

Interaction with members of the heterochromatin protein 1 (HP1) family and histone deacetylation are differentially involved in transcriptional silencing by members of the TIF1 family

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Mammalian TIF1 α and TIF1 β (KAP-1/KRIP-1) are related transcriptional intermediary factors that possess intrinsic silencing activity. TIF1 α is believed to be a euchromatic target for liganded nuclear receptors, while TIF1 β may serve as a co-repressor for the large family of KRAB domain-containing zinc finger proteins. Here, we report an association of TIF1 β with both heterochromatin and euchromatin in interphase nuclei. Co-immunoprecipitation of nuclear extracts shows that endogenous TIF1 β , but not TIF1 α , is associated with members of the heterochromatin protein 1 (HP1) family. However, *in vitro*, both TIF1 α and TIF1 β interact with and phosphorylate the HP1 proteins. This interaction involves a conserved amino acid motif, which is critical for the silencing activity of TIF1 β but not TIF1 α . We further show that trichostatin A, an inhibitor of histone deacetylases, can interfere with both TIF1 and HP1 silencing. The silencing activity of TIF1 α appears to result chiefly from histone deacetylation, whereas that of TIF1 β may be mediated via both HP1 binding and histone deacetylation.

Keywords: chromatin remodelling/KRAB domain/
phosphorylation/protein kinase/transcriptional repression

Introduction

Transcriptional regulation of gene expression in eukaryotes in response to developmental or environmental signals is a complex multi-step process that requires the concerted action of many cellular factors. Central players in this elaborate process are sequence-specific transcription factors that positively and/or negatively control transcription through interactions with transcriptional intermediary factors (TIFs; also designated as

co-activators and co-repressors), whose function is to remodel chromatin structure (reviewed in Kornberg and Lorch, 1999; Wolffe and Hayes, 1999), to stimulate or inhibit (pre)initiation complex formation (reviewed in Orphanides *et al.*, 1996; Hampsey and Reinberg, 1999 and references therein) or to associate target genes with specialized nuclear compartments that confer transcriptional activation or repression (Brown *et al.*, 1997, 1999; Cocktell and Gasser, 1999).

Mammalian TIF1 α and TIF1 β are members of a family of TIFs that are believed to function at the level of the chromatin template (Le Douarin *et al.*, 1996). These proteins display a characteristic domain structure comprising an N-terminal RBCC (RING finger, B boxes, coiled coil) motif, a poorly conserved central region, and a C-terminal region that contains a PHD finger and a bromodomain (see Figure 5A). Whereas RING fingers are present in many proteins with diverse functions (Freemont, 1993; Saurin *et al.*, 1996), PHD fingers and bromodomains are restricted to nuclear proteins, including transcriptional cofactors and chromatin-remodelling proteins (Aasland *et al.*, 1995; Jeanmougin *et al.*, 1997 and references therein). Initially identified in a yeast genetic screen for proteins increasing the transactivation potential of retinoid X receptors (RXRs) (Le Douarin *et al.*, 1995a), TIF1 α was subsequently found: (i) to interact *in vitro* with members of the nuclear receptor superfamily in the presence of their cognate agonistic ligand (Le Douarin *et al.*, 1995a; vom Baur *et al.*, 1996); (ii) to be a phosphoprotein that undergoes a ligand-dependent hyperphosphorylation as a consequence of nuclear receptor binding *in vivo* (Fraser *et al.*, 1998); (iii) to possess an intrinsic protein kinase activity (Fraser *et al.*, 1998); (iv) to be tightly and preferentially associated with highly accessible euchromatic regions (Remboutsika *et al.*, 1999); and (v) to be a particularly abundant protein in toti- and pluripotent cells, suggesting that it may represent a marker for numerous euchromatic sites in the genome of these cells (Remboutsika *et al.*, 1999). Supporting the notion that TIF1 α could play a dual role in transcription, being involved in both activation and repression, TIF1 α was also reported to silence transcription when tethered to DNA through fusion to a heterologous DNA binding domain (DBD) and to interact *in vitro* with members of the heterochromatin protein 1 (HP1) family (Le Douarin *et al.*, 1996), a subfamily of the chromatin organization modifier (chromo) superfamily (Koonin *et al.*, 1995), which is thought to be important for regulating heterochromatin-mediated silencing and chromosome structure. The prototype of this family is *Drosophila* HP1, which is preferentially localized in pericentric heterochromatin (James and Elgin, 1986) and involved in position effect variegation (PEV) (Eissenberg *et al.*, 1990), a well-known epigenetic silencing activity exhibited by

euchromatic genes placed within or near heterochromatin (Wakimoto, 1998). In mammals, three HP1 homologues have been described: HP1 α , HP1 β (MOD1/M31) and HP1 γ (MOD2/M32) (Singh *et al.*, 1991; Saunders *et al.*, 1993). We and others found that HP1 α and HP1 γ can significantly repress transcription when fused to a heterologous DBD in transiently transfected cells (Le Douarin *et al.*, 1996; Lehming *et al.*, 1998; Seeler *et al.*, 1998).

Using HP1 α as bait in a two-hybrid screen (Le Douarin *et al.*, 1996), we isolated TIF1 β , also referred to as KAP-1 (KRAB-associated protein 1) or KRIP-1 (KRAB-interacting protein 1), by virtue of its ability to interact with the transcriptional silencing KRAB domain of human zinc finger proteins, including KOX1 and Kid-1 (Friedman *et al.*, 1996; Kim *et al.*, 1996; Moosmann *et al.*, 1996). The so-called KRAB domain, which is present in about one-third of the 300–700 human zinc finger proteins of the Krüppel Cys2His2-type (Bellefroid *et al.*, 1991), is one of the most widely distributed transcriptional silencing domains identified yet in vertebrates (Margolin *et al.*, 1994; Witzgall *et al.*, 1994; Vissing *et al.*, 1995). When fused to a heterologous DBD, it silences both basal and activated transcription in a dose-dependent manner and over large distances (Pengue *et al.*, 1994; Deuschle *et al.*, 1995; Moosmann *et al.*, 1997). TIF1 β interacts with numerous KRAB domains both in yeast and *in vitro*, but not with KRAB mutants that do not repress transcription *in vivo*. An overexpression of TIF1 β can enhance KRAB-mediated repression, and TIF1 β itself acts as a repressor when tethered to DNA (Friedman *et al.*, 1996; Kim *et al.*, 1996; Moosmann *et al.*, 1996). Thus, TIF1 β exhibits properties expected for a transcriptional co-repressor of the large family of KRAB domain-containing zinc finger proteins (KRAB ZFPs).

The molecular mechanisms by which TIF1 α and TIF1 β may exert their repressing effects on transcription have not yet been elucidated. However, both proteins share structural and functional features that strongly favour chromatin-mediated mechanisms. Supporting this view, our present results provide evidence for a direct role of the HP1 proteins in TIF1 β silencing and implicate histone deacetylation in both TIF1 α - and TIF1 β -mediated silencing.

