Protein Synthesis

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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 the total chemical synthesis of proteins to prepare H2AY57p efficiently and study the molecular details of this regulation. This analogue, together with semisynthetically prepared ubiquitinated H2B, enabled us to provide direct evidence for the cross-talk between those two marks and the inhibition of SAGA activity by H2AY57p.*

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] Monoubiquitination 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 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 coactivator complex that contains a four-protein subcomplex (Ubp8/Sgf11/Sus1/Sgf73) known as the deubiquitinating module (DUBm), which 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] whereas phosphorylation of Ser1 inhibits transcription on a chromatin template.[9] 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 insight into the impact of phosphorylated H2AY57 on deubiquitination by the SAGA DUBm is the difficulty in obtaining H2A homogenously phosphorylated at Tyr57, as well as the absence of any suitable amino acid mimic of phosphotyrosine. Such a study would also require H2B homogenously 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, the synthesis of 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] in which 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 in 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 to N- or C-terminal modifications. For example, our research group[13] as well as the Muir group[14] used semisynthesis to assemble H2B bearing ubiquitin in its C-terminal region to assist numerous studies aimed at deciphering the role of ubiquitination in the context of chromatin. On the other hand, total chemical

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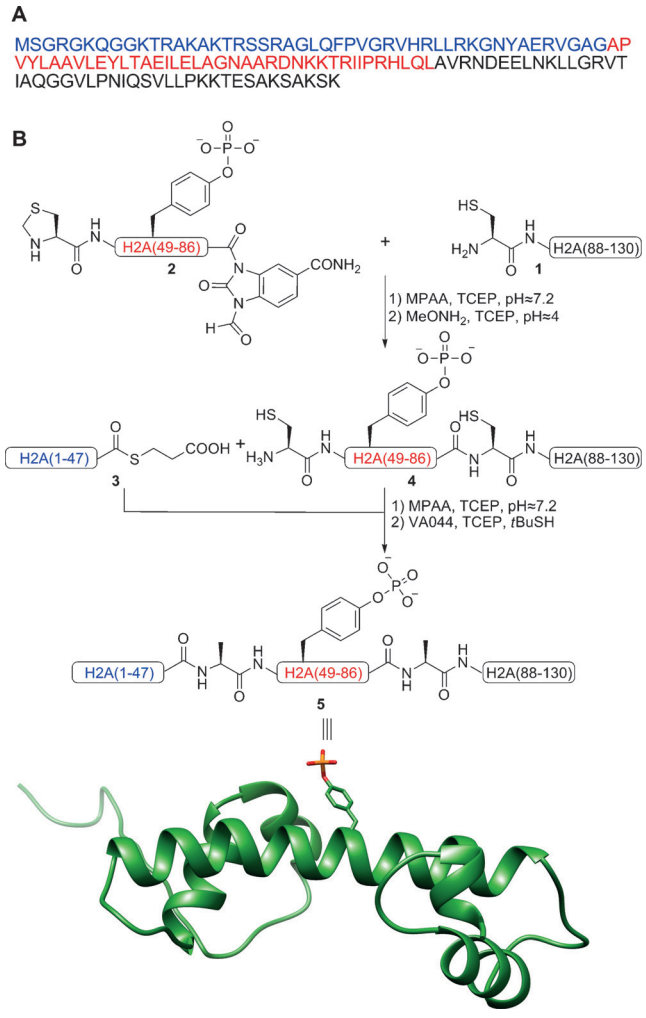
synthesis, whereby the entire modified protein is synthesized by the assembly of synthetic peptides generated by solid-phase peptide synthesis (SPPS), makes it possible to incorporate unlimited variations in the protein sequence, including the installation of PTMs at any desired position(s).[15]

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

For the chemical synthesis of **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 residues in fragments **2** and **3** were temporarily mutated to Cys to enable NCL, for which the N-terminal Cys residue of fragment **2** was protected as thiazolidine (Thz) to prevent cyclization or self-ligation during the ligation process. Fragments **2** and **3**, in their thioester forms, were prepared by the use of the *N*-acylurea[16] and *N*-methylcysteine[17] method, respectively. Notably, in the case of fragment **2**, the cyclization of 3,4-diaminobenzoic acid (Dbz) was unsuccessful under standard conditions in dichloromethane. However, the use of *N*,*N*-dimethylformamide allowed the efficient cyclization of Dbz and afforded the *N*-acylurea peptide with an additional mass of 28 Da owing to formylation of the *N*-acylurea moiety by the Vilsmeier–Haack reaction.[18] On the other hand, fragment **3**, which bears a C-terminal Gly residue, 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 9-fluorenylmethoxycarbonyl-based solid-phase peptide synthesis (Fmoc-SPPS).[20] All peptides were synthesized by Fmoc-SPPS in high purity and good yields (ca. 40–50%; see the Supporting Information).



