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A Novel Histone Methyltransferase, Kodo7 Induces Histone H3-K9 Methylation and Mediates Apoptotic Cell Death

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SET (Suppressor of variegation, Enhancer of zeste, and the Trithorax) domain-containing proteins are known to have methyltransferase activity at lysine residues of histone proteins. In this study, we identified a novel SET domain-containing protein from mouse and named Kodo7. Indeed, Kodo7 has methyltransferase activity at K9 residue of the H3 protein as demonstrated by a histone methyl-transferse activity assay using GST-tagged Kodo7. Confocal microscopy showed that Kodo7 is co-localized with histones in the nucleus. Interestingly, ectopic expression of Kodo7 by transient transfection induced cell death and treatment of the transfectants with a caspase-3 inhibitor, Ac-DEVD-AFC decreased Kodo7-induced apoptosis. These results suggest that Kodo7 induces apoptotic cell death through increased methylation of histones leading to transcriptional repression.

Keywords: histone, methyltransferase, transcription, apoptosis

Introduction

In the nuclei of eukaryotic cells, DNA is highly compacted with histone and nonhistone proteins called chromatin. Transcriptionally active region of the genome residues in euchromatin, wherease inactive region residues heterochromatin. The basic repeating unit of chromatin is the nucleosome octamer, which is built by the core histones H2A, H2B, H3, and H4 wrapping 147 bp of DNA (Luger *et al.*, 1997). Histone N-terminal tails are flexible and protrude from the nucleosome octamer. Histone octamer is subjected to posttranslational modifications, including acetylation, methylation, phosphorylation, ubiquitination, sumoylation, and ADP-ribosylation (Turner *et al.*, 2000; Strahl and Allis, 2002).

Covalent modifications have been demonstrated to play important roles in the regulation of chromatin structure and gene activity (Lachner *et al.*, 2002; Sims *et al.*, 2003, Fischle *et al.*, 2003). Histone acetylation and deacetylation have gained an interest since gene activity was first correlated with histone acetylation. Euchromatin and heterochromatin exhibit different acetylation patterns. Euchromatin is the chromatin that decondenses during interphase of the cell cycle and which contains most of the genes coding for cellular proteins. Heterochromatin is condensed even in interphase, often contain repetitive DNA, and is generally silent (Grunstein, 1997).

Methylation of specific lysines of histone proteins can target chromatin for activation or repression of transcription (Kouzarides, 2002). In histone H3, five lysine residues (K4, K9, K27, K36, and K79) and K20 in histone H4 may become methylated. Methylation of H3-K4, H3-K36, and H3-K79 has been correlated with transcriptional activation, whereas methylation of H3-K9, H3-K27, and H4-K20 is marks of repressive chromatin stated (Lachner *et al.*, 2003). In particular, H3-K9 methylation is well known for its role at the heterochromatin formation, transcriptional silencing, X chromosome inactivation, and DNA methylation (Richards and Elgin, 2002; Schotta *et al.*, 2004).

Histone methyltransferase (HMTase) is the enzymes responsible for this modification and is specific to either lysine or arginine residues. Arginine methylation is catalyzed by the PRMT/CARM family of HMTase, whereas most lysine

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methylation events are mediated by SET-domain containing HMTase. The first lysine HMTase identified was the Suv39h1 enzyme, which catalyzes methylation of histone H3 at lysine 9 (Rea *et al*., 2000) and another close relative, Suv39h2 was also identified (O'Carroll *et al*., 2000). In addition to the Suv39H family enzymes, G9a, ESET, and Eu-HMTase 1 have been reported that mammalian H3-K9 HMTase (Tachibana *et al*., 2001; Schultz *et al*., 2002; Ogawa *et al*., 2002).

A recent search for the evolutionarily highly conserved SET domain indicates that ≥ 200 'SET-domain' sequences occur in databases, ranging from entries for viruses, bacteria, plants and yeast to man. Evolutionary conserved SET domains were originally identified in three Drosophila proteins: Suppressor of variegation (Sur(var)3-9), Enhancer of zeste (E(z)), and the Trithorox (Tschiersch *et al*., 1994; Stassen *et al*., 1995; Jones *et al*., 1993). Interestingly, the mammalian homolog of trithorox, Mll/HRX, is frequently translocated in leukemias, resulting in oncogenic fusion proteins that lack the C-terminal SET domain (Waring *et al*., 1997). Another protein containing a SET domain, NSD1 (Kurotaki *et al*., 2001), has been associated with Wolf-Hirschhorn syndrome, suggesting that some members of the SET family might be linked to human pathologies.

