Phosphorylation of Histone H3 at Serine 10 Cannot Account for the Detachment of Human Heterochromatin Protein 1γ from Mitotic Chromosomes in Plant Cells*

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Heterochromatin protein 1 (HP1) controls heterochromatin formation in animal cells, at least partly through interaction with lysine 9 (Lys-9)-methylated histone H3. We aimed to determine whether a structurally conserved human HP1 protein exhibits conserved heterochromatin localization in plant cells and studied its relation to modified histone H3. We generated transgenic tobacco plants and cycling cells expressing the human HP1γ fused to green fluorescent protein (GFP) and followed its association with chromatin. Plants expressing GFP-HP1γ showed no phenotypic perturbations. We found that GFP-HP1γ is preferentially associated with the transcriptionally “inactive” heterochromatin fraction, a fraction enriched in Lys-9-methylated histone H3. During mitosis GFP-HP1γ is detached from chromosomes concomitantly with phosphorylation of histone H3 at serine 10 and reassembles as cells exit mitosis. However, this phosphorylation cannot directly account for the dissociation of GFP-HP1γ from mitotic chromosomes inasmuch as phosphorylation does not interfere with binding to HP1γ. It is, therefore, possible that phosphorylation at serine 10 creates a “code” that is read by as yet an unknown factor(s), eventually leading to detachment of GFP-HP1γ from mitotic chromosomes. Together, our results suggest that chromatin organization in plants and animals is conserved, being controlled at least partly by the association of HP1 proteins with methylated histone H3.

The packaging of DNA into nucleoprotein complexes that make up chromatin is highly conserved among eukaryotes (1). The fundamental structural unit of chromatin, the nucleosome, is made up of two copies of each of the four core histone proteins, H2A, H2B, H3, and H4. This basic structure of chromatin can be further organized into higher order chromatin structure by the aid of various proteins including histone H1, polycomb proteins, and heterochromatin protein 1 (HP1). Dynamic changes in chromatin structure are directly influenced by post-translational modifications of histone amino-terminal tails. Within histone tails there are specific amino acids (arginine, lysine, serine) that undergo a number of post-translational modifications, including acetylation, methylation, phosphorylation, ubiquitination, and poly(ADP-ribosylation) (2, 3). The “histone code” hypothesis predicts that modifications on histone amino-terminal tails act sequentially or in combination to form a code for recruitment of various proteins involved in controlling the diverse chromatin functions such as gene expression, recombination, and DNA replication (4).

The effect of the chromatin state of condensation on gene transcription is exemplified by the so-called position effect variegation phenomenon in Drosophila. Position effect variegation occurs when euchromatic genes are translocated to heterochromatic regions, resulting in a variegated (mosaic) expression pattern (5, 6). One of the best-studied modifiers of position effect variegation is the Su(var)2–5 gene encoding the HP1 protein (7, 8), having a predominant heterochromatic distribution (9, 10). HP1-like proteins are composed of two related functional domains, an amino-terminal chromo domain (chromatin organization modifier) (CD) and a carboxyl-terminal chromo shadow domain (CSD) (11). Current models suggest that HP1 proteins function as chromatin organizers, participating in the assembly of multi-protein complexes that promote silencing of euchromatic genes and the expression of heterochromatin genes interspersed in heterochromatids (12).

Some of the molecular events leading to HP1-induced heterochromatinization have been recently resolved with the discovery of histone methyltransferase activity associated with the human SUV39H1 and the fission yeast Clr4 proteins (13). These enzymes specifically methylate histone H3 at lysine 9 residue, generating a binding site for HP1 in euchromatin (14, 15), thereby inducing heterochromatin formation (17). Methylated (Lys-9) histone H3, however, may not be the sole factor in targeting HP1 to chromosomal DNA (18); alternative pathways have been suggested (19–22). In addition to methylation, other types of covalent modifications were extensively studied including acetylation, which is associated with transcriptional activation (23), and histone H3 phosphorylation at serine 10, a modification strictly occurring during meiosis and mitosis both in plants and animals, although its function is unknown (24).

