Inactivation of a Histone Methyltransferase by Mutations in Human Cancers

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Abstract

Histone methyltransferase (HMT)3 class enzymes that methylate lysine residues of histones or proteins contain a conserved catalytic core termed the SET domain, which shares sequence homology with an independently described sequence motif, the PR domain. Intact PR or SET sequence is required for tumor suppression functions, but it remains unclear whether it is histone methyltransferase activity that underlies tumor suppression. We now show that tumor suppressor RIZ1 (PRDM2) methylates histone H3 on lysine 9, and this activity is reduced by mutations in the PR domain found in human cancers. Also, S-adenosylhomocysteine or methyl donor deficiency inhibits RIZ1 and other H3 lysine 9 methylation activities. These results support the hypothesis that H3 lysine 9 methylation activities of a PR/SET domain have tumor suppression functions and may underlie carcinogenesis associated with dietary methyl donor deficiency.

Introduction

The retinoblastoma protein-interacting zinc finger gene RIZ was isolated in a functional screening for retinoblastoma-binding proteins (1), also independently isolated as a DNA-binding protein MTB-ZI (2), a GATA3 transcription factor binding protein G3B (3), and a coactivator of estrogen receptor (4). RIZ contains the canonical retinoblastoma-binding motif LXXC4 and the nuclear hormone receptor-binding motif LXXLL. In addition, RIZ contains a novel domain, called the PR (PRDI-BF1 and RIZ homology) domain, which shows similarity to the catalytic motif or the SET domain of histone/protein-methyltransferase (5–9). There are ~20 PR domain-containing genes and ~30 SET domain-containing genes in humans (10).

The RIZ gene expresses two products, RIZ1 that contains the PR domain and RIZ2 that lacks the domain (11). RIZ1 but not RIZ2 has tumor suppressive properties. The gene maps to chromosome 1p36, a region commonly deleted in more than a dozen different types of human cancers (12). RIZ1 expression, but not RIZ2, is commonly silenced in many types of human tumors, including breast cancer, liver cancer, colon cancer, neuroblastoma, melanoma, lung cancer, and osteosarcoma (13–15). Silencing is correlated with CpG island promoter DNA methylation (16, 17). RIZ1 missense inactivating mutations also occur in human cancer tissues and cell lines, and all target the PR domain (18). Frequent frameshift mutations of RIZ gene are common in microsatellite instability-positive tumors and truncate a PR-interacting domain (15, 19, 20). In keeping with the role as a tumor suppressor gene, RIZ1 has strong tumor suppressive activities. Adenovirus-mediated RIZ1 expression causes G2-M cell cycle arrest and/or apoptosis in breast cancer, liver cancer, and microsatellite instability-positive colon cancer cells (13–15). Adenovirus RIZ1 can also inhibit growth of colon cancer xenografts (21). Finally, gene knockout animals that lack RIZ1 but retain RIZ2 expression are prone to develop B-cell lymphomas (18). These studies suggest an important role for an intact PR domain in tumor suppression functions.

In this article, we examined whether the PR domain of RIZ1 has the suspected (HMT) activity and whether such activity may be inactivated by naturally occurring mutations.

Materials and Methods

Reagents. DHCA (kindly provided by Chong Yuan of Diazyme Lab., San Diego, CA) was prepared as 10 μM stock solutions in DMSO. Protein A-Sepharose was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Peptides and methylation-specific antibodies were from Upstate Biotech (Charlottesville, VA). Histones were extracted from U2OS cells by standard acid extraction procedures and used as substrates. Nucleosomes were prepared from HeLa cells by standard protocol for use as methylation substrates.

