SET Domain-containing Protein, G9a, Is a Novel Lysine-preferring Mammalian Histone Methyltransferase with Hyperactivity and Specific Selectivity to Lysines 9 and 27 of Histone H3*

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The covalent modification of histone tails has regulatory roles in various nuclear processes, such as control of transcription and mitotic chromosome condensation. Among the different groups of enzymes known to catalyze the covalent modification, the most recent additions are the histone methyltransferases (HMTases), whose functions are now being characterized. Here we show that a SET domain-containing protein, G9a, is a novel mammalian lysine-preferring HMTase. Like Suv39 h1, the first identified lysine-preferring mammalian HMTase, G9a transfers methyl groups to the lysine residues of histone H3, but with a 10–20-fold higher activity. It was reported that lysines 4, 9, and 27 in H3 are methylated in mammalian cells. G9a was able to add methyl groups to lysine 27 as well as 9 in H3, compared with Suv39 h1, which was only able to methylate lysine 9. Our data clearly demonstrated that G9a has an enzymatic nature distinct from Suv39 h1 and its homologue h2. Finally, fluorescent protein-labeled G9a was shown to be localized in the nucleus but not in the repressive chromatin domains of centromeric loci, in which Suv39 h1 family proteins were localized. This finding indicates that G9a may contribute to the organization of the higher order chromatin structure of non-centromeric loci.

In eukaryotes, organization of higher order chromatin structure is thought to be essential for both epigenetic gene control and proper chromosome condensation in mitosis. Targeted covalent modification of the amino-terminal tails of the core histones in nucleosomes has emerged as one of the important mechanisms in this process. Posttranslational acetylation and phosphorylation of histone tails are intensively studied among these modifications. Transcriptionally active euchromatin regions of the chromosome are often associated with hyperacetylated histones, and silent heterochromatic regions are often associated with hypoacetylated forms (1). Steady-state levels of histone acetylation correlate closely with chromosomal condensation and was shown to be required for proper chromosome condensation and segregation (4, 5). The phosphorylation of serine 10 also correlated with transcriptional activation of immediate-early genes in a rapid and transient manner upon mitogen stimulation (6, 7). Recently, several kinases have been identified as the enzymes that contribute to the mitogen-stimulated histone H3 phosphorylation (8, 9).

In addition to acetylation and phosphorylation, it has also been reported that core histones, especially H3 and H4, are methylated (10, 11). In mammalian HeLa cells, lysines 4, 9, and 27 in H3 and lysine 20 in H4 were shown to be methylated (12). Although the biological significance of histone methylation remains unclear, several histone methyltransferases (HMTases)1 have recently been identified (12–14). Among these enzymes, Suv39 h1 and its homologue, h2, are the first known mammalian lysine-preferring HMTases and SET domain-containing proteins. Both enzymes preferentially methylate H3 in a mixture of histones in vitro, and their catalytic activities are dependent on the SET domain. By using a peptide encoding the first 20 residues of the H3 amino terminus, it was shown that the Suv39 h protein could transfer methyl groups to lysine 9. The enzymes that catalyze methylation of the other residues, lysines 4 and 27 in H3 and lysine 20 in H4, remain to be identified.

The SET domains in Suv39 h1 and h2 are evolutionarily conserved from yeast to mammals and are found in many nuclear proteins called “chromatin modulators” (13). Therefore, SET domain-containing proteins have diverse functions. For example, some members of the Drosophila polycomb and trithorax group (Pc-G and trx-G) protein families, which have been shown to act as repressors and activators, respectively, of the homeotic selector genes, contain the SET domain in their carboxy terminal (14). Among several SET domain-containing protein families, Suv39 h family proteins are characterized as a group possessing a chromo-domain, which is also considered as a chromatin regulator motif. Members of this family were identified as CLR4 in yeast (15), SuuVAR3–9 in fruit fly (16), Suv39 h1 and h2 in mouse, and SUV39H1 in human (17, 18). The Suv39 h family proteins have been considered to contribute to the organization of repressive chromatin regions such as the centromere (13).

