Isoform-specific Intermolecular Disulfide Bond Formation of Heterochromatin Protein 1 (HP1)

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Three mammalian isoforms of heterochromatin protein 1 (HP1), α, β, and γ, play diverse roles in gene regulation. Despite their structural similarity, the diverse functions of these isoforms imply that they are additionally regulated by post-transcriptional modifications. Here, we have identified intermolecular disulfide bond formation of HP1 cysteines in an isoform-specific manner. Cysteine 133 in HP1α and cysteine 177 in HP1γ were involved in intermolecular homodimerization. Although both HP1α and HP1γ contain reactive cysteine residues, only HP1γ readily and reversibly formed disulfide homodimers under oxidative conditions. Oxidatively dimerized HP1γ strongly and transiently interacted with TIF1β, a universal transcriptional co-repressor. Under oxidative conditions, HP1γ dimerized and held TIF1β in a chromatin component and inhibited its repression ability. Our results highlight a novel, isoform-specific role for HP1 as a sensor of the cellular redox state.

Heterochromatin protein 1 (HP1) was originally characterized as an abundant protein that binds pericentric heterochromatin (1). HP1 acts as a scaffold-like molecule, which is composed of two conserved domains as follows: the chromodomain (CD) and the chromoshadow domain (CSD). The variable hinge region separates these two domains (2). The CD recognizes methylated lysine 9 of histone H3 (H3K9), which recruits HP1 homodimer formation and provides a surface for interaction with a variety of other chromatin proteins (6, 7). Although genetic experiments previously revealed that HP1 works as a repressor of gene activation by propagation of a heterochromatin structure, emerging evidence has elucidated its diverse functions other than gene silencing (8). Some of these functions are regulated in an isoform-specific manner (9).

In vertebrates, three isoforms of HP1 exist as follows: α, β, and γ, all of which share highly conserved domains. Tethering any HP1 isoform upstream of a promoter equally triggers gene silencing concomitant with local chromatin condensation and an increase in H3K9 methylation (10–12), indicating their common silencing ability. However, nonredundant functions (13, 14), different binding affinities to other proteins (15–17) and different localizations in tissues (18, 19), of these three HP1 isoforms imply that α, β, and γ are functionally diverse. Furthermore, recent evidence clarified apparently opposite functions of HP1 isoforms, e.g., a role in transcriptional activation or in transcriptional elongation (20, 21). One mechanism that could account for such functional diversity of HP1 isoforms is post-translational modification, which could cause conformational changes in the molecule. In fact, reversible modifications of HP1 (e.g. phosphorylation) can modulate its function in response to various stimuli or cellular environments, suggesting an active role for HP1 beyond its known function as a marker of heterochromatin (17, 22). However, the precise modulatory mechanism across three HP1 isoforms that leads to functional differences remains to be elucidated.

Here, we identified isoform-specific disulfide bond formation as a novel post-translational modification of HP1. We analyzed the biochemical and functional characteristics of this oxidative modification. These data may offer a new insight into a novel role for HP1 during the cellular response to oxidative stress.

EXPERIMENTAL PROCEDURES

Materials—We used the following commercially available materials for Western blotting: anti-HP1α (H2164, Sigma; 19s2, Millipore); anti-HP1β (MAB3448, Chemicon); anti-HP1γ (42s2, Millipore); anti-FLAG M2-peroxidase antibody (Sigma); anti-histone H3 (ab1791, Abcam); anti-GAPDH (MAB374, Chemicon); and anti-TIF1β (4123, Cell Signaling). We also used anti-FLAG M2 affinity gel for immunoprecipitation. We used menadione (Sigma), H2O2 (Wako), and hydroxytamoxifen (4-OHT) (Sigma) for cell treatment.

Cell Fractionation—Cells were lysed with hypotonic lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, and 10 mM KCl) with 0.5% Nonidet P-40 and centrifuged at 20,000 × g for 5 min.

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The supernatant was collected as the cytosolic fraction. Extraction buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 0.42 mM NaCl, 0.2 mM EDTA, 25% glycerol) was added to the pellet, and ultrasonic agitation was performed (30-s sonication with 30-s interval, 4–6 times at 0 °C; Bioruptor, CosmoBio). The suspension was incubated for 15 min at 4 °C and centrifuged at 20,000 × g for 10 min. The supernatant was collected as the nuclear extract.

