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Total Chemical Synthesis of Phosphorylated Histone H2A at Tyr57 Reveals Insight into the Inhibition Mode of the SAGA Deubiquitinating Module

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Abstract

Monoubiquitination of histone H2B plays a central role in transcription activation and is required for downstream histone methylation events. Deubiquitination of H2B by the Spt-Ada-Gcn5 acetyltransferase (SAGA) coactivator complex is regulated by a recently discovered histone mark, phosphorylated H2AY57 (H2AY57p), which inhibits deubiquitination of H2B by the SAGA complex as well as restricting demethylation of H3 and increasing its acetylation. Evidence for the effect of H2AY57p however, was indirect and was investigated in vivo by monitoring the effects of chemical inhibition of Tyr kinase CK2 or by mutating the phosphorylation site. We applied total chemical synthesis of proteins to prepare H2AY57p efficiently and study the molecular details of this regulation. This analogue, together with the semisynthetically prepared ubiquitinated H2B enabled us to provide direct evidence on the cross talk between those two marks and the inhibition of SAGA activity by H2AY57p.

Two in one

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Total chemical synthesis of proteins enabled the efficient preparation of homogeneously phosphorylated H2A at Tyr57 via one-pot synthesis. The assembly of phosphorylated H2A and ubiquitinated H2B with H3 and H4 into nucleosome, provided direct evidence for the inhibition of the activity of SAGA complex known to cleave ubiquitin from H2B.

Keywords

H2A histone; Phosphorylation; Deubiquitination; Chemical protein synthesis; SAGA DUB module

Genetic information is stored in eukaryotes as a nucleoprotein complex known as chromatin, whose repeating unit is called the nucleosome.^[1] Each nucleosome comprises approximately 146 base pairs of DNA wrapped twice around a histone octamer core consisting of two copies each of the four histone proteins, H2A, H2B, H3 and H4. Reversible posttranslational modifications (PTMs) of histones such as methylation, acetylation, phosphorylation and ubiquitination play a central role in regulating all processes requiring access to the genomic DNA in its chromatin context.^[2] Mono-ubiquitination of histone H2B, for example, plays important roles in transcription activation, elongation, mRNA splicing and export, as well as in DNA replication and DNA damage repair.^[3] H2B ubiquitination at Lys120 (**H2BK120Ub**) stimulates methylation of Lys79 methylation of H3 by the DOT1 histone methyl transferase^[4] and Lys4 methylation of H3 by Set1.^[5] Histone H2BK120 (in humans; Lys123 in yeast) is deubiquitinated by the Spt-Ada-Gcn5 acetyltransferase (SAGA) complex, a transcriptional co-activator complex that contains a four protein subcomplex

(Ubp8/Sgf11/Sus1/Sgf73), known as the deubiquitinating module (DUBm) that cleaves ubiquitin from histone H2B.^[6]

Several studies have shown that phosphorylation of various H2A residues is involved in regulating a variety of biological processes, such as DNA double-strand break repair.^[7] Phosphorylation of Ser121 of H2A, for example, was reported to prevent chromosome instability^[8] while phosphorylation of Ser1 inhibits transcription on a chromatin template.^[9] Very recently, a new conserved phosphorylation site in human histone H2A at Tyr57 (**H2AY57p**) was discovered.^[10] Phosphorylation of H2AY57 was found to inhibit deubiquitination of H2B by the SAGA complex as well as restricting demethylation of histone marks Lys4 and Lys79 and increasing acetylation of Lys27 in H3.^[10] Evidence for the effect of phosphorylated H2AY57 however, was indirect and was investigated in vivo by monitoring the effects of chemical inhibition of Tyr kinase CK2 or by mutating the phosphorylation site.^[10] A barrier to obtaining molecular insights into the impact of phosphorylated H2AY57 on deubiquitination by the SAGA DUBm is the difficulty in obtaining H2A homogeneously phosphorylated at Tyr57, as well as the absence of any suitable amino acid mimic of phosphotyrosine. Such a study would also require H2B homogeneously modified by the covalent attachment of ubiquitin to Lys120 (**H2BK120Ub**) and the assembly of nucleosomes containing the two desired modifications, which could then be used for further biochemical analysis. To answer this question, among many others, obtaining homogeneous **H2AY57p** and **H2BK120Ub** is an essential starting point.

