

PRMT 3, a Type I Protein Arginine *N*-Methyltransferase That Differs from PRMT1 in Its Oligomerization, Subcellular Localization, Substrate Specificity, and Regulation*

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Methylation is one of the many post-translational modifications that modulate protein function. Although asymmetric N^G,N^G -dimethylation of arginine residues in glycine-arginine-rich domains of eucaryotic proteins, catalyzed by type I protein arginine *N*-methyltransferases (PRMT), has been known for some time, members of this enzyme class have only recently been cloned. The first example of this type of enzyme, designated PRMT1, cloned because of its ability to interact with the mammalian TIS21 immediate-early protein, was then shown to have protein arginine methyltransferase activity. We have now isolated rat and human cDNA orthologues that encode proteins with substantial sequence similarity to PRMT1. A recombinant glutathione *S*-transferase (GST) fusion product of this new rat protein, named PRMT3, asymmetrically dimethylates arginine residues present both in the designed substrate GST-GAR and in substrate proteins present in hypomethylated extracts of a yeast *rmt1* mutant that lacks type I arginine methyltransferase activity; PRMT3 is thus a functional type I protein arginine *N*-methyltransferase. However, rat PRMT1 and PRMT3 glutathione *S*-transferase fusion proteins have distinct enzyme specificities for substrates present in both hypomethylated *rmt1* yeast extract and hypomethylated RAT1 embryo cell extract. TIS21 protein modulates the enzymatic activity of recombinant GST-PRMT1 fusion protein but not the activity of GST-PRMT3. Western blot analysis of gel filtration fractions suggests that PRMT3 is present as a monomer in RAT1 cell extracts. In contrast, PRMT1 is present in an oligomeric complex. Immunofluorescence analysis localized PRMT1 predominantly to the nucleus of RAT1 cells. In contrast, PRMT3 is predominantly cytoplasmic.

Protein function is often modulated by post-translational covalent modifications. One such modification is the *N*-methylation of the side chain of guanidino arginine residues (1–3). Type I protein arginine *N*-methyltransferase (PRMT)¹ enzymes

catalyze the formation of asymmetric N^G,N^G -dimethylarginine residues in proteins by transferring methyl groups from *S*-adenosylmethionine (AdoMet) to the guanidino nitrogen atoms of arginine residues. These enzymes generally methylate arginines found in RGG consensus sequences in the context of GAR (glycine and arginine-rich) domains (1, 4–6). The first cDNA for a mammalian type I PRMT enzyme to be cloned, PRMT1, was identified by a yeast two-hybrid screen as a rat cDNA encoding a protein that interacts with the product of the mammalian TIS21 immediate-early gene (7). TIS21 (also known as PC3 and BTG2) is a member of a family proteins (TIS21, BTG1, BTG3, and TOB) thought to be involved in negative control of the cell cycle (8, 9). Association of recombinant TIS21 protein with a fusion protein between glutathione *S*-transferase and rat PRMT1, GST-PRMT1, modulates PRMT1 activity *in vitro* (7), suggesting that interaction between transiently induced TIS21 (10–14) and constitutively expressed PRMT1 (7) may modulate PRMT1 enzyme activity *in vivo*. Moreover, human PRMT1 was independently isolated as a protein that interacts with the intracellular domain of the interferon- α , β receptor (15). The cytoplasmic domain of this receptor is also a docking site for many signaling proteins such as tyrosine kinases, serine/threonine kinases, and STAT transcription factors (16). PRMT1 antisense oligonucleotides relieve interferon- β -induced growth inhibition (15). The interaction of PRMT1 with both the TIS21 immediate-early gene product and with a transmembrane receptor suggests a role for this enzyme in cell signaling.

Human and rat PRMT1 are 96% identical at the amino acid level. Rmt1, the yeast PRMT1 homologue, is 45% identical to the human and rat PRMT1 proteins and is the predominant protein arginine methyltransferase activity in *Saccharomyces cerevisiae*, accounting for more than 85% of yeast PRMT activity (17, 18). Rmt1 is also a type I enzyme, catalyzing the formation of N^G,N^G -dimethylarginine residues. Data base searches have identified a distinct human gene, *HRMT1L1* (19), whose predicted open reading frame encodes a protein containing a potential protein arginine *N*-methyltransferase domain that has 33% of its amino acid residues identical to and 61% of its amino acid residues similar to human PRMT1.

The calculated molecular mass for the PRMT1 polypeptide, based on the translated cDNA sequence, is 40.5 kDa (7). However, protein arginine methyltransferase activity from several mammalian tissues and cell lines migrates on gel filtration columns at apparent sizes ranging between 150 and 500 kDa (4, 7, 20, 21), suggesting that PRMT1 is predominantly present as a component of a polypeptide complex. By using a yeast

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AF059530 and AF059531 for the rat PRMT3 and human PRMT3, respectively.

