Heterochromatin Protein 1 Is Extensively Decorated with Histone Code-like Post-translational Modifications*⒮

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Heterochromatin protein 1 (HP1) family members (α, β, and γ) bind histone H3 methylated at Lys-9, leading to gene silencing and heterochromatin formation. Several previous reports have suggested that HP1s are post-translationally modified, yet sites of modification have not yet been exhaustively determined. Here we perform the first comprehensive proteomic analysis of all HP1 isoforms using tandem mass spectrometry. Our data reveal that all HP1 isoforms are highly modified in a manner analogous to histones including phosphorylation, acetylation, methylation, and formylation, including several sites having multiple different types of modifications. Additionally, many of these modifications are found in both the chromo- and chromoshadow domains, suggesting that they may have an important role in modulating HP1 interactions or functions. These studies are the first to systematically map the abundant sites of covalent modifications on HP1 isoforms and provide the foundation for future investigations to test whether these modifications are essential in heterochromatin maintenance or other nuclear processes.   *Molecular & Cellular Proteomics 8: 2432–2442, 2009.*

The mammalian HP1α, β, and γ proteins are a family of chromatin-associated proteins that are homologs of the *Drosophila* heterochromatin protein 1 (HP1)[1], originally identified as a protein required for position effect variegation. Position effect variegation is the silencing phenomenon that occurs when a block of euchromatin is placed adjacent to a region of heterochromatin (1). HP1α, β, and γ proteins all contain a N-terminal chromodomain (CD) and a C-terminal chromoshadow domain (CSD) that are linked together by a flexible hinge region (2). The CD in HP1 is one of many specialized domains found in a number of other chromatin-associated proteins that recognizes histone post-translational modifications (PTMs). In particular, x-ray crystallographic studies have demonstrated that the HP1 chromodomain binds directly to the histone H3 protein trimethylated on Lys-9 (3).

The C-terminal CSD domain is structurally similar to the CD and is thought to be responsible for dimerization and interactions with other proteins. The mammalian HP1α, β, and γ proteins have been shown to form both homo- and heterodimers with each other (4). Although the HP1α, β, and γ proteins have similar structures and dimerize with each other, studies suggest they have non-overlapping functions. As the name implies, the HP1 proteins are generally associated with constitutive heterochromatic loci; however, HP1γ localizes with both hetero- and euchromatin (5). Moreover, although microscopy analysis has demonstrated that HP1α and HP1β localize together at many heterochromatic loci, they do not completely overlap, further suggesting that they can function independently (6). Furthermore, HP1γ was found to be associated with the DNA of actively transcribed genes by chromatin immunoprecipitation (7).

All three of the mammalian HP1 proteins can complex with the co-repressor protein, KAP-1/Tif1β. Interestingly, this protein is also a post-translational code-reading protein that contains a bromodomain (acetyl-lysine binding) and a plant homeodomain (methyl-lysine binding). While the majority of KAP-1/Tif1β co-localizes with HP1α and HP1β, there are some non-overlapping foci, suggesting the bromodomain and plant homeodomain of KAP-1/Tif1β is reading a sub-code found within some heterochromatic loci (8, 9). Although it is not clear how the HP1 proteins complex with KAP-1/Tif1β, the CSD is required for this interaction (10). Interestingly, it has been reported that KAP-1/Tif1β is a protein kinase that can phosphorylate all three of the mammalian HP1s and autophosphorylate itself (11). The HP1 proteins have also been shown to interact with a number of other proteins/complexes that are involved with chromatin metabolism such as; the CAF-1 complex, a histone deposition chaperone; the origin recognition complex, a protein complex required for DNA replication that binds to origins of replication; and the BRG1 complex, an ATP-dependent chromatin remodeling machine (12–14).

Like the target histones, the HP1 proteins themselves have been suggested to contain numerous PTMs such as phos-

[1] The abbreviations used are: HP1, heterochromatin protein 1; CD, chromodomain; CSD, C-terminal chromoshadow domain; PTM, post-translational modification; HEK, human embryonic kidney; MS, mass spectrometry; LC-MS/MS, liquid chromatography tandem mass spectrometry.

phorylation, acetylation, or methylation. For example, it is well known that there are phosphorylation sites within a *Drosophila* HP1 protein mediated by several kinases, and that hyperphosphorylation of this HP1 is associated with heterochromatin formation and affects chromosomal localization (15). Meanwhile, mutations at these HP1 phosphorylation sites attenuate or abolish silencing activity (16). Interestingly, a hypophosphorylated form of *Drosophila* HP1 has also been shown to associate with the subunits of the origin recognition complex, while the hyperphosphorylated form is more tightly associated with interphase chromatin indicating that phosphorylation may regulate HP1 function (17). HP1γ phosphorylation at Ser-83 is found to be an exclusive euchromatin mark interacting with the regulatory protein Ku70 and this modified HP1γ isoform also demonstrates impaired silencing properties and is targeted to areas of transcriptional elongation (18). Recently, HP1β phosphorylation at Thr-51 has been shown to be important during initiation of the DNA damage response mechanism, as suppression of the relevant kinase (casein kinase 2) causes a reduction in HP1 mobilization at repair sites and further inhibits downstream events (19). The HP1 proteins have also been shown to possess other covalent modifications such as acetylation, methylation, and ubiquination by immunoassay with various non-residue specific antibodies (18).

Despite all this evidence, only a handful of actual HP1 modification sites have ever been characterized. Therefore, as no comprehensive mass spectrometric analysis has heretofore identified all HP1 PTMs, we sought to systematically map the PTMs on the three mammalian HP1 homologs (α, β, and γ) using nanoflow liquid chromatography coupled to a high-resolution Orbitrap mass spectrometer. Our results showed that all HP1s are highly modified on many sites with many distinct types of modifications. Several of the sites we found are sites within the chromo- and chromoshadow domains. Interestingly, many HP1 residues were found to be multiply modified with different PTMs, a feature reminiscent of histones. This data will be useful for future studies designed to differentiate the functions of the individual HP1α, β, and γ proteins as well as their targeting to different regions of chromatin.

