ABSTRACT. Three subtypes of HP1, a conserved non-histone chromosomal protein enriched in heterochromatin, have been identified in humans, HP1α, β and γ. In the present study, we utilized a Drosophila system to characterize human HP1 functions. Over-expression of HP1β in eye imaginal discs caused abnormally patterned eyes, with reduced numbers of ommatidia, and over-expression of HP1γ in wing imaginal discs caused abnormal wings, in which L4 veins were gapped. These phenotypes were specific to the HP1 subtypes and appear to reflect suppressed gene expression. To determine the molecular domains of HP1 required for each specific phenotype, we constructed a series of chimeric molecules with HP1β and HP1γ. Our data show that the C-terminal chromo shadow domain (CSD) of HP1γ is necessary for HP1γ-type phenotype, whereas for the HP1β-type phenotype both the chromo domain and the CSD are required. These results suggest human HP1 subtypes use different domains to suppress gene expression in Drosophila cells.

Key words: heterochromatin/chromo domain/chromo shadow domain/transgenic fly

Introduction

Heterochromatin protein 1 (HP1) is a major constituent of heterochromatin and plays a key role in its formation and maintenance (Eissenberg and Elgin, 2000; Grewal and Elgin, 2002). HP1 is highly conserved from yeast to human (Li et al., 2002). HP1 was first identified as a gene product of an allele of Su(var)2-5 in Drosophila melanogaster (James and Elgin, 1986; Eissenberg et al., 1990). In Drosophila, it is localized in pericentric and telomeric heterochromatin and plays a role in condensation and segregation of chromosomes and telomere capping (Kellum and Alberts, 1995; Fanti et al., 2003; Perrini et al., 2004). Recruitment of HP1 to certain sites of the genome involves interaction with various chromatin components and depends on methylation of lysine 9 on histone H3 (H3K9) (Petters et al., 2003; Thiru et al., 2004; Stewart et al., 2005). Both trimethylated H3K9 and HP1 appear to be important for establishing and maintaining domains of heterochromatin (Sims et al., 2003). In human cells, three subtypes of HP1 have been identified and designated as HP1α, HP1β and HP1γ (Singh et al., 1991; Saunders et al., 1993; Ye and Worman, 1996). All of these HP1 family proteins consist of two highly conserved regions, the N-terminal chromo domain (CD) and the structurally related C-terminal chromo shadow domain (CSD), connected by a hinge region (Eissenberg and Elgin, 2000). The CSD motif appears unique to the HP1 family. In contrast, the CD motif is also found in Su(var)3-9 and Polycomb family proteins (Jones et al., 2000). It is reported that HP1 family members interact
with various other proteins through CSD (Le Douarin et al., 1996; Murzina et al., 1999; Li et al., 2002; Vassallo and Tanese, 2002), while the hinge region appears to be important for nuclear targeting (Smothers and Henikoff, 2001) and RNA-binding (Muchardt et al., 2002). In addition, CD is known to mediate the interaction of HP1 family proteins with H3K9 (Bannister et al., 2001; Lachner et al., 2001).

HP1α and β are concentrated in pericentric heterochromatin, but HP1γ may exhibit differential localization, always with some accumulation in pericentromeric heterochromatin (Minc et al., 1999; Minc et al., 2000). HP1β and γ can also be found in euchromatic regions (Minc et al., 1999; Minc et al., 2000; Nielsen et al., 2001a), where they are presumably involved in gene repression (Nielsen et al., 2001b; Li et al., 2002). Intracellular localization of HP1 subtypes dynamically changes during the cell cycle (Hayakawa et al., 2003). HP1β is replaced at the centromere by HP1α as the cell enters metaphase (Hayakawa et al., 2003). The N-terminal region of HP1β, including the CD, is responsible for localization to interphase centromeres and the C-terminal region, including the CSD, is responsible for localization to metaphase centromeres (Hayakawa et al., 2003). However, it has yet to be determined in detail whether differences in sub-cellular localization of HP1 family proteins relate to their functions in tissues of multi-cellular organisms.

