Functional analysis of the N- and C-terminus of mammalian G9a histone H3 methyltransferase

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Received April 14, 2005; Revised and Accepted May 16, 2005

ABSTRACT

Methylation of lysine 9 (K9) in the N-terminus tail of histone H3 (H3) in chromatin is associated with transcriptionally silenced genes and is mediated by histone methyltransferases. Murine G9a is a 1263 amino acid H3-K9 methyltransferase that possesses characteristic SET domain and ANK repeats. In this paper, we have used a series of green fluorescent protein-tagged deletion constructs to identify two nuclear localization signals (NLS), the first NLS embedded between amino acids 24 and 109 and the second between amino acids 394 and 401 of murine G9a. Our data show that both long and short G9a isoforms were capable of entering the nucleus to methylate chromatin. Full-length or N-terminus-deleted G9a isoforms were also catalytically active enzymes that methylated recombinant H3 or synthetic peptides representing the N-terminus tail of H3. In vitro methylation reactions using N-terminus tail peptides resulted in tri-methylation of K9 that remained processive, even in G9a enzymes that lacked an N-terminus region by deletion. Co-expression of G9a and H3 resulted in di- and tri-methylation of H3-K9, while siRNA-mediated knockdown of G9a in HeLa cells resulted in reduction of global H3-K9 di- and tri-methylation. A recombinant deletion mutant enzyme fused with maltose-binding protein (MBP-G9aΔ634) was used for steady-state kinetic analysis with various substrates and was compared with full-length G9a (G9aFL). Turnover numbers of MBP-G9aΔ634 for various substrates was ~3-fold less compared with G9aFL, while their Michaelis constants (Km) for recombinant H3 were similar. The K_adm/Met for MBP-G9aΔ634 was ~2.3–2.65 μM with various substrates. Catalytic efficiencies (k_cat/K_m) for both MBP-G9aΔ634 and G9aFL were similar, suggesting that the N-terminus is not essential for catalysis. Furthermore, mutation of conserved amino acids R1097A, W1103A, Y1120A, Y1138A and R1162A, or the metal binding C1168A in the catalytic region, resulted in catalytically impaired enzymes, thereby confirming the involvement of the C-terminus of G9a in catalysis. Thus, distinct domains modulate nuclear targeting and catalytic functions of G9a.

INTRODUCTION

In eukaryotic nuclei, DNA associates with proteins to generate the heritable genetic information contained within chromatin. Transcriptionally active regions of the genome reside in euchromatin, whereas inactive regions reside in heterochromatin. Regulation of euchromatic and heterochromatic regions forms the basis of epigenetic gene expression control (1) and proper chromosome condensation during meiosis (2,3). Alterations in chromatin structure have a vital role in changing the gene expression. Many alterations are targeted by specific chromatin remodeling complexes (4) and/or by DNA modification enzymes (5) directed at nucleosomes, the fundamental structural unit of histone chromatin. Nucleosomes are composed of 146 bp of DNA wrapped around an octamer of core histone proteins H2A, H2B, H3 and H4 (6) that are often modified at specific amino acid residues (7). These post-translational modifications include acetylation, phosphorylation, poly-ADP-ribosylation, ubiquitination and methylation (8). Such modifications have major roles in regulating gene transcription, heterochromatin formation, X chromosome inactivation, DNA replication, DNA repair and cellular memory (8–13).

Histones are small basic proteins with globular core domains and flexible, charged N-terminus regions that protrude out of the nucleosome core leaving them susceptible to
post-translational modifications. In histone H3, five lysine residues (K4, K9, K27, K36 and K79) and K20 in histone H4 may become methylated. Of the different site-specific H3 methylating sites, K9 methylation is well known for its role in heterochromatin formation, transcriptional silencing, X chromosome inactivation and DNA methylation (14). Several enzymes for K9 methylation have been purified and studied. The first was Su39h1, cloned and expressed by Jennewein and co-workers (15). Another close relative, Su39h2, was also cloned and characterized (16). Both Su39h1 and h2 contain SET ([Su(var), Enhancer of zeste, Trithorax] domains and their catalytic activities are dependent on this domain. The SET domain has a unique structure (17), rich in β-sheets, with each small sheet containing only a few strands. Although these K9 methyltransferases use AdoMet as a cofactor, no similarities with the canonical α/β AdoMet-dependent methyltransferases have been described previously (18). Furthermore, both Su39h1 and h2 contain a chromo domain that is considered a chromatin regulatory motif. The SUV39H family of proteins contributes to the organization of repressive chromatin regions, such as the centromeres (19), and is co-localized with heterochromatin-binding protein 1 (HP1) in heterochromatic regions of the mammalian genome (20).

Apart from the SUV39H family enzymes, three additional mammalian H3-K9 methyltransferases—G9a, ESET/SETDB1 and Eu-HMTase1—are have been reported previously (21–23). Unlike the SUV39H family, these proteins participate in H3-K9 methylation in euchromatic regions of the genome (24). Along with enzymatic catalysis, Eu-HMTase 1 also associates with E2F-6, Mga and Max proteins and may be involved in the silencing of E2F and Myc responsive genes in quiescent cells (23). The fact that multiple histone methyltransferases are capable of the same target lysine methylation raises the issue of redundancy in their functions. Targeted inactivation of G9a resulted in growth retardation and early embryonic lethality, suggesting that G9a-mediated H3-K9 methylation in euchromatic regions is essential for development (25) and cannot be compensated for by the other three known K9 methyltransferases. It is possible that different histone methyltransferases methylate K9 at different times during the cell-cycle or development. Another H3-K9 methyltransferase, ESET, participates in early mammalian development (26). Xin et al. (27) also demonstrated that H3-K9 methylation of the Prader-Willi syndrome imprinting center is reduced in G9a-null mouse ES cells, and maintenance of Cpg methylation in the Prader-Willi syndrome imprinting center requires G9a function, bringing two global epigenetic modifiers, DNA and histone methylation, together. Furthermore, G9a is a transcriptional repressor of PRDL-BF1, a DNA-binding protein involved in post-induction repression of interferon-β gene transcription in response to viral infections (28).

