Selective interaction between the chromatin-remodeling factor BRG1 and the heterochromatin-associated protein HP1 α

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Mammalian heterochromatin protein 1 (HP1) α , HP1 β and HP1 γ are closely related non-histone chromosomal proteins that function in gene silencing, presumably by organizing higher order chromatin structures. Here, we show by co-immunoprecipitation that HP1 α , but neither HP1 β nor HP1 γ , forms a complex with the BRG1 chromatin-remodeling factor in HeLa cells. *In vitro*, BRG1 interacts directly and preferentially with HP1 α . The region conferring this preferential binding has been mapped to residues 106–180 of the HP1 α C-terminal chromoshadow domain. Using site-directed mutagenesis, we have identified three amino acid residues I113, A114 and C133 in HP1 α (K, P and S in HP1 β and HP1 γ) that are essential for the selective interaction of HP1 α with BRG1. Interestingly, these residues were also shown to be critical for the silencing activity of HP1 α . Taken together, these results demonstrate that mammalian HP1 proteins are biochemically distinct and suggest an entirely novel function for BRG1 in modulating HP1 α -containing heterochromatic structures.

Keywords: chromatin remodeling/chromoshadow domain/heterochromatin assembly/HP1/SWI–SNF

Introduction

Members of the heterochromatin protein 1 (HP1) family represent a class of non-histone chromosomal proteins that have been implicated in the establishment and maintenance of higher order chromatin structures playing a role in nuclear organization, chromosome segregation and gene silencing (Eissenberg and Elgin, 2000; Li *et al.*, 2002). Yet, the molecular mechanisms by which these proteins are packaged into and operate in heterochromatin remain to be understood.

HP1 proteins have been identified in a variety of organisms, including *Drosophila* (HP1/HP1a, HP1b and HP1c), *Schizosaccharomyces pombe* (SWI6), *Xenopus*

(Xhp1 α and Xhp1), chicken (CHCB1, CHCB2 and CHCB3), mouse (HP1 α , HP1 β /MOD1/M31 and HP1 γ /MOD2/M32) and human (HP1^{Hs} α , HP1^{Hs} β and HP1^{Hs} γ) (Li *et al.*, 2002). Each member of the family contains an N-terminal chromodomain and a structurally related C-terminal chromoshadow domain (Cavalli and Paro, 1998; and references therein). These two domains consist of three-stranded antiparallel β -sheets folding back against α -helices, and show remarkable similarity to two archaeobacterial histone-like proteins (Ball *et al.*, 1997; Brasher *et al.*, 2000; Cowieson *et al.*, 2000). Recently, the chromodomains from HP1 α and the polycomb group protein Pc1/M33 have been demonstrated to possess histone H3-binding activity, providing a clear connection to chromatin structure (Bannister *et al.*, 2001; Lachner *et al.*, 2001; Nielsen *et al.*, 2001a).

Drosophila HP1, the founding member of the family, was identified originally as a heterochromatin-associated protein (James and Elgin, 1986) and subsequently was shown to exhibit dosage-dependent effects on position-effect variegation (PEV), a phenomenon associated with chromosomal rearrangements that cause mosaic expression of euchromatic genes when relocated next to or within heterochromatin (Wallrath, 1998). PEV is thought to be caused by the ability of the condensed transcriptionally silent heterochromatin to spread into, or sequester, the neighboring euchromatin in some cells (reviewed in Wallrath, 1998). The HP1-encoding gene *Su(var)2-5* has been demonstrated to suppress PEV when deleted and to enhance PEV when duplicated (Eissenberg *et al.*, 1992), indicating that HP1 is an essential component of heterochromatin required in a precise stoichiometry in order properly to set and/or maintain the inactivated state of genes subject to heterochromatic position effects.

Supporting the notion that mammalian HP1s could also play a role in heterochromatin-mediated silencing, they have been reported (i) to be associated, although not exclusively, with pericentromeric heterochromatin (Minc *et al.*, 1999, 2000; Nielsen *et al.*, 1999); (ii) to silence transcription in a deacetylase activity-dependent manner when directly tethered to DNA (Nielsen *et al.*, 1999); (iii) to cause dose-responsive silencing of centromeric transgenes (Festenstein *et al.*, 1999); (iv) to co-localize with inactive genes in B-cell lines (Brown *et al.*, 1997); and (v) to exhibit conserved heterochromatin targeting and silencing properties when ectopically expressed in *Drosophila* (Ma *et al.*, 2001). Recently, a network of protein–protein interactions has been described involving the HP1 proteins themselves as well as HP1 contacts with the methylated histone H3 N-terminal tail (Bannister *et al.*, 2001; Lachner *et al.*, 2001) and the globular part of the nucleosomes (Nielsen *et al.*, 2001a), interactions that could be relevant to heterochromatin formation and silencing.

Several non-histone proteins have also been implicated in direct or indirect interactions with the HP1 proteins (reviewed in Eissenberg and Elgin, 2000; Li *et al.*, 2002). These HP1-binding proteins include the histone H3-methyltransferase Suv39h1 (Aagaard *et al.*, 1999), the inner nuclear membrane protein lamin B receptor (Ye and Worman, 1996), the inner centromere protein INCENP (Ainsztein *et al.*, 1998), the origin recognition complex ORC (Pak *et al.*, 1997), the transcriptional cofactors TIF1 α and TIF1 β (Le Douarin *et al.*, 1996; Nielsen *et al.*, 1999), the retinoblastoma (Rb) protein (Nielsen *et al.*, 2001b), the TBP-associated factor TAF_{II}130 (Vassallo and Tanese, 2002), the nuclear body component SP100 (Seeler *et al.*, 1998), the 70 kDa subunit of the Ku factor (Song *et al.*, 2001) and the p150 subunit of chromatin assembly factor 1 CAF-1 (Murzina *et al.*, 1999). Two-hybrid interactions between HP1 α and the chromatin-remodeling factor BRG1 have also been reported (Le Douarin *et al.*, 1996). BRG1 (also known as SNF2 β) and its family member BRM/SNF2 α are mammalian homologs of the yeast ATPase SWI2/SNF2 (Muchardt and Yaniv, 1999). These two related proteins have been shown to be part of distinct multisubunit complexes, which possess DNA- and nucleosome-stimulated ATPase activity and have the ability to remodel nucleosomal arrays in an ATP-dependent manner (Wang *et al.*, 1996).

