Phosphorylation Site Mutations in Heterochromatin Protein 1 (HP1) Reduce or Eliminate Silencing Activity*

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HP1 is an essential heterochromatin-associated protein in Drosophila. HP1 has dosage-dependent effects on the silencing of euchromatic genes that are mislocalized to heterochromatin and is required for the normal expression of at least two heterochromatic genes. HP1 is multiply phosphorylated in vivo, and HP1 hyperphosphorylation is correlated with heterochromatin assembly during development. The purpose of this study was to test whether HP1 phosphorylation modifies biological activity and biochemical properties of HP1. To determine sites of HP1 phosphorylation in vivo and whether phosphorylation affects any biochemical properties of HP1, we expressed Drosophila HP1 in lepidopteran cultured cells using a recombinant baculovirus vector. Phosphopeptides were identified by matrix-assisted laser desorption ionization/time of flight mass spectroscopy; these peptides contain target sites for casein kinase II, protein tyrosine kinase, and PIM-1 kinase. Purified HP1 from bacterial (unphosphorylated) and lepidopteran (phosphorylated) cells has similar secondary structure. Phosphorylation has no effect on HP1 self-association but alters the DNA binding properties of HP1, suggesting that phosphorylation could differentially regulate HP1-dependent interactions. Serine-to-alanine and serine-to-glutamate substitutions at consensus protein kinase motifs resulted in reduction or loss of silencing activity of mutant HP1 in transgenic flies. These results suggest that dynamic phosphorylation/dephosphorylation regulates HP1 activity in heterochromatic silencing.

Heterochromatin protein 1 (HP1) is a heterochromatin-associated protein in Drosophila melanogaster (1) and a context-dependent regulator of transcription. HP1 has dosage-dependent effects on the silencing of euchromatic genes (3–5), and it is required for the proper expression of certain heterochromatic genes (6). HP1 is essential in Drosophila (5); mutations cause recessive late larval lethality, associated with misregulation of essential heterochromatin genes and mitotic defects (6–8).

HP1-like proteins have been identified in yeast, nematodes, insects, amphibians, and mammals (reviewed in Ref. 9). HP1 family proteins in Schizosaccharomyces pombe and mouse have also been shown to participate in position-dependent silencing (10, 11).

HP1 contains two copies of a structural motif called the chromo domain, connected by a short “hinge domain.” The chromo domain structure consists of three β-strands packed against an α-helix (12, 13), a motif found in an archeal DNA-binding protein (14, 15). The C-terminal chromo domain self-assembles in vitro (16). HP1 binds DNA and nucleosomes in vitro (17). Heterotypic interactions between HP1 family proteins and silencing factors (18–22), nuclear membrane proteins (23, 24), and replication factors (25, 26) have also been reported, suggesting that HP1 family proteins may participate in multiple distinct nucleoprotein complexes in vivo.

HP1 is multiply phosphorylated by serine-threonine kinases in Drosophila (27). Hyperphosphorylation of HP1 correlates with heterochromatin assembly during development. In Tetrahymena, the HP1 family protein Hhp1p becomes hyperphosphorylated in response to starvation; this is associated with reduction in macronuclear volume and increased chromatin condensation (28). Human HP1 family proteins are phosphorylated in vivo (29) and have been shown to be substrates in vitro for multiple kinases (21, 30).

Huang et al. (31) recovered differentially phosphorylated HP1 in distinct protein complexes from Drosophila embryo extracts, suggesting that phosphorylation could regulate the assembly of HP1 into higher order chromatin structures. This study also found a correlation between the extent of HP1 phosphorylation and salt extractability from embryo nuclei. These findings suggest that HP1 phosphorylation regulates distinct macromolecular interactions in vivo.

Consensus kinase target sites are found near the N- and C-terminal ends of Drosophila HP1 and in the hinge domain between the chromo domain motifs. We recently reported that casein kinase II (CKII) from embryo nuclear extract phosphorylates HP1 at three target sites in vitro and that alanine substitutions for CKII target serines interfere with efficient heterochromatin targeting in a transient expression assay (32). Our study did not test the transcriptional regulatory activity of mutant HP1 proteins.