Human HP1α, HP1β, and HP1γ have been successfully expressed in transgenic Drosophila and are associated with heterochromatin in Drosophila chromosomes (Ma et al., 2001; Norwood et al., 2004). Ectopic expression of HP1α and HP1γ significantly enhanced heterochromatic silencing in Drosophila, but that of HP1β failed (Ma et al., 2001; Norwood et al., 2004). In addition, expression of HP1α rescued the lethality of homozygous Su(var)2-5 mutants lacking HP1 (Norwood et al., 2004). These results indicate that at least some aspects of heterochromatic structure are highly conserved throughout the evolution of eukaryotes.

In the present study, we utilized a Drosophila system to characterize actions of human HP1α, HP1β and HP1γ when over-expressed in different tissues and developmental stages. Our results suggest that HP1β and γ each cause tissue-specific phenotypes due to suppressed gene expression. To determine the molecular domains of HP1 required for these actions, we constructed a series of chimeric molecules between HP1β and HP1γ. Our data show that CSD of HP1γ is necessary for the HP1γ-type phenotype (wing phenotype), while both CD and CSD are required for the HP1β-type phenotype (eye phenotype). These results suggest human HP1 subtypes use different domains to suppress gene expression in Drosophila cells.

**Materials and Methods**

**Fly stocks**

Fly stocks were maintained at 25°C on standard food. The Canton S fly was used as the wild-type strain. The GMR-GAL4 line (number 16) was described previously (Takahashi et al., 1999). The following enhancer-GAL4 lines were kindly provided by Dr N. Dyson (MGH Cancer Center): decapentaplegic-GAL4 (dpp-GAL4), sevenless-GAL4 (sev-GAL4), eyeless-GAL4 (ey-Gal4), 22B-GAL4, 71B-GAL4, Hsp70-GAL4 (hs-GAL4), engrailed-GAL4 (en-GAL4), apterous-GAL4 (ap-GAL4), twist-GAL4, Act88F-GAL4, veinlet-GAL4 (ve-GAL4), wingless-GAL4 (wg-GAL4), 3735-GAL4, 2689-GAL4, Sg-GAL4.

**Plasmids**

To construct the plasmids pUAST-HP1α, pUAST-HP1β, pUAST-HP1γ and pUAST-dHP1α, cDNA fragments of human HP1α, HP1β, HP1γ and Drosophila HP1α (dHP1α) were obtained by PCR using appropriate oligonucleotide primers with EcoRI and Xhol linkers and HP1α, HP1β, HP1γ and dHP1α cDNAs as templates. The PCR products were digested with EcoRI and XhoI and inserted into the pUAST vector. To construct chimera HP1s, the following regions were used: BG 2, amino acids 1 to 116 of HP1α and amino acids 76 to 173 of HP1γ; BG1, amino acids 1 to 75 of HP1γ and amino acids 111 to 173 of HP1γ; GB2, amino acids 1 to 110 of HP1γ and amino acids 117 to 185 of HP1γ; and GB1, amino acids 1 to 75 of HP1γ and amino acids 77 to 185 of HP1β (Hayakawa et al., 2003).

**Establishment of transgenic flies**

Transgenic lines for pUAST-HP1α, -HP1β, -HP1γ, -dHP1α and various chimera HA-tagged HP1s were generated by P element-mediated germ line transformation as described earlier (Spradling, 1986). F1 transformants were selected on the basis of white eye color rescue (Robertson et al., 1988). Several independent lines for each construct were established and confirmed to exhibit essentially the same phenotype.

**Immunostaining of polytene chromosomes**

Salivary glands from third instar larvae were dissected in 0.7% NaCl, fixed in 45% acetic acid and squashed in 33.3% lactic acid/50% acetic acid. Squashes were incubated with mouse monoclonal antibody specific for HP1β and HP1γ (Euromedex, Souffelweyersheim, France) at 1:1,000 dilution, or culture supernatant of hybridoma cells-producing mouse anti-βA monoclonal antibody (supplied by M. Inagaki, Aichi Cancer Center Research Institute) (1:100) at 25°C for 2 h. After extensive washing with PBS containing 0.2% Tween 20 and 1% BSA, samples were incubated at 25°C for 2 h with anti-mouse IgG conjugated with Alexa488 (Molecular Probes [Invitrogen], Carlsbad, CA, USA) at 1:400 dilution. Slides were mounted in Vectashield (Vector Laboratory, Peterborough,
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England) with 4,6-diamidino-2-phenylindole (DAPI) (0.05 µg/ml) for microscopic observation. The microscopic images were obtained by Olympus BX-50 microscope equipped with cooled CCD camera (Hamamatsu Photo., Shizuoka, Japan).

