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Specific Acetylation Patterns of H2A.Z Form Transient Interactions with the BPTF Bromodomain

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Abstract

Post-translational lysine acetylation of histone tails affects both chromatin accessibility and recruitment of multifunctional bromodomain-containing proteins for modulating transcription. The bromodomain- and PHD finger-containing transcription factor (BPTF) regulates transcription but has also been implicated in high gene expression levels in a variety of cancers. In this report, the histone variant H2A.Z, which replaces H2A in chromatin, is evaluated for its affinity for BPTF with a specific recognition pattern of acetylated lysine residues of the N-terminal tail region. Although BPTF immunoprecipitates H2A.Z-containing nucleosomes, a direct interaction with its bromodomain has not been reported. Using protein-observed fluorine nuclear magnetic resonance (PrOF NMR) spectroscopy, we identified a diacetylation of H2A.Z on lysine residues 4 and 11, with the highest affinity for BPTF with a K_d of 780 μ M. A combination of subsequent ¹H NMR Carr–Purcell–Meiboom–Gill experiments and photo-cross-linking further confirmed the specificity of the diacetylation pattern at lysines 4 and 11. Because of an adjacent PHD domain, this transient interaction may contribute to a higher-affinity bivalent interaction. Further evaluation of specificity toward a set of bromodomains, including two BET bromodomains (Brd4 and BrdT) and two *Plasmodium falciparum* bromodomains, resulted in one midmicromolar affinity binder, PfGCN5 (K_d = 650 μ M). With these biochemical experiments, we have identified a direct interaction of histone H2A.Z with bromodomains with a specific acetylation pattern that further supports the role of H2A.Z in epigenetic regulation.

Graphical abstract

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ASSOCIATED CONTENT

Supporting Information

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Peptide characterization, MALDI MS peptide data, PrOF NMR data, BPTF binding isotherm with bromosporine and bromodomain-containing proteins, synthesis of NH₂-AU1, small molecule NMR spectra, inhibition curve of NH₂-AU1, and HPLC quality control spectra (PDF)

The authors declare no competing financial interest.

Chromatin structure is dynamically regulated via post-translational modifications to both DNA and bound histone proteins. The nucleosome particle comprises approximately 147 bp of DNA surrounding an octameric bundle of four conserved histones: H2A, H2B, H3, and H4. This assembly provides a scaffold for recruiting transcription factors to accessible genomic regions for modulating transcription.¹ Histone acetylation is one such modification recognized by bromodomain effector modules and subsequently results in the recruitment of the transcriptional machinery. In addition to conserved histones, histone variants can be exchanged into nucleosomes, dramatically affecting chromatin stability,² and provide an additional mechanism for transcriptional control.³ Here, we evaluate interactions of the histone variant H2A.Z with a select set of bromodomains.

H2A.Z is a nonconserved histone variant commonly found at transcription start sites,^{4,5} leading to a destabilized nucleosome.⁶ H2A.Z levels positively correlate with the activating histone mark, H3K4me3, and negatively correlate with the repressive modification, H3K27me3.^{7–9} H2A.Z histones are spread throughout the promoter of inactive genes in the unacetylated state, but they are acetylated near highly localized transcription start sites (TSS).¹⁰ In yeast, the acetyltransferase GCN5 acetylates N-terminal lysines of H2A.Z with a preference for acetylation of K14.¹¹ Accumulation of hyperacetylated levels of H2A.Z at the TSS has been correlated with high levels of proliferative gene expression in bladder cancer,¹² melanoma,¹³ and liver cancer.¹⁴ However, the role and mechanisms employed by H2A.Z in regulating disease states are still being uncovered.

Histone chaperones also play a key role in the removal and addition of the H2A variant.^{15,16} Recently, the histone chaperone ANP32e was shown to selectively remove H2A.Z from the nucleosome,¹⁷ and the SWR1 complex was shown to catalyze the exchange of H2A with H2A.Z. The recruitment of SWR1 is partly directed through the bromodomain subunit of SWR1, Bdf1, which identifies acetylated histone tails on H3 and H4.^{18,19} While the acetylation state of H2A.Z correlates with disease states¹⁰ and epigenetic control,²⁰ to date direct interactions of the bromodomain with acetylated H2A.Z histones have not been systematically evaluated. An understanding of which, if any, acetylation states on H2A.Z are necessary to bind bromodomains would further elucidate the role H2A.Z plays in gene regulation.