In this study, we found a novel protein containing SET domains using research in Sanger and NCBI EST data base and Swissprot blast. Respected SET domain-containing protein was named Kodo7 and functional experiments were performed. We found that this Kodo7 enzyme methylates the histones with cofactors in vitro and overexpression of Kodo7 induces H3K9 methylation and decrease of cell viability.

Materials and methods

Plasmid construction

The coding sequence of SET domain-containing protein, Kodo7 was using blast at Sanger institute and NCBI web sites. A full length open redeading frame of Kodo7 was PCR amplified from mouse cDNA library (Clontech, Mountain view, USA) and the Kodo7 PCR products were inserted into bacterial expression vector pGEX-4T1 (Amersham Pharmacia Biotech., Piscataway, USA) to express GST tagged Kodo7 protein and inserted into eukaryotic expression vector pcDNA3.1-HisTOPO (Invitrogen, Carlsbad, CA) to express His tagged Kodo7 protein in eukaryotic cell line. The Kodo7 PCR products were also inserted into pEGFP-C1 vector (Amersham Pharmacia Biotech., Piscataway, USA) to express green-fluorescent protein recombinant Kodo7 in eukaryotic cell lines.

Expression and purification of recombinant proteins

The expression plasmids were transformed in *E. coli* BL21 (DE3) (Invitrogen, Carlsbad, CA) and GST tagged fusion proteins of Kodo7 were expressed by incubating at 28°C overnight. After IPTG induction for 2 hrs, the cells were lysed in NET buffer (50 mM Tris-HCl (pH7.8), 0.5 M NaCl, 1 mM EDTA, 0.01% Triton X-100, 1㆏protease inhibitor cocktail, and 0.5 ug/ul PMSF) and bacterial lysates were applied to glutathione sepharose beads with incubation for overnight. Glutathione sepharose-bound Kodo7 was washed thoroughly with NETN buffer (50 mM Tris-HCl (pH 7.8), 0.5 M NaCl, 1 mM EDTA, 0.01% Triton X-100, 1 ㆏protease inhibitor cocktail, and 0.5 ug/ul PMSF, 0.05% NP40) and eluted with the washing buffer. Purified recombinant Kodo7 proteins were used in methylation assays.

Histone methyltransferase assay

Histone methyltrasferase assays were carried out according to the protocol published previously with some modifications (Rea *et al*., 2000). For each methylation reaction, the total volume was 40 ul and contained 1 ug/ul histone (Sigma, St. Louis, USA) as a substrate, 150 nCi of S-adenosyl-[methyl-^{14}C]-$_L$-methionine (^{14}C-SAM) (15 Ci/mmol, Amersham Pharmacia Biotech., Piscataway, USA) as a methyl donor, and enzyme (Kodo7) in HMTase buffer (50 mM Tris-HCl (pH 8.5), 20 mM KCl 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1.25 M Sucrose). After incubation at 28°C for overnight, 10 ul 5 X SDS-PAGE sample loading buffer was added to each reaction mixture, and then the total 50 ul was loaded in SDS-PAGE. SDS-PAGE gels were dried with a gel dryer and the activity was analysed by Cyclone image analyser (Perkin Elmer, Boston, USA).

Cell culture and transient transfection

NIH3T3 cells were grown in DMEM media with 10% fetal bovine serum (GIBCO, Gaithersburg, USA) and 0.05% antibiotics in CO_2 incubator at 37°C. For transient transfection, NIH3T3 cells were plated in 6-well plates at 2 ㆏10^4 cells per well the day before transfection. Cells were transfected with 5 ug of plasmid DNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After 48-72 hrs culture, cells were collected and subjected to Western blot, apoptosis-related assay, and immunofluorescence assay.

Western blot analysis

The transiently-transfected cells were lysed and whole cell lysates were fractionated by SDS-PAGE, transferred to nitrocellulose (NC) membrane, blocked with 5% Skim milk, and probed with the α-His (1:500) or α-H3-K9-dimethyl (1:3000) antibodies from rabbit for overnight rotating at 4°C. Following washes, the NC membrane was incubated with an AP-conjugated goat α-rabbit secondary antibodies (1:2000) for 1 hrs at RT and washed 3 times. The blots were developed using BCIP/NBT solution (Sigma, St. Louis, USA) as a substrate for AP.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium

bromide) assay

Antiproliferative effects were measured by MTT assay using a commercial MTT stock (Amesco, Denver, USA). About 1×10^4 cells per well were plated in 96-well plates and incubated overnight in 100 ul of culture media and then cells were transfected with pcDNA3.1-HisTOPO-Kodo7. Ten microlitters of MTT (5 ug/ml) in PBS) and 90 ul media were then added to each well, and the cells were further incubated for overnight. The supernatant was removed and 100 ul of DMSO was added to each well, and then incubated for 5 min. The absorbance at wavelength of 570 nm was measured by spectrophotometry.