EXPERIMENTAL PROCEDURES

Cell Culture and Protein Isolation—HP1α, HP1β, and HP1γ coding sequences were amplified by PCR from a human cDNA library. Expression of the corresponding cDNAs cloned into the CβF expression vector with an N-terminal FLAG tag was directed by the hCMV promoter (20). The vectors were transfected into HEK293 cells grown in 10% calf serum/Dulbecco's modified Eagle's medium by calcium phosphate precipitation. After 36 h the FLAG-tagged proteins were isolated. All buffers used in the purification process contained histone deacetylase and phosphatase inhibitors (3 mM sodium butyrate, 1 mM sodium pyrophosphate, 1 mM β-phosphoglycerate, and 1 mM sodium vanadate). For protein purification, nuclei were prepared by hypotonic lysis in TMSD buffer (10 mM Tris-HCl, pH 7.8, 1.5 mM MgCl$_2$, 0.25 M sucrose, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride
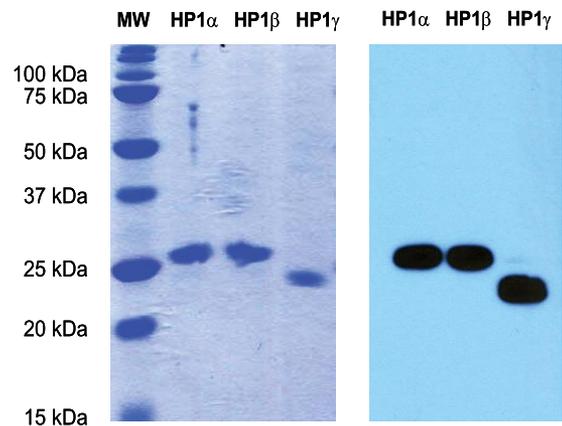

FIG. 1. **Purification of HP1.** *Left panel*, immunoprecipitated HP1s ($\sim$5 pmol each) separated on a 15% SDS-PAGE gel with Coomassie Blue staining. *Right panel*, Western blot of immunoprecipitated HP1s using anti-FLAG antibody. Peptide digests were then analyzed using nanoflow LC-MS/MS experiments on an LTQ-Orbitrap mass spectrometer.

HP1α (CBX5)

MGKKTKR**T**$_{phos}$AD**S**$_{phos}$**S**$_{phos}$**S**$_{phos}$**S**$_{phos}$EDEEEYVVEKVLDRRVV**K**$_{f/me1}$GQVEYLL**K**$_{fml/me1/ac}$W**K**$_f$GFS$_{fml}$EEHNTWEPE**K**$_{ac}$NLDCPELIS$_{fml}$EFMKKYK**K**MKEGENNKPR EKSESNKR**K**$_{ac/fml/me1}$S$_{fml}$NFS$_{fml}$NS$_{phos/f}$ADDI**K**$_f$S**K**K**K**$_{ac}$REQSNDIARGFERGLEPEKIIGATDSCGDLMFLMKW**K**$_{fmVac/me1}$DTDEADLVLAKEANV**K**$_{fml}$CPQIVIAFYEERL TWHAYPEDAEN**K**$_{fml}$EKETAK**S**

HP1β (CBX1)

MGKKQNK**K K**$_{me1}$VEEVLEEEEEYVVEKVLDRRVV **K**$_{ac/fml}$G**K**$_{ac/me1/fml}$VEYLL**K**$_{ac/fml}$WKGF SDEDNTWEPEENLDCPDLIAEFLQSQKTAHETDK SEGGKRK**AD**SDSEDKGEESKPK**KKK** EESEKPRGFARGLEPERIIGATDSSGELMFLMKW **K**$_{fmVac}$NSDEADLVPA**K**$_{fml}$EANVKCPQV VISFYEERLTWHSYPSEDDDKKDDKN

HP1γ (CBX3)

MASNK**TT**LQKMGKK QNGKSK**K**$_{fml/ac/me1}$VEEAEPEEFVVE**K**$_{fml/ac}$VLDRRVVNG**K**$_{fml/me1/ac}$VEYFL**K**$_{fml}$WKGFTDADNTWEPEENLDCPELIEAFLNSQKAGKEKD GTKR**K**S$_{phos}$S$_{phos}$DSES DDSKSK KK**R**DAADKPR GFARGLDPERIIGATDSSGELMFLMKW **K**$_{fml/ac/me1}$DS$_{fml}$DEADLV LAKEANMKCPQIVIAFYEERLTWH S$_{phos/fml}$CPEDEAQ

FIG. 2. **PTM maps of HP1 modifications.** Over 90% sequence coverage was obtained on all isoforms (shown in *red*). Residues with PTMs are shown in *black bold type*. PTMs identified include phosphorylation (*phos*), acetylation (*ac*), monomethylation (*me1*), and formylation (*fml*).

(21). Proteins were then extracted from the nuclei by dounce homogenation in immunoprecipitation buffer (20 mM Tris-HCl, 0.5 M NaCl, 0.1 mM phenylmethylsulfonyl fluoride, 0.2 mM EDTA, and 10% glycerol (v/v) pH 7.5). The extract was clarified by centrifugation at 13000 rpm for 15 min, and the FLAG-tagged proteins were purified by incubating the extracts with M2 FLAG beads (Sigma) overnight at 4 °C with rotation. The beads then were washed three times with IP buffer, and the proteins were eluted by incubation with FLAG peptide at 4 °C for 12 h. The eluted proteins were then resolved by 15% SDS-PAGE and visualized after staining with Coomassie Blue. Formaldehyde was not utilized in any step.

Sample Preparation for Mass Spectrometry—HP1s were subjected to both in-solution and in-gel digestion with multiple enzymes. For in-solution digestion, HP1 was diluted with 50 μl of 100 mM ammonium bicarbonate and reduced with 10 mM dithiothreitol for 1 h at 51 °C followed by alkylation with iodoacetamide (25 mM) for 45 min at room temperature in the dark. At this point, HP1 was digested for 6–12 h at 37 °C using either trypsin at a protein:enzyme ratio of 20:1, LysC at a protein:enzyme ratio of 10:1, or chymotrypsin at a protein:enzyme ratio of 20:1. Additionally, HP1 was separated on a 15% SDS-polyacrylamide gel, and in gel digested after dithiothreitol and iodoacetamide treatment (same concentrations as above) with tryp-

A

88	201	368	483	570	699	786	901	1016	1103	1249
S	L	S_{phos}	D	S	E	S	D	D	S	K
1249	1082	1049	882	767	680	551	464	349	234	147

