S-Adenosyl-L-methionine-dependent protein arginine N-methyltransferases (PRMTs) catalyze the methylation of arginine residues within a variety of proteins. At least four distinct mammalian family members have now been described, including PRMT1, PRMT3, CARM1/PRMT4, and JBP1/PRMT5. To more fully define the physiological role of PRMT3, we characterized its unique putative zinc-finger domain and how it can affect its enzymatic activity. Here we show that PRMT3 does contain a single zinc-finger domain in its amino terminus. Although the zinc-liganded form of this domain is not required for methylation of an artificial substrate such as the glutathione S-transferase-fibrillarin amino-terminal fusion protein (GST-GAR), it is required for the enzyme to recognize RNA-associated substrates in rat1 cell extracts. The recombinant form of PRMT3 is inhibited by high concentrations of ZnCl2 as well as N-ethylmaleimide, reagents that can modify cysteine sulfhydryl groups. We found that we could distinguish PRMT family members by their sensitivity to these reagents; JBP1/PRMT5 and Hsl7 methyltransferases were inhibited in a similar manner as PRMT3, whereas Rmt1, PRMT1, and CARM1/PRMT4 were not affected. We were also able to define differences in these enzymes by their sensitivity to inhibition by Tris and free arginine. Finally, we found that the treatment of rat1 cell extracts with N-ethylmaleimide leads to a loss of the major PRMT1-associated activity that was immune to inhibition under the same conditions as a GST fusion protein. These results suggest that native forms of PRMTs can have different properties than their GST-catalytic chain fusion protein counterparts, which may lack associated noncatalytic subunits.

PRMT3 Is a Distinct Member of the Protein Arginine N-Methyltransferase Family

CONFERRAL OF SUBSTRATE SPECIFICITY BY A ZINC-FINGER DOMAIN* 

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S-Adenosyl-L-methionine-dependent protein arginine N-methyltransferases (PRMTs) catalyze the methylation of arginine residues within a variety of proteins. At least four distinct mammalian family members have now been described, including PRMT1, PRMT3, CARM1/PRMT4, and JBP1/PRMT5. To more fully define the physiological role of PRMT3, we characterized its unique putative zinc-finger domain and how it can affect its enzymatic activity. Here we show that PRMT3 does contain a single zinc-finger domain in its amino terminus. Although the zinc-liganded form of this domain is not required for methylation of an artificial substrate such as the glutathione S-transferase-fibrillarin amino-terminal fusion protein (GST-GAR), it is required for the enzyme to recognize RNA-associated substrates in rat1 cell extracts. The recombinant form of PRMT3 is inhibited by high concentrations of ZnCl2 as well as N-ethylmaleimide, reagents that can modify cysteine sulfhydryl groups. We found that we could distinguish PRMT family members by their sensitivity to these reagents; JBP1/PRMT5 and Hsl7 methyltransferases were inhibited in a similar manner as PRMT3, whereas Rmt1, PRMT1, and CARM1/PRMT4 were not affected. We were also able to define differences in these enzymes by their sensitivity to inhibition by Tris and free arginine. Finally, we found that the treatment of rat1 cell extracts with N-ethylmaleimide leads to a loss of the major PRMT1-associated activity that was immune to inhibition under the same conditions as a GST fusion protein. These results suggest that native forms of PRMTs can have different properties than their GST-catalytic chain fusion protein counterparts, which may lack associated noncatalytic subunits.

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† The abbreviations used are: PRMT, protein arginine N-methyltransferase; AdoMet, S-adenosyl-L-methionine; [3H]AdoMet, S-adenosyl-L-[3H]methionine; MMA, N\(^{\text{\text{-}}}\)-monomethylarginine; ADMA, asymmetric N\(^{\text{\text{-}}}\),N\(^{\text{\text{-}}}\)-dimethylarginine; SDMA, symmetric N\(^{\text{\text{-}}}\),N\(^{\text{\text{-}}}\)-dimethylarginine; GAR, glycine- and arginine-rich; GST, glutathione S-transferase; ZF, zinc finger; hnRNP, heterogeneous nuclear ribonucleoprotein; AdoX, adenosine dialdehyde; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; ICP-AE, inductively coupled plasma-atomic emission; kb, kilobase(s).