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1 The abbreviations used are: HMTase, histone methyltransferase; h, human; kb, kilobase; PCR, polymerase chain reaction; EGFP, enhanced green fluorescent protein; DeRed, Discosoma striato red; HP1, heterochromatic protein 1; m, mouse; GST, glutathione S-transferase; CENP, centromere protein; TRF, telomeric repeat binding factor; ANK, ankyrin.

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In this study, a SET domain-containing protein, G9a, was identified as a novel mammalian lysine-prefering HMTase. Compared with Suvs91 h, G9a possesses an apparently unique molecular nature. It is much more efficient at transferring methyl groups to histone H3 in vitro. In addition, not only lysine 9, but also lysine 27 in H3, is a target for methylation by G9a. Finally, the nuclear localization of ectopically expressed G9a differs significantly from that of Suvs91 h1, suggesting that G9a may not be involved in the organization of repressive chromatin domains but may contribute to the regulation of chromatin structure in other loci.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Full-length human G9a (hG9a) cDNA (19) was obtained by ligation of a 1.2-kb BamH I/ApoI cDNA fragment encoding amino acids 1-339 to a BamH I/ApoI-digested partial hG9a cDNA (encoding amino acids 350 to termination), which was previously cloned into pBluescript (Stratagene). The former was PCR-amplified from a Jurkat leukemia cDNA library using specific primers 5'-GCATGAGCTC-GATGGCGGAAAATTTAAAAG and 5'-TCAGGCGACAGTACACTGGCC. For protein expression in mammalian cells, the resulting 3.0-kb HindIII/NotI-digested cDNA fragment containing the entire hG9a open reading frame was ligated into the respective site of pEGFP-N1 (Clontech) to generate an Aequorea Victoria green fluorescent protein (EGFP) (20) fusion protein. Full-length mouse Suvs9a h1 cDNA was PCR-amplified from a mouse B cell leukemia cDNA library using specific primers 5'-ATAAGATTCGATGGCGGAAAATTTAAAAG and 5'-ATAAGATTCGATGGCGGAAAATTTAAAAG and 5'-ATAAGATTCGATGGCGGAAAATTTAAAAG. The obtained cDNA fragment was inserted into the EcoRI sites of pEGFP-C1 and pDsRed1-C1 vectors (CLONTECH) in an in-frame fashion. Expression plasmids for EGFP and DsRed and isoform (HP1aa) (22) fusion molecules were constructed by similar procedures.

For protein expression in Escherichia coli, mouse G9a (mG9a) cDNA was isolated from a uni-ZAP murine testis cDNA library (Stratagene) using the 1.2-kb hG9a cDNA described above as a probe. A 1.4-kb EcoRI/XhoI cDNA fragment encoding amino acids 621-1000 of mG9a was subcloned into the bacterial expression vector pGEX-4T-3 (Amerham Pharmacia Biotech) to obtain an amino-terminal glutathione S-transferase (GST)-mG9a fusion molecule. A 1.0-kb cDNA encoding amino acids 82-412 of Suvs91 h1 was PCR-amplified and inserted into the EcoRI site of pGEX4T-3. Mouse histone H3 cDNA (H3.1, GenBank accession number 193861) was PCR-amplified from mouse embryonic stem cell line, T72 cDNA library using specific primers 5'-TAAAGATTCGATGGCGGAAAATTTAAAAG and 5'-ATAAGATTCGATGGCGGAAAATTTAAAAG. The obtained PCR product was digested with EcoRI/XhoI and subcloned into the respective sites of pZErO 2-1 (Invitrogen). A 180-base pair EcoRI/SalI fragment of the histone H3 cDNA encoding amino acids 1-57 was introduced into pGEX-4T-3. All PCR-amplified cDNA products were confirmed by sequencing. Point mutations within the SET domain of mG9a and Suvs9a h1 and the amino terminus of histone H3 were engineered by the standard double PCR mutagenesis method. The amino terminus (residues 1-52) of human centromere protein-A (CENP-A) and a full-length human centromere protein-A (CENP-A) and a full-length mouse histone H3 were engineered by the method described above. After 3 days of culture, cells were harvested and lysed in phosphate-buffered saline containing 0.5% Nonidet P-40 and a mixture of protease inhibitors (Roche Molecular Biochemicals). After sonication and centrifugation (16,000 × g, 15 min), supernatants were incubated with anti-EGFP specific antibodies (CLONTECH). The immune complexes were collected with protein-A-Sepharose beads (Amerham Pharmacia Biotech) and used in the Western blotting and in vitro HMTase assays.

