Published in final edited form as:

FEBS Lett. 2008 April 30; 582(10): 1501–1507. doi:10.1016/j.febslet.2008.03.044.

Intracellular delivery of acetyl-histone peptides inhibits native bromodomain-chromatin interactions and impairs mitotic progression

Akira Nishiyama^{a,+}, Kazuki Mochizuki^a, Florian Mueller^b, Tatiana Karpova^b, James G. McNally^b, and Keiko Ozato^{a,*}

^aLaboratory of Molecular Growth Regulation, Genomics and Differentiation Program, National Institutes of Child Health and Human Development

^bLaboratory of Receptor Biology and Gene Expression, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

Abstract

Bromodomains present in Brd4 and other chromatin proteins interact with acetylated histones to regulate transcription and cell growth. To study Brd4-chromatin interactions in vivo, histone H4 tail peptides were fused to a synthetic protein transduction domain (PTD) derived from the human immunodeficiency virus Tat and delivered into cultured cells. Acetyl-H4 peptides, but not unacetylated H4 peptides inhibited real time Brd4-chromatin interactions in living cells as assessed by fluorescence recovery after photobleaching assays. The acetyl-H4 peptides also inhibited an interaction of Brd4 with chromosomes during mitosis and reduced cell growth potential. Together, PTD-based delivery of histone tail peptides offers a novel means to study the mechanism and biological significance of bromodomain-chromatin interactions in vivo.

Keywords

Peptide transduction; acetyl histone tail; bromodomain; Brd4; FRAP

1. Introduction

Acetylation of various lysine (K) residues in the histone tails generates diverse information relevant to gene regulation [1]. The conserved bromodomains present in many chromatin binding proteins recognize acetyl-Ks on histone tails and each bromodomain is thought to distinguish residue specific acetylation [2]. Brd4, a ubiquitous nuclear protein, carries two bromodomains through which it interacts with acetylated histones and regulates

*Correspondence: Keiko Ozato, Laboratory of Molecular Growth Regulation, Genomics and Differentiation Program, National Institutes of Child Health and Human Development, National Institutes of Health, Building 6, Room 2A01, 6 Center Drive, Bethesda MD, 20892-2753 TEL: (301) 496-9184, FAX: (301) 402-2974 e-mail: ozatok@nih.gov.

⁺Present address; Laboratory of Genetics, National Institute on Aging, National Institutes of Health, Baltimore Maryland, 21224

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

transcription and cell growth [3,4]. We previously showed that the interaction of Brd4 with acetylated chromatin persists even during mitosis [3,5]. While bromodomain-acetyl histone interactions have been extensively studied in vitro [2], studies of this interaction in living cells have been difficult to undertake, due to the lack of appropriate in vivo methods.

The PTDs represent small, positively charged peptides that can penetrate through the cell membrane. When fused, PTDs are capable of delivering cargo proteins of many sizes into cultured cells and animals [6,7]. A HIV Tat peptide and its derivatives, extensively studied among various PTDs, are shown to be rapidly and efficiently incorporated into cultured cells even when fused to large cargo proteins [8]. Using a synthetic PTD, we introduced unacetylated and acetylated histone H4 tail peptides into cultured cells. Our results indicate that PTD-delivered acetyl H4 peptides, but not unacetylated peptides interact with endogenous Brd4 in vivo, resulting in an inhibition of Brd4-chromatin interactions during interphase and mitosis, profoundly affecting cell growth properties.

2. Materials and methods

2.1 PTD-histone tail peptides uptake

PTD peptides were synthesized by Alpha Diagnostic International (Fig. 1A). P19 embryonal carcinoma cells and NIH3T3 fibroblasts were incubated with 100– 500 nM of peptides diluted in complete media for 4 to 6 h at 37°C. For flow cytometry, cells were incubated with FITC-conjugated PTD-peptides, trypsinized and washed before analysis.