In this report, we compare the biochemical properties of phosphorylated and unphosphorylated HP1. We map sites of phosphorylation in HP1 by expressing Drosophila HP1 in lepidopteran cells using a recombinant baculovirus. MALDI-TOF mass analysis of proteolytic cleavage products identified products consistent with phosphorylation at consensus casein kinase II sites and a protein tyrosine kinase consensus, as well as a phosphopeptide containing a putative PIM-1 kinase target site. We find that phosphorylation leads to no significant change in HP1 secondary structure or self-association but in-

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† The abbreviations used are: HP1, heterochromatin protein 1; MALDI-TOF, matrix-assisted laser desorption ionization/time of flight; CKII, casein kinase II; PAGE, polyacrylamide gel electrophoresis; bp, base pair; PTK, protein tyrosine kinase; PKA, cAMP-dependent protein kinase; CaCKII, calcium/calmodulin-dependent kinase II.

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hibits HP1 binding to DNA in vitro. Amino acid substitutions in consensus phosphorylation sites reduced or eliminated silencing activity in transgenic flies, implicating phosphorylation in the regulation of HP1 in vivo.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant HP1—HP1 was expressed in *Escherichia coli* and purified as described previously (32). The BacPak™ Baculovirus Expression system (CLONTECH) was used to express HP1 in Sf21 cells. An XbaI-KpnI fragment with HP1 cDNA was inserted into the pBacPAK5 vector, and infections were performed according to the manufacturer’s instructions. Cells were grown at room temperature. After 5 days of infection, the cells were harvested, and HP1 expression was verified by Western blot analysis. For both the bacterially expressed and baculovirus expressed proteins, the glycine residue that is normally Gly52 in HP1 is preceded by the amino acid sequence MARVDL in both recombinant proteins.

To purify HP1 from the infected cells, 10 75-cm² flasks of cells or 500 ml of suspension cell culture was infected with passage two virus. After 5 days, the cells were collected by centrifugation at 1000 g for 5 min. The cell pellet was lysed in a buffer of 20 mM HEPES, pH 7.4, 0.1 M NaCl, 5 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 0.1 mM Na3VO4, 0.1% Triton X-100 by sonication on ice. The lysate was centrifuged at 16,000 rpm in an SS34 rotor (Sorvall) for 30 min, and the supernatant liquid was applied to a 1-mL DEAE column. Proteins were eluted with 0.5 M NaCl in lysin buffer from the column and diluted to 0.1 M NaCl in the same buffer. The sample was then loaded onto a 1-mL Mono Q column on a Smart System high pressure liquid chromatography (Amersham Pharmacia Biotech). The column was developed with a 0.1–0.5 M NaCl gradient, with subsequent fractions collected. The purity of HP1 was confirmed by SDS-PAGE and Western blot analysis.

Phosphatase treatment of baculovirus-expressed HP1 was performed as described (27). Briefly, 2 mg of potato acid phosphatase (0.8 units/mg; Sigma) was added to a 20-mL solution of 50 mM Tris-HCl, pH 8.0, containing 2 mg baculovirus-expressed HP1, and the mixture was incubated at room temperature for 15 min. After the reaction was stopped by adding SDS-PAGE sample buffer and boiling for 5 min. The samples were electrophoresed on a 10% polyacrylamide gel. After electrophoresis, the proteins were transferred briefly and the supernatant liquid was recovered. The membrane fragment was further sliced into small pieces, washed in water, and excised the region of membrane-containing protein. The protein samples were then loaded onto a 1-mL Mono Q column on a Smart System high pressure liquid chromatography (Amersham Pharmacia Biotech). The column was developed with a 0.1–0.5 M NaCl gradient, with subsequent fractions collected. The purity of HP1 was confirmed by SDS-PAGE and Western blot analysis.