‡ A. Frankel and S. Clarke, unpublished results.
pGEF-JBP1 was constructed by PCR-amplifying from pGALFLAG-JBP1 a 1.9-kb fragment with primers JBP1-N1 (5'-GTCGACATGGCCGATGCGGTGCTGGTTGGAGGA-3') and JBP1-C1 (5'-GGCGCTCGTACAGGACCAATGTAATGAAGCGCGCTGGTTGTTGAGGA-3') and ligating the plasmid pGALFLAGHSIL7 to a 2.5-kb fragment with primers HSIL7-N1 (5'-GTCGACATGCAAGCGATTTGTTTGGTGTTGAGGA-3') and HSIL7-C1 (5'-GGCGCGCTCAGAGGGCGAGAAAGGGAGTGCACGACAGATTAGTT-3'). JBP1- and HSIL7-PCR products were digested with SalI and NotI and ligated into pGEX(NS) at SalI/NotI sites (32). The correct coding sequence was confirmed by DNA sequence analysis. All GST fusion proteins were expressed in Escherichia coli DH5α cells (Life Technologies, Inc.) upon induction with a final concentration of 0.4 mM isopropyl-β-D-thiogalactopyranoside. Washed cells were re-suspended in 2 mL of phosphate-buffered saline and 100 μM phenylmethylsulfonyl fluoride per gram of cells and broken by four 30-s sonicator pulses (50% duty; setting “4”) on ice with a Sonifier cell disruptor W-350 (SmithKline Corp.). The resulting lysate was centrifuged at 23,000 × g for 40 min at 4°C. GST fusion proteins were then purified from soluble extracts by binding to glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. Proteins were eluted with 30 mM glutathione, 50 mM Tris-HCl, pH 7.5, 120 mM NaCl, and 2% glycerol, and then were desalted on Sephadex G-25 gel filtration column (13.5-cm column height × 2.5-cm inner diameter; 60-mL bed volume) in 50 mM sodium phosphate, pH 7.5. For the metal-deficient (apo) forms of PRMT3 and GST-ZF, the loaded beads were first washed with 250 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA prior to glutathione elution. Proteins were stored at −80°C.

Protein Concentration Determination—A modification of the Lowry procedure was used to determine protein concentrations of GST fusion proteins and RAT1 cell extracts following precipitation with 1.0 mL of 10% (w/v) trichloroacetic acid (33). A stock solution of bovine serum albumin was used as a protein standard.

Zn2+ Concentration Determination—Desalted GST fusion proteins were added to a 0.1% HNO3 solution resulting in final concentrations of 0.70–0.86% HNO3. The metal content was then measured at an absorbance of 206.2 nm in a Thermo Jarrel Ash Iris 1000 ICP-AE (inductively coupled plasma–atomic emission) spectrometer per the manufacturer’s instructions.