B

88	202	349	436	550	717	788	903	1018	1131	1277
S	N	F	S	N	S_{phos}	A	D	D	I	K
1277	1190	1076	929	842	728	561	490	375	260	147
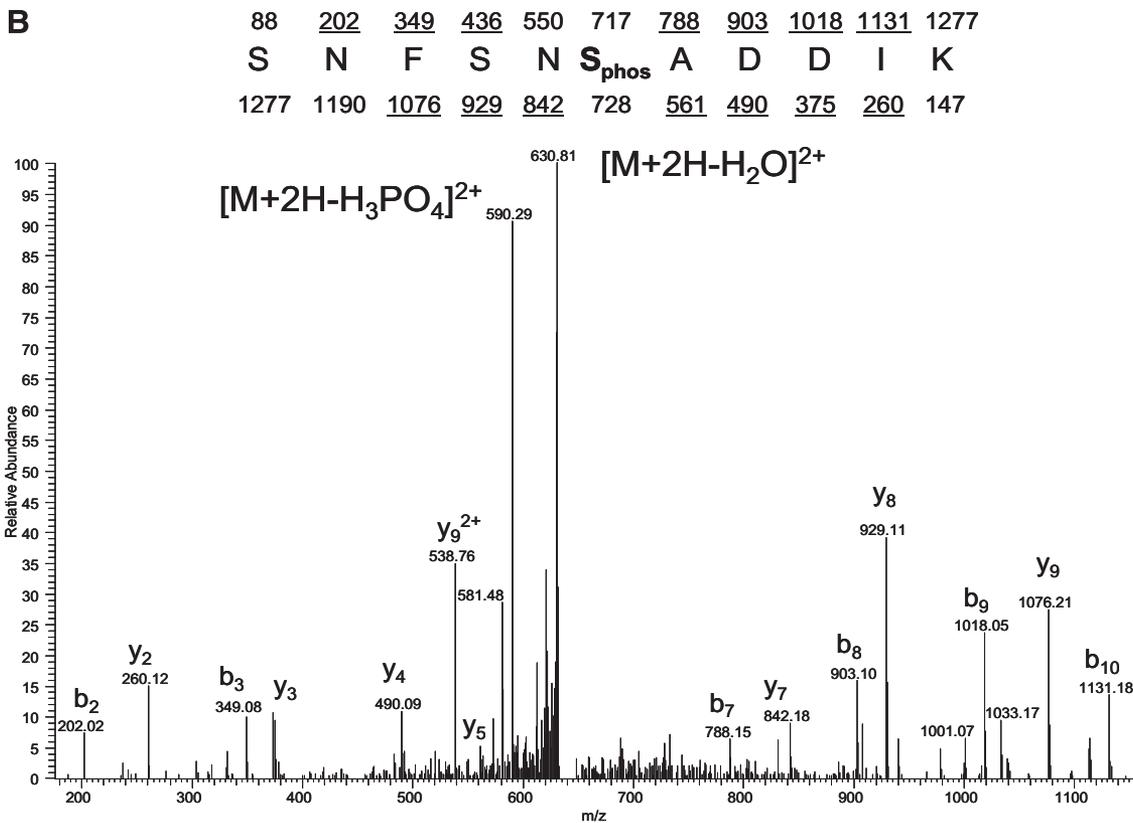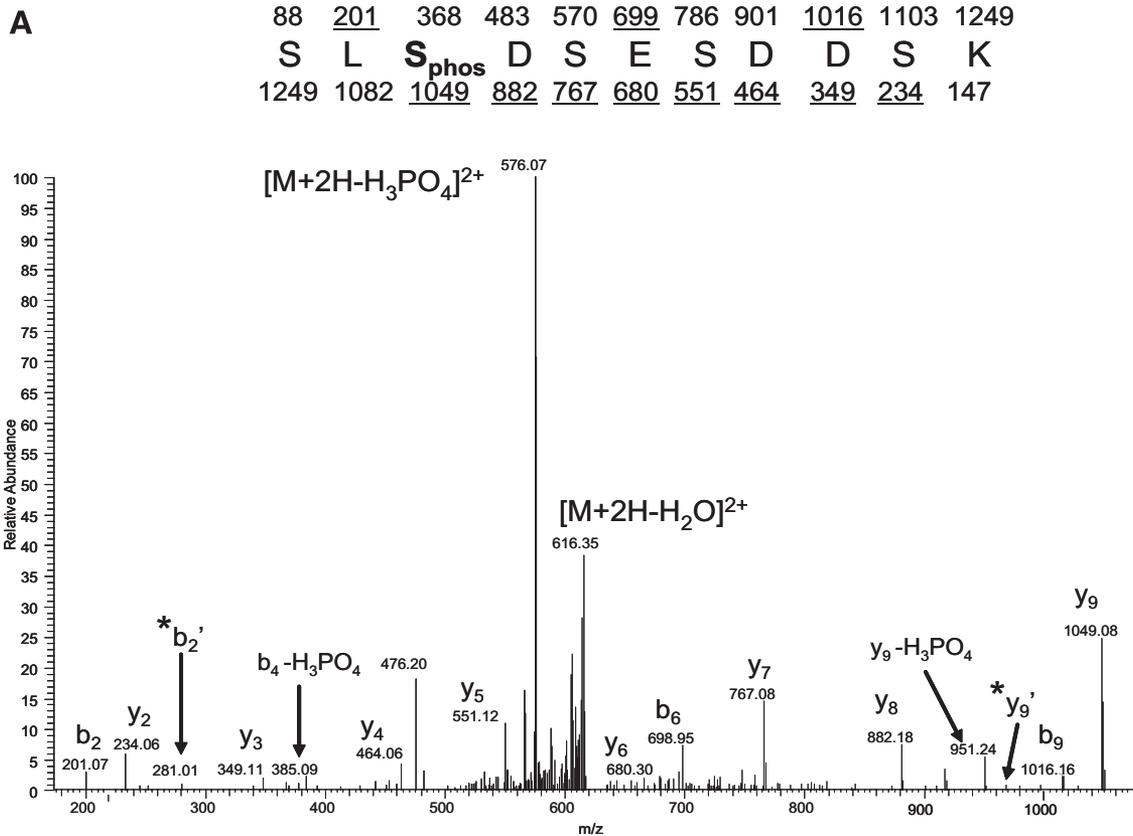
sin, LysC, or chymotrypsin as described previously (22). The resulting peptides were extracted according to standard in-gel digestion protocols (22). All peptide digests were desalted using homemade C18 STAGE tips (23), dried to near dryness in a vacuum centrifuge, and diluted in 10 μl of 0.1% acetic acid for MS analysis. Formic acid was not employed in any preparation step.

Liquid Chromatography and Mass Spectrometry—Nanoflow liquid chromatography tandem mass spectrometry (LC-MS/MS) was performed on a hybrid linear quadrupole ion trap-Orbitrap mass spectrometer (Thermo Electron, San Jose, CA) coupled to an Agilent 1200 Series binary HPLC pump (Agilent Technologies, Palo Alto, CA) and an Eksigent AS2 autosampler (Eksigent Technologies). Peptides samples (2 μl) were loaded on 75-μm inner diameter-fused silica capillary columns constructed with an integrated electrospray tip that were packed with 15 cm of C18 reversed phase resin (Magic C18, 5-μm particles, 200-Å pore size; Michrom BioResources, Auburn, CA). Peptides were separated by RP-HPLC using a gradient from 2 to 45% Buffer B (Buffer A, 0.1 M acetic acid; Buffer B, 70% acetonitrile in 0.1 M acetic acid) at a flow rate of ~200 nl/min for 70 min. The Orbitrap instrument was operated in data-dependent mode using a resolution setting of 30,000 to obtain a full MS spectrum followed by ten MS/MS spectra obtained in the ion trap. Peptides selected for MS/MS interrogation were then placed on an exclusion list for 30 s to limit duplicate spectra. The MS scans were collected with an automatic gain control target value of 5×10^5 and maximum injection time of 100 ms over a mass range of 300–1650 m/z. MS/MS scans were collected using an automatic gain control value of 4×10^4 and a threshold energy of 35% for collision-activated dissociation.

