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Characterization of the effects of phosphorylation by CK2 on the structure and binding properties of human HP1β

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ABSTRACT

Proteins of the Heterochromatin Protein 1 (HP1) family are regulators of chromatin structure and genome function in eukaryotes. Post-translational modifications expand the repertoire of the chemical diversity of HP1 proteins and regulate their activity. Here, we investigated the effect of phosphorylation by Casein kinase 2 (CK2) on the structure, dynamics and binding activity of human HP1β. We show that Ser89 in the hinge region is the most effective substrate, followed by Ser175 at the C-terminal tail. Phosphorylation at these sites results in localized conformational changes in HP1β that do not compromise the ability of the protein to bind chromatin.
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1. Introduction

The compaction of chromatin into different structural states, a fundamental mechanism whereby eukaryotes tune the functions of the genome, is a highly dynamic process subjected to different levels of regulation. One of the most important is the post-translational modification of histone tails. Methylation of lysine 9 in histone H3 (H3K9me) is specifically recognized by Heterochromatin Protein 1 (HP1), a key player in chromatin condensation and gene silencing. By means of its two distinct protein–protein interaction modules, the chromo domain (CD) and the chromoshadow domain (CSD), HP1 links a variety of proteins involved in chromatin function. CD engages the H3K9me mark in nucleosomes [1], while the dimeric CSD recruits different partners via recognition of a consensus pentameric sequence PXVXL found in numerous nuclear proteins [2–4]. The long hinge region connecting CD to CSD is highly dynamic [5,6] and has nucleic acid binding activity [7,8]. Given the central role of HP1 in chromatin biology it is important to understand how the protein is regulated.

Recent studies revealed that HP1 proteins are modified in mammalian cells with the same variety of post-translational modifications that mark the histones, such as phosphorylation, acetylation and methylation [9–13]. Phosphorylation is one of the prevalent modifications in HP1. Since the human HP1 homologs α, β and γ differ in the number of phospho-acceptors, differential phosphorylation could regulate their activity and localization. Indeed, the three human HP1 proteins showed remarkable differences in phosphorylation patterns during the cell cycle [14].

Phosphorylation of HP1γ by PKA [9] regulates HP1γ silencing activity, and HP1 proteins can be phosphorylated by members of the TIF1 family and participate in their transcriptional repression function [15]. In fission yeast, N-terminal phosphorylation of HP1 homolog Swi6 by Casein kinase 2 (CK2) was shown to control gene silencing in heterochromatin [16]. The phosphorylation of the N-terminal region of mammalian HP1α [17] and HP1 in Drosophila [18] by CK2 regulates the binding and the proper targeting of these proteins to heterochromatin. In addition, phosphorylation of T51 in

Abbreviations: CK2, casein kinase 2; CD, chromo domain; CSD, chromoshadow domain; HP1, heterochromatin protein 1; MS, mass spectrometry; NMR, nuclear magnetic resonance; SAXS, small angle X-ray scattering

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HP1β by CK2 regulates HP1β mobilization from chromatin after DNA damage by impairing the interaction of CD with methylated histone [19]. Thus, phosphorylation by CK2, a pleiotropic and constitutively active serine–threonine kinase with a global pro-survival activity [20], constitutes an important mechanism of regulation of HP1 function. Identification of the sites of CK2-mediated phosphorylation, the degree of phosphorylation and the investigation of the effects on the protein structure are therefore important to understand possible changes in the activity of HP1 at the molecular level.

Here, we studied the effects of phosphorylation on the structural and binding activity properties of human HP1β. We show that S89 in the hinge region and S175, which is unique to HP1β among the three human homologs, are the principal sites of modification. Upon phosphorylation, conformational changes occur around the site of modification. HP1β phosphorylated at S89 and S175 retains its key activity of binding to histones and chromatin.