**Column Chromatography**—For anion exchange, whole cells were lysed with buffer A (20 mM Tris, pH 8.0, 5% acetonitrile) containing 5 mM EDTA and 1% Nonidet P-40 and incubated at 4 °C for 15 min. The lysate was centrifuged at 20,000 × g for 5 min, and the supernatant was filtered and loaded onto an anion-exchange column (Q-Sepharose High Performance, GE Healthcare) pre-equilibrated with buffer A. After unbound samples were washed, protein was eluted with a linear gradient (0–100%) of buffer B (buffer A with 1.0 M NaCl). For reverse-phase HPLC, purified protein samples and nuclear extracts were prepared with 0.3% trifluoroacetic acid (TFA) and 20% acetonitrile and applied to a phenyl reverse-phase column (2.1 × 250 mm; Nakalai Tesque). Bound proteins were eluted by a segmented linear gradient of increasing concentrations of buffer B (acetonitrile and 0.1% TFA) in buffer A (0.1% TFA) at a flow rate of 0.5 ml/min. Buffer B was increased at a rate of 1.0%/fraction (fast gradient) or 0.2%/fraction (slow gradient). Collected fractions were dried by a centrifugal evaporator and reconstituted with SDS sample buffer with or without 2.5% 2-mercaptoethanol (reducing or nonreducing conditions, respectively).

**Triton Extraction**—Triton extraction was carried out as described previously with modification (23). Cells were lysed with a hypotonic lysis buffer with 0.5% Nonidet P-40 and centrifuged at 20,000 × g for 5 min (as described above). The pellet was lysed in extraction buffer with 0.2% Triton X-100, incubated on ice for 30 min, and centrifuged at 20,000 × g for 5 min. The supernatant was kept as a Triton-soluble fraction. The remaining pellet was lysed in SDS sample buffer (250 mM Tris, 5% SDS, and 5% glycerol) with or without 2.5% 2-mercaptoethanol (reducing or nonreducing conditions, respectively), and ultrasonic agitation was performed as described above. After centrifugation at 20,000 × g for 5 min, the supernatant was kept as a Triton-insoluble fraction.

**RNAi Knockdowns and Generation of HEK293T Stable Cells**—Lentiviral particles derived from the pLKO.1-puro-containing shRNA sequence were purchased from the Mission shRNA library (Sigma). The oligonucleotide sequences of the shRNA were as follows: shRNA-6, CGACGTGTAGTGAATGG-GAAA; and shRNA-7, GCCCTTCTAATTACTTGAGAAA. Lentiviral particles were used to transduce human umbilical vein endothelial cells (HUVECs) or HEK293T cells in the presence of 8 µg/ml Polybrene. To generate a HEK293T stable cell line, the infected cells were selected with 1 µg/ml puromycin. The stable cells in which HP1γ was almost completely depleted were next transfected with pEF-DEST51 HP1γ-FLAG WT or a C177S mutant (cloned from murine cDNA and resistant to shRNA), and the stable cells were selected with 5 µg/ml blasticidin.

**GAL4-luciferase Reporter Assay**—pC3-ERHBD-GAL4 or pC3-ERHBD-GAL4-KAP1 (TIF1β) with pGL4.31-PSV40-GAL4UAS were transfected using Lipofectamine 2000 into subconfluent HEK293T stable cells that were passaged 1 day before transfection. After 24 h, 0.04% ethanol or 4-OHT (500 nM) was added to the culture medium. Forty eight h after transfection, luciferase activity was measured by a luminometer (Lumat LB9507). Intranuclear mRNA levels of luciferase were measured as follows. Twenty four h after transfection, 4-OHT (500 nM) was added to the culture medium. Forty eight h after transfection, cells were lysed with a hypotonic lysis buffer with 0.5% Nonidet P-40 and centrifuged at 20,000 × g for 5 min (as described above). From the nuclear pellet, total RNA was isolated using RNA-Bee (Cosmo Bio). Total RNA was treated with DNase (Turbo DNA-free, Applied Biosystems) and was reverse-transcribed using a high capacity cDNA reverse transcription kit (Applied Biosystems). Luciferase mRNA levels were measured by real time quantitative PCR (SYBR Green ER, Invitrogen). Firefly luciferase cDNA was amplified using the following primers: 5′-TACCCACTCAGAGCAGG-3′ and 5′-ACTCGGCTAGGTAATGTCCTACCCCTC-3′. Human 18 S ribosomal RNA was measured using the following primers: 5′-GATCCCGTTGAAACACCCATT-3′ and 5′-CCATCCAATCCTAGTACCG-3′. The relative levels of luciferase mRNA were normalized to the mRNA levels of 18 S ribosomal RNA.