Posttranslationally modified histones can be prepared homogeneously either by total chemical synthesis or by semisynthetic methods^[11] where native chemical ligation (NCL) is applied to assemble the peptide fragments and construct the full protein sequence.^[12] In semisynthetic methods, the histone-derived peptides containing the desired PTMs (generally at the N- or C-terminal region) are synthesized chemically and subsequently ligated with recombinantly prepared complementary fragments. Thus, the application of this method is limited mainly for N- or C-terminally modifications. For example, our group^[13] as well as the Muir group^[14] used semisynthesis to assemble H2B bearing ubiquitin at its C-terminal region to assist numerous studies aiming at deciphering the role of ubiquitination in chromatin context. On the other hand, a total chemical synthesis approach, whereby the entire modified protein is synthesized via the assembly of synthetic peptides generated by solid phase peptide synthesis (SPPS), makes it possible to incorporate unlimited variations in the protein sequence, including the installation of PTMs at any desired position(s).^[15]

In thinking about the preparation of **H2AY57p** modified histone, it was obvious to us that chemical total chemical protein synthesis would be the most efficient way to prepare this relatively small protein, which is composed of 130 residues. Here we report the efficient total chemical synthesis of the **H2AY57p**, in one-pot fashion, which allowed the characterization of the SAGA deubiquitination of **H2BK120Ub** in the context of a nucleosome.

To chemically synthesize **H2AY57p**, the polypeptide was divided into three fragments, Cys-H2A(88–130), fragment **1**, Thz-H2AY57p(49–86), fragment **2**, and H2A(1–47), fragment **3**, (Scheme 1).

The N-terminal Ala in fragments **2** and **3** were temporary mutated to Cys to enable NCL, wherein the N-terminal Cys of fragment **2** was protected as thiazolidine (Thz) to prevent cyclization or self-ligation during ligation process. The fragments **2** and **3**, in their thioester forms, were prepared using *N*-acylurea^[16] and *N*-methylcysteine^[17] methods, respectively. Notably, in case of fragment **2**, the cyclization of 3,4-diaminobenzoic acid (Dbz) was unsuccessful via standard conditions in dichloromethane. However, the use of *N,N*-dimethylformamide allowed the efficient cyclization of the Dbz and afforded the *N*-acylurea peptide with the additional 28 Da mass due to formylation of the *N*-acylurea moiety via Vilsmeier–Haack reaction.^[18] On the other hand, fragment **3**, which bears the C-terminal Gly, was prepared by the *N*-methylcysteine strategy, since the *N*-acylurea method in this case would require extra protection/deprotection steps^[19] of the free amine on Dbz to avoid any possible branching during Fmoc-based solid phase peptide (Fmoc-SPPS).^[20] All peptides were synthesized using Fmoc-SPPS in high purity and with good yields (~40–50%) (Supporting Information).

With all fragments in hand, the first ligation between peptides **1** and **2** was performed in the presence of 4-mercaptophenylacetic acid (MPAA) and tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl) in 6 M Gn·HCl, pH~7.2 (Figure 1). After completion of the ligation reaction in 4h, the mixture was treated with MeONH₂·HCl at pH~4 for 10 h to convert Thz protecting group in ligation product to Cys-H₂A(48–130), **4**. Prior to the second ligation, the pH of the reaction mixture was adjusted to ~7.2 using NaOH. Fragment **3** was dissolved in a freshly prepared MPAA/TCEP solution and was added to the ligation mixture to enable the second ligation in one-pot manner, which furnished the ligation product **5**, H₂AY57p(1–130), in 1h. Notably, despite the presence of methoxylamine, in the second ligation only a minor amount of the side product generated from the attack of methoxylamine on fragment **3** was observed.^[15a, 15b, 21] This is probably due to the rapid ligation reaction between fragments **3** and **4**. Subsequently, one-pot desulfurization^[22] was carried out in presence of the initiator 2,2'-Azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044), TCEP and *t*-BuSH for 3h to produce the full length histone **H2AY57p** in ~25% isolated yield (Figure 1). The purified synthetic **H2AY57p** (Figure 2A) was examined by circular dichroism (CD), which exhibited the expected CD signature of folded H2A (Figure 2B).

In order to prepare the nucleosomal substrate needed to test the effect of **H2AY57p** on deubiquitination of **H2BK120Ub**, the synthesized **H2AY57p** was assembled into nucleosomes together with recombinant *Xenopus laevis* histones H3 and H4, **H2BK120Ub** and the Widom 601 DNA sequence.^[23] Notably, **H2BK120Ub** was prepared using our previously reported semisynthetic approaches.^[24] Histones **H2BK120Ub**, H3, H4, and either native H2A or synthetic **H2AY57p** were reconstituted into octamers and purified by size-exclusion chromatography as described (Figure 2C).^[25] Histone octamers could be assembled with similar efficiencies irrespective of whether or not they contained unmodified or phosphorylated H2A, indicating that the modification does not interfere with histone refolding. The histone octamers were further reconstituted into nucleosome core particles (NCPs) by gradient salt dialysis in the presence of the Widom 601 DNA purified using standard methods.^[25]

