Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 300 (2003) 135–140
BBRC
www.elsevier.com/locate/ybbrc

Dimerization of the Polycomb-group protein Mel-18 is regulated by PKC phosphorylation

Seiji Fujisaki,[a,b] Yuichi Ninomiya,[a] Hiroto Ishihara,[a] Masaki Miyazaki,[a] Rieko Kanno,[a,c] Toshimasa Asahara,[b] and Masamoto Kanno[a,c,*]

[a] *Department of Immunology, Graduate School of Science, Hiroshima University, 1-2-3, Kasumi, Minami-ku, Hiroshima 734-8551, Japan*
[b] *Department of Surgery, Graduate School of Sciences, Hiroshima University, Hiroshima 734-8551, Japan*
[c] *CREST (Core Research for Evolutional Science and Technology) of Japan Science and Technology Corporation, Japan*

Received 7 November 2002

Abstract

The Polycomb-group (Pc-G) gene products form complexes via protein–protein interactions and maintain the transcriptional repression of genes involved in embryogenesis, cell cycle, and tumorigenesis. Previously, we have shown that mouse Mel-18, a Pc-G protein, has tumor suppressor gene-like activity and negatively regulates transcription. Here, we show in vitro by pull-down assays and in vivo in transiently transfected COS-7 cells that Mel-18 forms homodimers. Deletion analysis revealed that the N-terminal RING-finger and α-helix domains are required for homodimer formation. In addition, we demonstrated that Mel-18 homodimerization is regulated by protein kinase C (PKC) and protein phosphatases, such that dephosphorylated Mel-18 is able to homodimerize. These results suggest that the stoichiometry and/or equilibrium of subunits of the class II Polycomb complex containing Mel-18 might be regulated by changes in phosphorylation status via the PKC signaling pathway.

Keywords: mel-18; Polycomb; Protein–protein interactions; Phosphorylation

Genes of the Polycomb and Trithorax groups (Pc-G and Trx-G) are part of a cellular memory system that maintains homeotic gene expression in the inactive and active state, respectively. This molecular mechanism of cell determination is phylogenetically conserved and involved not only in the repression of gene expression, but also in cell proliferation and cell death [3–5,8,11–14,16,17,20,24–26,30–33,35,37]. Pc-G proteins act on chromatin as a huge complex and these complexes have been categorized into classes, e.g., class I, class II, and class III, by complex purification [2,23]. The presence of multiple protein–protein interacting domains in most Pc-G proteins permits their association in homo- and/or heteromeric structures. For example, using yeast two-hybrid analysis, mammalian Mph1/Rae28 protein was found to directly interact with Bmi-1, Mel-18, and itself [2,7]. Bmi-1, in turn, interacts with itself and with M33 and Ring1A and Ring1B [9,10,30–32]. In addition, Enx1/EZH2 and EED, mammalian homologs of the *Drosophila* Pc-G protein enhancer of zeste (E(z)) and extra sex combs (esc), appear to be part of a distinct Pc-G complex. Enx1/EZH2 and EED co-immunoprecipitate and co-localize with one another, like their *Drosophila* homologs [15,21,34,38]. Moreover, *Xenopus* M33 self-associates, based on in vitro binding assays [27].

In general, the interacting surfaces of Pc-G proteins consist of highly conserved domains [18,19] and provide a variety of contacts, which may facilitate the self-organization and interaction of Pc-G complexes. Therefore Pc-Gs function through multiprotein complexes. This is consistent with the observation that *Drosophila* embryos with mutations in two or more Pc-G genes show a more severe phenotype than those with mutations in single genes [1,6]. Some class II Polycomb proteins (e.g., Bmi-1, Mph1, M33, etc.) are known to form homodimers and to be post-translationally modified. Here, we report that one class II Polycomb protein,

* Corresponding author. Fax: +81-82-257-5179.
E-mail address: mkanno@hiroshima-u.ac.jp (M. Kanno).

0006-291X/02/$ - see front matter © 2002 Elsevier Science (USA). All rights reserved.
PII: S0006-291X(02)02791-2

Mel-18, also forms homodimers and that its dimerization is regulated by PKC phosphorylation.