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¹ The abbreviations used are: PRMT, protein arginine *N*-methyltransferase; PCR, polymerase chain reaction; AdoMet, *S*-adenosyl-L-

methionine; GST, glutathione *S*-transferase; GAR, glycine- and arginine-rich region; PAGE, polyacrylamide gel electrophoresis; PFA, paraformaldehyde; PBS, phosphate-buffered saline; NAR, N-terminal acidic amino acid-rich.

two-hybrid screen to identify proteins that interact with PRMT1, we have identified a previously unknown protein whose carboxyl-terminal 365 residues share substantial sequence similarity with PRMT1. This protein, which has protein arginine *N*-methyltransferase activity, has been named PRMT3. Although PRMT3 and PRMT1 are both type I protein arginine *N*-methyltransferases, they differ in their substrate specificities, oligomerization properties, interaction with TIS21, and subcellular localization.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Analysis to Identify Proteins That Interact with Rat PRMT1—The rat PRMT1 cDNA was amplified by polymerase chain reaction (PCR), using pPC86-PRMT1 (7) as the template and the primers 5'CCCCGGAATTCATGGCGGCAGCCGAGGCC3' and 5'CTGGACGTCGACTCAGCGCATCCG3'. This cDNA was subcloned into pLexA(L) (7) at an *EcoRI/SalI* site to create the "bait" plasmid pLexA(L)-PRMT1. pLexA(L) contains a leucine-selective marker and encodes a LexA fusion protein that will bind to the LexA operator sequence. A rat pPC86 cDNA library prepared from rat FAO hepatoma cell poly(A)⁺ mRNA (7) was used as "prey" plasmids for the screen. These plasmids contain a tryptophan-selective marker and encode fusion proteins with the GAL4 transcription factor activation domain.

Yeast two-hybrid screening to identify proteins that interact with PRMT1 was performed as described previously (7). Twenty-five cDNA clones showed positive interactions with PRMT1 in both histidine prototrophy and β -galactosidase assays. These 25 clones were separated into four groups by PCR amplification, restriction digestion analysis, and sequencing. 15 clones were identical to pPC86-PRMT1, 8 were identical to clone 2A, and the other 2 were independent clones 4A and 7A. Clones 2A, 4A, and 7A were sequenced using the Dye Deoxyterminator cycle sequencing kit and a 373 DNA sequencer (Perkin-Elmer). Homology searches were performed using the BLAST algorithm, through the National Center for Biotechnology Information (22).

FAO cDNA Library Screening for a cDNA Clone Containing the Entire Open Reading Frame of PRMT3—Sequence analysis of clone 2A revealed that it is an incomplete clone, lacking the 5'-end region encoding the amino-terminal end of the protein. To recover a cDNA encoding the entire open reading frame, a rat FAO cDNA library in Lambda-Zap vector was screened using a 700-base pair 5'-end fragment of 2A as a probe. A 2.4-kilobase pair cDNA insert was recovered in pBlue-scriptSK(-), between the *EcoRI* and *XhoI* sites, and sequenced using the dye cycle sequencing kit. This 2.4-kilobase pair cDNA clone corresponding to the 2A sequence is termed PRMT3.

Two-hybrid Analysis of Interactions among PRMT1, PRMT3, and TIS21—The cDNA region corresponding to the amino-terminal 184-amino acid fragment of PRMT3 cDNA was PCR-amplified using primers 5'GGTGGGTCGACCGCCATGTGTTCTGCTGGCG3' and 5'CCACAGGAAGACTCACTTCTTCG3' and template pBlue-script(SK)-PRMT3. The fragment was digested with *SmaI* and *SalI* and inserted into pGEX(SN)-PRMT3-(90–528) (see "Preparation of GST Fusion Proteins") at *SalI/SmaI* sites. This reaction resulted in the GST-PRMT3 full-length fusion construct, pGEX(SN)-PRMT3. The full-length PRMT3 cDNA was removed from pGEX(SN)-PRMT3 with *SalI* and *NotI* and inserted into pPC86 to make pPC86-PRMT3.

Plasmid pBTM117 encodes a LexA DNA binding domain and was used to create alternative bait constructs for yeast two-hybrid analysis (24). pBTM117-PRMT3-(214–528) was constructed as follows: DNA for amino acids 214–528 was PCR-amplified using primers 5'GTCGACG-AAGGATGGCGTCTACTTCAGCTC3' and 5'TTGATTGGAGACTTGA-CC3' from pPC86-PRMT3-(90–528). The amplified cDNA was cut with *SalI* and *NotI* and inserted into pBTM117, to create pBTM117-PRMT3-(214–528). pBTM117-PRMT3-(90–528) and pGAD425-PRMT3-(90–528) were constructed by inserting the PRMT3-(90–528) coding region from pPC86-PRMT3-(90–528) into pBTM117 and pGAD425 at the *SalI* and *NotI* sites.