HP1-like proteins have been recently characterized in plants (Ref. 25, Plant Chromatin Data Base (www.chromdb.org)). A mutation in LHP1 of Arabidopsis (lhp1) led to changes in flowering time, leaf development, and plant architecture (25), suggesting that developmental processes in plants are controlled at least partly by HP1-mediated formation of heterochromatin. Although a single HP1 gene was identified in the Arabidopsis genome, the HP1 family in human comprises three different protein with distinct chromosome distributions in interphase nuclei. The human HP1α and HP1β reside mainly in centromeres, whereas HP1γ is distributed in multiple small foci outside the nucleoli (26), similar to the nuclear distribu-
tion of the Arabidopsis LHP1 protein (25). To determine whether a structurally conserved human HP1 protein exhibits conserved heterochromatin localization in plant cells, we generated transgenic tobacco plants and BY-2 cycling cells expressing the human HP1\(\gamma\) fused to GFP under the constitutive 35 S promoter. We studied the dynamic association of GFP-HP1\(\gamma\) with chromatin with respect to histone H3 modifications. Plants overexpressing the GFP-HP1\(\gamma\) did not display phenotypic alterations and had wild type morphology. Our results show that GFP-HP1\(\gamma\) and methylated histone H3 are confined to heterochromatin in interphase nuclei. During mitosis GFP-HP1\(\gamma\) is detached from chromosomes concomitantly with phosphorylation of histone H3 at serine 10 and reassembles as cells exit mitosis. We provide evidence showing that the detachment of HP1\(\gamma\) from mitotic chromosomes cannot be accounted for by phosphorylation of histone H3 at serine 10 inasmuch as this phosphorylation does not interfere with binding of HP1\(\gamma\) to lysine 9-methylated histone H3. The implications of our findings for chromatin organization are discussed.

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids—Human HP1 (GenBank\(\text{TM}\) accession number U26812) was isolated from mammalian cDNA library by PCR using the primers HP1-sense (5'-CAATGAAATTCGATCCTGTGGGTAATACGACTCACTATAGG) and HP1-antisense (5'-CAATGAAATTCGATCCTGTGGGTAATACGACTCACTATAGG) primers for amplification of the CD and HP1 domain (CSD) were amplified by PCR using pBluescript SK (Stratagene) to generate pBS-HP1.**

The PCR product was digested by BamHI and EcoRI restriction endonucleases and subcloned into the same sites of either pBluescript SK (Stratagene) to generate pBs-HP1. The chromo domain (CD) and the chromo shadow domain (CSD) were amplified by PCR using pBS-HP1\(\gamma\) as template DNA. We used HP1-S and CD-AS (5'-AATGTTGCTCGTGGGATCAAGGGCGAGGAG) and GFP-AS (5'-GTCTGTCTGGATCCATGGTGAGCAAGGGCGAGGAG) for the CSD. The PCR fragments were digested with BamHI and EcoRI and subcloned into the same sites of pEGFP (CLONTECH) to generate pGEX-HP1. The PCR product was digested with BamHI and EcoRI and subcloned into the BamHI site of pbsHP1 upstream from HP1\(\gamma\) DNA fragment. Several clones were isolated and sequenced to select for the appropriate pbs-GFP-HP1\(\gamma\) clone. To subclone GFP-HP1\(\gamma\) into a binary vector for plant transformation, the GFP-HP1\(\gamma\) fragment was amplified by PCR using pBS-GFP-HP1\(\gamma\) as a template. The PCR fragment was digested with XbaI and Smal and subcloned into the same sites of p2PZP111–3HA (27) to introduce GFP-HP1\(\gamma\) downstream from the 35 S promoter and in-frame with the HA epitope. The plasmid p2PZP111–GFP-HP1\(\gamma\) was introduced into tobacco BY-2 cells and Nicotiana tabacum using the Agrobacterium methodology. All PCR reactions were performed using the thermostable DNA polymerase Pwo (Roche Molecular Biochemicals), which has a proofreading activity. The identity and integrity of the PCR products were verified by sequencing. In experiments presented here we used F3 homozygous plants for 35S::GFP-HP1\(\gamma\).