Materials and methods

Plasmids. Plasmid manipulations were performed according to established methods [28]. To synthesize histidine-tagged Mel-18 (His-Mel), full-length mouse mel-18 cDNA [36] was subcloned into the pET-16b vector (Novagen) (pET16b/mel). A series of [^{35}S]methionine-labeled Mel-18 ([^{35}S]Mel/FL-Mel, ΔC11-Mel (amino acids (AA) 1–229); ΔC12-Mel (AA 1–202); ΔC13-Mel (AA 1–158); ΔC14-Mel (AA 1–97); and ΔN1-Mel (AA 1–10, 79–342)) deletion mutants were generated by in vitro mutagenesis with the pALTER-1 system (Promega) and designated pALTER-1/mel, pALTER-1/ΔC11, pALTER-1/ΔC11, pALTER-1/ΔC12, pALTER-1/ΔC13, pALTER-1/ΔC14, and pALTER-1/ΔN1. [^{35}S]methionine-labeled Bmi-1 ([^{35}S]Bmi) and luciferase ([^{35}S]Luc) were synthesized as above. The full-length mouse bmi-1 [39] and luciferase cDNAs were subcloned into the pALTER-1 vector to generate pALTER-1/bmi1 and pALTER-1/luc. To express Xpress-tagged Mel-18 (Xpress-Mel) in COS-7 cells, full-length Mel-18 was generated by PCR and subcloned into pEF4/HisB (Invitrogen) (pEF4B/Xpress-Mel). To express FLAG- and Myc-tagged Mel-18 (FLAG-Mel-Myc) in COS-7 cells, FLAG epitope plus full-length Mel-18 was generated by PCR and subcloned into pcDNA3.1(−)/Myc-HisB (Invitrogen) (FLAG-Mel-Myc). All constructs were verified by sequencing. All plasmids were CsCl-purified or isolated using Qiagen columns (Qiagen).

In vitro pull-down assay. [^{35}S]methionine-labeled Mel-18, Bmi-1, and luciferase proteins were synthesized using the TNT-coupled transcription and translation system (Promega) from linearized pALTER-1/mel, pALTER-1/bmi, and pALTER-1/luc, according to the manufacturer's instructions. To produce His-Mel from *Escherichia coli* transformed with the pET16b/mel-18 construct, *E. coli* were induced for 3 h at 30 °C with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG), as instructed by the manufacturer. His-Mel protein was purified from the cell extract using a Hi-Trap chelating column with zinc ions (Amersham BioScience). His-Mel protein was preincubated with 1 μg [^{35}S]Mel, [^{35}S]Bmi, or [^{35}S]Luc, respectively, for 30 min on ice. Following the addition of Zn Hi-Trap chelating Sepharose beads (Zn Sepharose beads), the samples were incubated for another 30 min at 4 °C. The Zn Sepharose beads were washed four times in high salt buffer (20 nM Na_2HPO_4, 0.5 M KCl, pH 7.2) or low salt buffer (20 nM Na_2HPO_4, 0.12 M KCl, pH 7.2), and bound proteins were eluted by boiling in 2× SDS buffer for 5 min and loaded onto 10% SDS–polyacrylamide gels for electrophoresis. After electrophoresis, the gels were visualized by autoradiography.

Transfections. COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS). All transfections were performed using SuperFect (Qiagen), according to the manufacturer's instructions. FLAG- and Myc-tagged Mel-18 or FLAG- and Myc-tagged-empty vector (−) in the presence of pEF4B/Xpress-Mel or pEF4B/Xpress-(−) were co-transfected into COS-7 cells (5.0×10^5 per 10-cm dish). COS-7 cells were transfected with a total of 5 μg plasmid, and after 3 h, washed with fresh DMEM, and then cultured for 48 h in DMEM containing 10% FCS.

Immunoprecipitation. Forty-eight hours after transfection, the cells were washed twice with PBS and lysed in 1 ml HNMG buffer (25 mM Hepes buffer (pH 7.6) containing 0.1 M NaCl, 0.05 M $MgCl_2$, 5% glycerol, and protease inhibitor (EDTA free) cocktail). Each cell lysate was precipitated with an anti-Xpress antibody (Invitrogen) and protein G–Sepharose beads (Amersham). The proteins bound to protein G–Sepharose beads were washed twice with Hepes buffer and eluted from protein G–Sepharose beads with SDS buffer.

Western blot analysis. Proteins eluted from protein G–Sepharose beads were separated by 10% SDS–PAGE and transferred to PVDF membranes. After overnight incubation at 4 °C in PBSMT (phosphate-buffered saline (PBS) containing 0.05% Tween 20 and 5% nonfat dried milk), membranes were subsequently immunoblotted with 1/5000-diluted anti-Myc antibody (Invitrogen) in PBSMT for 2 h at room temperature. After washing, membranes were incubated with horseradish peroxidase-linked swine anti-mouse IgG1 (Transduction Laboratories), as a secondary antibody, in PBSMT for 1 h at room temperature. After washing, the membrane was incubated with ECL Plus (Amersham BioScience) for 3 min and visualized by fluorography.

Phosphorylation–dephosphorylation. [^{35}S]Mel was incubated in the presence or absence of His-Mel protein (1 μg) in 10 μl kinase reaction buffer PK (protein kinase) containing 1 mM ATP in addition to protein kinase C (a mixture of α, β, and γ isoforms; UBI) (0–20 ng) and peptide PKC inhibitor (Life Technologies) or phosphatases (purified PP1 and PP2A; UBI (0.02 U)) at 30 °C for the indicated time periods. Zn Sepharose beads were added to the reaction and incubated for another 30 min at 4 °C. The Zn Sepharose beads were washed four times in low salt buffer (20 nM Na_2HPO_4, 0.12 M KCl, pH 7.2), and bound proteins were eluted by boiling in 2× SDS buffer for 5 min and then loaded onto 10% SDS–polyacrylamide gels. After electrophoresis, the gels were visualized by autoradiography.