In order to determine the effect of **H2AY57p** on DUB module activity, we compared the ability of the yeast SAGA DUB module to cleave monoubiquitin from histone H2B using ubiquitinated nucleosomes that contained either **H2AY57p** or unmodified H2A (Figure 3). The yeast SAGA DUB module, comprising Ubp8, Sus1, Sgf11 and Sgf73 residues 1–104 was purified as described.^[26] DUB module at a concentration of 200 nM was incubated with 1 μ M nucleosome in buffer containing 50 mM HEPES pH 7.5, 150 mM NaCl, 5 mM dithiothreitol and 10 μ M ZnSO₄. Aliquots of the reaction mixture were removed at the indicated time points, quenched by the addition of sample buffer containing sodium dodecyl sulfate and β -mercaptoethanol and analyzed by gel electrophoresis as described.^[27] Cleavage was assessed by comparing the disappearance of the higher molecular weight H2BK120Ub band and appearance of free ubiquitin (Figure 3). The rate at which ubiquitin was cleaved from histone H2B was significantly lower (an approximate 30-fold reduction) for H2BK120Ub in nucleosomes containing **H2AY57p**, directly implicating this mark in regulating of DUB module activity against **H2BK120Ub**.

The ability of **H2AY57p** to interfere with cleavage of ubiquitin from H2BK120 agrees with our recently reported crystal structure of the SAGA DUB module bound to ubiquitinated nucleosomes.^[27] Residue H2AY57 lies at the interface between the DUB module and its contact surface with histones H2A and H2B (Figure 4). In particular, molecular modeling^[27] showed that a phosphate group covalently linked to H2AY57 is in a position to clash with Sgf11 residues Arg84 or Arg91, which are required for efficient H2B deubiquitinase activity.^[27]

In summary, the efficient total chemical synthesis of **H2AY57p** was achieved from three peptide fragments, which were combined together applying one-pot NCL-desulfurization to give the desired product in high homogeneity and in 25% overall isolated yield. Taking advantage of our ability to prepare **H2BK120Ub**, we were able for the first time to assemble nucleosomes containing these two unique modifications and study the effect of H2A phosphorylation on deubiquitination of histone H2B in a nucleosomal context. Our results provide the first evidence that phosphorylation of H2A directly inhibits deubiquitination by the SAGA DUB module and lays the foundation for further studies dissecting cross-talk between these two marks as well as other marks that are modulated by ubiquitination-deubiquitination. Our study also highlights the power of total chemical synthesis of proteins to understand the complex mechanism of the histone regulation by multiple reversible PTMs.

Supplementary Material

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Acknowledgments

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Figure 1.

Analytical HPLC and mass traces for chemically prepared **H2AY57p**. A) Ligation of fragments **1** and **2**, peak a corresponds to **1**; peak c corresponds to **2**, and peak b corresponds to ligation product **4** with the observed mass 9187.9 ± 0.8 Da, calcd 9186.7 Da (average isotopes). B) Ligation of fragments **3** and **4**, peak d corresponds to side product by methoxylamine attack on thioester in peptide **3**; peak e corresponds to **1** and the cross ligation product between fragments **1** and **3**; peak g corresponds to cross ligation product between **2** and **3**; peak h corresponds to **4**; peak f corresponds to final ligation product of **1**, **2** and **3** with the observed mass 14192.5 ± 1.2 Da calcd 14191.5 Da (average isotopes). C) Desulfurization of the ligation product: peak d' corresponds to the desulfurized side product d, peak e' corresponds to the desulfurized side product e, Peak i corresponds to the desulfurized protein **H2AY57p**, with the observed mass 14128.0 ± 1.3 Da, calcd 14127.3 Da (average isotopes).

Figure 2.

A) Analytical HPLC and mass traces for the purified **H2AY57p** with the observed mass 14128.0 ± 1.3 Da, calcd 14127.3 Da (average isotopes). B) CD spectra of **H2AY57p**. C) Histone octamers reconstituted with the indicated modifications show identical reconstitution efficiencies as determined by analytical size-exclusion chromatography. (* indicates the peak containing octamer, whereas ** corresponds to the H2A/H2B heterodimer peak).

Figure 3.
DUB module cleavage rates for ubiquitinated nucleosomes are significantly reduced in the presence of **H2AY57p**.

Figure 4.

Location of **H2AY57p** at the interface between the SAGA DUB module and the nucleosome. Modeling of **H2AY57p** into the structure of the DUB module bound to the ubiquitinated nucleosome (PDB ID: 4ZUX). Modeled side chain positions suggest that **H2AY57p** may interfere with docking of the DUB module on the nucleosome.^[27]

Scheme 1.

A) H2A sequence. B) Schematic presentation of the synthesis of **H2AY57p** in one pot fashion.