Results

Immunolocalization of TIF1 β and HP1 proteins

The intracellular localization of TIF1 β , HP1 α , HP1 β and HP1 γ was investigated in mouse NIH 3T3 cells using indirect immunofluorescence labelling and confocal microscopy. DNA-containing structures were visualized by Hoechst 33258 staining and endogenous proteins were detected using specific monoclonal antibodies (mAbs) and a Cy3-fluorochrome-conjugated secondary antibody (Figure 1). As previously reported (Wreggett *et al.*, 1994; Remboutsika *et al.*, 1999), HP1 α was detected predominantly in heterochromatic regions, revealed by Hoechst staining as spots of brighter fluorescence (Figure 1C and D). These large blocks of condensed heterochromatin were also labelled by HP1 β and HP1 γ antibodies; however, HP1 β and even more so HP1 γ were detected in many additional sites dispersed within the nucleus (Figure 1E–H). Note that the present distribution of HP1 γ is in contrast

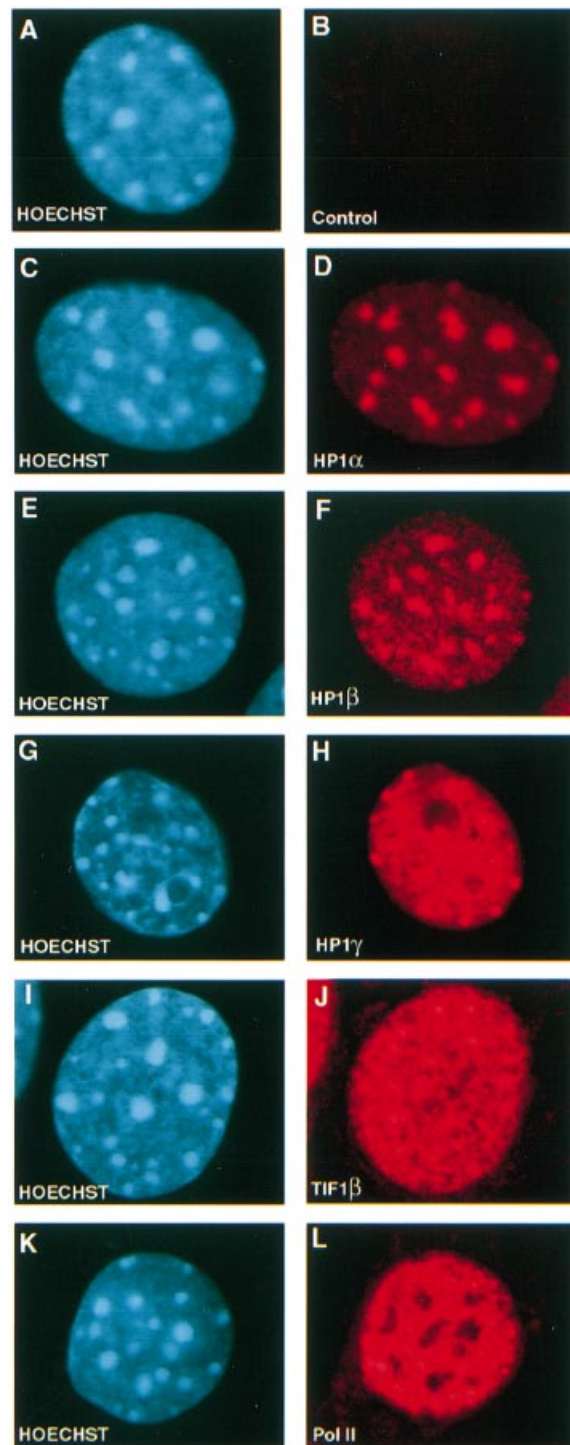


Fig. 1. Distribution of endogenous TIF1 β and HP1 proteins within the nuclei of NIH 3T3 cells. Confocal images of single optical sections through the nucleus of representative individual cells are shown. Left panels show the Hoechst DNA staining, and right panels correspond to immunodetection with specific mAbs, as indicated. A mouse IgG fraction was used as a negative control in (B).

to that observed in mouse C1271 interphase cells, in which HP1 γ appears to be largely excluded from heterochromatin (Horsley *et al.*, 1996).

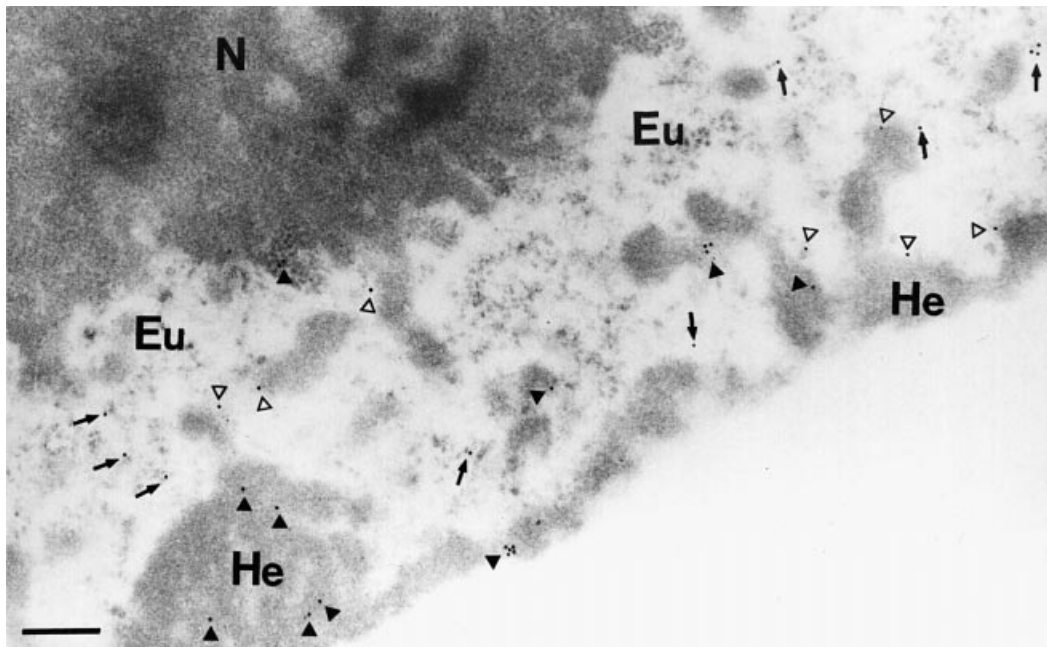


Fig. 2. Ultrastructural analysis of TIF1 β localization in P19 EC nuclei by indirect immunogold electron microscopy using a mouse anti-TIF1 β mAb and a 10 nM gold-conjugated secondary antibody. Eu, euchromatin; He, heterochromatin; N, nucleolus. Gold particles are seen as black dots. TIF1 β was localized in Eu (arrows), He (closed arrowheads), and at the borders between Eu and He (open arrowheads). No significant labelling was obtained when a polyclonal anti-mouse IgG fraction was used (data not shown). Scale bar, 0.25 μ m.

TIF1 β exhibited a diffuse as well as a punctate or speckled distribution throughout the nucleoplasm (Figure 1J). In contrast to the largest RPB1 subunit of RNA polymerase II (Pol II; Figure 1K and L), TIF1 β was not excluded from the regions of condensed heterochromatin, although the staining with the anti-TIF1 β antibody was less intense in the large blocks of heterochromatin than in the surrounding euchromatin (Figure 1I and J). TIF1 β was similarly distributed in nuclei of P19 and F9 EC cells (data not shown). In an attempt to localize TIF1 β further, immunogold electron microscopy was performed on P19 cell nuclei (Figure 2). TIF1 β was found in low-contrast areas of euchromatin (arrows), at the borders between euchromatin and heterochromatin (open arrowheads), and in electron-dense or heterochromatic regions located at the nuclear periphery, around nucleoli or dispersed as clumps in the nucleoplasm (Figure 2, closed arrowheads; and data not shown). In contrast, almost no signal was observed in the nucleoli or in the interchromatin granule clusters (Figure 2; and data not shown). These results, which are in keeping with the immunocytofluorescence data, indicate that TIF1 β is present in both euchromatin and heterochromatin.

Association of TIF1 α and TIF1 β with HP1 α , - β and - γ in mammalian cells

The above results and the previous observation that TIF1 β can interact with HP1 α , - β and - γ in the yeast two-hybrid system (Le Douarin *et al.*, 1996; Venturini *et al.*, 1999) prompted us to investigate whether these proteins could be physically associated in mammalian cells. When TIF1 β was immunoprecipitated from nuclear extracts of P19 EC cells, all three HP1s, HP1 α , - β and - γ , were found in the TIF1 β immunoprecipitate (Figure 3A, B and C, respectively). This co-immunoprecipitation was specific as none

of the HP1 proteins could be detected in control immunoprecipitations with an irrelevant antibody (Figure 3A–C, lane 2). A negative result was also obtained by using specific antibodies against TIF1 α (data not shown).

In a reciprocal experiment, P19 EC cell nuclear extracts were immunoprecipitated with either HP1 α , - β or - γ antibodies, and the immunoprecipitates were probed for the presence of TIF1 β . Co-immunoprecipitation of endogenous TIF1 β was clearly detected in each HP1 immunoprecipitate (Figure 2D, lanes 3–5), but not in controls (Figure 3D, lane 2). In contrast, we found no evidence for a co-immunoprecipitation of TIF1 α with HP1 antibodies (data not shown), indicating that unlike TIF1 β , TIF1 α may not be stably associated with HP1 proteins in P19 EC cells.

