated by enhanced van Holde-Weischet analysis (30). $s_{20,w}$ values were calculated by adjusting for a partial specific volume of 0.622 ml g$^{-1}$ for chromatin and buffer viscosity. Mean $s_{20,w}$ values were obtained by averaging over the dominant homogenous fraction. The integrity of octamers was confirmed by 15% SDS-PAGE at the end of each run.

Array Oligomerization Assays—Reconstituted arrays were dialyzed into measurement buffer (10 mM Tris, pH 7.8, 10 mM NaCl). The samples were subsequently mixed with equal volumes of MgCl$_2$ stock solutions at twice the final concentration, incubated for 10 min at 22 °C, and then centrifuged at 16,000 × g at 22 °C for 10 min. The amounts of arrays remaining in solution were determined by $A_{260}$ nm.
Electron Microscopy—Nucleosomal arrays were dialyzed into HEN buffer (10 mM HEPES, pH 7.8, 0.25 mM EDTA, and 2.5 mM NaCl), fixed with 0.1% glutaraldehyde for 4 h at 4 °C, and dialyzed overnight against HEN. Samples were diluted as needed, applied to glow-discharged carbon-coated grids, stained with 0.1% aqueous uranyl acetate, and washed extensively with water. Grids were examined in a Tecnai 12 TEM LaB6 filament operated at 100 kV in the tilted darkfield mode, and digital images were recorded using a TVIPS 2024/2024 CCD camera (31).

Single Molecule FRET Measurements—Quartz microscope slides were silanized and coated with polyethyleneglycol (PEG 5,000 M₉, Laysan Bio, Arab, AL). One percent of the PEG molecules have a biotin at one end (Laysan Bio). The slide surfaces were passivated with BSA before immobilizing nucleosomes through streptavidin-biotin conjugation. Nucleosomes were prepared as previously reported (20). To detect dinucleosome formation events, Cy3-labeled nucleosomes (601A) were immobilized on the surface, and Cy5-labeled nucleosomes (601B) were injected on the slide. The fluorescence signals were taken within 1 h after nucleosome immobilization to avoid nucleosome disassembly. A protocatechuate dioxygenase and protocatechuic acid (Sigma) mixture in addition to Trolox (2 mM) was used to elongate the dye photobleaching lifetime and stabilize the emission.

Fluorescence signals from single Cy3 and Cy5 fluorophores were collected with an electron multiplying CCD camera (EMCCD, iXon 897; Andor Technology, Belfast, UK) on a prism-coupled total internal reflection setup based on a commercial microscope (TE2000; Nikon, Tokyo, Japan). The excitation of the FRET donor (Cy3) was achieved with a laser at 532 nm (Laser Quantum). Fluorescence emission from FRET pairs (Cy3 and Cy5) were separated into two spectral regions (550–645 and 645–750 nm) with a dichroic mirror, and the two separate images of Cy3 and Cy5 regions were projected on a single EMCCD to collect fluorescence signals simultaneously from the two fluorophores in a time-resolved manner at a frame rate of 25 ms. The time series of fluorescence intensities of Cy3 and Cy5 were obtained from the series of fluorescence images and plotted against the elapsed time. FRET efficiency at each time point was calculated with a formula, $I_{cy5}/(I_{cy3}/I_{cy5})$. To construct the lifetime histogram of dinucleosomes, the duration of every FRET event was measured and each event was counted as one event of the measured duration. These events with their measured durations were constructed as the lifetime histogram of a dinucleosomal state. For dinucleosome formation frequency calculation, the total number of FRET events was counted and divided the number by the total observation time and the acceptor-labeled mononucleosome concentration.