The bromodomain- and PHD finger-containing transcription factor (BPTF) has recently been shown to serve an important regulatory role in cell cycle progression, proliferation,

development, and the immune response;^{21,22} furthermore, high levels of BPTF correlate with poor patient prognoses in various cancers.^{23–25} One role of BPTF as a potential oncoprotein has been supported via identification of a c-Myc protein–protein interaction at low-affinity interaction sites on chromatin, thus leading to BPTF's association with various Myc-dependent cancers.^{26,27} Recently, it has been shown that BPTF selectively immunoprecipitated H2A.Z-containing nucleosomes over H2A-containing nucleosomes.^{28,29} In addition, knockdown of either BPTF or H2A.Z decreased the mRNA levels of 10 elevated genes in bladder cancer that have H2A.Z enrichment near their TSS.²⁸ On the basis of these results, we sought to test if these effects were regulated via a direct interaction of the BPTF bromodomain with the acetylated H2A.Z histone as a potential mechanism for regulating transcription.

MATERIALS AND METHODS

For *Escherichia coli* growth, Luria-Bertani (LB) agar, LB medium, defined medium components such as unlabeled amino acids, uracil, thiamine hydrochloride, nicotinic acid, biotin, and buffer components were purchased from RPI Corp. 3-Fluorotyrosine, thymine, cytosine, and guanosine were purchased from Alfa Aesar. Magnesium chloride, manganese sulfate, succinic acid, calcium chloride, and 5-fluoroindole were purchased from Sigma-Aldrich.

Peptide Synthesis

Solid-phase peptide synthesis was performed using the procedure outlined below. 1-Hydroxybenzotriazole monohydrate (HOBt, Chem-Impex International), *O*-benzotriazol-1-yl-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU, Novabiochem), and *N,N*-diisopropylethylamine (DIEA, Sigma-Aldrich) were used as received without further purification and dissolved into reagent-grade dimethylformamide (DMF, Fisher Scientific). *N*- α -Fmoc-*N*- ϵ -Boc-L-lysine and *N*- α -Fmoc-*N*- ϵ -trifluoroacetyllysine were received from Chem-Impex. *N*- α -Fmoc-*O*-tBu-tyrosine, *N*- α -Fmoc-glycine, and *N*- α -Fmoc-*N*- ϵ -Boc-D-lysine were received from Novabiochem. Piperidine, trifluoroacetic acid, dichloromethane (Sigma-Aldrich), triisopropylsilane (TCI America), acetic anhydride (Fisher Scientific), 3-maleimidopropionic acid NHS (Chem impex), and triethylamine (Mallinckrodt) were all used as received. Peptides were made on a 25 μ mol scale on NovaSyn TGR resin in a CEM MARS microwave reactor. All peptides were synthesized using HOBt and HBTU coupling reagents according to published methods.³⁰ Following Fmoc deprotection of the final residue, the peptides were cleaved from the resin using a 95:2.5:2.5 TFA/TIPS/H₂O mixture and stirred for 2 h. The peptide cleavage solution was separated from the resin, and the solution was concentrated under a stream of N₂. The crude peptide was precipitated with ether, cooled to –20 °C for at least 15 min, and pelleted by centrifugation at 3000*g* for 5 min at 4 °C. The pellet was dissolved in a 60:40 0.1% TFA in H₂O/acetonitrile mixture and sufficient DMF to aid solubility. Dissolved peptides were purified on a Dionex Ultimate 3000 RP-HPLC system using a C-18 column on a 10 to 60% acetonitrile gradient over 60 min. Purified peptides were analyzed with an Ab-Sciex 5800 matrix-assisted laser desorption ionization (MALDI) time-of-flight mass spectrometer. Peptide theoretical and actual masses are listed in Table S1.