Data Analysis—All MS/MS spectra were processed with a demo on-line version of the Sorcerer™ software (Sage-N Research, Inc., San Jose, CA) or through the Bioworks 2.0 program both utilizing the Sequest algorithm. Parameters for MS/MS database searching included a precursor tolerance of 0.1 Da and fragment tolerance of 0.5 Da. Searches were performed using the appropriate enzyme (with up to 3 missed cleavages) or no enzyme specifications against a database created from downloading the HP1 protein sequences from the NCBI protein database and against the human protein database. A static modification of 57 Da for cysteine iodoacetamide treatment and dynamic lysine modifications of 14 Da (methylation), 28 Da (dimethylation or formylation), and 42 Da (trimethylation or acetylation), and serine/threonine phosphorylation (80 Da) were used in the database searches. The Scaffold program (version Scaffold_2.1.03, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted at greater than 95% probability as specified by the Peptide Prophet algorithm (24) or better than 0.01 peptide probability (Bioworks 2.0 program). MS/MS spectra were also searched against the reverse HP1 database to examine the false positive rate of peptide identifications (no modified peptides were found). Lastly, all data from modified peptides were manually inspected afterward.

RESULTS

HP1-FLAG fusion proteins were immunoprecipitated from asynchronously growing HEK (human embryonic kidney) cells using an anti-FLAG antibody. SDS-PAGE separation and Western blot of the IP eluant (Fig. 1) revealed that relatively pure protein isolations were achieved. Both HP1α and HP1β have slightly higher molecular mass (~29 kDa) compared with HP1γ (~24 kDa). When we subjected HP1 peptides to nanoLC-MS/MS experiments on an Orbitrap mass spectrometer, we achieved 95.3%, 93.5%, and 92.9% sequence coverage for HP1α, HP1β, and HP1γ, respectively (Fig. 2). Each isoform immunoprecipitation additionally isolated the other endogenous HP1 isoforms as well (data not shown), this is in agreement with previous biological data that HP1 isoforms interact with one another potentially via the chromoshadow domain (2). Searching of the MS data acquired using the Sequest algorithm detected a variety of PTMs on all three HP1 isoforms (Fig. 2 and supplemental Table 1). It should be noted that we also detect the same PTMs on endogenous HP1 isoforms that are pulled down with the FLAG-tagged HP1 proteins, thus we observe that our observations on the FLAG-HP1 PTMs are consistent with endogenous modification of HP1.

Consistent with previous reports, we found the HP1 proteins to be phosphorylated at various residues (15, 16, 25–27). Fig. 3A shows the tandem mass spectrum (MS/MS) of a peptide from HP1γ that was found to be phosphorylated, and Ser-95 was localized as the primary phosphorylation site indicated by the y_9 fragment at 1049.08 m/z and also by the b_2 fragment at 201 m/z (indicating the phosphate group was not primarily on Ser-93). However, it should also be noted that upon close inspection of the lower abundance fragments, we indeed have evidence in this tandem mass spectrum for a much lower level Ser-93 phosphorylation as well (b_2 fragment ion at 281 m/z and y_9 fragment ion at 969.16 m/z-labeled as *asterisks* in mass spectrum (Fig. 3A). Ser-93 phosphorylation (Ser-83 in *Drosophila*) has been previously found on HP1γ that is localized to euchromatin (18). We additionally found phosphorylation sites on other HP1 isoforms as well. The MS/MS of the peptide SNFSN**S**$_\mathbf{phos}$ADDIK from HP1α is shown in Fig. 3B. Interestingly, all phosphorylation sites we found belong to the unstructured hinge regions. Another cluster of phosphorylation sites were detected near the N terminus of HP1α (Fig. 2), and peptides with multiple phosphorylation sites in different combinations were also detected (supplemental Table 1).

Lomberk et al. (18) used antibody-based methods to suggest that HP1 proteins can be modified with other non-phosphorylation PTMs reminiscent of the "Histone Code" including acetylation, methylation, ubiquitination, and sumoylation. To explore this possibility, we also searched our data against various PTMs and discovered that HP1s were extensively

FIG. 3. **Identification of HP1 phosphorylation sites.** *A*, MS/MS spectrum of the 2+ charged precursor ion at 625.221 m/z corresponding to the SL**S**$_\mathbf{phos}$DSESDDSK peptide (Ser-95 phosphorylation) from HP1γ generated from a trypsin digest. Evidence for minor phosphorylation of Ser-93 can also be observed (b_2 ion at 281 m/z). *B*, MS/MS spectrum of the phosphorylated 2+ charged precursor peptide at 639.257 m/z, corresponding to the peptide SNFSN**S**$_\mathbf{phos}$ADDIK from HP1α generated from a trypsin digest. It should be noted that values *above* and *below* the sequence refers to nominal masses for predicted b and y fragment ions, respectively, with masses *underlined* indicating the observed fragments.

modified on lysines with several types of modifications. Fig. 4A shows the MS/MS spectrum of a peptide at 684.324 *m/z* from HP1α. The peptide was found to have a +42 Da shift on the N terminus, which the accuracy of the Orbitrap mass spectrometer revealed to be an acetylation (2.92 ppm error) rather than a methylation mark (23 ppm error). In general, HP1α was found to be highly acetylated compared with the other HP1 isoforms in agreement with the Lomberk *et al.* study (18). Lysine methylation was also found on the various isoforms as shown in Fig. 4B for a peptide at 655.382 *m/z* generated from HP1γ (VVNGK_{me1}VEYFLK). Fragment ions show that a monomethylation modification is located on Lys-44. Overall, monomethylation was found to be the most frequently identified degree of lysine methylation on the three isoforms. Unlike phosphorylation, the lysine acetylation and methylation sites were mostly localized to either the chromoshadow or chromodomains.