G9a is expressed ubiquitously and was originally mapped to the class III region of the major histocompatibility complex locus (29). Mouse G9a is 1263 amino acids long. The amino acid of G9a has very little similarity with any conserved protein domain. However, the carboxy half has homology with ANK (ankyrin) repeats, and contains preSET and SET domains. The SET domains are conserved in chromatin-modifying enzymes (22) that include histone methyltransferases (30), which recognize histone tails and modify specific lysine residues (31). A second distinguishing feature of G9a is the presence of several ANK repeats implicated in protein–protein interactions in very diverse protein families. G9a is also homologous to human SUV39H1 and yeast Clr4 family proteins.

Although G9a is crucial for transcriptional silencing and development, very little is known about its functional domains and its association with chromatin. Furthermore, there is emerging evidence for chromatin modifying enzymes, such as histone methyltransferases, in establishing and maintaining gene silencing either alone (32) or in cooperation with DNA methyltransferases (33). Here, we have evaluated the functional domains of mouse G9a for nuclear localization features and the minimal elements required for catalysis. The functional significance of conserved C-terminus amino acids for enzymatic activity was also determined.

**MATERIALS AND METHODS**

**Bacterial strains**

All protein expression constructs were either transformed into ER2566 [New England Biolabs (NEB)] or BL21DE3 (Invitrogen) Escherichia coli strains for protein purification. All other constructs were propagated in ER2502 E.coli strain (NEB).

**Green fluorescent protein (GFP) fusion gene constructs and cytochemical detection of fusion proteins**

All PCR amplifications were performed using Vent DNA Polymerase (NEB), and ligations were performed using Quick ligation kits (NEB). G9a full-length (G9aFL) and various deletion mutants were constructed with the pEGFPc2 back bone vector (BD Biosciences). The G9aFL construct (pZKmG9a) was based on the GenBank sequence accession no. AB077210 that was used as a template for PCR gene amplification to make various GFP fusion constructs. The list of primers used is available upon request.

For detailed analysis of the second conserved nuclear localization signal (NLS), PCR amplification and cloning was performed with forward primer GCCGAAATTTCAGAAACAG-GCGGAAAACGAG and reverse primer ACGGCTGACT-CAGTAGACACAGCCACCTAACTGCAC. PCR products were cloned into EcoRI and SalI sites. Annealing synthetic oligonucleotides and cloning into the pEFGPc2 vector resulted in G9aNLS2, G9aNLS-KP, G9aNLS-KD and G9aNLS-mKD constructs. All constructs were sequenced and confirmed.

For immunocytochemistry, COS-7 and HeLa cells were cultured on cover slips and transfected with a mixture of plasmid DNA and FuGENE6 (Roche) at a ratio of 1:3 μg/μl. Forty-eight hours post-transfection, the cells were fixed with 4% parafomaldehyde, washed once with 1× phosphate-buffered saline (PBS) (pH 7.4) and permeabilized with 0.2% Triton X-100. After three washes with PBS, nuclear staining was performed with Hoechst 33342 dye (Molecular Probes). GFP constructs were visualized using a fluorescence microscope with either a 63x or a 100x/1.4 oil Zeiss objective lens at 488 nm.

**Wild-type and mutant histone H3 constructs**

An in vitro vector, pTYB2 (NEB), was used for cloning and purification of recombinant human histone H3 protein and its mutants. The histone H3 was PCR amplified from human genomic DNA using the forward primer...
GGAATTCCATATGGCACGCACGAAGCAGAACAGCG and reverse primer CCGCTCGAGCCCCGGTCCCTCCTC- CGCGAATTCGGCC. The PCR amplified product was cloned into pcR2.1-TOPO (Invitrogen) and confirmed by restriction digestion and DNA sequencing. The histone H3 insert was recovered from the pcR2.1-TOPO vector by restriction digestion with NdeI and SmaI, followed by ligation into the pTYB2 vector digested with NdeI and SmaI, and then transformed into E.coli ER2566. This clone is pTYB2H3wt.

For PCR amplification of histone H3-K4A, H3-K9A and H3-K4AK9A mutants, the plasmid pcR2.1-TOPO::H3 was used as a template. pTYB2::H3-K9A and pTYB2::H3-K4AK9A were used to amplify NdeI/AgeI (~100 bp) fragments of histone H3 mutants, H3-K9AK27A and H3-K4AK9AK27A, respectively. For this, the pcR2.1-TOPO::H3 vector was digested with NdeI and AgeI and ligated with the ~100 bp PCR products digested with NdeI/AgeI. The inserts H3-K9AK27A and H3-K4AK9AK27A were excised from pcR2.1-TOPO::H3-K9AK27A and pcR2.1-TOPO::H3-K4AK9AK27A by restriction digestion with NdeI and AgeI and cloned into pTYB2. Wild-type, H3-K4A, H3-K9A and H3-K4AK9A had the same reverse primer; however, the forward primers were different. For H3-K4A: GGAATTTCCATATGGCACGCACGAAGCAAGCA- AACAGCTCGTCGTGGCTCAGTCG; and for H3-K4AK9A: GGAATTTCCATATGGCACGCACGGCGCAACACAGCTCGT- GGTCCTACGTC.

