Regulation of Protein Arginine Methyltransferase 8 (PRMT8) Activity by Its N-terminal Domain*§

Joyce Sayegh †, Kristofer Webb †, Donghang Cheng §, Mark T. Bedford ‡, and Steven G. Clarke §†

From the †Department of Chemistry and Biochemistry and the Molecular Biology Institute, University of California, Los Angeles, California 90095-1569 and the §Science Park-Research Division, the University of Texas M.D. Anderson Cancer Center, Smithville, Texas 78957

Human protein arginine methyltransferase PRMT8 has been recently described as a type I enzyme in brain that is localized to the plasma membrane by N-terminal myristoylation. The amino acid sequence of human PRMT8 is almost 80% identical to human PRMT1, the major protein arginine methyltransferase activity in mammalian cells. However, the activity of a recombinant PRMT8 GST fusion protein toward methyl-accepting substrates is much lower than that of a GST fusion of PRMT1. We show here that both His-tagged and GST fusion species lacking the initial 60 amino acid residues of PRMT8 have enhanced enzymatic activity, suggesting that the N-terminal domain may regulate PRMT8 activity. This conclusion is supported by limited proteolysis experiments showing an increase in the activity of the digested full-length protein, consistent with the loss of the N-terminal domain. In contrast, the activity of the N-terminal truncated protein was slightly diminished by limited proteolysis. Significantly, we detect automethylation at two sites in the N-terminal domain, as well as binding sites for SH3 domain-containing proteins. We suggest that the N-terminal domain may function as an autoregulator that may be displaced by interaction with one or more physiological inducers.

Protein arginine methylation is a post-translational modification that has a variety of biological roles in the cell (1–5). These roles include cell signaling (6, 7), transcriptional activation/mRNA splicing (8–13), transcriptional repression (14, 15), nucleocytoplasmic RNA transport (16), and ribosomal assembly (17, 18).

Methylation of arginine residues in proteins is catalyzed by four types of protein arginine methyltransferase enzymes (PRMTs). Type I, II, and III enzymes catalyze the transfer of methyl groups from S-adenosyl-L-methionine (AdoMet) to the ω-nitrogen of the guanidino group to produce ω-N\(^{\text{G}}\)-monomethylarginine (MMA), while type IV enzymes catalyze the addition of the methyl group to the internal δ-nitrogen of arginine residues (19, 20). Arginine residues are commonly dimethylated, where type I enzymes add a second methyl group to the same ω-nitrogen atom to produce asymmetric ω-N\(^{\text{G}},N\(^{\text{G}}\)-dimethylarginine (ADMA), and type II enzymes catalyze methylation of the second ω-nitrogen of arginine to produce symmetric ω-N\(^{\text{G}},N\(^{\text{G}}\)-dimethylarginine (SDMA) (19). PRMT enzymes that display type I activity are mammalian PRMT1 (21), PRMT3 (22), CARM1/PRMT4 (23), PRMT6 (24), and PRMT8 (25), while PRMT5 has been shown to be a type II enzyme (26). Evidence has been presented to suggest that FBXO11 may also a type II enzyme (27). It is unclear at present what type of methylation is catalyzed by PRMT7 (28, 29). No mammalian type IV enzymes have been characterized to date.

We have been interested in the PRMT8 enzyme. The gene encoding PRMT8 was identified in a search of genomic and cDNA databases (25, 30, 31). The amino acid sequence for human PRMT8 is closest to that of PRMT1 in the protein arginine methyltransferase family, although it contains a unique 76-amino acid N-terminal region (25). PRMT1 is the main type I arginine methyltransferase in mammalian cells (21), is localized in both the nucleus and cytosol (24, 32), and functions in multiple cellular processes (1). Because of the importance of PRMT1 in cellular physiology, we were thus interested in its close homolog PRMT8. PRMT8 differs, however, from both PRMT1 and other family members in that it has a limited tissue distribution and is associated with the plasma membrane (25). Northern blot analysis of PRMT1 and PRMT8 using RNAs from a variety of human tissues showed PRMT8 expression solely in the brain while PRMT1 expressed ubiquitously in these tissues (25). In addition, expressed sequence tag (EST) analysis of human and mouse PRMT8 revealed that the majority of sequence tags arose from brain cDNA libraries (25). In mice, in situ hybridization experiments suggest that the PRMT8 gene is only expressed in neurons (33). PRMT8 localizes to the plasma membrane via N-terminal myristoylation (25). This irreversible modification is achieved through the covalent addition of the 14 carbon saturated fatty acyl chain to the glycine residue adjacent to the N-terminal initiator methionine residue.
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idue. N-Myristoylation has been shown to be involved in modifying proteins in signal transduction cascades (34).