**RESULTS**

HP1α Forms Dimers via Disulfide Bonds through Cysteine 133—During purification of HP1α in our previous work (24), we found that endogenous HP1α separates into two peaks by fractionation using reverse-phase HPLC. To confirm this finding, we fractionated whole cell lysates from HEK293T cells using two-step column chromatography (Fig. 1A). Endogenous HP1α was eluted at a salt concentration ranging from 0.3 to 0.35 M on a Q-Sepharose anion-exchange column (Fig. 1D, top panel). We applied this single peak to a reverse-phase column. After elution with a fast gradient, HP1α was still detected as a single peak (Fig. 1D, 2nd panel). However, when eluted with a slow gradient, HP1α separated into two peaks representing a hydrophilic and a hydrophobic form (Fig. 1D, 3rd panel). Two other HP1α antibodies against different epitopes also detected both bands (data not shown), suggesting that these were biochemically different forms of HP1α. Even after direct fractionation of the nuclear extract, which includes the bulk of HP1α protein (Fig. 1B), endogenous HP1α showed a similar bimodal distribution (Fig. 1D, 4th panel). In other primary cells (HUVECs, neonatal rat cardiomyocytes, and rat cardiac fibroblasts), similar bimodal peaks were observed (supplemental Fig. S1). In contrast, recombinant HP1α expressed in *Escherichia coli* (Fig. 1C) exhibited only one peak with elution characteristics similar to those of the hydrophilic peak under the same separating condition used for the endogenous protein (Fig. 1D, bottom panel). These data suggest that two different forms of HP1α exist in multiple cell types and that the late-eluted hydrophobic species may be a post-translationally modified form.

To further elucidate the molecular characteristics of these two forms of HP1α, we used recombinant FLAG-tagged HP1α (HP1α-FLAG). As with endogenous HP1α, HP1α-FLAG existed mainly as a nuclear protein (Fig. 1E) and exhibited the
**FIGURE 1.** Endogenous HP1α shows a bimodal distribution after protein purification by reverse-phase HPLC. The late-eluted fraction of HP1α is oxidatively modified to form a disulfide bond. A, schematic representation of HP1α purification from cell lysates using sequential column chromatography. B, equal quantities of cytosolic and nuclear fractions from HEK293T cells were resolved by SDS-PAGE and probed with the indicated antibodies. C, GST-HP1α expressed in *E. coli* was purified and cleaved by Factor Xa (upper panel) and detected with anti-HP1α antibody (lower panel). D, HEK293T cell lysate was fractionated by a Q-Sepharose HP anion-exchange column. Eluted fractions were resolved by reducing SDS-PAGE and probed with anti-HP1α antibody (top panel). The x axis at the upper edge indicates salt concentration. HP1α fractions eluted from the anion-exchange column were next applied to a phenyl reverse-phase column. The fractions were eluted by a fast gradient (buffer B, 1.0% increase of acetonitrile concentration/fraction, 2nd panel from the top) or by a slow gradient (buffer B, 0.2%/fraction, 3rd panel from the top). Nuclear extraction from HEK293T cells (4th panel from the top) or HP1α purified from *E. coli* (bottom panel) was fractionated with the same slow gradient. The eluted fractions were resolved by reducing SDS-PAGE and probed with anti-HP1α antibody. E, equal quantities of cytosolic and nuclear fractions from HEK293T cells expressing HP1α-FLAG were resolved by SDS-PAGE and probed with the indicated antibodies. F, nuclear extract from HEK293T cells expressing HP1α-FLAG was directly applied to a reverse-phase column, and the eluted fractions were resolved by reducing SDS-PAGE and probed with the indicated antibodies. G, diagrams of the representative deletion mutant or point mutant of the HP1α protein during stepwise mutation analysis (left column). Nuclear extractions from HEK293T cells expressing each mutant protein were fractionated by reverse-phase HPLC, resolved by SDS-PAGE, and probed with anti-FLAG antibody (right column). H, endogenous HP1α was purified from the HEK293T cell lysate as shown in A. The fractions eluted from the reverse-phase column were resolved by SDS-PAGE under nonreducing conditions and probed with anti-HP1α antibody. I, nuclear extract from HEK293T cells was incubated with 2 mM DTT or 2.5% 2-mercaptoethanol (ME) for 30 min at 4 °C and then applied to a reverse-phase column. The eluted fractions were resolved by SDS-PAGE and probed with anti-HP1α antibody. D and F–I, the x axis at the lower edge indicates fraction numbers.
same bimodal distribution after reverse-phase HPLC (Fig. 1F). Thus, we concluded that HP1α-FLAG undergoes the same modification as endogenous HP1α, validating the use of the tagged protein for further analysis. Initially, we attempted to detect the specific modification directly by matrix-assisted laser desorption/ionization and time-of-flight mass spectrometry (MALDI-TOF/MS) (supplemental Fig. S2, A–C). Although we detected peptide masses from both fractions corresponding to ~75% of the entire HP1α sequence (supplemental Fig. S2B), we did not detect any distinct features in the mass spectra under two different digestion conditions (trypsin or Asp-N) (supplemental Fig. S2C). We next tried to detect a modified residue by making multiple, stepwise mutations throughout the entire HP1α molecule. We hypothesized that HP1α-FLAG lacking the modified residue would fractionate into a single peak by reverse-phase HPLC. First, we thoroughly screened the CD and hinge region, both of which are reported to be posttranslationally modified (17, 22). However, we could not determine any specific amino acid residue from the mutational analysis (supplemental Fig. S2D). Second, we screened the CSD (supplemental Fig. S2E) and found that a deletion mutant lacking residues 119–150 (Δ119–150) was eluted as a single peak. We further narrowed down the deleted sequence 119–150 and finally found that a mutant in which cysteine 133 (Cys-133) was replaced by alanine (C133A) was eluted as a single peak (Fig. 1G). These data suggest that the single cysteine 133 residue is responsible for the separation of the hydrophobic fraction of HP1α.