Scheme 1. A) H2A sequence. B) Synthesis of **H2AY57p** in a one-pot fashion.

With all fragments in hand, we performed the first ligation between peptides **1** and **2** 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 4 h, the mixture was treated with $MeONH_2$·HCl at pH ≈ 4 for 10 h to cleave the Thz protecting group in the ligation product and form Cys-H2A(48–130) (**4**). Prior to the second ligation, the pH value of the reaction mixture was adjusted to approximately 7.2 with 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 a one-pot manner, which furnished the ligation product H2AY57p(1–130) (**5**) in 1 h. 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,b,21] probably owing to the rapid ligation reaction between fragments **3** and **4**. Subsequently, one-pot desulfurization[22] was carried out in the presence of the initiator 2,2′-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044), TCEP, and *t*BuSH for 3 h to produce the full-length histone **H2AY57p**, which was isolated in approximately 25% 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).

To prepare the nucleosomal substrate needed to test the effect of **H2AY57p** on the deubiquitination of **H2BK120Ub**, we assembled the synthesized **H2AY57p** into nucleosomes together with recombinant *Xenopus laevis* histones H3 and H4, **H2BK120Ub**, and the Widom 601 DNA sequence.[23] Notably, **H2BK120Ub** was prepared by 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 efficiency irrespective of whether they contained unmodified or phosphorylated H2A, thus 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 by standard methods.[25]

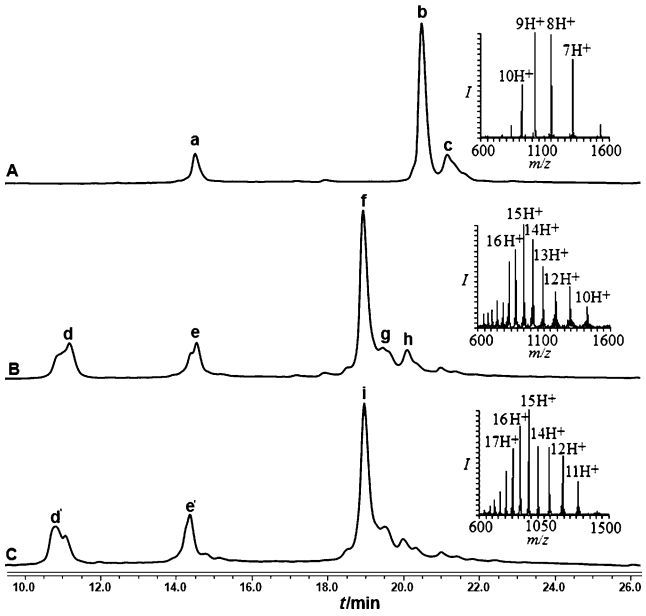


Figure 1. Analytical HPLC and mass traces for chemically prepared **H2AY57p**. A) Ligation of fragments **1** and **2**; peak a corresponds to **1**, peak c corresponds to **2**, and peak b corresponds to the ligation product **4** with the observed mass (9187.9 ± 0.8) Da (calcd: 9186.7 Da, average isotopes). B) Ligation of fragments **3** and **4**; peak d corresponds to the side product derived from methoxylamine attack on the thioester in peptide **3**, peak e corresponds to **1** and the cross-ligation product of fragments **1** and **3**, peak g corresponds to the cross-ligation product of **2** and **3**, peak h corresponds to **4**, peak f corresponds to the 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 product derived from the desulfurization of side product d, peak e′ corresponds to the product derived from the desulfurization of side product e, and peak i corresponds to the desulfurized protein **H2AY57p** with the observed mass (14128.0 ± 1.3) Da (calcd: 14127.3 Da, average isotopes).