In this work, we have examined the catalytic activity of full-length and N-terminal-truncated forms of PRMT8 that differ in their activity. We find that the presence of the N-terminal region attenuates the activity of PRMT8 and suggest that the activity of this enzyme may be regulated by altering the conformation of the protein in this region.

**EXPERIMENTAL PROCEDURES**

**PRMT8 Expression Vector Construction**—For this work, we prepared a plasmid expressing an N-terminal GST fusion of a N-terminal-truncated form of human PRMT8, which removes the first 60 amino acids of full-length PRMT8. The primers used to generate this fragment were: 5′-TAC GAA TTC AAG CTG CTG AAC CCA-3′ and 5′-AAG GTC GAC CTT AAC GCA TTT TGT AGT CAT TA-3′. EcoRI and Sall digestions were used to clone this fragment into pGex-6P-1. We also prepared plasmids expressing N-terminal His-tagged full-length and truncated PRMT8 proteins. We obtained a full-length cDNA clone of human PRMT8 (2024 nucleotides) from Invitrogen (Homo sapiens HMT1 hnRN methyltransferase-like 4 (Saccharomyces cerevisiae) mRNA; cDNA clone MGC: 26069; IMAGE:4796524; GenBank™ accession number BC022458). Purified plasmid DNA was used as a template to PCR amplify bases 36–1220 (full-length) and bases 216–1220 (Δ1–60 N-terminal truncation) using 20-mer primers. The forward primers to allow vector construction via PET Directional TOPO cloning (Invitrogen, pET 100/d-TOP Expression kit). PCR products were electrophoresed on a 1% agarose gel and purified using Quantum Prep Gel Slice kit (Bio-Rad). The TOPO cloning reaction was performed using 1 μl of purified PCR product (the full-length or truncated PRMT8 insert), 1 μl of salt solution (1.2 M NaCl, 0.06 M MgCl₂), 1 μl of TOPO vector (20 ng/μl), and 3 μl of water, incubated at room temperature for 10 min followed by transformation into One Shot Top10 Escherichia coli cells. Positive clones were screened by restriction digestion with BamHI enzyme followed by agarose gel electrophoresis. The nucleotide sequence of the coding region was confirmed by overlapping DNA sequencing of both strands (Davis Sequencing, Inc., Davis, CA). Positive clones were transformed into the BL21(DE3) E. coli strain. Full-length human PRMT8 construct expressing an N-terminal GST fusion protein has been previously described (25). This protein has a Tyr residue at position 45 and a C-terminal extension of QSTRAAAS.

**Purification of Full-length and Truncated His-PRMT8, GST-PRMT8, GST-PRMT1, and GST-GAR**—His-tagged full-length and truncated (Δ1–60) PRMT8 was expressed in BL21(DE3) E. coli cells grown at 37 °C in 2 liters of LB media with 100 μg/ml ampicillin in an optical density at 600 nm of 0.6 and then induced with 0.4 mM isopropyl-β-d-thiogalactopyranoside for 12 h at 25 °C. Cells were washed twice with phosphate-buffered saline solution (PBS) containing 137 mM NaCl, 2.7 mM KCl, 1.4 mM KH₂PO₄, and 4.3 mM NaHPO₄ pH 7.4. Protease inhibitor (100 μM phenylmethylsulfonyl fluoride from a 1000-fold concentrated stock in Me₂SO) was added, and cells were resuspended in 8 ml of PBS. Cells were lysed on ice by sonication using a Sonifier cell disrupter W-350 (Smith-Kline Corp, 50% duty cycle, setting 4) for seven cycles of 20 s of sonication with 1 min of cooling in between. Lysed cells were centrifuged at 23,000 × g at 4 °C, and the supernatant was applied to a 1.5-ml column of Ni²⁺-charged His-binding resin (Novagen His-Bind purification kit; Madison, WI). Bound His-PRMT8 proteins were washed with 10 ml of 0.5 M NaCl, 60 mM imidazole, 20 mM Tris-HCl, pH 7.9, and eluted with 5 ml of 1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9 in 1-ml fractions.

