Tethering of HP1 proteins to chromatin is relieved by phosphorylation of histone H3

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Histone H3 lysine 9 methylation is associated with long-term transcriptional repression through recruitment of heterochromatin protein 1 (HP1) proteins. These proteins are believed to promote the formation of dense chromatin structures interfering with DNA accessibility. During the G2 phase of the cell cycle, HP1 proteins are delocalized from foci of pericentromeric heterochromatin, while a wave of H3 serine 10 phosphorylation is initiated within these regions. Here, we show that in vivo phosphorylation of serine 10 in G2 can occur on histone tails methylated on lysine 9. Unexpectedly, this modification favours rather than prevents HP1 binding to chromatin. Dissociation of HP1 from the methylated histone H3 tails is observed only after a third modification by acetylation of lysine 14, which occurs in prophase. We propose that phosphorylation of histone H3 could be a general mechanism allowing the cell to overcome HP1-mediated transcriptional repression.

Keywords: kinetic; microinjection; mitosis; surface plasmon resonance

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INTRODUCTION

Heterochromatin protein 1 (HP1) proteins preferentially localize within condensed inactive heterochromatin. However, several recent observations suggest that this protein can also function as a co-repressor of genes located within active, transcribed chromatin (for a review, see Li et al., 2002). Recruitment of HP1 to chromatin is mediated by methylation of the histone H3 tail on the lysine at position 9 (H3 metK9). Methylation is a very stable modification and, to date, no histone demethylase has been identified. Yet, the cyclin E gene, for example, is targeted by HP1 upon Rb-induced repression (Nielsen et al., 2001) and nevertheless is re-activated later in the cell cycle. This observation clearly indicates that repression by HP1 can be reversed. Global delocalization of HP1 from chromatin has been observed at the G2/M transition, resulting in disappearance of pericentromeric accumulation of the protein and its increased extractability (Muzzina et al., 1999). This HP1 delocalization coincides with the wave of mitotic histone H3 serine 10 phosphorylation (H3 pS10) that initiates within the pericentromeric heterochromatin in late G2 and then spreads to the rest of the chromatin as M phase approaches. This observation suggested that H3 S10 phosphorylation may displace HP1 from methylated H3 tails (Fischle et al., 2003a). However, another report claimed that in vitro methylation of H3 K9 prevents phosphorylation of H3 S10 (Rea et al., 2000), suggesting that the effect of H3 pS10 on HP1 binding was more likely to be indirect. Here, we provide evidence that in vivo H3 metK9 and pS10 modifications can in fact occur concomitantly on the same histone tail. Surprisingly, this double modification does not prevent HP1 binding, and a third modification, namely acetylation of H3 lysine 14 (H3 acK14), is required for delocalization of the protein.

RESULTS

HP1 delocalization and H3 phosphorylation in G2

To dissect the mechanism leading to HP1 delocalization from the pericentromeric heterochromatin at the G2/M transition, we co-stained fixed NIH3T3 cells with anti-HP1x and anti-H3 pS10 antibodies. Using the pattern of anti-H3 pS10 staining as a criterion, we identified cells fixed at different steps in late G2 and early M (Fig 1A–L). As previously described (Muzzina et al., 1999), HP1 was delocalized from pericentromeric foci as cells entered M phase (Fig 1, compare A and C). However, careful observation revealed that early phases of H3 S10 phosphorylation were correlated with more concentrated rather than diffuse distribution of HP1 (Fig 1B,F). We also tested binding of a glutathione S-transferase (GST)–HP1x fusion protein in overlay assays. Fixed
cells were incubated in the presence of recombinant GST–HP1α, which in turn was detected using anti-GST antibodies (Muchardt et al., 2002). Similar to the endogenous HP1α, GST–HP1α associated well with the chromatin in late G2 after initiation of H3 S10 phosphorylation (Fig 1M,N,P,Q,S,T). Decreased binding of GST–HP1α (compared with interphasic cells) was observed only at later stages when the cells had entered prophase (Fig 1O,R,U). These observations suggested that delocalization of HP1 at the G2/M transition was likely to follow a more complex mechanism than simple inhibition of HP1 binding by phosphorylation of the H3 tails. As GST–HP1α was produced in *Escherichia coli*, they also showed that decreased mitotic chromatin binding of HP1 did not require any post-translational modification of the protein itself.