**Fluorescent Microscope Analysis**—Aliquots of cells were grown on glass coverslips in 35-mm dishes for 48 h, fixed with 4% paraformaldehyde/phosphate-buffered saline for 20 min, and treated with 0.1% Triton X-100 for 5 min. After staining with 4',6-diamidino-2-phenylindole (10 μg/ml) (14), cells were observed under a fluorescent microscope (Eclipse E600, Nikon) equipped with a PlanApo 60 x (NA 1.40) and a MicroMAX 1300Y cooled CCD camera (Princeton Instruments). Images were acquired by MetaMorph software (Universal Imaging Corp.) by collecting a Z-series of 0.5-μm optical sections.

**RESULTS**

**G9a Contains a SET Domain and Adjacent Cysteine-rich Regions, Essential for HMTase Activity**—Among SET domain-containing proteins, HMTase activity has so far been observed only in Suvs9a h family proteins including yeast CLR4 and mammalian Suvs9a h1 and h2. Because only Suvs9a h family proteins possess two cysteine-rich regions adjacent to the SET domain, Rea et al. (17) suggested that to exert HMTase activity, the SET domain requires combination with these adjacent cysteine-rich regions. Taking this viewpoint, we focused on G9a, a ubiquitously expressed SET domain-containing protein that was originally characterized as a molecule encoded by a gene mapped in the class III region of the human major histocompatibility complex locus (19). G9a resembles CLR4 and Suvs9a h, because it contains a SET domain flanked by two cysteine-rich regions in its carboxyl terminus, but other regions are quite different (illustrated in Fig. 1A).

Amino acid sequence alignment of the two cysteine-rich regions flanking the SET domain from hG9a and mouse Suvs9a h1 revealed that they are highly related, and the positions of their cysteine residues are highly conserved (Fig. 1B, top panel). Within the SET domain, the H/RR/φNHSC motif (where φ indicates a hydrophobic residue) was previously shown to be a possible catalytic core motif responsible for the enzymatic activity (shown boxed in Fig. 1B, bottom panel), because amino acid replacement of histidine 324 and cysteine 326 in Suvs9a h1 abolished enzymatic activity. Replacement of histidine 320 of Suvs9a h1 with arginine (Suvs9a H320R) results in a 20-fold increase in enzymatic activities. Yeast CLR4, which possesses arginine at this position of the putative catalytic core motif, exhibits hyperenzymatic activities similar to the Suvs9a H320R mutant, supporting the importance of this position in determining the strength of activity (17). H/RR/φNHSC motif of CLR4-type catalytic core motif in the SET domain (900R/φ/NHL9006), with arginine in the first position of the core motif. G9a Possesses Higher HMTase Activity—To investigate whether mG9a carboxyl terminus has HMTase activity, we performed an in vitro methylation assay using native histones as substrates. Recombinant GST-mG9a (residues 621-1000) and GST-Suvs9a h1 (residues 82-412) were incubated with...
G9a, Lysines 9 and 27-Preferring H3 Methyltransferase

We also performed the HMTase assay for G9a by using single native histone as a substrate. It was shown that not only H3 but also H1 can be efficiently methylated by Suv39 h1 if H1 was used alone. When purified native H1, H2A, and H3 were applied to the assay as single substrates, G9a also significantly methylated not only native H3 but also H1. Slightly methylated proteins were detected in the lane of H2A (Fig. 3, lane 2), possibly due to incomplete elimination of H1 and H3 during the purification processes. The physiological significance of the H1 methylation is currently unknown but poses an interesting issue for future studies.