GST-GAR is a fusion protein containing the first 148 amino acids of human fibrillarin (22). This protein, as well as GST-PRMT1 (35) and the full-length and truncated forms of GST-PRMT8 were expressed in DH5α E. coli cells to make extracts as described above. Cell lysates were added to 0.5 ml of glutathione-Sepharose 4B beads (GE Healthcare Biosciences AB, Uppsala, Sweden). Bound proteins were washed three times with 10 ml of PBS and eluted with 1 ml of 30 mM glutathione, 50 mM Tris-HCl, pH 7.5, and 120 mM NaCl.

**In Vitro Methylation of GST-GAR, Myelin Basic Protein, and Histones H2A and H4 by Recombinant PRMT1 and PRMT8 and SDS-PAGE and Amino Acid Analysis**—His- and GST-tagged PRMT8 proteins (2 μg of protein) were incubated with and without 10 μg of methyl-accepting proteins including myelin basic protein (MBP, purified from bovine brain, lyophilized powder, product M1891, Sigma), GST-GAR, and histones H2A and H4 (purified from calf thymus, Roche Applied Science). Incubations were performed in the presence of 0.61 μM Sadenosyl-L-[methyl-³H]methionine ([³H]AdoMet, 81.0 Ci/mmol, dilute HCl: ethanol (9:1), pH 2.0 to 2.5, Amersham Biosciences), and 100 mM sodium phosphate buffer (pH 7.5) in a final volume of 60 μl for 1 h at 37 °C.

For amino acid analysis, the reaction mixtures were precipitated with an equal volume of 25% (w/v) trichloroacetic acid with 20 μg of bovine serum albumin as a carrier protein and allowed to stand at room temperature for 30 min. Precipitated proteins were centrifuged at 1,000 × g for 30 min at room temperature, and the pellets were washed with acetone at 4 °C and centrifuged again for 30 min. The pellet was acid hydrolyzed with 100 μl of 6 M HCl at 110 °C for 20 h in vacuo in a Waters Pico-Tag Vapor Phase apparatus and dried. The hydrolyzed sample was resuspended in 50 μl of water and added to 500 μl of citrate buffer (0.2 M Na⁺, pH 2.2) along with 1.0 μmol each of unlabeled standards of ω-N⁶-monomethylarginine (acetate salt, Sigma) (MMMA) and ω-N⁶,ω-N⁶-dimethylarginine (hydrochloride, Sigma) (ADMA). The sample was loaded onto a cation-exchange column previously equilibrated with sodium citrate buffer (0.35 M Na⁺, pH 5.27) (column resin consisted of Beckman AA-15 sulfonated polystyrene beads; column length of 0.9 cm inner diameter × 7 cm column height), and eluted at 1 ml/min at 55 °C. To detect ³H radioactivity, 200 μl of each fraction was mixed with 400 μl of water and 5 ml of fluor (Safety Solve, Research Products International, Mount Prospect, IL). Radioactivity was determined using a Beckman LS6500 counter as an average of three 3-min counting cycles. Unlabeled standards were detected with a ninhydrin assay using 100 μl of each fraction (36).
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For SDS-PAGE analysis, methylation reactions were stopped with the addition of an equal volume of SDS gel sample buffer (180 mM Tris-HCl, pH 6.8, 4% SDS, 0.1% β-mercaptoethanol, 20% glycerol, and 0.002% bromphenol blue), heated at 100 °C for 3 min, and loaded on a 12.6% acrylamide and 0.43% N,N'-methylene bisacrylamide gel (1.5-mm thick, 10.5-cm resolving gel, 2-cm stacking gel). Using the Laemmli buffer system, electrophoresis was performed at 35 mA for 4.5 h. The gel was stained with Coomassie Brilliant Blue R-250 for 1 h and destained with 10% methanol and 5% acetic acid for 5 h. The destained gel was prepared for fluorography by incubation in EN3HANCE (PerkinElmer Life Sciences) for 1 h and washed with water for 20 min. The gel was dried at 70 °C in vacuo and exposed to Kodak BIOMAX XAR scientific imaging film at −80°C.