Plasmids and Protein Expression and Purification. Plasmids used for GST protein production were constructed by PCR cloning as follows. To express human RIZ1 protein 1–200 residue region, full-length human RIZ1 cDNA plasmid was used as a template for PCR (Pfu polymerase; Stratagene, San Diego, CA) by primers bRP109;2 (AAA CCA TGG ATC AGA ACA CTA CTG AG) and RP274 (CCG TAA GCT TCA TCG AGA GGT GAA ATC TGG C). The PCR fragment was cloned into the NcoI and HindIII sites of pKG-PBR vector by PCR to generate the plasmid pKG-H205. BL-21 cells were transformed by pKG-H205 to produce the GST-RIZ200 protein. To express RIZ1 protein 1–161 residue region, a stop codon was introduced at residue 162 in the plasmid pKG-HN1 by using the Quick-Change mutagenesis kit (Stratagene). The primers used are RP296P1 (CGA GCC AGC GCC CGG AGC TAA GCT TAA GGC GAG CTC CCC) and RP296P2, which has the compliment sequence of RP296P1. To generate longer peptide fragments of RIZ1 in bacteria, we first cloned the NH2-terminal 520 residue into the NcoI and HindIII sites of pKG-PBR vector by PCR to generate the plasmid pKGH205. However, this plasmid did not give high yields of the expected protein product. Next, we introduced a stop codon into this plasmid by Quickchange mutagenesis to generate pKGRIZ332, which produced high yields of RIZ1 NH2 terminus 323-residue peptide. The PCR primers used for mutagenesis are: RP307: GAT TTA TTA GAG GAA tgA AAA ACA ACT TCA GAA G, and RP308: CTT CTG AAG TGT TTT TcC ttt CCT CTG ATA ATC CCT. To generate missense mutant GST-RIZ200 or RIZ322 proteins, mutagenesis by Quick-Change was performed on the pKG-HN1 plasmid template using primers as described previously (18). Mutations were confirmed by DNA sequencing analysis. To purify GST fusion proteins, standard protocol was followed, except that RNase A was introduced at the step of cell lysis to degrade RNA.

Cell Culture and Immunoprecipitation. U2OS cells were grown in DMEM supplemented with 10% heat-inactivated FCS. U2OS cells were infected with an AdRIZ1 or the AdNull as described previously (13). Cells were then grown for an additional 48 h. Nuclear extracts were prepared using standard procedures (22) and proceeded to immunoprecipitation as described (1, 11). For the study of histone methylation in the presence and absence of DHCA, U2OS cells were treated with 5 μM DHCA added in the culture medium. Histones were extracted from U2OS cells by standard acid extraction procedures and analyzed by immunoblot analysis using dimethylation-specific H3 antibodies (Upstate Biotech). For examining DHCA effects on cell cycle, cells were treated with DHCA for 3 days and were then processed for FACScan analysis as described previously (13).
**Methylation Assays.** Methylation reactions ($30–40 \mu l$) contained 20 mM Tris-HCl (pH 8.0), 0.2 mM NaCl, 0.4 mM EDTA, 1 mM DTT, affinity-purified GST-RIZ1 or immunoprecipitation products, free histones (10 \mu g), or peptides, or nucleosomes, and 3 \mu l (1.65 \mu Ci and 21 pmol)[methyl-\(^3\)H]adenosyl-methionine (Amersham-Pharmacia). The mixture was incubated at 37°C for 1–2 h followed by SDS gel analysis followed by fluorography. Nuclear extracts were prepared according to standard procedures and used for immunoprecipitation. Antisera used for immunoprecipitation were anti-KG7.1S and preimmune serum as described previously (1). Similar HMT activity has also been observed with immunoprecipitates using a different serum (data not shown), 171S, which was described previously (18). Various amount of SAH (Sigma) were added in some methylation reactions.

To determine the methylated residues on H3, H3 NH\(_2\)-terminal 1–20 residue peptides, nonacetylated and acetylated on K9 and K14 (Upstate Biotech), were used as substrates for in vitro methylation reactions as described above. The reactions were resolved on Tris-Tricine 10–20% gel (Invitrogen) followed by fluorography. The \(^3\)H-labeled H3 peptides were purified by high-performance liquid chromatography and next sequenced from the NH\(_2\) termini in an Applied Biosystem model 477A Protein Sequencer (kindly performed by Chris Park and Wolfgang Fischer at the Salk Institute, La Jolla, CA). After conversion, the samples were collected for determination of radioactivity by scintillation counting.

**Fractionation of Nuclear Extracts.** Nuclear extracts were prepared by the standard Dignam procedure (22). The extracts were loaded onto a Sephacryl S-400 (Amersham Pharmacia) column pre-equilibrated in elution buffer containing 50 mM Tris HCl (pH 7.5) and 150 mM NaCl. RIZ1 knockout mouse embryo fibroblasts were from RIZ1 knockout mice in a p53-deficient genetic background (18).