Gel filtration chromatography. To purify Mel-18, C57BL/6 mouse embryo whole body was homogenized with HNMG buffer, pH 7.6, containing 25 mM Hepes, 0.1 M NaCl, 0.05 M $MgCl_2$, 5% glycerol, and protease inhibitor (EDTA free cocktail, Boehringer–Mannheim). After centrifugation (20 min, 60,000*g*), the supernatant was filtered. The concentrated sample was transferred to a column of Superose 12 (1 × 30 cm) equilibrated with HNMG buffer and fractionated by AKTA explorer 10S (Amersham BioScience).

Fractionated samples were separated by 10% SDS–PAGE and visualized by immunoblotting with anti-Mel-18 polyclonal antibodies [36]. The column was calibrated with a selection of protein markers from gel filtration calibration kits for high and low relative molecular masses (Amersham BioScience).

Results

Mel-18 proteins form homodimers both in vitro and in vivo

Pc-G proteins are known to form complexes through protein–protein interactions. Some members of Class II complexes, e.g., Mph1/Rae-28, Ring1A, and Bmi-1, have already been reported to form homodimers. Using an in vitro pull-down assay, we tested whether Mel-18 associates with itself, as Mel-18 is also a member of the Class II complex. His-tagged Mel-18 (His-Mel) was mixed with [^{35}S]methionine-labeled, in vitro translated Mel-18 ([^{35}S]Mel), Bmi-1 ([^{35}S]Bmi-1), or luciferase ([^{35}S]Luc, as a control) proteins, and then incubated with Zn Sepharose beads (See Materials and methods) to assess self-association. The reactions were washed with low salt buffer (0.12 M KCl buffer) and eluted samples were separated by SDS–PAGE electrophoresis (Fig. 1). A strong signal was observed from the combination of [^{35}S]Mel and His-Mel (Fig. 1, lane 4), and we also detected a weak signal from the combination of [^{35}S]Bmi-1 and His-Mel (Fig. 1, lane 5). We could not detect any signal from the combination of [^{35}S]Luc and His-Mel (Fig. 1, lane 6). Even with high salt buffer (0.5 M KCl buffer), we detected an interaction between Mel-18 protein and itself, [^{35}S]Mel and His-Mel, (Fig. 1,

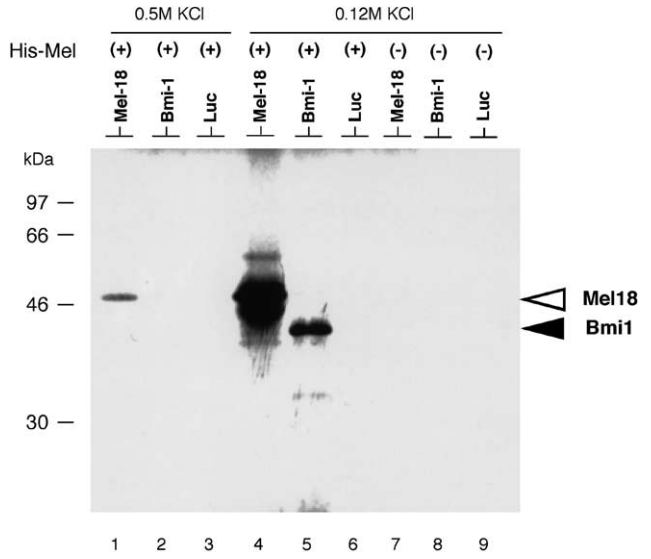

Fig. 1. Mel-18 strongly binds to itself based on in vitro pull-down assays. Full-length Mel-18, Bmi-1, and luciferase were synthesized in vitro, radio-labeled with [^{35}S]methionine, mixed with bacterially produced His-tagged Mel-18, and immobilized on Zn Sepharose beads. After incubating and washing with buffer containing 0.5 M KCl (high salt buffer) or 0.12 M KCl (low salt buffer), bound proteins were eluted and separated by SDS–PAGE (10% gels) and visualized by autoradiography. The samples in lanes 1–3 and lanes 4–9 were washed with high salt buffer and low salt buffer, respectively. Sizes of molecular weight markers (kilodalton) are indicated.