Among post-translational modifications of cysteine, oxidation is a common feature. The thiol side chain can be oxidized to sulfenic acid (–SOH), sulfenyl amide (–SN), a disulfide bond (C=O), or an irreversibly oxidized form (25). We examined the oxidative modification of HP1α under acidic conditions of the reverse-phase HPLC and under the overall structures are highly conserved, endogenous HP1α and HP1γ possess isoform-specific cysteine residues involved in the intermolecular disulfide bond formation. These two positions of the disulfide-linked cysteines are structurally sensitive to oxidation within the CSD.

HP1γ Is More Sensitive to Oxidation than HP1α in Vitro—We tested whether the differences in the positions of the modified cysteine residues between HP1α and HP1γ influenced their sensitivity to oxidation in vitro. Under mild oxidative conditions, only a low level of dimerized HP1α was detected even after a long exposure to air oxidation (Fig. 3A, left panels). In contrast, under the same conditions, HP1γ was easily oxidized to form disulfide bonds (Fig. 3A, right panels). Treatment with DTT reversed the disulfide formation of HP1γ. These data indicate that HP1γ is more sensitive to oxidation and more readily forms a disulfide dimer in vitro.

Using purified and oxidized HP1γ-FLAG, the intermolecular disulfide bond was confirmed by MALDI-TOF/MS analysis. The late-eluted dimerized fraction of HP1γ-FLAG was resolved by nonreducing SDS-PAGE, and the excised band was divided into two samples. One sample was reduced, carbamidomethylated with iodoacetamide, and digested by trypsin. The other sample was directly digested without pretreatment. The expected digested peptide, including Cys-177, consisted of the C terminus of HP1γ and lysine residue within the linker peptide (Fig. 3B). The mass spectrum peak of 3084.32, which was detected only in the nonreduced sample, corresponded to the estimated mass of the dimeric peptide connected by a disulfide bond via Cys-177 (3084.35) (Fig. 3C, upper panel). In contrast, the peak at 1600.68, which was detected only in the reduced sample, corresponded to the estimated mass of the monomeric peptide, including carbamidomethylated Cys-177 (1600.71) (Fig. 3C, lower panel). No other significant mass spectral peaks from the intermolecular disulfide bond were detected.

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Both HP1α and HP1γ possess isoform-specific cysteine residues that are oxidatively modified to form disulfide bonds. A, amino acid sequence alignment among mouse HP1 isoforms. Crosswise two-headed arrows indicate the N-terminal CD and C-terminal CSD. The bold blue arrow and bold white line along the CSD indicate β-sheet and α-helix, respectively. Blue highlights represent the following: two cysteine residues conserved among the HP1 family (Cys-59 and Cys-160; HP1α). Red highlights represent the following: position of the cysteine residue specific to HP1α (Cys-133) or HP1γ (Cys-177). The arrowhead indicates the position of the mutated HP1β serine residue (shown in E). B, nuclear extract from HEK293T cells was directly applied to a reverse-phase column, and the eluted fractions were resolved by SDS-PAGE and probed with anti-HP1α, -β, or -γ antibodies. The immunoblotting procedure was performed by consecutive stripping and reprobing with each antibody of the same membrane. The upper band of fraction 20 in the bottom panel (arrowhead) indicates the residual signal from hydrophilic HP1α. C, hydrophobic fractions of HP1γ purified from HEK293T cells (as shown in Fig. 1A) were resolved by SDS-PAGE under reducing or nonreducing conditions and probed with anti-HP1γ antibody. D, nuclear extract from HEK293T cells expressing each HP1α-FLAG (top three panels) or HP1γ-FLAG (bottom three panels) with a cysteine-to-serine mutation was fractionated by reverse-phase HPLC, resolved by SDS-PAGE, and probed with anti-FLAG antibody. E, nuclear extract from HEK293T cells expressing HP1β-FLAG with each serine-to-cysteine or tyrosine-to-cysteine mutation was fractionated by reverse-phase HPLC, resolved by SDS-PAGE, and probed with anti-FLAG antibody. F, late-eluted hydrophobic fraction of the HP1β-FLAG mutant (S129C or Y173C) was resolved by reducing or nonreducing SDS-PAGE and probed with anti-FLAG antibody. B–E, the x axis at the lower edge indicates fraction numbers.
was rapidly formed within minutes and was only formed via Cys-177 (Fig. 4C). The I165E mutation, which inhibits both noncovalent α-helix dimer formation and proper nuclear localization (6–7), decreased, but not completely, the amount of disulfide dimers of HP1γ (supplemental Fig. S3A). These data suggest that the oxidative dimerization of HP1γ requires the proper localization and formation of constitutive, noncovalent dimers.

