Phosphorylation of Heterochromatin Protein 1 by Casein Kinase II Is Required for Efficient Heterochromatin Binding in Drosophila*

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Heterochromatin-associated protein 1 (HP1) is a nonhistone chromosomal protein with a dose-dependent effect on heterochromatin mediated position-effect silencing. It is multiply phosphorylated in vivo. Hyperphosphorylation of HP1 is correlated with heterochromatin assembly. We report here that HP1 is phosphorylated by casein kinase II in vivo at three serine residues located at the N and C termini of the protein. Alanine substitution mutations in the casein kinase II target phosphorylation sites dramatically reduce the heterochromatin binding activity of HP1, whereas glutamate substitution mutations, which mimic the charge contributions of phosphorylated serine, have apparently wild-type binding activity. We propose that phosphorylation of HP1 promotes protein-protein interaction between HP1 and target binding proteins in heterochromatin.

In higher eukaryotes, chromosomes are composed of euchromatin and heterochromatin. Heterochromatin is distinguished from euchromatin in that it stays condensed throughout the cell cycle (1). In addition, it replicates late in S phase, is enriched in repetitive DNA, and is relatively poor in classical genes (2–4).

In Drosophila, when a euchromatic gene is placed next to or within heterochromatin by chromosomal rearrangement or transposition, the euchromatic gene usually undergoes cell-specific silencing, called "position-effect variegation" (5). This process indicates that heterochromatin interferes with euchromatic gene expression, which biochemical data suggest is caused by the acquisition of a distinct chromatin structure with reduced accessibility to DNA-binding proteins (6, 7). More than 50 different position-effect variegation regulators have been identified, including genes encoding chromatin proteins and protein modifiers (8). One such regulator encodes heterochromatin-associated protein 1 (HP1), a nonhistone chromosomal protein enriched in the pericentric heterochromatin of interphase nuclei (9, 10). HP1 exerts dosage-dependent effects on position-effect variegation (11, 12). HP1 localization during the cell cycle is complex, perhaps reflecting the changes in chromosome structures that accompany the cell cycle (13).

Current models suggest that HP1 functions as part of a chromosomal protein complex (14). Proteins thought to bind to HP1-like proteins include transcriptional intermediary factors (15), lamin B receptors (16), and origin recognition complex proteins (17, 18).

HP1 is multiply phosphorylated in vivo (19). Using two-dimensional gel electrophoresis, HP1 can be resolved into as many as eight charged isoforms. HP1 phosphorylation occurs predominantly at serines and threonines, and increased phosphorylation of HP1 is correlated with heterochromatin assembly during development. Phosphorylation may be correlated with the oligomeric state of HP1 in vivo (18). Phosphorylation of chromosomal proteins is implicated in the regulation of several nuclear functions. For example, histones are multiply phosphorylated during mitotic and developmentally programmed chromatin condensation (20), specific forms of phosphorylated histone H1 are correlated with specific heterochromatic satellite DNA sequences (21), and several transcription factors are regulated by phosphorylation (22).

As a first step toward defining the role of HP1 phosphorylation in heterochromatin assembly and position-effect variegation silencing, we mapped three of the phosphorylation sites at the N- and C-terminal domains of HP1. We present biochemical evidence for casein kinase II (CKII) phosphorylation of HP1 in vitro and in vivo. Alanine substitution mutation in the N-terminal CKII target site dramatically reduces heterochromatin binding activity of HP1, whereas glutamate substitutions mutations at either or both of the N- and C-terminal sites have apparently wild-type binding activity, suggesting that CKII phosphorylation is required for heterochromatin binding. We propose that phosphorylation of HP1 promotes protein-protein interaction between HP1 and target binding proteins within heterochromatin.