RESULTS

Chemical Synthesis of suH₄ss and H₄ K₁₆ac—To ascertain the effects of SUMO-3 modification at Lys-12 in histone H4, we first needed access to uniformly and site-specifically sumoylated histone H4. This was achieved by a disulfide-directed sumoylation strategy depicted in Fig. 2A (27). Briefly, a single Cys was introduced in heterologously expressed human histone H4 at the site of sumoylation (H₄ K₁₂C). The H₄ K₁₂C mutant, 1, was activated for disulfide formation by reaction with 2,2'-dithiobis(5-nitropyridine) to generate the mixed disulfide 2. Finally, reaction of 2 and 3 at pH 7.0 led to the formation of the disulfide-linked analog suH₄ss. This linkage strategy is known to be an effective mimic of the native isopeptide linkage between various target proteins and Ub or Smt3 (the yeast...
homolog of SUMO) (27, 32). Furthermore, the SUMO-3 C47S mutation, which was required for selective disulfide formation at the SUMO C terminus, does not lead to observable structural changes (33). Our semisynthetic strategy readily yielded milligram quantities of homogeneous suH4ss as seen by reversed-phase high performance liquid chromatography (RP-HPLC) and ESI-MS (Fig. 2, B and C).

As a benchmark for biophysical investigations, we also synthesized a thialysine analog of H4 K16ac, namely H4 K16ac. The thia-analog is indistinguishable from wild-type H4 K16ac in its ability to inhibit chromatin compaction mediated by Mg\(^2+\) (34). The synthesis of H4 K16ac employed radical-initiated thiol-ene coupling chemistry between a H4 K16C mutant and N-vinylacetamide (Fig. 3A) (35). A major advance over previous reports of this methodology was our utilization of a thermal, rather than UV light, activation strategy to generate the thyl radical. This prevented unwanted side reactions resulting from extended UV exposure of histones and led to clean reaction mixtures that were purified to homogeneity by RP-HPLC and characterized by ESI-MS (Fig. 3, B and C).

**Incorporation of suH4ss in Octamers and Nucleosome Arrays**—Homogenously modified suH4ss was incorporated intohistone octamers by mixing with equimolar quantities of heterologously expressed human histones H2A, H2B, and H3 C110A (27). The H3 C110A mutant is known to have no tail-less (22) and N-vinylacetamide (Fig. 3A) (35). A major advance over previous reports of this methodology was our utilization of a thermal, rather than UV light, activation strategy to generate the thyl radical. This prevented unwanted side reactions resulting from extended UV exposure of histones and led to clean reaction mixtures that were purified to homogeneity by RP-HPLC and characterized by ESI-MS (Fig. 3, B and C).

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To study the direct effect of sumoylation on chromatin structure, we assembled sumoylated nucleosomes on a fragment of concatenated dsDNA containing 12 repeats of the 601 sequence with a 30-bp intervening linker (the 12_177_601 sequence) (22). The complete occupancy of all 12 octamer binding sites was probed by multiple techniques including electron microscopy following staining by uranyl acetate (Fig. 4D) (38), by partial digestion with the nonspecific endonuclease M. nuclease, and by specific digestion with the endonuclease ScaI, which cuts within a unique AGTACT sequence incorporated in each linker region (Fig. 4E). Finally, two additional 12-mer arrays bearing either wild-type H4 or H4 K16ac that are known to compact maximally and minimally, respectively, were also assembled as benchmarks for array compaction experiments.