Preparation of RAT1 Cell Extracts—RAT1 fibroblast cells, provided by Dr. Harvey Herschman at UCLA, were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, grown to approximately 80–90% confluence, washed twice with PBS, and harvested, and the supernatant was desalted on a Sephadex G-25 gel filtration column (13.5-cm column height × 2.5-cm inner diameter; 60-mL bed volume) in 50 mM sodium phosphate, pH 7.5, with a commercial mixture of protease inhibitors (Roche Molecular Biochemicals, catalog no. 1836170; 50 μg/mL antipain, dihydrochelrolase, 40 μg/mL benstatin, 6–60 μg/mL chymostatin, 0.5–10 μg/mL E-64, 0.5 μg/mL leupeptin, 0.7 μg/mL pepstatin, 4–330 μg/mL phosphoramidon, 0.1–1 μg/mL Pefabloc SC, 0.06–2 μg/mL aprotinin). Washed cells were lysed by four 10-s sonicator pulses (50% duty; setting “2”) on ice, and subjected to a 30-min centrifugation at 18,000 × g at 4°C. The resulting supernatant was collected and stored at −80°C. Hypomethylated RAT1 cell extracts were made from RAT1 cells cultured in the presence of 20 μM adenosine dialdehyde (AdOx) for 48 h prior to harvesting; and the supernatant was desalted on a NAP-10 Sephadex G-25 gel filtration column (Amersham Pharmacia Biotech) in 50 mM sodium phosphate at pH 7.5. To abolish endogenous methyltransferase activity, AdOx-treated RAT1 cell extracts were heated for 10 min at 70°C. In some cases AdOx-treated RAT1 cell extracts were pretreated with bovine pancreatic RNase A (10 mg/mL; Sigma) at a 0.2 mg/mL final concentration for 30 min at ambient temperature prior to methyltransferase reactions (13).

In Vitro Methylation Reactions—Purified GST-methyltransferase fusion proteins were incubated with either GST-GAR or heat-denatured AdOx-treated RAT1 cell extracts in the presence of S-adenosyl-L-[methyl-3H]methionine ([3H]AdoMet; 77 μCi/mmol, from a 10.4 μM stock solution in dilute HCl/ethanol 9:1, pH 2.0–2.5; Amersham Pharmacia Bio- tech) for 1 h at 37°C. Each reaction mixture was buffered with 50 mM sodium phosphate at pH 7.5. Specific concentrations of proteins and [3H]AdoMet are indicated in each of the figure legends.

Methyltransferase inhibition by N-ethylmaleimide (Sigma) was done by incubating enzymes (either recombinant GST fusion proteins or endogenous proteins in RAT1 cell extracts) with an indicated concentra-
tivation of N-ethylmaleimide for 20 min at 37 °C as described previously (34).

Methylation reactions were quenched by the addition of an equal volume of 2× SDS-PAGE sample buffer (120 mM Tris-HCl, pH 6.8, 1.43 M 2-mercaptoethanol, 4% SDS, 24% glycerol, 0.002% bromphenol blue), heated at 100 °C for 5 min, and separated on slab gels prepared from 12.6% (w/v) acrylamide, 1.4% (w/v) N,N-methylenebisacrylamide (1.5-mm × 10.5-cm resolving gel) using the buffer system described by Laemmli at a constant current of 35 mA for approximately 4 h (35). Following electrophoresis, gels were stained in Coomassie Brilliant Blue R-250 for 20–30 min, destained in a 10% methanol (v/v), 5% acetic acid (v/v) solution to visualize protein bands, and then soaked in EN°HANCE (PerkinElmer Life Sciences) according to the manufacturer’s instructions. Gels were dried in vacuo, and radioactivity was visualized by fluorography (exposure time at −80 °C is indicated in the figure legends). Methylation was quantitated by scanning the fluorographs in a MultiImage light cabinet and using Alpha Imagere (Alpha Innotech Corp.) software to measure the amount of film exposure for each lane. The horizontal baseline was adjusted to the lowest densitometric measurement to subtract background from the results. The value determined for the control lane was set at 100%, and all subsequent values were set as a percentage of the control.