In contrast to HP1γ, an increase in dimerized HP1α was not observed under the same in vivo oxidative conditions (Fig. 4B, right panel). The dimerized forms of HP1α and HP1γ under basal conditions were almost undetectable without using the large scale purification shown in Fig. 1 because of their relatively low abundance before oxidant treatment. Menadione treatment promoted HP1γ dimerization in various cells, but the extent of dimerization varied among cell types (supplemental Fig. S3B), suggesting that the reactivity of HP1γ to reactive oxygen species stimulation varied according to cell type. In each cell, an increase in dimerized HP1α was not observed (data not shown). These results demonstrate that there is a clear difference in oxidation sensitivity among HP1 family members. Although both HP1α and HP1γ have oxidation-sensitive cysteines in their sequences, HP1γ perceives oxidative conditions and is able to more readily form a disulfide dimer than HP1α.

In HEK293T cells, the dimerized HP1γ was subsequently reduced to the monomer form after removal of the oxidant (Fig. 4D, upper panel), but HP1γ remained dimerized when continuously exposed to the oxidants (Fig. 4D, lower panel), suggesting that this oxidative modification was reversible.

H$_2$O$_2$, known as an endogenous source of reactive oxygen species, also promoted dimerization of HP1γ (Fig. 4E). This effect of H$_2$O$_2$ was relatively weak in HEK293T cells when compared with the treatment of menadione. However, the same concentration of H$_2$O$_2$ substantially increased the amount of dimerized HP1γ in HUVECs (Fig. 4E, lower panel). Therefore, we further examined the molecular characteristics of the disulfide dimerization of HP1γ using HUVECs.
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Under Oxidative Conditions, HP1γ Strongly and Transiently Interacts with TIF1 and Holds It in a Chromatin Component—The CSD of HP1, which includes Cys-177 at its C terminus, creates a binding surface for other proteins (27). Therefore, disulfide modification of HP1γ may affect the interactions between HP1 and HP1-binding proteins. Because many candidate effectors that bind to HP1 exist (8), we screened the interacting proteins of HP1γ under oxidative conditions using metabolically radiolabeled HUVECs expressing recombinant HP1γ-FLAG transduced with adenovirus. Among the co-immunoprecipitated proteins, one protein band was detected after treatment with H2O2 (Fig. 5A, arrowhead). The bound protein was purified and analyzed by MALDI-TOF/MS. The amino acid sequence of the digested peptides corresponded to TIF1γ (also known as TRIM28 or KAP1), which is a universal co-repressor of gene transcription and is a well-known interacting partner of HP1 (28–31). Co-immunoprecipitation analysis showed that endogenous HP1γ strongly interacted with TIF1γ under oxidative conditions (Fig. 5B). TIF1γ did not interact with HP1γ with a C177S mutation under oxidative conditions, suggesting that the disulfide bond formation of HP1γ enhanced the interaction of these proteins (Fig. 5C). When the oxidant was removed, TIF1γ dissociated again from HP1γ, suggesting that this enhanced endogenous interac-

FIGURE 4. HP1γ, but not HP1α, readily forms disulfide bonds under oxidative conditions. A, after treatment with DMSO or 50 μM menadione for 15 min, COS7 cells were stained with 20 μM dihydroethidium for 30 min and monitored by fluorescence microscopy. Bar, 100 μm. B, COS7 cells treated with DMSO or menadione under the indicated conditions were lysed, resolved by nonreducing (upper panel) or reducing (lower panel) SDS-PAGE, and probed with anti-HP1 antibody. The same membrane was reprobed with anti-HP1α antibody (right panel). C, COS7 cells expressing each cysteine-to-serine mutant HP1γ-FLAG were treated with 50 μM menadione for 15 min. Lysates were resolved by reducing (left panel) or nonreducing (right panel) SDS-PAGE and probed with anti-FLAG antibody. D, HEK293T cells were treated with 75 μM menadione for 15 min. Subsequently, the culture medium was exchanged for fresh medium (upper panel) or left unchanged (lower panel). After incubation for the indicated time, cell lysates were resolved by nonreducing SDS-PAGE and probed with anti-HP1γ antibody. E, HEK293T cells and HUVECs were treated with H2O2 under the indicated conditions. Cell lysates were resolved by SDS-PAGE and probed with anti-HP1γ antibody. B–E, cells were lysed with a buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1 mM EDTA, and 1% Nonidet P-40) containing 100 mM maleimide, a thiol-alkylating agent, to prevent artifactual oxidation.
Structurally, disulfide dimerization via Cys-177 is formed at the C terminus of the CSD, just adjacent to the binding interface for the PXVXL motif, which is a well-characterized binding sequence in HP1-interacting proteins, including TIF1γ/H9252 (Fig. 5E) (7).

