Selective Recognition of Acetylated Histones by Bromodomain Proteins Visualized in Living Cells

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4Present address: Molecular Immunology and Inflammation Branch, also their persistence during cell division in vivo. To achieve this, we employed a flow cytometric adaptation of the fluorescence resonance energy transfer technique (FRET) recently developed to study cell surface receptors (Siegel et al., 2000a, 2000b; Chan et al., 2001). FRET

Summary
Acetylation and other modifications on histones comprise histone codes that govern transcriptional regulatory processes in chromatin. Yet little is known how different histone codes are translated and put into action. Using fluorescence resonance energy transfer, we show that bromodomain-containing proteins recognize different patterns of acetylated histones in intact nuclei of living cells. The bromodomain protein Brd2 selectively interacted with acetylated lysine 12 on histone H4, whereas TAFII250 and PCAF recognized Brd2, Brd4, and Bdf1, and ATP-dependent chromatin remodeling factors such as BRG-1 (Jeanmougin et al., 1997). In yeast, bromodomains are involved in gene activation, and in antisilencing of genes at heterochromatin boundaries through chromatin association (Hassan et al., 2000; Ladurner et al., 2003; Matangkasombut and Buratowski, 2003). Brd4, a mammalian BET protein structurally similar to Brd2, has been shown to associate with chromatin (Dey et al., 2000, 2003). Also, an acetylation-dependent recruitment of bromodomain-protein complexes to chromatin-associated DNA targets has been demonstrated when nucleosomes were reconstituted in vitro (Agalioti et al., 2002; Hasan et al., 2002). A variety of HATs exhibit unique substrate specificity, discriminating between individual lysine residues of histones when assayed in vitro (Roth et al., 2001). Thus the potential exists for creating diverse patterns of acetylation, thereby establishing fine specificity within the histone code. This possibility is further strengthened by the implication of essential roles of H3 and H4 acetylations in transcription (Howe et al., 2000; Agalioti et al., 2002; An et al., 2002; Smith et al., 2002). Since bromodomains contained in a variety of transcriptional regulators and chromatin remodeling proteins recognize acetylated histones, they are predicted to possess the ability to recognize a diverse array of acetylated histone codes. Although several studies have recently addressed this question (Agalioti et al., 2002; Hassan et al., 2002; Ladurner et al., 2003; Matangkasombut and Buratowski, 2003), the levels of specificity and the mechanisms by which the bromodomains embedded in the whole protein recognize chromatin have yet to be fully resolved. The universal histone code created by acetylation as well as other modifications is likely to be complex. However, even a simple rule by which bromodomain proteins recognize specific acetylated histones in living cells has not been well established.

Introduction
Covalent modifications of histone proteins, such as acetylation, phosphorylation, methylation, and ubiquitination of the N-terminal tails of histones, have been hypothesized to constitute a histone code that controls patterns of gene expression (Strahl and Allis, 2000; Turner, 2002). Some histone modifications are short-lived and implicated in inducible gene activation or repression. Others may be inherited down through cell divisions in order to take part in stable transcriptional memory such as in developmental processes and cellular differentiation. Evidence suggests that the covalent modifications of histone tails serve as physical interaction surfaces on nucleosome assemblies to recruit chromatin remodeling proteins and transcription regulatory proteins (Strahl and Allis, 2000; Turner, 2002).

Acetylation of histones correlates with gene activation and can be recognized by chromatin-associated proteins containing bromodomains (Dhalluin et al., 1999; Jacobson et al., 2000; Owen et al., 2000). The bromodomain is a module of about 110 amino acids that is conserved in many chromatin-associated proteins including histone acetyltransferases (HATs) such as PCAF and TAF250, the BET family of nuclear proteins such as Brd2, Brd4, and Bdf1, and ATP-dependent chromatin remodeling factors such as BRG-1 (Jeanmougin et al., 1997). In yeast, bromodomains are involved in gene activation, and in antisilencing of genes at heterochromatin boundaries through chromatin association (Hassan et al., 2000; Ladurner et al., 2003; Matangkasombut and Buratowski, 2003). Brd4, a mammalian BET protein structurally similar to Brd2, has been shown to associate with chromatin (Dey et al., 2000, 2003). Also, an acetylation-dependent recruitment of bromodomain-protein complexes to chromatin-associated DNA targets has been demonstrated when nucleosomes were reconstituted in vitro (Agalioti et al., 2002; Hasan et al., 2002). A variety of HATs exhibit unique substrate specificity, discriminating between individual lysine residues of histones when assayed in vitro (Roth et al., 2001). Thus the potential exists for creating diverse patterns of acetylation, thereby establishing fine specificity within the histone code. This possibility is further strengthened by the implication of essential roles of H3 and H4 acetylations in transcription (Howe et al., 2000; Agalioti et al., 2002; An et al., 2002; Smith et al., 2002). Since bromodomains contained in a variety of transcriptional regulators and chromatin remodeling proteins recognize acetylated histones, they are predicted to possess the ability to recognize a diverse array of acetylated histone codes. Although several studies have recently addressed this question (Agalioti et al., 2002; Hassan et al., 2002; Ladurner et al., 2003; Matangkasombut and Buratowski, 2003), the levels of specificity and the mechanisms by which the bromodomains embedded in the whole protein recognize chromatin have yet to be fully resolved. The universal histone code created by acetylation as well as other modifications is likely to be complex. However, even a simple rule by which bromodomain proteins recognize specific acetylated histones in living cells has not been well established.