TIF1 α and TIF1 β bind directly to HP1 α , - β and - γ *in vitro*

To investigate whether the association of TIF1 β with the HP1 proteins in nuclear extracts corresponds to a direct interaction, binding assays were performed *in vitro* using purified recombinant proteins. N-terminally epitope-tagged His-TIF1 β was expressed in Sf9 cells using a baculovirus vector and purified by affinity chromatography. Glutathione *S*-transferase (GST)–HP1 fusion proteins expressed in *Escherichia coli* were immobilized on glutathione *S*-Sepharose beads and incubated with purified His-TIF1 β in the presence of various salt and detergent concentrations (Figure 4A). His-TIF1 β bound to GST–HP1 α (lanes 5–7), GST–HP1 β (lanes 8–10) and GST–HP1 γ (lanes 11–13), but not to GST alone (lanes 2–4). Interactions with all three HP1s were stable between 0.1 and 1 M KCl (upper panel) and between 0.5 and 5% NP-40 (Figure 4A, lower panel). Thus, TIF1 β interacts directly and stably with all three HP1s *in vitro*.

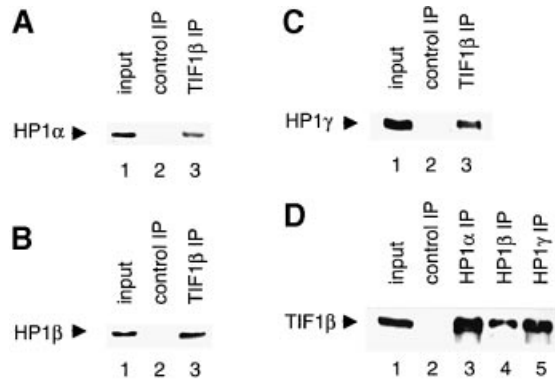


Fig. 3. P19 EC cell endogenous TIF1 β is associated with HP1 α , HP1 β and HP1 γ . (A–C) Detection of HP1 α , HP1 β and HP1 γ in TIF1 β immunoprecipitates. Nuclear extracts were analysed by Western blotting either directly (input) or following immunoprecipitation with a TIF1 β mAb (TIF1 β IP) or with an irrelevant antibody (anti-Flag antibody; control IP). Western blots were probed with either HP1 α (A), HP1 β (B) or HP1 γ (C) mAbs. Arrowheads indicate the position of the protein recognized by each mAb. (D) Detection of TIF1 β in HP1 immunoprecipitates. Nuclear extracts of P19 cells were used for IP with mAbs against HP1 α (lane 3), HP1 β (lane 4) or HP1 γ (lane 5) or an irrelevant anti-Flag mAb (lane 2). A Western blot of the immunoprecipitates probed with a TIF1 β mAb is shown. In all panels, lane 1 (input) corresponds to 1/20 the amount of nuclear extract used for immunoprecipitation.

His-tagged TIF1 α interacts with HP1 α and HP1 β *in vitro* (Le Douarin *et al.*, 1996). We show here that it can also bind to HP1 γ , whatever the concentration of salt and detergent (Figure 4B). Thus, although no association with HP1 proteins could be detected in mammalian cells, TIF1 α interacts in a stable manner with all three HP1s (α , β and γ) *in vitro*.

The HP1-interacting domains of TIF1 α and TIF1 β lie within a conserved 25-amino-acid region

To identify the HP1-interacting domain of TIF1 β , a deletion analysis of TIF1 β was performed using the yeast two-hybrid system (Figure 5). Various TIF1 β deletion mutants expressed as fusion proteins with the VP16 acidic activation domain (denoted AAD) were tested for interaction with HP1 α fused to the DBD of the oestrogen receptor (ER α ; amino acid residues 176–282) in a yeast strain containing an integrated URA3 reporter driven by three ER α binding sites (Le Douarin *et al.*, 1995b). As indicated by the orotidine 5'-monophosphate decarboxylase (OMPdecase) activity of the URA3 gene product (Figure 5B), this deletion analysis showed that an HP1 α -interacting domain is present between residues 468 and 535 of TIF1 β . Interestingly, the TIF1 β sequence from residues 484–506 is markedly similar to the HP1 box of TIF1 α , which is sufficient for interaction with HP1 α (Le Douarin *et al.*, 1996; Figure 5C). An interaction was effectively observed when DBD–TIF1 β (483–507) was co-expressed with either AAD–HP1 α , AAD–HP1 β or AAD–HP1 γ in PL3 (Figure 5D). To demonstrate that TIF1 β actually binds HP1 proteins through this HP1 box, an internal deletion from residues 484–493 was introduced into full-length TIF1 β . No interaction with DBD–HP1 α in yeast (Figure 5B, AAD–TIF1 β Δ 484–493) and no binding to GST–HP1 α *in vitro* could be detected, while binding to the KRAB domain of KOX1 was not affected

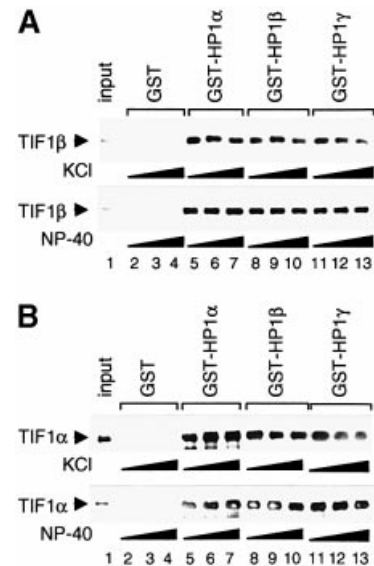


Fig. 4. TIF1 α and TIF1 β bind to HP1 α , HP1 β and HP1 γ *in vitro*. Purified His-TIF1 β (A) or His-TIF1 α (B) was incubated in a batch assay with 'control' GST (lanes 2–4), GST–HP1 α (lanes 5–7), GST–HP1 β (lanes 8–10) or GST–HP1 γ (lanes 11–13) in the presence of various concentrations of salt (0.1, 0.5 and 1 M KCl; upper panels) and detergent (0.5, 2 and 5% NP-40; lower panels). Bound TIF1 was detected by Western blotting. Lane 1 shows one-fifth of the amount of input His-TIF1, the position of which is indicated by an arrowhead.

(Figure 5E, TIF1 β Δ HP1 box). As expected, an internal deletion of the HP1 box in TIF1 α (from residues 678–687) resulted in a similar lack of binding to GST–HP1 α , while binding to the liganded oestrogen receptor [GST–ER α (DEF)] was not affected (Figure 5F). Taken together, these data indicate that TIF1 α and TIF1 β both contain a conserved HP1 box that is necessary and sufficient for specific interactions with the HP1 proteins.

TIF1 α and TIF1 β are autophosphorylatable protein kinases that also phosphorylate HP1 α , - β and - γ

We previously demonstrated that recombinant TIF1 α possesses an intrinsic kinase activity catalysing *in vitro* autophosphorylation, as well as phosphorylation of several proteins such as TFIIIE α , TAF $_{II}55$ and TAF $_{II}28$ (Fraser *et al.*, 1998). We therefore investigated whether TIF1 β could also be a protein kinase, and whether both TIF1 α and TIF1 β may phosphorylate HP1 α , - β and - γ to which they bind directly. Recombinant epitope-tagged full-length His-TIF1 α and His-TIF1 β proteins were expressed in Sf9 cells, purified to homogeneity by affinity chromatography and immunoprecipitation, and subjected to *in vitro* kinase assays (Figure 6). His-TIF1 α eluted from immunoprecipitates obtained with three distinct mAbs was phosphorylated (Figure 6A, lanes 1–4), confirming the autokinase activity of TIF1 α (Fraser *et al.*, 1998). Interestingly, recombinant GST–HP1 α (lanes 13–16) and GST–HP1 β (lanes 17–20), as well as His-HP1 γ (lanes 21–24), but not 'control' GST (lanes 9–12), were phosphorylated efficiently by TIF1 α (Figure 6A). Using the same kinase reaction conditions, TIF1 α was also tested for its ability to phosphorylate a well established kinase substrate, histone H1, to which TIF1 α also binds (our unpublished results). Under similar conditions, neither calf thymus histone H1 (Figure 6A,