**Results**

The sequence homology of SET domain with protein lysine methyltransferases of plants suggests that SET domain proteins may have similar functions as the plant enzymes (8, 9), and indeed, several SET proteins have methyltransferase activities on histones (23–26). PR domain is similarly related to plant enzymes as SET domain, and there are ~30 SET proteins and ~20 PR proteins in humans (10). Therefore, we examined whether the PR domain-containing protein RIZ1 may have histone methylation activity. Human osteosarcoma U2OS cells with low-level expression of RIZ1 (13) were infected with AdRIZ1 virus or control AdNull virus, and nuclear extracts from virus-infected cells were immunoprecipitated with RIZ1 serum anti-KG7.1S or preimmune serum as described previously (1). Immunoprecipitated products were used in methylation assay reactions containing free histones and S-adenosyl-[methyl-\(^3\)H]methylthionine. Reaction products were separated by SDS-PAGE and visualized by fluorography. RIZ1 immunoprecipitates of AdRIZ1-infected cells showed higher H3 methylation relative to that of control virus infected cells (Fig. 1A, Lane 4), suggesting that RIZ1 has methylation activity toward histone H3.

To determine the methylation site on H3, we next performed peptide methylation and sequencing experiment. A peptide consisting of the NH\(_2\)-terminal 1–20 residues of H3 was methylated in vitro by immunoprecipitated RIZ1 protein (Fig. 1B). The same peptide synthesized with methylated K4 also served as a substrate, albeit less well. In contrast, peptides synthesized with methylated K9 or acetylated K9/K14 were not methylated by the RIZ1 immunoprecipitates, consistent with K9 but not K4 as a target of RIZ1.

The methylated peptide was next subjected to sequencing analysis. A radioactive peak at lysine 9 was observed (Fig. 1C), suggesting that H3-K9 was methylated by RIZ1. Furthermore, recombinant GST-H3 fusion protein (containing H3 residue 1–50) was methylated by RIZ1, whereas a GST-H3 (1–50) containing a K9R mutation was not (Fig. 1D), suggesting that lysine 9 may be the only site methylated by RIZ1 among lysine residues located within H3 NH\(_2\)-terminal tail sequences.

We noted reduced methylation for the K4R mutant of GST-H3 protein (Fig. 1D), which was similar to the result with the K4-methylated peptide (Fig. 1B). These results suggest that a nonmodified K4 residue may facilitate H3K9 methylation. Taken collectively, the data suggest that RIZ1 has H3-K9 methylating activity. This activity may play a role in transcriptional repression, because H3-K9 methylation is known to be associated with repression (27–30).

To determine whether RIZ1 is part of a protein complex, we prepared nuclear extracts from AdRIZ1-infected U2OS cells and fractionated the extracts on a Sephacryl S-400 column. Immunoblot analysis of the collected fractions showed that a majority of RIZ1 protein exists in a complex of M\(_r\) ~800,000, whereas a minor portion is in a complex of M\(_r\) ~1,800,000 (Fig. 2A). We next assayed these fractions for HMT activity on histone substrates. When nuclear extracts from RIZ1 virus-infected cells (Fig. 2B, top) were compared with that from control virus-infected cells (Fig. 2B, bottom), RIZ1 overexpression increased H3 methylation activity in RIZ1 peak fractions (compare fractions 38 and 26 of top versus bottom in Fig. 2B). By contrast, H1 and H4 methylation activities were not significantly affected by RIZ1 overexpression. We additionally compared HMT activity profiles of fractionated nuclear extracts of RIZ1 knockout and wild-type mouse embryo fibroblasts (Fig. 3C). RIZ1 deficiency decreased H3 methylation activity of the M\(_r\) ~800,000 RIZ1 peak fraction (fraction 38). H1 and H4 methylation activity profiles were not affected by RIZ1 knockout. These results suggest that RIZ1-containing protein complex has H3 methylation activity.