The H3-K9AK27A forward primer is the same as the wild-type H3-K9 primer, whereas the reverse primer is CACGCACCGTGCTGGCGGAGGCC. For this construct, pTYB2H3-K9A was used as a template. For H3-K4AK9AK27A, the reverse primer was the same as for H3-K9AK27A, whereas the forward primer was the same as for H3-K4A.

Purification of histone H3 from E.coli

ER2566 cells containing the histone H3 expression constructs were grown in Luria–Bertani medium with 100 mg/l ampicillin followed by 0.5 mM isoprropyl-β-D-thiogalactopyranoside (IPTG) induction. The cells were lysed in 50 mM Tris–HCl, pH 7.8, 500 mM NaCl, 1 mM EDTA and 0.01% Triton X-100 with a protease inhibitor cocktail (Sigma) and 0.7 µg/µl phenylmethylsulfonyl fluoride (PMSF). Cleared lysates were incubated with chitin beads and were washed with lysis buffer. The H3 protein was cleaved from chitin beads by incubation with one volume of elution buffer (resuspension buffer supplemented with 50 mM DTT) overnight.

For co-expression of G9a and histone H3, pMALc2X::G9a1900 and pR976H3CBD were transformed into E.coli ER2566 (NEB). Histone H3 was purified after washing the chitin beads with 50 mM Tris–HCl, pH 7.8, 1500 mM NaCl, 1 mM EDTA and 0.01% Triton X-100 with a protease inhibitor cocktail (Sigma) and 0.7 µg/µl PMSF and eluted after overnight incubation with 50 mM DTT as described previously.

Gene construction and purification of maltose-binding protein (MBP)-G9a fusion protein from E.coli

A G9aFL cDNA insert was released from pEGFPc2::G9a by restriction digestion with EcoRI and cloned into the pMALc2X vector. After confirming the correct orientation of the insert by restriction digestion, the new plasmid was digested with BamHI and treated with klenow polymerase. After clean up with a spin column, the vector (now with a stop codon) was treated with T4 DNA quick ligase and transformed into E.coli BL21DE3 (Invitrogen).

The PCR products for G9aΔ634 and G9aΔ775 (PCR products G9a1 and G9a2 were described earlier for pEGFPc2 cloning) and vector pMALc2X were digested with EcoRI and Sall, purified with spin columns and ligated. Ligation mixtures were transformed into E.coli BL21DE3 (Invitrogen) and these new vectors were designated as pMALc2X::G9a1900 (G9aΔ634) and pMALc2X::G9a2324 (G9aΔ775). Bacteriophage expression of the G9aFL is described elsewhere (34).

Overnight cultures of MBP fusion proteins were inoculated in 1 liter of Rich medium supplemented with 2 g/l glucose and 100 mg/l ampicillin. After 2–3 h, protein expression was induced with 0.5 mM IPTG overnight, shaking at 16°C. The cells were resuspended in 20 ml of MBP column buffer (20 mM Tris–HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA with a protease inhibitor cocktail and 0.7 µg/µl PMSF). After sonication and centrifugation, the cleared lysate was loaded onto the column. The bound amylase resin was washed with 60 volumes of column buffer, and the fusion protein was eluted by adding 10 ml of MBP elution buffer (column buffer supplemented with 10 mM Maltose).

To generate G9a point mutants, two sets of PCR primers were used. The reverse primer in the first set contained a SapI site with a triplet codon for the desired mutation. The forward primer of the second set contained a SapI site followed by the complement triplet codon. After SapI digestion both fragments were ligated to obtain the desired mutations. All constructs were confirmed by restriction digestion and sequencing. The primer sequences used for point mutant constructs are available upon request.

Histone methyltransferase assay

Histone methyltransferase assays were carried out at 25°C for 3 min to overnight in duplicate in a total volume of 25 µl. A typical reaction contained S-adenosyl-l-[methyl-3H] methionine (AdoMet) (specific activity 15 Ci/mmol; Amersham), substrate histone/peptide and enzyme in assay buffer (50 mM Tris–HCl, pH 9.0, 5 mM MgCl2, 4 mM DTT and 7 µg/ml PMSF). For kinetic analysis, 1 nM peptide with one target lysine is equal to 1 nM methyl group (34). The efficiency of [3H] DNA measurements was ~55% and all calculations were corrected accordingly. Data obtained were plotted by regression analysis using the GraphPad PRISM program version 4b (GraphPad Software Inc.).

To determine the number of methyl groups added in MBP-G9aΔ634 catalyzed reactions, 640 nM of enzyme along with 192 µM synthetic peptide substrate CARTAQTARKSTG-GY(K-e-Biotin) (K4AK9) and 1.0 mM AdoMet was added in a total reaction volume of 300 µl. At 0, 15, 30 and 120 min intervals, 50 µl of the samples were withdrawn and 5 µl of trifluoro acetic acid (TFA) was added to stop the reaction. For completion of methylation, the last sample was incubated overnight (15 h). As a control, G9a enzyme was heat inactivated for 15 min at 95°C and incubated with K4AK9 and 1 mM AdoMet for 15 h. A blank reaction was performed.
with K4AK9 and 1 mM AdoMet. All the reactions were performed at 25°C.

**Analysis of peptide methylation by mass spectrometry**

The K4AK9 peptide was prepared using FMOC chemistry. Lysine methylation of K4AK9 was monitored by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. The samples were prepared by mixing 1 μl of sample and 3,5-dimethoxy-4-hydroxycinnamic acid (AHCA) matrix dissolved in 50:50 acetonitrile: water containing 0.1% TFA and air drying. The spectra were obtained on an Applied Biosystems Voyager DE MALDI-TOF mass spectrometry instrument.