2.2 Fluorescence recovery after photobleaching (FRAP)

FRAP assays were performed with NIH3T3 cells transduced with retroviral vectors for Brd4 small hairpin (sh) RNA and for a green fluorescent protein (GFP) fused Brd4 resistant to sh RNA [9]. FRAP assays were performed essentially as described [3,10]. Photobleaching was carried out on a small circular area (0.5-μm radius, 25 pixels) in the nucleus at the maximum laser power. Thirty prebleach images were acquired before a bleach pulse of 24 ms. Fluorescence recovery was monitored at low laser intensity (0.1% of a 45-mW laser) at 49 ms intervals for 14.68 s. Data points were binned so that later time points were evenly distributed on a logarithmic time scale [11]. Hierarchical clustering was used to identify FRAP curves with similar behavior. The distance between any pair of FRAP curves was defined as the sum of the squared residuals computed at each of the logarithmically distributed time points. Cluster trees were generated using the Matlab routine "linkage" with the "group average" method. FRAP curves within the same cluster were averaged and then fit with the function

$$frap(t)=\theta+(1-\theta)e^{-\frac{1}{\rho t}}\left[I_0\left(\frac{1}{\rho t}\right)+I_1\left(\frac{1}{\rho t}\right)\right],$$

where I_0 and I_1 are Bessel functions of the first kind, θ is the bleach depth and ρ is the recovery rate. θ and ρ are free parameters determined by fitting the averaged FRAP data with the Matlab routine *nlinfit*.

To assess the difference in FRAP curves, we also applied the extra sum-of-squares F-test [12]. Averaged FRAP curves from two clusters were fit either with the same ρ (null-hypothesis model) or two independent ρ 's (alternative model). The squared sum of residuals and the degrees of freedom for each model were combined to calculate an F value. P values were then computed from the F distribution. The best fits for the alternative model were found using the Matlab routine *nlinfit*, while the best global fit for the null hypothesis model was found using the Matlab routine *fminsearch* which minimized the sum of squared residuals for the concurrent fit.

2.3 Immunostaining of mitotic cells and colony forming assay

P19 cells were treated with nocodazole (100 ng/ml) for 6 h. Mitotic cells collected by mitotic shake-off were incubated in the presence of PTD-peptides for 45 min at 37°C. Cells were stained with Brd4 as in [5].

3. Results and Discussion

3.1 Intracellular delivery of PTD-histone tail peptides

With the aim of modulating Brd4-chromatin interactions in living cells, histone H4 tail peptides listed in Fig. 1A were fused to PTD-4, a synthetic peptide derived from HIV Tat, shown to be more efficient in cellular uptake than the original peptide [8]. To increase nuclear delivery, a nuclear translocation signal (NLS) from the simian virus 40 large T antigen was placed in between. NIH3T3 and P19 cells were incubated with FITC-labeled versions of peptides for 4 h, trypsinized to remove unincorporated peptides, and fluorescence uptake was measured by flow cytometry. Results showed dose dependent single peak uptake patterns (Fig. 1B). Time course analysis in Fig. 1C showed that peptides were taken up rapidly in the initial 1 h, but reached steady state at a lower level by 4 h, indicating that equilibrium between incorporation and processing was attained by this time. Upon microscopic inspection, FITC signals were found both in the cytoplasm and the nucleus (Fig. 1D), as reported for other peptides [7]. In Fig. 1E, uptake of PTD-acetyl H4 peptides (acetylated at K4, K8, K12, K16) was lower than that of unacetylated peptides. This is likely to be due to an alteration of overall charges in the peptides caused by acetylation [6].