**RESULTS**

**PRMT3 Binds Zinc in an Amino-terminal Zinc-finger Domain**—PRMT3 has been postulated to contain an amino-terminal C2H2-type zinc-finger domain (15). Such domains are present in a wide variety of proteins and often lead to the interaction of an a helix of the domain with the major groove of DNA (36), although other interactions with proteins and RNAs also occur (36, 37). To demonstrate that the amino-terminal region of PRMT3 does in fact bind to zinc, we purified several recombinant forms of PRMT3 as GST fusion proteins as indicated in Table I. The proteins were then subjected to ICP-AE spectroscopy to quantify zinc content in the different protein preparations. Neither GST nor the GST fusion protein of PRMT3 without its putative zinc-finger domain, GST-PRMT3azar (containing only amino acids 184–528 of rat PRMT3), were found to contain significant amounts of zinc. However, GST fusion proteins of full-length PRMT3 or its putative zinc-finger domain alone (amino acids 1–183) bound zinc almost stoichiometrically (Table I). Furthermore, when GST-PRMT3 was purified in the presence of chelators prior to desalting it by size-exclusion chromatography, its zinc content could be reduced by approximately half (Table I). These results clearly substantiate the presence of a zinc-finger domain in PRMT3. We found no difference in the metaltransferase activity toward the GST-GAR methyl-accepting substrate between the zinc-containing enzyme GST-PRMT3 and GST-PRMT3azar, but did observe an approximately 34% decrease in activity in the zinc-deficient GST-PRMT3 enzyme (data not shown).

**PRMT3 Zinc-finger Domain Confers Its Substrate Specificity**—The unique zinc-finger domain of PRMT3 may be involved in the regulation of the enzyme or in the recognition of specific methyl-accepting substrates. To test these hypotheses, GST-PRMT3, GST-PRMT3azar, GST-PRMT1, and GST-ZF-PRMT1 were incubated with heat-denatured hypomethylated extracts from AdOx-treated RAT1 cells as the methyl-acceptor (Fig. 1A). We observed large differences in their respective abilities to methylene substrates in RAT1 cell extracts. For example, neither the full-length GST-PRMT3 or the GST fusion protein lacking the zinc-finger domain appeared to recognize substrates in RAT1 cell extracts not treated with RNase (lanes 2 and 3), whereas GST-PRMT1 and a PRMT1 construct where the zinc-finger domain of PRMT3 was added to its amino terminus both methylated a large number of polypeptides (lanes 4 and 5). However, when RAT1 cell extracts were treated with RNase to uncover new methyl-acceptors (13), we found that GST-PRMT3 appeared to methylate at least six substrates in AdOx-treated RAT1 cell extracts incubated with RNase (lane 7). Significantly, no methylation was seen with the PRMT3 construct lacking the zinc-finger domain, indicating that this domain was required for the ability of PRMT3 to recognize these endogenous substrates. The effect of the zinc-finger domain was unique to PRMT3, because the addition of the domain to PRMT1 resulted in little change of its substrate specificity with either RAT1 cell extracts or RAT1 cell extracts treated with RNase (compare lanes 4 and 5 with 9 and 10). These results show that the zinc-finger domain of PRMT3 was responsible for its substrate specificity and suggest that PRMT3 may specifically methylate proteins that associate with RNA.

To explore the possibility that PRMT3 recognizes its substrates through interactions with its zinc-finger domain, we decided to test whether or not the zinc-bound (holoform) form of the zinc-finger domain can compete with GST-PRMT3 for substrate recognition by monitoring the methylation of GST-GAR (Fig. 1B). Using 0.48 μM GST-PRMT3, we found that its enzymatic activity decreased by 49% in the presence of 1 μM GST-ZF (holoform) (Fig. 1B, lane 2). At the same concentration, the apoform (metal-deficient) of GST-ZF (lane 7) and GST alone (lane 12) decreased GST-PRMT3 activity by 15% and increased its activity by 10%, respectively. Even at higher concentrations of competitor, it was the holoform (zinc-containing form) of GST-ZF that most dramatically reduced GST-PRMT3 activity (Fig. 1B, lanes 2–6). This same experiment was repeated with AdOx-treated RAT1 cell extracts (Fig. 1C). Although the methylation of polypeptides at 23, 31, 33, and 36 kDa was not uniquely inhibited by the holoform of GST-ZF (lanes 2–6) as compared with the other competitors (lanes 7–16), methylation of polypeptides at 15, 16, 60, 80, and 110 kDa was more inhibited by the holoform of GST-ZF than the other competitors at each respective concentration. These substrates appeared to represent a subclass of potential PRMT3-specific methyl-acceptors whose recognition was achieved primarily through the zinc-finger domain of the metaltransferase.