2. Material and methods

Details on samples preparation and HP1β–chromatin binding studies can be found in Supplementary information. For in vitro phosphorylation of HP1β and its fragments we used recombinant CK2 enzyme purchased from New England BioLabs. CK2 reactions were done at room temperature in 20 mM Tris–HCl pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, and ATP (Sigma) in molar excess with respect to the substrate concentration (typically 10:1). Incubation time was 4 days for isotope-labeled HP1β, 5 days for unlabeled HP1β and 7 days for CD, CSD and hinge fragments.

The NMR kinetic experiment was done at 298 K on a 0.17 ml sample of 0.08 mM ^{15}N-perdeuterated HP1β in the previously described CK2 buffer at pH 7 and with 0.6 mM ATP, by acquiring thirty ^{1}H–^{15}N HSQC of 40 min each after addition of 2500U of CK2. A 3 mm tube and a 700 MHz spectrometer equipped with cryoprobe were used. To derive the characteristic signal decay times, the intensity of original peaks as a function of incubation time was fitted to a single-exponential decay equation.

Additional experimental details can be found in Supplementary information.

3. Results and discussion

An overview of the phosphorylated sites found in human HP1β and occurring in cells is available at the "*PhosphoSitePlus*®, www.phosphosite.org" resource [21], and is illustrated in Fig. 1A. Part of the phosphorylated sites conforms to the consensus motif of CK2 substrates. Multiple acidic residues between the −1 and +4 position relative to the phospho-acceptor (Ser is favored over Thr) define a strong motif for CK2. The most relevant acidic determinant is at +3, followed by the +1 position. Basic residues are negative determinants between −1 and +4, as well as Pro at +1 [20]. Following these criteria, S46, S89 and S175 are closest to the optimal consensus phosphorylation sequence. Based on the general accepted motif S/TXXE/D, T73, S128 and S162 are additional potential CK2 substrates, although they lack the favoring +1 acidic determinant. Since variants like SXE/D or sites that depend on previous phosphorylation events, were also found among the hundreds of CK2 protein substrates [20], additional CK2-sites might be present in HP1β. Indeed T51, although residing in an atypical motif, was found to be phosphorylated by CK2 [19].

The ability of CK2 to phosphorylate recombinant human HP1β in our experimental conditions was initially assessed by mass spectrometry (MS). After in vitro phosphorylation of HP1β by CK2, MS analysis revealed a main double- phosphorylated species. The second notable signal indicated also the presence of triple-phosphorylated isoforms (Fig. 1B). To identify the phospho-acceptor sites, quantify the modification level and investigate at atomic resolution the possible structural changes derived from phosphorylation of HP1, we used NMR spectroscopy. NMR is an excellent method for the study of post-translational modifications of proteins as the covalent addition of chemical groups can be probed by highly specific changes in both the position and intensity of signals uniquely associated with specific residues. In particular, ^{1}H–^{15}N correlations are highly sensitive to local changes of chemical environment directed by protein phosphorylation. The reaction of CK2 on ^{15}N-perdeuterated HP1β sample resulted in changes for a subset of signals in the ^{1}H–^{15}N HSQC spectrum. The region including serine signals was mainly affected, and two new intense peaks appeared in the downfield direction (Fig. 2A), suggesting the occurrence of a main double-phosphorylated isoform, in agreement with MS. Nearby, two very small peaks also appeared (Fig. S1A), indicating the presence of rare isoforms that can correspond to the triple-phosphorylated species detected by MS.

NMR signals of residues within the CD and CSD were mainly unperturbed, suggesting that the obtained phosphorylation caused little effect on the globular domains of HP1β. Changes were observed primarily in the part of the spectrum belonging to the unfolded regions. Since peaks shift not only as a consequence of direct phosphorylation but also in consequence of indirect changes induced by the nearby phosphorylation sites, we carried out multidimensional triple-resonance experiments to unambiguously assign the backbone resonances of the prevalent double-phosphorylated isoform. Fig. 3A shows the plot of HP1β ^{1}H–^{15}N-backbone chemical shifts changes as a consequence of protein phosphorylation. While the N-terminal tail, the CD and most of the CSD did not show significant changes, the hinge region and the C-terminal part, including the very end of CSD, were perturbed.