3.2 PTD-acetyl histone peptides increase real time Brd4 mobility

FRAP analysis is an excellent way to assess real time interactions between histones and chromatin binding proteins in vivo [10,13]. To test the effect of PTD-H4 peptides on Brd4 FRAP mobility, we first constructed NIH3T3 cells expressing exogenous GFP-Brd4 in which endogenous Brd4 expression was reduced by Brd4 shRNA. Fig. 2A and B show that double-transduced cells expressed GFP-Brd4 with markedly reduced endogenous Brd4. The levels of GFP-Brd4 in these cells were comparable to those of endogenous Brd4 in control cells. GFP-Brd4 and endogenous Brd4 displayed comparable salt extraction profiles (Fig. 2C). Also, these cells showed similar patterns of histone H3 and H4 acetylation and grew at a comparable rate (Fig. 2D and E). These tests validated the use of these cells in FRAP analysis. Importantly, the use of these cells allowed us to avoid testing overexpressed Brd4 in our FRAP assays. For photobleaching, we selected circular spots in the nuclei of GFP-

Brd4 cells in regions showing a diffuse, homogeneous fluorescence distribution (Fig. 3A, red circle), and avoided nucleoli (dark regions) or regions containing bright puncta. Typical images from a fluorescence recovery are shown at 0.25s and 2.5 s (Fig. 3). Using such images, we measured average fluorescent intensities within the bleach spot as a function of time to generate individual FRAP curves for each cell. Typically for a given treatment ~15 such curves were averaged to produce the representative curves shown in Fig. 4A and B for cells incubated with different PTD-acetyl H4 peptides. Averaging of many individual FRAP curves from a particular treatment resulted in very small standard error bars on every point on the curves, enabling us to distinguish closely spaced curves. For example, even allowing for experimental error, the tetra-acetylated H4 peptide produced a Brd4 FRAP curve which was distinctly faster than the control, with every point in the tetra-acetylated H4 peptide curve clearly above the control curve (Fig. 4C). The same was true for most of the intermediate time points for the mono-acetylated H4 curve (H4AcK12, Fig. 4D). These and all of the other differences reported here were also highly reproducible from one day to the next. All differences reported here were consistently seen in at least three separate experiments. It is known that small differences in FRAP curves can reflect large differences in binding interactions [10], since FRAP curves reflect both binding and diffusion, and the latter process is not significantly altered by most biological perturbations.

The results showed that the tetra-acetyl H4 peptides consistently increased Brd4 mobility, while the unacetylated peptides had no effect, as the mobility was indistinguishable from that of control cells treated with medium alone. Among monoacetylated H4 peptides, those with AcK5 and AcK12, but not with AcK8 and K16, yielded consistently faster recovery compared with unacetylated peptides, with the difference somewhat smaller than that seen with tetra-acetylated peptides (Fig. 4B,D vs. 4A,C). These results indicated that Brd4 specifically interacted with H4 peptides containing AcK5 and AcK12. Significantly, this specificity agrees with that observed with *in vitro* Brd4-peptide interactions [3].

A quantitative analysis of the data in Fig. 4A using a clustering algorithm showed that control samples (medium alone) and those with the unacetylated H4 peptides formed a single cluster, while those with tetra-acetylated H4 peptides formed a separate cluster (Fig. 4E). In addition, peptides with monoacetylation at K5 and K12 displayed a distinct cluster from those acetylated at K8 and K16 (Fig. 4F). These results support the notion that the above differences in FRAP recoveries are significant. To further assess the significance of the data, the FRAP curves were averaged and fit with a mathematical formula for FRAP recoveries that contained a free parameter ρ that defines the rate of the FRAP recovery. An F test was used to determine the statistical significance of the groupings in Fig. 4E and F. Highly significant P values were obtained in all cases indicating that the changes observed in the FRAP recoveries are statistically significant (Fig. 4G and H). These analyses demonstrated that PTD-tetra-acetyl H4 peptide increased Brd4 mobility most, while AcK5 and AcK12 caused a less pronounced increase in Brd4 mobility. On the other hand, AcK8, AcK16 and unacetylated peptides did not have an effect. The analyses supported the idea that PTD-H4 peptides, through specific acetyl residues, bound to Brd4 in living cells, leading to partial inhibition of Brd4-chromatin interactions *in vivo*.