We wished to develop an approach that would allow the delineation of general patterns of the interactions between bromodomains and acetylated histones and also their persistence during cell division in vivo. To this end, we employed a flow cytometric adaptation of the fluorescence resonance energy transfer technique (FRET) recently developed to study cell surface receptors (Siegel et al., 2000a, 2000b; Chan et al., 2001). FRET
is based on the ability of a high-energy donor fluorophore to transfer energy directly to a lower energy acceptor fluorophore. In FC-FRET, spectral variants of the green fluorescent protein (GFP) that carry out energy transfer are quantified by flow cytometry in living cells. A FRET signal between two GFP variants requires proximity at the Angstrom level and can identify specific protein-protein interactions. This technique could allow the detection of bimolecular interactions between bromodomain proteins in macromolecular complexes and target histones within nucleosomal histone octamers in the intact nuclei of living cells.

Results

FRET Reveals Specific Histone-Nuclear Protein Interactions in Living Cells

To perform FC-FRET analysis, bromodomain proteins were fused to cyan fluorescent protein (CFP) as a donor, and histones were fused to yellow fluorescent protein (YFP) as an acceptor. We first expressed YFP-histone H1 (YFP-H1) or YFP-histone H4 (YFP-H4) in HeLa cells and found that these histones localized to the nucleus during interphase and to chromosomes during mitosis (Figure 1A). YFP-H4 was in the nucleosomal fraction and acetylated as efficiently as the endogenous H4 (Figure 1B), as in the previous reports (Lever et al., 2000; Kimura and Cook, 2001; Ahmad and Henikoff, 2002). Importantly, lysines K5, K8, K12, and K16 were all acetylated in YFP-H4, as reported for endogenous H4 (Turner et al., 1989). All other YFP-histones, including mutants used in the present study, were also incorporated into chromatin (data not shown). Thus, ectopically expressed YFP-histones behaved identically to the endogenous histones.

We first tested a bromodomain protein, Brd2 (formerly Ring3) (Denis and Green, 1996), as an effector molecule that may recognize acetylated histones. Brd2 belongs to the BET family of transcriptional regulators conserved from yeast to humans (Dey et al., 2000; Ladurner et al., 2003; Matangkasombut and Buratowski, 2003). Transfected CFP-Brd2 was expressed in reasonable proportion to endogenous Brd2 (Figure 1C), localized to the nucleus (Figure 1A), and was associated with an essential component of Mediator, TRAP220 (data not shown) in a similar way to endogenous Brd2 (Jiang et al., 1998). We then performed FC-FRET between YFP-H4 and CFP-Brd2 in living HeLa cells. Three fluorescent signals were separately measured in each cell: CFP, YFP, and FRET, where FRET is fluorescence in the YFP emission channel triggered by CFP excitation that was optimally compensated as described (Siegel et al., 2000a, 2000b; Chan et al., 2001). In Figure 1D, a substantial FRET signal was observed between CFP-Brd2 and YFP-H4, but not between CFP-Brd2 and YFP-H1. To compare FC-FRET results among different combinations of proteins, we selected cell populations expressing equivalent levels of CFP- and YFP-proteins (Figure 1E). When CFP-Brd2 and YFP-H4 were cotransfected, 23% of gated CFP/YFP double-positive cells showed FRET. On the other hand, FRET was not detected between CFP-Brd2 and YFP-H1. We also tested the latency-associated nuclear antigen (LANA) of Kaposi’s sarcoma-associated herpes-
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Selectivity of Brd2-Histone Interactions

We next determined whether Brd2 interacts with other core histones (Figure 2A). H2B produced a significant FRET signal, although consistently less than H4. In contrast, H3 and H2A yielded little or no FRET signals. Thus Brd2 selectively interacts with H4 and, to a lesser degree, H2B. Given the short range over which energy transfer can occur, it is highly unlikely that the Brd2-H4 interaction is indirect (Siegel et al., 2000b). Further supporting a direct, specific recognition of H4 acetylations by Brd2, we found that removing the N-terminal tail (H4ΔN) abolished FRET, indicating that the N-terminal tail of H4 is essential for interaction (Figure 2A). In the H4 tail, mutations in lysines K5 and K12, but not K8 and K16, effectively abolished FRET (Figure 2A), indicating that K5 and/or K12 are the main residues involved in the interaction with Brd2 in vivo. Importantly, the levels of acetylation at unaltered lysines in these mutants were similar to those of wild-type (Figure 2B; and data not shown), indicating that the selective abolition of acetylation at mutated residues accounted for the loss of FRET signal.