We further investigated the substrate specificity of G9a as a methyltransferase using several other nuclear proteins, which were shown to be covalently modified by other enzymes such as kinases or are possible substrate candidates of these enzymes. We introduced several recombinant GST-fused nuclear proteins, which were human CENP-A amino terminus (residues 1–52) (23) and full-length constructs of human CENP-B (24), human CENP-C (25), human HP1\(^{\alpha}\) (22), mouse TRF1 (26), mouse TRF2 (27), and human p53 (28), into the in vitro methyltransferase assays. Among these substrates, only the GST-fused histone H3 amino-terminal (residues 1–57) molecule (H3N, see Fig. 4A) was actively methylated by GST-mG9a, whereas methylation of the other GST fusion molecules was almost undetectable (Fig. 3 and data not shown). In addition, H3N was a more efficient substrate of G9a than was the native purified histone H3, presumably due to the pre-existence of methyl groups on the targeted residue(s) of native H3.

**G9a Transferred Methyl Groups to Lysines 9 and 27 of Histone H3**—Because methylation of H3 occurs dominantly at lysines 4, 9, and 27 in HeLa cells (12), and Suv39 h1 and h2 proteins were shown to methylate only lysine 9 of H3 (18), the enzymes involved in the reactions at lysines 4 and 27 in H3 remained to be identified. We next examined the residues of H3 targeted by G9a, focusing on these lysines. Because recombinant H3N was found to be a more suitable substrate than the native histone H3, we introduced several lysine-to-arginine replacements into H3N, as summarized in Fig. 4A. When we used NT as a substrate in which all three lysine residues were replaced with arginine, no methyl groups were introduced by GST-mG9a (Figs. 4B and 6A), suggesting that the G9a target site(s) in H3 is also lysine. When single residue-replaced mutants 4R, 9R, and 27R were used for the assay, Suv39 h1 only methylated mutants in which lysine 4 or 27 was replaced with arginine (4R or 27R) but not 9R (lysine 9 to arginine replacement), consistent with previous results (4). However, all the single lysine-replaced mutants including 9R were clearly methylated by GST-mG9a, although the methylation efficiency of 9R was only 20% of N3H (Fig. 6A). This result suggested that G9a...
could methylate two or more lysine sites. Further methylation assays using double lysine to arginine-replaced H3N mutants demonstrated this clearly. Not only N9 (lysines 4 and 27 were changed to arginine), but also N27 (lysines 4 and 9 were changed to arginine) was substantially methylated by GST-mG9a (Figs. 4B and 5A). Methylation efficiencies of N9 and N27 by GST-mG9a were about 50 and 20% of H3N, respectively. In contrast, N4 (lysines 9 and 27 were replaced with arginine) was again introduced into the above in vitro HMTase assays with 20 μg of free core histones (left four lanes) and 5 μg of sole histone H3 (right four lanes) as substrates and S-adenosyl-[methyl-14C]-L-methionine as a methyl donor. Coomassie Brilliant Blue R-250 staining (top panel) shows enzymes (arrows) and purified histones. Autoradiography (bottom panels) indicates specific methylation of H3 and H1 by GST-mG9a. All the presented data of methyltransferase assays are representative of multiple independent studies. E, enzyme.

Lysine 27 Could Not Be Methylated by Hyperactive Suv39 h1 Mutant—Because the first position of the catalytic core motif in the mG9a SET domain (899R→NHLC905) is arginine, which also exists in the core motif of the active HMTase CLR4 (406R→φNHSC412), we speculated that arginine 899 might be responsible for the strong HMTase activity of G9a. A previous observation that a single amino acid replacement of histidine 320 in the stringent HMTase Suv39 h1 (carrying 320H→φNHSC326) with arginine produced a hyperactive enzyme that possessed more than 20 times higher enzymatic activity than the original (17) supported this possibility. In addition to the higher enzymatic activity, we found that G9a could catalyze the methylation of lysines 9 and 27 in H3N in vitro, whereas Suv39 h1 was limited to lysine 9. Therefore, we also investigated the possibility that the first amino acid residue of the catalytic core motif of G9a, called the “hyperactive position” (shown shaded in gray in Fig. 1B, bottom panel), might also contribute to the target specificity of G9a, especially with regard to lysine 27 of H3. To address these issues, we constructed two recombinant enzymes, in which the amino acid at the hyperactive position was substituted by site-directed mutagenesis. G9aR899H protein was produced by amino acid substitution of arginine 899 in mG9a with histidine, and SuvH320R was produced by replacement of histidine 320 in Suv39 h1 with arginine. As expected, the catalytic activity of G9aR899H was dramatically reduced to an almost undetectable level even though 10 times more protein was used (Fig. 5A, right panel). Because of this poor enzymatic activity of G9aR899H, lysine selectivity could not be determined. In contrast to this result, SuvH320R gained hyperactivity by this substitution, as described previously, and quite efficiently methylated H3N and N9 but remained unable to transfer methyl groups to N27 (Fig. 5B, right panel and summarized in Fig. 6C). Therefore, these data indicate that the hyperactive position may not influence the target selectivity of lysine residues in H3, at least in the case of Suv39 h1.