We next examined the localization changes of these proteins before and after oxidant treatment. No remarkable change in localization was detected by immunostaining (data not shown). However, biochemical analysis using Triton extraction verified the TIF1β translocation. HP1γ existed mainly in the Triton-insoluble chromatin component, whereas TIF1β was distributed both in the soluble and the insoluble components (Fig. 5F). Under oxidative conditions, HP1γ dimerized and was maintained in the insoluble components. Concomitant with HP1γ dimerization, the insoluble component of TIF1β transiently increased. The knockdown of endogenous HP1γ combined with the replacement by a C177S mutant of HP1γ inhibited the translocation of TIF1β, suggesting that HP1γ held TIF1β on chromatin only when oxidized via Cys-177 (Fig. 5G). These data suggest that the intracellular redox state is transduced to the conformational and localization change of the repressor complex via oxidative modification of HP1γ.

**FIGURE 5.** HP1γ strongly interacts with TIF1β and promotes translocation of TIF1β to a chromatin component when dimerized under oxidative conditions. A, HUVECs expressing HP1γ-FLAG (transduced by adenovirus) were metabolically labeled with [35S]cysteine and -methionine for 6 h. Nontransduced cells were also labeled as a negative control. Forty-eight h after transduction, the cells were treated with 100 μM H2O2 or control (water) for 10 min, lysed, and immunoprecipitated with anti-FLAG M2 affinity gel. Bound samples were resolved by reducing SDS-PAGE and visualized by autoradiography. The arrow indicates the protein band co-immunoprecipitated with HP1γ-FLAG only under the oxidative conditions. B, lysates from HUVECs treated with control (water) or H2O2 for 10 min were immunoprecipitated (IP) with anti-HP1γ antibody. Bound samples were resolved by SDS-PAGE and probed with the indicated antibodies. C, HUVECs expressing WT or C177S HP1γ-FLAG (transduced by adenovirus) were treated with 100 μM H2O2 for 10 min. Lysates were immunoprecipitated with anti-FLAG M2 affinity gel, and bound samples were resolved by SDS-PAGE and probed with the indicated antibodies. D, HUVECs were transiently treated with 100 μM H2O2. Lysates were immunoprecipitated with anti-HP1γ antibody, and bound samples were resolved by SDS-PAGE and probed with the indicated antibodies. E, structure of HP1 CSD noncovalent homodimer (blue and cyan) and PXVXL motif (yellow) complex (Protein Data Bank code 1s4z, modified using the WEB tool (43)). The position of Cys-177 in HP1γ is highlighted in red (black arrows). F, HUVECs were transiently treated with 100 μM H2O2. Soluble and insoluble nuclear fractions were obtained using Triton extraction. Each sample was resolved by SDS-PAGE and probed with the indicated antibodies. G, HUVECs were transduced with lentivirus expressing shRNA against HP1γ and adenovirus expressing shRNA-resistant HP1γ-FLAG WT or C177S mutant. Triton-insoluble fractions from these cells after H2O2 treatment were resolved by SDS-PAGE and probed with the indicated antibodies.
Dimerized HP1γ under Oxidative Conditions Inhibits the Repression Ability of TIF1β—To clarify whether the repression ability of TIF1β was promoted or inhibited when trapped by HP1γ under oxidative conditions, we used a GAL4-based transcriptional reporter assay. In HEK293T cells, menadione treatment promoted the disulfide dimerization of HP1γ and the interaction between HP1γ and TIF1β more prominently than H2O2 treatment (supplemental Fig. S3 and Figs. 4 and 6A). Therefore, we used menadione treatment for further analysis in HEK293T cells. We generated HEK293T cells stably expressing shRNA for HP1γ and shRNA-resistant recombinant FLAG-tagged HP1γ WT or C177S mutant. In these cells, endogenous HP1γ was almost completely depleted and was replaced by the dimerizable or undimerizable recombinant proteins (Fig. 6B).