3.3 PTD-acetyl H4 peptides inhibit association of Brd4 with mitotic chromosomes and reduce colony formation

Brd4 remains bound on chromosomes even during mitosis, since the interaction with acetyl histones persists throughout cell cycle [3,5]. Brd4-mitotic chromosome interactions are transiently disrupted by anti-mitotic drugs such as nocodazole, temporally releasing Brd4 from chromosomes, although the drug withdrawal rapidly restores Brd4-chromosome interactions [5]. Restoration of Brd4-chromosome interactions tested after drug removal provides a sensitive way to assess the effect of PTD-acetyl H4 peptides on mitotic Brd4-histone interactions. P19 cells pre-treated with nocodazole were then incubated with PTD-H4 peptides and Brd4-chromosome interactions were monitored by microscopic inspection (Fig. 5A). In the presence of unacetylated peptides, Brd4 (red) bound to chromosomes, colocalizing with DNA (blue) in most cells (Fig. 5B). In contrast, in the presence of acetyl peptides, Brd4 remained outside of chromosomes in a large fraction of cells. Quantification in Fig. 5C confirmed these results, indicating that acetyl-H4 peptides inhibited Brd4-mitotic chromosome interactions. It should be noted here that due to the nuclear membrane breakdown, PTD-H4 peptides may be more efficiently delivered during mitosis than during interphase, likely allowing PTD peptides to gain direct access to chromatin. Nocodazole treatment has been shown to reduce the proliferative potential of cells [5,14]. We tested whether PTD-H4 peptides affect colony formation after nocodazole treatment (Fig. 6A). Cells incubated with unacetylated H4 peptides produced comparable numbers of colonies as cells treated with nocodazole alone (Fig. 6B). In contrast, significantly fewer colonies were generated when cells were incubated with tetra-acetyl H4 peptides, indicating that acetyl-H4 peptides further reduced cell's growth potential. In Fig. 6C, K5 monoacetylated H4 also reduced colony formation, although other monoacetylated peptides did not have an effect. Thus, inhibition of colony formation generally correlated with the ability of peptides to inhibit Brd4-chromatin interactions tested in FRAP assays.

The use of the PTD approach provided a new means to address the role of histone acetylation in vivo. While various proteins have been delivered into the cells by this method [6,7,15], to our knowledge, delivery of chromatin constituents has not been reported to date. It seems clear that under the conditions employed here, PTD-acetyl H4 peptides bound to Brd4 with sufficient affinity to alter Brd4 real time mobility, leading to the inhibition of global Brd4-chromatin interactions in vivo. This study also revealed that Brd4 bound to H4 peptides with exquisite acetyl residue specificity in vivo, the type of information difficult to obtain by standard biochemistry. An additional piece of interesting information generated in this work is that Brd4 binds free acetyl H4 tails without a globular nucleosomal base. The use of PTD-based acetyl H4 delivery allowed us to verify the biological significance of Brd4-acetyl chromatin interactions, as evidenced by the inhibition of cell growth. Given that histone tails are modified by numerous ways, creating diverse information on transcription and convey epigenetic memory, PTD-histone peptides may provide a versatile new avenue to study the role of histone modifications in gene expression and epigenetic regulation.

Acknowledgments

We thank A. Dey, T. Tamura, and T. Kanno for helpful discussions and critical reading of the manuscript. This work was supported by the Intramural Programs of NICHD and NCI, NIH.