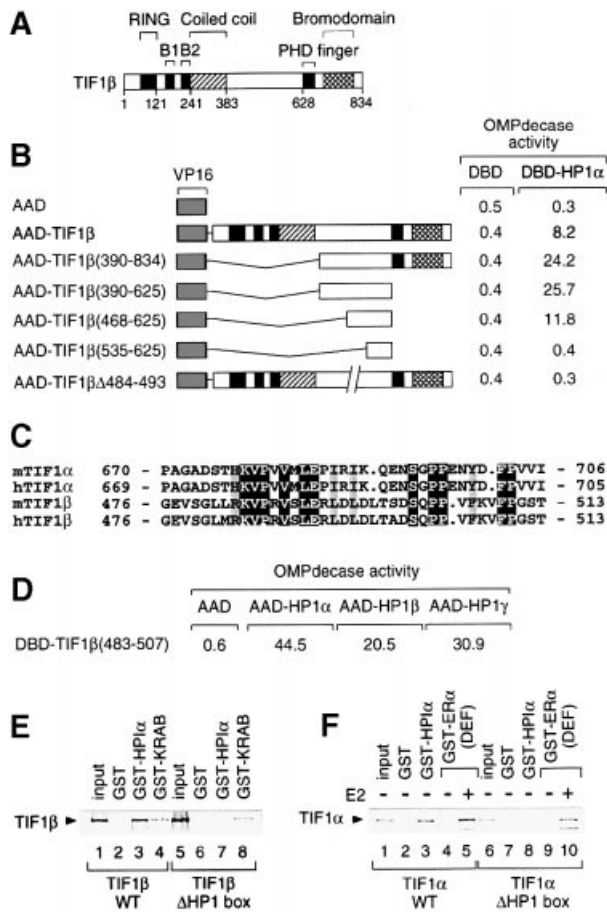


Fig. 5. Mapping of the HP1-interacting domain in mouse TIF1β. (A) Domain organization of mouse TIF1β. Numbers refer to amino acid positions (see Le Douarin *et al.*, 1996). (B) Residues 484–493 of TIF1β are required for interaction with HP1α in yeast. Various deletion mutants of TIF1β were fused to the VP16 AAD and assayed for interaction with the ‘unfused’ ERα DBD or a DBD fusion of HP1α in the yeast strain PL3, which contains a URA3 reporter gene driven by three oestrogen response elements (EREs) (Pierrat *et al.*, 1992). Transformants were grown in liquid medium containing uracil. OMPdecase activities determined for each cell-free extract are expressed in nmol substrate/min/mg protein. The values ($\pm 20\%$) are the average of at least three independent transformants. Note that expression of all fusion proteins was confirmed by Western blotting using the antibody 2GV4 against VP16 (data not shown). (C) Alignment of the HP1-interacting domain of TIF1α (Le Douarin *et al.*, 1996) with TIF1β amino acids 468–535 reveals a highly conserved region, referred to as the HP1 box. Invariant amino acids are highlighted in bold. Shading indicates conserved amino acid residues. Database accession nos: mouse TIF1α (mTIF1α, S78219); human TIF1α (hTIF1α, AF119042); mouse TIF1β (mTIF1β, X99644); human TIF1β (hTIF1β, U95040). (D) Residues 483–507 of TIF1β are sufficient for interaction with all three HP1s in yeast. DBD-TIF1β(483–507) was co-expressed with either the ‘unfused’ VP16 AAD (negative control) or the VP16 AAD fused to HP1α, HP1β or HP1γ in yeast strain PL3. OMPdecase activities are given as in (B). (E and F) The HP1 box is required for binding to HP1α *in vitro*. In (E), *in vitro* ^{35}S -labelled TIF1β (lanes 1–4) and TIF1βΔHP1box lacking residues 484–493 (lanes 5–9) were incubated in a batch assay with immobilized ‘control’ GST, GST-HP1α or GST-KRAB containing the KRAB repression domain of KOX1 (residues 1–97). After extensive washing, the bound TIF1β protein was eluted with SDS, resolved on SDS-PAGE and visualized by autoradiography. Lanes 1 and 5 represent one-tenth of the amount of input labelled TIF1β and TIF1βΔHP1box, the positions of which are indicated by an arrowhead. In (F), similar GST ‘pull-down’ assays were performed with *in vitro* ^{35}S -labelled TIF1α (lanes 1–5) or TIF1αΔHP1box lacking residues 678–687 (lanes 6–10) and ‘control’ GST or GST-HP1α or GST-ERα(DEF). A 10^{-6} M concentration of oestradiol (E2) was added to the incubations as indicated.

lanes 5–8) nor a recombinant GST–mouse histone H1 fusion (data not shown) were phosphorylated by TIF1α (Figure 6A). Similarly, affinity-purified and immunoprecipitated recombinant His-TIF1β exhibited an autokinase activity (Figure 6B, lane 3) and phosphorylated HP1α (lane 6), HP1β (lane 9) and HP1γ (lane 12), thus indicating that TIF1β, like TIF1α, has an intrinsic protein kinase activity.

To extend the above results obtained with recombinant proteins to more physiological conditions, we also investigated whether endogenous TIF1α and TIF1β exhibited kinase activity when immunopurified from nuclear extracts from P19 EC cells. As shown in Figure 6C and D, autokinase and HP1 kinase activities were specifically co-immunoprecipitated with mAbs against TIF1α and TIF1β, respectively, but not with a control antibody. Thus, endogenous P19 EC cell TIF1α and TIF1β also appear to be associated with HP1 kinase activity.

Involvement of HP1 interaction and histone deacetylation in transcriptional repression by TIF1α and TIF1β

Similar to HP1 proteins (see below and the references in Introduction), TIF1α and TIF1β can repress transcription when fused to a heterologous DBD such as the GAL4 DBD (hereafter designated as GAL4–TIF1 fusion proteins) (Le Douarin *et al.*, 1996; Moosmann *et al.*, 1996). To investigate whether an interaction with HP1s is required for the silencing activity of TIF1α and TIF1β, GAL4–TIF1 fusion derivatives containing an internal deletion of the HP1 box, GAL4–TIF1αΔHP1box and GAL4–TIF1βΔHP1box, were generated and tested in transiently transfected Cos-1 cells for repression of the chimeric transactivator ER(C)-VP16 using a GAL4 reporter containing two GAL4 DNA binding sites (UAS, 17M2) and an oestrogen response element (ERE) in front of a β-globin (G) promoter–CAT fusion (17M2-ERE-G–CAT; Figure 7A). GAL4–TIF1αΔHP1box repressed the reporter gene activity to the same degree as the wild-type TIF1α fusion, indicating that an intact HP1 box is not required for the silencing activity of TIF1α (Figure 7B). This finding is consistent with our previous conclusion that point mutations in the HP1 box (V681E/V682E; Le Douarin *et al.*, 1996) impair the interaction of TIF1α with HP1α in yeast, but not its transcriptional silencing effect in transfected mammalian cells. In contrast, deletion of the HP1 box in TIF1β resulted in a marked decrease in repression (with no effect on the expression level of the GAL4 fusion protein; Figure 7C; and data not shown), suggesting that the silencing effect of TIF1β requires an interaction with the HP1 proteins. However, additional silencing mechanism(s) appear to be involved as a residual level of repression was reproducibly retained by the GAL4–TIF1βΔHP1box mutant (Figure 7C).

