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Combined bottom-up and top-down mass spectrometry analyses of the pattern of post-translational modifications of *Drosophila melanogaster* linker histone H1

Carles Bonet-Costa[a,b,1], *Marta Vilaseca*[c,1], *Claudio Diema*[c], *Olivera Vujatovic*[a,b], *Alejandro Vaquero*[a,b,2], *Núria Omeñaca*[c], *Lucía Castejón*[a,b], *Jordi Bernués*[a,b], *Ernest Giralt*[b,d,*], *Fernando Azorín*[a,b,*]

[a]*Institute of Molecular Biology of Barcelona, CSIC, 08028 Barcelona, Spain*
[b]*Institute for Research in Biomedicine, IRB Barcelona, 08028 Barcelona, Spain*
[c]*Mass Spectrometry Core Facility, Institute for Research in Biomedicine, IRB Barcelona, 08028 Barcelona, Spain*
[d]*Department of Organic Chemistry, University of Barcelona, 08028 Barcelona, Spain*

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ABSTRACT

Linker histone H1 is a major chromatin component that binds internucleosomal DNA and mediates the folding of nucleosomes into a higher-order structure, namely the 30-nm chromatin fiber. Multiple post-translational modifications (PTMs) of core histones H2A, H2B, H3 and H4 have been identified and their important contribution to the regulation of chromatin structure and function is firmly established. In contrast, little is known about histone H1 modifications and their function. Here we address this question in *Drosophila melanogaster*, which, in contrast to most eukaryotic species, contains a single histone H1 variant, dH1. For this purpose, we combined bottom-up and top-down mass-spectrometry strategies. Our results indicated that dH1 is extensively modified by phosphorylation, methylation, acetylation and ubiquitination, with most PTMs falling in the N-terminal domain. Interestingly, several dH1 N-terminal modifications have also been reported in specific human and/or mouse H1 variants, suggesting that they have conserved functions. In this regard, we also provide evidence for the contribution of one of such conserved PTMs, dimethylation of K27, to heterochromatin organization during mitosis. Furthermore, our results also identified multiple dH1 isoforms carrying several phosphorylations and/or methylations, illustrating the high structural heterogeneity of dH1. In particular, we identified several non-CDK sites at the N-terminal domain that appear to be hierarchically phosphorylated. This study provides the most comprehensive PTM characterization of any histone H1 variant to date.

* *Corresponding authors at:* Institute of Molecular Biology of Barcelona, CSIC, Institute for Research in Biomedicine, IRB Barcelona, Barcelona Science Park, Baldiri Rexac, 10. 08028 Barcelona, Spain. Tel.: +34 93 4034958; fax: +34 93 4034979.
E-mail address: fambmc@ibmb.csic.es (F. Azorín).
[1] These authors contributed equally to this work.
[2] Present address: Chromatin Biology Laboratory, Cancer Epigenetics and Biology Program (PEBC), Bellvitge Biomedical Research Institute (IDIBELL), 08908 L'Hospitalet de Llobregat, Spain.

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1. Introduction

Eukaryotic chromatin is primarily organized as nucleosomes, a nucleoprotein complex composed by 146 bp of DNA wrapped around a histone octamer formed by two copies of each core histone H2A, H2B, H3 and H4. Chromatin, however, exists mainly as a 30-nm fiber that results from the folding of nucleosomes into a higher-order structure (reviewed in [1–5]). The formation and stability of this higher-order chromatin structure depends on a fifth protein, histone H1, which binds the linker DNA connecting adjacent nucleosomes. In contrast to core histones that are highly conserved through evolution, histone H1 shows much higher variability [6], with most species containing several variants that appear to play both specific and redundant functions [7,8].

Core histones are extensively modified at multiple residues. Known post-translational modifications (PTMs) of core histones include: K-acetylation, K-methylation (mono-, di- and tri-), R-methylation (mono-, di-symmetric and di-asymmetric), S/T-phosphorylation, K-ubiquitination, K-sumoylation, E-ADP ribosylation, R-deimination and P-isomerization (reviewed in [9,10]). In contrast, information about the PTM pattern of histone H1 is limited, as it has been analyzed only in few species and variants. In addition, although recent studies have identified multiple PTMs in human, mouse and chicken H1 variants [11,12], most other published work focuses mainly on the mapping of phosphorylation sites [13–17]. Like core histone modifications, which contribute to the regulation of multiple genomic processes (reviewed in [9,10]), modifications on histone H1 are likely to play important regulatory functions. Little is known, however, about their functional significance. In this regard, given its remarkable lack of evolutionary conservation, describing histone H1 PTMs in different species appears essential.