**Sumoylation at H4 K12 Inhibits Intra- and Inter-array Interactions Key to Chromatin Compaction**—We first tested the effect of SUMO-3 on intra-array compaction by measuring changes in the sedimentation coefficients of sumoylated arrays in the absence of Mg\(^2+\) and presence of 1 mM Mg\(^2+\). The presence of Mg\(^2+\) induces formation of compact fibers akin to condensed 30-nm chromatin, even in the absence of linker histone H1 (39). Surprisingly, suH4ss prevented array compaction to a similar degree as H4 K16ac (Δs\(_{20,w}\) = 9.5 ± 1.0 S for suH4ss and Δs\(_{20,w}\) = 10.0 ± 1.0 S for H4 K16ac), and both modifications significantly inhibited compaction in comparison with wild-type unmodified 12-mer arrays (Δs\(_{20,w}\) = 16.9 ± 0.9 S, Fig. 5A). H4 K16ac was previously shown to be indistinguishable from wild-type H4 K16ac in sedimentation velocity measurements (34). This implies that sumoylation at Lys-12 and acetylation at Lys-16 have identical in cis effects on chromatin compaction. To confirm that the observed effect upon sumoylation was not due to the H4 K12C mutation alone, we reduced disulfide linkage and found that the desumoylated arrays underwent compaction similar to unmodified arrays (Δs\(_{20,w}\) = 22.6 ± 1.7 S, Fig. 5B). Some heterogeneity in the sedimentation profiles of the resultant H4 K12C arrays, in comparison with the initial suH4ss arrays, was a result of disulfide formation after removal of the reducing agent and SUMO-3 by overnight dialysis. We also measured the effect of sumoylation on higher-order inter-array interactions that are observable at >1 mM Mg\(^2+\) concentrations and that underlie chromatin fiber formation (40). Consistent with their inhibitory effect on internucleosomal interactions, we observed that both suH4ss and H4 K16ac inhibited higher-order inter-array interactions. This follows from the fact that at 2 mM Mg\(^2+\), a concentration at which ~80% of wild-type arrays formed higher order oligomers that sedimented upon centrifugation, sumoylated and acetylated nucleosome arrays remained more soluble (Fig. 5C). Thus, our results indicate that suH4ss has similar inhibitory effects on both intra-array compaction and inter-array interactions as H4 K16ac, a modification that marks active genes.

There are two copies of H4 per nucleosome and sumoylation may occur asymmetrically on one tail, or symmetrically on both tails. PTMs are found in both symmetric and asymmetric forms in nucleosomes from multiple cell types (41). Therefore, we wondered if the inhibitory effects of symmetric sumoylation extend to asymmetrically sumoylated arrays. To answer this question, a pool of octamers containing a 1:1 ratio of suH4ss to...
wild-type H4 was generated and employed to assemble 12-mer arrays (Fig. 6A). These 50% sumoylated arrays were subjected to Mg$^{2+}$/H11001-mediated compaction and oligomerization assays. Although 50% sumoylation did not exhibit an inhibitory effect on chromatin compaction ($s_{20,w} = 20.4 \pm 0.6$ S for 1:1, suH4ss: H4, Fig. 6B), an intermediate degree of inhibition was observed toward inter-array interactions (Fig. 6C). Therefore, the threshold degree of suH4ss in chromatin that would be required to completely prevent compaction and higher-order interactions appears to be greater than 50%.

**Sumoylation and Acetylation at H4 Lys-12 Have Differing Effects on Array Compaction**—One mechanism by which SUMO-3 may inhibit internucleosomal interactions is by neutralizing the positively charged side chain of Lys-12 that could inhibit histone H4 tail interactions with adjacent nucleosomes. However, Richmond and co-workers (22) demonstrated that residues 1–12 in the H4 tail are entirely dispensable for mediating chromatin compaction in vitro, suggesting that this region of H4 does not contribute significantly to internucleosomal interactions. This suggests an additional possibility whereby SUMO may act as a steric wedge that prevents the close apposition of adjacent nucleosomes. Such a mechanism was suggested to be important for chromatin decompaction by uH2B (18). To test this mechanism, we utilized disulfide-directed chemistry to replace SUMO with Ub at H4 Lys-12. We refer to this H4 Lys-12 ubiquitylated histone as uH4ss. SUMO-3

**FIGURE 4.** Generation of sumoylated octamers, mononucleosomes, and 12-mer nucleosome arrays. A. Coomassie-stained 15% SDS-PAGE of octamers containing wild-type (Wt) or sumoylated histone H4 (suH4ss). B. C4 analytical RP-HPLC of wild-type (top) and sumoylated (bottom) octamers. C. ethidium bromide-stained 5% TBE gel of wild-type and suH4ss-containing MNs. Lane 3 shows the result of reducing the disulfide-linkage in suH4ss to confirm core histone composition of the MN. D. electron micrographs showing full occupancy of sumoylated arrays assembled on a 12_177_601 DNA substrate (mean occupancy = 11.7 \pm 0.56, n = 17, and the error is S.D.). E. ethidium bromide-stained 5% TBE gel showing Scal digestion products of wild-type and modified 12-mer arrays. An asterisk indicates both partial and complete reduction of the disulfide-linkage in suH4ss by thiols present in the digestion buffer.