To establish independent evidence for the interaction between Brd2 and acetylated H4 observed with FC-FRET, we examined the biochemical association of Brd2 with nucleosomes (Figure 2C). Mononucleosomes were prepared from HeLa cells expressing FLAG-tagged Brd2 or control FLAG constructs by digesting isolated nuclei with micrococcal nuclease. Anti-FLAG antibody coprecipitated nucleosomes containing acetylated H4 from cells transfected with FLAG-Brd2, but not FLAG control cells (Figure 2C, right panel). Similar results were obtained for endogenous Brd2-nucleosome complexes immunoprecipitated by anti-Brd2 antibody (data not shown). Thus, Brd2 is associated with nucleosomes in vivo. However, the nucleosomal histones that directly contact with Brd2 cannot be determined by this type of coprecipitation assays, since all core histones are precipitated as a complex. Therefore, to identify the residues on the H4 tail that actually contact Brd2, we performed in vitro peptide-precipitation assays. HeLa nuclear extracts were incubated with biotinylated H4 tail peptides, either unacetylated or acetylated at various lysines, and bound complexes were precipitated by biotin-avidin interactions. Endogenous Brd2 selectively bound to tetra-acetylated H4 (Figure 2D) and H4 peptides di-acetylated at K5/K12 but not at K8/16 (Figure 2E). In blocking experiments, unbiotinylated H4 peptides di-acetylated at K5/12 but not K8/16 blocked Brd2 binding to tetra-acetylated H4 peptides (Figure 2E). Further analysis showed that CFP-Brd2 bound strongly to H4 peptides acetylated at K12, but very weakly to those acetylated at K8 or K16 (revealed only after long exposure), and not at all to those acetylated at K5 (Figure 2F). In contrast, Brd2 did not bind to H3 peptides unmodified or acetylated at K9 and/or K14 (Figure 2F). These results provide strong biochemical validation of our FRET data and show that Brd2 specifically recognizes acetylated H4 through K12. Indeed, the single amino acid substitution of YFP-H4 at K12, but not at K5, with arginine effectively abolished FRET with CFP-Brd2 (Figure 2G). Also, the weak FRET with H2B represented a weak interaction of Brd2 with acetylated H2B, since removing the N-terminal tail (H2BΔN) significantly reduced FRET (Figure 2H), and in peptide binding experiments Brd2 bound to acetyl-lysine at K5 and K12 but not K15 or K20 of H2B (Figure 2I).

HATs Bromodomains Interact with H3 as Well as H4

Given that H3 is a preferred substrate for many HATs in vitro assays (Roth et al., 2001), the absence of FRET between H3 and Brd2 was striking. This could not be explained by a lack of H3 acetylation, since HeLa cells contained significant amounts of acetylated H3 (Figure 3A). This led us to test another bromodomain protein, TAF$_{	ext{II}250}$, for interaction with H3 (Figure 3B). TAF$_{	ext{II}250}$ is a component of the basal transcription factor TFIIID, and its double bromodomain module binds to an acetylated H4 peptide in vitro (Jacobson et al., 2000) and acetylated H3 in a reconstituted chromatin target (Agalioti et al., 2002). Transfected CFP-TAF$_{	ext{II}250}$ associated with other components of TFIIID in HeLa cells, indicating that it was incorporated into a stable TFIIID complex (data not shown). We found that TAF$_{	ext{II}250}$ produced a significant FRET signal with H3 as well as H4 and to a lesser extent H2B but not with H2A (Figure 3C). Because TAF$_{	ext{II}250}$ has a HAT activity and contains a histone recognition site outside the bromodomains (Mizzen et al., 1996), we tested deletions that removed the HAT region, but retained the bromodomains (Figure 3B). The deletions produced FRET with H3 and H4 as well as did full length TAF$_{	ext{II}250}$ (Figure 3C; and data not shown). It is of note that these deletions also lacked the TBP binding surface, and TAF$_{	ext{II}250}$-BD is further devoid of the putative HMG-like region, indicating that DNA binding of TAF$_{	ext{II}250}$ is dispensable for its histone association. Thus, H3 is recognized by the bromodomains of TAF$_{	ext{II}250}$ but not of Brd2. Moreover, in contrast to FRET with Brd2, both mutants H4-K(5,12)G and H4-K(8,16)G partially reduced FRET with TAF$_{	ext{II}250}$, indicating that K5/K12 and K8/K16 of H4 virus, which is known to interact with H1 (Cotter and Robertson, 1999). A positive FRET signal was observed between CFP-LANA and YFP-H1, but not between CFP-LANA and YFP-H4. Also, cells transfected with CFP-proteins alone or YFP-histone alone were negative for FRET. Thus, Brd2 interacts with the core histone H4, but not with the linker histone H1, while LANA interacts with H1, but not with H4. These data show that FC-FRET allows detection of specific bimolecular interactions between histones and nuclear proteins in the native chromatin. These interactions could not be attributed to overexpression of the transfected proteins driving spurious interactions, since the amount of YFP-H4 was about 3% of endogenous H4 whereas the amount of CFP-Brd2 was about 50% of endogenous Brd2 (see Figure 1C legend). Competition between the CFP-labeled Brd2 as well as YFP-labeled histones with large amounts of their endogenous counterparts most likely resulted in the observed population of cells that exhibited FRET of less than 100%. In all the FRET analyses performed in the present study, gated cell populations expressing equivalent levels of CFP- and YFP-proteins were compared, and threshold lines were drawn to yield <1% FRET-positive cells on singly transfected cells in the same set of experiments.
Figure 2. Brd2-Histone Interactions Require K12 of H4 or K5/K12 of H2B

(A) FRET analysis of Brd2 interaction with H2A, H2B, H3, or H4 N-terminal tail mutants. Histograms shown are gated on CFP- and YFP-fusion protein-expressing cells as in Figure 1.

