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Histone H4 acetylation during interleukin-2 stimulation of mouse T cells

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Abstract Proliferation and cell cycle progression of eukaryotic cells are closely linked to changes in chromatin structure and gene expression. By reversible histone acetylation the cell is able to modulate chromatin condensation and accessibility of specific regions within the chromatin. Here, we examined histone H4 acetylation patterns during growth induction of the murine interleukin-2 dependent T cell line B6.1. In order to detect acetylation on each of the four potential target residues we produced a set of antibodies recognizing specifically acetylated lysine 5, 8, 12 and 16 in the N-terminal tail of histone H4. Acetylation was generally low in resting T cells, but increased after stimulation with a specific kinetics for each lysine. Lysine 16 was acetylated during the G1 phase and deacetylated during S phase. H4 acetylation on lysine 5, 8 and 12, in contrast, was induced before cells started to replicate, and persisted until cells entered mitosis. Treatment of resting B6.1 cells with the specific deacetylase inhibitor trichostatin A (TSA) led to H4 hyperacetylation at all four lysine residues indicating that the histone modification can occur in the absence of replication. After release from TSA treatment normal H4 acetylation levels were reestablished by extremely rapid deacetylation of lysines 5, 8, 12 and 16. The deacetylation step was 60–100 times faster than TSA induced acetylation and equally efficient in resting and exponentially growing T cells. Our results indicate the presence of cell cycle regulated lysine specific acetylating and deacetylating activities in mouse T cells.

Key words: Chromatin; Histone acetylation; Histone H4; Growth induction; Cell cycle; Acetyllysine specific antibody

1. Introduction

Reversible acetylation of the four nucleosomal core histones H2A, H2B, H3 and H4 plays an important role in chromatin structure and gene expression. Highly conserved lysine residues within the N-terminal domains of core histones are the targets for this modification. The level of histone acetylation is controlled by histone acetyltransferases and histone deacetylases. There is compelling evidence for a link between transcriptionally active chromatin and histone acetylation (reviewed in [1–7]). Recently it was shown that transcriptional regulators repress transcription by recruiting histone deacetylases to specific promoters [8–18]. In addition the transcriptional repressor MeCP2 that binds to methylated DNA has been found to be associated with histone deacetylase activity [19,20].

Previously we identified mouse histone deacetylase 1 (HDAC1) by differential display as a growth factor inducible gene in murine T cells [21]. The levels of HDAC1 mRNA, protein and enzyme activity increased when resting lymphocytes were growth-stimulated by interleukin-2 (IL-2). Several biological processes like spherulation of the slime mold *Physarum polycephalum* [22], and spermatogenesis in rat [23], trout [24], rooster [25] and *Drosophila* [26] are accompanied by changes in histone acetylation levels. Furthermore, studies in *P. polycephalum* revealed cell cycle specific differences in the acetylation patterns of core histones [27,28]. In particular changes in the acetylation levels of specific H4 lysine residues were shown to be crucial for certain cellular processes like silencing [29], chromatin assembly [30,31], X chromosome inactivation [32], mitosis [33–35] and transcriptional regulation of specific genes [36]. Therefore we examined how histone H4 acetylation changes during the re-entry of resting T cells into the cell cycle. Antibodies directed against acetylated lysines in the N-terminal tail of core histones have been shown to serve as excellent tools to analyze histone acetylation in a variety of cellular systems [33,37–41].

To study lysine site specific acetylation we raised antibodies against four short peptides representing parts of the N-terminal tail of histone H4, each mono-acetylated at lysine 5, 8, 12 or 16. Using this set of site specific H4 acetyllysine antibodies we analyzed in this report H4 acetylation in T cells during the re-entry into the cell cycle. Our study reveals different kinetics for the acetylation and deacetylation of the four target lysine residues and significant differences in the efficiency between acetylation and deacetylation in resting T cells.