Although the human BRG1 and BRM complexes have been widely associated with transcriptional activation (Muchardt and Yaniv, 1999; Vignali *et al.*, 2000; and references therein), there are now indications that each complex is also implicated in transcriptional repression (Varga-Weisz, 2001). BRG1 has been reported to co-operate with the Rb protein to form a repressor complex, which inhibits transcription of genes for cyclin E and A (Zhang *et al.*, 2000). BRG1 and BRM complexes have been purified from HeLa nuclear extracts that contain components of the SIN3 complex, which is known to be involved in the repression of transcription by a variety of transcription factors (Sif *et al.*, 2001). A recent study has also reported an association of BRG1 with a co-repressor multiprotein complex, N-CoR1, that possesses histone deacetylase activity and includes the HP1-interacting protein TIF1 β (Underhill *et al.*, 2000). These findings prompted us to investigate further the two-hybrid interaction detected between BRG1 and HP1 α in yeast (Le Douarin *et al.*, 1996).

Here, we provide evidence that BRG1 and HP1 α associate in mammalian cells and interact directly with each other *in vitro*, under conditions where very little interaction was observed between BRG1 and HP1 β or HP1 γ . We have examined the molecular basis for this selectivity and identified three amino acid residues in the chromoshadow domain of HP1 α , - β and - γ that play a major role in determining the efficiency of interaction with BRG1. Mutation of these residues not only impairs the ability of HP1 α to interact with BRG1, but also significantly reduces HP1 α -mediated repression. These results provide the first evidence for a physical and functional interaction between a chromatin-remodeling factor and a specific heterochromatin-associated HP1 isoform, and thus have implications for the establishment and/or maintenance of heterochromatin or heterochromatin-like structures *in vivo*.

Fig. 1. Subcellular distribution of endogenous (HP1) and ectopically expressed FLAG epitope-tagged HP1 (f:HP1) proteins in either the parental HeLa cells (control) or the three distinct f:HP1-expressing HeLa-derived cell lines. Cells were lysed by the addition of 0.3% NP-40 in a buffer containing 5 mM MgCl₂ to yield nuclei and the cytoplasmic fraction (C). Nuclei were lysed in a hypotonic buffer containing 10 mM EDTA to separate the nucleoplasmic fraction (NU) from the chromatin pellet (CH). Equivalent samples from each fraction were analyzed by western blotting with mAbs against HP1 α (in A), HP1 β (in B) and HP1 γ (in C). Arrowheads indicate the position of the proteins recognized by each mAb.

Results

BRG1 interacts specifically with HP1 α in mammalian cells

The previous identification of a mouse cDNA encoding amino acids 295–634 of BRG1/SNF2 β in a yeast two-hybrid screen using HP1 α as a bait (Le Douarin *et al.*, 1996) led us to investigate whether these proteins could be physically associated in mammalian cells. Three HeLa-derived cell lines, that stably expressed FLAG epitope-tagged HP1 α , HP1 β or HP1 γ (f:HP1s), were generated (see Materials and methods). These f:HP1-expressing cell lines initially were subjected to a subcellular fractionation procedure to investigate the distribution pattern of the FLAG-tagged proteins. As a control, subcellular fractions from the parental HeLa cell line were also analyzed. When cytoplasmic (C), nucleoplasmic (NU) and chromatin (CH) fractions were tested by western blot analysis (see legend to Figure 1), all three FLAG-tagged proteins, f:HP1 α (31 kDa; Figure 1A), f:HP1 β (32 kDa; Figure 1B) and f:HP1 γ (25 kDa; Figure 1C), were found predominantly associated with the chromatin fraction (lanes 6). Similar chromatin association was observed for the three endogenous HP1s, HP1 α (Figure 1A, lanes 3 and 6), HP1 β (Figure 1B) and HP1 γ (Figure 1C). HP1 α and HP1 β were almost absent from the cytoplasmic and nucleoplasmic fractions (lanes 1 and 2, and 4 and 5 in Figure 1A and B, respectively). In contrast, a small amount of HP1 γ protein was detected reproducibly in these fractions (Figure 1C, lanes 1, 2, 4 and 5), indicating that HP1 γ is partially soluble or more loosely attached to the chromatin than HP1 α and HP1 β . Interestingly, similar results were obtained with the corresponding f:HP1 γ fractions (Figure 1C, lanes 4 and 5). Thus, the distribution pattern

Fig. 2. Selective interaction of BRG1 with HP1 α *in vivo* as well as *in vitro*. (A) Detection of endogenous TIF1 β and BRG1 in f:HP1 immunoprecipitates. Nuclear extracts from the parental HeLa cells (control) or from each of the three HeLa-derived cell lines that express the tagged proteins (f:HP1 α , f:HP1 β and f:HP1 γ) were analyzed by western blotting either directly (input) or following immunoprecipitation with the M2 anti-FLAG antibody (FLAG IP). Western blots were probed with mAbs against TIF1 β or BRG1. The input corresponds to one-twentieth the amount of nuclear extract used for immunoprecipitation. (B) *In vivo* association of BRG1 with wild-type HP1 α . Nuclear extracts from untagged HeLa cells were used for immunoprecipitation with a specific HP1 α mAb (HP1 α IP) or with an irrelevant antibody (anti-FLAG antibody; control IP). A western blot of the immunoprecipitates probed with an anti-BRG1 mAb is shown. Lane 1 (input) corresponds to one-twentieth the amount of nuclear extract used for immunoprecipitation. (C) *In vitro* binding of BRG1 to HP1s. *In vitro* ³⁵S-labeled BRG1 was incubated in a batch assay with 'control' GST (lane 2), GST-HP1 α (lane 3), GST-HP1 β (lane 4) or GST-HP1 γ (lane 5). Bound BRG1 was resolved on SDS-PAGE and visualized by autoradiography. Lane 1 represents one-tenth the amount of input labeled BRG1.

of the FLAG-tagged HP1 proteins is similar to that of the endogenous HP1 proteins.