**Synchronization of BY-2 Cells—**Tobacco BY-2 cells stably transformed with 35S::GFP-HP1\(\gamma\) were synchronized (partly) with aphidicolin, an inhibitor of DNA polymerase \(\alpha\) (28). Briefly, BY-2 cells at the stationary phase (7 ml) were transferred into 50 ml of fresh medium containing 2 \(\mu\)g/ml 2,4-dichlorophenoxacyclic acid (2,4-D) and 7.5 \(\mu\)g/ml aphidicolin and incubated for 24 h at 26 °C with gentle shaking. Aphidicolin was removed by extensive washing of cells with fresh medium. Cells were then transferred into fresh medium supplemented with 2,4-D, incubated as above, and sampled at various time points after the release of each synchronized cell line. Each sample was incubated on ice for 10 min to let the cells settle down, medium was removed, and the sample was kept frozen at −20 °C. Cell cycle progression was determined in each sample by the frequency of mitotic figures (occurrence of metaphases and anaphases) after staining with propidium iodide (PI, 1 mg/ml in 0.1% Triton X 100). Cells were inspected by a fluorescence microscope (Olympus) equipped with a CCD camera (Imago, Photonic) or by a Zeiss confocal microscope (510 scanning system). Images were pseudo-colored and merged using TILL Vision version 3.3 software. Olympus filters U-MW1B2, U-MNG, and U-MNU were used to detect GFP, propidium iodide (PI), and 4,6-diamidino-2-phenylindole signals, respectively. Synchronization was verified by analyzing SUC1-associated histone H1 kinase activity (29).

**Nuclei Preparation and Chromatin Fractionation—**Nuclei were prepared by a combination of methods described by van Blokland et al. (30) and Remboutsika et al. (31). Leaves from 3SS::GFP-HP1\(\gamma\) transgenic tobacco plants were ground using pestle and mortar in buffer N (15 mM Tris–HCl, pH 7.5, 60 mM KCl, 15 mM MgCl\(_2\), 1 mM CaCl\(_2\), 1 mM dithiothreitol, 2 mM sodium vanadate, 250 mM sucrose, and protease inhibitors (5 \(\mu\)g/ml aprotinin, 5 \(\mu\)g/ml leupeptin, and 2.5 \(\mu\)g/ml pepstatin; Sigma). The homogenate was gently stirred for 30–60 min on ice and filtered through a 150-μm nylon mesh followed by centrifugation (4 °C, 1,000 \(\times\) g, 10 min). The pellet was gently washed to remove the upper chloroplast layer, nuclei pellets were recovered, and nuclei were washed twice with buffer N. Fractionation of chromatin was performed essentially as described (31, 32). Briefly, nuclei (75 \(\mu\)l) were digested with increasing amounts (2, 5, and 10 units per 2 × 10\(^5\) nuclei) of micrococcal nuclease (MBI Fermentas) at room temperature for 12 min. A portion of the digested nuclei was used for DNA preparation as described (33). The remaining micrococcal nuclease (MNsase)-digested nuclei were cooled on ice for 10 min before centrifugation at 13,000 \(\times\) g for 10 min at 4 °C. The supernatant (S1) was collected, and the pellet was resuspended in 50 \(\mu\)l of ice-cold 2 mM EDTA, pH 8.0. After a 10-min incubation on ice samples were centrifuged as above, the supernatant (S2) was collected, and the pellet (P) was resuspended in 65 \(\mu\)l of SDS buffer. Equivalent samples were resolved on 15% SDS-PAGE and immunoblotted using anti-HA and anti-dimethylated (Lys-9) histone H3. DNA was run on 1.5% agarose gel followed by ethidium bromide staining.