To analyze interactions, strain L40 was transformed with bait and prey plasmids in different combinations. The transformed yeast were plated on appropriate selective media and incubated at 30 °C. Individual colonies were patched and assayed for β -galactosidase activity.

Preparation of GST Fusion Proteins—pGST-GAR was constructed by fusing the *Schistosoma japonicum* glutathione *S*-transferase protein to the first 148 amino acids of human fibrillarin, a region containing 14 arginine residues in a glycine-rich region. The human fibrillarin coding sequence was PCR-amplified from a combined liver and spleen cDNA library and cloned into the GST fusion vector pGEX-2T (Amersham

Pharmacia Biotech), utilizing *BamHI* and *EcoRI* restriction sites engineered into the PCR primers. The resulting construct, pGST-FIB, was digested with *NheI* and *NdeI* to remove the carboxyl-terminal portion of fibrillarin. The 5'-overhangs remaining from the digest were filled in with the Klenow large fragment polymerase, and the plasmid was religated and introduced into *Escherichia coli* DH5a cells (Life Technologies, Inc.).

The multiple cloning site of pGEX-2T (Amersham Pharmacia Biotech) was modified to accept cDNA fragments at *SalI* and *NotI* sites and termed pGEX(SN). To make pGEX(SN)-PRMT3-(184–528), the DNA encoding the sequence between these amino acids of PRMT3 was amplified from pPC86-PRMT3, using primers 5'GGTGGGTCGACCAT-GAAACAATTTGCTCAGGACTTT3' and 5'TTGATTGGAGACTTGCAC3'. The amplified fragment was digested with *SalI* and *NotI* and inserted into pGEX(SN) at the *SalI/NotI* sites. pGEX(SN)-PRMT3-(90–528) was constructed by inserting the PRMT3-(90–528) DNA sequence from pPC86-PRMT3-(90–528) into pGEX(SN) at the *SalI/NotI* sites. pGEX(SN)-PRMT1 and pGEX-RMT1 were described previously (7, 17).

All GST fusion proteins were expressed and purified as described by the manufacturer (Amersham Pharmacia Biotech). SDS-PAGE analysis of each purified protein, with the exception of GST-GAR, indicated that only a single major band was visible by Coomassie staining. Although full-length GST-GAR is a 41-kDa polypeptide, the major methylated product electrophoreses as a broad band at apparent sizes of 28–36 kDa, with a major species at 28 kDa. In addition, a 14-kDa polypeptide is also methylated. These results suggest that the fusion protein may be sensitive to proteolysis during its expression or purification. Purified fusion proteins were stored at –80 °C.

Protein Concentration Determinations—RAT1 cell lysate protein concentration was determined by the bicinchoninic acid assay (Pierce). Other protein samples were assayed using a modified Lowry procedure (23), after precipitation with 1 ml of 10% (w/v) trichloroacetic acid. Bovine serum albumin was used as the standard for both procedures.

Preparation and Characterization of Rabbit Polyclonal Antibodies against PRMT1 and PRMT3—Polyclonal antibodies against PRMT1 and PRMT3 were raised in rabbits, using TrpE fusion proteins as antigens. The cDNA for the TrpE-PRMT1 fusion protein was constructed as follows: cDNA of PRMT1 was PCR-amplified from pPC86-PRMT1, using the primers 5'CCCCGGAATTCATGGCGGCAGCCGAGGCC3' and 5'GTCGACTCTAGAGTGATGGTGATGGTGATGGTGATGGCGCATCCGGTAGTCGGTGG3'. The amplified fragment was digested with *EcoRI* and *XbaI* and inserted into pATH11 (24) to make pATH11-PRMT1. The plasmid for TrpE-PRMT3-(166–528) fusion protein expression, pATH11-PRMT3-(166–528), was constructed by inserting the *XmaI* and *XbaI* fragment of the PRMT3 (from base pair 575 to 2070 in the PRMT3 cDNA), in-frame, into pATH11. TrpE fusion proteins were expressed as described previously (24). Proteins from inclusion bodies were extracted with 6 M urea and subjected to SDS-PAGE. The fusion proteins were excised from the gel and electroeluted in 10 mM NaHCO₃, 3 mM Na₂CO₃, pH 9.9, containing 0.06% SDS. The purified proteins, which appeared homogenous on SDS-PAGE, were used to immunize New Zealand White rabbits for antibody production (Cocalico Biologicals, Co., Reamstown, PA).