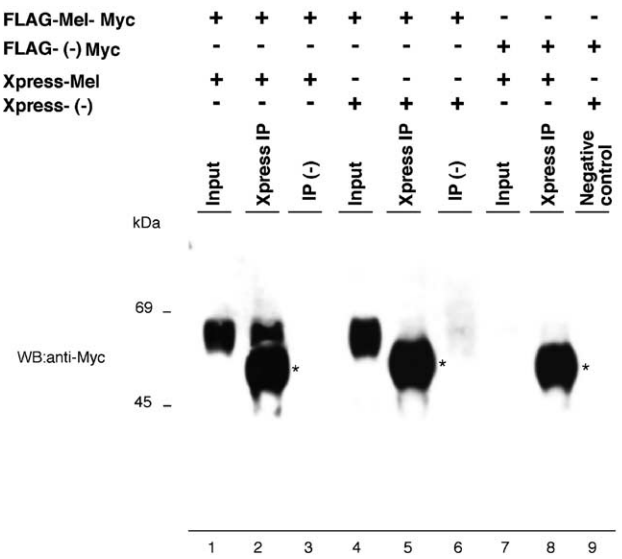

Fig. 2. Mel-18 is able to bind to itself in vivo. COS-7 cells were transiently transfected with an expression vector for FLAG-Myc-tagged-Mel-18 or a vector with no insert, as a negative control, in the presence and absence of Xpress-tagged Mel-18. Each cell lysate was immunoprecipitated with an anti-Xpress antibody and protein G–Sepharose beads and then washed with HNMG buffer. The bound proteins were eluted and separated by SDS–PAGE (10% gels) and immunoblotted with anti-Myc antibody. The IgG heavy chain is denoted by an asterisk.

lane 1), but not between Mel-18 and Bmi-1 (Fig. 1, lane 2). These results show that Mel-18 protein strongly self-associates. To confirm whether Mel-18 can form homodimers in mammalian cells, as it does in vitro, we expressed FLAG-Myc-tagged Mel-18 (FLAG-Mel-Myc) and/or Xpress-tagged Mel-18 (Xpress-Mel) proteins in COS-7 cells by transient co-transfection. We used anti-Xpress antibody to immunoprecipitate from whole cell lysate and then immunoblotted with anti-Myc antibody (Fig. 2). In cases where both Xpress-Mel and FLAG-Mel-Myc proteins were co-expressed, we detected a signal from FLAG-Mel-Myc (Fig. 2, lane 2), but no other control combination (lanes 5 and 8). This result clearly indicates that Xpress-Mel associates with FLAG-Mel-Myc, and therefore, we conclude that Mel-18 can also homo-dimerize in mammalian cells.

Mel-18 forms homodimers through the N-terminal domain containing RING-finger motifs

Next, we constructed a series of deletion mutants to determine which domain(s) are required for dimerization (Fig. 3A). A series of [^{35}S]Mel proteins with different portions deleted were incubated with His-Mel and then we performed the same pull-down assay as in Fig. 1. Full-length Mel-18 (FL-Mel), ΔC11-Mel, ΔC12-Mel, and ΔC13-Mel could interact with His-Mel (Fig. 3B, lanes 2–5). In contrast, ΔC14-Mel, that had all α-helix domains deleted, and ΔN1-Mel, that did not possess a RING-finger domain, could not interact with His-Mel (Fig. 3B, lanes 6 and 7). These results suggest that an N-terminal domain, containing a RING-finger motif and first/second α-helix domains, is necessary for Mel-18 homodimer formation.

Mel-18 homodimers are disrupted by protein kinase C

By computer PROSITE analysis, we noticed that the putative dimerization domains have numerous target sites for protein kinase C (PKC) phosphorylation. We therefore examined whether Mel-18 homodimer formation is affected by PKC and/or protein phosphatases. Recombinant His-Mel and in vitro translated [^{35}S]Mel proteins were mixed in conditions with or without PKC, a PKC inhibitor, and phosphatases (Fig. 4). Mel-18 homodimer formation was not detected upon treatment with PKC (Fig. 4, lane 3). Signals indicating homodimerization, however, reappeared in the presence of a PKC inhibitor (Fig. 4, lane 4) or in the presence of protein phosphatase 1+2A (Fig. 4, lane 5). Thus, Mel-18 dimerization may be regulated by its phosphorylation status, with dephosphorylated Mel-18 forming homodimers, which dissociate when phosphorylated by PKC. However we do not know whether heterodimer formation between phosphorylated- and dephosphorylated- Mel-18 is possible, therefore we could not rule out this possibility in our in vivo experiments.