**In Vitro Histone Methyltransferase Assay—**In vitro histone methyltransferase reactions were performed with glutathione-Sepharose containing GST-SUV39H1/H320R (kindly provided by T. Jenuwein and S. Opravil) essentially as described (13). Briefly, reactions were carried out in a final volume of 50 \(\mu\)l in methylase buffer (50 mM Tris-HCl, pH 8.0, 10 mM DTT, 10% glycerol, 1 mM ATP, 10% DMSO, 100 mM 32P-γGTP, 100 units of nuclease S1, 0.5 μg/ml of GST-SUV39H1/H320R, 0.25 μM H3-K9/E14/E18Ac, or 0.25 μM H3-K9/K14/K18Ac) for 20 min at 30 °C. Then the reaction was stopped by the addition of 100 μl of stop buffer (50 mM Tris-HCl, pH 7.5, 500 mM KCl, 0.5% Triton X-100) and the assay products were resolved on 10% SDS-PAGE. The reaction products were visualized by exposure to autoradiographic film.
Figs. 2A–2C. GFP-HP1γ is preferentially associated with transcriptionally inactive heterochromatin fraction. A, an outline of the chromatin fractionation procedure. Nuclei from GFP-HP1γ-expressing plants were digested with 2, 5, and 10 units of MNase at room temperature for 12 min and processed to yield the soluble fractions S1 and S2 and the pellet (P) fraction. B, nucleosomal ladder of MNase-digested nuclei. DNA was prepared from nuclei treated with the indicated concentrations of MNase and separated on 1.5% agarose gel followed by CBB Green I staining. The position of the mononucleosome is indicated (IN). The molecular weight markers are indicated in kilobases on the left. C, DNA from equivalent S1, S2, and P nuclear samples (initially digested with 5 units of MNase) was resolved by 1.5% agarose gel followed by staining with PI. T indicates total DNA after digestion with 5 units of MNase for 12 min. D, GFP-HP1γ and methylated Lys-9 histone H3 are associated with the transcriptionally inactive fraction. Equivalent samples from S1, S2, and P fractions were resolved by 15% SDS-PAGE and immunoblotted using anti-HA (to detect GFP-HP1γ) and anti-dimethylated (Lys-9) histone H3 (dmK9H3). T indicates total protein extract of mesophyll cells.

8.5, 20 mM KCl, 10 mM MgCl2, 10 mM β-mercaptoethanol, and 250 mM sucrose) containing as substrates free histones prepared from tobacco leaves (34) and 300 nCi of S-adenosyl-L-[methyl-3H]methionine (25 μCi/ml) (Amersham Biosciences) as the methyl donor. After incubation at 37 °C for 60 min, reactions were added SDS loading buffer and boiled for 5 min, and proteins were resolved by 15–18% SDS-PAGE and visualized by Coomassie Blue staining and fluorography.

RESULTS

GFP-HP1γ Is Localized to the Nucleus of Tobacco Cells—We generated transgenic tobacco plants and BY-2-cycling cells expressing HA-tagged GFP-HP1γ under the control of the 35 S promoter. BY-2 cells expressing GFP alone (Fig. 1, A and B) showed the GFP signal both in the nucleus and the cytoplasm. However, the GFP-HP1γ fusion protein was exclusively localized to the nucleus both in BY-2 cells (Fig. 1, C and D) and in tobacco mesophyll cells (Fig. 1F), indicating that HP1γ directed GFP into the nucleus. Often GFP-HP1γ showed a speckle-like distribution in the nucleus (not shown). Immunoblotting analysis using anti-HA confirmed the production of GFP-HP1γ fusion protein in transgenic BY-2 cells (Fig. 1E) and in tobacco mesophyll cells (Fig. 1G). Neither BY-2 cycling cells nor tobacco plants expressing GFP-HP1γ showed any change in their growth kinetics and morphology compared with their wild type counterparts (data not shown).