Site-directed Mutagenesis and Construction of the Transgene Expression Vectors—Site-directed mutagenesis was performed using the Transgenemix™ site-directed mutagenesis kit (CLONTECH) according to the manufacturer’s instructions. Site-directed mutations were performed directly on a pYCI1.8-containing HP1 cDNA under the Hsp70 heat shock promoter (34).

Genetic Assays of Phosphorylation Site Mutations—To measure the ability of the different kinase target mutations to affect heterochromatic position-effect silencing, the following crosses were performed: *In (1)wm4/CyO, Ubx;w*;[InCyRoi] × *u*;transgene; *ro~5506* (when the transgene is on the second chromosome) or *In 1(1)wm4/CyO, Ubx;w*;[InCyRoi] × *u*;transgene; *ro~5506/Shy~506* (when the transgene is on the third chromosome), where “transgene” refers to either wild-type or mutant HP1 cDNA transgene constructs. Progeny from each cross were heat-shocked twice daily at 37 °C for 45 min throughout development. Heat shocks were spaced at least 8 h apart. Sons were collected, aged for 3 or more days, and red eye pigment was extracted and measured spectrophotometrically according to the method of Ephrussi and Herold (35). Pigment values are expressed as percentage of wild-type (*Canton S*) red eye pigment, and each measurement was made using a minimum of 30 fly heads.

RESULTS

**HP1 Is Multiply Phosphorylated When Expressed from Recombinant Baculovirus**—Most of HP1 phosphorylation occurs at serines and threonines (27). Inspection of the amino acid sequence of HP1 revealed consensus target sites for several different protein kinases (Fig. 1). We previously showed that the N- and C-terminal CKII consensus sites are phosphorylated in *in vitro* by an embryo nuclear extract CKII activity, suggesting that these sites are used in vivo. However, the CKII sites cannot explain the 7–8 distinct HP1 isoforms previously...
observed in whole tissue extracts (27). To identify sites of HP1 phosphorylation, we expressed HP1 in Sf21 lepidopteran (Spodoptera frugiperda; the fall armyworm) cells using recombinant baculovirus. We reasoned that since HP1 is highly conserved, and since lepidoptera are insects, these cells would likely phosphorylate HP1 at the same sites as for HP1 expressed in Drosophila. Fig. 2 shows two-dimensional gel analysis of total cellular protein from Sf21 cells infected with recombinant baculovirus expressing HP1. At least seven HP1 isoforms are visible, the most basic of which represents unphosphorylated HP1. We refer to the baculovirus-expressed recombinant HP1 as “rHP1.” Previous work (32) suggested that rHP1 does not significantly affect HP1 secondary structure.

After purifying SfHP1 from infected cells (see “Experimental Procedures”), we used matrix-assisted laser-desorption/ionization time-of-flight (MALDI-TOF) mass spectroscopy to examine the posttranslational modifications in SfHP1. A single major mass is present in the rHP1 preparation (Fig. 3A) corresponding to the expected mass of HP1 minus the N-terminal methionine, confirming that rHP1 is not posttranslationally modified beyond N-terminal processing. In contrast, the SfHP1 is distributed over multiple masses (Fig. 3B), many separated by the mass of one phosphate (80) and all major masses larger than the mass of HP1. After phosphatase treatment, the SfHP1 mass profile collapses into a single major peak whose mass is equivalent to that of HP1 minus the N-terminal methionine (Fig. 3C). Thus, SfHP1, like the endogenous HP1 in Drosophila, is multiply phosphorylated. This analysis also shows that there are no significant posttranslational modifications in SfHP1 besides phosphorylation and N-terminal processing.

Mapping Sites of Phosphorylation in HP1 Using MALDI-TOF—To determine whether consensus kinase sites in HP1 are phosphorylated in vivo, we looked for evidence that these motifs are phosphorylated in SfHP1. We digested purified SfHP1 with different endopeptidases and subjected the peptides to MALDI-TOF mass analysis to identify phosphopeptides (36).