Initially, our database searches yielded a large amount of dimethylated lysine residues on all of the HP1 proteins. Given the unusual low occurrence of monomethylated peptides from the same searches, a closer inspection of the accurate peptide masses was warranted. We determined that the lysine residues were not dimethylated, but rather formylated (Δm = 0.0364 Da). Fig. 5A shows an MS/MS spectrum coming from the sequence GK_{fml}VEYLLK from HP1β. The accurate mass of this peptide (489.289 *m/z*) agrees with formylation (489.287 *m/z* calculated) rather than dimethylation (489.305 *m/z* calculated). The MS/MS spectrum of another formylation from HP1α, (LTWHAYPEDAENK_{fml}EK) is shown in Fig. 5B. Fragment ions show that Lys-184 is the modified lysine residue. As we did not use formaldehyde or formic acid in any stage of the experiment, we concluded that these formylation modifications must be generated *in vivo*. The majority of the formyl groups were detected on peptides from the chromoshadow and chromodomain, often on the same sites where acetylation or methylation were found. Lastly, we detected serine and threonine formylations on HP1 proteins. Fig. 6A shows the MS/MS spectrum of the 722.294 *m/z* precursor ion, and accurate mass of the precursor and fragment ions point to the formylation of HP1γ at Ser-176 as the modified site. Interestingly, Ser-176 was also found to be phosphorylated (Fig. 6B). Formylation of proteins has recently emerged as an *in vivo* covalent modification on histones and other nuclear proteins (28, 29), and these findings will fuel future work on the subject in the context of its importance in epigenetic silencing through nuclear- or chromatin-mediated pathways.

DISCUSSION

HP1 is an important nuclear protein first shown to be required for position-effect variegation and is enriched in heterochromatic regions of the genome. The CD of HP1 has been shown to bind histone H3K9 methylation, particularly trimethyl (3), as well as histone H1.4K26 methylation (30), which trigger

a silencing mechanism. Since its discovery, researchers have suggested that the role of HP1 in heterochromatin formation may be influenced by post-translational modifications. Early studies have indeed focused on the function of HP1 phosphorylation under a variety of physiological conditions. Using 32P labeling and two-dimensional gel electrophoresis, Eissenberg *et al.* (15) showed that in *Drosophila* embryos, HP1 is initially hypophosphorylated, but that phosphorylation levels increase during development (with multiple phosphorylated HP1 isoforms present). Additionally, HP1 phosphorylation is first seen during the nuclear 10–11 cycle, which is the exact time when heterochromatin first becomes cytologically visible. This suggests a role for HP1 phosphorylation in heterochromatin assembly. Our proteomic data are consistent with HP1 being phosphorylated at multiple sites *in vivo*, and we have even identified multiply phosphorylated HP1 peptides (such as near the N-terminal of HP1α). Inspection of the HP1 general sequences indicates that HP1 might be a target by several kinases such as casein kinase II and cAMP-dependent kinase or protein kinase C. Pim-1 has been implicated as a kinase involved in HP1γ phosphorylation that was identified through a yeast two-hybrid screen and shown to bind HP1γ via the chromoshadow domain (25). Pim-1 promotes the phosphorylation around the sequence KSLSDSESDDSKS, and abolishing this sequence enhanced transcriptional repression activity (25). We have mapped two phosphorylation sites in this region, supporting the *in vivo* phosphorylation of this serine cluster.

Casein kinase II also has been shown to phosphorylate *Drosophila* HP1 near both the N terminus and also C terminus and that some of these sites have an influence on the silencing activity of HP1 or interfere with HP1-mediated heterochromatin formation (27). We have confirmed these are potential sites of phosphorylation on HP1α; many of those sites are near an acidic patch (the preferred casein kinase II motif) further supporting this association. None of the detected phosphorylation sites were found in either the chromodomain or chromoshadow domain, but rather in the hinge regions suggesting that phosphorylation may not be a mechanism for modulating HP1 interactions with histones or itself. In agreement, Kellum and co-workers (31) previously found that site-directed mutagenesis targeting the few suspected sites of phosphorylation in those regions does not affect *Drosophila* HP1 binding to H3K9me2 or homo-dimerization of HP1. Additionally, those studies also determined that mutations mimicking constitutively phosphorylated HP1 in the hinge actually affect the binding of HP1 to the proteins involved in the origin recognition complex.