Protein Expression Conditions for Unlabeled or Fluorine-Labeled Bromodomains Brd4(1), BrdT(1), BPTF, *Pf* GCN5, and PFA0510w

The pNIC28-BSA4 plasmid containing the Brd4(1) and BPTF genes was a kind gift from the laboratory of S. Knapp, whereas the plasmid containing the gene of *Pf*GCN5 and PFA0510w were kind gifts from R. Hui at SGC Toronto.

Unlabeled Bromodomains

For unlabeled protein expression of BPTF and *Pf*GCN5 bromodomains, the *E. coli* Rosetta (DE3) strain (Novagen) was first transformed with the pRARE (Novagen) plasmid. The transformed cells were selected using chloramphenicol. Calcium competent pRARE-containing *E. coli* cells were transformed with the respective plasmids. Co-transformed cells were selected by using 35 mg/L chloramphenicol and 100 mg/L kanamycin to select for the pRARE plasmid and bromodomain, respectively. Following overnight incubation at 37 °C, a single colony was selected from the agar plate and inoculated in 50 mL of LB medium containing kanamycin (100 mg/L) and chloramphenicol (35 mg/L). The primary culture was grown overnight at 37 °C while being shaken at 250 rpm. For secondary culture growth, 1 L of LB medium containing kanamycin (100 mg/L) was inoculated with the primary culture and incubated at 37 °C while being shaken at 250 rpm. When the OD of the culture at 600 nm reached 0.6, the shaker temperature was reduced to 20 °C. After 30 min, expression was induced with 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) overnight for 12–16 h. Cells were harvested by centrifugation and stored at –80 °C.

Fluorine-Labeled Bromodomains

3-Fluorotyrosine- and 5-fluorotryptophan-labeled proteins were expressed using previously published methods^{31,32} and briefly described here. To express the labeled protein, the secondary culture in LB medium was grown until the OD at 600 nm reached 0.6, and then the cells were harvested at 3000g for 10 min. Cells were resuspended in the defined medium of Muchmore et al.³³ containing 3F-tyrosine (10 mg per tyrosine residue in the respective protein per liter of defined medium) in place of tyrosine or 5-fluoroindole (60 mg/L) in place of tryptophan. The resuspended *E. coli* cells were incubated at 37 °C while being shaken for 1 h followed by cooling to 20 °C and equilibration of the temperature of the medium for 30 min. Protein expression was induced by 1 mM IPTG overnight (14–16 h) at 20 °C. The cells were harvested and stored at –80 °C. All proteins were purified as previously described.³¹

Protein-Observed Fluorine Nuclear Magnetic Resonance (NMR)

Protein-observed one-dimensional ¹⁹F NMR samples were obtained as previously described^{31,34} using a frequency window of –120 to –130 ppm, a D_1 of 0.7 s, an acquisition time of 0.05 s, a SW of 30 ppm, an NS of 1000, and a 90° pulse width. Peptide stock solutions were prepared in Milli-Q water at ~20 mM. Peptides were titrated into bromodomain protein [protein solutions at 40–50 μ M in 50 mM Tris, 100 mM NaCl, and 5% D₂O (pH 7.4)].

¹H CPMG NMR Spectroscopy

¹H CPMG NMR experiments were performed on a Bruker HD-500 NMR instrument. A series of ¹H CPMG NMR spectra were recorded during experiments performed in a single NMR tube with the initial run of 100 μM peptide, peptide with 10 μM protein, or peptide with protein and 100–800 μM competitor bromosporine or NH₂-AU1 (with a filter length of 1.2 s, an interpulse delay of 2.5 ms, and a *D*₂₀ of 25 ms, with the loop counter at 160).

Photo-Cross Linking with H2A.Z (unAcK, AcK15, and AcK4,11) Peptides

The procedure for protein expression and photo-cross-linking of BPTF-W2950AzF has been described previously.³⁵ W2950, reported previously, corresponds to W2824 used in this study. In brief, for photo-cross-linking experiments, 0.5–1 mM biotin-labeled histone H2A.Z peptides (unAcK, AcK15, and AcK4,11) were preincubated with 30 μM BPTF-W2950AzF in a buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20, and 0.5 mM TCEP. After incubation at room temperature for 30 min, samples were subjected to ultraviolet (UV) irradiation at 365 nm for 15 min at 4 °C. Negative controls were not subjected to UV irradiation. Samples were then bound to Ni-NTA agarose resin and incubated for 1 h at room temperature with gentle agitation. To remove un-cross-linked peptide, samples were washed 10 times each with 1 mL of washing buffer [50 mM Tris-HCl (pH 8.0), 200 mM NaCl, and 1% Triton X-100]. Finally, cross-linked proteins were eluted with a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM β-mercaptoethanol, and 400 mM imidazole. The eluted proteins were separated on a 4 to 12% Criterion XT precast sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel (Bio-Rad Laboratories) and analyzed by Western blotting as described below.