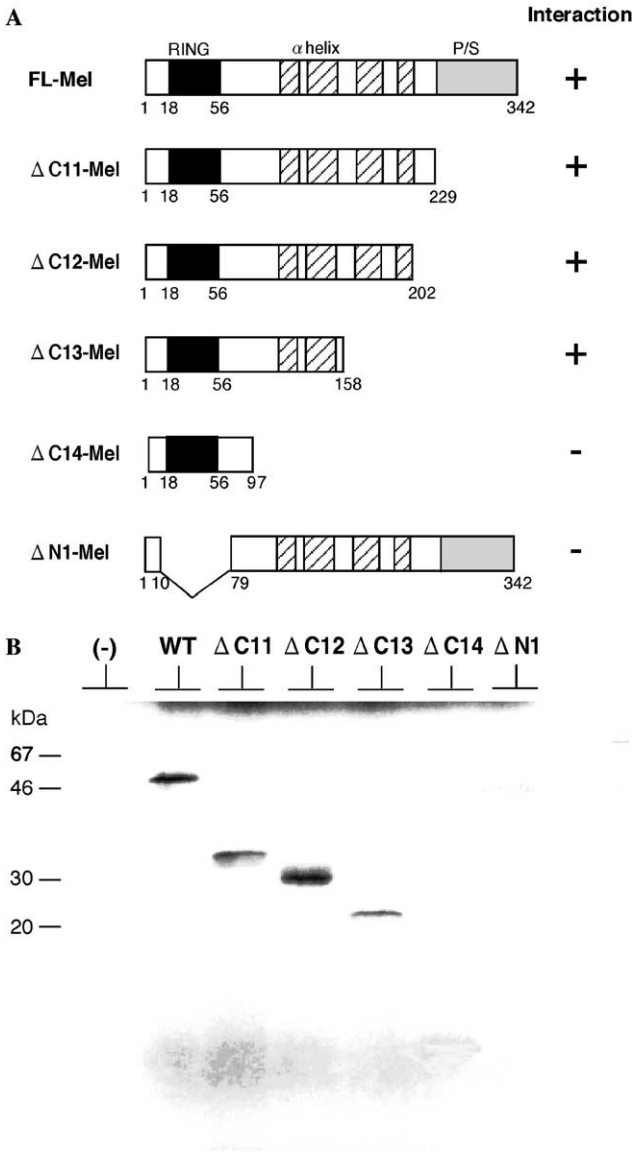

Fig. 3. Mel-18 associates with itself through its N-terminal domain containing a RING-finger and the near putative helix–turn–helix structure. (A) Schematic representation of the deletion series of Mel-18 proteins used to determine the binding domain. The black box represents the RING finger domain; the hatched boxes represent the putative helix–turn–helix region; and the light-shaded box represents proline/serine-rich region (P/S). (B) The proteins shown in (A) were [^{35}S]methionine-labeled and synthesized in vitro. They were then mixed with bacterially produced His-tagged Mel-18 and immobilized on Zn Sepharose beads. After incubating and washing with buffer, the bound proteins were eluted and separated by SDS–PAGE (10% gels) and visualized by autoradiography. Sizes of molecular weight markers (kilodalton) are indicated.

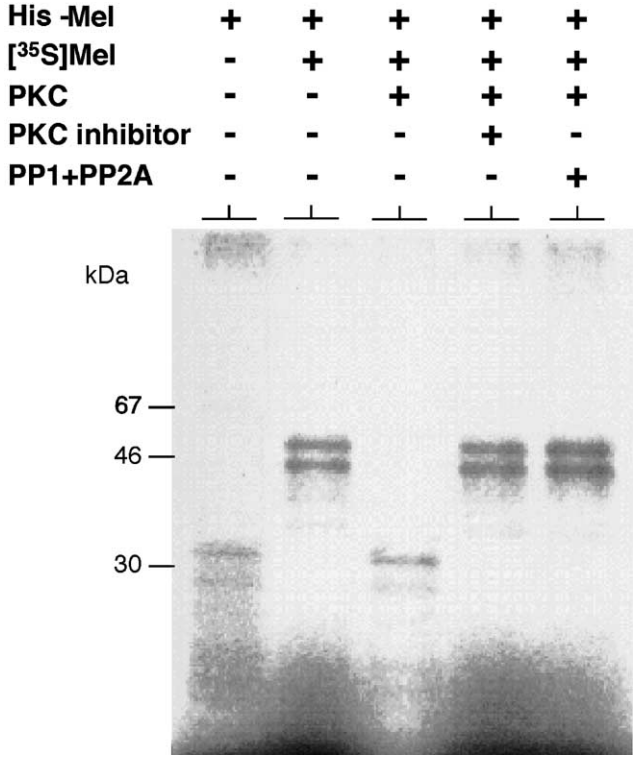

Fig. 4. Mel-18 homodimer is disrupted by protein kinase C (PKC). [^{35}S]methionine-labeled Mel-18 proteins were mixed with bacterially produced His-tagged Mel-18 and immobilized on Zn Sepharose beads in the presence or absence of PKC, PKC inhibitor, and phosphatases. After incubating and washing with buffer, bound proteins were eluted and separated by SDS–PAGE (10% gels) and visualized by autoradiography. Sizes of molecular weight markers (kilodalton) are indicated.

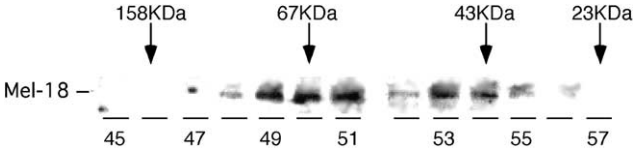

Fig. 5. Gel filtration analysis of 18.5 dpc mouse embryo extract. Whole cell extracts were fractionated by Superose 12 chromatography. Fraction numbers are indicated at the bottom. Detection of Mel-18 by immunoblotting. The elution positions of molecular mass standards are indicated by arrows.

Moreover, we explored whether dimers form in natural physiological conditions. We prepared 18.5 dpc mouse whole embryo extracts, subjected them to simple gel filtration using Superose12 columns (Amersham BioScience), and performed Western blot analysis on each fraction with anti-Mel-18 antibody. We detected Mel-18 signals in both the monomer (~36–40 kDa) and dimer fractions (~70–90 kDa). Therefore, we concluded that there are at least two forms of Mel-18, monomers and homo- or heterodimers, in the mouse embryo (Fig. 5).