**PRMT3, Hsl7, and PRMT5 Contain Reactive Sulfhydryl Groups**—We wanted to then test whether the addition of zinc to PRMT3 might affect its activity. We found that the addition of 500 μM ZnCl2 to the apoform of PRMT3 resulted in an approximate 75% reduction in its enzymatic activity toward GST-GAR (Fig. 2A, compare lanes 5 with 8). On the other hand, GST-PRMT1, lacking a zinc-finger domain, was not affected at all (lanes 1–4). To determine if the zinc-finger domain of PRMT3 was responsible for the inhibitory effect of zinc, the zinc-finger domain-lacking fusion protein GST-PRMT3azar was the PRMT1 fusion protein with the zinc-finger domain of PRMT3
We found that the addition of the PRMT3 zinc-finger domain to PRMT1 did not result in sensitivity to zinc (Fig. 2A, lanes 13–16). However, PRMT3 lacking the zinc-finger domain was equally sensitive to zinc as the intact protein (lanes 9–12). These results clearly show that the methyltransferase activity exhibited by PRMT3 was inhibited by zinc ion independent of its zinc-finger domain.

Based on these results, we surmised that PRMT3 enzymatic activity may be sensitive to ZnCl$_2$ due to reactive sulfhydryl groups within the regions of the protein containing the common catalytic domain. We thus assayed whether PRMT3 and other GST-methyltransferases might be sensitive to either ZnCl$_2$, or
N-ethylmaleimide, a fairly specific reagent for modifying sulfhydryl residues. We found that GST-Rmt1 and GST-PRMT1 did not exhibit any sensitivity to either inhibitor (Fig. 2B, lanes 1–14), and GST-PRMT4 was not inhibited by N-ethylmaleimide (lanes 15 and 16). Thus the sensitivity was not common to all members of the methyltransferase family. As anticipated, GST-PRMT3 was inhibited by N-ethylmaleimide in addition to ZnCl2 (Fig. 2B, lanes 31–37). Significantly, both GST fusion proteins of PRMTs were tested for their sensitivity to both Zn2+ and N-ethylmaleimide. Methylation reactions contained 1.0 μg of recombinant fusion proteins GST-Rmt1, GST-PRMT1, GST-PRMT4, GST-Hsl7, GST-PRMT5, and GST-PRMT3 incubated with 0.87 μM [3H]AdoMet, 8.5 μg of GST-GAR, and different amounts of ZnCl2 and N-ethylmaleimide as indicated in the figure in a final volume of 30 μl. Film exposures were 1 h (lanes 8–14 and 31–37), 3 h (lanes 1–7), 15 h (lanes 17–23), and 5 days (lanes 15 and 16).

**Fig. 2. PRMT sensitivity to Zn2+ and N-ethylmaleimide.** A, Zn2+ inhibited the GST-PRMT3-catalyzed methylation of GST-GAR independent of its zinc-finger domain. Methylation reactions included 1.0 μg of the recombinant fusion proteins GST-PRMT1, GST-PRMT3, GST-PRMT3ΔZF, and GST-ZF-PRMT1 incubated with 0.58 μM [3H]AdoMet, 10 μg of GST-GAR, and different concentrations of ZnCl2 in a final volume of 45 μl. Polypeptides were separated by SDS-gel electrophoresis; a 4-h fluorograph of the gel is shown. No precipitation of proteins was observed at any of the concentrations of ZnCl2 used in the assay. B, several GST fusion proteins of PRMTs were tested for their sensitivity to both Zn2+ and N-ethylmaleimide. Methylation reactions contained 1.0 μg of recombinant fusion proteins GST-PRMT1, GST-PRMT4, GST-Hsl7, GST-PRMT5, and GST-PRMT3 incubated with 0.87 μM [3H]AdoMet, 8.5 μg of GST-GAR, and different amounts of ZnCl2 and N-ethylmaleimide as indicated in the figure in a final volume of 30 μl. Film exposures were 1 h (lanes 8–14 and 31–37), 3 h (lanes 1–7), 15 h (lanes 17–23), and 5 days (lanes 15 and 16).