Because, in many instances, recruitment of histone deacetylases (HDACs) has been shown to be involved in transcriptional repression (Pazin and Kadonaga, 1997; Ashraf and Ip, 1998; Kuo and Allis, 1998 and references therein), we analysed the effects of the specific inhibitor of HDAC, trichostatin A (TSA; Yoshida *et al.*, 1995), on repression by GAL4–TIF1 fusion proteins. Repression by both GAL4–TIF1α and GAL4–TIF1αΔHP1box was almost fully relieved upon treatment with TSA

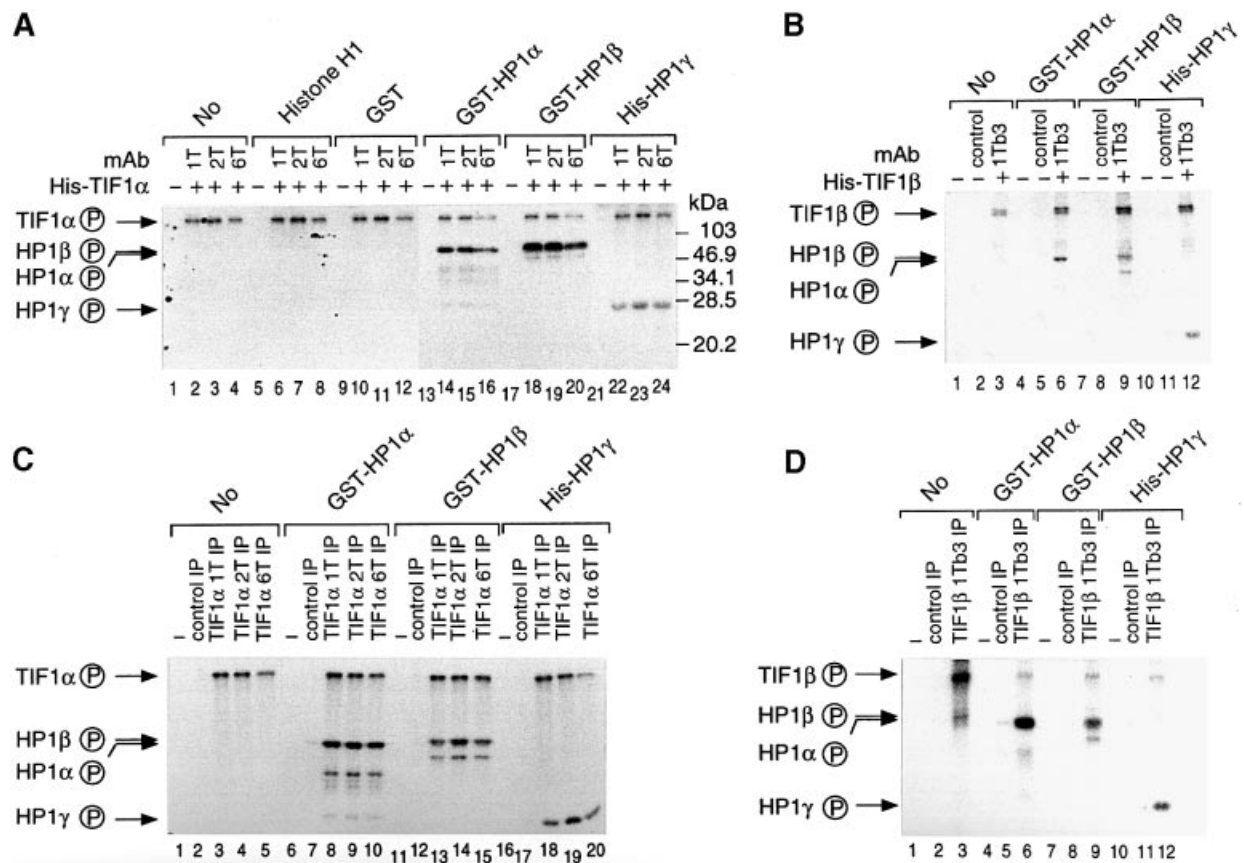


Fig. 6. Association of autokinase and HP1 kinase activity with recombinant and endogenous TIF1 α and TIF1 β . (A) Recombinant His-TIF1 α expressed in Sf9 cells and purified by nickel chelate affinity chromatography was immunoprecipitated with three different TIF1 α mAbs (mAb1T, mAb2T and mAb6T) as indicated. TIF1 α was eluted from the three immunoprecipitates with the respective epitope peptides, and the peptide eluates were individually tested for autokinase activity (lanes 1–4) and transphosphorylation activity using calf thymus histone H1 (lanes 5–8), purified *E.coli*-expressed unfused GST (lanes 9–12), GST-HP1 α (lanes 13–16), GST-HP1 β (lanes 17–20) and His-HP1 γ (lanes 21–24). As a negative control, *in vitro* kinase reactions were performed in the absence of His-TIF1 α (buffer alone; lanes 1, 5, 9, 13, 17 and 21). The proteins were resolved by SDS-PAGE and analysed by Coomassie Blue staining (data not shown) and autoradiography. The sizes of protein markers are indicated on the right side of the figure. Arrows mark positions of phosphorylated TIF1 α and HP1s. (B) An *in vitro* kinase assay was performed as in (A) in the absence (buffer alone; lanes 1, 4, 7 and 10) or presence of recombinant His-TIF1 β expressed from Sf9 cells, purified by nickel chelate affinity chromatography and immunoprecipitated with a specific TIF1 β antibody (mAb TIF1 β 1Tb3; lanes 3, 6, 9 and 12). As an additional control, an Sf9 extract containing His-TIF1 β was processed in the presence of an irrelevant anti-Flag mAb (control mAb; lanes 2, 5, 8 and 11). The positions of phosphorylated TIF1 β and HP1s are shown. The identity of the upper band as His-TIF1 β was confirmed by Western blot analysis (data not shown). (C) Co-immunoprecipitation of HP1 kinase activity with endogenous TIF1 α . Nuclear extracts from P19 EC cells were immunoprecipitated with antibodies against TIF1 α (TIF1 α 1T, 2T or 6T IP) or an irrelevant anti-Flag antibody (control IP), and the resulting immunoprecipitates were tested for autokinase activity (lanes 2–5) and kinase activity using recombinant GST-HP1 α (lanes 7–10), GST-HP1 β (lanes 12–15) and His-HP1 γ (lanes 17–20). *In vitro* kinase reactions were also performed in the absence of immunoprecipitates (buffer alone, lanes 1, 6, 11 and 16). (D) Co-immunoprecipitation of HP1 kinase activity with endogenous TIF1 β . Nuclear extracts from P19 EC cells were immunoprecipitated with a TIF1 β antibody (anti-TIF1 β 1Tb3 IP) or an irrelevant anti-Flag antibody (control IP), and the resulting immunoprecipitates were tested for kinase activity as in (C).

(Figure 8A), suggesting that histone deacetylation is chiefly involved in TIF1 α -mediated repression. In contrast, under similar conditions, the silencing activity of GAL4-TIF1 β was only partially inhibited by TSA treatment (Figure 8B). However, an almost complete relief of repression was achieved upon TSA treatment of GAL4-TIF1 β Δ HP1box-expressing cells (Figure 8B), thus suggesting that repression by TIF1 β involves both the binding of HP1 and histone deacetylation.

TSA treatment partially relieves HP1-dependent repression

HP1 α (Le Douarin *et al.*, 1996; Lehming *et al.*, 1998; Seeler *et al.*, 1998) and HP1 γ (Lehming *et al.*, 1998) have been shown to repress transcription when directly tethered to a promoter in mammalian cells. We show

here that a GAL4-HP1 β fusion protein can also function as a transcriptional repressor in transiently transfected Cos-1 cells (Figure 8C). We reproducibly found that GAL4-HP1 α was a more efficient repressor than either GAL4-HP1 β or GAL4-HP1 γ (Figure 8C); Western blot analysis using an antibody against the GAL4 DBD indicated similar expression levels for all three fusion proteins (data not shown). Interestingly, treatment of the transfected cells with TSA significantly reduced the silencing activity of each GAL4-HP1 (~3- to 4-fold; Figure 8C), indicating that HP1-dependent repression also involves a histone deacetylase activity. Note, however, that repression was not fully relieved by TSA (Figure 8C; and data not shown), suggesting that a deacetylase-independent mechanism is also involved in HP1-mediated silencing.