EXPERIMENTAL PROCEDURES

Fly Culture and Tissue Preparation—Fly stocks were maintained at room temperature on standard cornmeal-yeast-sucrose-agar medium containing methylparaben as a mold inhibitor. Third-instar larvae were collected immediately before dissection and kept on ice until dissection. Expression and Purification of Recombinant HP1—A Xhol-BamHI fragment containing Drosophila HP1 cDNA was cloned into expression vector pET11a, and the recombinant plasmid was transformed into Escherichia coli BL21(DE3) cells. A single colony of transformed cells was grown in LB medium until A600 = 0.6–1.0. Expression of HP1 was induced by 1 mM isopropyl-1-thio-β-D-galactopyranoside at 30 °C for 2–3 h. Cells were spun down at 5,000 × g for 5 min in 4 °C, and the cell pellet was frozen and stored at −70 °C. For protein purification, the pellet was thawed on ice and suspended in 40 ml of lysis buffer (100 mM Tris-HCl, pH 7.3, 4 mM EDTA, 0.4 mM EGTA, 4 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). All subsequent steps were done on ice or at 4 °C unless indicated otherwise. 24 mg of lysozyme was added to the sample and incubated on ice for 20 min. Cells were lysed either by freezing and thawing or by sonication. NaCl was added to a final concentration of 0.3 M to get maximum HP1 solubilization. After centrifugation at 18,000 rpm for 30 min in a Sorvall SS-34 rotor, the supernatant was diluted 3-fold and applied to a DEAE-Sepharose col-

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Heterochromatin Binding of HP1 Requires CKII Phosphorylation

**Results**

**Procedures**

Pooled HP1-enriched fractions are shown; MWm, molecular weight markers. Sizes in kDa are given to the left of the gel. Lane 1, DEAE-Sepharose fraction; lane 2, Resource Q FPLC fraction; lane 3, S-200 Sephacryl FPLC fraction; lane 4, Mono Q FPLC fraction. Fractions enriched for recombinant HP1 were resolved by 12% SDS-PAGE and stained with Comassie Brilliant Blue R.

The anti-HP1 serum was a gift of Drs. R. F. Clark and S. C. R. Elgin.

**Preparation of Drosophila Nuclear Extract—**Five g of frozen 0–6-h-old embryos (gift of Dr. S. C. R. Elgin) were used to make the nuclear extract. Drosophila embryo nuclei were prepared according to Wu et al. (23). The nuclear extract was made as described (9), with modifications. Briefly, nuclei were suspended in 4 ml of extraction buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM β-mercaptoethanol, 0.25 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, 0.4% Triton X-100) and homogenized at 4 °C with a glass-Teflon homogenizer. Homogenate was centrifuged at top speed in an Eppendorf centrifuge for 5 min. The supernatant was stored at −20 °C before peptide mapping. Tryptic peptide mapping was done on a 40% PAGE under alkaline conditions as described (26, 27). The gel was dried and subjected to autoradiography or PhosphorImager analysis to visualize the phosphopeptides.

**Sequence of Radiolabeled Tryptic Peptides and Identification of Radioactive Amino Acids—**Tryptic phosphatepeptide bands on the dried gel were excised with a surgical blade. Peptide was eluted from the gel slices with Milli-Q water. Peptides in the extract were purified and desalted with a C-18 reverse phase HPLC column (130A Separation System, Applied Biosystems) and subjected to protein sequencing (477A Protein Sequencer, Applied Biosystems). Cleaved phenylthiohydantoin derivatives from each cycle were collected, and radioactive amino acid residues were determined by: 1) counting in a scintillation counter (1500 Tri-Carb liquid scintillation analyzer, Packard Instrument Co.) for the radioactivity and 2) concentrating to <10 μl and spotting onto a 0.1-mm cellulose thin layer electrophoresis plate (EM Separations) for autoradiography.

**Anti-CKII Immunodepletion of Extracts—**Antibody depletion of CKII was done according to Birnbaum et al. (28) with modifications. 5 μl of nuclear extract was incubated with 4 μl of rabbit antiserum against native Drosophila CKII, 16 μl of NET buffer (140 mM NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, and 0.05% Nonidet P-40), and 25 μg of bovine serum albumin at 4 °C for 1 h. 25 μl of protein A-acrylic beads suspension (Sigma) was added, and the mixture was incubated at 4 °C for an additional 20 min. Following a brief centrifugation, the supernatant was used in the in vitro phosphorylation assay. As a control, 5 μl of the extract was treated with rabbit nonimmune serum (no preimmune serum remains), and the sample was also used in the in vitro phosphorylation assay.