Discussion

In this study, we have shown that Mel-18 protein is able to bind itself both in vitro and in vivo in mammalian cells (Figs. 1 and 2). Additionally, we have shown that homodimer formation requires the N-terminal region (AA 10–158) containing RING-finger and

the first/second α-helix of the putative helix–turn–helix (HTH) domain. This region of Mel-18 is also well conserved among murine Bmi-1 (AA 10–158) and *Drosophila* posterior sex combs (Psc; its mammalian counterparts are Mel-18 and Bmi-1 (AA 250–473)). In *Drosophila*, this region of Psc has been reported to interact with Psc, Polycomb (Pc), and Polyhomeotic (Ph) Pc-G proteins, using the yeast two-hybrid system [18,19]. In contrast, Bmi-1 is able to bind itself via its C-terminal region which contains a proline/serine-rich domain and an HTH domain, whereas homo-dimerization via its N-terminal region was possible but very weak. Therefore, the N-terminal RING-finger and first/second α-helix domains might not be required for strong Bmi-1 homo-dimerization [9,29]. At present, we do not have an explanation why different domains (N-terminal and C-terminal regions) are required for Mel-18, Psc, and Bmi-1 homodimer formation. It is possible that the RING-finger and/or α-helix domains of these Polycomb proteins possess different three-dimensional conformations, which play a pivotal role in protein–protein interactions.

To understand the different domain requirements for homo-dimerization on Mel-18 and Bmi-1, we tried to discover the amino acid difference on these regions (RING-finger and α-helix domains). From precise comparisons of amino acid sequences of RING-finger domain between the Mel-18 and Bmi-1 (amino acid sequences on RING-finger domain), we noticed that there are only six amino acid differences in the protein domain from AA 10 to 79 (corresponding to ΔN1 for RING-finger). This six AA difference in the RING-finger domain might be critical for conferring a functional difference between Mel-18 and Bmi-1. Indeed, this domain (AA 10–79, corresponding to ΔN1 for RING-finger) of Mel-18 was necessary for strong homodimer formation but that of Bmi-1 interacted itself with weakly.

On the other hand, first/second α-helix domain (AA 97–158, corresponds to ΔC14 for α-helix domain) was also necessary for strong homo-dimerization of Mel-18 but weak interaction of Bmi-1. There are 30 amino acid differences within the AA 97–158 domain (corresponds to ΔC14 for α-helix) between Mel-18 and Bmi-1. We assume that a completely different helix structure between these two Polycomb proteins may explain the different biological functions, because approximately 50% of the amino acids differ in this 62 AA stretch in the α-helix domain.

Intracellular localization and/or chromatin association of Pc-G proteins have been reported to be controlled by phosphorylation status. For example, Bmi-1 is specifically retained in the chromatin-associated nuclear protein fraction at G1/S of the cell cycle, but phosphorylated Bmi-1 is not chromatin-bound during G2/M [40]. In the case of mouse Polycomb M33 protein, hyperphosphorylated M33 localizes to the nucleus, whereas hypophosphorylated M33 localizes to the cytoplasm [22]. In addition, the phosphorylation status of Polycomb proteins is likely important for nuclear localization and/or function as a transcriptional repressor or cell cycle regulator. In this study, we have shown that Mel-18 homodimer formation is inhibited in the presence of PKC and stimulated when in its dephosphorylated form, e.g., in the presence of PKC inhibitors or protein phosphatases. Thus, we assume that these domains, for phosphorylation and dimerization, of Mel-18 (and Bmi-1) regulate the stoichiometry of class II Polycomb protein complexes by sequestering interacting proteins from this complex. This sequestering function of Mel-18 might depend on the equilibrium between monomer and homodimer controlled by phosphorylation status. Therefore, the regulation of equilibrium between monomers and dimers might control the function and specificity of class II Polycomb protein complexes by regulating the subunit composition of the complex.

The transcription silencing assay using purified Polycomb protein complexes and/or monomer/dimmer form of Mel-18 is underway in our laboratory. Further study is required to explore the biological significance of dimer formation and the mechanism by which dimer formation affects the stability of class II Polycomb protein complexes.

Acknowledgments

We thank Hiroko Inoue, Ruriko Sakai, and Mayumi Ninomiya for technical assistance. This study was supported by CREST (Core Research for Evolutional Science and Technology) of Japan Science and Technology Corporation, a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (12215108, 12490025 for M.K. and 13037025, 13780558 for H.I.), and the Uehara Life Science Foundation.

References

[1] P.N. Adler, E.C. Martin, J. Charlton, K. Jones, Phenotypic consequences and genetic interactions of a null mutation in the *Drosophila* posterior sex combs gene, Dev. Genet. 12 (1991) 349–361.