(B) YFP-H4, YFP-H4-K(5,12)G, YFP-H4-K(8,16)G, or YFP was acid-extracted from transfected HeLa cells and blotted with the indicated antibodies.

(C) Mononucleosome preparations from HeLa cells expressing FLAG-Brd2 or control FLAG were immunoprecipitated with FLAG antibody followed by elution with FLAG peptide. Eluted nucleosomes were analyzed by immunoblot with tetra-acetyl H4 antibody (the right panel). For input samples, nucleosomal DNA staining in agarose gel after proteinase K digestion, and immunoblots of FLAG-Brd2 and acetyl H4 are shown. An asterisk indicates nonspecific bands.

(D) HeLa nuclear extract was incubated with biotinylated H4 peptides, either unmodified (H4) or tetra-acetylated (AcH4), and precipitated with streptavidin beads followed by immunoblot with Brd2 antibody.

(E) Peptide precipitation assays as in (D) with biotinylated H4 peptides di-acetylated at K8/K16 or K5/K12 (the left two lanes). In the right two lanes, extracts were incubated with unconjugated H4 peptides di-acetylated at K8/K16 or K5/K12 followed by incubation with biotin-conjugated tetra-acetylated H4.

(F) Peptide precipitation assays with extracts from CFP-Brd2 transfected HeLa cells and mono-acetylated H4, or mono- or di-acetylated H3 peptides. CFP-Brd2 was detected with GFP antibody.

(G and H) FRET analysis of Brd2 interaction with H4 mutants (G) or a H2B mutant (H).

(I) Peptide precipitation assays with extracts from CFP-Brd2-transfected HeLa cells and mono-acetylated H2B peptides.
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Figure 4. Specificity of Histone Recognition Is Intrinsic to Bromodomains

(A) Diagram of chimeric proteins. BD, bromodomain; NLS, nuclear localization signal; ET, the ET domain; B1 and B2, the bromodomains #1 and #2 of Brd2, respectively; T1 and T2, the bromodomains #1 and #2 of TAFII250, respectively; LB, the linker region of Brd2; LT, the linker region of TAFII250.

(B) FRET analysis of the interaction between the indicated core histone and TAFII250 or TAFII250BD.

Figure 3. TAFII250 Bromodomains Interact with H3 as Well as H4

(A) Nucleosomal preparations from HeLa cells and those expressing YFP-H3 were analyzed by immunoblot with di-acetyl H3 antibody.

(B) Diagram of wild-type TAFII250 and truncated constructs. HAT, histone acetyltransferase; NLS, nuclear localization signal; BD, bromodomain.

(C) FRET analysis of the interaction between the indicated core histone and TAFII250 or TAFII250BD.

(D) FRET analysis for TAFII250 and H4 N-terminal tail mutants.

(E) Peptide precipitation assays using extracts from CFP-TAFII250-transfected HeLa cells and the indicated H4 or H3 peptides. CFP-TAFII250 was detected with GFP antibody.

(F) FRET analysis of the interaction between PCAF and the indicated core histone. Histograms in (C), (D), and (F) were generated as in Figure 1.

Figure 4. Specificity of Histone Recognition Is Intrinsic to Bromodomains

(A) Diagram of chimeric proteins. BD, bromodomain; NLS, nuclear localization signal; ET, the ET domain; B1 and B2, the bromodomains #1 and #2 of Brd2, respectively; T1 and T2, the bromodomains #1 and #2 of TAFII250, respectively; LB, the linker region of Brd2; LT, the linker region of TAFII250.

(B) CFP-tagged bromodomain chimeric proteins were tested for FRET with YFP-histones and analyzed as in Figure 1.

tides acetylated at K14 (Figure 3E). We conclude that specific lysine residues of both H3 and H4 are recognized by the TAFII250 bromodomains in vivo and that TAFII250 has broader recognition specificity than Brd2.

As the third bromodomain protein, we tested PCAF, a HAT that has a single bromodomain. PCAF produced FRET signals with both H3 and H4 but not H2A or H2B (Figure 3F). Peptide-precipitation assays revealed PCAF binding to both acetylated H3 and H4 (data not shown), consistent with the previous study (Dhalluin et al., 1999). These results collectively indicate that there is fine specificity among bromodomains in recognizing differentially acetylated histones and that bromodomains of HAT proteins constitute a discrete recognition group different from Brd2.