The most affected signals correspond to S89 and S175 and the neighboring residues, which comply with the optimal consensus sequence motif of CK2. As shown in Fig. 2A, S89 and S175 experienced large downfield backbone ^{1}H/^{15}N chemical shift changes (0.51/1.54 ppm and 0.44/0.87 ppm, respectively), with the magnitude and direction being typical for phosphorylated serine residues in solvent exposed regions [22]. These features, together with the observation that the original peaks corresponding to the unmodified variant almost vanished (94% and 92% were phosphorylated, respectively), indicate that S89 and S175 are the two principal sites of phosphorylation. The small additional signals appearing next to pS89, probably belonging to the triple-phosphorylated isoforms detected by MS, could not be assigned due to low signal intensity (4% relative to pS89), indicative of sub-stoichiometric levels of modification in the NMR sample.

To identify the position of the third phospho-acceptor site, we incubated CK2 with two different HP1 fragments containing the residues that were mainly perturbed as shown by the previous NMR analysis of full-length HP1β (2–185): a hinge region peptide, spanning residues 80–109, and a CSD containing protein, spanning residues 107–185. For the latter construct, one phosphorylated site was detected by MS and NMR and confirmed to be S175 (Figs. 1B and 4A). The incubation of CK2 with the hinge peptide resulted in a major mono-phosphorylated species together with a secondary component corresponding to a double-phosphorylated peptide (Fig. 1B). Thus, a third phosphorylation site of full-length HP1β is located in the hinge region. It might correspond to S91, which is within a sequence variant substrate (SXE/D) for CK2 and was found, in addition to S89 and S175 [10,12,13], to be phosphorylated in human cells [12,13]. However, NMR data obtained in the context of full-length HP1β indicated that S91 remained mainly in the unmodified state and isoforms phosphorylated at this site were relatively rare. Indeed for S91 we did not observe the large downfield chemical shift typical of phospho-serine (Fig. 2A). Its moderate shift instead reflects the proximity to the neighboring pS89 (see below).