To verify that f:HP1s are competent for protein-protein interactions *in vivo*, nuclear extracts from the respective HeLa-derived cell lines were immunoprecipitated with the anti-FLAG antibody M2, and the immunoprecipitates were probed for the presence of the silencing cofactor TIF1 β , which was shown previously to be associated with endogenous HP1 α , - β and - γ in P19 EC nuclear extracts (Nielsen *et al.*, 1999). Co-immunoprecipitation of TIF1 β was clearly detected in each f:HP1 immunoprecipitate (Figure 2A, lanes 4, 6 and 8), but not in control immunoprecipitates with nuclear extract from the parental HeLa cells (Figure 2A, lane 2). Thus, f:HP1 proteins, like their endogenous counterparts, are associated with TIF1 β .

We then attempted to detect an association of the HP1 proteins with BRG1 by probing the f:HP1 immunoprecipitates with a specific anti-BRG1 monoclonal antibody (mAb). BRG1 was found in the f:HP1 α immunoprecipitate (Figure 2A, lane 12). BRG1 was not detected in control immunoprecipitations (lane 2). Also, no signal of BRG1 co-purifying with HP1 β or HP1 γ was detected in immunoprecipitates from f:HP1 β and f:HP1 γ nuclear extracts (lanes 14 and 16, respectively). Comparison of BRG1 levels in the load material (input) versus pellets (FLAG IP) indicated that ~2–4% of total BRG1 could be

immunoprecipitated with f:HP1 α (Figure 2A), under conditions where recovery of f:HP1 α was ~20% efficient (data not shown). Similar co-immunoprecipitation was obtained with untagged HP1 α isolated from nuclear extracts of wild-type HeLa cells (Figure 2B, lane 3). Taken together, these results demonstrate that a small but significant fraction of BRG1 is associated with HP1 α in HeLa nuclear extracts.

BRG1 binds to HP1 α *in vitro*

Binding assays between BRG1 and HP1 α were performed *in vitro*. In the same assays, we also tested HP1 β and HP1 γ to assess further the specificity of the interactions observed in mammalian cells. GST-HP1 fusion proteins, GST-HP1 α , GST-HP1 β and GST-HP1 γ , were expressed in *Escherichia coli*, immobilized on glutathione-Sepharose beads and subsequently incubated with *in vitro* synthesized ³⁵S-labeled BRG1. After extensive washing, the matrix-associated BRG1 protein was eluted and visualized by SDS-PAGE and autoradiography. As shown in Figure 2C, only BRG1 and HP1 α demonstrated a significant interaction (lane 3). In contrast, we detected very little binding of BRG1 to GST-HP1 β (lane 4) and GST-HP1 γ (lane 5), and no interaction of BRG1 with GST alone (lane 2). A binding assay carried out with a purified *E.coli*-expressed histidine-tagged BRG1 fragment bearing residues 295–634 [referred to here as His-BRG1(295–634) in Figure 4E] demonstrated direct binding to HP1 α (lane 3), but not to HP1 β (lane 5) and HP1 γ (lane 7), whereas, under similar conditions, purified calf thymus histone H3 and recombinant TIF1 β bound to all three GST-HP1s (Figure 4F and data not shown). Taken together, these results demonstrate that HP1 α , but neither HP1 β nor HP1 γ , can interact directly with BRG1 *in vitro*.

The chromoshadow domain of HP1 α and residues 390–444 of BRG1 are sufficient for interaction

To identify the region of HP1 α to which BRG1 binds, a deletion analysis of HP1 α was performed using the yeast two-hybrid system. Various HP1 α deletion mutants expressed as fusion proteins with the DNA-binding domain (DBD) of the estrogen receptor ER α (Le Douarin *et al.*, 1996) were tested for interaction with BRG1 fused to the acidic activation domain (AAD) of VP16 in a yeast strain containing a *URA3* reporter gene controlled by three ER α -binding sites (PL3; Le Douarin *et al.*, 1995; Figure 3A). As indicated by the orotidine 5'-monophosphate decarboxylase (OMPdecase) activity of the *URA3* gene product, full-length HP1 α interacted with BRG1 (Figure 3B), thus confirming the results obtained in mammalian cells as well as *in vitro*. No interaction was detected with a DBD fusion protein bearing HP1 α residues 1–66 or 67–113, which include the chromodomain and the less conserved central region, respectively (Figure 3B). In contrast, an interaction was observed in the presence of an N-terminally truncated fusion protein lacking the chromodomain [DBD-HP1 α (67–191); Figure 3B] or in the presence of a fusion protein containing the chromoshadow domain only [DBD-HP1 α (106–180); Figure 3B]. Thus, the chromoshadow domain of HP1 α is sufficient for its binding to BRG1.