[2] M.J. Alkema, M. Bronk, E. Verhoeven, A. Otte, L.J. van't Veer, A. Berns, M. van Lohuizen, Identification of Bmi1-interacting proteins as constituents of a multimeric mammalian Polycomb complex, Genes Dev. 11 (1997) 226–240.

[3] M. Bienz, J. Muller, Transcriptional silencing of homeotic genes in *Drosophila*, Bioessays 17 (1995) 775–784.

[4] A. Breiling, B.M. Turner, M.E. Bianchi, V. Orlando, General transcription factors bind promoters repressed by Polycomb group proteins, Nature 412 (2001) 651–655.

[5] G. Cavalli, R. Paro, Chromo-domain proteins: linking chromatin structure to epigenetic regulation, Curr. Opin. Cell Biol. 10 (1998) 354–360.

[6] N.N. Cheng, D.A. Sinclair, R.B. Campbell, H.W. Brock, Interactions of Polyhomeotic with Polycomb group genes of *Drosophila melanogaster*, Genetics 138 (1994) 1151–1162.
[7] M.J. Gunster, D.P. Satijn, K.M. Hamer, J.L. den Blaauwen, D. de Bruijn, M.J. Alkema, M. van Lohuizen, R. van Driel, A.P. Otte, Identification and characterization of interactions between the vertebrate Polycomb-group protein BMI1 and human homologs of Polyhomeotic, Mol. Cell. Biol. 17 (1997) 2326–2335.
[8] M. Hasegawa, O. Tetsu, R. Kanno, H. Inoue, H. Ishihara, M. Kamiyasu, M. Taniguchi, M. Kanno, Mammalian Polycomb group genes are categorized as a new type of early response gene induced by B-cell receptor cross-linking, Mol. Immunol. 35 (1998) 559–563.
[9] N. Hashimoto, H.W. Brock, M. Nomura, M. Kyba, J. Hodgson, Y. Fujita, Y. Takihara, K. Shimada, T. Higashinakagawa, RAE28, BMI1, and M33 are members of heterogeneous multimeric mammalian Polycomb group complexes, Biochem. Biophys. Res. Commun. 245 (1998) 356–365.
[10] C.S. Hemenway, B.W. Halligan, L.S. Levy, The Bmi-1 oncoprotein interacts with dinG and MPh2, the role of RING finger domains, Oncogene 16 (1998) 2541–2547.
[11] A. Ishida, H. Asano, M. Hasegawa, H. Koseki, T. Ono, M.C. Yoshida, M. Taniguchi, M. Kanno, Cloning and chromosome mapping of the human Mel-18 gene which encodes a DNA-binding protein with a new 'RING-finger' motif, Gene 129 (1993) 249–255.
[12] J.J. Jacobs, P. Keblusek, E. Robanus-Maandag, P. Kristel, M. Lingbeek, P.M. Nederlof, T. van Welsem, M.J. van de Vijver, E.Y. Koh, G.Q. Daley, M. van Lohuizen, Senescence bypass screen identifies TBX2, which represses Cdkn2a (p19(ARF)) and is amplified in a subset of human breast cancers, Nat. Genet. 26 (2000) 291–299.
[13] J.J. Jacobs, B. Scheijen, J.W. Voncken, K. Kieboom, A. Berns, M. van Lohuizen, Bmi-1 collaborates with c-Myc in tumorigenesis by inhibiting c-Myc-induced apoptosis via INK4a/ARF, Genes Dev. 13 (1999) 2678–2690.
[14] J.J. Jacobs, M. van Lohuizen, Cellular memory of transcriptional states by Polycomb-group proteins, Semin. Cell Dev. Biol. 10 (1999) 227–235.
[15] C.A. Jones, J. Ng, A.J. Peterson, K. Morgan, J. Simon, R.S. Jones, The *Drosophila* esc and E(z) proteins are direct partners in Polycomb group-mediated repression, Mol. Cell. Biol. 18 (1998) 2825–2834.
[16] M. Kanno, M. Hasegawa, A. Ishida, K. Isono, M. Taniguchi, mel-18, a Polycomb group-related mammalian gene, encodes a transcriptional negative regulator with tumor suppressive activity, EMBO J. 14 (1995) 5672–5678.
[17] J.A. Kennison, The Polycomb and trithorax group proteins of *D*rosophila: trans-regulators of homeotic gene function, Annu. Rev. Genet. 29 (1995) 289–303.
[18] M. Kyba, H.W. Brock, The *Drosophila* Polycomb group protein Psc contacts ph and Pc through specific conserved domains, Mol. Cell. Biol. 18 (1998) 2712–2720.
[19] M. Kyba, H.W. Brock, The SAM domain of Polyhomeotic, RAE28, and scm mediates specific interactions through conserved residues, Dev. Genet. 22 (1998) 74–84.
[20] J. Lessard, A. Schumacher, U. Thorsteinsdottir, M. van Lohuizen, T. Magnuson, G. Sauvageau, Functional antagonism of the Polycomb-group genes eed and Bmi1 in hemopoietic cell proliferation, Genes Dev. 13 (1999) 2691–2703.