The specificities of anti-PRMT1 and anti-PRMT3 antisera were analyzed in immunoblotting experiments, using RAT1 cell extracts (7) and GST fusion proteins. Anti-PRMT1 antiserum recognized recombinant GST-PRMT1 but not GST-PRMT3-(90–528). Anti-PRMT3 antiserum recognized recombinant GST-PRMT3-(90–528) but not GST-PRMT1. The anti-PRMT1 antiserum recognized PRMT1 present in a RAT1 cell lysate as a 42-kDa band on SDS-PAGE. Immunoreactivity of the anti-PRMT1 antiserum with this 42-kDa antigen band was immunodepleted by GST-PRMT1 absorption but not by GST-PRMT3-(90–528) absorption. The anti-PRMT3 antiserum recognized a 60-kDa antigen in RAT1 cell extracts. Immunoreactivity was depleted from the anti-PRMT3 antiserum by absorption with GST-PRMT3-(90–528) fusion protein but not by absorption with GST-PRMT1 fusion protein.

Northern Analyses of RNA from Rat Organs and PC12 Cells—Total RNA from tissues was prepared using guanidinium thiocyanate phenol/chloroform (25). Total RNA from PC12 cells was isolated using lithium chloride (26). RNA was subjected to electrophoresis and Northern blotting as described previously (9).

Immunolocalization of PRMT1 and PRMT3 in RAT1 Cells—RAT1 cells in four-cell chamber slides were washed twice with phosphate-buffered saline solution (PBS, 1.5 mM KH₂PO₄, 8.1 mM Na₂HPO₄, 137 mM NaCl, 2.6 mM KCl, pH 7.4) and fixed with 2% paraformaldehyde (PFA) in PBS for 15 min, followed by a 15-min incubation in 2% PFA in PBS containing 0.1% Triton X-100. Alternatively, cells were fixed and