Western Blotting

Equal volumes of the pulled-down samples (both UV-irradiated and nonirradiated) were separated via SDS–PAGE and transferred onto a 0.2 μm supported nitrocellulose membrane (Bio-Rad Laboratories) at a constant current of 67 mA for 16 h at 4 °C. Membranes were blocked with 20 mL of TBST buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.01% Tween 20] with 5% nonfat dry milk for 1 h at room temperature while being gently shaken. The blocking buffer was then removed, and membranes were washed with 20 mL of TBST buffer. Immunoblotting was performed with a 1:500 primary antibody dilution (Biotin pAb, catalog no. PA1-26792, Invitrogen) for 16 h at 4 °C. The antibody solutions were removed, and membranes were washed three times with TBST buffer at room temperature. The blots were then incubated with HRP-conjugated secondary antibody donkey anti-goat IgG (H+L) pAb (catalog no. NBP1-74815, Novus) with 5% nonfat dry milk (1:500 dilution) in TBST for 1 h at room temperature. After similar washing, protein bands were visualized by chemiluminescence using VISIGLO HRP Chemiluminescent substrates A and B (catalog nos. N252-120ML and N253-120ML, respectively, aMReSCO) following the manufacturer's protocol.

RESULTS

Evaluation of Acetylation Patterns on H2A.Z with 5FW-BPTF Using ProF NMR

Bromodomain–histone interactions are characteristically weak interactions with measured affinities at midmicromolar to millimolar levels.³⁶ For example, the BPTF bromodomain has been characterized to recognize acetylated lysine 16 of histone H4 (H4acK16) with a K_d of 99–210 μ M by ITC and fluorescence polarization, whereas the adjacent PHD domain of BPTF binds H3K4Me3 with a K_d of 1.6–2.7 μ M (Figure 1).³⁷ Therefore, to characterize weak binding interactions with the BPTF bromodomain, we turned to NMR spectroscopy, which can be used to accurately quantitate micromolar and millimolar dissociation constants.

Protein-observed ^{19}F NMR (ProF NMR) has become a useful method for characterizing protein–ligand interaction binding sites and quantifying affinity, particularly for weakly binding ligands.^{38,39} In the case of several bromodomains, there is a tryptophan that is part of a three-residue hydrophobic region called the WPF shelf. This tryptophan is near the histone binding site and has been used to detect binding events by ProF NMR, including with BPTF.³⁴ For studies presented here, a single fluorinated BPTF bromodomain was expressed with W2824 fluorine-labeled as 5-fluorotryptophan using previously reported methods and defined as 5FW-BPTF for this report.³¹ This protein construct was used to characterize the affinity of all peptides described in this report.

To benchmark our method, we first characterized the interaction between the BPTF bromodomain and a 20-residue peptide of the N-terminal region of Histone H4, H4AcK16. Titration of this peptide with the fluorinated bromodomain and measurement of the change in chemical shift of W2824 of BPTF yielded a K_d of 300 μ M (Figure 2A), comparable to the reported affinity determined by fluorescence polarization of 210 μ M.³⁷ The slight difference may correspond to experimental conditions, or a small perturbation from fluorine incorporation. We next turned our attention to H2A.Z. H2A.Z is found in two isoforms, I and II. We elected to study a 20-residue peptide of H2A.Z I with an N-terminal tyrosine residue as a chromophore for accurate concentration determination, as isoform I has been studied more extensively than isoform II. This peptide will be called H2A.Z hereafter. There are five lysine residues in the active portion of H2A.Z that can potentially be acetylated and “read” by BPTF (K4, K7, K11, K13, and K15). To identify which, if any, acetylation state was necessary to bind BPTF, we acetylated the lysine residues in various mono-, di-, tri-, tetra-, and penta-acetylated patterns. Using ^{19}F NMR, we followed the shift of a single fluorine resonance from the 5-fluorotryptophan incorporated into BPTF (Figure 3A). An initial study of singly acetylated lysine residues showed H2A.Z AcK4, AcK7, and AcK11 to have the greatest affinities [$\sim$ 1.2–1.3 mM (Figure 3B,C)], whereas H2A.Z AcK15 and the unacetylated histone displayed either nonsaturating binding or no binding behavior.