2. Materials and methods

2.1. Cell culture and media

B6.1 is an IL-2 dependent cytolytic mouse T cell line [42]. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% heat inactivated fetal calf serum, 50 mM 2-mercaptoethanol, 10 mM L-glutamine, and 100 U/ml of recombinant IL-2 (generous gift from M. Nabholz, ISREC, Switzerland). To arrest B6.1 cells IL-2 was removed from the medium for 30 h. Growth arrest and re-entry into the cell cycle during IL-2 stimulation were monitored by fluorescence activated cell sorter (FACS) analysis using a Partec PAS-II sorter. To induce histone hyperacetylation in arrested B6.1 cells the medium was supplemented with 20 ng/ml and 40 ng/ml trichostatin A (Wako Pure Chemical Industries) or 5 mM sodium butyrate. HeLa cells were treated with 5 mM sodium butyrate for 24 h.

2.2. Histone isolation

Cells were harvested by centrifugation at $700\times g$ and washed once with ice-cold PBS. The pellet was resuspended in 1 ml lysis buffer (10 mM Tris-HCl pH 6.5, 50 mM sodium disulfite, 10 mM $MgCl_2$, 10 mM sodium butyrate, 8.6% sucrose, 1% Triton X-100) and centrifuged at $1000\times g$. After three washes in lysis buffer the pellet was resuspended in 10 mM Tris-HCl pH 7.4, 13 mM EDTA. After this wash the pellet was resuspended in cold distilled water and H_2SO_4 was added to 0.4 N final concentration. Incubation on ice for 1 h was followed by a centrifugation at $10\,000\times g$ for 5 min. From the supernatant total histones were precipitated with $10\times$ volumes of acetone at −20°C overnight. The precipitated histones were collected by centrifugation, dried and resuspended in distilled water.

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2.3. Peptides and antibodies

To obtain specific antibodies directed against each acetylated lysine residue in the N-terminal tail of histone H4, the following peptides were used for the immunization of rabbits: anti-acetyllysine 5: H_2N-SGR G**acK**G GKG LYC-$CONH_2$ (K5); anti-acetyllysine 8: H_2N-SGR GKG G**acK**G LGK YC-$CONH_2$ (K8); anti-acetyllysine 12: H_2N-GKG LG**acK** GGA KYC-$CONH_2$ (K12); anti-acetyllysine 16: H_2N-GLG KGG A**acK**R HYC-$CONH_2$ (K16).

The peptides were used to immunize rabbits by standard immunological techniques (Eurogentec, Seraing, Belgium). The corresponding antibodies were named K5, K8, K12 and K16, respectively. For affinity purification 1 mg of the corresponding peptide was coupled to a SulfoLink column (Pierce). Antiserum (1 ml) was loaded on the column and bound antibodies were eluted with 100 mM glycine pH 2.5. Each fraction (1 ml) was buffered with 50 μl of 1 M sodium phosphate pH 8.0 and supplemented with sodium azide (0.02% final concentration).

2.4. Western blot analysis

Equal amounts of histones (5–10 μg) were electrophoresed on a SDS-18% polyacrylamide gel. Gels were blotted on nitrocellulose membranes and detected by Western blot analysis using an ECL kit (NEN) as previously described [21].

3. Results and discussion

3.1. Preparation and characterization of antisera

In order to study histone H4 acetylation on each of the potential target sites we raised antisera against acetyllysines 5, 8, 12 and 16. The corresponding antibodies were named K5, K8, K12 and K16, respectively. As shown in Fig. 1A, antibodies obtained from rabbits immunized with short peptides of H4 are highly specific for the corresponding mono-acetylated peptides. No cross-reaction between these four antibodies was detected by dot-blot analysis. In addition, all four antibodies were specific for acetylated H4 isoforms since a non-acetylated peptide encompassing residues 1–18 of histone H4 was not recognized by any of the antibodies (data not shown). To test if the antibodies also recognize the acetylated lysines on histone H4 protein, Western blots with HeLa histones isolated before and after treatment with sodium butyrate were incubated with each antibody. All four antibodies recognized hyperacetylated H4 histones from butyrate treated cells with higher affinity than H4 histones from untreated cells (Fig. 1B). In addition antibodies K5, K8 and K12 have been shown to recognize mono-, di-, tri- and tetra-acetylated H4 histone from chicken erythrocytes (D. Kölle et al., submitted). None of the affinity-purified antibodies recognized other histones or non-histone proteins in whole cell extracts (data not shown). Thus, the four antibodies are suitable for studies of lysine site specific H4 acetylation patterns.