Ectopically Introduced EGFP-G9a Localizes in Nuclear Loci Different from Suv39 h1—The above experiments clearly demonstrated that G9a possesses a distinct enzymatic nature in vitro compared with the first identified lysine-prefering HMTase, Suv39 h1. To estimate the in vivo function of G9a, we examined the nuclear localization of ectopically introduced G9a in mammalian cells. Expression constructs of EGFP-hG9a and DsRed fused with HP1HISα were cointroduced into 293 cells, and the stably expressed fluorescent tagged proteins were simultaneously observed. HP1HISα has been shown to be localized to the centromeric heterochromatin region in human cells (29). We observed that DsRed-HP1HISα was also localized in the centromeric regions in metaphase chromosomes (data not shown), similar to previous reports of EGFP-HP1HISα (30). As shown in Fig. 7, left panels, G9a (green) was strictly localized in nuclei and visualized as discrete clear speckles, whereas HP1HISα (red) appeared as heterogeneous dots in the interphase nuclei. When the two images were merged, almost no overlapping of foci was seen except in a few cases (shown as yellow dots in upper nuclei in Fig. 7D). We also established 293 cells expressing both EGFP-Suv39 h1 and DsRed-HP1HISα (Fig. 7, right panels). In the interphase nuclei, Suv39 h1 and HP1HISα signals were observed as completely overlapped dots (Fig. 7L), consistent with a similar report (31). When EGFP-G9a and DsRed-Suv39 h1 were cointroduced, again these two signals were found to be mainly independent and non-overlapping (Fig. 7, middle panels). These data indicated that most of
the G9a molecules are outside of the heterochromatin domains around centromeric loci, in which both Suv39 h1 and HP1 proteins have been shown to be localized.

**DISCUSSION**

In this report, we demonstrated that G9a is a lysine-prefering H3 HMTase that shows stronger HMTase activity than Suv39 h1. Mutagenesis experiments demonstrated that the hyperenzymatic activity of G9a is also dependent on the first residue of the core motif in the SET domain, arginine 899 in mouse, as shown by the change of histidine 320 to arginine at this position in Suv39 h1 (17).

More importantly, G9a was found to be able to transfer methyl groups to not only lysine 9 but also lysine 27 in H3 in vitro, suggesting that G9a may be the responsible enzyme for the lysine 27 methylation described in HeLa cells. Furthermore, studies with the mutant SuvH320R suggested that the target lysine specificity of G9a is probably independent of its hyperenzymatic activity. From the in vitro HMTase assays using various H3N mutants, the target lysine residue preference of G9a was examined. GST-mG9a transferred methyl groups to lysine 27 even in the H3N mutant N27 (lysines 4 and 9 were replaced with arginine), the most preferred target site of G9a was lysine 9, and N9 (lysines 4 and 27 were replaced) gave the highest methyl-14C counts among the double lysine-replaced mutants (Fig. 6A). It is also worth noting that the total incorporation rate of methyl-14C from the double lysine-replaced H3N substrates never reached the levels of the wild-type H3N for G9a and also Suv39 h1 (Fig. 6, A and B), which suggests that methylation of lysine residues, at least at positions 9 and 27, may synergistically affect each other.