To investigate whether the chromoshadow domain of HP1 α was also sufficient to promote BRG1 binding in the

context of HP1 β and HP1 γ , a chimera composed of the N-terminal moiety of HP1 β and HP1 γ fused to the C-terminal moiety of HP1 α (amino acids 106–191) was generated (HP1 β/α and HP1 γ/α) and assayed for interaction with BRG1 and TIF1 β in yeast (Figure 3C). When co-expressed with AAD-BRG1, both DBD-HP1 β and DBD-HP1 γ wild-type activated the *URA3* reporter above the AAD background (Figure 3C). However, this activation was much weaker than that observed with DBD-HP1 α (Figure 3C). It is noteworthy that, under similar conditions, co-expression of each DBD-HP1 $\alpha/\beta/\gamma$ fusion with AAD-TIF1 β resulted in the same level of reporter activation (Figure 3C). Thus, as observed *in vitro*, BRG1 interacts preferentially with HP1 α in yeast. Interestingly, DBD-HP1 β/α and DBD-HP1 γ/α chimeras interacted with BRG1 as strongly as DBD-HP1 α (Figure 3C). These results clearly demonstrate that the determinants contributing to BRG1 binding are entirely contained within the chromoshadow domain of HP1 α .

In an attempt to identify the minimal domain in BRG1 that is sufficient for HP1 α interaction, several deletions were introduced into the original BRG1 cDNA clone that had been isolated in the yeast two-hybrid screen using HP1 α as bait [designated HP1-BP72 in Le Douarin *et al.* (1996); and referred to here as AAD-BRG1(295–634)], and the resulting AAD-BRG1 fusion proteins were assayed for interaction with HP1 α in the yeast reporter strain PL3 (Figure 3D). Based on the reporter gene activity, we identified a central region (amino acids 390–517) that was sufficient for interaction. In addition, we found that the C-terminal residues of this region (amino acids 445–517) were dispensable for the interaction, but their presence increased the interaction of

BRG1(390–444) with HP1 α up to 2.2-fold. BRG1(390–444) was also tested for interaction with HP1 β and HP1 γ (Figure 3E). When co-expressed with AAD-BRG1(390–444), DBD-HP1 β and DBD-HP1 γ did not activate the reporter gene above the AAD background (Figure 3E), indicating that, similarly to full-length BRG1, BRG1(390–444) interacts selectively with HP1 α .

Converting HP1 β and HP1 γ into BRG1-interacting proteins

To gain greater insight into the molecular basis underlying the differential interactions of HP1 α , - β and - γ with BRG1, we compared the amino acid sequence of the HP1 α chromoshadow domain with that of HP1 β and HP1 γ . As illustrated in Figure 4A, the HP1 α chromoshadow domain

Fig. 3. The chromoshadow domain of HP1 α and residues 390–444 of BRG1 are sufficient for interaction. (A) Schematic representation of the yeast two-hybrid system used in this study. The DBD of the estrogen receptor ER α (amino acids 176–282) and the AAD of VP16 (amino acids 411–490) unfused or fused to the proteins tested for interaction are shown. The HP1 chromodomain (CD) and chromoshadow domain (CSD) are represented. In the BRG1 protein, boxes indicate the HP1 α -interacting domain initially identified in the yeast two-hybrid screen (amino acids 295–634; Le Douarin *et al.*, 1996) and the DNA-dependent ATPase domain. The *URA3* reporter gene, which is regulated by three estrogen response elements (ERE3X) in the yeast reporter strain PL3, is represented below. (B) BRG1 interacts with the chromoshadow domain of HP1 α . Plasmids expressing individual regions of HP1 α fused to the ER α DBD (as indicated) were introduced into PL3 together with either the 'unfused' VP16 AAD or an AAD fusion containing BRG1 (AAD-BRG1). Transformants were grown in liquid medium containing uracil. Extracts were prepared and assayed for OMPdecase activity, which is expressed in nmol substrate/min/mg protein. (C) The C-terminal chromoshadow domain of HP1 α is sufficient to promote BRG1 binding to HP1 β and HP1 γ . A schematic diagram of the chimeras is shown on the left. Wild-type and chimeras were expressed as DBD fusions in PL3 together with either the VP16 AAD or the VP16 AAD fused to BRG1 or TIF1 β . Two-hybrid interaction assays were performed as in (B). (D) Mapping of the HP1 α -interacting domain in BRG1. The indicated AAD-BRG1 fusions were assayed for two-hybrid interaction with DBD-HP1 α as in (B). (E) Residues 390–444 of BRG1 are sufficient for interaction with HP1 α , but not with HP1 β or HP1 γ . PL3 transformants expressing the indicated DBD and AAD fusion proteins were treated as in (B). Note that in all cases expression of the DBD and AAD fusion proteins was confirmed by western blotting using the antibodies F3 against the F region of ER α and 2GV4 against VP16, respectively (data not shown). In all panels, the values ($\pm$ 10%) are the average of at least three independent experiments.

Fig. 4. Identification of residues within the chromoshadow domain of HP1 α that confer the selectivity of the interaction of HP1 α with BRG1. (A) Amino acid alignment of the chromoshadow domains. The sequences were aligned using the Clustal W program. Secondary structure elements shown were derived from the mouse HP1 β chromoshadow domain (Brasher *et al.*, 2000). Numbers refer to amino acid positions in the corresponding proteins. Residues that form the hydrophobic core and the dimer interface are boxed and shaded, respectively. Mutations introduced into HP1 α are indicated below the alignment. (B) HP1 α interacts with BRG1 in a chromoshadow domain integrity-dependent manner. PL3 transformants expressing the indicated DBD and AAD fusion proteins were treated as described in Figure 3B. OMPdecase activities are given in nmol substrate/min/mg protein. Expression of the DBD fusion proteins was confirmed by western blotting (data not shown). (C) The ability of HP1 α to interact with itself and with HP1 β and HP1 γ in yeast is not affected by the triple substitution mutation I113K/A114P/C133S. Two-hybrid interaction assays were performed as in (B) with the indicated DBD and AAD fusion proteins. (D–F) GST pull-down assays with the various wild-type (WT) and mutant HP1s. *In vitro* ^{35} S-labeled full-length BRG1 (D), purified His-tagged BRG1(295–634) (E) or purified calf thymus histone H3 (F) were incubated in a batch assay with ‘control’ GST (lane 2) or GST fusions containing the indicated wild-type and mutant HP1s (lanes 3–8). Bound BRG1 was resolved by SDS–PAGE and visualized by autoradiography (D) or by western blotting with an anti-His antibody (E). Bound H3 was detected by SDS–PAGE and silver staining (F). Lane 1 shows one-tenth of the amount of input. (G) A triple substitution mutation in the chromoshadow domains of HP1 β and HP1 γ increases BRG1 binding. Two-hybrid interaction assays were performed as in (B) with wild-type and chromoshadow domain mutants for HP1 β and HP1 γ .