**Cell culture and siRNA-mediated knockdown of G9a**

HeLa cells (ATCC) were grown in DMEM media with 10% serum (GIBCO) and transfected with 20 nM of fluorescein-siRNA transfection control (non-specific siRNA; NEB) or siRNA specific for G9a (si-G9a) using siRNA transfection reagent Lipofectamine 2000 (Invitrogen). The double-stranded RNA was made using HiScribe RnaI transcription kit (NEB) and siRNA mixes were produced using ShortCut siRNA kit (NEB). The double-stranded RNA was based on GenBank accession no. bc020970.gb_pr and the fragment spanned from 590 and 1000 nt.

Western blot analysis of cell extracts was performed with anti-actin (Sigma), anti-G9a (NEB), anti-H3 (CST), anti-di or tri-methyl H3-K9 and di-methyl H3-K27 (Upstate) antibodies according to the manufacturer’s recommendations.

**RESULTS**

**N-terminus of G9a participates in nuclear localization**

Functional characterization of different domains of mouse G9a was performed with deletion mutants from either the 5’ or the 3’ region of the gene. These mutants were cloned in-frame with GFP as shown in Figure 1A, resulting in green fluorescent protein (GFP) fusion constructs. Before cytochemical analysis, all the fusion constructs were transfected into COS-7 cells, the extracts were separated on SDS–PAGE gels, western blotted and probed with monoclonal anti-GFP antibodies. All the constructs expressed fusion proteins of the expected sizes. A representative panel for some of the expression construct fusion proteins is shown (Figure 1B). GFP fusion constructs transfected into COS-7 and HeLa cells were used to identify amino acids responsible for nuclear localization and chromatin binding. Full-length GFP-G9a fusion proteins localized exclusively in the nucleus and were clearly enriched at specific regions as evidenced by punctate staining patterns. G9a enzymes were excluded in some cells from nucleoli, the site of ribosome synthesis. However, a small proportion of GFP-G9a fusion proteins were also found in nucleoli (data not shown). A majority of GFP-G9a fusion proteins were found in pericentric heterochromatin, as observed by complete overlap of nuclear staining with GFP fluorescence patterns. Pericentric heterochromatin is transcriptionally silent and the presence of G9a may facilitate gene repression. Gradual deletions from the G9a C-terminus were performed to determine the region(s) necessary for nuclear import and heterochromatin localization. Deletion of 75 C-terminus amino acids (G9a3) did not impact the co-localization of G9a in heterochromatin. However, deletion of SET and preSET domains (G9a4 and G9a5) decreased heterochromatin localization of the enzyme. Deletion of amino acids 464–598 (G9a6) resulted in a diffuse nuclear distribution, suggesting the presence of a heterochromatin-binding sequence. Furthermore, deletion mutant G9a10, which lacks amino acids 464–598, also localized to heterochromatin, suggesting that a sequence other than amino acids 464–598 may also be involved in heterochromatin binding. Several other amino terminus deletions (G9a11, G9a1 and G9a2) were also examined and compared. While G9a11 containing amino acids 109–1263 was transported into the nucleus and localized on heterochromatin, G9a1 and G9a2 remained cytoplasmic. This is possible if ANK repeats participate in heterochromatin binding. Comparing the localization profiles of G9a4, G9a5, and G9a6, we hypothesize that amino acids between 464 and 890 that includes ANK repeats are essential for heterochromatin localization in G9a.

Surprisingly, G9a7 expressing amino acids 1–311 and G9a8 expressing amino acids 1–109 of G9a also localize in the nucleus. Nuclear localization was disrupted in G9a12, which contains only amino acids 1–24, suggesting a NLS between 24 and 109 in the G9a N-terminus. However, G9a11 that lacks the first 109 amino acids also localizes in the nucleus. This suggests the presence of at least one additional NLS embedded in the protein. Additional N-terminus deletion mutants representing amino acids 264–640 (G9a12a), 383–640 (G9a13), 394–640 (G9a14) and 416–640 (G9a15) were evaluated. Fusion proteins G9a12a, G9a13 and G9a14 were nuclear localized (data not shown for G9a12a and G9a13) but G9a15 was excluded from the nucleus, indicating a second NLS between amino acids 394 and 416. This region contains two amino acid stretches that are rich in basic lysine and arginine residues (392–398, RRKAKKK and 409–415, RKRRKR) that have been shown to act as NLSs. Thus, murine G9a contains at least two NLS sequences at the N-terminus of the long G9a isoform, with the second NLS conserved between both long and short isoforms of the enzyme in human and mouse (Figure 1D).

**Mutational analysis of conserved NLS**

Since G9a translocates to the nucleus to methylate H3-K9, we devised a strategy to determine the amino acids in the

