Tethering of HP1 proteins to chromatin is relieved by phosphoacetylation 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 phosphoacetylation of histone H3 could be a general mechanism allowing the cell to overcome HP1-mediated transcriptional repression.

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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 (Murzina *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 prevented 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-HP1 α 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 (Murzina *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)–HP1 α fusion protein in overlay assays. Fixed

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Fig 1 | Mitotic phosphorylation of histone H3 is associated first with concentration, then with delocalization of HP1 from pericentromeric heterochromatin. (A–L) Fixed NIH3T3 cells were labelled with the indicated antibodies and DAPI. (M–U) NIH3T3 cells were extracted with Triton X-100, fixed, incubated with recombinant GST–HP1 α and then labelled with anti-GST (M–O), anti-H3 pS10 (P–R) and DAPI (S–U). (a–i) NIH3T3 cells were extracted with Triton X-100, fixed and labelled with the indicated antibodies and DAPI. Scale bars, 10 μ m. The presented nuclei were ordered according to the levels of H3 S10 phosphorylation and chromatin compaction. The arrows indicate interphasic cells.

cells were incubated in the presence of recombinant GST–HP1 α , which in turn was detected using anti-GST antibodies (Muchardt *et al*, 2002). Similar to the endogenous HP1 α , GST–HP1 α 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–HP1 α (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–HP1 α 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-HP1 α and anti-H3 pS10 antibodies surprisingly showed that in early stages of the G2/M transition, HP1 α was less, rather than more, extractable than in interphasic cells (Fig 1a,d,g; compare HP1 α 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 HP1 α is not fully extractable (Fig 1b,e,h), suggesting that HP1 α 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 HP1 α 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

Fig 2 | H3 histone tails can be phosphorylated on S10 and methylated on K9 *in vivo*. (A–L) Fixed NIH3T3 cells were labelled with antibodies raised against an H3 peptide carrying a dimethylation on K9 and a phosphorylation on S10. To test specificity, the antibodies were challenged with an excess of the indicated peptides. Panels show mitotic cells strongly labelled by the antibodies. (a–o) Fixed NIH3T3 cells were labelled with the indicated antibodies and DAPI. The presented nuclei were ordered according to the level of H3 S10 phosphorylation and chromatin compaction. The arrows point to an interphasic cell. Scale bars, 10 µm.

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-HP1 α and anti-H3 AcK14 antibodies. Cells fixed at different steps in late G2 and M were identified based on HP1 α 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 HP1 α 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

Fig 3 | HP1 extractability in M phase is correlated with increased H3 K14 acetylation. (A–L) NIH3T3 cells were extracted with Triton X-100, fixed and labelled with the indicated antibodies and DAPI. The presented nuclei were ordered according to the level of HP1 extractability and chromatin compaction. The arrows indicate a cell in interphase. Scale bar, 10 μ m. (M) Total extracts from HeLa cells either asynchronous (lanes 1 and 3) or arrested in M phase with nocodazole (lanes 2 and 4) were resolved by 18% SDS–polyacrylamide gel electrophoresis, and levels of H3 K14 acetylation were estimated by western blot.

on K9 (Fig 4C,E,F,J,L,M). As expected, no competition was observed with either unmodified or H3 pS10 peptide (Fig 4B,D,I,K). These observations suggested that a triple modification of the H3 histone tail might be necessary for inhibition of HP1 binding. We therefore tested an H3 peptide carrying a metK9–pS10–acK14 modification. This peptide failed to compete for HP1 binding (Fig 4G,N), suggesting that it is the combined effect of phosphorylation and acetylation of the histone H3 tails that interferes with HP1 binding. Overlays on peptides spotted on nitrocellulose membrane confirmed that HP1 could bind metK9, metK9–pS10 and metK9–acK14, but not metK9–pS10–acK14 peptide (Fig 4O). Interestingly, in this assay, we noted some preference of HP1 for the metK9–pS10 peptide (Fig 4O, compare lane 4 with lanes 2 and 5). To examine HP1 peptide binding *in vivo*, we microinjected the various peptides into the nucleus of an NIH3T3-derived cell line expressing a green fluorescent protein (GFP)–HP1 β fusion protein. Injection of H3 metK9 peptide led to rapid delocalization of GFP–HP1 β (Fig 4a,b). Most probably due to rapid diffusion of the injected peptide out of the nucleus, this delocalization was only transient and relocalization of GFP–HP1 β to pericentromeric heterochromatin was observed within 135 s, with images captured every 15 s (Fig 4c,d). A similar delocalization was observed after injection