On the basis of the singly acetylated histone results and the known effects of hyperacetylated histones at TSS,⁴⁰ we then tested an array of di-, tri-, tetra-, and penta-acetylated histone peptides (Figure 3D). From these titrations, diacetylated histone H2A.Z AcK4,11 showed the highest affinity for 5FW-BPTF with a K_d of 780 μ M and modest selectivity over the other acetylation patterns. H2A.Z AcK4,7 also had increased affinity compared to those of

other acetylation patterns, with a K_d of 990 μ M. Higher acetylation states, which included AcK4 and -11, led to attenuated binding, indicating avidity effects from hyperacetylation also do not promote this interaction. We conclude from these studies that the BPTF bromodomain is capable of binding acetylated H2A.Z histones, albeit more weakly than it binds conserved histone H4AcK16.

Various Bromodomains Evaluated for Affinity for H2A.Z AcK4,11

We selected four additional bromodomains as an initial evaluation of whether H2A.Z AcK4,11 was a preferred acetylation pattern for BPTF over other bromodomains. Two bromodomains we selected from the bromodomain and extraterminal (BET) family, Brd4 and BrdT, contain a WPF shelf similar to BPTF. We selected another bromodomain, GCN5 from *Plasmodium falciparum* (*PfGCN5*), which also contains a WPF shelf and is predicted to be structurally related to BPTF based on the phylogenetic tree analysis with human GCN5L2 and BPTF. A fourth unrelated malarial bromodomain that does not contain a WPF shelf, PFA0510w, was also evaluated. Malarial bromodomains were selected because within *P. falciparum*, *PfH2A.Z* is enriched at active var gene promoters, indicating these acetylated variants may have a specific functionality with regard to *P. falciparum* virulence.⁴¹

A ProOF NMR titration indicated that H2A.Z AcK4,11 exhibited a weaker affinity for the BET bromodomains (Figure 4A,B) with K_d values of 1.5 and 4.2 mM for the N-terminal bromodomains of Brd4 and BrdT, respectively. In the case of the Brd4 bromodomain, only the resonance for W81 was perturbed, consistent with binding near the histone recognition site. However, in the case of BrdT, the WPF shelf resonance for W50 was not affected, and only the resonance for W44 was perturbed. This latter interaction with BrdT may thus be a weak nonspecific binding event. Alternatively, *PfGCN5* showed a significant perturbation of the downfield fluorinated tryptophan resonance at -125.5 ppm, resulting in an affinity of 0.65 mM, similar to our results with BPTF (Figure 4C,D). We tentatively assign this resonance to the tryptophan in the WPF shelf, based on similar perturbations of this resonance from ProOF NMR experiments with the pan-bromodomain inhibitor, bromosporine (Figure S1A). Finally, because the second PFA0510w bromodomain lacks a WPF shelf, we labeled this protein with 3-fluorotyrosine, because of the presence of a conserved tyrosine within the binding site (Y1235). This final titration experiment with H2A.Z AcK4,11 exhibited little chemical shift perturbation, with only slight broadening of the upfield resonance at high concentrations, which we attribute to a nonspecific effect and did not pursue further (Figure S1B). Although the affinity is modest, these studies with H2A.Z AcK4,11 support a preference for the BPTF bromodomain and the structurally related *PfGCN5* bromodomain, although a larger panel of bromodomains would need to be evaluated to define a selectivity profile.