Limited Trypsin Digestion of Recombinant PRMT8—His- and GST-tagged PRMT8 full-length and truncated proteins (10 µg of protein) were incubated in a final volume of 50 µl with 0.06 µg of trypsin (TPCK-treated, from bovine pancreas, type XII, Sigma) and 100 mM sodium phosphate, pH 7.5, for 5, 10, 60, and 120 min at 37 °C. The reaction was stopped by the addition of 0.6 µg of soybean trypsin inhibitor (Calbiochem, La Jolla, CA). Control samples were incubated at 0 °C without trypsin or with the PRMT8 protein pretreated for 5 min at 0 °C with 0.6 µg of soybean trypsin inhibitor before the addition of trypsin. Samples from each time point containing 5 µg of protein were analyzed by SDS-PAGE as described above. Separate samples containing 2 µg of digested protein from each time point were incubated with 10 µg of GST-GAR, 0.61 µM [3H]AdoMet, and 100 mM sodium phosphate buffer (pH 7.5) for 1 h at 37 °C. Samples were analyzed by SDS-PAGE and fluorography as described above.

Auto-methylation of GST-PRMT8—Purified GST-PRMT8 (20 µg) was incubated with [3H]AdoMet (0.34 µM) and 100 mM sodium phosphate buffer (pH 7.5) in a final volume of 50 µl for 1 h at 37 °C. The methylation reaction was stopped with the addition of 60 µl of sample buffer and resolved by SDS-PAGE as described above. The corresponding GST-PRMT8 protein band was gel extracted, acid hydrolyzed, and subjected to amino acid analysis as described above with the addition of an additional unlabeled standard of 1 µmol of ω-N6,N8-dimethylarginine (di(p-hydroxyazobenzene-p'-sulfonate) salt, Sigma) (SDMA).

Analysis of Methylated Peptides by Liquid Chromatography Tandem Mass Spectrometry (LC-MSMS)—Full-length GST-PRMT8 bands were excised from four lanes of a Coomassie-stained SDS gel (200 µg of protein loaded per lane) and subjected to in-gel trypsin digestion using the following procedure. Gel slices were diced into 3 mm × 3 mm pieces and destained by vortexing for 10 min in 225 µl of 25 mM ammonium bicarbonate pH 8.0 and 225 µl of acetonitrile, then discarding the supernatant. This procedure was followed by a similar treatment with 450 µl of 25 mM ammonium bicarbonate, and the entire destaining process was repeated twice more. The gel slices were then dried under vacuum. 250 µl of 10 mM dithiothreitol was incubated with the sample for 1 h at 56 °C followed by 1 ml of 55 mM iodoacetamide for 1 h at room temperature to reduce and alkylate the free cysteine residues. Reagents were removed by repeating the destain protocol twice more. The gel slices were again dried and 4 µg of trypsin (Sequencing grade modified trypsin; Promega, Madison, WI) dissolved in 250 µl of 25 mM ammonium bicarbonate pH 8.0 was added. After 16 h at 37 °C, peptides were extracted in 500 µl of 50% acetonitrile, 5% formic acid, and 45% 25 mM ammonium bicarbonate at pH 8.0 with sonication for 5 min. The lyophilized extract was brought up in 30 µl of water, and three 10-µl injections were analyzed using LC-MS/MS data-dependent acquisition on a Thermo LTQ ion trap instrument. A reverse-phase column (150 mm × 1 mm; Develosil RP-Aqueous-AR[C30] 5 µm, 140 Å; Phenomenex, Torrance, CA) was equilibrated for 30 min at 50 µl/min with 100% A (A, 0.1% formic acid in water;
Methylation of GST-GAR

Enzyme Only

A. His-(Δ1-60) PRMT8

B. His-PRMT8

Twenty-four hours after transfection, the cells were labeled using a previously described in vivo methylation assay (38). The cells were lysed in radioimmune precipitation assay buffer, and immunoprecipitations were performed with α-GFP antibodies. Samples were separated on a 10% SDS-PAGE, transferred to a polyvinylidene difluoride membrane, sprayed with Enhance, and exposed to film.