Rat PRMT3	1	MCSLAAGNGQ	GAELGPEPLE	LSDSGDDAGW	EDEDADAEP	QGRQHTF	<u>CLF</u>	<u>CDRLFRSAEE</u>
Human PRMT3	1	-----	-----DEPE	LSDSGDEAAW	EDE-DDADLP	HGKQQT	<u>CLF</u>	<u>CNRLFTSAEE</u>
Rat PRMT1		-----	-----	-----	-----	-----	-----	-----
Yeast Rmt1		-----	-----	-----	-----	-----	-----	-----
2A								
Rat PRMT3	61	<u>TFSSHCKLEH</u>	FNIDGMVHKH	GLEFYGYIKL	INFIRLKNPT	VEYMNSIYNP	VPWDKDEYLK	
Human PRMT3	44	<u>TFSSHCKSEH</u>	FNIDSMVHKH	GLEFYGYIKL	INFIRLKNPT	VEYMNSIYNP	VPWEKEEYLK	
Rat PRMT1		-----	-----	-----	-----	-----	-----	-----
Yeast Rmt1		-----	-----	-----	-----	-----	-----	-----
Rat PRMT3	121	PVLEDDLLQ	FDVEDLYEPV	SAPFTYPNGL	SENTSAVEKL	KLMEARALSA	EAALARARED	
Human PRMT3	104	PVLEDDLLQ	FDVEDLYEPV	SVFFSYNGL	SENTSVVEKL	KHMEARALSA	EAALARARED	
Rat PRMT1		-----	-----	-----	-----	-----	-----	-----
Yeast Rmt1		-----	-----	-----	-----	-----	-----	-----
Rat PRMT3	181	LQMKQFAQD	FVMNVDVRTC	-SSTTTIADL	QEDDGVYFS	SYGHYGIHEE	MLKDKVRTES	
Human PRMT3	164	LQMKQFAQD	FVMHTDVRTC	SSSTSVIADL	QEDDGVYFS	SYGHYGIHEE	MLKDKIRTES	
Rat PRMT1	1	--MAAAEAA	CIMEVSCGQA	ESSEKPAED	MTSKD-YYFD	SYAHFGIHEE	MLKDEVRTL	
Yeast Rmt1	1	-----	----MSKTAV	KDSATEKTKL	SESEQ-HYFN	SYDHYGIHEE	MLQDVTRTL	
post I								
Rat PRMT3	240	YRDFIYNPH	IFKDKVVLVDV	GCGTGILSMF	AAKAGAKKVI	AVDQSEILYQ	AMDIIRLNKL	
Human PRMT3	224	YRDFIYNPH	IFKDKVVLVDV	GCGTGILSMF	AAKAGAKKVL	GVDQSEILYQ	AMDIIRLNKL	
Rat PRMT1	57	YRNSMFHNRH	LFKDKVVLVDV	GSGTGILCMF	AAKAGARKVI	GIECSSISDY	AVKIVKANKL	
Yeast Rmt1	46	YRNAIQNKD	LFKDKIVLVDV	GCGTGILSMF	AAKHGAKHVI	GVDMSIIEM	AKELVELNGF	
II								
Rat PRMT3	300	EDTIVLIKGR	IEEVSLPVEK	VDVISEWMG	YFLLFESMLD	SVLYAKSKYL	AKGGSVYPDI	
Human PRMT3	284	EDTITLIKGR	IEEVHLPVEK	VDVISEWMG	YFLLFESMLD	SVLYAKNKYL	AKGGSVYPDI	
Rat PRMT1	117	DHVVTIIRGR	VEEVSLPVEK	VDVISEWMG	YCLFYESMLN	TVLHARDKWL	APDGLIFPDR	
Yeast Rmt1	106	SDKITLLRGR	LEDVHLPEK	VDVISEWMG	YFLFYESMMD	TVLYARDHYL	VEGGLIFPDR	
III								
Rat PRMT3	360	CTISLVAUSD	VSKHADRIAF	WDDVYGFNMS	CMKKAVIPEA	VVEVDHKTLL	ISDPCDIKHI	
Human PRMT3	344	CTISLVAUSD	VNKHADRIAF	WDDVYGFNMS	CMKKAVIPEA	VVEVLDPKTL	ISEPCGIKHI	
Rat PRMT1	177	ATLYVTAIED	RQYKDYKIHW	WENVYGFNMS	CIKDVAIPEA	LVDVVDPKTL	VTNACLIKEV	
Yeast Rmt1	166	CSIHLAGLED	SQYKDEKLN	WQDVYGFNMS	PFVPLVLHEP	IVDTVERNNV	NTTSDKLIEF	
Rat PRMT3	420	DCHTTSISDL	EFSSDFTLRT	TKTAMCTAVA	GYFDIYFEKN	-CHNRVVFST	GPQSTKTHWK	
Human PRMT3	404	DCHTTSISDL	EFSSDFTLKI	TRTSMCTAIA	GYFDIYFEKN	-CHNRVVFST	GPQSTKTHWK	
Rat PRMT1	237	DIYTVKVEDL	TFTSPFCLQV	KRNDYVHALV	AYFNIEFTTR	-CHKRTGFST	SPESPYTHWK	
Yeast Rmt1	226	DLNTVKISDL	AFKSNFKLTA	KRQDMINGIV	TFWDIVFPAP	KGKRPVEFST	GPSPYTHWK	
Rat PRMT3	479	QTIFLLEKPF	PVKAGEALKG	KITVHKNNKD	PRSLIVTLTL	NSSTQTYSLQ	-----	
Human PRMT3	443	QTVFLEKPF	SVKAGEALKG	KVTVHKNNKD	PRSLTVTLTL	NNSTQTYGLQ	-----	
Rat PRMT1	295	QTVFYMEDYL	TVKTGEEIFG	TIGMRPNAKN	NRDLDFITDL	DFKGQLCELS	CST-----DY	
Yeast Rmt1	286	QTIFYFPDDL	DAETGDTIEG	ELVCSNPEKN	NRDLNIKISY	KFESNGIDGN	SRSRKNESGY	
Rat PRMT3		---	---	---	---	---	---	
Human PRMT3		---	---	---	---	---	---	
Rat PRMT1	351	RMR						
Yeast Rmt1	346	LMH						

FIG. 1. Amino acid sequences of rat and human PRMT3, rat PRMT1, and yeast Rmt1. The deduced amino acid sequence of the rat PRMT3 protein was obtained from the sequence of the PRMT3 cDNA clone isolated by screening a rat FAO hepatoma cell cDNA library with the 2A clone. The arrow indicates the start of the 2A clone. The deduced sequence of the human PRMT3 protein was obtained from the sequence, determined in our laboratory, of the cDNA (GenBank™ clone AA307385). Rat PRMT1 and yeast Rmt1 sequences are reported previously (7, 17). Amino acid sequence alignment was performed using Clustal W (50). Conserved amino acids are indicated by * for identical residues and by · for similar residues. The first boxed segment (Cys⁴⁸–His⁶⁹) of PRMT3 represents a putative C2H2 zinc finger motif. The second box (Tyr⁹⁵–Phe¹⁰³) includes a tyrosine phosphorylation consensus sequence. The signature methyltransferase regions I, post I, II, and III are indicated, and are in boldface and are underlined.

permeabilized with 100% methanol for 15 min at -20°C . The PFA-fixed cells were washed twice with PBS containing 0.1 M glycine and 0.1% Triton X-100 and then blocked with goat serum (1:20 dilution in PBS containing 0.2% Tween 20). For methanol fixation, cells were blocked with goat serum. After blocking, cells were incubated for 2 h in primary antibody dilutions (1:350 for anti-PRMT1, 1:200 for anti-PRMT3) in PBS containing 0.2% Tween 20 and goat serum diluted 1:50. Then cells were washed three times with PBS containing 0.2% Tween 20 and incubated in the secondary antibody solutions (1:100 diluted fluorescein isothiocyanate-labeled anti-rabbit IgG in PBS containing 0.2% Tween 20 and goat serum diluted 1:50). After 1 h, cells were washed four times in PBS containing 0.2% Tween 20 and mounted in fluoromount (Southern Biotechnology Associates, Birmingham, AL) and examined with a Zeiss fluorescence microscope.