Caspase-3 assay

Caspase assay was performed with Caspase-3 fluorescent assay kit (ApoProbe-3, Peptron, Daejon, Korea). Cells were grown on 6-well plates and transiently transfected with pcDNA3.1-HisTOPO-Kodo7 using lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 48 hrs, cells were precipitated by centrifugation at 1500 rpm for 5 min. Cell pellet was resuspended and incubated on ice for 10 min. Cell lysates were centrifuged at 12,000 rpm for 3 min at 4°C and the supernatant was transferred to new microcentrifuge tubes. Induced samples were incubated with 2 ul of 2.5 mM inhibitor before adding substrate for 5 min at RT and added 50 ul of 2X reaction buffer containing freshly added 10 mM DTT to each reactions. Then, 2 ul of 2.5 mM substrate was added to each tube and incubated at 37°C for 30 min. Protease activity was detected by fluorimeter equipped with a 360 nm excitation and 460 nm emission filter.

Confocal microscopy using immunofluorescence

Cells were grown on glass cover slips, and transfected with pEGFP-Kodo7 plasmid using lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 48 hrs culture, cells were fixed with 4% formaldehyde for 15 min, and permeabilized with 0.25% Triton X-100 for 5 min. Cells were blocked with 2% serum in PBS for 30 min and incubated with the α-histone (1:500) antibodies (Upstate Biotechnology, Charlottesville, USA) in 5% skim milk for 1 hr at 37°C, followed by incubation with a Cy3-conjugated goat α-rabbit secondary antibodies (JacksonLaboratory, Bar Harbor, USA) and then mounted. Staining was visualized using a confocal laser scanning microscope (Leica, Allendale, USA).

Results and Discussion

Kodo7 has methyltransferase activity when immunoprecipitated with NIH3T3 cell extracts.

To investigate whether the SET domain of Kodo7 has enzyme activity, we tested histone as substrates for *in vitro* methylation. Full length Kodo7 proteins were immunoprecipitated with NIH3T3 cell extracts and used *in vitro* HMTase reaction and probed for activity to transfer a labeled methyl group from ^{14}C-SAM to free histones. The HMTase activities were detected where Kodo7 proteins were immunoprecipitated for overnight at 4 but no signals were detected that Kodo7 proteins were not immunoprecipitated (Fig. 1). These results suggest that Kodo7 need cofactors probably contained in cell extracts to function as a HMTase.

As shown in Fig. 1, HMTase activity of Kodo7 has different methylation pattern compared with PRMT1 (histone methyltransferase that methylates Arg3 on histone H4). Kodo7 methylated histone H3 while PRMT1 methylated H2B and H4 of histone. These different methylation patterns demonstrate that Kodo7 has the substrate specificity for histone.

The SET domain of Kodo7 predominantly methylates the H3K9 of histones in vivo.

The above results indicated that the HMTase activity of Kodo7 proteins is selective for H3 of histones. To examine this finding in more detail, we carried out immunoblot analysis using Kodo7 proteins in the overexpressed cell lines. After transient transfection with Kodo7, cell lysates were subjected to immunoblot analysis with specific antibodies to each dimethylated histone at lysine residues. Using specific antibodies for each dimethylated histone lysines, lysine specificity of Kodo7 was inverstigated (Fig. 2). Compare to the other lysines methylated tested, the increased methylation of histone H3K9 was detected. Based