Generation of Protein Microarrays, Peptide Synthesis, and Labeling—The generation of protein microarrays and the labeling of peptide probes has been described previously (39). The peptide (Biotin-STEVSPPSQPPQPVPQPKVQ) was synthesized by the W.M. Keck Center (New Haven, CT).

GST Pull-down Experiments—HeLa cells were transiently transfected with the GFP-PRMT8(G→A) construct. This construct expresses a form of PRMT8 that cannot be myristoylated and is thus not membrane bound. HeLa cells were lysed in 0.5 ml of mild lysis buffer (150 mM NaCl, 5 mM EDTA, 1% Triton X-100,
10 mM Tris/HCl, pH 7.5). 15 μg of GST fusion protein bound to beads was incubated with HeLa cell extracts (10-cm plate) for 2.5 h at 4 °C. After five washes with lysis buffer, the beads were boiled in loading buffer and separated by SDS/PAGE and transferred onto polyvinylidene difluoride membranes.

RESULTS AND DISCUSSION

GST-PRMT8 Has a Much Lower Enzymatic Activity than GST-PRMT1—The amino acid sequence of PRMT8 is 83% identical to that of PRMT1 over 325 residues of its 347 residue total length (25). We were surprised, however, to find that the full-length N-terminal GST fusion of PRMT8 had much lower activity than the N-terminal GST fusion of PRMT1 when either GST-GAR (22) or myelin basic protein were used as methyl-accepting substrates (Fig. 1).

N-terminal Truncated Polypeptide of PRMT8 Displays Enhanced Enzymatic Activity in Vitro—The major difference in the sequence of PRMT1 and PRMT8 occurs at the N terminus where a unique additional sequence of about 65 residues is present on PRMT8 (25). We thus prepared both GST fusion and His-tagged constructs of PRMT8 with and without its N-terminal 60 amino acid residues. The truncated PRMT8 proteins would then closely match the sequence of PRMT1 (25). We could then compare the activity of GST- and His-tagged species with and without the unique N-terminal domain of PRMT8.

We first measured methyltransferase activity of the PRMT8 constructs toward the GST-GAR methyl-accepting substrate using amino acid analysis after incubation with [3H]AdoMet. We compared the activity of the full-length PRMT8 His-tagged fusion protein with the comparable (Δ1–60) PRMT8 species. We found that the formation of both [3H]MMA and [3H]ADMA derivatives were more than 10-fold increased when the truncated construct was used compared with the full-length form (Fig. 2). Control experiments in the absence of the GST-GAR substrate revealed only trace amounts of radiolabeled products by SDS-PAGE and fluorography (4-h exposure). Molecular weight standards were electrophoresed in the left hand lane (mw) as in Fig. 1. The GST-PRMT8 and GST-(Δ1–60) PRMT8 fluorograph was exposed for 5 days while the His-PRMT8 and His-(Δ1–60) PRMT8 fluorograph was exposed for 1 day.

FIGURE 4. Limited trypsin digestion allows activation of GST-PRMT8 methyltransferase activity in vitro. Trypsin digestion of GST-PRMT8 and GST-(Δ1–60) PRMT8 proteins (5 μg) at 37 °C for 5, 10, 60, or 120 min. The 0, NT and 0, T time points indicate no trypsin (NT) digestion and trypsin (T) digestion after 5 min of incubation with soybean trypsin inhibitor. Digested GST-PRMT8 and GST-(Δ1–60) PRMT8 proteins were resolved by SDS-PAGE. Digested GST-PRMT8 and GST-(Δ1–60) PRMT8 (2 μg of protein) were incubated with 10 μg of GST-GAR and [3H]AdoMet (0.61 μM) at 37 °C for 1 h, followed by analysis of methylated products by SDS-PAGE and fluorography (1 day exposure).