Here we analyzed the PTM pattern of histone H1 in the fruit-fly *Drosophila melanogaster* that, in contrast to most eukaryotic species, contains a single histone H1 variant, dH1, which is encoded by the multicopy *His1* gene [18]. For this purpose, we used a combined bottom-up and top-down mass-spectrometry (MS) approach. Bottom-up and top-down strategies for MS-based protein characterization are complementary. In bottom-up strategies, proteomic measurements at the peptide level offer a basis for unambiguous PTM identification. On the other hand, top-down proteomics, which does not require the proteolysis of proteins to generate peptides [19–22], can provide an integrated view of PTM occupancy at the intact protein level. Top-down MS has been used to characterize several purified proteins, including some core histones [23–29]. However, this approach is currently envisaged as a complement of the classical bottom-up strategy since, despite recent progress in protein separation methods [30,31], the analysis of complex mixtures of proteins by top-down MS remains a challenge. In fact, histone H1 PTMs have been analyzed mainly by bottom-up MS [12,13,15–17] and, only in *Tetrahymena thermophila*, histone H1 has been partially analyzed by top-down MS [14].

Here, we report on the identification of multiple PTMs in dH1, including: seven phosphorylated S/T; three mono- and eight dimethylated K; three acetylated K, and four ubiquitinated K. Interestingly, most of these modifications are located at the N-terminal domain, some being conserved in specific human and/or mouse variants, suggesting that they play conserved functions. Our results also showed that dimethylation of K27, a main dH1 PTM that is conserved in vertebrates, accumulates at pericentromeric heterochromatin in metaphase, suggesting a functional contribution to heterochromatin organization and function during mitosis. In addition, we also present evidence of the co-existence of different PTMs in the same dH1 molecule. This study, which illustrates the usefulness of combining bottom-up and top-down analytical approaches, highlights the structural heterogeneity of individual dH1 molecules. It must also be noted that, previous to this study, only a single major phosphorylation site was mapped in dH1 [17].

2. Materials and methods

2.1. Obtaining dH1

dH1 was extracted from purified nuclei from *D. melanogaster* S2 cultured cells by treatment with 10% perchloric acid (PCA) for 1 h [32] (Fig. 1A). After extraction, dH1 was precipitated by addition of 50% trichloroacetic acid (TCA) to a final concentration of 20%, washed with acetone, and air-dried under vacuum.

2.2. Chemical oxidation of dH1

40 μl (8 μg) of dH1 was dissolved to a final volume of 150 μl in a freshly prepared mixture of 3% aqueous H_2O_2 and 3% formic acid (FA) following previously described conditions for mild performic acid treatment (MPA) [33].

2.3. Reverse phase high performance liquid chromatography (RP-HPLC) purification of dH1

For RP-HPLC purification, 8–10 μg of dH1, with and without prior oxidation, was subjected to chromatography on a BioSuite pPhenyl 1000 column (Waters) (10 μm RPC, 4.6×75 mm), using a linear gradient of 5% to 80% B in 60 min (A=0.1% FA in H_2O, B=0.1% FA in CH_3CN) at a flow rate of 1 ml/min (Fig. 1B). An Acquity UPLC chromatographic system (Waters) was used. The LC eluent was coupled after a 1/10 split post-column to a LCT-Premier XE mass spectrometer (Waters-Micromess) provided with an ESI source. A BioRad model 2110 fraction collector was used to collect the fractions of interest. Fractions were lyophilized and stored at −20 °C for further analysis.

2.4. CNBr treatment of dH1 and RP-HPLC purification of dNtH1 peptide

Non-oxidized RP-HPLC-purified dH1 (8 μg) was resuspended in 95 μl 0.1% aqueous FA. The solution was heated at 80 °C for 1 h and 10 μl of 5 M CNBr (Sigma-Aldrich) in acetonitrile was added. Digestion was allowed to proceed overnight at room temperature. dNtH1 peptide was purified using a BioSuite pPhenyl 1000 column (Waters) (10 μm RPC, 2.0×75 mm) and a linear gradient of 5% to 80% B in 60 min (A=0.1% FA in H_2O, B=0.1% FA in CH_3CN) at a flow rate of 100 μl/min. A Finnigan Micro AS autosampler and a Surveyor Finnigan MS quaternary