Histone Recognition Specificity Is an Intrinsic Property of Bromodomains

To gain insight into the basis of distinct recognition of H3 and H4 by different bromodomain proteins, we constructed two chimeric proteins in which both of the bromodomains in Brd2 were replaced with those from TAFII250 in the Brd2 backbone (Figure 4A). In Brd2-TAFBD1/BD2, the linker region between the original Brd2 bromodomains was preserved, while in Brd2-TAFBD-unit, the whole double bromodomain unit from...
helices with two loops: one between α2 and αA, and the other between αB and αC (Figure 5A) (Zeng and Zhou, 2002). Conserved residues V, Y, Y, and Y (boxed in Figure 5A) in the loops in PCAF are critical for the formation of a hydrophobic pocket that accepts an acetylated lysine (Dhalluin et al., 1999), and the first tyrosine residue in the corresponding residues of Gcn5p makes a strong hydrogen bond with acetyl-lysine (Owen et al., 2000). We therefore assessed which amino acid residues of the two Brd2 bromodomains were important for the specific interactions with acetylated histones. First, mutations were introduced into the four conserved amino acids in each of the bromodomains (BD1-m4, BD2-m4), or all eight in both domains (BD-m8) (Figure 5B). BD-m8 failed to produce FRET with YFP-H4, whereas BD1-m4 and BD2-m4 showed reduced, but significant, levels of FRET (Figure 5C). The histone tail deleted control (H4ΔN) showed the background level of FRET. Thus, one intact Brd2 bromodomain is sufficient for a partial interaction with H4, and mutations in both bromodomains abrogate the interaction. Peptide precipitation assays confirmed the complete loss of acetyl-H4 binding activity by BD-m8 and that the association of H4 with Brd2 depended on the acetylation (Figure 5D). We then tested Brd2 bromodomain mutants with fewer substitutions (Figures 5B and 5E) and found that single substitutions of the critical tyrosine to phenylalanine in both bromodomains were sufficient to abolish FRET with H4, which is dependent on H4 K12. These results reveal the importance of the conserved residues of the bromodomains for interaction with acetyl-H4 and show that the observed FRET signal is mediated by a direct interaction between the hydrophobic pockets of the Brd2 bromodomains and the acetylated K12 of H4 tail. The Brd2 mutants with the substitutions in both bromodomains also failed to produce FRET with YFP-H2B (data not shown), indicating that the interaction with H2B is also mediated by the hydrophobic pocket of the Brd2 bromodomains.

Brd2-Histone Interaction and Transcription

We next explored a link between the Brd2-acetylated histone interaction and transcriptional regulation. Previously, it was reported that Brd2 regulates Ras-mediated cyclin E promoter activity (Denis et al., 2000). We found that the wild-type Brd2 but not its double bromodomain mutant BD-m8 enhanced the promoter activity in response to constitutively active Ras in a Brd2 dose-dependent manner (Figure 6A). Interestingly, Ras expression led to a marked increase in H4 acetylation at K12 in these cells, the specific residue that promotes Brd2-H4 association (Figure 6C). The effect of Ras was selective for K12 of H4, in that acetylation levels of K8 on H4 and K9/K14 on H3 remained unchanged. Therefore, we conclude that the interaction of its bromodomains with acetylated histone H4, especially K12, is essential for the role of Brd2 in amplifying Ras-mediated cyclin E transcription.

Brd2-Histone Interaction during Mitosis

Some BET family proteins persist on mitotic chromosomes (Chua and Roeder, 1995; Dey et al., 2000, 2003), while other bromodomain proteins leave chromosomes during mitosis (Muchardt et al., 1996; Kruhlak et al., 2001). YFP-tagged Brd2 mostly localized on mitotic chromosomes, whereas the double-bromodomain mutants, BD(1-2)-Y/F and BD-m8, which did not interact with acetylated H4 in FRET analysis, were dispersed into the extrachromosomal space (Figure 7A). The single-bromodomain mutant BD2-Y/F that interacted with H4 retained the ability to associate with mitotic chromosomes (Figure 7A). These data indicate that bromodomain-mediated recognition of acetylated H4 is crucial for the retention of Brd2 on mitotic chromosomes. In contrast, we found that YFP-TAF250 and YFP-PCAF were dispersed into the nonchromosomal space during mitosis (Figure 7B; and data not shown), consistent with the previous study for the endogenous counterparts (Kruhlak et al., 2001). To further assess the ability of the TAF250 bromodomains to associate with mitotic chromosomes, we tested the two chimeric Brd2 constructs that have their bromodomains substituted with the TAF250 bromodomains. Both of them no longer associated with mitotic chromosomes and were dispersed into the nonchromosomal space (Figure 7B). These results indicate that it is the bromodomain structures unique to Brd2 that govern the retention on mitotic chromosomes.

To gain further insight into the basis of the bromodomain-mediated mitotic chromosomal retention of Brd2, we wished to visualize interactions under microscope. To do so, we used bimolecular fluorescence complementation (BiFC) (Hu et al., 2002). In this technique, the full length YFP is split into two fragments, amino-terminal YFP [Y(N)] and carboxy-terminal YFP [Y(C)], both of which are nonfluorescent. When these two nonfluorescent YFP fragments closely associate, they complement each other and emit YFP fluorescence, which can be visualized in living cells. Complementation of YFP signal is achieved only when the two fragments are individually fused to a pair of directly interacting protein partners. We therefore constructed Y(N)-Brd2 and Y(C)-H4 to attempt to visualize the Brd2-H4 interaction. When transfected alone, neither Y(N)-Brd2 nor Y(C)-H4 produced a YFP signal, as expected (Figure 7C). However, coexpression of the two proteins generated a clear YFP (BiFC-YFP) signal seen throughout the interphase nucleus with the exception of nucleoli (Figure 7C). Control experiments using Y(N)-Brd2-BDm8, Y(C)-H4ΔN, or
Figure 5. Point Mutations in the Bromodomain Abolish H4 Interations

(A) Diagram of bromodomains. Dashed boxes indicate α helices.

(B) Diagram showing wild-type Brd2 and its mutants. BD, bromodomain. Positions of point mutations are marked as “A” (alanine substitutions) or “F” (phenylalanine substitutions).

(C and E) Brd2 bromodomain mutants were tested for FRET.