To confirm that the HMT activity associated with RIZ1 protein complex is intrinsic to RIZ1, we prepared recombinant GST fusion proteins of RIZ1 NH\(_2\)-terminal region that contains the PR domain. The purified fusion proteins were assayed for HMT activity using histones as substrates. The top shows fluorography indicating methylation of histones and the bottom shows Coomassie blue staining. B, methylation of H3 NH\(_2\)-terminal peptide. The methylated peptides were resolved by 16% Tris-Tricine SDS gel followed by fluorography. C, automated sequencing of the H3 peptide. The tritium incorporation of individual amino acids identified at each successive round of microsequencing is shown. D, methylation of GST-H3N (1–50) proteins. GST, GST-H3N (wild-type 1–50 residues), and two K to R mutant proteins K4R and K9R, were used as substrates in methylation reactions. Top, fluorography. Bottom, Coomassie blue staining.
We examined the activity of RIZ1 proteins with missense mutations that have been found in human cancers (18). Three such mutations, C106Y from SAOS2 osteosarcoma cells, A159V from a neuroblastoma cell line, and I188V, which is found in 30% of primary diffuse large B-cell lymphomas, were introduced into the GST-RIZ-N200 fusion protein. These mutations decrease RIZ1 transcription factor activities (18). We found that HMT enzyme activity was abolished by I188V mutation and partially affected by the other two mutations (Fig. 3, compare Lane 3 with Lanes 4–6). Both C106Y and A159V were each found in a single tumor cell line and not detected in primary tumors. The possibility of these variants as polymorphisms with only minor effects on enzyme activity cannot be excluded by our studies.

By contrast, I188V was commonly found in primary B cell tumors and appeared to abolish HMT activity (18). Our results here suggest that RIZ1 enzyme activity is reduced in cancers by the I188V mutation, which in turn supports the hypothesis that HMT activity of a PR/SET domain plays an important role in tumor suppression.

SAH is a competitive product inhibitor of methyltransferases and is induced to high levels by methyl-deficient and carcinogenic diet (10). We examined whether SAH can inhibit RIZ1 activity. Methylation assay was performed in the presence of 0.42 μM of [3H]SAM and physiologically relevant levels of SAH (μM range). The activity of immunoprecipitated RIZ1 was partially inhibited by SAH at 0.5 μM and completely at 5 μM (Fig. 4A). The results suggest that SAH is a potent inhibitor of RIZ1. We then examined whether HMT activity in general may be sensitive to modest elevation in SAH levels in vivo. The compound DHCA was used to inhibit SAH hydrolase, leading to elevation of SAH (31, 32). The effect of DHCA on histone methylation in U2OS cells was examined by Western blot using methylation-specific antibodies. H3-K9 methylation was inhibited by DHCA treatment (Fig. 4B). By contrast, H3-K4 and H3-K36 were not significantly affected. These data suggest that H3-K9 methyltransferase activity is inhibited when SAM:SAH ratio is low. DHCA treatment caused a slight increase of cell population at G2-M phase and a decrease in the number of cells at G1 phase (data not shown). Overexpression of RIZ1 is known to cause G2-M arrest (13). This function of RIZ1 was not blocked by DHCA treatment (data not shown), probably because any inhibition of RIZ1 function may be cancelled by DHCA-induced G2-M arrest.

Fig. 2. H3 methylation activity of RIZ1 protein complex. A, nuclear extracts from AdRIZ1 virus infected cells were fractionated on a Sephacryl S-400 column. Collected fractions were analyzed by immunoblot analysis using RIZ1 antibody. Fraction numbers are indicated at the bottom of each lane. The fractionation profile of molecular weight markers (Sigma) is indicated. B, RIZ1 overexpression increased H3 methylation activity. Collected fractions from sizing column were used as enzymes for methylation of histone substrates. Top represents results from AdRIZ1-infected cells and bottom is from control AdNull virus-infected cells. C, RIZ1 deficiency decreased H3 methylation activity. Nuclear extracts from RIZ1+/+ (top) and RIZ1−/− (bottom) mouse embryo fibroblasts were fractionated and assayed as described above.