**Western immunoblot analysis**

To perform Western blots, third instar larvae were homogenized in SDS sample buffer and heated at 95°C for 10 min. Proteins were applied to SDS-polyacrylamide gels containing 12% acrylamide. After SDS-PAGE, proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). The blotted membranes were incubated with mouse monoclonal anti-HP1β antibody (Euromedex, Souffelweyersheim, France) at 1:2,000 dilution, mouse monoclonal anti-HP1γ antibody (Euromedex, Souffelweyersheim, France) at 1:4,000 dilution or mouse monoclonal anti-α tubulin antibody (Sigma, St. Louis, MO, USA) at 1:2,000 dilution. After washing, the membranes were incubated with HRP-conjugated anti-mouse IgG (GE Healthcare, Buckinghamshire, England). Visualization was carried out using ECL (GE Healthcare, Buckinghamshire, England) and images were analyzed with a Lumivision Pro HSII image analyzer (Aisin Seiki, Aichi, Japan).

**Over-expression experiments**

Transgenic lines carrying various UAS-HP1s or UAS-dHP1a were crossed with the different GAL4-driver lines described above and raised at 28°C. For heat shock induction of proteins, third instar larvae were heat-shocked for 1 h at 37°C, recovered for 2 h at 25°C, then subjected for either immunostaining or Western immunoblot analyses.

**Results**

**Functional differences in human HP1 isoforms**

In order to examine the effects of human HP1 isoforms on *Drosophila* development, we generated transgenic fly lines carrying UAS-HP1α, UAS-HP1β or UAS-HP1γ and crossed them with fifteen different GAL4-driver strains. HP1β and HP1γ generated abnormality in specific tissues (Table I). Over-expression of HP1β with the ey-GAL4 driver caused abnormally patterned eyes (100% penetrance) (Fig. 1A), with an approximately 50% reduction in the number of ommatidia, and an abnormal head phenotype (Fig. 1B) (the HP1β-type phenotype). In contrast, no changes were observed with over-expression of HP1α and γ under control of the ey-GAL4 driver (Table I).

With 32B-GAL4/UAS-HP1γ and en-GAL4/UAS-HP1γ, flies exhibited abnormal wings, in which wing vein L4 was gapped (32B-GAL4: male 100% (n=32), female 19.1% (n=62), en-GAL4: male 40.9% (n=44), female 60% (n=60)) (Fig. 1C, arrows) (the HP1γ-type phenotype). In addition, a curled wing phenotype was observed with dpp-GAL4/UAS-HP1γ (data not shown). In contrast, over-expression of HP1α and β with the 32B-GAL4, the en-Gal4 or the dpp-

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**Table I. SUMMARY OF EFFECT OF hHP1β, γ EXPRESSION WITH EACH GAL4 DRIVER LINES**

<table>
<thead>
<tr>
<th>GAL4 line</th>
<th>Chromosome linkage</th>
<th>Phenotype</th>
<th>hHP1α</th>
<th>hHP1β</th>
<th>hHP1γ</th>
<th>dHP1a</th>
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<tbody>
<tr>
<td>GMR</td>
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<tr>
<td>eyeless</td>
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<td>small eye</td>
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<td>—</td>
<td>vein</td>
<td>wing</td>
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<tr>
<td>en</td>
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<td>vein</td>
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<tr>
<td>32B</td>
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<td>vein</td>
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<td>71B</td>
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<td>act88F</td>
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<td>—</td>
<td>—</td>
<td>ND</td>
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</table>

— No detectable phenotype, ND: Not determined
GAL4 did not have any discernible influence on the phenotype (Table I). These results suggest that human HP1 isoforms have functional differences in *Drosophila* as observed with cultured mammalian cells (Hayakawa et al., 2003).