Recent reports have identified a single key phosphorylation site each on HP1β and HP1γ (18, 19). Mammalian HP1γ was found to be phosphorylated at Ser-83 (Ser-93 human), and this mark was found only on a small population of HP1γ that was exclusively located in euchromatin and had impaired silencing activity. Our mass spectrometric analyses indeed
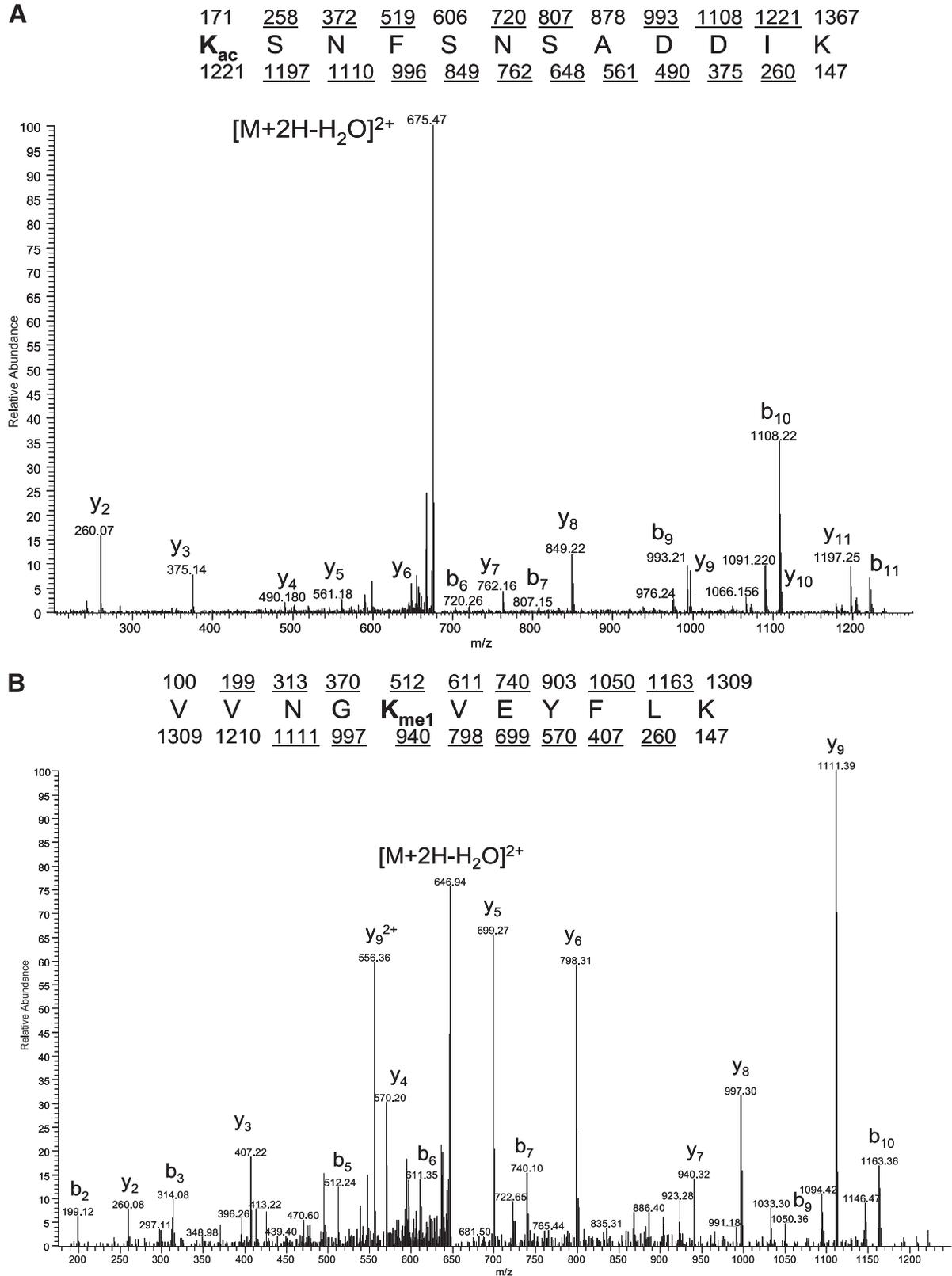
FIG. 4. **Characterization of lysine modifications on HP1.** *A*, MS/MS spectrum of the 2+ charged peptide ion at 684.324 *m/z* corresponding to the peptide with the sequence **K**$_{ac}$SNFSNSADDIK from HP1α generated from a trypsin digest. *B*, MS/MS spectrum of the [M+2H]$^{2+}$ ion showing that Lys-44 is monomethylated on HP1γ (VVNG**K**$_{me1}$VEYFLK peptide generated from a LysC digest). Values *above* and *below* the sequence refers to nominal masses for predicted b and y fragment ions, respectively, with masses *underlined* indicating the observed fragments.
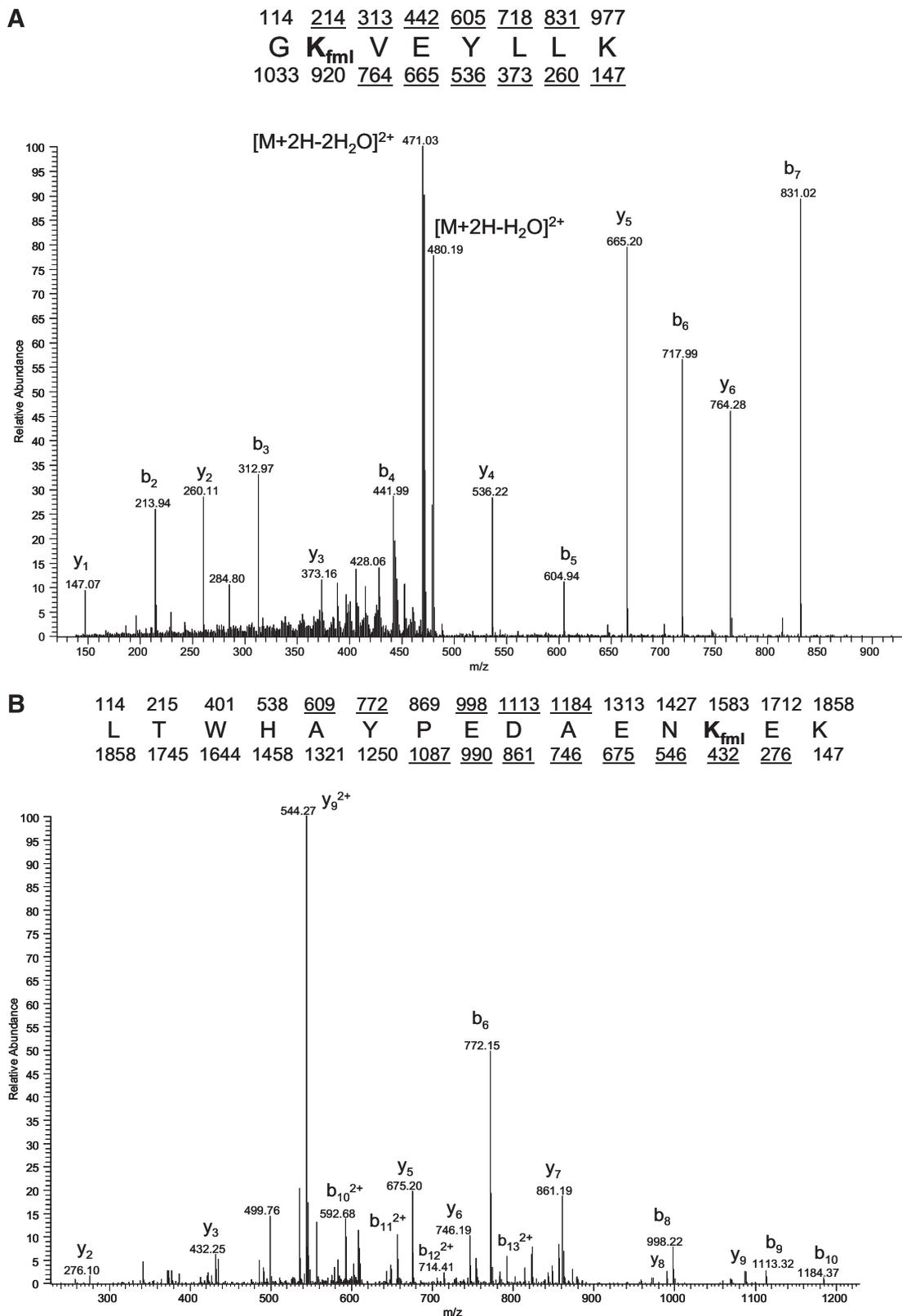
A

	114	214	313	442	605	718	831	977
	G	K_fml	V	E	Y	L	L	K
	1033	920	764	665	536	373	260	147

B

	114	215	401	538	609	772	869	998	1113	1184	1313	1427	1583	1712	1858
	L	T	W	H	A	Y	P	E	D	A	E	N	K_fml	E	K
	1858	1745	1644	1458	1321	1250	1087	990	861	746	675	546	432	276	147

