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Short communication

Neutrophil Elastase in the capacity of the "H2A-specific protease"

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ABSTRACT

The amino-terminal tail of histones and the carboxy-tail of histone H2A protrude from the nucleosome and can become modified by many different posttranslational modifications (PTM). During a mass spectrometric proteome analysis on haematopoietic cells we encountered a histone PTM that has received only little attention since its discovery over 35 years ago: truncation of the histone H2A C-tail at V_{114} which is mediated by the "H2A specific protease" (H2Asp). This enzyme is still referenced today but it was never identified. We first developed a sensitive AQUA approach for specific quantitation of the $H2AV_{114}$ clipping. This clipping was found only in myeloid cells and further cellular fractionation lead to the annotation of the H2Asp as Neutrophil Elastase (NE). Ultimate proof was provided by NE incubation experiments and by studying histone extracts from NE *Null* mice. The annotation of the H2Asp not only is an indispensable first step in elucidating the potential biological role of this enzymatic interaction but equally provides the necessary background to critically revise earlier reports of H2A clipping.

1. Introduction

The evolutionary conserved histone proteins are intimately associated with DNA to form the chromatin. Their amino-terminal tail and the carboxy-tail of histone H2A protrude from the nucleosome and can become modified by many different PTM. One somewhat dramatic PTM is the enzymatic clipping of histone tails, which was recently shown to play an epigenetic role in differentiation (Duncan et al., 2008; Osley, 2008). Although many previous reports on histone clipping have speculated on its potential to influence transcription (Duncan and Allis, 2011), histone truncation has equally been described in the context of other biological processes, such as neutrophil extracellular trap or NET formation (Papayannopoulos et al., 2010).

During a proteome analysis on haematopoietic cells, we came across a histone clipping event that was first described over 35 years ago in calf thymus histone extracts: truncation of the C-tail of histone H2A at V_{114} (Eickbush et al., 1976). The responsible enzyme was named the 'H2A specific protease' (H2Asp) and its involvement in transcriptional regulation was soon hypothesized but it was never sequenced nor identified (Davie et al., 1986; Eickbush et al., 1988; Eickbush and Moudrianakis, 1978; Elia and Moudrianakis, 1988; Watson and Moudrianakis, 1982). Structurally, this truncation has been suggested to modulate chromatin dynamics and it was shown to be induced during macrophage differentiation (Minami et al., 2007; Vogler et al., 2010). Even today, references to this histone H2A C-tail clipping and the H2Asp still recur, yet no reports have thus far questioned the identity of the enzyme or its involvement in epigenetics (Azad and Tomar, 2014; Mandal et al., 2012; Okawa et al., 2003; Santos-Rosa et al., 2009).

Here we show that the H2Asp actually is Neutrophil Elastase (NE). While we continue to search for the biological potential of this truncation in health or disease, we emphasize that the clipping of the histone H2A C-tail shows remarkable parallels to the more epigenetically established clipping of the H3 N-tail, but could equally be involved in other biological processes such as NET formation. Importantly, we caution that most experimental approaches still do not anticipate on these clipping events while high enzyme kinetics and strong association between nucleosomes could greatly hamper efficient enzyme inhibition in any protocol.

Abbreviations: H2Asp, H2A-specific protease; NE, Neutrophil Elastase; CL, cathepsin L; mESC, mouse embryonic stem cells; cH2A, cleaved histone H2A; PTM, posttranslational modification; MS, mass spectrometry; PBMC, peripheral blood monocytes; AQUA, Absolute Quantitation.

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2. Materials and methods

2.1. Cells and reagents

Phosphate buffered saline (PBS), media, L-glutamine, Foetal bovine serum (FBS), penicillin/streptomycine, Dynabeads and Sypro Ruby were from Invitrogen (San Diego, CA), ammonium bicarbonate (ABC), sodium dodecyl sulfate (SDS), N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) and Tween-20 from Millipore (Billerica, MA). All other reagents were purchased from Sigma Aldrich (St. Louis, MI, USA). Bovine histones (cat. no. 223565) were from Roche (Basel, Switzerland), recombinant human H2A (M2502S) from New England Biolabs (Ipswich, MA) and purified Neutrophil Elastase from Abcam (Ab80475, Cambridge, UK). Raji and Jurkat cells were obtained from ATCC. Total leukocytes were obtained by red blood cell lysis on whole blood (Qiagen, Venlo, Netherlands). Lymphocytes were isolated from healthy volunteers using Ficoll-Paque. T-cells were purified by means of the RosetteSep® Human T Cell Enrichment Cocktail (Stem cell Technologies, Grenoble, France). Cells were cultured in Dulbecco's Modified Eagle medium supplemented with 2% (w/v) L-glutamine, 10% (w/v) FBS and 50 IU/ml penicillin/streptomycine at 37 °C. Apoptosis in Raji's was induced by o/n incubation in 0.2 μg/ml MHCII antibody or 100 ng/ml PMA at 37 °C or by 15 min UV irradiation.

2.2. Mice

Null mice *stock# 006112 strain# B6.129X1-Elanetm1Sds/J* and wild type control mice *stock# 005304 Strain# C57BL/6NJ* were purchased from The Jackson Laboratory (Bar Harbour, MA, USA) and bred at the animal facility of Ghent University Hospital. All experimental procedures were approved by the local ethics committee according to national animal welfare legislation. All mice were genotyped as instructed by the supplier. Blood samples were obtained by cardiac puncture of mice under terminal anaesthesia. Leukocytes were recovered by red blood cell lysis and histones were isolated as described below.