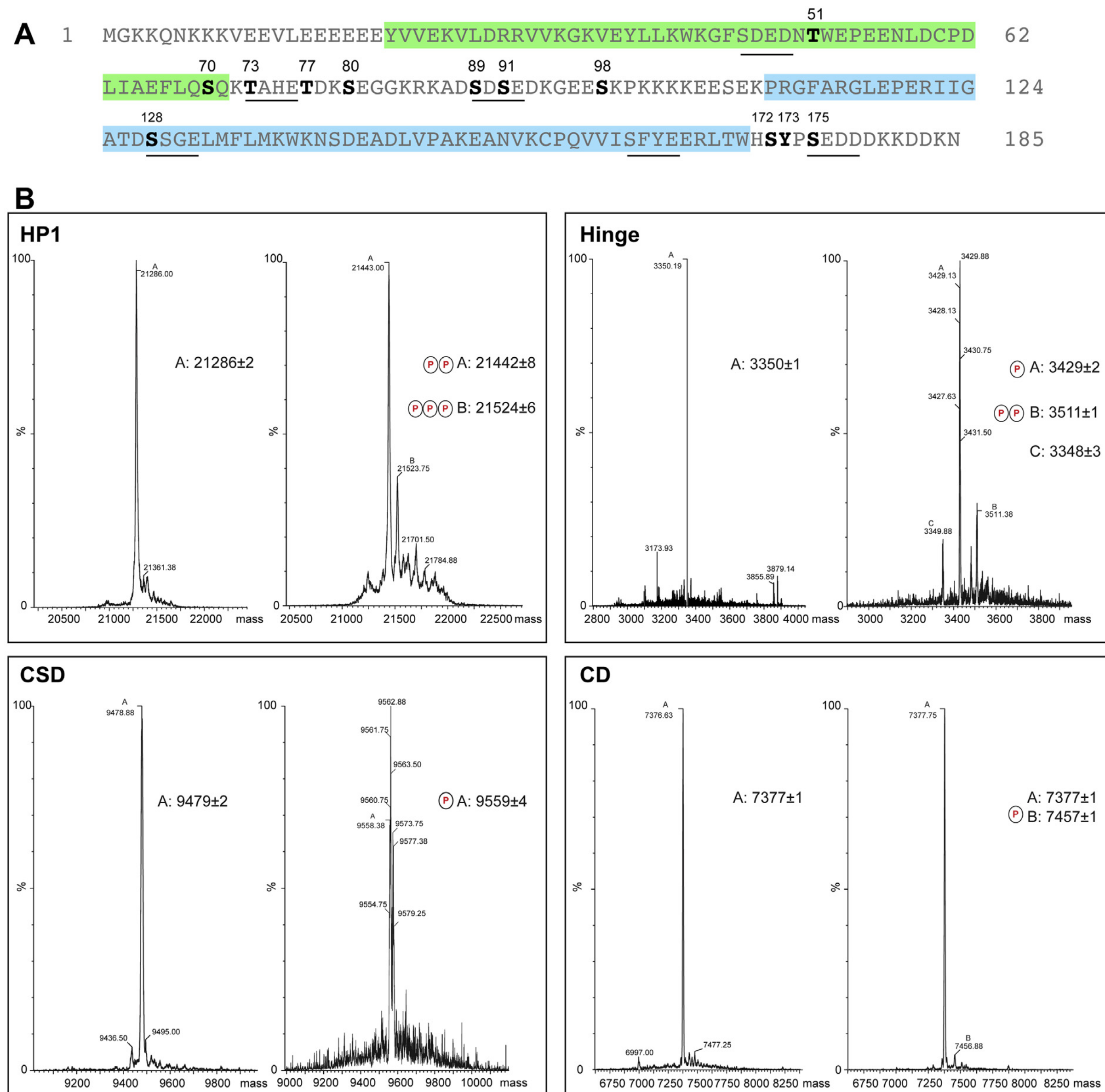

Fig. 1. Phosphorylation of human HP1β. (A) Primary structure of human HP1β (UniProt ID: P83916). Previously identified phosphorylation sites listed at "*PhosphoSitePlus*®, www.phosphosite.org" [21] are numbered and marked in black. Regions corresponding to the CK2 consensus motif 'S/TXXD/E' are underlined. CD and CSD are highlighted in green and blue respectively. (B) MS analysis of proteins before (left side of each panel) and after (right side of each panel) incubation with CK2. HP1: HP1β(2–185); Hinge: synthetic peptide Hinge(80–109); CSD: ^{15}N-CSD(GHM107–185); CD: ^{15}N-CD(G19–79). Note the contribution of ^{15}N-labeling (close to 100%) to the mass of CD and CSD samples.

Next, we analyzed the kinetics of phosphorylation at each site using NMR spectroscopy. During incubation for 1 day, the kinetics of "substrate consumption" upon phosphorylation differed significantly at individual sites (Fig. 2B). The signal decay times of S89 and S175 upon reaction were 5.2 ± 0.1 and 9.7 ± 0.7 h, respectively. Thus, phosphorylation of S89 in the hinge region is faster than that of S175. This difference may arise from better solvent exposure of the hinge region, while access to S175 might be hindered by the neighboring CSD. The signal decay time of S91 was 5.2 ± 0.2 h (Fig. S1B). The similarity of the decay times of S91 and S89 suggests that the signal perturbation observed for S91 is caused by a change of chemical environment due to phosphorylation at S89 and not due to a direct modification.