GFP-HP1γ Is Preferentially Associated with a Transcriptionally “Inactive” Chromatin Fraction during Interphase—HP1γ proteins are preferentially associated with heterochromatic regions in animal interphase nuclei (12). To determine the chromatin distribution of GFP-HP1γ in transgenic plants, nuclear fractions enriched with transcriptionally “active” or inactive chromatin were prepared from transformed mesophyll cell nuclei subjected to mild MNase digestion in a low salt sucrose-containing solution (31, 32). This treatment yields a soluble fraction (S1, shown in Fig. 2A) enriched in oligonucleosomes representing the potentially transcriptionally active chromatin (euchromatin). Hypotonic extraction of the MNase-digested nuclei pellet yields a soluble fraction (S2, shown in Fig. 2A), which is enriched in polynucleosomes, representing the transcriptionally inactive chromatin (heterochromatin) (32). Incubation of nuclei with increasing concentrations of MNase resulted in the formation of a typical nucleosomal ladder, with the mononucleosome fraction enriched at the higher MNase concentrations (Fig. 2B). To determine whether GFP-HP1γ was associated with euchromatin (S1) or heterochromatin (S2), equivalent samples of S1, S2, and the pellet fraction (P) were immunoblotted either with anti-HA to detect GFP-HP1γ or with anti-dimethylated (Lys-9) histone H3. Fig. 2D shows that the GFP-HP1γ was preferentially associated with the S2 and P fractions but not with the S1 fraction. Methylated (Lys-9) histone H3 showed a similar distribution. These results demonstrated the localization of both GFP-HP1γ and methylated (Lys-9) histone H3 to the heterochromatin fraction.

GFP-HP1γ Is Detached from Chromosomes during Mitosis—To determine the GFP-HP1γ distribution during various phases of the cell cycle, BY-2 cells expressing GFP-HP1γ were synchronized by incubating them for 24 h with aphidicolin. After removal of aphidicolin, cells were sampled at various time points, and cell cycle progression was determined by fluorescence microscopy after staining the cells with PI. More than 50% of BY-2 cells reached mitosis 9 h after aphidicolin removal, with the majority exhibiting anaphase appearance (data not shown). Synchronization was also verified by histone H1 kinase activity purified by glutathione-Sepharose containing GST-SUC1 (data not shown). Fluorescence microscopy analyses showed uneven distribution of GFP-HP1γ in the nucleus during interphase (Fig. 3; see also panel 5 Fig. 4A). Notably, although PI stained nucleoli quite intensely, the GFP-HP1γ protein was hardly noticeable in this nuclear compartment, consistent with a previous report (26). During mitosis, the
GFP-HP1γ protein was absent from chromosomes; prophase, metaphase, and anaphase chromosomes were stained with PI only (Fig. 3). However, as cells completed mitosis, the GFP-HP1γ protein reassociated with chromosomes (Fig. 3, Telophase). Confocal microscopy analysis (Fig. 4A) verified that GFP-HP1γ was not associated with mitotic chromosomes; a diffused GFP signal could be detected in the cytoplasm. Western blot analysis verified that the absence of GFP-HP1γ protein from mitotic chromosomes was not due to degradation of the protein but was due to degradation of the protein because the GFP-HP1γ remained stable throughout the cell cycle (Fig. 4B).