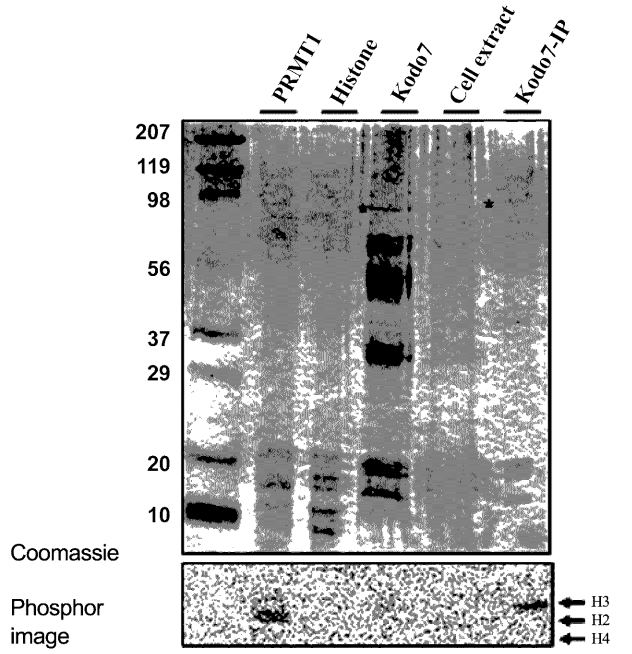

Fig. 1. Kodo7 can methylate histones in the presence of NIH3T3 cell lysates. Kodo7-IP and PRMT1 were detected C^{14}-labeled histones. Kodo7-IP was immunoprecipitated with NIH3T3 cell extracts for overnight at 4 and PRMT1 is positive control and Histone (no enzyme) and cell extract are negative control. Upper panel is the coomassie-staining and lower panel is the autoradiograph developed by a phosphor imager.

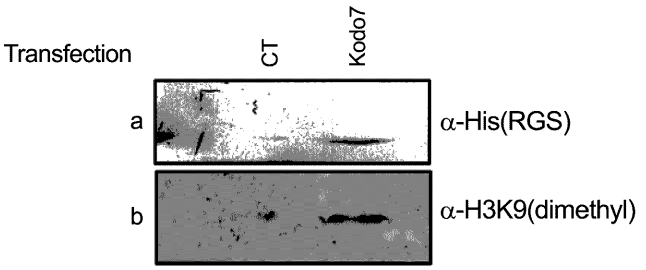

Fig. 2. Overexpression of Kodo7 increases dimethylation of the H3K9 in NIH3T3 cells. Transient transfected cell extracts were immunoblot against anti-dimethyl H3K9. Anti-His antibodies detected the expression of pcDNA3.1-HisTOPO-Kodo7.

on these results, two possible explanations can be made. The one possibility is that Kodo7 proteins directly methylates the H3K9 dimethylation *in vivo*. The other is that Kodo7 induce heterochromatin formation or silencing pathway and H3K9 dimethylation is increased by another H3K9-specific HMTases.

Kodo7 induces cell death through caspase-3 activation.

In previous studies, the functional significance of HMTases in cell cycle regulation was demonstrated by impaired embryonic development and abnormal cell differentiation in mutant organisms ranging from C. elegans to mice (Martin *et al.*, 2005). For example, G9a mutant mice had significant increases in apoptotic cell death (Tachibana *et al.*, 2002).

A series of experiments were performed to examine the ability of Kodo7 to induce apoptotic cell death. The cell growth inhibition of Kodo7 was determined at 48-72 hrs by direct cell count (Fig. 3). Cell number was 1.6×10^4 cell/ml in control and 1.1×10^4 cell/ml in Kodo7-transfected cells (Fig. 3A). Kodo7 expression decreased cell viability by 30% compared with control group. The MTT assay was also done to clear the effect of overexpressed Kodo7 proteins at cell viability. The MTT analysis provides a general measurement of mitochondrial dehydrogenase activity and cell viability. The MTT assay is based on the reduction of the soluble yellow MTT tetrazolium salt to a blue MTT formazan product by mitochondrial dehydrogenases. As shown Fig. 3B, the MTT activity of Kodo7-overexpressed cells were lower than that of control cells by about 20%. The direct cell count and MTT assay revealed that Kodo7 decreases cell viability and induces cell death.

As caspase-3 plays a key role in apoptosis, we investigated whether it was also involved in Kodo7-induced cell death. Caspase-3 activity was assayed by cleavage of Ac-DEVD-AFC, a fluorogenic substrate based on the peptide sequence at the caspase-3 cleavage site of poly (ADP-ribose) polymerase. The cells were transfected with Kodo7 for 48 hrs, caspase-3 activity was then determined using the fluorogenic tetrapeptide substrate Ac-DEVD-AFC in the presence or absence of a caspase-3 inhibitor (Ac-DEVD-CHO). Fig. 3C shows that overexpression of Kodo7 resulted