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Figure 1. Dissection of nuclear localization signals of murine G9a. (A) A schematic diagram representing different GFP fusion proteins used to determine NLS. GFP is fused at the amino terminus of each fragment. Poly E (En), ANK repeats, preSET and SET domains are indicated. Amino acid residues are indicated below each construct. Nomenclature for the constructs is indicated on the left. The effects of deletions are summarized at the right, Nu (nuclear), Cy (cytoplasmic) and H (heterochromatic). (B) Western blot analysis of representative constructs from transfected cell extracts. Constructs are indicated on top and fusion proteins are marked by an arrow. Anti-GFP monoclonal antibody was used to probe the blot. (C) Cytochemical detection of GFP fusion proteins. Deletion constructs are indicated on the left. Nuclear staining is shown in purple, GFP-G9a fusions are in green and merge of both purple and green as light blue. Hoechst stain was used for nuclear staining. (D) Mapping and identification of NLS of G9a. Both NLS1 and NLS2 are shown in red. The translated protein sequence was based on the following GenBank accession numbers—murine G9a (mG9a): AB077210_gb_ro, human long isoform (hG9a) and short isoform G9a (shortG9a) are NM_006709 and BC020970_gb_pr, respectively.
conserved NLS regions that are critical (Figure 2A). Control (empty vector) and GFP fusion protein expression plasmids containing amino acids 394 to the native stop codon (G9a394) were transfected into cells and examined for nuclear localization. The control GFP construct remained predominately in the cytoplasm and G9a394, containing one putative NLS (amino acids 394–425), displayed predominantly a nuclear localization (data not shown). Furthermore, a GFP fusion construct containing amino acids 394–415 (G9aNLS2) was localized to the nucleus, suggesting that these amino acids are indeed crucial for nuclear localization. In order to functionally dissect the first Lys-Arg-rich region (amino acids 394–401) from the second, functional NLS of G9a, we further confirmed this observation by transfecting and localizing a GFP fusion construct containing only amino acids 409–415 of G9a (G9aNLS-KD) along with an identical construct containing Lys to Ala mutations at amino acids 396, 397 and 398 (G9aNLS-mKD). Indeed, the GFP construct containing the wild-type amino acids was localized exclusively in the nucleus, whereas the mutant remained in both the nucleus and cytoplasm. This result confirmed that mutation of amino acids K396, K397 and K398 disrupted nuclear targeting.

C-terminus of G9a participates in histone methylation

In order to determine the structural organization and role of the N-terminus of G9a in enzymatic catalysis, full-length G9a (G9aFL) and two N-terminus deletion mutants (Δ634 and Δ775) were cloned into the baculovirus expression vector pVIC1, or into the MBP fusion expression vector, pMALc2X. Mutant Δ634 and Δ775 were selected because they contained either the full or partial ANK repeat that is implicated in protein–protein interactions. The pVIC construct containing G9aFL has a C-terminus intein chitin binding domain tag. The mutants Δ634 and Δ775 were cloned to the C-terminus of MBP and were expressed as fusion proteins. G9aFL and deletion mutants MBP-G9aΔ634 and MBP-G9aΔ775 were expressed and enzymes purified to >90% homogeneity (Figure 3A). Catalytic activity, as determined by methyl group transfer, of both wild-type and MBP-G9aΔ634 on a synthetic H3 tail (Wt-H3) was similar. MBP-G9aΔ775 was less active compared with either the wild-type or MBP-G9aΔ634 enzyme (Figure 3B). Thus, we chose MBP-G9aΔ634 to perform additional enzymatic studies. All the dialyzed, purified proteins (wt, MBP-G9aΔ634 and MBP-G9aΔ775) were stored at −20°C and remained catalytically active for 6 months with minimal loss of activity.

To determine the functional role of the N-terminus region of the recombinant G9a in catalysis, steady-state kinetic properties between wild-type G9aFL and MBP-G9aΔ634 were compared. The time course for methylation of H3 at constant enzyme and variable histone H3 concentrations was measured. The reaction was linear for the first 10 min (data not shown), with each enzyme molecule potentially adding one, two or three methyl group(s) to lysine during this time. Analysis of the optimal enzyme and substrate concentrations for this assessment was described previously (34). Various amounts of recombinant H3 with G9aFL or MBP-G9aΔ634 were incubated with excess tritiated AdoMet, and the radioactive methyl group incorporation was measured and plotted against the substrate concentration. A Michaelis–Menten plot for the data was generated by nonlinear least-square fit of the data using an equation for a hyperbolic curve. A representative Michaelis–Menten plot for MBP-G9aΔ634 on recombinant histone H3 substrate is shown in Figure 3C. Similar graphs were plotted with additional substrates and estimates for V_{max} and K_m were determined for the MBP-G9aΔ634 mutant. The k_cat and catalytic efficiency were calculated and compared with G9aFL as presented in Table 1. In general, N-terminus deletion mutant MBP-G9aΔ634 displayed reduced turnover numbers for all four substrates examined compared with G9aFL. The Michaelis constant for AdoMet remained similar for both full-length and deletion mutants, suggesting that the N-terminus of G9a is not essential for AdoMet binding. However, the Michaelis constant for substrate peptides and rH3 were reduced except with K4AK9 substrates. These results suggest that the N-terminus of G9a may play a role in substrate recognition and binding, although similar catalytic
**Figure 3.** Catalytically active recombinant G9a and its amino terminus deletion mutants (A) Purified G9a full-length (G9aFL) along with amino terminus deletion mutants MBP-G9aΔ634 and MBP-G9aΔ775 resolved on SDS–PAGE. Molecular masses of the markers are indicated on the left. (B) Amino terminus deletion mutants and G9aFL are capable of histone methylation. Equimolar amounts of enzymes were used in this assay. The background value is deducted from the experimental result. (C) Representative initial substrate velocity curve for MBP-G9aΔ634 versus substrate concentration. MBP-G9aΔ634 activity with recombinant full-length histone H3 substrate is shown. Methylation reactions were performed with substrate concentrations of 0.03, 0.033, 0.0375, 0.05, 0.06, 0.075, 0.1, 0.15, 0.3, 0.44 and 0.55 mM and fixed enzyme and AdoMet concentrations of 25 nM and 5 µM, respectively. Product formation is plotted versus substrate concentration and nonlinear regression was performed for the determination of $K_m$ values. The inset shows the Lineweaver–Burke plot of the substrate velocity. $V_{max}$ and $K_m$ were calculated from the substrate velocity plot. (D) Purified wild-type H3 and point mutants shown in a Coomassie-stained denaturing gel on top panel. Histone methylation catalyzed by full-length baculovirus expressed G9a. The substrates were purified histones with different point mutations as indicated at the bottom. Background values were deducted and each bar represents two duplicates. (E) A similar assay as in (C), but the enzyme is MBP-G9aΔ634.
efficiencies were observed for both full-length and mutant enzymes.