H3 S10 phosphorylation has also been correlated with increased HP1 extractability (Murzina et al., 1999). To re-investigate this issue, we used cells that were treated with the non-ionic detergent Triton X-100 before fixation. Labelling with anti-HP1α and anti-H3 pS10 antibodies surprisingly showed that in early stages of the G2/M transition, HP1α was less, rather than more, extractable than in interphasic cells (Fig 1a,d,g; compare HP1α signals in the interphasic (arrow) and pS10-positive cells). High extractability was observed in cells selected at later stages, and extractability appeared to increase gradually as M phase progressed (Fig 1a–i; see also Fig 3 for more intermediate steps). We also noted that in prophase the diffusely distributed HP1α is not fully extractable (Fig 1b,e,h), suggesting that HP1α nuclear redistribution and its release from chromatin are two uncoupled events.

Taken together, these experiments show that mitotic phosphorylation of H3 S10 cannot be simply correlated either with diffused HP1α distribution or increased extractability.

### Concomitant methylation and phosphorylation

To determine whether mitotic phosphorylation of histone H3 on S10 can occur on histone tails already methylated on K9, we generated antibodies directed against the double H3 metK9–pS10 modification. Competition experiments using synthetic peptides mimicking modified H3 histone tails showed that these antibodies specifically recognized the double modification and not unmodified or singly modified histone tails carrying either the metK9 or the pS10 modifications (Fig 2A–E,G–K). We noted some cross-reactivity with a peptide carrying a metK27–pS28 modification (Fig 2F,L). Competition with this peptide was, however, significantly less efficient than with the metK9–pS10 peptide (Fig 2, compare E and F). Double staining with anti-H3 metK9–pS10 and anti-H3 pS10 showed simultaneous appearance and disappearance of the two signals during G2 and M phases (Fig 2a–o). We also double-stained cells with anti-H3 metK9–pS10 and anti-H3 pS28 antibodies. Whereas anti-H3 metK9–pS10 signal was detected in late G2, anti-pS28 signal was observed only as starting in prophase (supplementary Fig 1 online). This is consistent with earlier observations showing that phosphorylation occurs later on H3 S28 than on H3 S10 (Goto et al., 1999). From these experiments, we concluded that although the anti-H3 metK9–pS10 antibodies potentially crossreacted with H3 metK27–pS28, histone H3 tails carrying the double metK9–pS10 modification were detected with certainty in late G2. Besides, the phosphorylation of methylated H3 tails appeared to follow a
pattern similar to that of nonmethylated tails. It must be noted here that the anti-H3 pS10 antibodies used did not recognize the double modification (supplementary Fig 2 online).

**Acetylation and HP1 extractability in mitosis**

The histone deacetylase inhibitor trichostatin A can induce HP1 delocalization from pericentromeric heterochromatin (Taddei et al., 2001; Cimini et al., 2003) and reduces HP1-mediated transcriptional repression (Nielsen et al., 1999). We therefore examined H3 acetylation during mitosis with a focus on K14, which is located in close proximity to the methylated K9. Cells were extracted with Triton X-100 before fixation and then stained with anti-HP1α and anti-H3 AcK14 antibodies. Cells fixed at different steps in late G2 and M were identified based on HP1α extractability and degree of chromatin condensation observed by 4,6-diamidino-2-phenylindole (DAPI) staining of the DNA. AcK14 staining was detected in all cells including interphasic cells (Fig 3, arrows). It was unchanged during the early phases of the G2/M transition, then increased in M phase (Fig 3F–H). Interestingly, this increase was well correlated with augmented HP1α extractability (Fig 3B–D). As a confirmation, we also detected increased mitotic H3 K14 acetylation by western blot on extracts from HeLa cells blocked in prophase with nocodazole (Fig 3M, lanes 3 and 4).

**Acetylation inhibits HP1 binding**

To characterize the effect of H3 S10 phosphorylation and K14 acetylation on HP1 binding to H3 metK9, we performed overlay assays in the presence of excess of various H3 peptides. In addition to H3 metK9, HP1 binding to chromatin was competed for by H3 metK9–pS10 and H3 metK9–acK14 peptides, indicating that neither S10 phosphorylation nor K14 acetylation could prevent HP1 from associating with an H3 peptide also methylated in vivo.
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**DISCUSSION**