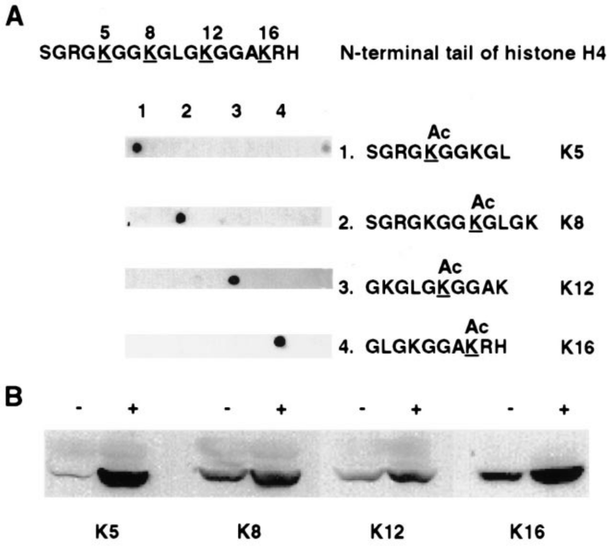

Fig. 1. Specificity of rabbit polyclonal antibodies against acetylated lysine residues of histone H4. A: Dot blot analysis of the different H4 antibodies. The four peptides (2 ng) used for immunization were spotted on nitrocellulose membranes and probed with the different antisera. B: Western blot analysis using affinity to hyperacetylated histone H4. Equal amounts (10 μg) of histones isolated from HeLa cells before (−) and after (+) treatment with the deacetylase inhibitor butyrate were transferred to a nitrocellulose membrane and detected with the H4 specific antibodies as indicated.

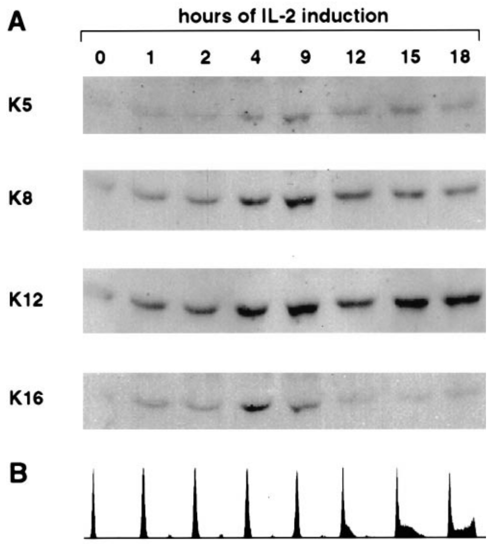

Fig. 2. Changes in histone H4 acetylation pattern during IL-2 stimulation of murine T cells. B6.1 cells were arrested by IL-2 deprivation for 30 h and restimulated with fresh medium containing 100 U/ml recombinant IL-2 for different periods of time (indicated in hours). Total histones were isolated at indicated time points. A: Equal amounts of histones were separated on a SDS-18% polyacrylamide gel and blotted on nitrocellulose. Staining of the membrane with Ponceau S indicated that the 12 h lane contained slightly less histone. The membrane was sequentially incubated with antibodies directed against acetylated lysine 5 (K5), 8 (K8), 12 (K12) and 16 (K16) of histone H4. B: FACS analysis of the DNA content of B6.1 cells during IL-2 stimulation.