**Distribution patterns of HP1β and HP1γ on polytene chromosomes**

For some reason, no alteration was observed on ectopic expression of HP1α with any of the GAL4 drivers used. We therefore focused on differences between HP1β and HP1γ by immunostaining salivary gland polytene chromosomes from transgenic larvae (hs-GAL4/UAS-HP1β or hs-GAL4/ UAS-HP1γ) with HP1β- and HP1γ-specific antibodies (Figs. 2A and 3A). Neither the anti-HP1β nor the anti-HP1γ antibodies are cross-reactive to *Drosophila* HP1 family proteins, since neither of these antibodies detected the endogenous HP1 family proteins on the yw salivary gland polytene chromosomes in immunostaining (Figs. 2A and 3A, g to I) and Western immunoblot analyses (Figs. 2B and 3B). HP1β and HP1γ were both associated primarily with heterochromatic chromocenters (Figs. 2A and 3A, arrows), localized in the telomeric regions of the chromosomes (Figs. 2A and 3A, arrowheads).

While both were found in euchromatic regions, the
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HP1β-staining was in sharp bands (Fig. 2A, d to f). In contrast, HP1γ-staining was more diffuse (Fig. 3A, d to f). To test the possibility that HP1γ does not bind tightly to chromatin or chromosome, we decreased the amount of HP1γ expression in salivary gland cells. Without heat shock induction with the hs-GAL4 driver, only 38% of HP1γ was expressed compared with the heat-shocked samples (Fig. 3B). Under these conditions, HP1γ was mainly localized at...
With heat shock induction, HP1γ localization was expanded through the euchromatic region (Fig. 3A, d to f). In contrast, no difference of HP1β localization on the chromosomes was observed with and without heat shock and only the signal intensity changed (Fig. 2A, a and d), despite the 2.6 fold increase of HP1β after the heat shock (Fig. 2B). These data suggest that HP1β and HP1γ localize on chromosomes differently and bind in different ways.

**The CSD of HP1γ is required for the HP1γ-specific phenotype**

Although HP1 isoforms share significant similarities in their amino acid sequences (Fig. 4A), their over-expression using several GAL4 drivers in *Drosophila* here resulted in...
different phenotypes. Thus, we determined the necessary molecular domains of HP1 required for each specific phenotype. To this end, we constructed a series of chimeric molecules between HP1β and HP1γ (Fig. 5A). Over-expression of BG1 (HP1β CD+HP1γ hinge and CSD) and BG2 (HP1β CD and hinge+HP1γ CSD) using 32B-GAL4 driver resulted in the HP1γ-specific wing phenotype (BG1; males 55.8% (n=43), females 30.3% (n=79), BG2; males 82.4% (n=34), females 23.5% (n=51)) (Fig. 5B, a and b). In contrast, no detectable wing phenotype in adult external morphology was observed on over-expression of GB1 (HP1γ CD and HP1β hinge+CSD) or GB2 (HP1γ CD+hinge and HP1β CSD) using 32B-GAL4 driver (Fig. 5B, c and d). These results suggest that CSD of HP1γ is required for the HP1γ-specific wing phenotype.

Next we examined the molecular domains of HP1 required for the HP1β-specific eye phenotype induced by over-expression with eyeless-GAL4. However, expression of chimeric proteins resulted in no detectable phenotype (Fig. 5A). These results suggest that the whole of HP1β is required for the HP1β-specific eye phenotype, although the possibility remains that the hinge domain is also indispensable for it can be excluded.
One possible explanation for the above-described results is that the specific localizations of HP1 isoforms cause HP1 isoform-specific phenotypes. To address this question, we examined the localization of HP1 chimeric molecules on polytene chromosomes. Over-expression was attained using the hs-GAL4 driver. Immunostaining with anti-HA antibodies demonstrated localization of all chimeric HP1 proteins in chromocenter and telomeric regions (Fig. 6). However, the sharp and restricted bands in euchromatic regions specifically observed with HP1β (HP1β-specific localization) (Fig. 2A) were not apparent with any of the chimeric proteins (Fig. 6). These results again suggest that the whole region of HP1β is required for the HP1β-specific localization on polytene chromosomes. In addition, we could not observe any variation in the localization of chimeric proteins on polytene chromosomes (Fig. 6). The localization of HP1 chimeric proteins therefore does not explain the HP1γ-specific phenotype.