FIG. 5. **Characterization of lysine formylation on HP1.** *A*, MS/MS spectrum of the 2+ charged precursor peptide ion at 489.289 *m/z* corresponding to the peptide with the sequence GK_fmlVEYLLK from HP1β generated from a LysC digest. *B*, MS/MS spectrum produced from the 3+ charged peptide ion at 620.293 *m/z* from HP1α generated from a trypsin digest. Values *above* and *below* the sequence refers to nominal masses for predicted b and y fragment ions, respectively, with masses *underlined* indicating the observed fragments.
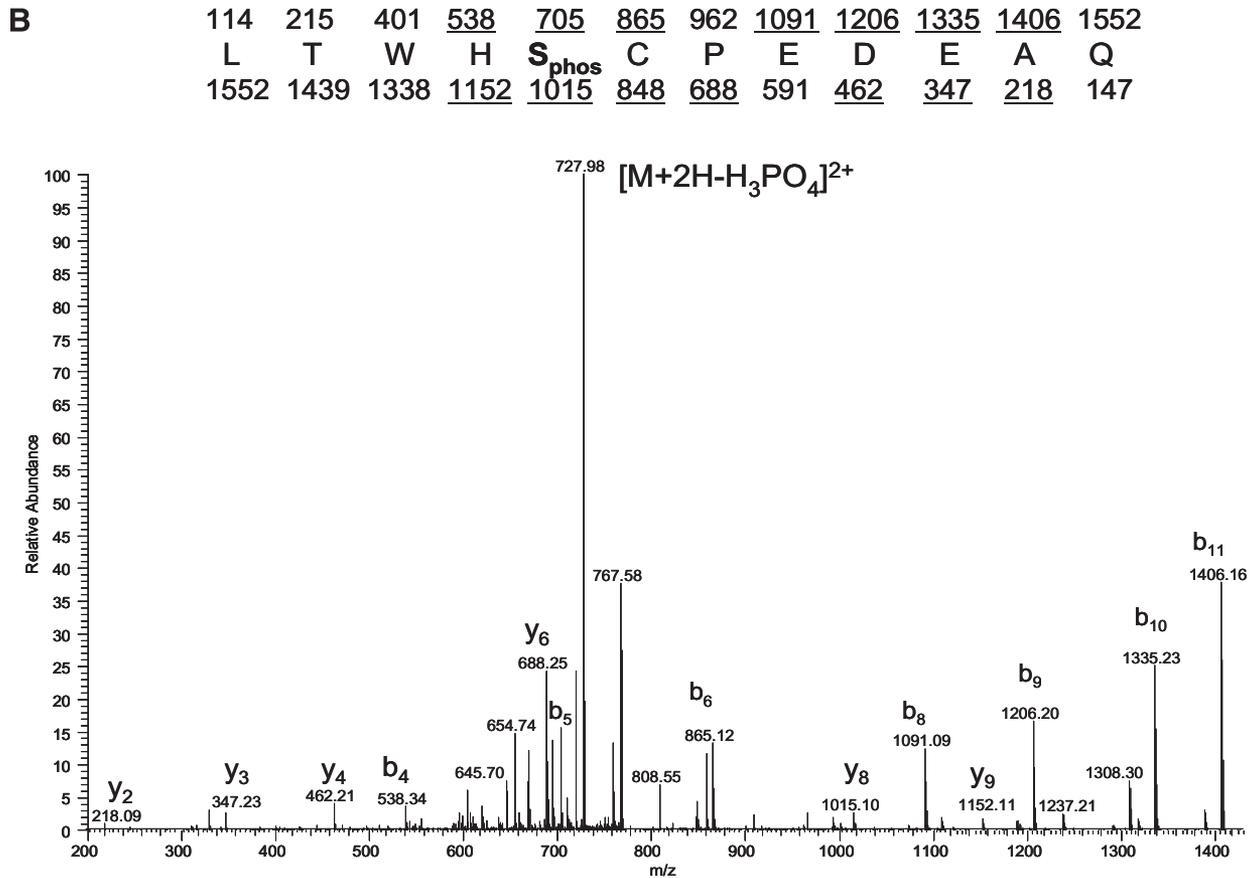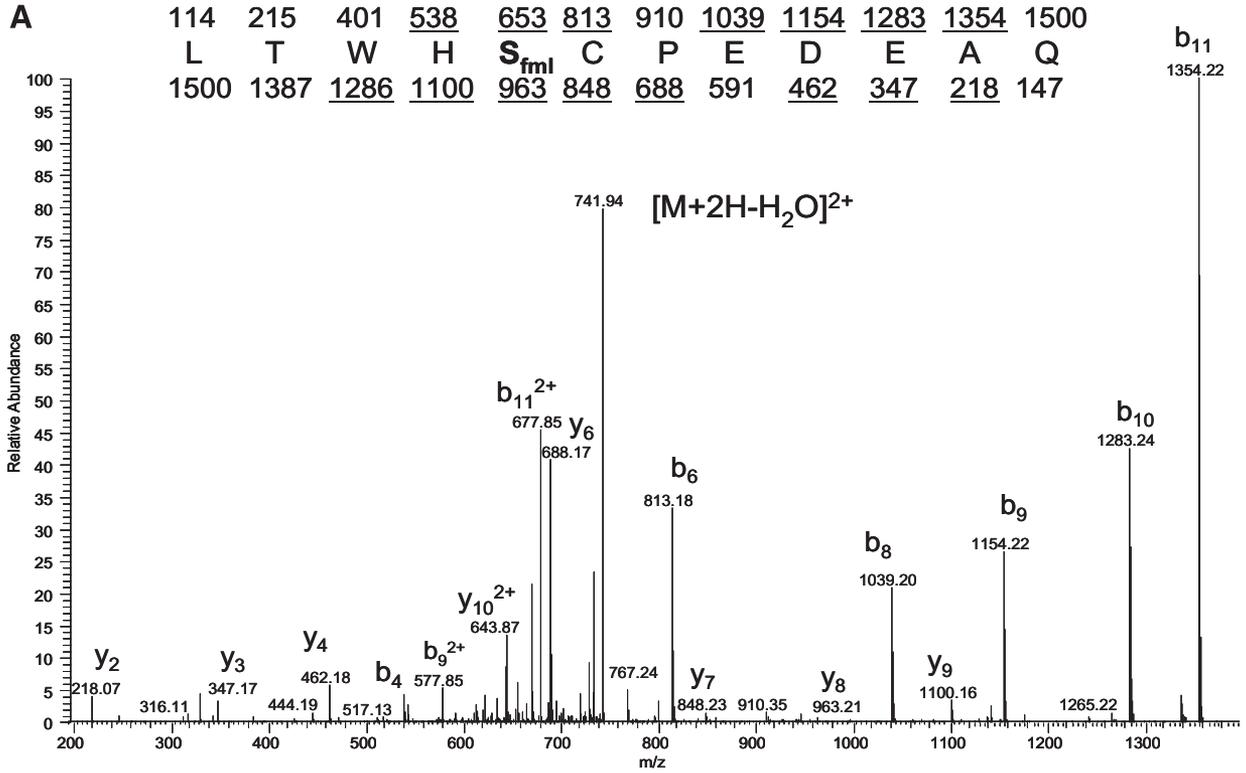
A

	114	215	401	<u>538</u>	<u>653</u>	813	910	<u>1039</u>	<u>1154</u>	<u>1283</u>	<u>1354</u>	1500
	L	T	W	H	**S_{fml}**	C	P	E	D	E	A	Q
	1500	1387	<u>1286</u>	<u>1100</u>	<u>963</u>	<u>848</u>	<u>688</u>	591	<u>462</u>	<u>347</u>	<u>218</u>	147