Ligand-Observed ^1H NMR Competition Experiments

Having established an acetylation pattern and preference for the *PfGCN5* and BPTF bromodomains, we further tested bromodomain binding site engagement versus a less specific binding mode using a ligand-observed ^1H NMR method, the CPMG competition experiment. The ^1H CPMG NMR experiment utilizes the transfer in the transverse relaxation time (T_2) of the protein to the ligand in the bound state that is subsequently

filtered out. This transfer is observed as a drop in resonance signal intensity when a ligand binds to the protein of interest. Recovery of the signal can be induced by addition of a high-affinity competitor.⁴² An initial experiment with only H2A.Z AcK4,11 shows the two acetyl singlet resonances near 1.88 ppm. When either *Pf*GCN5 or the BPTF bromodomain was added, a significant decrease in resonance intensity is observed, as expected if the ligand were binding to the bromodomain. In a separate experiment, we validated the pan-bromodomain inhibitor bromosporine binding to the *Pf*GCN5 bromodomain by PrOF NMR with high affinity (Figure S1). When we added bromosporine to *Pf*GCN5, a full recovery of resonance intensity was observed for *Pf*GCN5 with 400 μ M bromosporine (Figure 5A). However, in the case of BPTF, only partial recovery (34%) was seen upon addition of 400 μ M bromosporine. Titration of bromosporine with BPTF using PrOF NMR yielded a K_d of 37 μ M (Figure S2), which may be too weak for full displacement of H2A.Z from BPTF, or the compound may become aggregated and not soluble at high concentrations. We recently reported on a BPTF bromodomain inhibitor AU1 with a K_d of 2.8 μ M.³⁴ However, this compound is also not soluble at high concentrations. We therefore used an analogue capable of binding to the BPTF bromodomain, but with a protonatable benzylic amine for solubility, NH₂-AU1. In this case, addition of the inhibitor could dose-dependently restore the resonance intensity (75% at 800 μ M). The CPMG experiment corroborates our initial assessment in that H2A.Z AcK4,11 binds to both BPTF and *Pf*GCN5 and can be competitively displaced with small molecule inhibitors that engage the histone binding pocket.

Photo-Cross Linking Captures a Transient Bromodomain Interaction

Sudhamalla et al. recently showed that photo-cross linking experiments were capable of capturing transient bromodomain histone interactions using a BPTF bromodomain analogue in which the tryptophan in the WPF shelf was replaced with a *p*-azidophenylalanine (BPTF-*p*AzF).³⁵ Without higher-resolution structural biology data, we sought to capture the transient bromodomain histone interaction demonstrating bromodomain engagement near the WPF shelf. In this final set of experiments, we incubated three biotinylated H2A.Z peptide constructs with BPTF-*p*AzF-unacetylated H2A.Z, monoacetylated AcK15, and diacetylated AcK4,11. Using 0.5 and 1.0 mM peptide, each solution with BPTF was irradiated with 365 nm light. Photo-cross linked protein-peptide adducts were resolved via gel electrophoresis and detected via Western blotting with an anti-biotin antibody (Figure 6). In the case of the unacetylated H2A.Z, no cross-linking was observed. For H2A.Z AcK15, low levels of cross-linking were observed, which were consistent with the previously detected weak interactions from PrOF NMR titrations (Figure 3B,C). However, for diacetylated H2A.Z AcK4,11, an increased level of cross-linking in a dose-dependent fashion was observed. Control experiments showed no cross linking was detected in the absence of photoirradiation in all cases. We conclude from these studies that diacetylated H2A.Z AcK4,11 engages the BPTF bromodomain in a manner consistent with previously characterized histone interactions.

DISCUSSION

Nucleosomes with histone variant H2A.Z can alter chromatin accessibility, particularly near transcriptional start sites, leading to altered transcriptional programming.⁴³ Acetylation of H2A.Z was identified to play a fundamental role in changing chromatin states. SWR-1-Com, NuA4, and GCN5 have been shown to acetylate H2A.Z,^{16,44} and ANP32e is able to evict H2A.Z from the nucleosome.¹⁷ However, to date, no direct interaction of H2A.Z with a bromodomain had been characterized. Here, we identified specific acetylation states of the H2A.Z histone variant that can form transient interactions with the bromodomain of BPTF. All five lysine residues within the H2A.Z N-terminal tail were modified in a variety of mono- and multiacetylated patterns. ProOF NMR identified H2A.Z AcK4,11 to have high a micromolar affinity for BPTF ($K_d = 780 \mu\text{M}$). Acetylation of lysine 15 did not exhibit saturating binding behavior, arguing against a promiscuous acetyllysine recognition motif.