As a negative control, other residues were analyzed. Interestingly S46, that fits the CK2 criteria to be an optimal substrate, does not show any change in signal position, nor in intensity. This is probably because the consensus sequence is embedded in the CD fold and therefore not accessible. Also T51, identified as CK2 substrate in a recent study by different experimental strategies [19], did not show any clear NMR signal change in our experimental conditions. To explore the possibility that in full-length protein the strong competition with the main sites located in the hinge region and C-terminus impeded phosphorylation in CD, we assayed CK2 activity on the isolated construct CD (19–79). MS results suggested the occurrence of partial phosphorylation (Fig. 1B). However, the majority of the protein remained unmodified in our experimental conditions, suggesting that phosphorylation of CD was not efficient. The fact that T51 is within the CD fold and resides in an atypical sequence for CK2 recognition is consistent with the low level of modification at this site. In a cellular context phosphorylation of T51 by CK2 might be boosted by specific molecular events. For example, possible protein–protein interactions induced

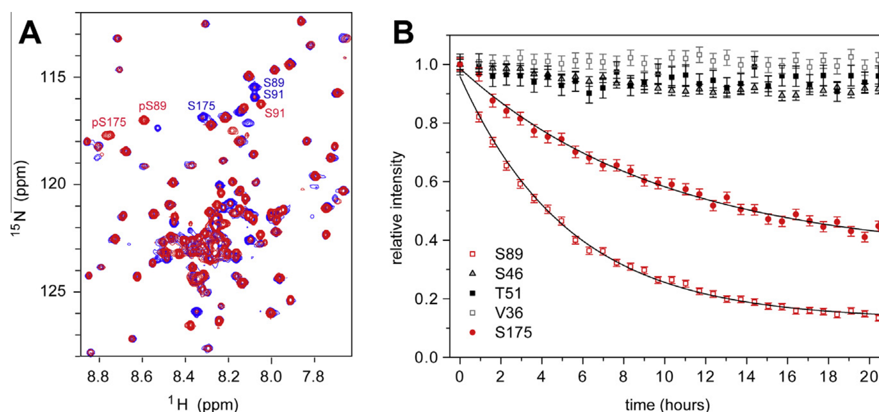

Fig. 2. NMR analysis of HP1β phosphorylation by CK2. (A) Superposition of a selected region from the ^{1}H–^{15}N TROSY HSQC spectrum of HP1β (blue) and HP1β after phosphorylation by CK2 (red). The new signals, corresponding to the phosphorylated counterpart of S89 and S175, are labeled with pS89 and pS175. (B) Time course of the phosphorylation reaction monitored by substrate consumption based on the relative changes in peak intensity. Errors were determined through propagation of the noise in the NMR spectra.

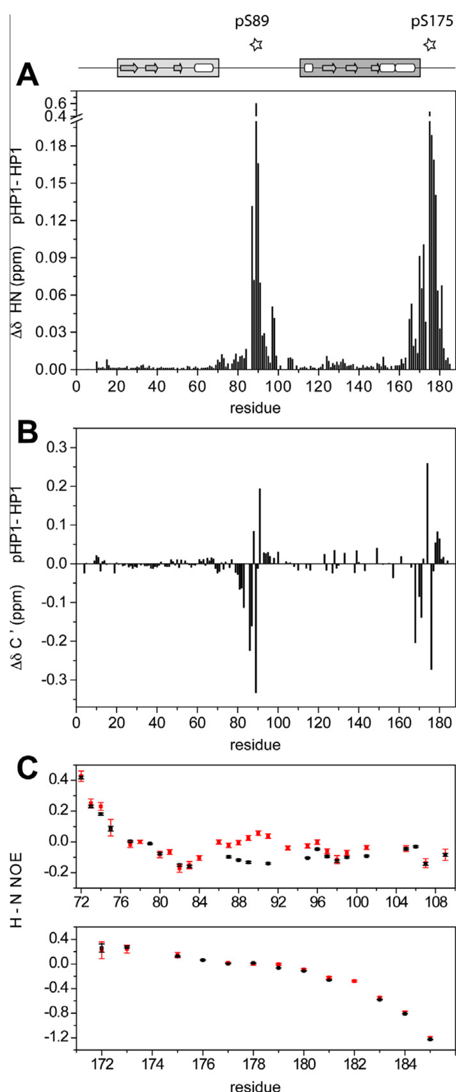

Fig. 3. Conformational analysis of phosphorylated HP1β (pHP1β). (A) Combined ^{1}H–^{15}N chemical shift differences between pHP1β and HP1β. (B) ^{13}C carbonyl chemical shift differences between pHP1β and HP1β. The scatter observed in the CSD is due to the lower signal-to-noise ratio caused by the slower tumbling of the dimeric CSD. Signals affected by severe overlap or broadening were excluded from the analysis. (C) Steady-state ^{1}H–^{15}N hetNOE measured on full-length HP1β (black) and pHP1β (red) using 10 s recycle delay.

by the DNA damage signaling could favor the recruitment of CK2 at the CD or induce local conformational changes that make T51 more accessible for the enzyme catalysis.