B

	114	215	401	<u>538</u>	<u>705</u>	<u>865</u>	962	<u>1091</u>	<u>1206</u>	<u>1335</u>	<u>1406</u>	1552
	L	T	W	H	**S_{phos}**	C	P	E	D	E	A	Q
	1552	1439	1338	<u>1152</u>	<u>1015</u>	<u>848</u>	<u>688</u>	591	<u>462</u>	<u>347</u>	<u>218</u>	147

validate that Ser-83 (Ser-93 human) is a modification site on HP1γ. However, we found that this site was phosphorylated at a very low level, and another nearby residue (Ser-95) was actually much more abundant. This might signify that Ser-83 (Ser-93) may be truly limited to a small population of euchromatic HP1γ, whereas Ser-95 might be a mark that is needed on the more abundant HP1γ heterochromatin population. Lastly, Thr-51 was found to be phosphorylated on HP1β during DNA damage by casein kinase II and upon phosphorylation was found to be released from chromatin influencing H2A.X phosphorylation (a hallmark signal of DNA damage). Unfortunately, we did not identify this site as an *in vivo* phosphorylation site, perhaps due to its role in DNA damage pathways and hence low level in asynchronously growing cells. In agreement with Lomberk *et al.* (18)*,* we found that HP1β had the lowest general level of phosphorylation of all the HP1 isoforms. Lastly, we note that two large-scale mass spectrometry-based phosphoproteomic analyses of human cells have also yielded data on the phosphorylation of HP1α on the peptides (RTADSSSSEDEEEYVVEK, multiple sites) and on HP1γ at Ser-93, Ser-95, and Ser-176, in support of our results (32, 33).

Urrutia and co-workers (18) showed by non-residue specific antibody methods that certain HP1 isoforms seem to be acetylated, methylated, ubiquitinated, or sumoylated. In our experiments, we also identified several sites of acetylation and methylation on all HP1 isoforms. We did not detect any sites of sumoylation or ubiquitination on any HP1 isoform, presumably due to their low abundance in asynchronously grown HEK cells causing them to be expressed at levels below our current detection limits. Additionally, these sumoylated or ubiquitinated HP1 forms may also be biological condition-specific as well. Interestingly, in agreement with results from Lomberk *et al.* (18), we found that the largest number of acetylation sites is on the HP1α isoform. Acetylation and methylation sites were more evenly distributed throughout the protein sequences including the hinge domains, chromodomains, and chromoshadow domains. The significance of these modifications remains to be uncovered. All HP1 isoforms are known to complex with the histone H3K9 methyltransferase Suvar39H1 through a CSD interaction (34), so it is tempting to speculate that the methylation sites on HP1 in that region may be a direct result of that interaction. Alternatively, all HP1 isoforms directly interact with the co-repressor KAP-1/Tif1β, and KAP-1/Tif1β binds SETDB1, a SET domain protein with histone H3K9-specific methyltransferase activity (35). Therefore, a secondary effect of the KAP-1/Tif1β: SETDB1 interaction may be the methylation of HP1. Recently, Eskeland *et al.* (36) showed that while HP1 binds strongly to methylated H3 peptides, it only seems to bind weakly to methylated chromatin. However, adding recombinant methyltransferases seems to increase HP1 binding affinity for chromatin, and the researchers propose that HP1 may have other binding sites on chromatin besides H3K9 that are enhanced with the methyltransferase addition. These results could also be explained if methylation of HP1 itself is needed before it can bind chromatin. Our data also clearly showed that HP1 methylation and acetylation often occur on the same residue, sparking comparisons to similar observations on histones (such as histone H3K9 or H3K27 acetylation or methylation). These different modifications occurring at the same residues may act as "switches" to alter HP1 function in the way that certain "switches" may regulate histone H3 function, as in the case of H3K9ac (associated with gene activation), and H3K9me3 (associated with transcriptional repression and heterochromatin) (37, 38).

This study demonstrated that HP1 proteins are also highly formylated on multiple residues. The formylation of proteins can occur in sample preparation by the use of formaldehyde described under "Experimental Procedures" (39) or gel silver staining protocols (40), and by using excessive formic acid (41). Thus, we did not use any of these reagents in any of our preparations. Endogenous protein formylation has been reported on histones, high mobility group proteins, and other nuclear proteins (28). Jiang *et al.* (29) demonstrated that the reactive 3′-formylphosphate residues produced from the 5′-oxidation of deoxyribose in DNA under oxidative stress can cause a secondary modification on the side chains of lysine residues on histones. While this could be a potential source of formylation on HP1 proteins, especially since HP1 has recently been shown to be involved in DNA damage initiation (or be associated with the DNA methyltransferase Dmnt3a/b) (19, 42), another intriguing possibility could occur with regards to HP1 biology since HP1 proteins themselves do not bind DNA directly. HP1 is well known to bind histone H3K9me3 and in the last several years, many histone demethylases have been described with varying specificity. Many of the demethylases known catalyze the removal of higher degrees of methylation from Lys-9 on histone H3 yielding a final byproduct of formaldehyde per methyl reduction (43). We postulate that as histones are demethylated by demethylases, they release formaldehyde, elevating local concentration of formaldehyde that could react with nearby proteins, including histones and HP1.

Recently, it has been published that *Drosophila* HP1α interacts with the histone H3K36 demethylase dKDM4A through its chromoshadow domain and stimulates the activity of dKDM4A (44). Loss of HP1α also leads to increased

FIG. 6. **Serine 176 on HP1γ is multiply modified.** *A*, MS/MS spectrum of the precursor peptide ion at 722.294 *m/z* generated from a trypsin digest clearly showing that the formylation modification is on the serine residue (LTWH**S$_{fml}$**CPEDEAQ). *B*, MS/MS spectrum of the precursor ion at 748.286 *m/z* with the sequence LTWH**S$_{phos}$**CPEDEAQ generated from a trypsin digest. Values *above* and *below* the sequence refers to nominal masses for predicted b and y fragment ions, respectively, with masses *underlined* indicating the observed fragments.

H3K36me3 *in vivo*. It is interesting to note that many of the formylation sites we detected lie in the chromodomains on HP1s, including the sequence (…VEYLL**K**$_{fml}$W**K**$_{fml}$…) that directly binds H3K9me3. Formylation of this region may potentially sterically hinder HP1 from further binding to H3K9me3 marks. The majority of the formylated sites were seen on lysine residues that are also methylated and/or acetylated, as previously noted for histone formylation (28). These observations imply that HP1 lysine formylation may potentially interfere with epigenetic mechanisms controlling chromatin structure depending on the frequency and reversibility of this reaction. Finally, we gather evidence for the formylation of Ser and Thr residues on HP1, which to our knowledge has not been described to date. Again, we demonstrate that the Ser residues observed to be formylated can also be phosphorylated as well, bringing up the possibility that these residues may influence HP1 function through two distinct PTMs.

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