**FIGURE 5.** Histone H4 sumoylation impairs chromatin array compaction and fiber formation. A, sedimentation coefficient distributions of nucleosomal arrays containing unmodified histone H4, H4 K16ac, or suH4ss were determined by sedimentation velocity experiments and van Holde-Weischet analysis at 0 and 1 mM Mg$^{2+}$ (open and solid symbols, respectively). To allow direct comparison, the $s_{20,w}$ values for SUMOylated arrays were corrected for the different molecular weights of the modified and unmodified arrays (22). Single representative traces from n = 4 are shown. B, sedimentation coefficient distributions of arrays containing suH4ss, or where SUMO-3 was removed by reduction with dithiothreitol (DTT), were determined by sedimentation velocity experiments and van Holde-Weischet analysis at 0 and 1 mM Mg$^{2+}$ (open and solid symbols, respectively). Single representative traces from n = 2 are shown. C, oligomerization behavior of nucleosomal arrays containing unmodified histone H4, H4 K16ac, or suH4ss was assessed by determining Mg$^{2+}$-dependent solubility. n = 14–16 and error bars show S.E.
Asymmetric sumoylation of nucleosomes has differing effects on compaction and fiber formation. A, Coomassie-stained 15% SDS-PAGE of asymmetric octamers containing a 1:1 ratio of wild-type histone H4 and suH4ss. B, sedimentation coefficient distributions of nucleosomal arrays reconstituted with the octamers shown in A were determined by sedimentation velocity experiments and van Holde-Weischet analysis at 0 and 1 mM Mg2+ (open and solid symbols, respectively). To allow direct comparison, the $s_{20,w}$ values for sumoylated arrays were corrected for the different molecular weights of the modified and unmodified arrays (22). Single representative traces from $n = 2–4$ are shown. C, oligomerization behavior of nucleosomal arrays containing unmodified histone H4, 1:1 suH4ss:H4, or suH4ss was assessed by determining Mg2+-dependent solubility. $n = 7–14$ and error bars show S.E.

Sumoylation at H4 Lys-12 Inhibits Long-range Internucleosomal Interactions—In human cells, the length of linker and nucleosomal DNA between adjacent nucleosomes, known as the nucleosome repeat length (NRL), varies from ~167 to ~237 bp (43), whereas a uniform NRL is often employed in biophysical studies of chromatin compaction (17, 18, 22). It was previously shown that the chromatin structure is directly affected by NRL (39, 44, 45). Furthermore, the angle of rotation between two adjacent nucleosomes, which varies with NRL, also affects the degree of chromatin fiber formation when shorter linkers of ~167–172 bp are employed (46). This raises the possibility that the structural effect of SUMO, or indeed any histone PTM, measured in uniformly spaced arrays in vitro may not reflect the true effect in cells where NRL varies. Faced with this challenge, we sought to investigate whether the observed effect of suH4ss on internucleosomal interactions is truly independent of NRL.

To study internucleosomal interactions at the mono-nucleosome level without the influence of linker length, we employed a single-molecule fluorescence method (20). With two sets of fluorescently labeled MNs, each with a FRET donor or an acceptor, we followed the association of two MNs by monitoring the Förster resonance energy transfer (FRET) between two fluorophores (Fig. 8). This method measured dinucleosome formation frequencies, the stabilities of formed dinucleosomes, and consequently, the free energy changes during dinucleosome formation. Measured in this platform, a difference in the free energy captures a difference only in the enthalpy benefit of internucleosomal interactions, assuming a constant diffusion of MNs with small chemical modifications such as acetylation and sumoylation. Thus, this measurement is free from concerns regarding changes in linker conformations during internucleosomal compaction, which is affected by NRL. The single-molecule method was applied to measure the association between pairs of MNs containing suH4ss, H4 K12ac, H4 K16ac, and wild-type H4.