NMR was further used to investigate the effects of S89 and S175 modification on the conformational properties of the prevalent double-phosphorylated HP1β isoform. Analysis of combined ^{1}H–^{15}N chemical shift differences (Fig. 3A) indicated that the overall structure of neither CD nor CSD was affected. On the other hand, analysis of ^{13}C carbonyl (^{13}C’) chemical shifts, highly sensitive probes of protein conformation, showed that phosphorylation at S89 and S175 induced local conformational changes (Fig. 3B) around the phospho-sites. The decrease of ^{13}C’ chemical shift values observed for the residues preceding the phosphorylated serines of pHP1, relative to the unmodified protein, suggests a shift in the conformational ensemble of this region toward more extended structures. A reverse effect was observed for the residues following the phospho-sites. Moreover, steady-state ^{1}H–^{15}N heteronuclear NOEs analysis, reporting on NH-bond motion in the picosecond-to-nanosecond timescale, showed a localized alteration in the hinge region (Figs. 3C and S1C). Between residues 87 and 95, a ∼0.1–0.2 rise was observed, indicating that upon S89 phosphorylation, this segment partially loses its mobility. This feature seems to be site-specific, as residues around S175 at the C-terminal tail did not show the same effect. The local conformational changes observed around the phospho-sites were not accompanied by a perturbation in the global size of the molecule or its assembly state, as the SAXS-derived radius of gyration of HP1β and pHP1β was comparable (Fig. S1D).

The chemical shift analysis (Fig. 3A and B) suggested that the conformational perturbation in proximity to pS175 might expand up to the C-terminus of the CSD. In addition, the C-terminal tail was shown to cooperate with CSD for binding selectivity in HP1a of *Drosophila melanogaster* [23]. To evaluate a possible influence of S175 phosphorylation on CSD binding activity, we carried out NMR interaction experiments using a CSD construct, which extends up to the HP1β C-terminus (residues 107–185), and a histone H3 (35–67) peptide containing the CSD binding ‘shadock’ motif [24,25]. Fig. 4A–C shows that the interaction of CSD/pCSD with the peptide significantly perturbed residues belonging to the two groups 124–127 and 163–173, consistent with the specific PXVXL binding interface described for the NMR structure of CSD bound to CAF-1 peptide [4]. Thus, phosphorylation at S175 does not compromise the shadock peptide binding functionality of CSD. However, a small difference in the relative intensities suggests that binding of pCSD is slightly less efficient. To evaluate the effect of phosphorylation on the binding of full-length HP1β to its cognate