To evaluate the transcriptional repression ability of TIF1β, we transfected the plasmids encoding ERHBD-GAL4 as a control or ERHBD-GAL4-TIF1β fusion protein with the reporter plasmids. Twenty four h after transfection, 4-OHT was added to culture medium (500 nM). Forty eight h after transfection, luciferase activity was measured (right panel). The relative value was corrected by the value of the cells transfected with ERHBD-GAL4 without 4-OHT induction. Student’s t test; *, **, p < 0.01. D, HEK293T stable cells expressing ERHBD-GAL4-TIF1β were treated with DMSO or 75 μM menadione for 15 min. Lysates were immunoprecipitated with anti-FLAG affinity gel. Bound samples were immunoblotted with anti-GAL4 antibody. D indicates DMSO, and M indicates 75 μM menadione. E, under the same conditions as C, luciferase transcription levels were determined both by protein enzymatic activity and intranuclear mRNA levels measured by quantitative PCR. Student’s t test; *, **, p < 0.01. F, HEK293T stable cells expressing ERHBD-GAL4-TIF1β with 500 nM 4-OHT induction were treated with DMSO for 60 min or 75 μM menadione for the indicated time. Intranuclear luciferase mRNA levels at each time point were measured by quantitative PCR. The relative value was corrected by the value of the cells treated with DMSO. Two-way repeated measure analysis of variance; *, p < 0.05. The means ± S.D. as indicated by the error bars were determined from three independent experiments.

FIGURE 6. Dimerized HP1γ under oxidative conditions inhibits the repression ability of TIF1β. A, lysates from HEK293T cells treated with DMSO, 75 μM menadione, water, or 200 μM H2O2 for 15 min were immunoprecipitated (IP) with anti-HP1γ antibody. Bound samples were resolved by SDS-PAGE and probed with the indicated antibodies. B, HEK293T cells stably expressing shRNA for HP1γ and shRNA-resistant recombinant FLAG-tagged HP1γ were treated with DMSO or 75 μM menadione for 15 min. Cell lysates were resolved by nonreducing or reducing SDS-PAGE and probed with anti-HP1γ antibody. D indicates DMSO, and M indicates 75 μM menadione. C, lysates from HEK293T stable cells transfected with an ERHBD-GAL4 or ERHBD-GAL4-TIF1β fusion protein were resolved by SDS-PAGE and probed with the indicated antibodies (left panel). HEK293T stable cells were transfected with the plasmids encoding ERHBD-GAL4 or ERHBD-GAL4-TIF1β with the reporter plasmids. Twenty four h after transfection, 4-OHT was added to culture medium (500 nM). Forty eight h after transfection, luciferase activity was measured (right panel). The relative value was corrected by the value of the cells transfected with ERHBD-GAL4 without 4-OHT induction. Student’s t test; *, **, p < 0.01. D, HEK293T stable cells expressing ERHBD-GAL4-TIF1β were treated with DMSO or 75 μM menadione for 15 min. Lysates were immunoprecipitated with anti-FLAG affinity gel. Bound samples were immunoblotted with anti-GAL4 antibody. D indicates DMSO, and M indicates 75 μM menadione. E, under the same conditions as C, luciferase transcription levels were determined both by protein enzymatic activity and intranuclear mRNA levels measured by quantitative PCR. Student’s t test; *, **, p < 0.01. F, HEK293T stable cells expressing ERHBD-GAL4-TIF1β with 500 nM 4-OHT induction were treated with DMSO for 60 min or 75 μM menadione for the indicated time. Intranuclear luciferase mRNA levels at each time point were measured by quantitative PCR. The relative value was corrected by the value of the cells treated with DMSO. Two-way repeated measure analysis of variance; *, p < 0.05. The means ± S.D. as indicated by the error bars were determined from three independent experiments.
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...tional change of the luciferase gene under oxidative conditions, we measured the intranuclear mRNA levels of luciferase by quantitative PCR instead of luciferase protein enzymatic activity (Fig. 6E). We chose this end point because the oxidation of HP1γ was too rapid to properly evaluate its effect on luciferase transcription by measuring luciferase protein enzymatic activity. Under these conditions, menadione treatment relieved the levels of luciferase transcription repressed by ERHBD-GAL4-TIF1β in the cells expressing HP1γ-FLAG WT but did not relieve the levels in the cells expressing the C177S mutant (Fig. 6F). These data suggest that dimerized HP1γ under oxidative conditions inhibits the repression ability of TIF1β.

It remained unclear whether the intranuclear redox-sensing mechanism through the oxidative modification of HP1γ plays a role in the cellular response to extrinsic oxidative stress. Therefore, we assessed the effect of this modification on cell survival under oxidative conditions using HEK293T cells stably expressing shRNA against HP1γ (supplemental Fig. S4A). Depletion of HP1γ using shRNA uniformly decreased cell viability under oxidative conditions induced by menadione treatment (supplemental Fig. S4B). For a rescue experiment, these stable clones were transduced with an adenoviral vector encoding WT HP1γ-FLAG or C177S HP1γ-FLAG. Both HP1γ vectors were cloned from murine cDNA and were resistant to shRNA against human HP1γ. Transduction of both adenoviral constructs at a multiplicity of infection of 20 resulted in nearly equal expression of recombinant HP1γ with endogenous HP1γ and yielded a similar disulfide dimerization pattern (supplemental Fig. S4C). Under these conditions, WT HP1γ-FLAG rescued cell viability after menadione treatment in each stable clone, but the C177S HP1γ-FLAG mutant did not rescue cell viability (supplemental Fig. S4D). These results suggest that HP1γ disulfide dimerization plays a pivotal role in cell survival under oxidative conditions.