HP1γ Interacts with Plant Histone H3 Methylated at Lysine 9—The possibility existed that the release of GFP-HP1γ from chromatin during mitosis resulted from a post-translational modification(s) of its interacting protein(s). The preferential association of HP1γ and methylated (Lys-9) histone H3 with the transcriptionally inactive fraction (shown in Fig. 2D) as well as recent publications showing the interaction between HP1 proteins and histone H3 methylated at lysine 9 (14, 15) prompted us to examine the capability of HP1γ to bind histone H3 from tobacco leaves. GST pull-down assays were performed with glutathione-Sepharose containing GST alone, GST-HP1γ, GST-CSD (containing HP1 chromo shadow domain), and GST-CD (containing HP1 chromo domain). As shown (Fig. 5A, upper panel), the tobacco leaf acid-soluble fraction contained three polypeptides (ranging from 14 to 16 kDa) that reacted with anti-H3 (lane 1). GST-HP1γ (Fig. 5A, lane 2) bound two of the three histone H3 polyepitopes, an interaction that required the HP1γ chromo (GST-CD, lane 5) but not the chromo shadow domain (GST-CSD, lane 4). By using anti-dimethylated (Lys-9) histone H3 antibodies, we showed that the histone H3 polyepitopes pulled down by GST-HP1γ were methylated at lysine 9 (Fig. 5A, lower panel). The capability of GST-HP1γ to pull-down in vitro methylated (lysine 9) histone H3 was examined by using the hyperactive mutated form of the human histone methyltransferase, SUV39H1 (H320R) (13). Hence, acid-soluble fraction prepared from tobacco leaves was subjected to in vitro histone methyltransferase assay in the presence of glutathione-Sepharose containing GST alone, GST-SUV39H1 (H320R), GST-E1A, and GST-HP1γ. As shown in Fig. 5B, methylation of histone H3 occurred only with GST-SUV39H1 (H320R), and the resultant methylated form was bound by GST-HP1γ but not by GST alone (Fig. 5C).

Phosphorylation of Histone H3 at Serine 10 Does Not Interfere with Binding of HP1γ to Lys-9-Methylated Histone H3—We next investigated whether detachment of HP1γ from chromosomes during mitosis was related to phosphorylation of histone H3 at serine 10, a modification previously reported to occur strictly during mitosis both in plants and animals (24, 35, 36). Histone proteins (acid-soluble fraction) from BY-2 cells at various time points after the release from aphidicolin were resolved by SDS-PAGE and stained with Coomassie Blue (Fig. 6A) or immunoblotted using anti-phospho (S10) histone H3 antibodies (Fig. 6B). Indeed, the phosphorylated form of histone H3 was found only during mitosis (Fig. 6B, 9 and 10 h), concomitantly with dissociation of HP1γ from chromosomes (shown in Fig. 3 and Fig. 4A). This temporal correlation could have suggested that phosphorylation of histone H3 during mitosis renders GFP-HP1γ incapable of binding histone H3, hence leading to its release from chromosomes. We therefore investigated whether the detachment of GFP-HP1γ from chromosomes during mitosis resulted from its failure to interact with histone H3.
H3 Phosphorylation (S10) Does Not Disrupt Binding to HP1

The phosphorylation of histone H3 at serine 10 residue (14, 15). This methylation event, carried out by the histone methyltransferase SUV39H1 (13), is efficiently inhibited when histone H3 is first phosphorylated at serine 10 residue (14, 15). This methylation event, carried out by the histone methyltransferase SUV39H1 (13), is efficiently inhibited when histone H3 is first phosphorylated at serine 10 residue (14, 15). This methylation event, carried out by the histone methyltransferase SUV39H1 (13), is efficiently inhibited when histone H3 is first phosphorylated at serine 10 residue (14, 15). This methylation event, carried out by the histone methyltransferase SUV39H1 (13), is efficiently inhibited when histone H3 is first phosphorylated at serine 10 residue (14, 15). This methylation event, carried out by the histone methyltransferase SUV39H1 (13), is efficiently inhibited when histone H3 is first phosphorylated at serine 10 residue (14, 15). This methylation event, carried out by the histone methyltransferase SUV39H1 (13), is efficiently inhibited when histone H3 is first phosphorylated at serine 10 residue (14, 15). This methylation event, carried out by the histone methyltransferase SUV39H1 (13), is efficiently inhibited when histone H3 is first phosphorylated at serine 10 residue (14, 15). This methylation event, carried out by the histone methyltransferase SUV39H1 (13), is efficiently inhibited when histone H3 is first phosphorylated at serine 10 residue (14, 15). This methylation event, carried out by the histone methyltransferase SUV39H1 (13), is efficiently inhibited when histone H3 is first phosphorylated at serine 10 residue (14, 15). This methylation event, carried out by the histone methyltransferase SUV39H1 (13), is efficiently inhibited when histone H3 is first phosphorylated at serine 10 residue (14, 15). This methylation event, carried out by the histone methyltransferase SUV39H1 (13), is efficiently inhibited when histone H3 is first phosphorylated at serine 10 residue (14, 15).