[21] J. Ng, C.M. Hart, K. Morgan, J.A. Simon, A *Drosophila* ESC-E(Z) protein complex is distinct from other Polycomb group complexes and contains covalently modified ESC, Mol. Cell. Biol. 20 (2000) 3069–3078.
[22] K. Noguchi, R. Shiurba, T. Higashinakagawa, Nuclear translocation of mouse Polycomb m33 protein in regenerating liver, Biochem. Biophys. Res. Commun. 291 (2002) 508–515.
[23] H. Ogawa, K. Ishiguro, S. Gaubatz, D.M. Livingston, Y. Nakatani, A complex with chromatin modifiers that occupies E2F- and Myc-responsive genes in G0 cells, Science 296 (2002) 1132–1136.
[24] V. Pirrotta, Chromatin-silencing mechanisms in *Drosophila* maintain patterns of gene expression, Trends Genet. 13 (1997) 314–318.
[25] V. Pirrotta, PcG complexes and chromatin silencing, Curr. Opin. Genet. Dev. 7 (1997) 249–258.
[26] V. Pirrotta, Polycombing the genome: PcG, trxG, and chromatin silencing, Cell 93 (1998) 333–336.
[27] M.J. Reijnen, K.M. Hamer, J.L. den Blaauwen, C. Lambrechts, I. Schoneveld, R. van Driel, A.P. Otte, Polycomb and bmi-1 homologs are expressed in overlapping patterns in *Xenopus* embryos and are able to interact with each other, Mech. Dev. 53 (1995) 35–46.
[28] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
[29] D.P. Satijn, M.J. Gunster, J. van der Vlag, K.M. Hamer, W. Schul, M.J. Alkema, A.J. Saurin, P.S. Freemont, R. van Driel, A.P. Otte, RING1 is associated with the Polycomb group protein complex and acts as a transcriptional repressor, Mol. Cell. Biol. 17 (1997) 4105–4113.
[30] D.P. Satijn, K.M. Hamer, J. den Blaauwen, A.P. Otte, The Polycomb group protein EED interacts with YY1, and both proteins induce neural tissue in *Xenopus* embryos, Mol. Cell. Biol. 21 (2001) 1360–1369.
[31] D.P. Satijn, A.P. Otte, Polycomb group protein complexes: do different complexes regulate distinct target genes?, Biochim. Biophys. Acta 1447 (1999) 1–16.
[32] D.P. Satijn, A.P. Otte, RING1 interacts with multiple Polycomb-group proteins and displays tumorigenic activity, Mol. Cell. Biol. 19 (1999) 57–68.
[33] A.J. Saurin, Z. Shao, H. Erdjument-Bromage, P. Tempst, R.E. Kingston, A *Drosophila* Polycomb group complex includes Zeste and dTAFII proteins, Nature 412 (2001) 655–660.
[34] R.G. Sewalt, J. van der Vlag, M.J. Gunster, K.M. Hamer, J.L. den Blaauwen, D.P. Satijn, T. Hendrix, R. van Driel, A.P. Otte, Characterization of interactions between the mammalian Polycomb-group proteins Enx1/EZH2 and EED suggests the existence of different mammalian Polycomb-group protein complexes, Mol. Cell. Biol. 18 (1998) 3586–3595.
[35] J. Simon, Locking in stable states of gene expression: transcriptional control during *Drosophila* development, Curr. Opin. Cell Biol. 7 (1995) 376–385.
[36] M. Tagawa, T. Sakamoto, K. Shigemoto, H. Matsubara, Y. Tamura, T. Ito, I. Nakamura, A. Okitsu, K. Imai, M. Taniguchi, Expression of novel DNA-binding protein with zinc finger structure in various tumor cells, J. Biol. Chem. 265 (1990) 20021–20026.
[37] O. Tetsu, H. Ishihara, R. Kanno, M. Kamiyasu, H. Inoue, T. Tokuhisa, M. Taniguchi, M. Kanno, mel-18 negatively regulates cell cycle progression upon B cell antigen receptor stimulation through a cascade leading to c-myc/cdc25, Immunity 9 (1998) 439–448.
[38] M. van Lohuizen, M. Tijms, J.W. Voncken, A. Schumacher, T. Magnuson, E. Wientjens, Interaction of mouse Polycomb-group (Pc-G) proteins Enx1 and Enx2 with Eed: indication for separate Pc-G complexes, Mol. Cell. Biol. 18 (1998) 3572–3579.
[39] M. van Lohuizen, S. Verbeek, B. Scheijen, E. Wientjens, H. van der Gulden, A. Berns, Identification of cooperating oncogenes in E mu-myc transgenic mice by provirus tagging, Cell 65 (1991) 737–752.
[40] J.W. Voncken, D. Schweizer, L. Aagaard, L. Sattler, M.F. Jantsch, M. van Lohuizen, Chromatin-association of the Polycomb group protein BMI1 is cell cycle-regulated and correlates with its phosphorylation status, J. Cell Sci. 112 (1999) 4627–4639.