Phosphopeptides were identified that contained each of the consensus CKII sites (Fig. 4, A and B). We previously showed that these sites are phosphorylated in bHP1 by an embryo nuclear extract in vitro; these results strongly suggest that the CKII consensus sites are significantly phosphorylated in vivo. Interestingly, a LysC peptide containing the N-terminal CKII site was found to be doubly phosphorylated (Fig. 4A). This peptide also contains a consensus protein tyrosine kinase (PTK) motif. Platero et al. (34) showed that the conservative substitution of phenylalanine for tyrosine in this PTK motif resulted in loss of silencing activity of the mutant protein. The detection of a doubly-phosphorylated peptide containing both a CKII and PTK motif suggests that the consensus PTK site is used in vivo.

Unexpectedly, we found a LysC peptide whose mass corresponds to that of a singly phosphorylated internal peptide of HP1 without a consensus kinase motif. While this study was in progress, Koike et al. (30) reported that the human HP1 family protein HP1\(^{\text{b}}\) is a target for the pim-1 kinase proto-oncogene. Multiple sites of Pim-1 phosphorylation occur in HP1\(^{\text{b}}\), at and downstream from the sequence motif KRKS in the hinge region. Fig. 4C shows that a peptide containing the single KRKS motif, which also occurs in the hinge domain of Drosophila HP1, is singly phosphorylated.

We found no consistent evidence for phosphorylation at either of the two PKA/CaCKII consensus sequences or at the PKC consensus sequence motif.

Phosphorylation Does Not Affect HP1 Secondary Structure or Dimerization in Vitro—To test whether phosphorylation causes significant changes in HP1 structure in solution, circular dichroism spectra were obtained using purified rHP1 (unphosphorylated) and SfHP1 (phosphorylated). No significant differences were observed (Fig. 5A), indicating that phosphorylation does not significantly affect HP1 secondary structure.

The HP1 C-terminal chromo domain can dimerize in vitro...
(16), which could account for self-association of rHP1 in solution (17). To test whether phosphorylation can affect HP1 selfassociation, we exposed solutions of purified rHP1 and SfHP1 to the bifunctional cross-linking agent disuccinimidyl suberate and then separated proteins by SDS-PAGE and immunolocalized HP1 by Western blot. As shown in Fig. 5B (compare lanes 2 and 4), both SfHP1 and rHP1 preparations are crosslinkable as dimers to similar extents, indicating that phosphorylation has no significant effect on the efficiency of HP1 self-association.

Phosphorylation Inhibits HP1 Binding to DNA—We showed previously that rHP1 binds to DNA in vitro (17). To test whether phosphorylation affects DNA binding activity of HP1, we compared rHP1 and SfHP1 binding to a 146-bp DNA fragment in vitro. As shown in Fig. 6A, only rHP1 detectably binds DNA. To test whether the SfHP1 protein preparation contains an inhibitor of DNA binding, equal amounts of each protein were mixed and challenged to bind DNA; the mixture retards DNA mobility somewhat more than rHP1 alone, suggesting that SfHP1 contributes to a band shift, but only in the presence of unphosphorylated HP1. A likely interpretation of this is that SfHP1 can form heterotypic complexes with rHP1 but does not contribute directly to DNA binding.

Surprisingly, phosphatase treatment of SfHP1 fails to restore DNA binding activity (data not shown). Judging from the MALDI mass analysis, phosphatase treatment of SfHP1 yields a spectrum nearly identical to that of unphosphorylated rHP1, with no evidence of residual modifications. This suggests that phosphorylation causes tertiary conformational changes in HP1 structure that inhibit DNA binding and that this structural modification remains after phosphatase treatment. Consistent with this possibility, we find that dephosphorylated SfHP1 becomes insoluble.

To test the contribution of CKII phosphorylation to HP1 DNA binding activity, mutant HP1 protein in which both target serines were substituted with either alanine or glutamate (to mimic phosphorylation) were expressed in E. coli and purified. As shown in Fig. 6B, the double alanine mutation band-shifts labeled DNA with similar efficiency to wild-type HP1, but the double glutamate mutation band-shifts DNA very poorly and with a distinct mobility from the wild-type protein. This suggests that CKII phosphorylation of HP1 contributes significantly to the loss of DNA binding in phosphorylated HP1 protein.