(D) Peptide precipitation assays using H4 peptides (unmodified or tetra-acetylated) and extracts from HeLa cells expressing CFP-Brd2 or CFP-Brd2-BDm8.

Y(C)-H3 showed negligible reconstitution of YFP signals. To quantitate the specific complementation by Y(N)-Brd2 and Y(C)-H4 in comparison with these controls, the complemented YFP signals were measured by flow cytometry. Only the combination of Y(N)-Brd2 and Y(C)-H4 produced significant YFP signals over background levels (Figure 7D). Thus it is the specific Brd2-H4 interaction that was visualized in living cells by BiFC. In cells undergoing mitosis, coexpression of Y(N)-Brd2 and Y(C)-H4 showed clear Brd2-H4 interaction on mitotic chromosomes (Figure 7E). Moreover, Y(N)-Brd2 and Y(C)-H4-K(8,16)G complemented each other and produced BiFC-YFP signals on mitotic chromosomes, indicating that K8 and K16 of H4 are not critically required for the interaction between Brd2 and H4 on mitotic chromosomes (Figure 7E).

Discussion

In the present study, we found highly selective recognition of acetylated histones by different bromodomain proteins in the physiological context of living cells. The concept of a histone code raises the question of the generality of the information contained in various his-
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tone modifications and how it is interpreted by the nuclear transcription machinery. Although the histone code is likely to be very complex and difficult to fully unravel, our approaches with FC-FRET and BiFC allowed us to observe patterns of selective recognition of acetylated histones by different bromodomains taking place in living cell nuclei. The fact that these recognition patterns were assayed independently of any particular gene or promoter, and that they were fully consistent with in vitro peptide binding assays, suggests that they may represent general patterns that guide bromodomain-histone interactions involving many genes in vivo.

To detect interactions between bromodomains and nucleosomal histones in living cells, we utilized a recently developed FC-FRET technique. The advantages of this technique include (1) avoiding fixation or destruction of cells and (2) allowing analyses of individual interactions occurring in or between protein complexes in the physiological environment. The N-terminal tail of each core histone protrudes from the nucleosome core in a similar manner, allowing direct comparisons between them for the interaction with bromodomain proteins. We observed specific binding patterns with selected histone tails by each of the bromodomain proteins analyzed.

Because of the tight packing of nucleosomes and the potential flexibility of N-terminal histone tails, however, it may be theoretically possible that binding to one histone tail or even other types of nucleosome binding such as direct DNA binding might juxtapose a bromodomain.

Figure 6. Point Mutations in the Bromodomain Abolish the Transcriptional Function of Brd2

(A) Luciferase assays were performed in triplicate in NIH 3T3 cells using a cyclin E promoter reporter, expression vectors for active Ras (rasV12), and increasing amounts of wild-type Brd2 or Brd2-BDm8. (B) Comparable expression of Brd2 and Brd2-BDm8 was confirmed by immunoblot.

(C) NIH 3T3 cells and those expressing rasV12 were acid extracted and analyzed by immunoblot with the indicated antibodies.

Figure 7. Brd2-H4 Interactions Persist during Mitosis

(A and B) Microscopic images of YFP-tagged constructs on mitotic P19 cells and the corresponding DNA staining.

(C) BiFC between Y(N)-Brd2 and Y(C)-H4 in living HeLa cells visualized by a confocal microscope tuned for YFP signals.

(D) BiFC signals (fluorescence in the YFP emission channel) in living HeLa cells transfected with indicated constructs were analyzed by flow cytometry. Threshold lines are drawn to yield <0.5% YFP-positive cells on singly transfected cells.

(E) BiFC analysis was performed on living P19 cells undergoing mitosis.
protein to noninteracting histone tails in the same or a neighboring nucleosome and produce "false" FRET. However, our domain-swap and mutagenesis experiments argue against this possibility. For Brd2, point mutations introduced in either H4 or the Brd2 bromodomains abrogated FRET signals, indicating a specific interaction with H4. The absence of FRET by Brd2 with H1, H2A, H3, and the H4 and H2B mutants indicates that the potential juxtaposition of these histones to Brd2 when it was bound to native H4 was not sufficient to produce FRET. For TAF,250, although it binds to DNA in the TFIID complex, the truncated versions devoid of DNA binding still produced specific and comparable FRET signals as the full length TAF,250, indicating that DNA binding was unlikely to account for FRET signals. These observations lend credence to the proposition that only direct interactions between bromodomains and histone tails produced significant FRET in the present study. Another important consideration is that FRET depends on both the distance between fluorophores and their relative orientation so that the conformation as well as the size of a fluorophore-tagged protein may affect the FRET efficiency. Therefore it was important to confirm that the absence of FRET between YFP-H3 and CFP-Brd2 was not due to an unfavorable three-dimensional conformation of Brd2. The chimeric proteins in which bromodomains of Brd2 were exchanged with those of TAF,250 exhibited FRET with H3 in the Brd2 backbone, eliminating the possibility that a unique structure of Brd2 prevented FRET with H3. Together, the present study demonstrates that the FRET technique can be applied to the study of protein-protein interactions in the native chromatin environment. In addition, the flow cytometric method employed in this work allows an analysis of thousands of cells simultaneously. The data collected by FC-FRET represent statistical values of in vivo interactions and allow quantitative comparisons among different combinations of interactions. Importantly, the same specificity of acetyl-histone recognition by bromodomains was found using peptide precipitation assays.