**Site-directed Mutagenesis and Construction of the Transgene Expression Vector—**Site-directed mutagenesis was performed using the Transform^™ site-directed mutagenesis kit (CLONTECH) according to manufacturer’s instructions. Mutagenesis was done in a pBluescript SK(+) plasmid (Strategene) with Drosophila HP1 cDNA inserted between the XbaI and BamHI sites. Mutant cDNA was then excised with SacI and BglII and cloned into vector SPI (29). A KpnI fragment from the SPI vector was cloned into transformation vector pvJ206 in which HP1 cDNA is fused downstream of and in frame with E. coli lacZ under the Drosophila Hsp70 heat-shock promoter (29), inserted in the P-element vector, pYCl1.8 (30).

**Expression and Localization of β-Galactosidase Fusion Protein—**Germine transformation was performed by injecting v episomal β-galactosidase fusion constructs, heat-shocked at 37 °C for 30 min, and recovered at room temperature for 1 h. Salivary glands were dissected and stained with X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) as described (35). Stained tissue was placed on a microscope slide and mounted in 95% glycerol, 5% phosphate-buffered saline.

2 C. V. C. Glover, personal communication.
Heterochromatin Binding of HP1 Requires CKII Phosphorylation

**RESULTS**

*Heterochromatin Binding of HP1 Requires CKII Phosphorylation*

**Drosophila Embryo Nuclear Extract Phosphorylates Recombinant HP1 in Vitro.—**HP1 is multiply phosphorylated in vivo, and hyperphosphorylation is correlated with heterochromatin assembly (19). As a first step toward understanding the functional significance of HP1 phosphorylation, we mapped phosphorylation sites in HP1. Our strategy was to use recombinant HP1 (rHP1) as a substrate for phosphorylation by *Drosophila* nuclear extract in *vitro* and then map the sites of phosphorylation corresponding to sites used in *vivo*. *Drosophila* HP1 cDNA was expressed in *E. coli* and purified to >95% homogeneity (Fig. 1). To determine whether rHP1 is phosphorylated in bacteria, we compared its mobility in two-dimensional gel electrophoresis to that of HP1 from *Drosophila* (dHP1). Multiple dHP1 isoforms are detected in Western blots of total *Drosophila* protein after two-dimensional gel electrophoresis (Ref. 19; Fig. 2A, top). Similar Western blots of rHP1 revealed a single major HP1 isoform (Fig. 2A, middle). When rHP1 is added to total *Drosophila* protein and co-electrophoresed, the rHP1 migrates at the extreme basic end of the dHP1 pattern (Fig. 2A, bottom), consistent with unmodified HP1. Hyperphosphorylated isoforms of HP1 begin to appear by 2 h of embryonic development (19). Therefore, we used a nuclear extract from 0–6-h *Drosophila* embryos to phosphorylate rHP1 in *vitro*. When rHP1 is added to nuclear extract in the presence of [γ-32P]ATP, rHP1 is efficiently radiolabeled. Co-electrophoresis with total *Drosophila* protein demonstrates that radiola-

**Fig. 1.** Mapping of phosphorylation sites in HP1 using recombinant HP1 protein. A. Western blots of two-dimensional gels, aligned to show multiple dHP1 isoforms in larval tissue (top), the single major isoform of rHP1 (middle), and the co-electrophoresis of rHP1 with the most basic dHP1 isoforms (bottom). B. Two-dimensional gel analysis of rHP1 after *in vitro* phosphorylation. Western blot of a two-dimensional gel containing a mixture of dHP1 and phosphorylated rHP1 (top) and an autoradiograph of the same blot show the relative mobility of phosphorylated rHP1 (bottom). C. co-electrophoresis of the major *in vitro* and *in vivo* tryptic phosphopeptides. Phosphorylated rHP1 and dHP1 was labeled, purified, and analyzed as described under “Experimental Procedures.” Phosphopeptides were imaged using a Molecular Dynamics PhosphorImager. The band between peptides 1 and 2 was also observed in some digests of *in vitro* phosphorylated samples and probably represents an incomplete digestion product. D, positions of phosphorylated residues detected by sequence analysis.