Mutations in HP1 Phosphorylation Sites Abolish or Antagonize Silencing Activity—To test whether HP1 phosphorylation is required for heterochromatin-mediated gene silencing in vivo, we replaced serines with either alanines (to block phos-
phorylation) or glutamate (to mimic constitutive phosphorylation) at sites containing serine/threonine kinase consensus motifs. Ser15 and Ser202 fall within consensus motifs for CKII. Furthermore, these sites were shown previously to be phosphorylated in vitro by Drosophila embryo nuclear extract (32). To test the role of CKII consensus motifs in silencing, serine codons 15 and 202 were replaced with alanine or glutamate codons. Similarly, serines 89–91 and 102–104 are found within consensus motifs for PKA and CaCKII. In these cases, three serines were simultaneously replaced with three alanines or three glutamates. Transgenic lines were established for these mutant HP1 proteins using P-element mediated germ line transformation.

Mutant proteins were tested by expression under an Hsp70 heat shock promoter in transgenic flies carrying the white variating chromosome inversion In(1)wm4. The dominant suppressor of position effect variegation Su(var)2-1 was included in the background to give a strong background of pigmentation on which enhancement can readily be detected. If the mutant protein retains wild-type silencing function, transgene expression will enhance the silencing of the white gene in the In(1)wm4 inversion, giving flies with reduced levels of red eye pigment compared with sibs that lack the transgene. Heat shock resulted in similarly elevated levels of HP1 antigen by Western blot in all transgenic lines, indicating that mutant proteins were expressed at comparable levels (data not shown).

FIG. 5. Phosphorylation does not affect HP1 secondary structure or self-association. A, circular dichroism was determined for rHP1 (dashed line) and SHP1 (solid line). Each line represents the average of five separate measurements. B, purified recombinant HP1 expressed in bacteria (lanes 1 and 2) or in SF21 cells (lanes 3 and 4) was cross-linked in solution. Cross-linked samples are shown in lanes 2 and 4. Proteins were then resolved by 10% SDS-PAGE and subjected to Western blot analysis using an anti-HP1 serum. Lane M shows the relative mobilities of protein standards whose masses are given on the left of the panel.

FIG. 6. Phosphorylation inhibits HP1 binding to DNA. A, 10 μM bacterially expressed (1st and 4th lanes) or baculovirus expressed protein (3rd and 4th lanes) was mixed with 10 nM Cy5-labeled 146-bp DNA and then electrophoresed in a 8% native polyacrylamide gel. Note that the baculovirus expressed protein alone does not shift the DNA but enhances the shift caused by bacterially expressed HP1 (compare 2nd and 4th lanes). B, 10 μM wild-type HP1, HP1 (S15A/S202A), or HP1 (S15E/S202E) was mixed with 10 nM Cy5-labeled 146-bp DNA and electrophoresed in an 8% native polyacrylamide gel.

FIG. 7. Mutations in HP1 phosphorylation sites reduce or abolish silencing activity. Males hemizygous for a transgene expressing wild-type or mutant HP1 were crossed to In(1)wm4; In(1)wm4; Su(var)2-101/InCyRoi females. Vials were heat-shocked twice daily throughout development to express the transgenic HP1. Red eye pigment was extracted from sons with and without the transgene. A minimum of 30 flies was used for each genotype, and typical standard deviations were 10% (never more than 20%). For each cross, a ratio of transgene-bearing to control siblings was calculated. This ratio is log-transformed for graphical presentation.
for wild-type HP1 overexpression from the same promoter (−3.5-fold), suggesting that the CKII mutant proteins are not fully functional in this assay. Similarly, an S15E/S202A double mutation also has reduced silencing activity.