differs from that of HP1 β and HP1 γ at only eight positions between amino acids 106 and 180. The importance of these particular residues for BRG1 interaction was investigated by site-directed mutagenesis. Specifically, these residues, I113, A114, E118, K125, C133, D135, T145 and A176, were replaced with the corresponding residues (K, P, A, R, S, E, S and S, respectively) present in HP1 β and HP1 γ . The mutant proteins were then assayed for their ability to interact with BRG1 and TIF1 β in yeast

(Figure 4B). Mutation of E118 (DBD-HP1 α E118A), K125 (DBD-HP1 α K125R), D135 (DBD-HP1 α D135E), T145 (DBD-HP1 α T145S) or A176 (DBD-HP1 α A176S) had no detectable effect on interaction with BRG1 or TIF1 β (Figure 4B). In contrast, mutation of I113 (DBD-HP1 α I113K), A114 (DBD-HP1 α A114P) and C133 (DBD-HP1 α C133S) reduced the binding of HP1 α to BRG1, with C133S having the most pronounced effect (an ~2.5-fold reduction), whereas the binding to TIF1 β was

not affected (Figure 4B). We next examined the interaction properties of a I113K/A114P/C133S triple substitution mutant. As compared with wild-type HP1 α , HP1 α I113K/A114P/C133S was not affected in its ability to interact with TIF1 β (Figure 4B) and to form homodimers and heterodimers with HP1 β and HP1 γ (Figure 4C). In contrast, a 5-fold reduction in BRG1 interaction was observed (Figure 4B). This interaction was also examined in a GST pull-down assay (Figure 4D and E). In agreement with the two-hybrid interaction data, HP1 α I113K/A114P/C133S showed a decreased binding to *in vitro* translated full-length BRG1 (Figure 4D, compare lanes 3 and 4). No interaction was detected with purified His-BRG1(295–634) (Figure 4E), whereas under similar conditions the binding to H3 was not affected (Figure 4F). These results clearly demonstrate that amino acid residues I113, A114 and C133 of the chromoshadow domain of HP1 α are critical for interaction with BRG1.

The reciprocal K to I, P to A and S to C triple substitution mutation was then introduced into full-length HP1 β and HP1 γ . HP1 β K109I/P110A/S129C interacted with BRG1 more efficiently than wild-type HP1 β in yeast (Figure 4G) and *in vitro* (Figure 4D and E, compare lanes 5 and 6), under conditions in which the binding to H3 was not affected (Figure 4F). The same mutation in the corresponding residues of HP1 γ produced an HP1 γ variant (HP1 γ K103I/P104A/S123C) that became as potent as HP1 α in binding BRG1 (Figure 4D, E and G). Thus, we have identified three amino acid residues in the chromoshadow domain of HP1 α , - β and - γ that play a major role in determining the efficiency of the interaction with BRG1.

BRG1 interaction enhances HP1-mediated transcriptional repression

HP1 α , - β and - γ have been shown previously to repress transcription when directly tethered to a promoter in cell culture assays (Nielsen *et al.*, 1999; and references therein). Because in these assays HP1 α was found reproducibly to be more efficient than HP1 β and HP1 γ in repressing transcription, we investigated whether HP1 α requires BRG1 interaction for full repression. To this end, the repressive activity of wild-type HP1 α was compared with that of HP1 α mutants, which were impaired in their ability to interact with BRG1. GAL4-HP1 α fusion derivatives bearing the single C133S amino acid change or the triple I113K/A114P/C133S mutation 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 estrogen response element (ERE) in front of a β -globin (G) promoter–CAT fusion (17M2-ERE-G-CAT; Figure 5A). As described previously (Nielsen *et al.*, 1999), expression of the GAL4-HP1 α protein resulted in a significant, dose-dependent repression of the reporter (Figure 5B). In contrast, a much weaker repression was observed in the presence of the mutant GAL4-HP1 α C133S and GAL4-HP1 α I113K/A114P/C133S proteins (Figure 5B); an ~100-fold repression was seen with saturating concentrations of GAL4-HP1 α WT expression vector (Figure 5B), whereas under the same conditions GAL4-HP1 α C133S and GAL4-HP1 α I113K/A114P/C133S repressed by only ~20- and

Fig. 5. BRG1 contributes to HP1 α -mediated transcriptional repression. (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) BRG1 interaction is required for maximal HP1 α -mediated repression. Cos-1 cells were transiently transfected with 1 μ g of 17M2-ERE-G-CAT reporter, 1 μ g of pCH110 (expressing β -galactosidase), 100 ng of ER(C)-VP16 and increasing amounts (2–1000 ng) of expression vectors coding for the indicated wild-type (WT) and mutated GAL4-HP1 α chimera. The CAT activity achieved is expressed relative to the CAT activity seen after co-transfection with the expression vector coding for the unfused GAL4 DBD. Values ($\pm$ 10%) represent the average of three independent duplicated transfections after normalization to the internal control β -galactosidase activity of pCH110. (C and D) The triply mutated HP1 β (C) and HP1 γ (D) mutants that interact with BRG1 are more efficient than the wild type in repressing transcription when tethered to DNA. Co-transfection assays were performed as in (B) with the indicated wild-type and mutated GAL4-HP1 chimera. Fold repression was calculated as shown in (B). In all panels, expression of the fusion proteins was confirmed by western blot using the antibody 2GV3 against the GAL4 DBD (data not shown).