3.2. H4 acetylation during IL-2 induction of resting B6.1 cells

Previously we showed that murine HDAC1 is an IL-2 inducible gene in B6.1 cells. HDAC1 protein levels and enzymatic activity both were up-regulated during T cell activation. Therefore we asked if the increased histone deacetylase activity correlates with changes in general H4 acetylation pattern during the IL-2 induction of resting T cells. B6.1 cells were arrested by IL-2 deprivation for 30 h and restimulated with recombinant IL-2 for different periods of time. Re-entry of resting T cells into the cell cycle was monitored by FACS analysis (Fig. 2B). In IL-2 deprived B6.1 cells H4 acetylation on all four lysine residues was low (Fig. 2A). Growth stimulation by IL-2 led to cell cycle phase specific changes in H4 acetylation patterns. Acetyllysine 16 levels increased early and peaked during G1 phase, 4 h after IL-2 addition. When B6.1 cells started to replicate steady state acetylation levels of lysine 16 decreased and stayed low from mid-S phase to mitosis. Lysine residues 8 and 12 were acetylated with comparable kinetics reaching the highest levels at the beginning of the S phase 9 h after IL-2 stimulation. Lysine 5 showed delayed acetylation kinetics with maximal acetylation between 9 and 15 h after IL-2 stimulation corresponding to the S phase. These findings are in good agreement with the previously observed S phase specific acetylation of de novo synthesized H4 on lysines 5 and 12 [43] and recently published data that showed sequential acetylation of lysines 8 and 5 by HAT-B enzymes from maize and rat liver [44]. H4 isoforms acetylated on lysines 5, 8 and 12 (but not on lysine 16) were also found to be associated with the chromatin assembly factor CAF-1 [30]. Interestingly the preferred order of acetylation for H4 lysine residues during the onset of proliferation was similar to that found by Turner and colleagues [33] in human cells after treatment with the histone deacetylase inhibitor butyrate. Acetylation levels of lysines 5 and 8 decreased between mid-S phase and mitosis with the order lysine 8 followed by lysine 5, while lysine 12 acetylation stayed high throughout the remaining part of the cell cycle. This result suggests the presence of lysine site specific deacetylating activities during late S phase of the cell cycle.

3.3. *TSA induced hyperacetylation of histone H4 in resting B6.1 cells*

Next we asked if growth arrest by IL-2 deprivation affects the capability of histone acetyltransferases to modify all four lysine residues in the N-terminal domain of histone H4. B6.1 cells were arrested by removal of IL-2 from the culture medium and histone deacetylase activities were blocked by the specific inhibitor TSA [45]. H4 acetylation on lysines 12 and 16 started to rise 1 h after TSA addition followed by acetylation on lysines 5 and 8 (2 h after TSA addition) to reach maximum levels after 9 h TSA treatment (Fig. 3A). Longer incubation with the histone deacetylase inhibitor in the absence of IL-2 led to a dramatic reduction of the viability of B6.1 cells (data not shown). Similar acetylation kinetics in B6.1 cells were observed by inhibiting histone deacetylation with sodium butyrate (J. Taplick, unpublished observations).

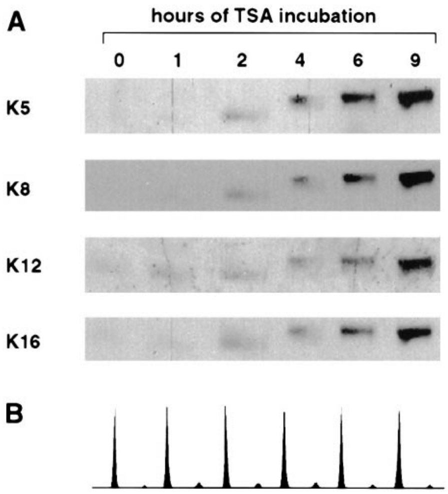

Fig. 3. TSA induced hyperacetylation of H4 histones in resting B6.1 cells. A: B6.1 cells were IL-2 starved for 30 h followed by addition of 20 ng/ml TSA to the medium. At different time points (indicated in hours) histones were isolated and analyzed on a Western blot with acetyllysine specific antibodies. B: FACS profile of resting B6.1 cells during TSA incubation.