We further investigated whether N-terminus deletions contribute to different substrate specificities. G9a is responsible for K9 and K27 methylation of histone H3 tails in vitro (21). The tail of histone H3 also has a lysine at K4 that is methylated or acetylated. Thus, we mutated K4, K9 and K27 of the histone H3 tail in various combinations and expressed recombinant H3 as intein fusion proteins. These fusions were cleaved on affinity columns with 50 mM DTT and stored with 50% glycerol without any apparent degradation for 6 months. Wild-type human H3 K4, K9 and K27 mutants (H3-K4A, H3-K9A, H3-K27A, H3-K4AK9A and H3-K4AK9AK27A) were separated on SDS gels and were >90% pure (Figure 3D, top panel). These recombinant H3 and mutant proteins were in vitro methylated using both G9aFL and MBP-G9aΔ634 (Figure 3D and E). As expected, mutant H3-K4AK9A, H3-K9AK27A and H3-K4AK9AK27A did not yield any tritium incorporation by either of the enzymes. However, H3 wild-type and H3-K4A were methylated to various extents. A mutation of K9A impaired the enzymatic methylation of the substrate (Figure 3D and E). Surprisingly, the K4A mutation also impaired the activity of the enzyme. The same set of experiments was conducted with G9a N-terminus mutant G9aΔ775 and a similar substrate methylation profile was observed (data not shown). Thus, amino acids 634 and 775 of the G9a N-terminus are redundant for catalytic functioning of the enzyme in vitro.

Lysine tri-methylation catalyzed by recombinant G9a deletion mutant in a processive manner

The epsilon amino group of lysine residues is prone to methylation. Biochemically, three methyl groups were added by the recombinant G9aFL histone methyltransferase (34). To elucidate the function of the N-terminus region of G9a in the methylation reaction and to determine the number of methyl groups transferred by deletion mutants of G9a, MALDI-TOF mass spectroscopy was used. A synthetic H3 peptide was in vitro methylated for different time intervals with cold AdoMet substrate. The molecular mass of the peptide was 2038 Da. Because one methyl group is 14 Da, a mono-, di- or tri-methylation will add 14, 28 or 42 Da onto the peptide substrate when enzymatic methylation occurs. Recombinant G9a lacking the first 633 amino acids methylated synthetic peptide in a time-dependent manner by transferring one methyl group at a time until all the available lysine residues were mono-methylated (molecular mass 2052). Once the peptides were converted to mono-methyl K9 peptides, a second methyl group was added until all the mono-methylated peptides were converted to di-methyl K9 (molecular mass 2066). Prolonged incubation (15 h) of the enzyme with the substrate converted di-methylated K9 to tri-methylated K9 peptides (molecular mass 2080) as shown in Figure 4, panels A–F. Incubation of G9a and synthetic H3 peptide without AdoMet for 15 h did not methylate the substrate peptide, suggesting that the reaction is AdoMet dependent. To differentiate between enzymatic and non-enzymatic methylation, heat-inactivated G9a was incubated with AdoMet and H3 peptide for 15 h followed by mass spectroscopic analysis. Indeed, there was no methyl group incorporation on the H3 peptide (Figure 4G). A blank incubation of AdoMet and peptide also gave similar result (data not shown), confirming the tri-methylation of K9 is enzymatic. Mutant G9aΔ634 yielded the same pattern of peptide methylation as that observed for G9aFL (34). These kinetic data suggest that both G9aFL and N-terminus-deleted G9a remained efficient in processive mono-, di- and tri-methylation reactions targeting methylation to the K9 residue of histone H3 tails (Figure 4 and data not shown).

G9a is capable of lysine tri-methylation in vivo

We further examined whether G9a can perform tri-methylation of lysine 9 in histone H3 in vivo. In the first set of experiments, an overexpressing plasmid clone containing MBP-G9aΔ634 was transformed along with an H3 overexpression clone into E.coli. Because E.coli does not possess histones and histone methyltransferases, it provides a unique system to study methylation reactions catalyzed by G9a in vivo in contrast to mammalian and insect cell systems. G9a enzymes co-purified with H3 even with 1.5 M NaCl washes, suggesting that the enzyme–substrate complex is strong (Figure 4H, top two panels). Western blot analysis was performed to determine the methylation status of H3 molecules. Indeed, both anti-K9 di-methyl and tri-methyl specific antibodies recognized the histone pool (Figure 4H, bottom two panels), suggesting that G9a performs di- and tri-methylation in vivo.