We compare our results with those of proteomics studies that have characterized abundant acetylation states of H2A.Z. In chicken erythrocytes, Dryhurst et al.⁴⁵ found a triacetylated version of H2A.Z to be the most abundant where it was specifically acetylated at lysine 4, 7, and 11. The second most abundant peptides found in their proteomic studies were diacetylated histones acetylated at lysine 4 and 7, lysine 7 and 11, and lysine 4 and 11. Proteomic analysis of H2A.Z from mouse embryonic stem cells revealed the monoacetylated lysine residues to be the most highly abundant with a preference for lysine 4, 7, and 11, while diacetylated lysines were the next most abundant. However, the diacetylation pattern for lysine 4 and 11 was detected at significant levels only when H2A.Z was ubiquitinated.⁹ These results demonstrate that diacetylation patterns on H2A.Z can be found under physiological conditions and that additional acetylation patterns in addition to lysine 4 and 11 may be relevant.

In our experiments, we attain only modest affinity of H2A.Z AcK4,11 for BPTF. This affinity should not be sufficient to facilitate H2A.Z nucleosome enrichment on its own as observed by Kim et al.²⁸ However, enhancement of H2A.Z deposition near TSS correlates strongly with H3K4me3, a known recognition site for the BPTF PHD domain.⁹ We believe the added affinity may strengthen a bivalent interaction (Figure 7), similar to how BPTF was demonstrated to interact with H4AcK16 and H3K4me3.³⁷ Ruthenburg et al. identified bivalent interactions of H4AcK16 with the bromodomain and H3K4me3 with the PHD domain of BPTF lead to an increase in total affinity greater than that seen with just one modification (2–3-fold enhancement of nucleosomal binding with both H4AcK16 and H3K4me3 over mononucleosomal binding with H3K4me3 alone).³⁷ While only modest increases were observed, the H3K4me3 interaction led to specificity toward only H4AcK16, even though H4AcK12 has a similar affinity alone. The interaction between H2A.Z AcK4,11 and BPTF may induce a similar bivalency with the PHD domain. This is a starting point in understanding the role acetylated H2A.Z plays in recognizing BPTF. Investigation of the bivalent interactions between the PHD and the bromodomain of BPTF with H2A.Z nucleosomes would be necessary to further understand their interactions in a more native-like environment.

Four other bromodomains were also evaluated in terms of their affinity for H2A.Z AcK4,11: Brd4, BrdT, *PfGCN5*, and PFA0510w. Of these proteins, only the *PfGCN5* bromodomain showed an affinity ($K_d = 650 \mu\text{M}$) comparable to that of BPTF. The human GCN5L2 bromodomain is structurally similar to BPTF, and both proteins bind H4AcK16. In *Saccharomyces cerevisiae*, GCN5 promotes acetylation and binds acetylated nucleosomes via its bromodomain.^{46,47} The human GCN5L2 bromodomain contains a deep hydrophobic cavity that binds N^e-acetylated lysines with modest affinity ($K_d \sim 900 \mu\text{M}$), with no affinity toward unacetylated peptides.⁴⁸ The *PfGCN5* bromodomain binds to H2A.Z AcK4,11 with an affinity similar to that of human GCN5L2 for H4AcK16. Although the human and *P. falciparum* H2A.Z sequences are not identical, these biochemical results for characterization of H2A.Z represent an interesting starting point for understanding the role of acetylated H2A.Z in regulating the virulence of the parasite.