In mammals, three lysine-prefering HMTases including G9a and some arginine methyltransferases that can methylate histones were identified. However, we still have very limited information about the physiological significance of histone methylation. A nuclear receptor coactivator-interacting protein, CARM1, which contains an arginine-specific methyltransferase motif and shows HMTase activity in vitro, provided suggestive evidence that histone methylation may contribute to transcriptional activation, although the significance of arginine methylation in H3 has not been defined (32). In addition, lysine-selective HMTase activities were detected in transcriptionally active *Tetrahymena* macronuclei but not in transcriptionally inert micronuclei (12). On the other hand, a possible correlation between methylation of lysine 9 in H3 and transcriptional silencing has also been proposed. If all of these findings are physiologically relevant, histone methylation may both positively and negatively regulate transcription. How might histone methylation regulate transcription? One possible mechanism was proposed by the studies of Suv39 h1 function, in which methylation of lysine 9 inhibited phosphorylation of serine 10 in the H3 amino terminus peptides and vice versa (17). The phosphorylated serine 10 was shown to accelerate histone acetyl transferase acetylation of lysine 14 in H3 (33). Therefore, it is proposed that HMTases may function through regulation of other histone-modifying activities, such as kinase and histone acetyl transferase, to positively or neg-

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**Fig. 3.** Native histones H1 and H3 and recombinant H3 amino terminus (residues 1–57) are suitable substrates for *in vitro* methylation by G9a. In vitro methylation assays were carried out using 10 μg each of purified histones H1, H2A, and H3 and several recombinant GST fusion proteins, such as 10 μg of CENP-A amino terminus (residues 1–52), 10 μg of histone H3 amino terminus (residues 1–57) (termed H3N, see Fig. 4A), 15 μg of human HP1α, 5 μg of mouse TRF1, and 5 μg of human p53, as substrates. The catalytic activity of G9a is highly specific to native H1, H3, and recombinant H3N. E, enzyme; S, substrate.
FIG. 4. G9a can transfer methyl groups to lysines 9 and 27 of histone H3. A, recombinant GST fusion histone H3 amino terminus (residues 1–57), H3N, and the mutants used as substrates for in vitro methylation assays. The amino acid sequence of the mouse H3 amino terminus is.

B

C

D

E

% Relative methylation

100 0 2.0 2.3 38.6 18.9 autoradiograph

| Enzyme                  | 1 μg G9a-C(621–1000) |
|-------------------------|----------------------|
| Substrate               | E         | S         | E         | S         |
| 4R GST                  | 50 kDa    | 29 kDa    | 50 kDa    | 29 kDa    |
| 9R GST                  | 50 kDa    | 29 kDa    | 50 kDa    | 29 kDa    |
| 27R GST                 | 50 kDa    | 29 kDa    | 50 kDa    | 29 kDa    |
| NT GST                  | 50 kDa    | 29 kDa    | 50 kDa    | 29 kDa    |
| N4 GST                  | 50 kDa    | 29 kDa    | 50 kDa    | 29 kDa    |
| N9 GST                  | 50 kDa    | 29 kDa    | 50 kDa    | 29 kDa    |
| N27 GST                 | 50 kDa    | 29 kDa    | 50 kDa    | 29 kDa    |

| Enzyme                  | 10 μg Suv39h1(82–412) |
|-------------------------|----------------------|
| Substrate               | E         | S         | E         | S         |
| 4R GST                  | 50 kDa    | 29 kDa    | 50 kDa    | 29 kDa    |
| 9R GST                  | 50 kDa    | 29 kDa    | 50 kDa    | 29 kDa    |
| 27R GST                 | 50 kDa    | 29 kDa    | 50 kDa    | 29 kDa    |
| NT GST                  | 50 kDa    | 29 kDa    | 50 kDa    | 29 kDa    |
| N4 GST                  | 50 kDa    | 29 kDa    | 50 kDa    | 29 kDa    |
| N9 GST                  | 50 kDa    | 29 kDa    | 50 kDa    | 29 kDa    |
| N27 GST                 | 50 kDa    | 29 kDa    | 50 kDa    | 29 kDa    |

B

C

D

E

% Relative methylation

100 0 2.0 2.3 38.6 18.9 autoradiograph
atively regulate transcription (33, 34). Interestingly, lysine 27 of H3 is embedded within a similar sequence context as lysine 9 (RKS), and their adjoining serines 10 and 28 were both capable of being phosphorylated in mitotic chromosomal condensation (35). In this context, G9a may also suppress phosphorylation of serine 28 by methylation of lysine 27 and control serine 28 phosphorylation–mediated function. An essential function of HMTase may be simply to mark specific histone residues with methyl groups, as previously proposed (2). The specific methyl marking may recruit various regulatory molecule(s) involved in transcription or higher order chromatin organization. If so, H3 methylation by G9a and Suv39 h1 may result in recruitment of distinct molecule(s) to the nucleosome.