of the H3 metK9–pS10 peptide, confirming that this peptide can compete for chromatin binding of HP1 *in vivo* (Fig 4e,f). Interestingly, relocalization of HP1 was significantly slower than with H3 metK9 peptide, suggesting that HP1 associates more stably with the H3 metK9–pS10 than the H3 metK9 peptide (Fig 4g,h). No delocalization of GFP–HP1 β was observed when using either unmodified (data not shown) or metK9–pS10–acK14 peptide (Fig 4i–l), confirming that the triply modified peptide was unable to bind HP1.

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 $\times$ His–HP1 α bound to the sensor-chip surface (Fig 5). The equilibrium constant (K_d) of the interaction with the H3 metK9 peptide was $4.5 \pm 0.8 \mu$ M. This value is compatible with earlier measurements performed using other techniques (Nielsen *et al*, 2002; Fischle *et al*, 2003b). The affinity of 6 $\times$ His–HP1 α for H3 metK9–pS10 peptide was in the same range as that for H3 metK9 ($K_d = 16.2 \pm 4.2 \mu$ M). However, the kinetic parameters of both interactions were significantly different. Indeed, the dissociation rate of the HP1 α /H3metK9–pS10 complex ($k_{off} = (4.6 \pm 0.6) \times 10^{-3} s^{-1}$) was more than 20-fold lower than that of the HP1 α /H3metK9 complex ($k_{off} > 0.1 s^{-1}$), 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 $\times$ His–HP1 α fusion protein.

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 phosphorylated 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 (Muchardt *et al*, 2002), suggesting that a large fraction of the histone H3 tails methylated on K9 are potential binding sites not associated with an HP1 protein. HP1 proteins are known to be highly mobile and are able to recolonize quickly areas subject to laser-induced photobleaching (Cheutin *et al*, 2003). We therefore suggest that an early effect of mitotic S10 phosphorylation is to ‘trap’ HP1 on methylated histone H3 tails outside the heterochromatin foci, leading to diffuse HP1 localization. This model is compatible with the poor extractability of the diffused HP1 in late G2 (see, for example, Fig 3B).

Acetylation of H3 K14 gradually increases as M phase progresses and this increase is correlated with increased HP1 extractability. *In vitro*, we also find that an H3 peptide carrying a triple metK9–pS10–AcK14 modification no longer binds HP1. These observations strongly suggest that the combined effect of

Fig 4 | HP1 α is displaced from its chromatin targets by metK9, metK9-pS10 and metK9-acK14, but not by metK9-pS10-acK14 peptide. (A–N) Fixed NIH3T3 cells were incubated with recombinant GST-HP1 α in the presence of the indicated peptides. Cells were then labelled with anti-GST (A–G) and DAPI (H–N). (O) 1 μ g of the indicated peptides was spotted on a nitrocellulose membrane and stained with Ponceau (bottom panel). The membrane was then blocked with bovine serum albumin and incubated first with GST-HP1 α and then with anti-GST antibodies (top panel). (a–l) Indicated peptides were microinjected into the nuclei of an NIH3T3-derived cell line expressing GFP-HP1 β . Injected cells were then photographed every 15 s. Scale bars, 10 μ m.

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

Fig 5 | 6×His-HP1α 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)(pS)TGGKAPRKQLC. 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-HP1α 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: ARTKQ TARKSTGGKAPRC. The sequence of the dimetK27-pS28 peptide was as follows: QLATKAARKSAPATGGVC. Peptides were carefully quantified 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-HP1β 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 CM5 sensor chip. 6 × His-HP1α was captured on flow cell 1, and ten different concentrations of the

peptides (0.2–150 μ M) were then injected simultaneously on flow channels 1 and 2 for 2 min. The dissociation of the complexes was followed for 100 s. Flow cell 2 served as an anti-6 $\times$ His reference surface devoid of 6 $\times$ His-HP1 α . The residual signals obtained for the unmodified peptide were used as negative controls, and subtracted from the signals obtained for the modified peptides. Between binding cycles, the coated surfaces were regenerated by two injections of 10 mM HCl. The association and dissociation profiles were analysed with a nonlinear least-squares algorithm implemented in the Biaevaluation 3.0 software (Biacore AB).

Supplementary information is available at *EMBO reports* online (<http://www.emboports.org>).

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