In the present paper, we have followed HP1 localization and chromatin binding in late G2 and M phases, and we provide several observations suggesting that the mitotic redistribution of this protein is a multistep mechanism. First, we realized that extractability of HP1 is decreased after the onset of S10 phosphorylation in late G2 or early M. This can be paralleled with the slower dissociation of HP1 from an H3 metK9–pS10 peptide (compared to an H3 ‘metK9-only’ peptide) observed in both microinjection and SPR experiments. Taken together, these observations indicate that association of HP1 with phospho-methylated H3 tails is more stable than association with only methylated tails. Interestingly, the distribution of H3 methylated on K9 does not coincide with the distribution of HP1 on H3methylK9 complex (Koff = 0.1 s⁻¹), showing that the phosphorylation of S10 increases the stability and the half-life of the association between H3 and HP1. Once again, we observed no or little binding of the unmodified (data not shown), the H3 pS10 and H3 metK9–pS10–acK14 peptides to the immobilized 6 His–HP1α fusion protein.

**Effects of phosphorylation on the H3–HP1 complex**

To evaluate the affinity of HP1 for the H3 metK9 and H3 metK9–pS10 peptides, we performed surface plasmon resonance (SPR) assays with recombinant 6 His–HP1α bound to the sensor-chip surface (Fig 5). The affinity constant (Kd) of the interaction with the H3 metK9 peptide was 4.5 ± 0.8 μM. This value is compatible with earlier measurements performed using other techniques (Nielsen et al, 2002; Fischle et al, 2003b). The affinity of 6 His–HP1α for H3 metK9–pS10 peptide was in the same range as that for H3 metK9 (Kd = 16.2 ± 4.2 μM). However, the kinetic parameters of both interactions were significantly different. Indeed, the dissociation rate of the HP1α/H3metK9–pS10 complex (Kdoff = (4.6 ± 0.6) × 10⁻³ s⁻¹) was more than 20-fold lower than that of the HP1α/H3metK9 complex (Kdoff > 0.1 s⁻¹), showing that the phosphorylation of S10 increases the stability and the half-life of the association between H3 and HP1. Once again, we observed no or little binding of the unmodified (data not shown), the H3 pS10 and H3 metK9–pS10–acK14 peptides to the immobilized 6 His–HP1α fusion protein.

**Effects of acetylation on the H3–HP1 complex**

To evaluate the effect of acetylation on HP1, we performed assays using recombinant 6 His–HP1α bound to the sensor-chip surface (Fig 5). The affinity constant (Kd) of the interaction with the H3 acK14 peptide was 4.5 ± 0.8 μM. This value is compatible with earlier measurements performed using other techniques (Nielsen et al, 2002; Fischle et al, 2003b). The affinity of 6 His–HP1α for H3 acK14 peptide was in the same range as that for H3 acK14 (Kd = 6.2 ± 4.2 μM). However, the dissociation rate of the HP1α/H3metK9–pS10 complex (Kdoff = (4.6 ± 0.6) × 10⁻³ s⁻¹) was more than 20-fold lower than that of the HP1α/H3metK9 complex (Kdoff > 0.1 s⁻¹), showing that the phosphorylation of S10 increases the stability and the half-life of the association between H3 and HP1. Once again, we observed no or little binding of the unmodified (data not shown), the H3 pS10 and H3 metK9–pS10–acK14 peptides to the immobilized 6 His–HP1α fusion protein.

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S10 phosphorylation and K14 acetylation is responsible for the delocalization of HP1 from chromatin in mitosis. Acetylation of K14 alone did not affect binding of HP1 to H3 metK9, indicating that some histone H3 tails bound by HP1 may be acetylated before phosphorylation. However, the chronology that we observe suggests that K14 acetylation occurs after S10 phosphorylation.

As mentioned above, S10 phosphorylation, in the absence of K14 acetylation, surprisingly stabilizes the HP1–H3 complex. This
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Fig 5 16×His–HP1b binds in vitro to the metK9 and metK9–pS10 peptides, but not to the triple-modified metK9–pS10–acK14 peptide. A typical SPR measurement is shown for the different peptides at 100 μM. Note the slower dissociation phase observed for metK9–pS10 compared with metK9 peptide.