References

1. Fischle W, Wang Y, Allis CD. Histone and chromatin cross-talk. *Curr Opin Cell Biol.* 2003; 15:172–183. [PubMed: 12648673]
2. Zeng L, Zhou MM. Bromodomain: an acetyl-lysine binding domain. *FEBS Lett.* 2002; 513:124–128. [PubMed: 11911891]
3. Dey A, Chitsaz F, Abbasi A, Misteli T, Ozato K. The double bromodomain protein Brd4 binds to acetylated chromatin during interphase and mitosis. *Proc Natl Acad Sci U S A.* 2003; 100:8758–8763. [PubMed: 12840145]
4. Wu SY, Chiang CM. The double bromodomain-containing chromatin adaptor Brd4 and transcriptional regulation. *J Biol Chem.* 2007
5. Nishiyama A, Dey A, Miyazaki J, Ozato K. Brd4 is required for recovery from antimicrotubule drug-induced mitotic arrest: preservation of acetylated chromatin. *Mol Biol Cell.* 2006; 17:814–823. [PubMed: 16339075]
6. Wender PA, Mitchell DJ, Pattabiraman K, Pelkey ET, Steinman L, Rothbard JB. The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: peptoid molecular transporters. *Proc Natl Acad Sci U S A.* 2000; 97:13003–13008. [PubMed: 11087855]
7. Wadia JS, Dowdy SF. Protein transduction technology. *Curr Opin Biotechnol.* 2002; 13:52–56. [PubMed: 11849958]
8. Ho A, Schwarze SR, Mermelstein SJ, Waksman G, Dowdy SF. Synthetic protein transduction domains: enhanced transduction potential in vitro and in vivo. *Cancer Res.* 2001; 61:474–477. [PubMed: 11212234]
9. Mochizuki K, et al. The bromodomain protein Brd4 stimulates G1 gene transcription and promotes progression to S phase. *J Biol Chem.* 2008
10. Stavreva DA, Muller WG, Hager GL, Smith CL, McNally JG. Rapid glucocorticoid receptor exchange at a promoter is coupled to transcription and regulated by chaperones and proteasomes. *Mol Cell Biol.* 2004; 24:2682–2697. [PubMed: 15024059]
11. Waharte F, Brown CM, Coscoy S, Coudrier E, Amblard F. A two-photon FRAP analysis of the cytoskeleton dynamics in the microvilli of intestinal cells. *Biophys J.* 2005; 88:1467–1478. [PubMed: 15596489]
12. Motulsky H, Christopoulos A. Fitting models to biological data using linear and nonlinear regression A practical guide to curve fitting. 2004:160–162.
13. Phair RD, et al. Global nature of dynamic protein-chromatin interactions in vivo: three-dimensional genome scanning and dynamic interaction networks of chromatin proteins. *Mol Cell Biol.* 2004; 24:6393–6402. [PubMed: 15226439]
14. Bhalla KN. Microtubule-targeted anticancer agents and apoptosis. *Oncogene.* 2003; 22:9075–9086. [PubMed: 14663486]
15. Morris MC, Depollier J, Mery J, Heitz F, Divita G. A peptide carrier for the delivery of biologically active proteins into mammalian cells. *Nat Biotechnol.* 2001; 19:1173–1176. [PubMed: 11731788]

Abbreviations used in this paper

FRAP	fluorescence recovery after photobleaching
GFP	green fluorescent protein
PTD	peptide transduction domain

Figure 1. Delivery of PTD-acetyl histone tail peptides into cultured cells

A. Schematic diagram of PTD-peptides tested in this work. All peptides were acetylated at the N-terminus and aminated (NH₂) at the C-terminus for stability. The position of acetylated lysine is marked by Ac.

B. Flow cytometry analysis of PTD-peptide uptake. P19 and NIH3T3 cells were incubated with FITC-PTD-4 fused to unacetylated H4 for 4 h, trypsinized, and FITC signals were detected in flow cytometry.

C. Kinetics of PTD-peptide uptake. Cells were incubated with the above peptides for the indicated times and uptake was monitored as above.

D. NIH3T3 cells were incubated with FITC-PTD-4 fused to unacetylated H4 for 4 h and the intracellular distribution of FITC signals was viewed by confocal microscopy.

E. Cells were incubated with PTD-4 fused to unacetylated or tetra-acetylated H4 tail peptides for 4 h and uptake was monitored as above.