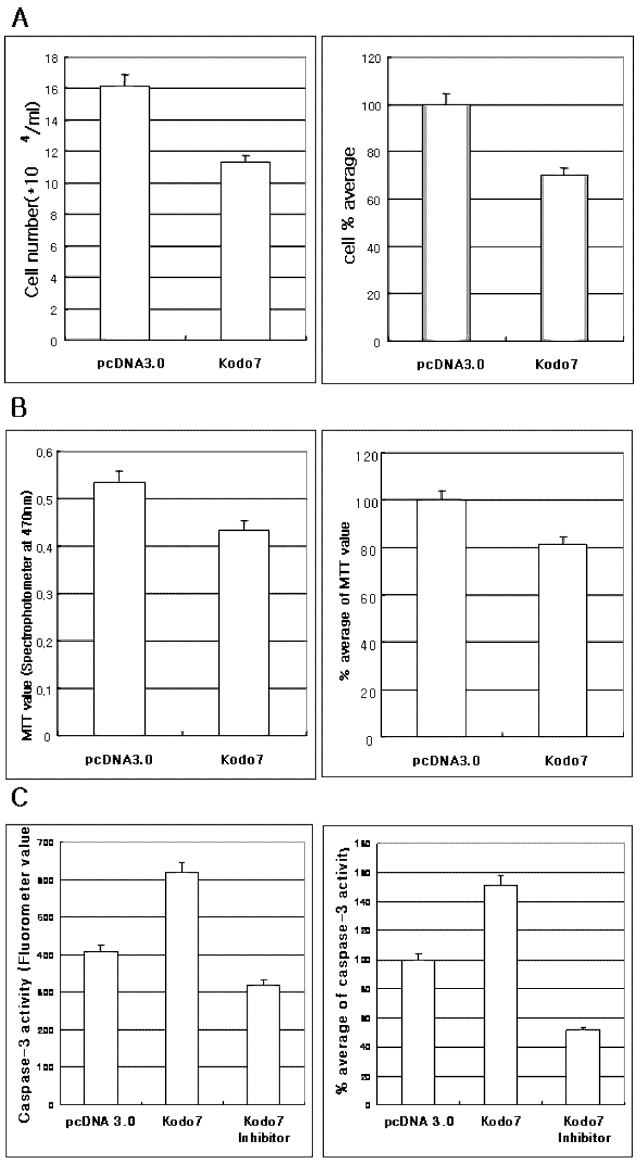

Fig. 3. Apoptosis assay. (A) Direct cell count. At 48 hrs after Kodo7 translocation, viable cells were coounted using trypan blue staining. Left panel represents viable cell numbers and right panel represents their percentage values (B) MTT assay. Cell growth of the cells were monitored by mitochondrial dehydrogenase activity after 48 hrs exposure periods. Left graph presents the values of spectrophotometry at 470 nm and right graph presents the relative value to control. (C) Caspase-3 assay. Caspase-3 activity was determined with fluorometer with a 360 nm excitation and 460 nm emission filter.

in an 1.5-fold increase in caspase-3 activity compared with control group. This activity was abolished in the presence of the caspase-3 inhibitor confirming the specificity of the assay. These results suggest that Kodo7-induced cytotoxicity is mediated by caspase-3 which is involved in apoptotic cell death.

Colocalization of Kodo7 with histones

The observation that Kodo7 methylates the histones with

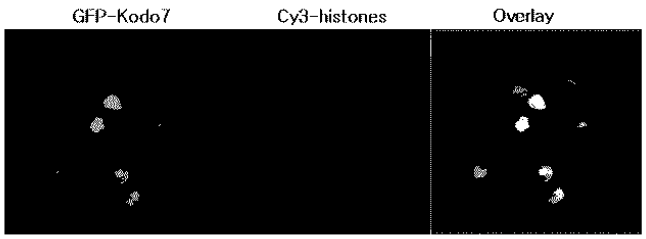

Fig. 4. Analysis of Kodo7 localization with histones. After transient transfection of pEGFPc1-Kodo7, cells were stained with antibodies against histone and Cy3-conjugated secondary antibodies. Kodo7 localization is visualized as green, antibody staining is visualized as red, and an overlap between two is visualized as yellow when overlayed.

cofactors and related with cell viability led us to investigate whether they would individually colocalize with histones *in vitro*. We generated GFP fusions of Kodo7 proteins and carried out colocalization studies to demonstrate the association between individual Kodo7 and histones in intact cells. The cells were transfected with expressing GFP-fused Kodo7 proteins and were immunostained with anti-histone antibodies and Cy3-conjugated secondary antibodies. Colocalization was detected by overlaying GFP and Cy3 images. The individual and overlay detection of Kodo7 and histones show both proteins are in the nucleus (Fig. 4). Colocalization of Kodo7 with histone implies that Kodo7 directly bind to and function to the histone proteins in the nucleus.

Collectively, The results show that Kodo7 methylates the histones with cofactors in vitro, overexpression of Kodo7 led to dimethylation of H3K9 and led to cell death *in vitro*. The findings suggest that the Kodo7 induces H3K9-dimethylation which might be involved in apoptotic cell death.

Acknowledgements

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