**DISCUSSION**

Similarly to HP1 in animal cells, the GFP-HP1γ protein has the tendency to target heterochromatic regions within the plant nucleus. This localization was confirmed in several ways as follows. (i) The GFP signal was absent from euchromatic, PI-stained regions (37), such as the nucleolus, a nuclear compartment engaged in transcription of ribosomal DNA both in plants and animals (38, 39). A similar behavior has been found for HP1γ in mammalian cells as well as for the Arabidopsis HP1 (LHP1) in tobacco cells (25, 26, 40). (ii) The GFP and the 4,6-diamidino-2-phenylindole signals overlap (data not shown). (iii) Chromatin fractionation assays showed that the GFP-HP1γ was localized to the transcriptionally inactive heterochromatic fraction. In mammalian cells, HP1γ was not found in constitutive heterochromatin but was spread along chromosomes (26, 41) or localized in discrete intranuclear 4,6-diamidino-2-phenylindole-stained foci (40). This suggests that HP1γ occupies interstitial heterochromatin subdomains located within native euchromatin and may be involved in heterochromatinization of euchromic genes (12, 22).

Our results are consistent with the distribution of HP1 (Mod1) in mouse cycling cells where HP1 protein dissociates from chromosomes just before mitosis and reassociates at the end of mitosis (20). The detachment of GFP-HP1γ from chromosomes during mitosis appears to be in contrast to a previous report where it has been shown by immunolocalization that a fraction of HP1γ remained associated with chromosomes during mitosis (26). As noted by the authors, however, this observation should be re-evaluated ("The association of HP1γ with the mitotic apparatus was difficult to assess since the antibodies directed against this isoform also reacted with an unidentified Mr, 45,000 cytoplasmic component") (26). In various eukaryotic systems the localization of HP1 proteins during mitosis may differ. The Drosophila HP1 protein as well as the human HP1α were reported to be associated with mitotic chromosomes (26, 42). In certain cases, only a fraction of the HP1 protein was found in association with mitotic chromosomes, whereas the remaining was dispersed throughout the cytoplasm (26, 42, 43). These differences in HP1 localization during mitosis may reflect different methodologies or may be related to differences either in cell type or in amino acid sequence of the various HP1 proteins (43).