FIGURE 5. Activation of PRMT8 activity by limited trypsin digestion of the full-length but not the N-terminal-truncated species. Trypsin digestion of His-tagged PRMT8 and His-(Δ1–60) PRMT8 proteins (5 μg) at 37 °C for 5, 10, 60, or 120 min. Digestion was terminated with the addition of soybean trypsin inhibitor as described under "Experimental Procedures." Controls were performed without trypsin or 37 °C incubation (0, NT), and with trypsin without incubation (0, T). Digested recombinant PRMT8 and (Δ1–60) PRMT8 proteins were resolved by SDS-PAGE. Digested His-PRMT8 and His-(Δ1–60) PRMT8 (2 μg of protein) were incubated with 10 μg of GST-GAR and [3H]AdoMet (0.61 μM) at 37 °C for 1 h, followed by analysis of methylated products by SDS-PAGE and fluorography (4-h exposure). Molecular weight standards were electrophoresed in the left hand lane (mw) as in Fig. 1.
thymus histones H2A and H4, and myelin basic protein. Here, the radiolabeled polypeptides were analyzed by SDS-PAGE (Fig. 3, right panels). We also repeated the experiment using the full-length and truncated GST-PRMT8 enzymes (Fig. 3, left panel). For all of these substrates, the recombinant truncated PRMT8 fusion proteins had increased activity in comparison to the full-length proteins, although the difference was much larger with the GST fusion proteins than with the His-tagged proteins.

**Increased Methyltransferase Activity upon Limited Trypsin Cleavage of the Full-length PRMT8 Protein**—Because the truncated forms of the recombinant PRMT8 enzyme are highly active, we decided to try to induce the enzymatic activity of the recombinant full-length forms with tryptic cleavage under native conditions. Full-length and (Δ1–60)-truncated GST-tagged PRMT8 proteins were subjected to such limited proteolysis, and the digestion products were analyzed by SDS-PAGE. After 5 min of digestion, the predominant band corresponding to the full-length enzyme is lost (Fig. 4, left panel). Under the same digestion conditions, we found that the recombinant (Δ1–60) truncated form of PRMT8 was relatively stable to mild trypsin digestion (Fig. 4, left panel). The digested full-length and truncated GST-tagged PRMT8 proteins were then used to methylate GST-GAR with [3H]AdoMet. Methylation products were analyzed by SDS-PAGE and fluorography (Fig. 4, right panel). We found little or no activity of the control digested full-length PRMT8 samples (absence of trypsin or pretreatment with soybean trypsin inhibitor). However, at 5 min of digestion, activity was detected that increased over the next 55 min of digestion (Fig. 4, right panel). Interestingly, digestion of the (Δ1–60)-truncated form resulted in actually a decrease in activity from the control samples to samples digested for 5 or 10 min and little activity was found in samples digested for 60 min or more. These results suggest that the N-terminal domain of full-length PRMT8 is susceptible to cleavage with trypsin under native conditions and that such cleavage can enhance the catalytic activity of the protein.

To verify that trypsin cleavage leads to activation of the full-length PRMT8 enzyme, the mild trypsin digestion experiment was repeated with the full-length and truncated His-tagged PRMT8 proteins (Fig. 5). Here, digestion of the full-length species resulted in the loss of the intact polypeptide, while the truncated form was much more stable (Fig. 5, left panel). The trypsin-digested products of the full-length and recombinant (Δ1–60) truncated His-tagged PRMT8 proteins were then used in the methylation assay with GST-GAR and analyzed by SDS-PAGE and fluorography (Fig. 5, right panel). In these experiments, we detected an increase in methyltransferase activity of the full-length form upon digestion, although a significant amount of activity was observed in the undigested controls. This activity increased with increasing exposure to trypsin over 60 min. The recombinant truncated His-PRMT8 enzyme showed little significant change in methyltransferase activity with exposure to trypsin cleavage.