DISCUSSION

In this study, we identified isoform-specific disulfide bond formation, which is a novel post-translational modification of HP1, using a unique column chromatography method. Biochemical analysis revealed two isoform-specific reactive cysteine residues, cysteine 133 in HP1α and cysteine 177 in HP1γ. In particular, HP1γ readily and reversibly formed disulfide dimers under oxidative conditions. Dimerized HP1γ strongly interacted with TIF1β and held it in a chromatin component. The GAL4 tethering repression assay revealed that the tight interaction of the repressor proteins had a reversing effect for transcriptional repression.

Several post-translational modifications of HP1 have been reported. Specifically, the linker region between the CSD and CD is highly amenable to post-translational modifications, especially phosphorylation that affects silencing activity or nuclear location of HP1 (17, 33–35). Also in the CD, Thr-51 of HP1β has been shown to be phosphorylated in response to DNA damage (22). More recently, a comprehensive proteomic analysis revealed that all HP1 isoforms are highly modified by phosphorylation, acetylation, methylation, and formylation both in the CD and in the CSD (36). Prior to this study, however, no oxidative modification of HP1 had been identified. Because oxidative modifications at cysteine residues would be easily disrupted under reducing conditions, such modifications may be detected only by the unique HPLC-based method used in this study and not by ordinary mass spectrometry analysis.

Both isoform-specific cysteines involved in forming disulfide bonds reside in a structurally flexible region of the CSD. Cys-133 of HP1α lies in the long loop between the β1 and β2 sheets, and Cys-177 of HP1γ lies in the C-terminal region. Introducing cysteine residues into these flexible sites of HP1β conferred the ability to form disulfide bonds, suggesting that these sites have specific structures in the oxidative center. Although both cysteines were reactive, a distinct difference of sensitivity to oxidation existed. Each location of reactive cysteine and the surrounding structure might determine the sensitivity of HP1α and -γ to oxidation. Under both in vitro and in vivo oxidative conditions, HP1γ readily formed disulfide bonds. In contrast, only minimal disulfide formation of HP1α by oxidation was observed under our experimental conditions. The reactivity of HP1α under oxidation might be observed under different conditions. Nonetheless, the isoform specificity and functional importance of Cys-133 in HP1α has been reported previously (15).

HP1 has been reported to form dimers via the CSD, but these dimers are not mediated by disulfide bonds or other covalent bonds (6, 37, 38). Thus, HP1 dimerizes in at least two ways. The interface of the noncovalently linked dimer involves a symmetrical interaction on helix α2 of the CSD (27) and creates a nonpolar groove structure, which is a binding site for the PXVXL motif in HP1-interacting proteins, such as TIF1β (Fig. 5E) (7). Because reactive cysteine 177 in HP1γ is located in the C terminus adjacent to the groove structure, disulfide bond formation at this site likely affects the binding affinity of HP1γ. Indeed, HP1γ strongly and transiently interacted with TIF1β and promoted its translocation to a chromatin component stringently depending on the oxidative status of cysteine 177. This rapid reacting mechanism to transduce cellular redox state to a conformational change like a clear “on-off switch” suggests that HP1γ is a functional redox sensor.

During the cellular response to oxidative stress, an increase in oxidants can trigger alterations in transcription levels through direct activation or by promoting a change in the subcellular localization of transcription factors by oxidizing reactive cysteine residues (25). Among these oxidative responses, the disulfide dimerization of HP1γ demonstrated in this study appears to be one of the most rapid transcriptional regulatory mechanisms. TIF1β is a universal co-repressor for the Krüppel-associated box domain containing the zinc finger protein (KRAB-ZNF) family of transcription factors, and it is the major protein binding the CSD of HP1 (28–31). TIF1β also works as a scaffold for the repressor complex, and its interaction with HP1 is essential for its repression activity (12, 39–41). Recent findings have revealed that the binding of HP1 to TIF1β is essential for their coordinated function on the promoter of the endogenous genes (42). Therefore, the reversing effect for the repressive ability of TIF1β caused by HP1γ disulfide dimerization might be required for a short period of adaptation against oxidative stress. Downstream genes regulated by these scaffold complexes remain to be clarified in the future analysis.
In conclusion, our study suggests that HP1 potentially acts as a rapid redox sensor, and it may connect the intracellular redox state with transcriptional regulation under various physiological conditions.

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