A triple alanine substitution at the upstream PKA/CaCKII consensus motif (S89A/S90A/S91A) results in loss of silencing activity, suggesting that these serines are used in vivo (Fig. 7). Strikingly, a triple alanine substitution at the downstream PKA/CaCKII consensus motif (S102A/S103A/S104A) results in protein that suppresses silencing in two of three transgenic lines (Fig. 7). Since this is the opposite of the normal activity of HP1, this mutation results in an antimorphic or “dominant negative” phenotype suggesting that the mutant proteins antagonize heterochromatin in vivo (37). Finally, a triple glutamate substitution at the downstream consensus also results in a protein defective in silencing. Thus, both PKA/CaCKII motifs are functionally important in HP1 silencing activity.

**DISCUSSION**

Our mutational studies (summarized in Table I) strongly implicate multiple kinases in the regulation of HP1 silencing activity in vivo. The fact that silencing activity is reduced or lost with mutations that block phosphorylation as well as with mutations that mimic constitutive phosphorylation suggests that both phosphorylated and unphosphorylated isoforms are functionally important and that HP1 phosphorylation is dynamic. This is consistent with a previous observation that HP1 phosphorylation continues to occur in the absence of nascent protein synthesis (27). We cannot exclude, however, that kinase target site mutations may exert their effects by affecting HP1 structure or HP1 protein-protein interactions required for silencing.

**Functional Significance of HP1 Phosphorylation—**A recent study found a correlation between the extent of HP1 phosphorylation in embryos and salt extractability (31). Extensively phosphorylated HP1 was extractable at relatively low salt, while high salt concentration was required to solubilize hypophosphorylated HP1. Hypophosphorylated HP1 was also found associated with a high molecular weight (≈1 MDa) complex, while extensively phosphorylated HP1 was found in small (<100 kDa) complexes. The functional significance of differential salt extractability and molecular complex formation is unknown, but these observations suggest that phosphorylation may regulate HP1 trafficking in the nucleus. We reported previously that mutations in CKII sites at either end of HP1 interfered with efficient heterochromatin targeting in vivo (32), consistent with this inference. The results reported here showing reduced HP1 silencing activity for CKII site alanine and glutamate substitutions further support the inference that CKII phosphorylation is important to the mechanism of HP1-mediated heterochromatin formation.

Pim-1 kinase was found to associate with the human HP1 family protein HP1Hue in a yeast two-hybrid protein assay. *pim-1* is a proto-oncogene with multiple targets (38, 39). The functional significance of Pim-1 phosphorylation is unknown, but Pim-1 expression is correlated with cell proliferation in several systems. Since previous work indicated that several serines are targeted downstream of the Pim-1 kinase consensus target motif in HP1Hue (30), we did not test mutations in the two serines and single threonine within the Pim-1 consensus peptide. The *D. melanogaster* genome contains three Pim-1 kinase homologs (CG3105, CG11870, and CG8201). No mutations are known for any of these genes, so it is not possible to test the role of these putative kinases in HP1 phosphorylation or heterochromatic silencing.

Human HP1 family proteins associate with the silencing transcriptional intermediary factors TIF1α and TIF1β, and all three human HP1-like proteins are substrates for the intrinsic kinase activity of these factors (21). The physiological significance of these associations, however, are unknown, as are the target sites for phosphorylation in any of the human HP1s. The *D. melanogaster* genome contains six putative genes with significant homology to TIF1 family proteins (*bonus, GH10646, CG 14306, CG 1624, CG 3815*, and *CG 8419*). Of these, only *bonus* has known mutant alleles (40). *bonus* is a late larval recessive lethal required for peripheral nervous system, midgut, fat body, and cuticle development (41). It will be interesting to test whether HP1 phosphorylation is affected in *bonus* mutant embryos or larvae.