**Fig. 2.** Mapping of phosphorylation sites in HP1 using recombinant HP1 protein. A. Western blots of two-dimensional gels, aligned to show multiple dHP1 isoforms in larval tissue (top), the single major isoform of rHP1 (middle), and the co-electrophoresis of rHP1 with the most basic dHP1 isoforms (bottom). B. Two-dimensional gel analysis of rHP1 after *in vitro* phosphorylation. Western blot of a two-dimensional gel containing a mixture of dHP1 and phosphorylated rHP1 (top) and an autoradiograph of the same blot show the relative mobility of phosphorylated rHP1 (bottom). C. co-electrophoresis of the major *in vitro* and *in vivo* tryptic phosphopeptides. Phosphorylated rHP1 and dHP1 was labeled, purified, and analyzed as described under “Experimental Procedures.” Phosphopeptides were imaged using a Molecular Dynamics PhosphorImager. The band between peptides 1 and 2 was also observed in some digests of *in vitro* phosphorylated samples and probably represents an incomplete digestion product. D, positions of phosphorylated residues detected by sequence analysis.
and charge contributions of phosphorylated serine; Refs. 38 and 39). Five mutations were assayed (Fig. 4): replacement of the N-terminal serine by alanine (S15A) or by glutamate (S15E), replacement of the very C-terminal serine by alanine (S202A) or by glutamate (S202E), and a combination of both Ser → Ala mutations (S15A,S202A). The mutant HP1 cDNA were fused downstream from, and in frame with, E. coli lacZ, and the fusion cDNA was placed under the control of the Drosophila Hsp70 heat shock promoter. P-element-mediated germline transformations were generated for the two glutamate substituted mutants. Transgenic larvae were subjected to heat shock, and larval polytene tissue was stained with X-gal to localize the fusion protein. β-Galactosidase alone is a cytoplasmic protein when expressed in Drosophila cells (Fig. 4a). As reported previously (29, 40), full-length HP1-β-galactosidase fusion protein targets β-galactosidase activity to the nucleus and decorates the heterochromatin with β-galactosidase; this results in blue-staining nuclei with X-gal, with one or two intensely staining dark blue spots corresponding to the pericentric heterochromatin (Fig. 4c). For both the S15E and S202E mutations, X-gal staining showed that these two mutant HP1 fusion proteins resulted in nuclear localization and heterochromatin binding indistinguishable from wild-type HP1 fusions (Compare Fig. 4, a and g–c; see also Refs. 29 and 40).

For the S15A, S202A, and S15A,S202A mutations, comparable efforts to obtain germline transformants resulted in no transgenic lines (occasionally, transformed phaerate adults were observed, but these failed to hatch or died shortly after hatching). To assay fusion protein targeting for these mutations, we resorted to somatic transformation. Plasmid DNA injected into early embryos stably endures in somatic tissues through the third-instar larval stage, displaying correct tissue-specific expression (33, 34). We had employed somatic transformation in previous studies to assay HP1-β-galactosidase fusions (29, 40). Somatically transformed third-instar larvae were heat-shocked, and polytene tissues were dissected out and stained with X-gal. By this assay, the S202A mutant appeared similar to the wild type (Fig. 4f), but the S15A mutant showed decreased heterochromatin binding activity visible only in highly expressing cells (Fig. 4d). Only in overstained nuclei did we observe the darkly staining spots that represent heterochromatin binding. For the double mutation (S15A,S202A), the loss of heterochromatin targeting was even more dramatic. Although the fusion protein still appeared to concentrate in the nuclei, it no longer bound to heterochromatin (Fig. 4h). These results demonstrate a requirement for CKII phosphorylation for efficient heterochromatin binding.