observation is compatible with an earlier study showing that phosphorylation of H3 S10 does not prevent binding of plant HP1γ in GST pull-down assays (Fass et al., 2002). In the structure derived from co-crystals of the HP1 chromodomain and a methylated histone H3 tail (Jacobs & Khorasanizadeh, 2002; Nielsen et al., 2002), S10 appears to point outwards from the HP1–H3 interface. This positioning of S10 would sterically allow the accommodation of an additional phosphate group. The crystal structure also shows that HP1 undergoes a conformational change when binding to methylated H3 tails. As suggested by the decreased off-rate of HP1 bound to H3 metK9–pS10 peptide in the SPR experiments, further conformational changes may occur upon binding to phosphomethylated H3 tails. Possibly, the presence of an acetyl group on H3 K14 may prevent HP1 from performing a proper conformational shift upon contact with histone H3. The mitotic H3 S10 kinase Ipl1/Aurora, when assayed in vitro, poorly phosphorylates an H3 peptide methylated on K9 (Rea et al., 2000). However, using the anti-H3 metK9–pS10 antibodies that we developed, we find that S10 phosphorylation of nonmethylated and K9-methylated histone tails initiates simultaneously during the late G2 phase of the cell cycle. This observation shows that in vivo methylation of K9 is not an obstacle for the phosphorylation of the neighbouring residue. It is possible that, in vivo, S10 is displayed in a conformation that is more favourable for phosphorylation by Ipl1/Aurora. Alternatively, other kinases, such as the NIMA-related kinases, may phosphorylate methylated histone H3 tails in vivo (Roig et al., 2002).

The data presented here have been collected from cells at the G2/M transition. Nevertheless, the mechanism of HP1 delocalization that we have uncovered may apply in other phases of the cell cycle. In particular, the transition from growth arrest (G0) to G1 is a time of extensive re-expression of transiently silenced genes. This transition, when induced by mitogenic stimulation, is followed by a wave of H3 S10 phosphorylation and K14 acetylation (Cheung et al., 2000; Clayton & Mahadevan, 2003). At the same time, we also observe a wave of phosphomethylation using the anti-H3 metK9–pS10 antibodies (data not shown). It is therefore tempting to speculate that in addition to recruitment of chromatin remodelling and transcription factors, these modifications serve as ways to overcome HP1-mediated repression. Redistribution of HP1 has recently been observed upon dedifferentiation in plant cells (Williams et al., 2003). Further studies will be required to determine whether re-entry into the cell cycle also affects HP1 targeting in mammalian cells.

METHODS

Antibodies and peptides. Anti-H3 metK9–pS10 antibodies were produced in rabbits using a peptide coupled to KLH with the following sequence: AR(di-metK)TGGKAPRKLQ. These antibodies were purified by protein A-affinity columns and labelled with Fluorescein-EX (from Molecular Probes). Rabbit anti-H3 pS10 and anti-H3 acK14 antibodies were purchased from Upstate. Anti-H3 acK14 can recognize dimetK9–pS10–acK14 peptide. Monoclonal mouse anti-HP1b clone 1H5 was purchased from Euromedex. Rat anti-H3 pS28 (H9908) was purchased from Sigma. DNA was labelled with DAPI at 150 ng/ml. Competition experiments were performed with the following peptides either unmodified or with dimetK9, pS10, dimetK9–pS10, dimetK9–acK14 or dimetK9–pS10–acK14 modifications: ARTKQA

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TARKSTGGKAPRCL.

The sequence of the dimetK27–pS28 peptide was as follows: QLATKARSKAPATGGVC. Peptides were carefully purified by amino-acid analysis and the presence of the modifications was confirmed by mass spectrometry.

Immunocytochemistry and microinjection. Immunofluorescent labelling, Triton extractions and overlay assays were performed using NIH3T3 cells as previously described (Muchardt et al., 2002). For microinjection, a GFP–HP1b expression construct (gift from C. Gazin) was stably integrated into NIH3T3 cells. Nuclear microinjection was performed using a Transjector 3246 (Eppendorf). All peptides were resuspended at 90 μM in water supplemented with Texas red dextran (Molecular Probes). Imaging was performed on an Axiovert 200M microscope (Zeiss) coupled with an RS2000 Nipkow-disk confocal system (Perkin Elmer).

In vitro binding studies by SPR. The assays were performed on a Biacore 2000 instrument equilibrated at 25 °C with PBS + 0.005% Tween 20 at a flow rate of 20 μl/min. The Penta-His monoclonal antibody (Qiagen) was covalently immobilized, using the Amine Coupling Kit (Biacore AB), on the carboxymethylated surface of flow cells 1 and 2 of a CMS sensor chip. 6 × His–HP1b was captured on flow cell 1, and ten different concentrations of the
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