Two sets of 601 DNA sequences were constructed as previously reported (20). One 147-bp sequence was labeled with Cy3 at the 129th base (601A) and the other was labeled with Cy5 at the 60th base (601B). MNs were reconstituted with the appropriate octamers and fluorophore-labeled DNA sequences. The Cy3-labeled MNs were then immobilized on a streptavidin-coated microscope slide, and a dilute solution of Cy5-labeled MNs (10 nM) was injected onto the slide. The lifetimes of FRET events and the frequency of FRET events in the presence of 1 mM Mg2+ reflect dinucleosome stability and their formation frequency, respectively. We first constructed FRET lifetime histograms (Fig. 9) to measure the stabilities of dinucleosomes. The first column of a lifetime histogram was excluded from data analysis because there are always undetectable short-lived FRET events, which would make the lifetime measurements inaccurate. The incorporation of suH4ss in an MN reduced the lifetime of dinucleosomes to 0.34 ± 0.019 s compared with longer lifetimes of 0.47 ± 0.016 s observed for wild-type dinucleosomes (Table 1). Reductions in the lifetimes to 0.28 ± 0.016 and 0.28 ± 0.009 s were also observed for dinucleosomes containing H4 K16ac and H4 K12ac, respectively. We also counted the number of dinucleosome formation events in each case and converted the number to a rate constant. According to these results, wild-type, suH4ss, H4 K16ac, and H4 K12ac MNs, respectively, formed compact dinucleosomes 26,200,
8,740, 9,710, and 13,800 times/s/mol (Table 1). Combining the lifetimes \((1/k_{reverse})\) and formation frequencies \((k_{forward})\) of dinucleosomes together, we calculated the free energy changes during dinucleosome compaction \(\Delta G^0 = -RT \ln(\frac{k_{forward}}{k_{reverse}})\), Table 1). The free energy changes of compaction for wild-type, suH4ss, H4 Ks16ac, and H4 Ks12ac MNs are 23.3 ± 0.2, 19.8 ± 0.2, 19.5 ± 0.2, and 20.5 ± 0.4 kJ/mol, respectively. These changes in \(\Delta G^0\) are significantly greater than the thermal energy \((RT)\) at 37 °C of 2.6 kJ/mol. Consequently, these \(\Delta G^0\) changes clearly reflect the differences in the array compaction efficiency. In particular, the difference between either H4 Ks16ac or suH4ss and H4 Ks12ac, although small, is statistically significant and clearly observable in the sedimentation coefficients of the corresponding arrays at 1 mM Mg2+ (Fig. 7A). This may be ascribed, in part, to the small differences in dinucleosome formation being amplified by multiple simultaneous internucleosomal interactions in 12-mer arrays.

The observed FRET event frequencies and durations further indicate that the mechanism for inhibition of internucleosomal compaction by suH4ss is different from that of acetylation at either Lys-12 or Lys-16. The frequency of dinucleosome formation is lower with suH4ss or H4 Ks16ac than with H4 Ks12ac, whereas the lifetime of the dinucleosome state is longer with suH4ss than with H4 Ks12ac or H4 Ks16ac. This indicates that once formed, suH4ss dinucleosomes are inherently more stable than the acetylated dinucleosomes and argues against SUMO-3 acting as a steric wedge to pry nucleosomes apart. On the other hand, dinucleosomes containing suH4ss or H4 Ks16ac are harder to assemble than those with H4 Ks12ac. It then follows that suH4ss and H4 Ks16ac inhibit array compaction more by attenuating the initial formation of dinucleosomes than by destabilizing dinucleosome stacks, when compared with H4 Ks12ac. Thus, suH4ss and H4 Ks16ac may inhibit long-range internucleosomal interactions that are responsible for the initial formation of dinucleosome stacks.