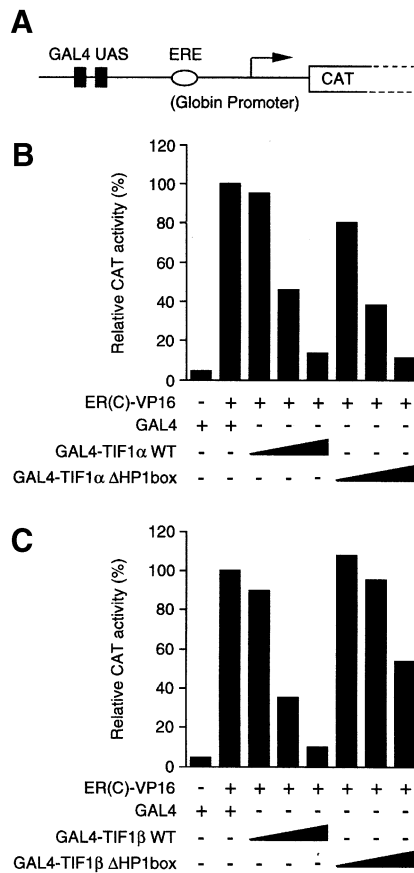


Fig. 7. Effects of HP1 box deletion on the repression activity of TIF1 α and TIF1 β . (A) Schematic representation of the 17M2-ERE-G-CAT reporter gene used. GAL4 UAS motifs are represented by filled squares, the ERE by an open oval and the transcription initiation site by an arrow. (B) TIF1 α -mediated repression is not affected by deletion of the HP1 box. Increasing amounts of GAL4-TIF1 α wild type (WT) and GAL4-TIF1 α lacking the HP1 box (Δ HP1box) expression plasmids (0.2, 2 or 20 ng) were transiently transfected into Cos-1 cells with 1 μ g of 17M2-ERE-G-CAT reporter, 1 μ g of pCH110 (expressing β -galactosidase) and 100 ng of ER(C)-VP16. CAT activities are expressed relative to that measured in the presence of the unfused GAL4 expression vector (20 ng; taken as 100%). Values (\pm 10%) represent the averages of two independent triplicated transfections after normalization for the internal control β -galactosidase activity of pCH110. (C) HP1 box deletion reduces the repression activity of TIF1 β . Cos-1 cells were transiently co-transfected with 1 μ g of 17M2-ERE-G-CAT reporter, 1 μ g of pCH110, 100 ng of ER(C)-VP16, and 0.2, 2 or 20 ng of GAL4-TIF1 β WT and GAL4-TIF1 β Δ HP1box expression plasmids. CAT activities are expressed as in (B).

Discussion

HP1 proteins are differentially involved in the silencing activity of members of the TIF1 family

In an attempt to characterize the molecular mechanism(s) underlying the silencing activity of TIF1 α and TIF1 β , we investigated the possibility that they may interact physically and functionally with members of the HP1 gene family, a class of non-histone chromosomal proteins with a well established epigenetic gene silencing function in *Drosophila* (for reviews, see Elgin, 1996; Wallrath, 1998). Using yeast as well as GST pull-down experiments, we have demonstrated that both TIF1 α and TIF1 β can bind to HP1 α , HP1 β and HP1 γ , and we have identified a

conserved amino acid motif within the central region of TIF1 α and TIF1 β that is both necessary and sufficient for HP1 binding (HP1 box). However, in mouse P19 cells, an association with HP1 proteins was detected for TIF1 β only, suggesting that the interaction between HP1s and TIF1 α *in vivo* might be transient and/or involve only a small subset of the proteins. We have also demonstrated that TIF1 β , but not TIF1 α , requires an intact HP1 box for full repression activity, indicating that the formation of a complex with HP1 proteins is instrumental in transcriptional repression by TIF1 β . After completion of this manuscript, a study was published by Ryan *et al.* (1999) in which *in vitro* studies confirm that TIF1 β (designated KAP-1) is capable of interacting directly with the HP1 proteins. Interestingly, the authors observed a co-localization of TIF1 β with HP1 β and HP1 γ in subnuclear territories of pericentromeric heterochromatin and in numerous punctate euchromatic domains, respectively (Ryan *et al.*, 1999), supporting our co-immunoprecipitation data showing that endogenous TIF1 β and members of the HP1 family are associated.

Taken together with the observation that, similarly to TIF1 β , HP1 proteins function as potent transcriptional repressors when tethered to DNA through fusion to a GAL4 DBD (Le Douarin *et al.*, 1996; Lehming *et al.*, 1998; Seeler *et al.*, 1998; this study), our results raise the possibility that HP1 proteins may mediate, at least in part, the TIF1 β silencing effect, whereas these proteins may not be involved in TIF1 α repression. A novel member of the TIF1 gene family, termed TIF1 γ , has been identified recently (Venturini *et al.*, 1999). TIF1 γ resembles TIF1 α and TIF1 β in that it possesses an intrinsic silencing effect (Venturini *et al.*, 1999). However, in contrast to TIF1 α and TIF1 β , TIF1 γ has no HP1 box and does not interact with HP1 proteins in yeast or *in vitro* (Venturini *et al.*, 1999; our unpublished results). Thus, TIF1 β may represent the only member of the TIF1 gene family to silence transcription through an interaction with HP1 proteins.

Histone deacetylation is involved in the silencing activity of TIF1s and HP1s

We have shown that TSA, a specific and potent inhibitor of HDACs, can interfere with TIF1 α - and TIF1 β -mediated repression. In the case of TIF1 α , the repression was almost fully relieved by HDAC inhibition, suggesting that histone deacetylation is a key determinant in TIF1 α -mediated silencing. In the case of TIF1 β , HP1-independent repression (as exhibited by the TIF1 β Δ HP1box mutant) was also almost abrogated by HDAC inhibition, while repression by the wild-type protein was only partially relieved, suggesting that TIF1 β repression requires both HP1 binding and histone deacetylation. In the case of TIF1 γ , our preliminary results indicate that repression by TIF1 γ can also be relieved, at least partially, by treating cells with TSA (J.Ortiz, R.Losson and P.Chambon, unpublished data). Thus, as observed in many cases of transcriptional repression (see Ashraf and Ip, 1998 and references therein), histone deacetylation appears to be involved in repression by all three TIF1 family members. Several transcriptional co-repressors have recently been shown to be components of or to recruit multi-protein complexes that contain HDACs (Kuo and Allis, 1998; Pazin and Kadonaga, 1998 and references therein). Once tethered to target promoters,

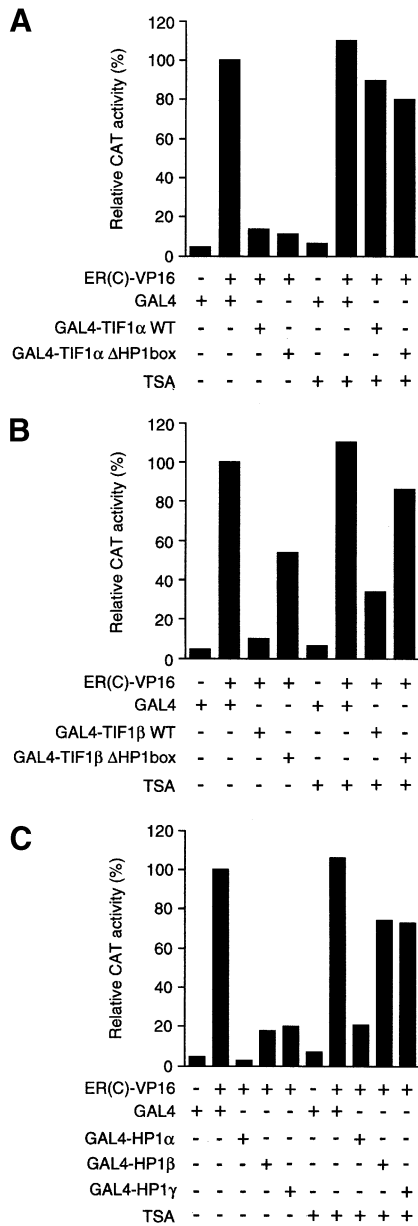


Fig. 8. Relief of TIF1- and HP1-mediated repression of transcription by the histone deacetylase inhibitor TSA. (A) TSA treatment relieves the repression activity of TIF1 α WT and TIF1 α Δ HP1box. Cos-1 cells were transiently co-transfected with 1 μ g of 17M2-ERE-G-CAT reporter, 1 μ g of pCH110, 100 ng of ER(C)-VP16 and 20 ng of the indicated GAL4 expression vectors. For TSA treatment, 300 nM TSA was added 24 h before collecting the cells. CAT activities are expressed as in Figure 7A. (B) TSA and HP1 box deletion have additive relieving effects on TIF1 β -mediated repression in Cos-1 cells transfected and treated as described in (A). CAT activities are expressed as in Figure 7A. (C) TSA treatment partially relieves HP1 repression. Cos-1 cells were transiently co-transfected with 1 μ g of 17M2-ERE-G-CAT reporter, 1 μ g of pCH110, 100 ng of ER(C)-VP16 and 200 ng of the indicated GAL4 expression vectors. For TSA treatment, 300 nM TSA was added 24 h before collecting the cells. CAT activities are expressed as in Figure 7A. In each panel, values (\pm 10%) represent the averages of two independent triplicated transfections after normalization for the internal control β -galactosidase activity of pCH110.

these HDACs are thought to interfere with transcription by inducing local repressive chromatin conformations as a consequence of deacetylation of nucleosomal histones. Future experiments will show whether TIF1 α , - β and - γ can

interact with one of these HDAC-containing complexes (Sin3-HDAC, MI2-HDAC; Zhang *et al.*, 1997, 1998).