Fig. 3. Inactivation of RIZ1 HMT activity by mutations. Mutations in the PR domain of RIZ1 reduce HMT activity. GST fusion proteins of RIZ1 were assayed for HMT activity toward nucleosome substrates N322, N200, and N161 represent GST fusion proteins of wild-type RIZ1 containing residues 1–322, 1–200, and 1–161, respectively. C106Y, I188V, and A159V represent mutant N200 proteins. Top shows fluorography indicating methylation of H3. Bottom shows Coomassie blue staining of reaction products.
Discussion

An intact PR domain is known to be required for RIZ1 tumor suppression function in mice (18). RIZ1 knockout mice are tumor prone and are deficient only in the PR domain as these animals express the PR-deficient product RIZ2. The presence of naturally occurring mutations in the PR domain that altered RIZ1 transcription factor function additionally suggests an important role for this domain in human cancer (18). These observations, taken together with the present findings of HMT activity for the PR domain and the inactivation of this activity by naturally occurring mutations, suggest that HMT activity is important to RIZ1 tumor suppression function. The H3-K9 methylation activity of RIZ1 may play a similar role as other HMTs in the epigenetic control of gene silencing. Several HMTs are known to be key players in the mitotic inheritance of cell fates and gene expression patterns (33–38). Some of these enzymes are commonly involved in cancer (10). Altered epigenetic control of gene expression pattern may lead to changes in cell fate and acquisition/inheritance of malignant phenotypes, and may underlie carcinogenesis associated with HMT or RIZ1.

About a third of all human cancer deaths in the United States is linked with the typical Western diet that is rich in meat/fat and low in vegetables/fruits. Numerous dietary factors have been hypothesized to influence cancer risks in Western countries, but for the moment the evidence for any one of these remains unclear, except for the methyl donor folic acid (39). High consumption of meat and low intake of folic acid in the form of fruits/vegetables seems to be the chief carcinogenic element of diet (39). The typical Western diet lowers the ratio of SAM versus SAH, and a low SAM:SAH ratio is known to inhibit the activity of many methyltransferases (10). Our results here show that histone H3-K9 methylation is also sensitive to inhibition by a low SAM:SAH ratio. A reduction in H3-K9 methylation may be critical to the initiation of carcinogenesis because it is also the biochemical outcome of naturally occurring mutations in the RIZ1 tumor suppressor gene. However, we do not expect all of the H3-K9 methyltransferases (there are likely to be many) to be inhibited to the same extent by a low SAM:SAH ratio. Some H3-K9 methylation activity will be present in tumor cells and such activity may even be necessary to maintain malignant phenotypes. The presence of H3-K9 methylation may serve to silence tumor suppressor genes (40–42). The observation that nearly all of the H3-K9 methylation activity is inhibited by DHCA treatment suggests that a high level of SAH is induced by DHCA treatment. Such level may be much higher than those that may be encountered during carcinogenesis. So, the effect of DHCA does not suggest that different H3-K9 methyltransferase will be inhibited to the same extent when the level of SAH is only modestly elevated. In addition to RIZ1, there are five other mammalian HMTs that are known to methylate H3-K9, including Suv39H1 (9) and Suv39h2 (43), G9a (44), Eu-HMTase1 (45), and ESET/SETDB1 (46, 47). It will be interesting to determine in the future whether these enzymes have different sensitivity to SAH inhibition.

A persistent inhibition of protein function at the gene product level is essentially similar to mutational inactivation. Because genetic inactivation of HMTs causes cancer (18, 48, 49), persistent inhibition of HMTs at the protein level will likely do the same. Either way, reduced HMT function may lead to mitotically inheritable changes in chromatin methylation and gene expression patterns. Such abnormal chromatin patterns may result in the generation of neoplastic phenotypes, which may be inherited from one cell to its progeny. Because methylation is stable, transient fluctuation in HMT activity may not pose a serious health risk, because it may not affect the methylation status of slow turnover substrates such as histones. However, persistent inhibition of HMT activity would cause heritable, potentially pathological effects. These observations suggest a hypothetical pathway of dietary carcinogenesis as follows: typical Western diet, chronic low SAM:SAH ratio, persistent inhibition of a subset of H3-K9 methyltransferases, inactivation of HMT tumor suppressors at the protein level, heritable changes in chromatin methylation and gene expression pattern, and acquisition and inheritance of malignant phenotypes. However, this explanation of dietary carcinogenesis by no means excludes other mechanisms such as mutations and DNA methylation.

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