Furthermore, we also used siRNA-mediated G9a knock-down in HeLa cells to determine the status of global H3-K9 tri-methylation. Although a non-specific siRNA did not reduce G9a enzyme and H3-K9 tri-methylation levels, a G9a-specific siRNA (si-G9a) reduced the level of H3-K9 di- and tri-methylation (Figure 4I) up to 25 and 40%, respectively.
Figure 4. Mass spectroscopic determination of methylation reaction progression by recombinant MBP-G9aΔ634 (panels A–F). Spectra were taken at time (A) 0, (B) 15 min, (C) 30 min, (D) 120 min, (E) 8 h and (F) 15 h. Percentage intensity is shown at the left side and mass (m/z) on the bottom. Single methyl group as mono, double methyl groups as di and triple methyl groups as tri indicated on top of each peak with respective molecular masses of 2052 (mono), 2066 (di) and 2080 (tri) daltons. (G) Control experiment with a heat-inactivated enzyme is shown. (H) Western blot analysis of methylation status of lysine 9 histone H3 coexpressed in the presence of MBP-G9aΔ634 in E.coli. H3 alone or H3 plus MBP-G9aΔ634 as indicated on top were expressed. Top two panels show Coomassie staining of the co-eluted MBP-G9aΔ634 and H3 as well as the gradient of H3 loading. The bottom panels are western blot analysis of H3 as indicated under each panel for specific antibody. (I) siRNA-mediated knockdown of G9a resulted in the reduction of global di- and tri-methylated H3-K9 but not di-methylated H3-K27. siRNAs used for knockdown are indicated on top and antibodies for each panel on the bottom. Molecular mass is indicated as kDa. Densitometric scan of methylated histones is shown on the right.
as determined by densitometry. Neither of the siRNAs had any effect on the level of other known H3-K9 tri-methylases, Suv39H1 (Figure 4I) or on di-methylated H3-K27. These data confirm that reduction of G9a alone is sufficient to reduce H3-K9 di- and tri-methylation.

Mutational analysis of conserved G9a C-terminus amino acid residues

Both G9a and SUV39H histone methyltransferase families may tri-methylate K9 residues in histone H3 tails. However, there is little amino acid similarity between these methyltransferase families, except within the SET and post-SET regions (Figure 5A). Recent structural analysis of DIM-5, a SUV39H family H3-K9 methyltransferase from Neurospora crassa, demonstrates the involvement of SET domain conserved amino acids R155, W161, Y204 and R234 in forming a concave pocket (35). This concave pocket is large and can accommodate more than one cofactor, suggesting that this pocket may participate in the exchange of the reaction products AdoMet and AdoHcy, thereby facilitating a processive methylation reaction on H3-K9. A comparison of the primary

Figure 5. Mutation of the conserved amino acids in the SET domain abolishes catalytic activity of G9a. (A) Sequence comparison between the Lys9 trimethylase DIM-5 of N. crassa and mouse G9a as indicated. Amino acids used for point mutation analysis are underlined. Cys residues shown in gray participate in metal binding in DIM-5 and are conserved in mouse G9a. The histone methyltransferase signature motif NHXCDPN is shaded. (B) Purified mutants resolved on SDS–PAGE and stained are shown. Mutants are similar to MBP-G9aΔ634, except mutation of indicated amino acids is indicated on top. (C) Methylation of wild-type H3 peptide by purified recombinant wt (MBP-G9aΔ634) and mutants. Enzymes used for methylation are shown at the bottom. (D) Tritiated AdoMet binding by recombinant purified mutant G9a as compared with wild-type (MBP-G9aΔ634) enzyme with the same set of point mutation as that of (C). All experiments were performed in duplicate and the background values were deducted from experimental values.
structure between DIM-5 and G9a revealed identical amino acids at R1097, W1103, Y1138 and R1162 at the C-terminus region of these enzymes (Figure 5A). Thus, we determined whether any of these amino acids is involved in the G9a methyltransfer reaction. Point mutants using the MBP-G9aΔ634 back bone were made and proteins purified >90% (Figure 5B). Alanine-substituted point mutation of any of these conserved amino acids (R1097A, W1103A, Y1138A and R1162A) rendered the enzyme catalytically inactive (Figure 5C). We further examined whether these mutations affected cofactor AdoMet binding in the active site pocket by titrating tritiated AdoMet with purified G9a enzyme in solution. A saturating concentration of AdoMet (~2×K_m) was chosen for study. Indeed, the wild-type enzyme bound strongly to AdoMet. However, an ~40% decrease in AdoMet binding was observed in catalytically inactive mutants R1097A, W1103A, Y1138A and R1162A (Figure 5D), suggesting that AdoMet binding is compromised in these mutants.

DISCUSSION

G9a possesses a SET domain flanked by cysteine residues, which is the characteristic of all the histone methyltransferases. Two isoforms of G9a, 140 and 165 kDa, respectively, have been identified in mice by western blot analysis (25). In addition to the elements present in the human long G9a isoform, the mouse long isoform contains 53 more residues at its N-terminus. These additional amino acids in the mouse long G9a isoform have eight GR (Gly-Arg) repeats. GR motifs are involved in the nuclear accumulation of the large fibroblast growth factor isoform (36) and are present in hnRNA binding proteins (37) and nucleolin (38). Molecular analysis of the GR motifs in nucleolin suggests that this sequence functions as a nuclear retention signal (NRS). Our data suggest that GR repeats in murine G9a function as an NLS, since a GFP fusion construct with the first 100 G9a amino acids localized the GFP-G9a fusion protein to the nucleus. Mouse GFP-G9a fusion constructs with amino acids 409–1263 (G9a409) or 634–1263 (G9a1) showed cytoplasmic localization, although a deletion enzyme with identical amino acid residues was catalytically active, suggesting that NLS and catalytic domains are separable. Fine deletion analysis of the G9a N-terminus region revealed at least two NLS regions, with one between amino acids 24 and 109 and the other between 394 and 402. The second NLS is conserved between human and mouse and is common to both the long and short G9a isoforms. Thus, the GR repeat-containing long G9a isoform may have specialized functions that are yet to be discovered. Overall, mouse G9a appears to have at least three functional regions, including an N-terminus NLS, central ANK repeats for protein–protein interactions and a catalytic region at the C-terminus. Architecturally, the functional organization between G9a and other DNA (cytosine-5) methyltransferases, such as DNMT1, DNMT3a and DNMT3b, are similar with an NLS at the N-terminus region of the protein (39,40). The structural and functional relevance of the G9a poly-Glu residues is not yet determined. Furthermore, most of the fusion proteins tested here localized in both transcriptionally active euchromatin and transcriptionally inactive heterochromatin. Heterochromatin is the major location for tri-methylated H3-K9. G9a fusion proteins containing the first 698 amino acids, although catalytically inactive, were localized on euchromatic and heterochromatic regions in the nucleus.