**DISCUSSION**

Proteomic studies with human histones have revealed a myriad of histone modifications present in different combinations on the core histones (1, 47). Given the difficulty in isolating homogenously modified histones from tissues and cultured cells or in their generation by enzymatic means, ascribing specific functions to the bewildering array of histone PTMs is a major challenge for modern biology. Initial studies in HEK293 cells and *Saccharomyces cerevisiae* indicated that the H4 N-terminal tail is a target for modification by SUMO and more recent
proteomic studies have established Lys-12 as a specific site for modification. However, despite its discovery over a decade ago, nothing is known regarding the effect of histone sumoylation on chromatin structure, which is intimately linked to the later’s function. Therefore, we applied a semisynthetic strategy to address this critical question. Our disulfide-directed strategy yielded suH4ss, a close analog of wild-type histone H4 sumoylated at Lys-12, in multi-milligram quantities. This permitted a series of biophysical experiments aimed at gaining insight on the effect of suH4ss on chromatin compaction and chromatin fiber formation. Key to our success was the observation that suH4ss is efficiently incorporated into octamers and nucleosomes and forms stable nucleosomal arrays.

Based on genetic experiments in yeast and human cells, chromatin sumoylation was associated with reduced gene transcription, and was proposed to act by mediating the formation of a reversible heterochromatin structure (11, 13). A comparably bulky histone mark associated with gene repression is ubiquitylated H2A (uH2A). The Ring1B E3 ligase that is a component of the polycomb repressive complex 1 (PRC1) installs ubiquitin at Lys-119 in H2A (48) and this transcriptionally repressive mark correlates with silent chromatin. uH2A functions in part by inhibiting transcription elongation by RNA polII. In keeping with its role in gene repression, uH2A purified from calf thymus cells and reconstituted into nucleosomal arrays had no discernible effect on chromatin compaction as seen from sedimentation velocity analysis during analytical ultracentrifugation (49). Furthermore, because arrays containing uH2A precipitated at lower Mg2+/H11001 concentrations than unmodified arrays, uH2A appeared to facilitate higher-order structure formation. In contrast with uH2A, we found that suH4ss attenuated internucleosomal and inter-array interactions and consequently inhibited chromatin compaction. Hence it is unlikely that sumoylated H4 affects chromatin function through the same mechanisms as uH2A.

An investigation of the structural role for uH2B in chromatin revealed that the bulky Ub acts as a steric wedge to prevent chromatin compaction. SUMO-3 is structurally similar to Ub and its attachment at H4 K16ac introduces significant steric bulk and also neutralizes the Lys-12 side chain -amine. Interestingly, both sumoylation and acetylation at Lys-12 showed similar inhibitory effects on the compaction and aggregation of...
nucleosome arrays reconstituted with human histones. Therefore, we suggest that both H4 K16ac and H4 K12ac may directly regulate the structure of human chromatin. This is further supported by studies in yeast that found the H4 K12R mutation, which abrogates acetylation at this site, leads to a more condensed telomeric heterochromatin structure (50). Our mechanistic studies at the single-molecule level further revealed that sumoylation destabilizes the dinucleosomal state, but to a lesser degree than H4 K12ac or H4 K16ac. Given its sterically bulk, this is a surprising result and suggests that SUMO-3 on one nucleosome may bind an adjacent nucleosome to partially compensate for the destabilization arising from neutralization of the H4 Lys-12 electrostatic interaction. Sumoylation does, however, inhibit the rate of formation of dinucleosomes when compared with H4 K12ac. This is largely due to the attenuation of long-range internucleosomal interactions that slows the rate of formation of dinucleosome stacks.

Previous genetic studies could not address the direct site-specific effects of SUMO-3 in the context of nucleosomes and histones (11, 13). Our studies have revealed that suH4 forces chromatin to adopt an open structure rather than a compact histone (11, 13). Our studies have revealed that suH4 forces chromatin condensation. Given the number of SUMO-interacting proteins that are associated with chromatin, it is possible that SUMO-3 may instead recruit specific chromatin-modifying enzymes that change the chromatin modification state and repress gene transcription. Moreover, as SUMO-3 may form polySUMO chains, these may further increase the local concentration of histone-modifying enzymes at chromatin due to avidity effects. Hence, future studies in our laboratory will be aimed at interrogating the biochemical in trans roles for suH4 in dictating chromatin function.

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