Among the native histone molecules we used as a single substrate, H1 was also found to be a good in vitro substrate for G9a. A similar finding has also been reported for Suv39 h1 (17). Although H1 methylation is currently not well understood in mammalian cells, some H1 functions may be regulated by

2 In accordance with this idea, several groups showed most recently that the chromo-domain of HP1 could bind specifically to methylated lysine 9 of H3 (36–38).

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Histidine 320 of Suv39 h1 is responsible for hyper-HMTase activity but not for lysine selectivity in H3. To determine whether the amino acid residue at the hyperactive position also contributes to lysine selectivity, amino acid-substituted mutants of GST-mG9a and GST-Suv39 h1 were introduced in in vitro HMTase assays. A, HMTase activities of G9aR899H (arginine 899 replaced with histidine) were measured (right panel). Significant reduction of the HMTase activity of G9aR899H was observed, making estimation of substrate specificity difficult (right panel). B, recombinant GST-Suv39 h1-(82–412) and its hyperactive mutant SuvH320R (replaced histidine 320 with arginine) were assayed as shown in A. Although it gained in hyperactivity as previously reported (17), SuvH320R still methylated only lysine 9 (right panel), indicating that the amino acid residue at the hyperactive position may not contribute to lysine selectivity. E, enzyme; S, substrate.
methylation.

Other than H3N, none of the other analyzed GST-fused nuclear proteins, such as CENP-A, CENP-B, CENP-C, HP1α, TRF1, TRF2, and p53, were methylated by G9a under our conditions. However, another histone modification activity, acetylation, is not restricted to histones. For example, histone acetyl transferase activity was shown to catalyze p53 acetylation, and this modification is regarded to be physiologically relevant (28). Considering the fact that multiple SET domain-containing proteins, including functionally uncharacterized ones, exist within a single organism, it should not be surprising that SET domain-mediated methylation is not restricted to histones. Furthermore, we could not eliminate the possibility that some of the nuclear proteins we analyzed might be methylated by a different type of methyltransferase that regulates their functions in vivo.

Finally, we found that EGFP-hG9a had a quite different localization pattern in interphase nuclei than that of Suv39 h1. Endogenous Suv39 h1 and its human counterpart SUV39H1 were enriched at heterochromatic foci in interphase nuclei in mammalian cells (39) and associated with HP1α (40), indicating their likely contribution to the structural organization of the centromere. EGFP-hG9a colocalized with neither DsRed-Suv39 h1 nor HP1α, whereas EGFP-Suv39 h1 and DsRed-HP1α were strictly colocalized. Amino acid sequences between G9a and Suv39 h1 were quite different except for the conserved regions essential for HMTase activity (Fig. 1A). Suv39 h1 contains a 60-amino acid chromo-domain, and G9a contains six contiguous 33-amino acid ankyrin (ANK) repeats upstream of their respective SET domains. Whereas the chromo-domain directs euchromatic or heterochromatic localizations (41), ANK repeats have been identified in many proteins with various subcellular localizations, such as Notch protein in the inner face of the plasma membrane (42), NF-κB in the cytoplasm (43), and SW14 in the nucleus (44). ANK repeats are considered as a motif that serves to interact with other proteins; therefore G9a may be recruited to specific chromosomal loci via interaction between the ANK repeats of G9a and additional regulatory proteins. Although our data should be confirmed by specific antibodies against endogenous molecules, the current findings suggest the possibility that HMTase-mediated higher order chromatin structure may not be restricted to centromeric loci but may also occur in other chromosomal loci. Further characterization of the loci, in which G9a protein accumulation was observed, will facilitate the elucidation of the in vivo function of G9a.

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