7-fold, respectively (Figure 5B). Western blot analysis using an antibody against the GAL4 DBD indicated similar expression levels for each of these fusion proteins (see legend to Figure 5). Thus, the full repressing potential of HP1 α on transcription requires BRG1 binding.

In a converse experiment, we introduced the three HP1 α amino acids I113, A114 and C133 into the equivalent positions of the HP1 β and HP1 γ chromoshadow domains to generate triply mutated GAL4-HP1 β K109I/P110A/S129C and GAL4-HP1 γ K103I/P104A/S123C fusion proteins. The mutants repressed transcription more efficiently than the GAL4 chimera containing the corres-

Table I. BRG1 interaction and transcriptional repression activities of wild-type and mutant HP1 proteins

	Interaction with BRG1 ^a	Transcriptional repression ^b
HP1 α WT	+++	+++
HP1 α C133S	+	+
HP1 α I113K/A114P/C133S	+/-	+/-
HP1 β WT	+/-	+/-
HP1 β K109I/P110A/S129C	+	+
HP1 γ WT	+	+
HP1 γ K103I/P104A/S123C	+++	+++

+++, + and +/- denote decreased repression activities.

^aDetermined by the yeast two-hybrid system as described in Figure 4. The relative levels of interaction were scored from background level (-) to strong (+++).

^bDetermined in transient transfection assays as described in Figure 5.

ponding wild type (Figure 5C and D). GAL4-HP1 β K109I/P110A/S129C repressed transcription up to twice better than the wild type (Figure 5C), whereas GAL4-HP1 γ K103I/P104A/S123C repressed transcription to a level comparable with that seen with GAL4-HP1 α WT (Figure 5D). These results are consistent with the distinct ability of the various wild-type and mutated HP1 proteins to bind BRG1 (summarized in Table I), and therefore provide strong evidence for a correlation between the interaction of the mutated HP1s with BRG1 and their ability to repress transcription, indicating that this interaction is involved in transcriptional repression.

Discussion

The chromoshadow domains of HP1 α , HP1 β and HP1 γ can be distinguished by differential interactions with BRG1

Like other HP1 family proteins, HP1 α , - β and - γ possess an N-terminal chromodomain connected by a less conserved hinge region to a C-terminal chromoshadow domain (Eissenberg and Elgin, 2000; Li *et al.*, 2002). They display >80% sequence identity and, according to this high degree of structural similarity, they share several properties, including the ability to associate with pericentromeric constitutive heterochromatin (Minc *et al.*, 1999, 2000; Nielsen *et al.*, 1999), to possess nucleosome-binding activity (Nielsen *et al.*, 2001a), to form homo- as well as heteromers (Nielsen *et al.*, 2001a) and to silence transcription in a deacetylase-dependent manner when directly tethered to DNA (Nielsen *et al.*, 1999).

Despite these structural and biochemical similarities, HP1 α , - β and - γ may not be functionally redundant. Indeed, they also differ in some properties. In both human and mouse cells, they have been reported to exhibit differential patterns of subnuclear distribution. While all three isoforms are associated with pericentromeric heterochromatin, HP1 β and HP1 γ , but not HP1 α , also localize to euchromatin, and HP1 γ does so predominantly (Nielsen *et al.*, 1999; Minc *et al.*, 2000; and references therein). Thus, HP1 β and HP1 γ have less specificity for heterochromatin than HP1 α , suggesting that these HP1s may exert specialized functions in the euchromatic compartment. In contrast to HP1 α and HP1 β , HP1 γ distribution

was also reported to be highly dynamic during the cell cycle (Minc *et al.*, 1999) and, in accordance with this observation, we show here that HP1 γ is less tightly associated with chromatin than HP1 α and HP1 β . Another important characteristic that distinguishes HP1 α , - β and - γ is their cell cycle-related phosphorylation pattern; HP1 β is unphosphorylated throughout the cell cycle, while HP1 α and HP1 γ are phosphorylated during interphase and even hyperphosphorylated during mitosis (Minc *et al.*, 1999).

Family proteins that are functionally and biochemically distinct from each other are expected to exhibit specificity in their molecular interactions. We and others have identified a number of HP1-interacting proteins involved in chromatin silencing, DNA replication, histone deposition and subnuclear organization (reviewed in Li *et al.*, 2002; see also Introduction). Among these binding proteins, several have been reported to interact with each HP1 isotype (e.g. histone H3, TIF1 α , TIF1 β , CAF-1 and SP100). In contrast, TAF $_{II}$ 130 binds to HP1 α and HP1 γ , but not to HP1 β (Vassallo and Tanese, 2002), while the linker histone H1 interacts with HP1 α only (Nielsen *et al.*, 2001a). The region conferring this selective binding has been mapped to the hinge region separating the chromo- and chromoshadow domains (Nielsen *et al.*, 2001a). Here, we report differential association of the three HP1 proteins with the chromatin-remodeling BRG1 factor. HP1 α was found to interact with BRG1 both *in vitro* and *in vivo*, whereas HP1 β and HP1 γ interacted only weakly, if at all, with this protein. This result strongly supports the notion that HP1 α , HP1 β and HP1 γ participate in the formation of distinct complexes *in vivo* (Aagaard *et al.*, 1999) and provides new clues for functional differences between the various HP1 family proteins.