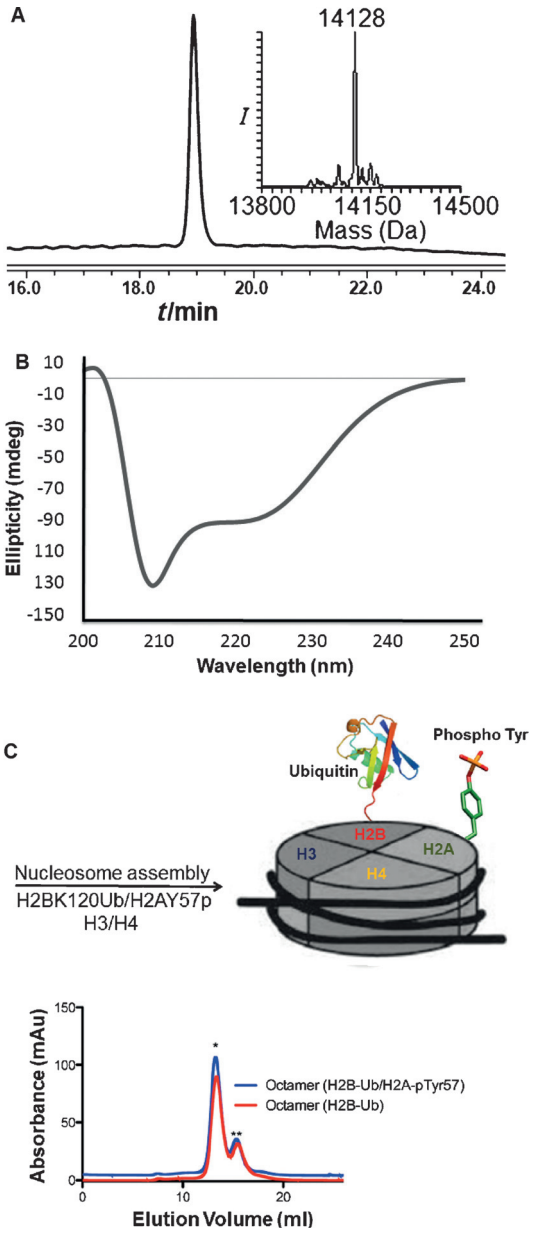


Figure 2. A) Analytical HPLC and mass traces for purified **H2AY57p** with the observed mass (14128.0 ± 1.3) Da (calcd: 14127.3 Da, average isotopes). B) CD spectrum of **H2AY57p**. C) Histone octamers reconstituted with the indicated modifications showed identical reconstitution efficiencies to those containing unmodified H2A, as determined by analytical size-exclusion chromatography (* indicates the peak corresponding to the octamer, whereas ** corresponds to the H2A/H2B heterodimer peak).

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 by 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] The DUB module at a concentration of 200 nM was incubated with the nucleosome (1 μM) in a buffer containing HEPES (50 mM, pH 7.5), NaCl (150 mM), dithiothreitol (5 mM), and $ZnSO_4$ (10 μM). 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 approximately 30-fold reduction) for **H2BK120Ub** in nucleosomes containing **H2AY57p**, thus directly implicating this mark in the regulation of DUB-module activity against **H2BK120Ub**.

The ability of **H2AY57p** to interfere with the 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]

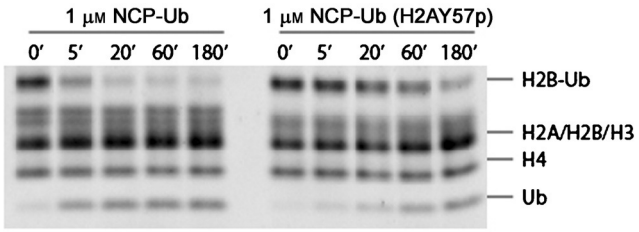


Figure 3. Gel electrophoresis showing that the DUB-module cleavage rates for ubiquitinated nucleosomes are significantly reduced in the presence of **H2AY57p**.

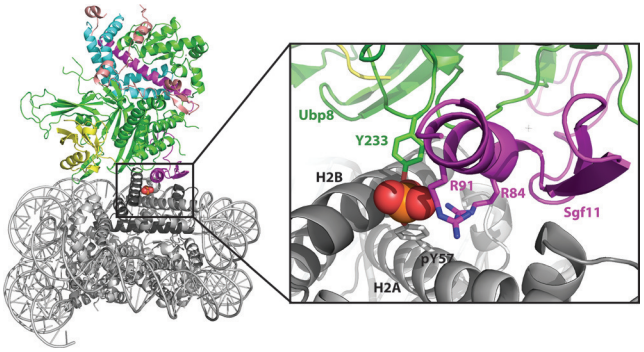


Figure 4. Location of **H2AY57p** at the interface between the SAGA DUB module and the nucleosome. Modeling of **H2AY57p** into the structure of the DUB module bound to the ubiquitinated nucleosome (PDB ID: 4ZUX). Modeled side-chain positions suggest that **H2AY57p** may interfere with docking of the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

In summary, we have described the efficient total chemical synthesis of **H2AY57p** from three peptide fragments, which were combined by applying one-pot NCL–desulfurization to give the desired product with high homogeneity and in 25 % overall yield. By taking advantage of our ability to prepare **H2BK120Ub**, we were able to assemble nucleosomes containing these two unique modifications and study the effect of H2A phosphorylation on the 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 lay the foundation for further studies to dissect cross-talk between these two marks as well as other marks that are modulated by ubiquitination–deubiquitination. Our study also highlights the power of the total chemical synthesis of proteins to enhance our understanding of the complex mechanism of histone regulation by multiple reversible PTMs.

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