Because ZnCl2 and N-ethylmaleimide sensitivity was observed for the GST fusion proteins of PRMT3, Hsl7, and PRMT5, we considered the possibility that at least one conserved cysteine residue may be found in these enzymes and not found in the unaffected proteins tested. Fig. 3 reveals that Hsl7 and PRMT5 appeared to share two conserved cysteine residues, one in the amino terminus (Cys-196 of PRMT5 and Cys-202 of Hsl7), and the other within the methyltransferase catalytic core (Cys-449 of PRMT5 and Cys-477 of Hsl7). It is possible that the inhibitory action of ZnCl2 and N-ethylmaleimide worked through the two sets of conserved residues indicated in Fig. 3, especially considering that both GST fusion proteins of Hsl7 and PRMT5 appeared to be similarly affected by both inhibitors (Fig. 2B). Cys-390 and Cys-414 in PRMT3, however, aligned with cysteine residues Cys-487 and Cys-518 in PRMT5, respectively, but these residues were also found to be conserved in the N-ethylmaleimide-insensitive enzyme PRMT1, which suggests that the inhibitory effect of ZnCl2 and N-ethylmaleimide on PRMT3 activity may act through different cysteine residues in PRMT3 as compared with those in Hsl7 and PRMT5.

**Effect of Methylation Inhibitors in RAT1 Cell Extracts**—Because the methylation activity toward the substrate GST-GAR of recombinant forms of Hsl7, PRMT5, and PRMT3 was inhibited by N-ethylmaleimide, we wanted to see if this compound could also inhibit endogenous methyltransferase activities in RAT1 cell extracts. Previously, Tang et al. (15) have shown by Western analysis of fractions from a size exclusion column that PRMT1 elutes as a protein complex of approximately 317 kDa,
whereas PRMT3 elutes as a roughly 37-kDa monomer. We thus wanted to ask whether the native complexes of PRMT1 and PRMT3 (as opposed to the GST fusion constructs) are sensitive or insensitive to PRMT3 (as opposed to the GST fusion constructs) are sensitive or insensitive to N-ethylmaleimide. We found that the bulk of methyltransferase activity eluted from a gel filtration column in the position expected for the larger PRMT1 complex (Fig. 4, A and B). Based on the results with the GST fusion enzymes (Fig. 2), we expected that the PRMT1 activity would not be affected by incubating with N-ethylmaleimide. However, when column fractions were preincubated with 1.0 mM N-ethylmaleimide prior to the methyltransferase assay, GST-GAR methylation was not detected for any column fractions (Fig. 4C). This result suggests that, although PRMT1 was not affected by N-ethylmaleimide as a recombinant GST fusion protein, it was susceptible to inhibition within its native enzyme complex. Differential Inhibition of Specific PRMT Methyltransferase Activity by Tris and Arginine—In our previous characterization of PRMT3 we had used Tris-HCl to buffer the enzymatic activity assays (15). In this work we found that some enzymes prepared in sodium phosphate buffer for zinc metal analysis had a greatly enhanced methyltransferase activity. We thus considered the possibility that Tris might be an inhibitor of the reaction, perhaps as a structural analogue of the arginyl side chain methyl-accepting substrate (Fig. 5A). Both Tris and arginine can form an extensive hydrogen-bonding network through either the amino groups of arginine (38), or the hydroxyl groups and single amino group of Tris. We tested the activity of GST fusion constructs of yeast proteins Rmt1 and Hsl7, and mammalian proteins PRMT1, PRMT3, and PRMT5 with the methyl-accepting fusion protein GST-GAR in the presence of increasing amounts of Tris, arginine, or arginine’s uncharged analogue, citrulline. We found that both Tris and arginine inhibited at least 50% of the enzymatic activity at 100 mM concentrations for all enzymes tested (Fig. 5B and C, respectively), with the notable exceptions of GST-Rmt1 and GST-PRMT3, whose activities do not appear to be affected by increasing amounts of Tris. PRMT1 activity appeared to be most strongly affected by both Tris and arginine in the reaction mixture with almost a full loss of activity at 100 mM concentration. Due to the resilience of PRMT3 activity and the dramatic inhibition of PRMT1 activity at high Tris concentrations, it might be possible to use Tris as a diagnostic marker of these activities in mammalian cell extracts. Interestingly, increasing amounts of the uncharged citrulline did not inhibit PRMT activity (Fig. 5D), but it seemed to slightly stimulate activity for GST-PRMT3 and GST-Rmt1, the only two enzymes tested that were inhibited by arginine and not Tris in this assay. Tris appeared to inhibit a significant amount of native PRMT activity in gel filtration column fractions tested (Fig. 4D), but some activity remained in the PRMT1-containing complex (fractions 38 and 40).