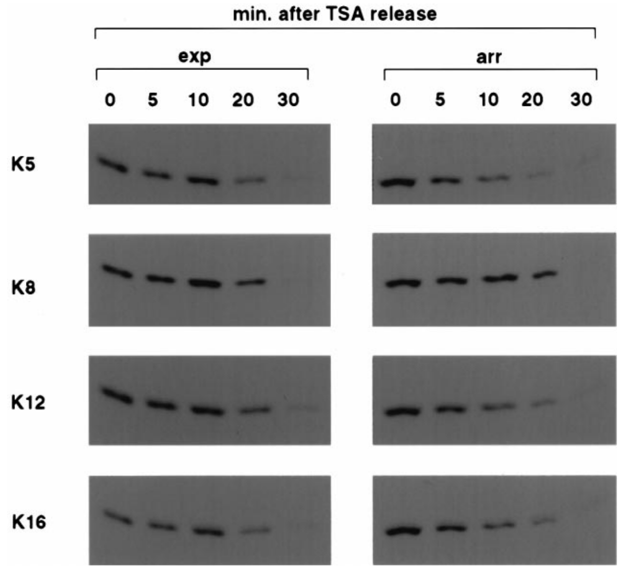

Fig. 4. H4 deacetylation kinetics after TSA release in arrested and exponentially growing B6.1 cells. IL-2 deprived (arr) and exponentially growing (exp) cells were treated with 40 ng/ml TSA for 4 h. The medium was then replaced by fresh TSA-free medium and cells were harvested at the indicated time points. Acetylated H4 histones were detected by Western blot analysis using antibodies directed against individual acetyllysine residues. The lane with histones from exponentially growing B6.1 cells 10 min after TSA release was slightly overloaded.

Our results demonstrate that by blocking deacetylating activities hyperacetylation on all four lysine residues can be induced in non-replicating T cells. The H4 hyperacetylation occurs in the absence of de novo histone synthesis and therefore seems to be catalyzed by nuclear histone acetyltransferases. To investigate if TSA induced histone hyperacetylation has an effect on growth arrest, we monitored the DNA content with FACS analysis. As shown in Fig. 3B, B6.1 cells showed no detectable change in the G0 DNA profile in the absence of IL-2.

3.4. *Deacetylation of hyperacetylated histone H4 in resting and cycling B6.1 cells*

Finally, we compared the acetylation levels for specific H4 lysine residues in resting and cycling B6.1 cells after TSA release. Histone hyperacetylation was induced in IL-2 deprived and exponentially growing cells by treatment with TSA (40 ng/ml) for 4 h and H4 acetylation was examined after removal of TSA from the culture medium. As shown in Fig. 4 all four lysine residues of the H4 N-terminal tail were deacetylated within 30 min after TSA release. In arrested cells deacetylation at lysine 5, 12 and 16 started 5 min after TSA was withdrawn. Acetyl residues were most efficiently removed from lysine 5 followed by lysines 12 and 16, while lysine 8 was deacetylated with delayed kinetics between 20 and 30 min after TSA release. In exponentially growing cells H4 deacetylation was slightly slower, starting around 20 min after TSA release with similar kinetics for lysine 5, 12 and 16. As in arrested cells lysine 8 was the last residue that was deacetylated. These small differences in the deacetylation process between G0 cells and cycling cells might reflect the presence of higher acetylating activity in proliferating cells. It is intriguing how fast deacetylation occurs when the enzyme inhibitor TSA is removed. In G0 cells removal of the acetyl residues is about 60–100 times faster than the hyperacetylation during TSA treatment. Since TSA induced hyperacetylation is an artificial condition for the cell, it might be important for the eukaryotic cell to restore normal acetylation levels.

This would indicate that the control of steady state levels of histone acetylation by an intracellular balance between acetylating and deacetylating activities is crucial for eukaryotic cells. This idea is supported by the fact that overexpression of histone deacetylases [21,46] or inhibition of a histone acetyltransferase function [47] has serious consequences for cell growth and cell cycle progression. The data also suggest that histone deacetylases are very effective even in the absence of growth factors and can be quickly activated by this control system. It also raises the question if and how the cells remember the default acetylation state of chromatin both long range and on particular nucleosomes within specific promoters. This cellular memory might be linked to factors that recognize the acetylation of core histones and/or to the methylation of genomic DNA [19,20,48].

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