In the present study, we found that bromodomains from Brd2 specifically recognized acetylated K12 of H4, whereas those in TAF,250 and PCAF recognized both acetylated H3 and H4 with broader acetyl-lysine specificities. The results suggest that each acetyl-lysine residue carries a distinct code that is recognized by distinct downstream transcriptional regulatory proteins containing bromodomains. Although several recent studies have addressed similar questions, these studies have not provided general principles governing bromodomain recognition of acetylated histones. A yeast bromodomain protein Bdf1 is reported to interact with both acetylated H3 and H4 in vitro (Ladurner et al., 2003; Matangkasombut and Buratowski, 2003). While Bdf1 and the closely related protein Bdf2 both interacted with unacetylated and acetylated H4, only Bdf1 exhibited preference for hyperacetylated forms of H4, and this H4 interaction was crucial for chromatin association and the support of viability by Bdf1 (Matangkasombut and Buratowski, 2003). Ladurner et al. (2003) demonstrated that the interaction with H4 but not H3 by Bdf1 was tight enough to protect acetyl-lysines from Sir2-mediated deacetylation. Hassan et al. (2002) measured in vitro binding of yeast SWI/SNF and SAGA complexes to reconstituted nucleosome arrays, and they reported that there was no difference between acetylated H3 and H4 in their ability to retain the entire protein complexes containing bromodomain proteins. However, one of the bromodomain proteins in the SAGA complex failed to interact with acetylated histones despite its intrinsic ability, indicating that specificity of bromodomain function is context dependent. Agalioti et al. (2002) demonstrated a differential requirement of distinct acetyl-lysines by bromodomain proteins in their nucleosomal association in the interferon-β promoter reconstituted in vitro. The recruitment of BRG-1 in the SWI/SNF complex specifically required acetylated K8 of H4, while the subsequent recruitment of TAF,250 required acetylated K9 and K14 of H3. However, the highly specific interaction of TAF,250 with H3 was observed only in the enhancer/promoter and chromatin context, and when tested in isolation, the TAF,250 bromodomains interacted with both H3 and H4. It is not clear to what extent the requirement of acetylated histones by bromodomain proteins observed for a model gene applies to the general behavior of bromodomains in the cell or to those on different genes/promoters.

Our FC-FRET analysis allows measurement of global bromodomain-histone interactions in a steady state in living cells. Although individual interactions may take place in a variety of contexts, average signals in each cell were acquired in this study. The present results clearly indicate the distinct recognition of acetyl-lysines on H3 and H4 by different bromodomains. As revealed by bromodomain swap experiments, recognition of specific acetyl-lysine on histone is an intrinsic property of the bromodomain. Thus, the bromodomains present in different proteins must harbor sufficient structural diversity to support specific recognition of acetylation codes, most likely located in the loop structures which form a hydrophobic pocket. What allows the bromodomains of TAF,250 and PCAF, but not those of Brd2, to interact with H3 is a question for future study. It is possible that amino acids near the critical acetyl-lysine of H3 contribute to the interaction specificity, as is the case for the PCAF-HIV Tat interaction (Mujtaba et al., 2002) or the Gcn5-H4 interaction (Owen et al., 2000; reviewed in Zeng and Zhou, 2002).

While the state of histone acetylation is shown to influence inducible gene expression in various organisms, what role they play in the heritable memory processes maintaining gene expression patterns across cell division is not fully understood (Turner, 2002; Smith et al., 2002; Ladurner et al., 2003). In yeast, the memory of the transcriptionally active state of telomeric genes correlates with acetylation of H4 K12 (Smith et al., 2002). In higher eukaryotes, transcription factors generally dissociate from chromatin during mitosis, and histones become hypoacetylated, coinciding with the general repression of transcription (Gottesfeld and Forbes, 1997). Nevertheless, significant levels of acetylation remain for K5 and K12 of H4 at metaphase (Turner and Fellows, 1989) but not for H2B (Kruhlak et al., 2001). We found that the selective interaction of Brd2 with acetylated K12 of H4 was stable enough to retain Brd2 on mitotic chromosomes. BiFC revealed that this association was distributed broadly throughout the length of mitotic
chromosomes, suggesting that it affected many different genes. Hence, K12 acetylation and the association of Brd2 may be a plausible molecular mechanism for epigenetic “marking” in mammalian cells. It is of note that mitotic retention is a property of a subfamily of bromodomains that includes Brd2, Bdf-1 (Chua and Roeder, 1995), and Brd4 (Dey et al., 2000, 2003) but does not include BRG-1 (Muchardt et al., 1996), p300, PCAF, or TAF250 (Kruhak et al., 2001). The inefficiency of the TAF250 bromodomains in the mitotic chromosome retention was confirmed by bromodomain swap experiments. It should be noted that although the majority of TFIIID including TBP, TAFI5, TAF20, TAF10, and TAF250 are displaced from mitotic chromosomes, a small subpopulation remains associated with mitotic chromosomes, possibly required for rapid reassambling of preinitiation complexes in the newly divided cells (Segil et al., 1996; Christova and Oegeschlager, 2001; Chen et al., 2002).