**DISCUSSION**

Each PRMT polypeptide chain identified to date contains a common central region of homology that appears to correspond to the catalytic core domain (Fig. 3). Outside of this domain, PRMTs can be quite dissimilar in amino acid sequence. Mammalian PRMT1 and its yeast homologue Rmt1 correspond to the shortest polypeptides in the PRMT family and appear to represent just the catalytic core, whereas CARM1/PRMT4, JBP1/PRMT5, and its yeast homologue Hsl7 contain amino- and/or carboxyl-terminal extensions to the catalytic core whose functions remain to be elucidated. PRMT2 contains an amino-terminal SH3 domain, but the catalytic activity of the enzyme has not yet been demonstrated (30, 31). We show here that PRMT3 is unique in that it contains a zinc-finger domain in its amino terminus, binding to $\text{Zn}^{2+}$ in a ratio approaching 1 mol of metal to 1 mol of protein.

Previously, Tang et al. (1999) suggested that PRMT3 activity appears to be modulated by its amino-terminal domain by comparing its activity as a full-length GST fusion protein to the amino-terminal deletion form of the enzyme with GST-GAR as the methyl-acceptor (15). In this work, however, we did not observe any difference in activities between the two forms of
the enzyme when GST-GAR was the substrate, and it is unclear why this discrepancy exists. Nevertheless, we did observe that the zinc-finger domain of PRMT3 was required for the enzyme to methylate substrates in hypomethylated AdOx-treated RAT1 cell extracts that have been subjected to RNase treatment prior to methylation. Furthermore, the zinc-bound form of the GST fusion construct of the PRMT3 zinc-finger domain was found to inhibit the PRMT3-catalyzed methylation of GST-GAR and hypomethylated RAT1 cell extracts to a greater extent than the zinc-deficient form. These results clearly demonstrate that the zinc-finger domain of PRMT3 contributes to its substrate specificity and suggests that its substrates appear to associate with RNA.

Although zinc-finger domains have been studied extensively as DNA-binding modules (36, 37), these domains have also been shown to interact with RNA (36), as well as to mediate both homo- and heterodimer formation between proteins (37). It is possible that the PRMT3 zinc-finger domain can help to specifically recognize its protein or protein-RNA targets for methylation. For example, mammalian transcriptional repressor proteins Zik1, Kid-1, and MZF-1, all containing zinc-finger domains, have been reported to bind hnRNP K, and this interaction is blocked when hnRNP K is complexed to poly(C) RNA (39, 40). Interestingly, hnRNP K contains three regions of amino acid sequence similarity to the PRMT substrate Sam68 (its GAR domain, KH domain, and SH3-binding regions), which suggests that it may be itself a candidate methyl-accepting species for PRMTs (8). Our observations that PRMT3 substrates in RAT1 cell extracts need to be exposed to RNase to be methylated, and that substrate recognition is zinc finger-dependent, intimate that a PRMT3 zinc-finger interaction with the free protein component of several methyl-accepting protein-RNA complexes may also be occurring in RAT1 cell extracts. We have previously shown that the RNase treatment of both yeast and mammalian cell extracts can modulate the activity of GST-Rmt1, GST-PRMT1, and GST-PRMT3 (13).