Fig. 5. Schematic representation of chimera HP1β and HP1γ proteins and summary of effects of chimera HP1 expression with ey-GAL4 and 32B-Gal4 driver lines (A). (B) Chimera HP1 was over-expressed with the following GAL4 drivers in wing discs. (a)32B-GAL4/UAS-BG1, (b) 32B-GAL4/UAS-BG2, (c) 32B-GAL4/UAS-GB1, and (d) 32B-GAL4/UAS-GB2. Arrows indicate loss of L4 wing veins.

Molecular domains in HP1 necessary for their localization on polytene chromosomes in Drosophila

One possible explanation for the above-described results is that the specific localizations of HP1 isoforms cause HP1 isoform-specific phenotypes. To address this question, we examined the localization of HP1 chimeric molecules on polytene chromosomes. Over-expression was attained using the hs-GAL4 driver. Immunostaining with anti-HA antibodies demonstrated localization of all chimeric HP1 proteins in chromocenter and telomeric regions (Fig. 6). However, the sharp and restricted bands in euchromatic regions specifically observed with HP1β (HP1β-specific localization) (Fig. 2A) were not apparent with any of the chimeric proteins (Fig. 6). These results again suggest that the whole region of HP1β is required for the HP1β-specific localization on polytene chromosomes. In addition, we could not observe any variation in the localization of chimeric proteins on polytene chromosomes (Fig. 6). The localization of HP1 chimeric proteins therefore does not explain the HP1γ-specific phenotype.
Discussion

Localization of human HP1 on Drosophila polytene chromosomes

While HP1γ is predominantly linked to heterochromatic chromocenters in Drosophila polytene chromosomes under weak expression conditions, it becomes more diffusely located among euchromatin regions, unlike HP1β (Fig. 3A). One possible mechanism by which HP1 associates with chromosomes is through an interaction of its CD with methylated H3K9, due to a modification generated by the Su(var)3-9 (Bannister et al., 2001; Lachner et al., 2001; Jacobs and Khorasanizadeh, 2002). This interaction is consistent with the histone code hypothesis stating that histone tail modifications serve as specific recognition markers for chromosomal proteins (Jenuwein and Allis, 2001).

However, other experimental data suggest that HP1 uses alternative mechanisms for localization in non-centric regions (Li et al., 2002). On Drosophila polytene chromosomes, methylated H3K9 and HP1 do not exhibit complete co-localization within euchromatin (Cowell et al., 2002; Li et al., 2002). Furthermore, a mutation in CD that abolishes interactions with methylated H3K9 does not eliminate association at telomeres (Fanti et al., 1998; Jacobs and Khorasanizadeh, 2002). In the present study, none of the chimera HP1s exhibited HP1β-specific localization on Drosophila polytene chromosomes, suggesting that both of CD and CSD are required for site determination.

What are the functional differences between HP1 subtypes?

In our chimera HP1γ experiment, CSD was required for the HP1γ-specific wing phenotype. A large number of CSD interaction partners contain a PXVXL motif, including: TIF1α, TIF1β and TAFII130, which regulate transcription; IDN3, which may play a role in chromosome segregation; and the large subunit of CAF1, which contributes to nucleosome assembly during DNA replication and repair (Le Douarin et al., 1996; Murzina et al., 1999; Vassallo and Tanese, 2002). The functions of many of these proteins rely on their interactions with CSD, linking them to H3K9 methylation and the establishment/maintenance of gene silencing.

Surprisingly, however, comparison of CSD amino acid sequences between HP1β and HP1γ showed only limited variation (Fig. 4B). In Drosophila, casein kinase II (CKII) phosphorylation of at least two distinct regions of dHP1a affects targeting to heterochromatin: Ser-15 in the N-terminal region as well as Ser-169 and Ser-172 at CSD (all HP1β numbering) (Zhao and Eissenberg, 1999). Ser-172 is within a consensus CKII site (S/TXXD/E), and it is thought that Ser-169 becomes a consensus CKII site following phosphorylation of Ser-172 (Zhao and Eissenberg, 1999). In HP1β, there are potentially three such interdependent phosphorylation sites, at Thr-169, Ser-172 and Ser-175. The last is missing in HP1γ, so that it could conceivably be responsible for the difference in affinity for CSD interactions and thus phenotypic differences in Drosophila tissues. Further analysis is necessary to clarify this point.
References


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