In summary, bromodomains demonstrate diverse fine specificity in recognition of acetylated histones and ability to remain on mitotic chromosomes. These properties may be critical in understanding how histone acetylation regulates the initiation and maintenance of gene expression patterns.

Experimental Procedures

Constructs

CFF- and YFP constructs were cloned in pECFP-C1 and pEYFP-C1 (Clontech), respectively, positioning the fluorescent tags at N termini. Inserted cDNAs were: mouse Brd2 (identical to gi 3041763, cloned from F9 x JAP library), LANA (from J. Jung), human TAF250 (from R.G. Roeder), human PCAF (from Y. Nakatani), human H1 (IMAGE:376009), human H2A (IMAGE:418370), human H2B (IMAGE:2339049, the 4th residue T therein was corrected to A), mouse H3 (gi 51299, from ATCC), and human H4 (IMAGE:2130477). IMAGE clones were purchased from ResGen, and nucleotide sequences were confirmed. Mutagenesis was performed using a Quick change site-directed mutagenesis kit (Stratagene). Y(N)-Brd2 and Y(C)-H4 were produced by deleting amino acids 155–238 and 2–154 from YFP-Brd2 and YFP-H4, respectively. In YFP-H4, amino acids 1–16 were deleted from YFP-H4. In H4-K(5,12G) and H4-(K8,16G), lysines K5 and K12, and lysines K8 and K12 were replaced with glycines, respectively. In H4-KSR and H4-K12R, lysines K5 and K12 of H4 were replaced with arginine, respectively. In YFP-H2B3N, amino acids 1–22 were deleted from YFP-H2B. TAF250,JN and TAF250BD contain amino acids 1207–1872 and 1351–1608 of TAF250, respectively. Brd2-TAFBD1/B2D was constructed by replacing amino acids 77–179 and 336–448 of Brd2 with amino acids 1381–1495 and 1502–1623 of TAF250, respectively. Brd2-TAFBD-1 unit was constructed by replacing amino acids 77–448 of Brd2 with amino acids 1381–1623 of TAF250. Brd2 constructs were also cloned in a CMV-driven expression vector to produce pCX-Brd2, pCX-Brd2-BD-m8, and pCX-FLAG-Brd2.

FRET and BiFC Analyses

For FRET analysis, 8 x 10^6 HeLa cells seeded on 6 cm dishes were transfected with expression vectors for a CFP-fusion protein (2.8 μg) and a YFP-histone protein (0.2 μg) using PolyFect reagent (Qiagen). Cells were harvested 24–48 hr after transfection, and immediately analyzed on a FACSCalibur cytometer (Becton Dickinson) as described (Siegel et al., 2000a, 2000b; Chan et al., 2001). Excitation lasers and filters for detection channels were: a krypton laser (413 nm) and a 470/20 nm bandpass filter for CFP; an argon laser (514 nm) and a 546/10 nm bandpass filter for YFP; and the 413 nm krypton laser and a 546/10 nm bandpass filter for FRET. All three signals were detected from the same individual cells by sequentially illuminating them with the 514 nm and 413 nm lasers. For BiFC analysis, HeLa cells were transfected on a chambered coverglass (Lab-Tek) and observed with a Leica TCS SP2 confocal microscope.

Luciferase Reporter Assays

NIH 3T3 cells on 24-well plates were transfected using FuGene 6 (Roche) with a cyclin E promoter-driven luciferase reporter (from K. Ohtani) (0.2 μg), an internal control vector coding for Renilla luciferase (0.002 μg), and a combination of expression vectors, including RsaV12 (0.2 μg), and Brd2 or Brd2-BD-m8 (0.2, 0.4, or 0.6 μg). Luciferase/Renilla luciferase ratios measured using a dual-luciferase reporter assay system (Promega) were expressed as relative luciferase activities.

Peptide Precipitation Assays

Biotinylated H4 peptides, unmodified or tetra-acetylated, and H3 peptides were obtained from Upstate Biotechnology. Other H4 peptides and H2B peptides were obtained from United States Biological. Extracts were incubated with a biotinylated peptide in a buffer containing 20 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.2% NP-40, and protease inhibitors at 4°C for 2–4 hr. Streptavidin-coupled magnetic beads (Dynal Biotech) were then added for 1 hr. Beads were washed three times and subjected to SDS-PAGE and immunoblotting.

Nucleosomal Preparations and FLAG Immunoprecipitation

For analysis of YFP-histone incorporation in the nucleosome (Figures 1B and 3A), polynucleosome fractions were prepared as described (Schnitzler, 2000). To obtain the mononucleosome fraction in Figure 2, HeLa nuclei isolated as described (Schnitzler, 2000) were digested with micrococcal nuclease and extracted in a low-salt buffer according to the method by Mizzen and Aliis (Mizzen et al., 1999). Immunoprecipitation with FLAG antibody and elution with FLAG peptide were performed using ANTI-FLAG M2 affinity gel system (Sigma).

Antibodies

Rabbit polyclonal antibody was produced against a baculovirus recombinant Brd2. The following antibodies were purchased from commercial sources: H4 tetra-acetylated (K5/K8/K12/K16), H4 acetylated at K8 or K12, and H3 di-acetylated (K9/K14) were from Upstate Biotechnology; H4 acetylated at K5 or K16 from Seropec; and GFP from Santa Cruz and Roche.

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References