We have also investigated whether histone deacetylation is involved in HP1-mediated silencing. TSA treatment of transfected cells partially relieved repression by HP1 α , - β and - γ . Thus, as observed in budding and fission yeast cells (Braunstein *et al.*, 1996; Ekwall *et al.*, 1997), a functional relationship between histone deacetylation and heterochromatin-mediated silencing may exist in mammals. At least two non-exclusive possibilities could account for our results. First, HP1 α is known to self-associate and to interact with HP1 β and HP1 γ in yeast as well as *in vitro* (Le Douarin *et al.*, 1996; A.L.Nielsen, unpublished data). Thus, by analogy with the known function of *Drosophila* HP1, which, presumably through self-association and the formation of a silencing complex, exerts dose-dependent effects on heterochromatin-mediated gene silencing (for reviews, see Eissenberg *et al.*, 1995; Weiler and Wakimoto, 1995; Elgin, 1996), HP1 α , - β and - γ could form dimers or higher order multimeric complexes, leading to the establishment of a repressive heterochromatin-like structure. The formation and/or stabilization of this structure may involve specific interactions between HP1 proteins (or HP1-associated proteins) and hypoacetylated histones. Note that interactions have been observed in budding yeast between histone tails and components of heterochromatin (i.e. the silent informant regulator SIR proteins), which, interestingly, are affected by acetylation (Hecht *et al.*, 1995, 1996). That HP1 proteins could be involved in the transition from a euchromatic to a heterochromatic state is supported by the observation that HP1 β and HP1 γ are not exclusively associated with heterochromatin (Horsley *et al.*, 1996; this study). A second possibility is suggested by the recent observation that in proliferating B cells Ikaros DNA-binding proteins are capable of recruiting repressed genes (and HDAC complexes) to regions of heterochromatin (Brown *et al.*, 1997, 1999; Hahm *et al.*, 1998; Klug *et al.*, 1998; Kim *et al.*, 1999). Similarly, HP1 proteins may recruit the DNA template to which they are bound into heterochromatic nuclear compartments where other HP1s and silencing proteins localize. The structure and/or function of these heterochromatic domains, which, in contrast to euchromatic domains, are rich in hypoacetylated histones (Grunstein, 1998 and references therein), might be affected by histone acetylation. This view is supported by the observation that treating fission yeast cells with TSA is sufficient to induce a hyperacetylated state in centromeric heterochromatin accompanied by a delocalization of the SWI6 protein and a loss of centromeric silencing (Ekwall *et al.*, 1997).

We also show here that all three HP1 proteins can be phosphorylated by both endogenous and recombinant TIF1 α and TIF1 β . Although the biological relevance of these phosphorylations has not yet been established, it is reminiscent of *Drosophila* HP1 phosphorylation, which has been correlated with heterochromatin assembly and/or maintenance (Eissenberg *et al.*, 1994). This phosphorylation of HP1 may favour self-association or interaction with other proteins. In the case of TIF1 α , which is mostly associated with euchromatic regions readily accessible to micrococcal nuclease digestion (Remboutsika *et al.*, 1999), it is tempting to speculate that phosphorylation of HP1s

by TIF1 α may favour their incorporation into heterochromatin, thereby preventing their association with euchromatic components. On the other hand, in the case of TIF1 β , which requires HP1 binding for full repression activity, our results raise the possibility that phosphorylation of HP1s by TIF1 β may increase their effects on gene silencing and/or chromatin condensation at specific loci.

The three members of the TIF1 family are differentially involved in transcriptional repression

Although our present study indicates that the silencing effect of TIF1 α and β is chiefly mediated through chromatin remodelling, it is not clear how these TIF1s could be recruited to chromatin as they have no known DNA binding motif and apparently no sequence-specific DNA binding activity (Remboutsika *et al.*, 1999). Therefore, their recruitment to chromatin may result from association(s) with other proteins. In the case of TIF1 β , this possibility is supported by a number of co-transfection and biochemical interaction data showing that TIF1 β is a putative mediator of transcriptional repression for the large class of KRAB-ZFPs (Friedman *et al.*, 1996; Kim *et al.*, 1996; Moosmann *et al.*, 1996; see Introduction). Although there is currently no known DNA/RNA recognition sequence for any of the KRAB ZFPs identified so far, these proteins possess Krüppel Cys2His2-type zinc fingers in their C-termini, which suggests the existence of a specific nucleic acid binding function (Holleman *et al.*, 1996 and references therein). Moreover, their differential spatial and temporal expression patterns indicate that these proteins may play an important role in regulating expression of specific genes during cell differentiation and development (Bellefroid *et al.*, 1993, 1998; Witzgall *et al.*, 1993; Mazarakis *et al.*, 1996; Poncelet *et al.*, 1998). Based on our present findings, we propose a molecular mechanism for the function of these proteins in transcriptional repression, which involves epigenetic effects resulting from association of TIF1 β with members of the HP1 family and targeted histone deacetylation. In this model, the KRAB domain of a DNA-bound KRAB ZFP recruits TIF1 β to specific loci. TIF1 β in turn binds HP1 proteins (and perhaps also directly HDACs) to silence transcription, via the local conversion of a euchromatic region into a heterochromatin-like structure and/or the recruitment of the chromatinic DNA template to heterochromatic compartments. That both mechanisms could operate within the same cell is strongly suggested by our present finding that TIF1 β localizes to both euchromatin and highly condensed heterochromatic compartments.

Similar to TIF1 β , TIF1 α has been reported to interact with the KRAB silencing domain of the human KOX1 protein both in yeast and *in vitro* (Moosmann *et al.*, 1996; our unpublished data). However, in contrast to TIF1 β , this interaction has not been reproduced with typical KRAB domains of other ZFPs (M.Ebrink, C.Lotta, J.Ortiz, C.Sanchez and R.Losson, unpublished data). TIF1 α , but not TIF1 β , was previously shown to interact with transcriptionally active nuclear receptors in an agonistic ligand- and activation function AF-2-dependent manner (Le Douarin *et al.*, 1995a; vom Baur *et al.*, 1996). Thus, TIF1 α could be recruited to DNA by agonist-bound nuclear receptors and be involved in ligand-dependent repression through a mechanism that involves histone deacetylation. How-

ever, as TIF1 α is tightly and preferentially associated with euchromatic sites in toti- and multipotent cells (Remboutsika *et al.*, 1999), we favour another model in which TIF1 α functions in euchromatin as a protein anchor to which nuclear receptors bind in the presence of their cognate agonistic ligands. As a consequence, TIF1 α would become hyperphosphorylated (Fraser *et al.*, 1998), and this modification would decrease its silencing activity, thus resulting in an activation of transcription.

To date, no interaction between TIF1 γ and DNA binding proteins has been described, but the fact that neither KRAB domains nor nuclear receptors interact with TIF1 γ , at least in yeast and *in vitro* (Venturini *et al.*, 1999), supports the view that TIF1 α , TIF1 β and TIF1 γ are functionally distinct. Gene knockouts are in progress to determine their respective biological role(s).