To immunodeplete PRMT1 and PRMT3 antisera, GST-PRMT1 and GST-PRMT3 (90–528) were isolated with glutathione-Sepharose beads from 350-ml *E. coli* cultures. The beads with the absorbed GST proteins were resuspended in 100 μl of PBS, and 10 μl of anti-PRMT1 or

anti-PRMT3 antisera was added. The mixtures were incubated at 4°C for 2 h. The supernatant fractions were recovered by passing the mixtures through 0.2- μm filters. Western blotting experiments demonstrated the specificity of immunodepletion.

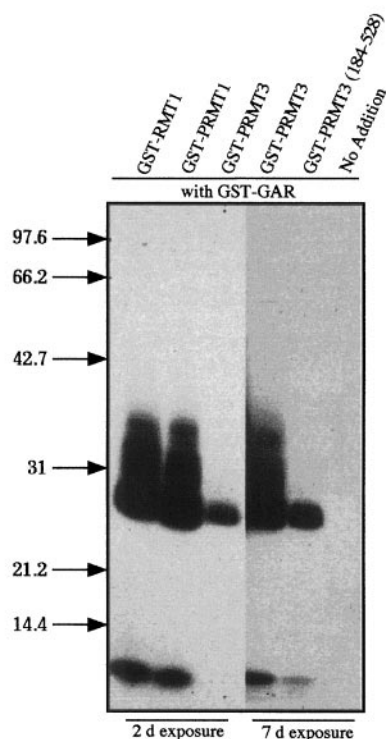
Preparation of a Methylation-deficient Yeast Extract—Hypomethylated yeast extract from *S. cerevisiae* rmt1 strain JDG9100-2, lacking the major yeast type I protein arginine *N*-methyltransferase, Rmt1, was prepared as described previously (17).

Analysis of Methylated Polypeptides by SDS-PAGE and Fluorography—Specific conditions for the methylation reactions are described in the relevant figure legends. Reactions were terminated by the addition of an equal volume of SDS-containing sample buffer (30) and heating to 100°C for 5 min. Samples were then subjected to SDS-PAGE (27), Coomassie Brilliant Blue R staining, and fluorography as described previously (7).

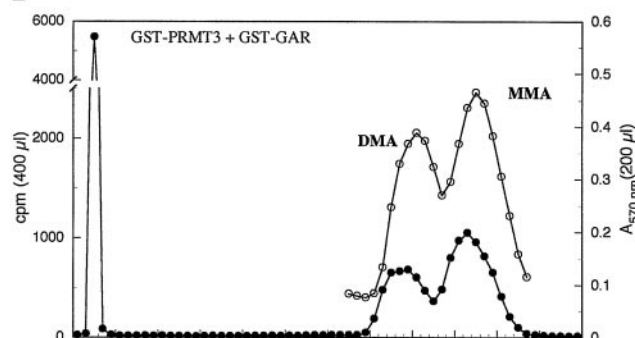
Western blot Analysis—Protein samples were subjected to SDS-PAGE and immunoblotting analysis as described previously (28).

RESULTS

A



B



C

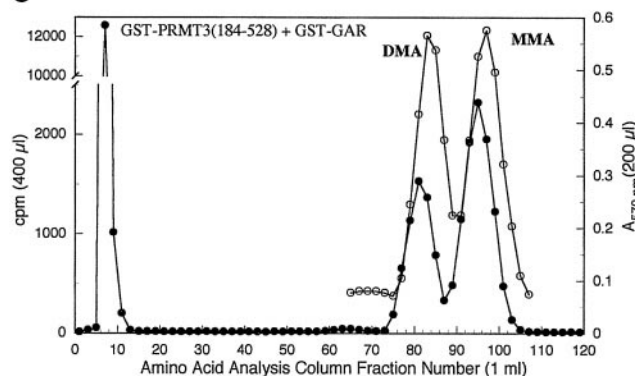


FIG. 2. PRMT3 encodes a type I protein arginine *N*-methyltransferase. *In vitro* methylation reactions were performed with GST fusion proteins of the putative rat PRMT3 protein arginine *N*-methyltransferase and the known protein arginine *N*-methyltransferases, yeast Rmt1 and rat PRMT1. [^3H]AdoMet was used as a methyl donor and GST-GAR as a methyl-accepting substrate. Reactions contained GST-GAR (0.7 μg), 0.75 μM (3.3 μCi) [^3H]AdoMet (NEN Life Science Products; 80 Ci/mmol, from a stock in 10 mM sulfuric acid solution: ethanol (9:1) at a concentration of 0.55 mCi/ml), and either 0.84 μg of GST-Rmt1, 0.77 μg of GST-PRMT1 protein, 2.07 μg of GST-PRMT3, or 10.6 μg of GST-PRMT3(184–528) in a final volume of 55 μl with a buffer of 25 mM Tris-HCl, 1 mM sodium EDTA, 1 mM sodium EGTA at