In studying the effects of exogenously added zinc on GST-PRMT3 activity, we found that at higher metal concentrations...
methyltransferase activity was inhibited in a zinc-finger domain-independent manner. Other recombinant GST-PRMTs tested for their zinc responsiveness revealed that PRMT5 and Hsl7 activities were also inhibited. Similar results were obtained by preincubating PRMTs with N-ethylmaleimide prior to the methylation reaction, suggesting that one or more cysteine sulfhydryl groups might be essential for the activity. However, some GST-methyltransferases were not inhibited by zinc or N-ethylmaleimide. We thus could distinguish between two different classes of recombinantly expressed PRMTs; Zn2+/N-ethylmaleimide-sensitive enzymes such as PRMT3, PRMT5, Hsl7, and Zn2+/N-ethylmaleimide-insensitive enzymes such as Rmt1, PRMT1, and PRMT4.

We found that the activities of recombinantly expressed PRMT enzymes were also sensitive to buffer conditions. We showed that GST-PRMTs exhibit similar activities toward GST-GAR when the proteins and substrate are desalted in 50 mM sodium phosphate, pH 7.5, and assayed in the same buffer.

Previous studies of GST-PRMTs employed Tris buffers in their assays (8, 9, 13–15), which we found to be an effective inhibitor of PRMT1, JBP1/PRMT5, and Hsl7 methyltransferase activities under these conditions. Furthermore, comparisons of GST-PRMT1 activities with hypomethylated yeast extracts as the methyl-acceptor typically resulted in the methylation of only a 55-kDa polypeptide (14, 15, 18), whereas in other assays GST-PRMT1 was capable of methylating more yeast proteins than just the 55-kDa species (13). These inconsistencies are perhaps explained by the fact that both high concentrations of Tris and arginine inhibit PRMT activity of recombinant GST fusion proteins, as well as the majority of PRMT activity in RAT1 cell extracts, presumably by competing with arginyl residues with methyl-accepting substrates. Recently, arginine derivatives found in Korean red ginseng as well as other polyamines were shown to effectively inhibit a purified mammalian PRMT activity (41). Although Tris and arginine are relatively poor methylation inhibitors, and free arginine does not appear to be a substrate as determined by thin layer chromatography (data not shown), their suspected mode of action suggests that PRMTs exhibit their substrate affinity toward protonated amino groups.

Based on the pattern of inhibition of GST-methyltransferase fusion proteins, we predicted that the major PRMT1 activity in mammalian extracts (20) would also be insensitive to inhibition by zinc ion and N-ethylmaleimide. However, we found that this was not the case. The possibility exists in that PRMT1 within cells may bind to additional subunits, which allow cysteine residues that are buried within the GST-PRMT1 fusion protein to become exposed and susceptible to N-ethylmaleimide modification as the native enzyme complex. Evidence has been presented that PRMT1 can be complexed (at least transiently) with several proteins (10, 14, 15, 29). GST fusion proteins of TIS21 and BTG1 can enhance the PRMT1-dependent methylation of hnRNP A1 and other methyl-acceptors in RAT1 cell extracts (14). Recently, Tang et al. (10) showed that ILF3 can interact with and serve as a substrate for PRMT1, in addition to demonstrating that GST-ILF3 can activate the GST-PRMT1 methylation of substrates in RAT1 cell extracts. It is plausible that the interactions PRMT1 shares with other proteins may affect not only its activity but also its responsiveness to inhibitors such as N-ethylmaleimide and Tris. These findings lead us to propose that recombinant forms of PRMT catalytic chains do not provide a completely accurate depiction of endogenous activity.
PRMT activity. To fully understand how these enzymes work in vivo, it will be important to explore the effect of their endogenous binding partners on their enzymatic activity and their methyl-acceptor specificity.

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