The determinants responsible for the selective binding of BRG1 to HP1 α reside within the C-terminal chromoshadow domain. This result was particularly intriguing because the chromoshadow domains of HP1 β and HP1 γ are highly homologous to that of HP1 α , having an amino acid identity of 88%. Most of the proteins interacting with HP1 α , - β and - γ have been reported to associate through the chromoshadow domain. Thus, although highly conserved among the various HP1 family members, this domain can be involved in both specific and common interactions. Recently, a pentapeptide sequence of consensus P[R Φ]V[S Φ]L (where Φ is a hydrophobic amino acid) was shown to be necessary and sufficient for binding of the chromoshadow domain by many HP1 effectors, including TIF1 β (Murzina *et al.*, 1999; Nielsen *et al.*, 1999; Smothers and Henikoff, 2000). This consensus motif is absent from the HP1 α -interacting domain of BRG1 (amino acids 390–444) and, consistent with this, we identified three amino acid residues in the chromoshadow domain of HP1 α (I113, A114 and C133) that were critical for BRG1, but not TIF1 β , interaction. Moreover, mutations in these residues did not change the ability of HP1 α to form homodimers as well as heterodimers with HP1 β and HP1 γ . In the three-dimensional structure of the chromoshadow domain from mouse HP1 β (Brasher *et al.*, 2000), HP1 α residues I113 and A114 and their HP1 β equivalents (K109 and P110) are located in the N-terminal tail, which is not as well structured as the rest of the domain, while the HP1 β S129 residue corresponding to HP1 α C133 lies in a surface-exposed loop between

β strands 1 and 2 (see Figure 4A). Thus, the mutations introduced in these residues may perturb a functionally important BRG1 contact, leaving other contacts in the chromoshadow domain unperturbed.

The role of BRG1 in the modulation of HP1 α -containing heterochromatic structures

The present study provides evidence for a functional and physical interaction between BRG1 and the heterochromatic HP1 α protein. Immunoprecipitation assays show that BRG1 and HP1 α form a complex *in vivo*. *In vitro* protein–protein interaction assays demonstrate that BRG1 and HP1 α interact directly with each other. Cell culture assays indicate that this interaction contributes to HP1 α -mediated repression. These results suggest a direct role for BRG1 in the modulation of repressive higher order chromatin structures containing HP1 α .

There are several mechanisms by which BRG1 could contribute to HP1 α -dependent silencing. Reconstitution studies have shown that on its own BRG1 can remodel nucleosomes in an ATP-dependent manner (Phelan *et al.*, 1999), indicating that the presence of this ATPase subunit within a complex is sufficient for chromatin remodeling. We have demonstrated previously that HP1 α can bind to nucleosomes *in vitro* and associates with chromatin *in vivo* through a direct interaction with the histone fold domain of histone H3 (Nielsen *et al.*, 2001a). Thus, the chromatin-remodeling activity of BRG1 may facilitate the binding of HP1 α to its nucleosomal sites. Recent studies have also described a specific binding of HP1 α to the methylated tail domain of H3, which is critical for its targeting to centromeric heterochromatin (Bannister *et al.*, 2001; Lachner *et al.*, 2001). Similarly, the underacetylated state of the histone tails is an important determinant for the maintenance of the HP1 α protein at heterochromatic sites (Taddei *et al.*, 2001) and for full silencing (Nielsen *et al.*, 1999). Thus, the BRG1 activity may enhance access of the histone tails to HP1 α -associated deacetylases and methyltransferases, which may, in turn, promote the formation of a local, heterochromatin environment that results in effective gene silencing. Another way in which BRG1 might participate in the assembly of HP1 α -dependent heterochromatic structures is to alter nucleosome spacing. In general, sequences with repressive chromatin domains are packaged into highly regular nucleosome arrays, the regularity of which correlates with gene silencing (Sun *et al.*, 2001). Thus, it is tempting to speculate that BRG1 may contribute to HP1 α -mediated silencing by manipulating nucleosomal spacing.

Silencing through nucleosome remodeling is probably a general property of the DNA-dependent ATPases of the SWI2/SNF2 superfamily. Recently, the *domino* gene of *Drosophila* has been demonstrated to encode two novel members of the SWI2/SNF2 family of proteins, which contribute to the silencing of homeotic genes (Ruhf *et al.*, 2001). *Drosophila* Mi-2, another DNA-dependent ATPase of the SWI2/SNF2 family, functions in Polycomb silencing (Kehle *et al.*, 1998). The human counterparts of Mi-2, Mi-2 α and Mi-2 β , have been found recently, together with the transcriptional regulator Ikaros, in toroidal structures that form around heterochromatin (Kim *et al.*, 1999). Along the same line, the *decreased DNA methylation 1* (*DDM1*) gene encodes a plant member of the SWI2/SNF2

protein family, that is required for maintenance of methylated gene silencing (Habu *et al.*, 2001; and references therein). *DDM1* mutations cause changes in patterns of DNA methylation, similar to mutations in the human *ATRX* gene (Gibbons *et al.*, 2000), which encodes an SWI2/SNF2-like protein. Interestingly, in a previous two-hybrid screen using HP1 α as bait (Le Douarin *et al.*, 1996), we have isolated mouse *ATRX* (referred to as HP1-BP38), indicating that HP1 α may interact not only with BRG1, but also with other members of the SWI2/SNF2 family.

Although our data provide support for a role of BRG1 in mediating HP1 α -dependent silencing, it remains possible that association of HP1 α with BRG1 is also involved in the selective targeting of BRG1 to unique heterochromatic sites, where the chromatin remodeling factor could relieve the overall repressed environment and maintain a transcriptionally active state. Relevant to this is the recent demonstration from genetic studies in *Drosophila* that HP1 is not only a dosage-dependent silencer of euchromatic genes exhibiting PEV, but also a dosage-dependent activator of heterochromatic genes (Eissenberg and Hilliker, 2000).