Identification of Proteins That Interact with PRMT1—A yeast strain expressing the bait fusion protein LexA-PRMT1 was transformed with a cDNA library in which the GAL4 activation domain (GAL4AD) is fused to cDNAs from rat liver-derived FAO cells (7). Twenty-five positive cDNA plasmids were isolated and separated into four groups by PCR analysis, restriction digest analysis, and sequencing; PRMT1 (15 isolates), 2A (8 isolates), 4A (1 isolate), and 7A (1 isolate). Sequence analysis indicated that 4A is homologous to a yeast alcohol dehydrogenase, and 7A encodes a homologue of a glycosylphosphatidylinositol-anchored extracellular membrane protein involved in transcytosis in Madin-Darby canine kidney cells.

The Open Reading Frame Encoded by the cDNA from Which the 2A Clone Is Derived Shows Significant Homology to PRMT1 and Is Termed “PRMT3”—A BLAST search (22) of protein sequence data bases indicated that the predicted open reading frame encoded by the 2A clone is closely related to mammalian PRMT1 (7, 15) and yeast Rmt1 (17, 18) (Fig. 1). Northern analysis of RNA from RAT1 fibroblast cells suggested that 2A is a truncated portion of a 2.4-kilobase pair transcript (data not shown). By using clone 2A as a probe, a longer cDNA was cloned from a RAT1 cDNA library. This cDNA encodes an open reading frame of 528 amino acids.

Comparison of PRMT1 and the “2A” putative methyltransferase domain (amino acids 195 to 528) reveals 46% identity and 67% similarity at the amino acid level. The designation “PRMT2” already exists as a GenBankTM entry (U80213) for a cDNA that shares some sequence identity with PRMT1. This human sequence was named HRMT1L1 (19). We have, therefore, named the polypeptide encoded by the full-length 2A transcript “protein arginine methyltransferase 3” or PRMT3. The high degree of identity among the Rmt1, PRMT1, and PRMT3 sequences suggests that PRMT3 might also be a protein arginine *N*-methyltransferase.

pH 7.5. Samples were incubated for 40 min at 37 °C. A, reaction mixtures were then subjected to SDS-PAGE and fluorography. Two exposure times (2 and 7 days) are shown; the lane in which GST-PRMT3 was tested is shown at both exposure times. The arrows indicate the position of the molecular size standards. B, the product from a reaction mixture containing GST-GAR and 2.07 μg of the GST-PRMT3 was analyzed for the presence of methylated arginine derivatives. Proteins were precipitated in 6 $\times$ 50-mm borosilicate vials with an equal volume (55 μl) of 25% (w/v) trichloroacetic acid in the presence of 30 μg of bovine serum albumin. The resulting pellet was washed with 2 volumes of acetone at –20 °C. The air-dried protein pellets were then hydrolyzed *in vacuo* at 110 °C for 20 h in a vapor-phase Waters Pico-Tag apparatus using 200 μl of 12 N HCl. The hydrolyzed material was resuspended in 50 μl of a diluted citrate buffer (0.1 M Na⁺, containing 1% (v/v) thioglycol and 0.05% phenol at pH 2.2) and analyzed as described previously (17). Prior to loading on the column, the sample was diluted with an equal volume of the concentrated citrate loading buffer (0.2 M Na⁺, containing 2% (v/v) thioglycol and 0.1% phenol at pH 2.2) and nonradiolabeled standards of *N*^G-monomethylarginine (MMA) and *N*^G,*N*^G-dimethylarginine (DMA) were added (1.0 μmol each, Sigma). The column (0.9 cm in diameter $\times$ 11 cm in length) was equilibrated and eluted isocratically at 1 ml/min in citrate buffer (0.35 M Na⁺, pH 5.27) at 55 °C. One-minute fractions were collected and ^3H radioactivity was detected by scintillation counting a 400- μl aliquot of the fractions (filled circles). The elution positions of the methylated arginine standards (open circles) were determined by analyzing a 200- μl aliquot of the fractions using the ninhydrin method (1). The column was regenerated between runs by washing with 0.2 N NaOH for 20 min and then re-equilibrating in the elution buffer. C, a reaction mixture containing GST-GAR and 10.6 μg of GST-PRMT3(184–528) was analyzed as in B. In a control experiment, we determined that incubation of GST-GAR with [^3H]AdoMet alone gives no ^3H -methylated arginine derivatives (data not shown). The large peak of radioactivity near fraction 10 may represent unreacted [^3H]AdoMet or its degradation products that were not completely removed by the acid precipitation step.