2.3. Histone isolation

All steps were done at 4 °C. Harvested cells were washed twice in PBS containing 1 mM PMSF and protease inhibitor cocktail. 10^7 cells/ml were resuspended in Triton extraction buffer (PBS containing 0.5% (v/v) Triton X 100, 1 mM PMSF and protease inhibitor cocktail) and lysed by gentle stirring. Pelleted nuclei were washed in PBS containing 1 mM PMSF and proteinase inhibitor cocktail. Histones were extracted overnight after benzonase treatment of the sonicated nuclei by acid extraction: incubation in 250 μl 0.2 M HCl at 4 °C with gentle stirring. Precipitated proteins were pelleted and the supernatant containing the histones was dried and stored at −80 °C until further use. Protein quantitation was done by Bradford Coomassie Assay from Thermo (St. Waltham, MA, USA).

2.4. Western blot analysis

3 μg of dried histone extract was resuspended in Laemmli buffer, incubated at 99 °C and run on a 15% Tris–HCl gel (Biorad, Hercules, CA) and transferred to nitrocellulose membrane in a 10 mM CAPS buffer with 20% MeOH (Merck) in 100 min at 120 V. Histone H2A (LS-C24265, LifeSpan BioScience, Seattle, WA) was used at a 1:1000 dilution in 0.3% Tween20, 1%BSA. For the detection of biotinylated H2A 1:10 000 Avidin-HRP (18-4100-94) (eBioscience, San Diego, CA) was used.

2.5. Mass spectrometry analysis

All tryptic digests were performed after reduction in freshly prepared 50 mM TCEP 25 mM TEABC solution for 2 h at 56 °C and alkylation in 200 mM MMTS solution for 1 h at room temperature. The samples were separated on an U3000 (Dionex) in a 70 min organic gradient from 4% to 100% buffer B (80% (v/v) ACN in 0.1% (v/v) FA) and analyzed on an ESI Q-TOF Premier (Waters, Wilmslow, UK). Data was searched against SwissProt database using Mascot 2.3 (Matrix Science, London, UK). For the specific analysis of the H2A V_{144} clipping, the isobaric peptides (AQUA1; AQUA2) were combined in a stock solution in a ten (AQUA1, Thermo) to one (AQUA2, Sigma Aldrich) ratio and added to 1 μg of histone extract right before MS analysis.

2.6. Sucrose gradient ultracentrifugation

Mechanical isolation of nuclei was done by lysing the cells in a cell plunger after 5 min of incubation in 10 mM Hepes pH 8, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT at 4 °C. After 10 strokes, nuclear isolation efficiency was found to be at least 90% by microscopy. The pelleted nuclei were resuspended in 3 ml buffer S1 (0.25 M sucrose, 10 mM $MgCl_2$) and brought on top of 3 ml buffer S2 (0.35 M sucrose, 0.5 mM $MgCl_2$). After 5 min at 572 × *g* at 4 °C, the pellet was resuspended in S2 for a second wash and the nuclei were lysed by sonication in 50 mM Tris–HCl pH 7.5 supplemented with 250 U of benzonases for 10 min for DNA degradation. The linear sucrose gradient (5 ml) 10–50% was prepared in 0.01 M Tris–HCl pH 7.5 with 0.001 M Na_2EDTA. The gradient was placed at 4 °C for 2 h. 200 μl of the nuclear extract was applied on top of the gradients and they were spun at 190,000 × *g* for 1040 min at 4 °C in a Centrikon T1080 ultracentrifuge. After centrifugation, 10 fractions of 500 μl were collected manually.

3. Results and discussion

3.1. High throughput quantitation of $H2AV_{114}$ clipping by AQUA

During a mass spectrometric proteome analysis on haematopietic cells, we detected only one semi-tryptic peptide out of >7400 annotated MSMS spectra that recurred in all 6 replicate runs: VTIAQGGVLPNIQAV, a fragment derived from histone H2A ending at V_{114} (clipped H2A or cH2A, Fig. 1A). Remarkably, this specific fragment was already described in calf thymus 1976 but the responsible enzyme, named H2A specific protease (H2Asp) was never identified (Eickbush et al., 1976). To be able to specifically quantify the V_{114} clipping, a sensitive mass spectrometry approach based on the AQUA (Absolute Quantitation) principle was developed using two isotopically labelled synthetic peptides (Fig. 1A and B). To our knowledge, this is the first description of a technique that can specifically quantify a histone clipping event in high throughput. To validate the efficiency of this approach, we quantified the amount of clipped H2A (cH2A) in 0.1–2.5 μg of bovine histones and consistently found it to be between 3% and 9%, despite the 25-fold loading difference (Fig. 1C and D). Note that this cH2A was detected in commercial calf thymus histones, the source from which the H2A specific protease was first isolated (Eickbush et al., 1976).

3.2. The H2A specific protease is Neutrophil Elastase

Screening larger cohorts of histone extracts from different haematopoietic origins indicated that cH2A prevalence was strongly related to myeloid content since PBMCs, leukocytes and the pellet from Ficoll-Paque separated haematopoietic cells showed the presence of cleaved H2A (Fig. 2A, left panel). In T-cells, Raji's and Jurkat cells only intact H2A was detected. Lymphoid