Figure 2. Dosage adjusted expression of GFP-Brd4 used for FRAP analysis

A. NIH3T3 cells were transduced with pMSCV-EGFP-Brd4 and pSuper-Brd4 shRNA (right lane, GFP-Brd4 + Brd4 shRNA). For control, cells were transduced with empty vectors (left lane, Mock). Cell lysates were tested by immunoblot.

B. The distribution of GFP-Brd4 in the transduced cells during interphase or mitosis is shown.

C. Extracts from mock and EGFP-Brd4/Brd4 shRNA transduced cells prepared with buffer of increasing NaCl concentrations (mM). Brd4 was detected by immunoblot with an anti-Brd4 antibody.

D. Histones from the above cells were tested for acetylation by immunoblot.

E. Mock transduced and EGFP-Brd4/Brd4 shRNA transduced NIH3T3 cells were cultured for the indicated days and total cell yields were plotted on the Y-axis.

Figure 3. FRAP procedure

A. Live-cell images of a cell before and after photobleaching. A circular spot (red circle) was photobleached for 24 ms in the nucleus of NIH3T3 cells expressing GFP-Brd4 (Figure 2) and incubated with PTD-acetyl H4. The first image after the photobleach is shown (0 s). At subsequent time points, fluorescence recovers within the photobleached spot (example images at 0.25 s and 2.5 s are shown). FRAP recovery curves were generated by computing the average fluorescent intensity within the region defined by the red circle at all time points, and then subtracting background (as measured from the average intensity inside the blue circle) and normalizing by the loss of fluorescence due to repeated imaging (green circle).

Figure 4. Altered mobility of GFP-Brd4 following PTD-acetyl peptide delivery

A. Cells were incubated with the indicated PTD-H4 peptides for 1 h prior to photobleaching. The curves represent the average recovery of 15 cells for each treatment.

B. Cells were incubated with the indicated PTD-peptides (500nM) and fluorescence recovery was monitored as in A.

C. Control and tetra-acetylated H4 curves from A shown with standard error bars on each time point. Note that the differences between the curves are much larger than the standard errors.

D. H4 and mono-acetylated H4AcK12 curves from B shown with standard error bars. The differences between the curves at intermediate time points are much larger than the standard errors.

E, F. Hierarchical cluster trees for the FRAP curves in A and B. Branch lengths on the tree reflect the distance between pairs of averaged FRAP curves defined by the sum of squared residuals between each pair of curves.

G, H. Averaged FRAP curves were computed for the clusters in C, D. The curves were fit with a mathematical model (see Experimental Procedures) to obtain an estimate of the recovery rate ρ . An F test was performed to determine whether the averaged curves could be described by the same ρ value (null hypothesis) or two distinct ρ values (alternative hypothesis).

Figure 5. PTD-acetyl histone peptides impair Brd4-chromosome interactions

A. P19 cells were treated with nocodazole, washed and mitotic cells were allowed to proceed in the presence of PTD-peptides for 45 min.

B. Cells incubated with or without peptides were immunostained with anti-Brd4 antibody and Hoechst 33342. Typical Brd4 distributions on mitotic chromosome (left panels) and outside of chromosome (right panels) are shown.

C. Quantification of Brd4-chromosome interactions. P19 cells treated with nocodazole were incubated with PTD-peptides at the indicated concentrations. The number of cells in which Brd4 was (or was not) associated with mitotic chromosomes were counted. Solid bars represent the percentage of mitotic cells with Brd4 on chromosomes. Open bars indicate the percentage of cells with Brd4 outside of mitotic chromosomes. At least 250 cells were counted for each sample. Similar results were observed in three independent experiments.

Figure 6. The effect of acetyl-peptides on the colony forming ability

A. Diagram of colony forming assays.

B. Cells treated with or without nocodazole were incubated with PTD with unacetylated or tetra-acetylated H4 peptides (500 nM). The number of colonies produced after PTD-peptide delivery was compared to that produced by untreated cells and is expressed as the percentage of colonies relative to control.

C. Cells treated with nocodazole (500 ng/ml) were incubated with the indicated PTD-peptides and colony forming assays were performed as above.