These results suggest that limited trypsin digestion can remove the N-terminal extension of PRMT8 and thus alleviate its normal inhibition of its activity in vitro, and that once cleaved, the enzyme can increase its activity to levels that may...
The automethylated band of 3H-methylated GST-PRMT8 was extracted, acid hydrolyzed, and fractionated stopped with the addition of equal volume of sample buffer and the polypeptides resolved by SDS-PAGE. PRMT fusion proteins (panel B). The same membrane was the stained with Ponceau-S to demonstrate roughly equal loading of the GST-brane, sprayed with Enhance™ (PerkinElmer Life Sciences) and exposed to film overnight (panel C). We developed a quantitative kinetic assay for PRMT enzymes by high-resolution cation exchange chromatography with unlabeled standards of ADMA, SDMA, and MMA, as described under “Experimental Procedures” and the legend to Fig. 2. C, different plates of HeLa cells were transiently transfected with GFP-PABP1, GFP-PRMT5, GFP-PRMT6, and PRMT8L-GFP. Methylated proteins were labeled and analyzed as described under “Experimental Procedures.” Immunoprecipitations (IP) were performed with α-GFP antibodies. The 3H-labeled proteins were exposed for visualization by fluorography (upper panel), and the same membrane was immunoblotted with α-GFP antibodies (lower panel). GFP-PABP1 is a known CARM1 substrate and serves as a positive control.

**Automethylation of PRMT8**—To ask whether the activation of the truncated form of GST-PRMT8 resulted from a change in the $K_m$ or the $V_{max}$ of the enzyme for its substrates, we developed a quantitative kinetic assay for PRMT enzymes using [3H]AdoMet as a methyl donor. We measured the radiolabeled GST-GAR product separated on SDS-PAGE as described in the legend of Fig. 6. For each enzyme preparation, we determined initial velocity conditions where the formation of product was proportional to both the amount of enzyme used and the time of incubation. We first tested this method with the well-characterized GST-PRMT1 enzyme and found that both AdoMet and the GST-GAR methyl-accepting substrate were recognized with high affinity ($K_m$ values of 0.35 μM and 1.5 μg/ml, respectively). We then compared the activities of the full-length GST-PRMT8 and the truncated GST-(Δ1–60) PRMT8 enzymes (Fig. 6). We found that the truncated enzyme had a marginally increased affinity for GST-GAR, a significantly increased affinity for AdoMet ($K_m$ value of 0.3 μM versus 2.1 μM), and most importantly, a large increase in the maximal velocity (18 pmol/min/mg versus 1.7 pmol/min/mg). Thus, it appears that the activation that occurs when the N-terminal domain of PRMT8 is removed is largely based on an increased binding affinity for AdoMet and an increase in the catalytic rate constant. It is of interest to note that under physiological levels of AdoMet (~20 μM in mammalian brain; Ref. 40), all of these enzymes would be expected to be saturated with the methyl donor and that the rate differences would result largely from changes in the catalytic rate constant.

**Kinetic Analyses of PRMT Enzymes**—To ask whether PRMT8 or other PRMTs also have the ability to methylate themselves we performed an in vitro methylation assay incubating a set of GST-PRMTs with [3H]AdoMet with no additional methyl-accepting substrate. Radio-labeled GST-PRMT proteins were analyzed by SDS-PAGE. Using this assay we find that PRMT6 is strongly methylated as expected (Fig. 7, panel A). In addition, the full-length form, but not the (Δ1–60)-truncated form, of PRMT8 is also strongly methylated, suggesting that automethylation is occurring at its N-terminal domain. We confirmed the automethylation of the full-length form of PRMT8 by amino acid analysis that showed the presence of radiolabeled ADMA and MMA (Fig. 7, panel B). PRMT1 and PRMT4 also display a weak degree of automethylation (Fig. 7, panel A).

To determine if automethylation of PRMT8 is taking place in a cellular context, we performed an in vivo methylation assay where HeLa cells were transiently transfected with four different GFP fusion proteins (PABP1, PRMT5, PRMT6, and PRMT8L), and methylated proteins were labeled with tritiated methionine in the presence of cycloheximide (Fig. 7, panel C). GFP-PABP1 is a known CARM1 substrate and serves as a positive control. GFP-PRMT5 is not labeled in this assay and serves as a negative control. Both PRMT6 and PRMT8 are labeled, indicating that these two PRMTs are automethylated in vivo as well as in vitro.