Heterochromatin not only plays a role in transcriptional regulation, but also contributes to centromere function. Studies in *S.pombe* and *Drosophila* have shown that SWI6/HP1 is required for sister centromere cohesion during cell division (Bernard *et al.*, 2001; Nonaka *et al.*, 2002) and proper chromosome segregation (Kellum and Alberts, 1995). Supporting the notion that mammalian HP1 proteins also have a centromere function, they are associated with centromeric heterochromatin in both interphase and mitotic cells (HP1 α and β ; Minc *et al.*, 1999; Remboutsika *et al.*, 1999; and references therein), interact with INCENP (HP1 α ; Ainsztein *et al.*, 1998) and cohesin (HP1 α ; Nonaka *et al.*, 2002), while Suv39h-deficient mice display enhanced chromosomal instability with a higher frequency of non-homologous pairing during male meiosis (Peters *et al.*, 2001). On the basis of these data, the association of BRG1 with HP1 α described here may be part of a nuclear event that occurs at the centromeres during cell division. In support of such a hypothesis is the recent finding that BRG1, and not the closely related member BRM which has no HP1-binding activity (Le Douarin *et al.*, 1996), is part of a high molecular weight complex, called PBAF (SWI/SNF-B), that localizes at the kinetochores of mitotic chromosomes (Xue *et al.*, 2000).

In conclusion, our results provide evidence indicating that HP1 family proteins are functionally distinct and point to a novel role for BRG1 in the organization of heterochromatic structures. The identification of the HP1 α amino acid residues that contribute to its selective interaction with BRG1 will allow us to assess the biological consequences of this interaction by site-directed mutagenesis *in vivo*, which should provide further insight into the role played by BRG1 in heterochromatin.

Materials and methods

Plasmids

Details on individual plasmid constructs, which were all verified by sequencing, are available upon request. Human BRG1 cDNA and mouse

cDNAs for HP1 α , HP1 β , HP1 γ and TIF1 β have been described previously (Le Douarin *et al.*, 1996; Ichinose *et al.*, 1997). To create FLAG-tagged HP1-expressing cell lines, the full-length coding sequences of HP1 α , β and γ were introduced in-frame into a modified version of the puromycin resistance-encoding vector pSG5puro (Nielsen *et al.*, 2001a). For GST pull-down assays, the indicated cDNAs were fused to GST in the pGEX2T plasmid (Pharmacia). For expression of ^{35}S -labeled BRG1, the coding sequence of BRG1 was inserted into the pSG5 vector, and coupled transcription/translation was performed using the T7 RNA polymerase with the TNT lysate system (Promega). The His-tagged BRG1(295–634) construct has been obtained by cloning the cDNA encoding amino acids 295–634 of BRG1 into the pRSET plasmid (Invitrogen). 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.*, 1995, 1996).

Antibodies

mAbs used include: anti-HP1 α mAbs, 2HP1-1H5 for immunoprecipitation and 2HP-2G9 for western blot analysis (Remboutsika *et al.*, 1999); anti-HP1 β mAb, 1Mod-1A9; anti-HP1 γ mAb, 2Mod-1G6; and anti-TIF1 β mAb 1Tb3 (Nielsen *et al.*, 1999); anti-BRG1 mAb, 2SNF-2E12 (Remboutsika *et al.*, 1999); anti-FLAG mAb, M2 (Kodak); anti-VP16 mAb, 2GV4; and anti-ER α mAb, F3, raised against the F region of human ER α (Le Douarin *et al.*, 1995). Anti-His antibody was from Santa Cruz Biotechnologies.

Cell line establishment

HeLa cells were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 5% fetal calf serum. A total of 5×10^5 cells were transfected by calcium phosphate precipitation with 10 μg of expression plasmids (FLAG-HP1 α , FLAG-HP1 β and FLAG-HP1 γ). Cells were selected with 0.3 $\mu\text{g}/\text{ml}$ puromycin (Sigma) added to the growth medium 48 h after transfection over a period of 2 weeks with regular medium changes. Several puromycin-resistant colonies were picked and expanded for western blot and immunoprecipitation experiments.

Cell fractionation

Cells were fractionated into cytosolic, nucleoplasmic and chromatin fractions as described previously (Remboutsika *et al.*, 1999).

Nuclear extract preparation and immunoprecipitation

Nuclear extracts from the wild-type and tagged HeLa cells were analyzed by western blotting either directly or following immunoprecipitation as described previously (Nielsen *et al.*, 1999).

In vitro binding assays

GST pull-down assays were performed as described previously (Nielsen *et al.*, 1999). GST and GST-HP1 fusion proteins were expressed in *E. coli* and purified on glutathione-Sepharose beads (Pharmacia), as described by the manufacturer.

Yeast two-hybrid interaction assays

Yeast two-hybrid experiments were carried out as described previously (Le Douarin *et al.*, 1995).

Acknowledgements

We are grateful to I. Michel for technical assistance, S. Vicaire for DNA sequencing, F. Ruffenach and A. Staub for oligonucleotide synthesis, and F. Cammas for critical reading of the manuscript. This work was supported by the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, l'Hôpital Universitaire de Strasbourg (H.U.S.), the association pour la Recherche sur le Cancer, the Collège de France, the Fondation pour la Recherche Médicale (FRM) and Bristol-Myers-Squibb. A.L.N. is the recipient of fellowships from EMBO and the Danish Cancer Society. C.S. was supported by the Fundación para el Futuro de Columbia and the ARC. H.I. was supported by a fellowship from INSERM.

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Received March 27, 2002; revised August 14, 2002;
accepted September 3, 2002