The detachment of GFP-HP1γ from mitotic chromosomes is temporally correlated with the phosphorylation of its interacting protein histone H3 at serine 10 residue. Indeed, in agreement with previous reports (24, 35, 36), our data showed that the phosphorylation of histone H3 at serine 10 occurs strictly during mitosis. Association of HP1 with chromatin is mediated by interaction with histone H3 methylated at the lysine 9 residue (14, 15). This methylation event, carried out by the histone methyltransferase SUV39H1 (13), is efficiently inhibited when histone H3 is first phosphorylated at serine 10 (13) and, thus, could render HP1γ incapable of binding histone H3 and, hence, of binding to chromosomes. Our results, however,
clearly demonstrate that dissociation of GFP-HP1 from mitotic chromosomes cannot be accounted for by the phosphorylation of histone H3. First, only a small fraction of histone H3 is phosphorylated at serine 10 during mitosis, and second, phosphorylated (S10) histone H3 was pulled down by GST-HP1. Our results indicate that the phosphorylated form (S10) of histone H3 is also methylated at lysine 9. This is in agreement with the capability of the Ipl1/aurora kinase to phosphorylate, although at low efficiency, histone H3 when methylated at lysine 9 residue (13). Furthermore, our finding that HP1 can bind histone H3, which is both methylated at lysine 9 and phosphorylated at serine 10, is consistent with the recently resolved structure of the HP1 chromo domain bound to Lys-9-methylated histone H3 (44, 45). Because serine 10 is found at the end of the interacting region, its phosphorylation is unlikely to introduce any steric hindrance that would prevent binding of a Lys-9-methylated histone H3 to HP1. Together, our results suggest that dissociation of GFP-HP1 from mitotic chromosomes is not directly related to histone H3 phosphorylation. It is possible that phosphorylation of histone H3 at serine 10 creates a code (4, 24) that is read by as yet an unknown factor(s) eventually leading to changes in chromatin structure and detachment of GFP-HP1 and perhaps other chromatin-associated factors from mitotic chromosomes (20, 46). Such a factor(s) may act during mitosis to bring about chromosome condensation, similarly to condensin (47).

Overexpression of GFP-HP1 in BY-2 cells did not disturb their growth kinetics because their cell cycle progression was indistinguishable from that of wild type cells (data not shown). Nor did overexpression of GFP-HP1 in tobacco plants lead to
obvious phenotypic alterations. Yet, Ingram et al. (48) showed that transgenic tobacco plants overexpressing the chromo domain derived from the Drosophila polycomb protein (PCcd) displayed developmental alterations. The lack of phenotypic perturbations in plants expressing GFP-HP1 might be explained in a number of ways. The subnuclear distribution and activity of HP1 are determined not only by the chromo domain but also by other regions of the protein, e.g., the chromo shadow domain (19). Also, other factors that interact with HP1 were in limiting amounts, thus restraining the effect of HP1 on chromatin structure and gene expression. It is possible, however, that changes may have occurred at the chromatin level without any obvious phenotypic perturbations as shown for cmt3 mutants of Arabidopsis. Likewise, loss-of-function of chromomethylase 3, a cytosine methyltransferase homolog, caused a genome wide decrease in CpXpG methylation and reactivation of endogenous retrotransposons with no changes in wild type morphology (49).

The similar localization of HP1γ in animal and plant cells lends biological significance for the results obtained by overexpressing human HPV1 in plant cells. In addition, the identification of HP1-like proteins in plants (Ref. 25, Plant Chromatin Data Base, www.chromdb.org) and the demonstration that the tomato HP1-like (LeHP1-L) protein can bind methylated (Lys-9) histone H3 (data not shown) through its chromo domain suggest evolutionary conservatism of the molecular machinery involved in heterochromatin formation. Based on the results presented here, the association of HP1γ with chromatin in plant cells can be explained by its direct interaction with methylated (Lys-9) histone H3. This is supported by the localization of HP1γ and Lys-9-methylated histone H3 to the transcriptionally inactive heterochromatin fraction (Fig. 2D). Alternatively, HP1γ may be recruited to chromatin in plant cells through interaction with other chromatin-associated proteins such as pRB (21, 22) or the large subunit of the chromatin assembly factor 1 (CAF-1) (24).

It appears that the molecular machinery involved in chromatin condensation during mitosis is distinct from the one regulating the formation of condensed heterochromatin subdomains in interphase nuclei. This also points to heterochromatin being a heterogeneous rather than a homogenous compartment, which can be specified not only by its level of compaction (33, 50) but also by the molecular machinery involved in its formation.

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