G9aFL-MBP fusion or G9a N-terminus deletion fusion proteins (634 and 775) resulted in catalytically active H3-specific methyltransferases. Tachibana et al. (21) had a similar observation with a 621 amino acid deletion mutant of G9a. These results suggest that the catalytic domain is functionally independent of the rest of the protein. Steady-state kinetic analysis of G9aFL and mutant G9aΔ634 showed similar catalytic efficiencies, although 3-fold less substrate turnover and differing Michaelis constants were statistically significant.

A similar observation has been noted for the Drosophila SU(VAR)3-9 histone methyltransferase (41). Thus, the possibility of N-terminus involvement in substrate binding or recruitment for catalysis is not excluded in our analysis. Alternatively, deletion of the N-terminus may affect protein stability or folding. In contrast to the histone methyltransferases G9a and SU(VAR)3-9, deletion of the first 580 amino acids in human (cytosine-5) DNA methyltransferase 1 resulted in catalytic activation (42) without changing the enzyme specificity to hemi-methylated DNA (43). Both G9aΔ634 and G9aΔ775 mutants were able to methylate their natural H3 substrates, and peptides mimicking the N-terminal tail of H3, confirming only the C-terminus is required for histone methylation, although the N-terminus influences the catalytic parameters.

We determined that recombinant G9aFL (34) or the mutant lacking the first 633 amino acids is capable of mono-, di- or tri-methylation of the target lysine residue by both mass spectrometry analysis and sequencing of the methylated peptide. In our assay conditions, we were able to tri-methylate all of the unmethylated substrate (Figure 4). This observation is in contrast with studies describing G9a as a H3-K9-specific mono- or di-methyltransferase in vivo by deleting G9a (44,45). However, Suv39h1 deletion showed a small amount of H3 tri-methylation, which is consistent with our observations. Since most pericentric heterochromatin is tri-methylated by Suv39h1 and a proportion of euchromatic genes are silenced by G9a mediated H3 methylation, the small amount of H3-K9 tri-methylation observed in Suv39h1 null cells might be maintained by G9a. However, associations with additional H3-K9-specific methyltransferases are not excluded by these studies.

In DIM-5, the hydroxyl group of a proximal tyrosine residue (Y178) forms a hydrogen bond with target lysine 9 and remains highly deprotonated at pH ~10. The deprotonation of Y178 facilitates the deprotonation of the amino group of K9, resulting in a nucleophilic attack on the positively charged AdoMet methylsulfonium. The corresponding amino acid residue in G9a is Y1120. Mutation of Y1120A resulted in a ~90% reduction in catalytic activity (data not shown), although AdoMet binding was not impaired. This observation suggests an essential role for Y1120 in catalysis, as observed for Y178 in DIM-5. Three conserved cysteines are embedded in the post-SET region of SETDB1, DIM-5 and Suv39 histone methyltransferases that are essential for histone lysine methyltransferase activity (15,26,35). Zhang et al. (31) proposed that these three Cys residues coupled with a fourth Cys situated at the active site pocket (signature motif HXXCPN) form a metal binding pocket. Indeed, in DIM-5 these residues participate in
coordinated Zn$^{2+}$ binding. Furthermore, the post-SET and active site regions are highly conserved between SET domain-containing proteins, and the metal-binding center is essential for enzymatic activity. To understand the functional association of Cys residues in the signature motif (HXCDDPN) of G9a, we mutated C1168A. The mutant enzyme was catalytically inactive, although it can bind AdoMet, confirming that C1168 and an intact metal-binding pocket are essential for catalysis (data not shown).

Mutational analysis of the conserved amino acids in the SET domain of G9a resulted in catalytically inactive (R1097A, W1103A, Y1138A and R1162A) or reduced activity (Y1120A) enzymes. These amino acids are conserved between N.crassa DIM-5 histone H3 lysine methyltransferase and G9a. In DIM-5, mutation of R155H, W161F, Y204F and R238H reduced the enzymatic activity from 75% to nearly inactive. The reduced activity in DIM-5 generally mirrored reduced AdoMet binding. A small reduction in AdoMet binding was also observed in G9a mutants (R1097A, W1103A, Y1138A and R1162A), but this did not mirror the enzymatic activity, suggesting that AdoMet docking into the enzyme for G9a and DIM-5 may not be similar. However, it is possible that AdoMet movement in G9a, like DIM-5, may permit exchange and DIM-5 may not be similar. However, it is possible that AdoMet movement in G9a, like DIM-5, may permit exchange of the reaction product AdoHcy with AdoMet without releasing the histone tail. This would allow the methyltransfer reaction to proceed in a processive manner to tri-methylation.

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**ACKNOWLEDGEMENTS**

The authors thank Dr Yoichi Shinkai for the murine G9a clone (pZKmG9a). The authors also thank Dr D. G. Comb, New England Biolabs, Inc. for his support and encouragement. This study was partially supported by NIH/NCI grants CA90571 and CA107300, CMISE (NASA URETI award New England Biolabs, Inc. for his support and encouragement. The authors thank Dr Yoichi Shinkai for the murine G9a histone H3 lysine methyltransferase and G9a.

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