Unexpectedly, serine-to-alanine substitutions in the hinge region connecting the chromo and chromo shadow domains result in proteins that interfere with heterochromatic silencing. This dominant negative activity suggests that mutant proteins are sequestering certain factors in heterochromatin but are unable to function in a competent silencing complex. Thus, phosphorylation in the hinge domain is also implicated in HP1 regulation in vivo, although we were unable to detect significant amounts of phosphopeptides containing either of the PKA/CaCKII consensus motifs using MALDI analysis of baculovirus-expressed HP1. DNA binding activity has been localized to the hinge domain in the human HP1 family protein HP1Hue (42), suggesting that phosphorylation in the hinge could act by regulating HP1-DNA contacts. We were unable to delimit DNA binding activity in rHP1 to a single domain (17), suggesting that multiple contacts mediating DNA binding are dispersed in *Drosophila* HP1. Further studies on HP1-DNA interaction, as well as interactions between HP1 and histone and nonhistone proteins, will be required to determine the mechanism of phosphorylation-mediated HP1 regulation.

**Identification of Sites of HP1 Phosphorylation in HP1—**We have identified phosphopeptides consistent with four distinct
sites of HP1 phosphorylation. Together with the unphosphorylated HP1 isoform, this accounts for five of the seven isoforms visible in the two-dimensional gel of baculovirus-expressed HP1. The mutational results suggest that both PKA/CakII consensus kinase sites may also be used, which could account for two more spots. There are several possible reasons why phosphopeptides containing these motifs were not detected in our MALDI-TOF analysis. 1) These sites may only be phosphorylated in a small fraction of the total purified protein. 2) Phosphopeptides containing these sites may not desorb and/or ionize efficiently during the MALDI procedure. 3) These sites may not be efficiently phosphorylated in S. frugiperda cells. Finally, our studies cannot rule out additional sites of phosphorylation in vitro.

The two-dimensional electrophoresis pattern only reveals the number of modifications in a given isoform, not the sites of modification, so in principle there could be more than six potential phosphorylation sites.

The distributions of phosphorylated peptides and consensus motifs implicate at least four classes of protein kinases in the phosphorylation of HP1: CkiII, PKA/CakII, protein tyrosine kinase, and Pim-1 kinase. All of these kinases are known to have multiple nuclear targets that include transcriptional regulators. Thus, HP1 joins a substantial fraction of nuclear factors as a transcriptional regulator subject to posttranslational regulation by phosphorylation.

Biochemical Consequences of HP1 Phosphorylation—We found that phosphorylation of HP1 has no detectable effect on HP1 secondary structure or dimerization but strongly interferes with HP1 binding to DNA. Serine-to-glutamate substitutions at the CkII consensus target serines in HP1 have a severe effect on DNA binding of rHP1 in vitro, suggesting that CkII phosphorylation contributes significantly to the loss of SHH1 DNA binding activity. A simple mechanism by which phosphorylation could alter DNA binding is by contributing to electrostatic repulsion between a phosphorylated domain and the DNA sugar-phosphate backbone. Such a mechanism would be expected to have little effect on the protein secondary structure, which is what we observe. Thus, phosphorylation can provide a mechanism to regulate selectively HP1-DNA interactions while preserving HP1-protein contacts.

Phosphorylation is known to control the activity of several transcription factors (43, 44). In some cases, phosphorylation has been shown to regulate DNA binding activity. For example, phosphorylation of the POU transcription factor GHP-1 inhibits its DNA binding activity (46), and phosphorylation of the zinc finger transcription factor SP1 by casein kinase II reduces its DNA binding activity (45). Phosphorylation of the architectural transcription factor HMG-1 reduces its binding to DNA and nucleosomes in vitro (47). Hyperphosphorylation of the yeast DNA damage-responsive transcription factor CrT1 inhibits its ability to bind DNA (48). Calcium-dependent phosphorylation inhibits DNA binding by the EtS-1 transcription factor 50-fold by stabilizing an inhibitory secondary structure in the EtS-1 protein (49). We found that phosphorylated HP1 lacks detectable DNA binding activity in vitro, suggesting that phosphorylation could regulate HP1 in vivo by modulating its ability to bind chromatin. In addition, phosphorylation may regulate interactions between HP1 and other heterochromatin-associating proteins. As candidates for HP1-associated proteins are identified, the role of HP1 phosphorylation in modulating such associations should be tested.

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