CONCLUSION

We have studied the effects of N-terminal acetylation of histone variant H2A.Z on the direct interaction with bromodomains. Of the peptides tested, we identified a diacetylated lysine versus single or higher acetylation states of the H2A.Z histone that is responsible for recognition of the BPTF bromodomain. Both BPTF and H2A.Z play a significant role in cancer disease progression, and recent findings show a dependent functional activity of both proteins in bladder cancer. Our findings offer a potential link for modulating function through a direct interaction with H2A.Z in chromatin but posit that a higher-affinity multivalent interaction through a neighboring PHD domain may be necessary. Similar bromodomain interactions between H2A.Z and the *PfGCN5* protein may also be present in *P. falciparum*. Synthetic nucleosomes incorporating particular acetylation patterns have been useful for characterizing effector domain interactions using single-domain and multidomain protein constructs. These constructs will be useful for further testing this molecular recognition event.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

BPTF	bromodomain- and PHD finger-containing protein
CPMG	Carr–Purcell–Meiboom–Gill
BET	bromodomain extra terminal

PrOF NMR protein-observed fluorine nuclear magnetic resonance

BRD bromodomain

DDT DNA binding homeobox and different transcription factors

PHD plant homeodomain

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Figure 1.

Proteins and ligands characterized in this study. (A) BPTF sequence with varying domains, including the DNA binding homeobox and different transcription factors (DDT), two plant homeodomain (PHD) fingers, and the bromodomain (BRD). W2824, which is modified in this study, is indicated for the sake of clarity. (B) BPTF bromodomain with W2824 highlighted (orange) in complex with H4AcK16 (purple) (Protein Data Bank entry 3QZS). (C) BPTF small molecule ligands NH₂-AU1 and bromosporine and peptide ligands H2A.Z and H4AcK16. Acetylatable lysines are colored purple.

Figure 2. ProOF NMR experiments with H4AcK16 binding to 5FW-BPTF. (A) Binding isotherm of H4AcK16 binding to 5FW-BPTF with a K_d of 300 μM . (B) Stacked ^{19}F NMR spectra with an increasing concentration of the H4AcK16 peptide with 47 μM BPTF.

H2A.Z: YAGGKAGKDSGKAKTKAVSR-NH2

Figure 3.

H2A.Z fluorine NMR binding experiments with BPTF. (A) ProOF NMR titration overlay with increasing concentrations of H2A.Z AcK4,11 shifting the 5-fluorotryptophan resonance on BPTF (50 μM protein). (B) H2A.Z histone peptide binding isotherms with various monoacetylated states. (C) Dissociation constants determined using ProOF NMR on H2A.Z acetylated histone variants binding to 5FW-BPTF (N.B., nonbinding; N.S., nonsaturating). (D) Array of di-, tri-, tetra-, and penta-acetylated H2A.Z peptide ProOF NMR binding isotherms with BPTF. Dissociation constants are indicated with corresponding fitting errors from nonlinear regression analysis.

Figure 4. H2A.Z AcK4,11 histone peptide ProOF NMR binding isotherms with bromodomains (A) Brd4, (B) BrdT, and (C) BPTF and (D) malarial bromodomain *PfGCN5*. Dissociation constants are indicated with corresponding fitting errors from nonlinear regression analysis.

Figure 5.

Ligand-observed ¹H NMR CPMG competition experiments for evaluating H2A.Z AcK4,11 histone binding site occupancy: (A) *P/GCN5* and (B and C) BPTF. Experimental spectra are shown for the histone alone (black), the histone with the bromodomain (red), and the histone with the bromodomain and increasing concentrations of the inhibitor (blue and green in panel B) for bromosporine and (blue, green, magenta, and gold in panel C) for BPTF inhibitor NH₂-AU1.

Figure 6.

(A) *p*-Azido-phenylalanine mutant replaces W2824 in BPTF for photo-cross-linking experiments. This residue is part of the acetylated lysine recognition site within the WPF shelf of BPTF. (B) Photo-cross-linking anti-biotin antibody Western blot analysis of biotinylated H2A.Z variant peptides irradiated with BPTF-*p*AzF. Unacetylated, monoacetylated, and diacetylated H2A.Z peptides (unAcK, AcK15, and AcK4,11, respectively) were tested at 0.5 and 1.0 mM in the presence and absence of UV light.

Figure 7.

Cartoon depicting two domains on the BPTF PHD domain and bromodomain that bind post-translational modifications on histone tails. The PHD domain binds H3K4me3 with low micromolar affinity, while the bromodomain (BRD) binds H2A.Z AcK4,11 with high micromolar affinity. These two interactions may facilitate a bivalent intranucleosomal interaction as proposed by Ruthenburg et al.³⁷