**DISCUSSION**

*Drosophila HP1 Is Phosphorylated by CKII in Vivo*—The methods we used to identify HP1 phosphorylation sites involved direct comparison of the in vivo and in vitro tryptic peptide map by high concentration PAGE, rHP1 phosphopeptide sequencing, and radioactivity detection of each amino acid derivative. This strategy has been used to identify phosphorylation sites on other proteins (26, 27, 41, 42). For all three sites common to phosphorylated rHP1 and dHP1, the targets are good fits to CKII consensus motifs, which, together with the sensitivity of rHP1 phosphorylation to spermine, heparin, and anti-Drosophila CKII serum, strongly suggests that HP1 is a substrate for CKII. CKII is an ubiquitous cyclic nucleotide-independent protein kinase that appears not to directly mediate known signaling pathways (43). CKII activity has been found to increase in response to some mitogens, and its substrates include a number of transcription factors involved in growth control (44). Because CKII is found both in the nucleus and the cytoplasm (36), and because we found that alanine substitution had no effect on nuclear targeting, HP1 phosphorylation by CKII could occur in either compartment.

CKII consensus target sites are found at the N and/or C terminus of HP1 homologs from *Drosophila virilis*, *Schizosaccharomyces pombe*, mealybug, mouse, and human. Not all HP1 homologs have CKII targets at both ends (some have neither), but in several such cases the homologous position is occupied by glutamate. Little or nothing is known about the functionalology between *Drosophila melanogaster* HP1 and its structural homologs in other species, but such apparent structural conservation suggests functional conservation. Nevertheless, the data presented here showing that CKII phosphorylation is required for efficient heterochromatin targeting by the unique *D. melanogaster* HP1 suggest that such structural conservation is likely to be functionally significant.

The only detailed structural information for any HP1 homo-
log is a solution NMR peptide structure based on the N-terminal chromo domain of a single mouse HP1 homolog (45), and the sequences corresponding to the targets of CKII lie outside of the solved structure. So far, we have found no effect of CKII phosphorylation on HP1 multimerization in solution.\(^3\) Although two of the CKII sites occur within a previously identified nuclear targeting domain (40), we observed no impairment in nuclear targeting for any of the CKII site mutant fusions. CKII phosphorylation could contribute to heterochromatin binding by HP1 by promoting a conformational shift that permits 1) additional kinases to phosphorylate internal targets in, for example, the HP1 linker region between the chromo domains; or 2) the exposure of sites for protein–protein interactions. Either of these results could facilitate heterochromatin assembly. As previously noted (19), this interval is serine/threonine-rich and includes two consensus targets for protein kinase A and one for protein kinase C.

**Phosphorylation on Both the N- and the C-terminal CKII Sites Is Required for Heterochromatin Binding**—Although the Ser→Ala mutation on the C-terminal site did not discernibly alter the heterochromatin binding activity of the mutant fusion protein, the Ser→Ala mutation on the N-terminal site conspicuously reduces heterochromatin binding. The double Ser→Ala mutation (S15A,S202A) almost completely eliminated heterochromatin binding, although the protein could still get into the nucleus. The double mutant appeared to have a generally more severe effect. However, care should be taken in interpreting quantitative differences, because levels of fusion protein expression vary from cell to cell in these assays. Although the effect of the single C-terminal substitution was not detectable by the X-gal staining method, it is possible that each mutation exerts some effect on HP1 heterochromatin binding activity because the combined mutations had the most dramatic effect on heterochromatin binding. Although there are two CKII sites at the C terminus of HP1, we chose to mutate the more downstream site. The upstream site is dependent on the phosphorylation of the downstream serine, so when we mutated the first serine to alanine, we also disabled the second one as a CKII target. Thus, in our assay, any effect attributable to the downstream serine could also reflect a requirement for phosphorylation on HP1 multimerization in solution.3 Al-

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\(^3\) T. Zhao and J. C. Eisenberg, unpublished observations.
Heterochromatin Binding of HP1 Requires CKII Phosphorylation

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