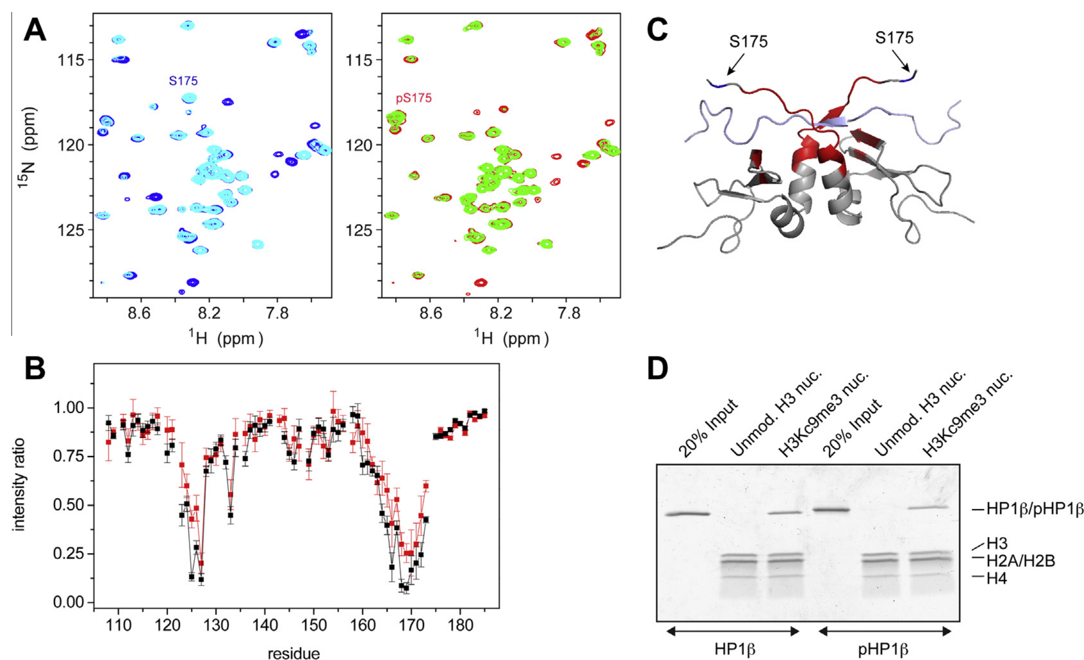

Fig. 4. Analysis of phosphorylated chromoshadow domain of HP1β. (A) Superposition of a selected region of the ^{1}H–^{15}N TROSY HSQC spectrum of CSD(107–185) in the free state (left, blue) and bound to shadock peptide (left, cyan), and pCSD(107–185) in the free state (right, red) and bound to shadock peptide (right, green). (B) Intensity loss of residues in CSD (black) and pCSD (red) in ^{1}H–^{15}N TROSY HSQC spectra upon addition of the shadock peptide (HP1:peptide at 10:1 molar ratio). (C) Intensity ratio values smaller than 0.7 from analysis of CSD in complex with peptide of panel B, are mapped in red onto the 3D structure of CSD in complex with CAF-1 peptide (marked in light blue) (PDB ID: 1s4z [4]). (D) Co-precipitation of HP1β/pHP1β with unmodified and H3Kc9me3 12-mer oligonucleosomal array. The Coomassie-stained SDS–PAGE gel is shown.

target, chromatin, we performed co-precipitation experiments using recombinant 12-mer oligonucleosomal arrays. The results in Fig. 4D show that HP1β phosphorylated at S89 and S175 fully retained its ability to bind the H3Kc9me3 chromatin template. This is in line with the dominant role of CD for this interaction [8].

In human HP1β, substitution of S89/S91 and S172/S175 by alanine did not impair its localization to chromatin [19]. In the Drosophila homolog of HP1, on the other hand, mutagenesis of the CK2 consensus sites S15 and S202 interfered with HP1 heterochromatin targeting and silencing activity, and impaired the binding to DNA [18,26]. In addition, the hinge region of HP1 was shown to bind non-specifically to nucleic acids [7,8]. Thus, phosphorylation of S89, in combination with additional phospho-acceptors such as S91, might modulate HP1β diffusion along chromatin due to altered protein–DNA electrostatic interactions. Phosphorylation of S175, which is not conserved in other human HP1 homologs, might also provide a way to selectively modulate the binding to partners that specifically recognize the human HP1β C-terminus. This would constitute a potential mechanism to regulate the signaling-dependent HP1 activities unique to the β variant.

In summary, we provided high-resolution information of the effects of CK2-mediated phosphorylation on the structure, dynamics and binding activity of human HP1β. NMR spectroscopy allowed us to identify the primary sites of CK2 and determine the enzyme preference for individual sites. Although protein modification by CK2 induces backbone conformational changes around S89 and S175, the main sites of phosphorylation, HP1β phosphorylated at these sites retains its fundamental activity of binding methylated chromatin.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.02.019.

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