Materials and methods

Plasmids

Details on individual plasmid constructs, which were all verified by sequencing, are available upon request. For *in vitro* binding assays, the indicated cDNAs were fused to GST in the pGEX2TK plasmid (Pharmacia; Le Douarin *et al.*, 1995a, 1996). For expression of ³⁵S-labelled proteins, the coding sequences of TIF1 α and β were inserted into the pSG5 vector and coupled transcription/translation was performed using T7 RNA polymerase with the TNT lysate system (Promega). The His-TIF1 α/β constructs have been cloned into a modified version of the pAcSGHisNT-B baculovirus expression vector (Pharmingen), in which the *Bam*HI-*Xho*I fragment was replaced with the *Bgl*II-*Xho*I fragment of pet15b, thus eliminating the protein kinase A site, but preserving the reading frame and the His₆ tag (Fraser *et al.*, 1998). For yeast two-hybrid assays, DBD and AAD fusion proteins were expressed from the yeast multicopy plasmids pBL1 and pASV3, respectively (Le Douarin *et al.*, 1995b). These plasmids express inserts under the control of the phosphoglycerate kinase (PGK) promoter. pBL1 contains the HIS3 marker and directs the synthesis of epitope (region F of ER α)-tagged ER α DBD fusion proteins. pASV3 contains the LEU2 marker and a cassette expressing a nuclear localized VP16 AAD, preceding a polylinker and stop codons in all reading frames. For transfection studies in mammalian cells, the GAL4(1-147) chimeras were constructed into pG4MpolyII (Le Douarin *et al.*, 1996). The chimeric protein ER(C)-VP16, which encodes amino acids 176-280 of ER α and amino acids 413-490 of VP16, has been described previously as well as the reporter gene 17M2-ERE-G-CAT (Le Douarin *et al.*, 1996).

Antibodies

mAbs used include: (i) anti-HP1 α mAbs, 2HP1-1H5 for immunocytochemistry and immunoprecipitation and 2HP-2G9 for Western blot analysis (no cross-reactivity with HP1 β or HP1 γ ; Remboutsika *et al.*, 1999); (ii) anti-HP1 β mAb, 1 Mod-1A9, raised against recombinant *E.coli*-expressed mouse HP1 β (no cross-reactivity with HP1 α or HP1 γ); (iii) anti-HP1 γ mAb, 2Mod-1G6, raised against recombinant *E.coli*-expressed mouse HP1 γ (no cross-reactivity with HP1 α or HP1 β); (iv) anti-TIF1 α mAbs, 5T-1E8, 1T, 2T and 6T (Le Douarin *et al.*, 1995a, 1996; Fraser *et al.*, 1998); (v) anti-TIF1 β mAb, 1Tb3, raised against TIF1 β (123-834); (vi) anti-RPB1 mAb, 1PB-7C2, raised against the heptad repeat CTD-containing peptide of the RPB1 largest subunit of the human RNA polymerase II (Nguyen *et al.*, 1996); (vii) anti-GAL4(1-147), 2GV3 (Le Douarin *et al.*, 1996); (viii) anti-VP16 mAb, 2GV4 (Le Douarin *et al.*, 1996); and (ix) anti-ER α F mAb, F3, raised against the F region of human ER α (Le Douarin *et al.*, 1996). Confocal immunofluorescence and immunoelectron microscopy were performed as described previously (Remboutsika *et al.*, 1999).

Nuclear extract preparation, immunoprecipitation and Western blot analysis

P19 EC cells were grown in monolayers in Dulbecco's modified Eagle's medium supplemented with 5% (v/v) normal and 5% delipidated fetal calf serum, and incubated in a 7% CO₂/93% air humidified atmosphere at 37°C. A total of 10⁷ cells were harvested, washed twice with ice-cold 10 mM phosphate-buffered saline (PBS) pH 7.2 and suspended in nuclei

isolation buffer [NIB; 15 mM Tris-HCl pH 7.5, 60 mM KCl, 15 mM NaCl, 5 mM MgCl₂, 1 mM CaCl₂, 1 mM dithiothreitol (DTT), 2 mM sodium vanadate, 250 mM sucrose, and protease inhibitor mixture]. An equal volume of NIB buffer containing 0.6% NP-40 was added to the cells, the suspension was gently mixed and incubated on ice for 5 min. Nuclei were pelleted by centrifugation at 2000 *g* for 5 min at 4°C and washed with NIB buffer before final suspension in the same buffer. DNA was estimated by measuring UV absorption at 260 nm (A₂₆₀) in a 2 M NaCl solution (3 A₂₆₀ units correspond to 100 µg DNA; Bellard *et al.*, 1989). After centrifugation, nuclei were suspended in nuclei extraction buffer (NEB; 25 mM Tris-HCl pH 8.0, 250 mM NaCl, 1 mM EDTA, 10% glycerol, 0.2% NP-40, and protease inhibitor mixture). A total of 250 U of DNase I and 5 mM MgCl₂ were added to 1 mg DNA equivalent. Samples were digested for 1 h at 4°C. After addition of 10 mM EDTA and centrifugation (at 15 000 *g* for 30 min at 4°C), the resulting nuclear extract (5 mg protein/10⁸ initial cells) was incubated with 50 µl of specific antibody coupled to protein G-Sepharose beads for 12 h at 4°C. The immunoprecipitates were washed five times with 800 µl of NEB buffer, and the final pellets were resuspended in 50 µl of Laemmli buffer (Laemmli, 1970); 10 µl were subjected to SDS-PAGE and electrotransferred to nitrocellulose filters. The filters were incubated with specific mAbs followed by a peroxidase-conjugated anti-mouse IgG secondary antibody and developed using an ECL detection kit (Amersham Pharmacia Biotech.).

In vitro binding assays

The assay was performed as described previously (Le Douarin *et al.*, 1995a). Briefly, GST or GST-HP1 fusion proteins were expressed in *E. coli* and purified on glutathione S-Sepharose (Pharmacia). Purified proteins were quantified by Coomassie staining after SDS-PAGE separation and by Bradford protein assay. Then 12 µg of GST or GST fusion proteins loaded on glutathione S-Sepharose beads for 2 h at 4°C in NEB buffer were incubated with 1 µg of baculovirus-expressed His-tagged TIF1β or 10 µl of *in vitro*-translated proteins. Incubation was carried out for 6 h at 4°C with gentle agitation. The beads were washed six times with 750 µl of NEB buffer, resuspended in 25 µl of Laemmli buffer, boiled for 10 min, and proteins analysed by SDS-PAGE. Bound proteins were detected by immunoblotting or autoradiography.

Transactivation assays

Yeast cells grown in yeast extract/peptone/dextrose (YPD) or selective medium were transformed by the lithium acetate procedure (Gietz *et al.*, 1995). Yeast PL3 (*Matα ura3-Δ1 his3-Δ200 leu2-Δ1 trp1::3ERE-URA3*; Le Douarin *et al.*, 1995b) transformants were grown exponentially for about five generations in selective medium supplemented with uracil. Yeast extracts were prepared and assayed for OMPdecase activity as described previously (Pierrat *et al.*, 1992). The transient transfection of mammalian cells and CAT assays were as described previously (Le Douarin *et al.*, 1996). TSA (Sigma) was added 24 h after transfection, and cells were harvested 24 h later.

In vitro phosphorylation

Recombinant His-TIF1α and His-TIF1β, expressed in SF9 cells using a baculovirus vector, were purified as described previously (Fraser *et al.*, 1998). Immunopurified TIF1α and TIF1β from P19 EC nuclear extracts were prepared as described above. *In vitro* phosphorylation was performed with 50 ng of recombinant purified His-TIF1α/β or 10 ng of immunopurified TIF1α/β in 20 mM Tris-HCl pH 7.9, 15 mM MgCl₂, 50 mM NaCl, 10 µM ATP, 1 mM DTT and 0.5 µCi of [^γ-³²P]ATP with or without substrate (1 µg) as indicated. Reactions were performed at 30°C for 30 min and stopped by addition of Laemmli buffer and boiling. The phosphorylated proteins were subjected to SDS-PAGE, Coomassie Blue staining and autoradiography.

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