The sites of PRMT8 automethylation were then localized by mass spectrometric analyses of GST-PRMT8 purified by SDS-PAGE (Fig. 8). MS/MS sequencing of tryptic peptides revealed the presence of dimethylarginine at positions 108 and 182 of the
GST protein and position 73 of the PRMT8 protein (Fig. 8A). These residues appear to be asymmetrically dimethylated because we find characteristic ions with a neutral loss of 45 daltons from the loss of dimethylamine, confirming the results of amino acid analysis (Fig. 7B) (41). Additionally, we find a site of monomethylarginine at position 58 of PRMT8 (Fig. 8A). With the exception of the GST site at position 182, we also detected each of these peptides in the unmodified form, suggesting that the automethylation reaction is generally incomplete. In Fig. 8B, we show that there appears to be no common sequence elements adjacent to the methylated sites. We suspect that the methylation of each of these residues reflects their proximity to the PRMT8 active site. At this point, it is unclear whether the same polypeptide is responsible for its own methylation or whether adjoining catalytic chains in a complex are involved.

It is not known at present what the effect of the automethylation reactions are on the activity of PRMT8. However, it is significant that the PRMT8 automethylation sites are localized in and near the N-terminal domain. The ability of PRMT8 to methylate its own N terminus points toward a possible mechanism for autoregulation. Clearly, if the N terminus folds back into its own active site, then subsequent substrate recognition may be impaired.

PRMT8 Is Bound by SH3 Domains—To investigate whether the N-terminal region of PRMT8 may harbor unique protein motifs, a ScanSite search was performed (42). We identified two proline-rich sequences that are predicted to bind SH3 domains and GST itself was arrayed on a nitrocellulose slide. The array was probed with a Cy3-labeled peptide representing PR-domain I. The array was subsequently probed with an αGST antibody and detected with a fluorescein isothiocyanate-conjugated secondary antibody to establish roughly equal loading.
formed a GST pull-down experiment with a set of six different GST-SH3 domains. HeLa cells were transfected with PRMT8-GFP and cell lysates were incubated with bacterially expressed GST-SH3 fusion proteins prebound to glutathione-Sepharose 4B beads. PRMT8-GFP pulled down by the fusion proteins was detected by Western blot analysis (Fig. 9B). We detected strong binding of PRMT8-GFP to the SH3 domains of Fyn and PRMT2, weak binding to the SH3 domains of Ptc and p85, and no binding to Src or Abl. To establish whether the proline-rich domains of PRMT8 are sufficient for binding to these SH3 domains, we synthesized a biotinylated peptide that harbored PR-domain I of PRMT8 (Fig. 9A). This peptide was Cy3-labeled and used to probe a SH3 domain microarray, using an approach that we recently developed (39, 43). We found that this proline-rich peptide binds strongly to the SH3 domains of both p85 and PRMT2 (Fig. 9C). It is intriguing that the SH3 domain of PRMT2, a member of the protein arginine methyltransferase family that has no known enzymatic activity (1), binds strongly both to PRMT8 and to the proline rich peptide. Finally, we asked whether GST-SH3 domains could alter the methyltransferase activity of GST-PRMT8 in an in vitro assay. However, we found no changes with any of the six GST-SH3 domains (data not shown). It is possible that the other domains of these proteins, or additional binding partners, may be necessary for regulatory activity.

Model of PRMT8 Activity—In Fig. 10, we summarize our findings on the methyltransferase activity of recombinant forms of human PRMT8 and suggest a model for its regulation in cells. We propose that the N-terminal domain may structurally inhibit the activity of the catalytic core domain, resulting in a full-length species with low activity. We have shown that the activity can be enhanced by either the removal of the N-terminal domain in the recombinant (Δ1–60) truncated forms or by limited proteolytic digestion. Recently, it has been suggested that N-terminal sequences can also affect the activity and the substrate specificity of PRMT1 (44). The human PRMT8 protein contains an N-terminal myristoyl modification (25) that is not found in our recombinant proteins expressed in E. coli. We suggest that the myristoyl residue may participate in the role of the N-terminal domain in limiting the activity of the enzyme. We speculate that binding of one or more physiological ligands may be able to displace the N-terminal domain from the catalytic domain and alleviate the inhibition of enzyme activity. N-terminal myristoylation has been previously shown to act in a regulatory fashion in the autoinhibition of the c-Abl kinase (45).

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