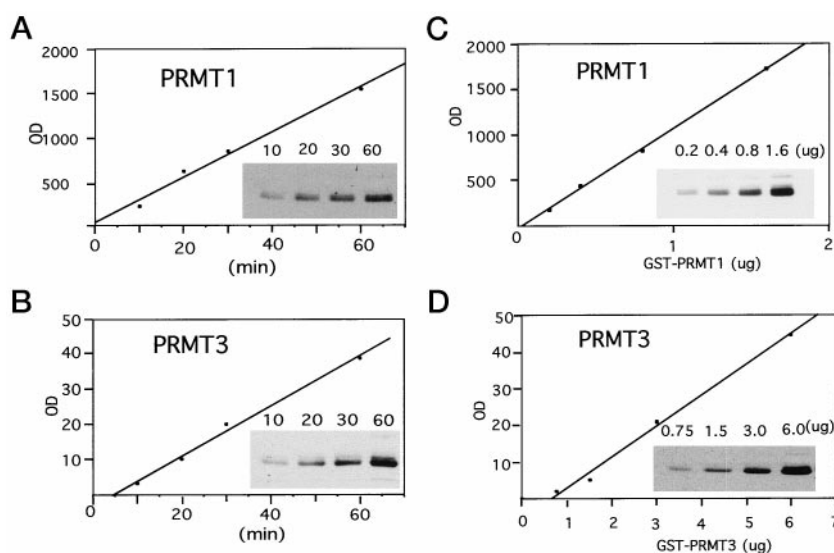


FIG. 3. Comparison of specific activities of recombinant PRMT1 and PRMT3, using GST-GAR as substrate and [3 H]AdoMet as methyl donor. A and B, 240- μ l reaction mixtures contained 48 μ g (6 μ M) of GST-GAR, 17.6 μ Ci of [3 H]AdoMet (0.9 μ M), and either 3.2 μ g of GST-PRMT1 or 12 μ g of GST-PRMT3 in 25 mM Tris-HCl, pH 7.5. Methylation reactions were performed at 37 $^{\circ}$ C. At each time point (10, 20, 30, and 60 min), 60- μ l aliquots (containing 0.8 μ g of GST-PRMT1 or 3 μ g of GST-PRMT3) were withdrawn; the reactions were stopped by addition of SDS-PAGE sample buffer and heating at 95 $^{\circ}$ C for 4 min, and the samples were subjected to SDS-PAGE. The GST-PRMT1 and GST-PRMT3 samples were subjected to fluorography for 15 min and 15 h, respectively. Methylation of GST-GAR was quantitated by densitometric scanning. Methylated GST-GAR is expressed as the densitometry units produced in 1 min of fluorography. The specific activity of GST-PRMT1 is 31 densitometer units/min/ μ g of protein; the specific activity of GST-PRMT3 is 0.24 densitometer units/min/ μ g of protein. C and D show the linear relationship between GST-PRMT1 and GST-PRMT3 concentrations and GST-GAR methylation. Reaction mixtures (60 μ l) contained 0.9 μ M [3 H]AdoMet, 6 μ M GST-GAR, and the amounts of GST-PRMT1 and GST-PRMT3 shown in the figures. Reactions were run for 30 min and analyzed as described for the data in A and B. The GST-PRMT1 and GST-PRMT3 samples were subjected to fluorography for 30 min and 15 h, respectively.

The amino-terminal 194-amino acid fragment of PRMT3, which does not resemble any known protein, is rich in acidic amino acid residues. It is, therefore, called the N-terminal acidic amino acid-rich (NAR) domain of PRMT3. The PRMT3 NAR domain contains two functional motifs, a putative C2H2 zinc finger motif (Cys⁴⁸–His⁶⁹) and a tyrosine phosphorylation consensus sequence (Tyr⁸⁵–Phe⁹³) similar to the STAT1 tyrosine phosphorylation site (29).

A BLAST search identified two human EST sequences (AA307385 and H38113) closely related to rat PRMT3 cDNA. Sequencing confirmed that AA307385 is the human PRMT3 orthologue but is missing the amino-terminal 10 amino acid residues (Fig. 1). At the amino acid level, human PRMT3 is 90% identical to rat PRMT3. Human PRMT3 contains the carboxyl-terminal putative methyltransferase domain and the amino-terminal NAR domain.

GST-PRMT1 and GST-PRMT3 Fusion Proteins Methylate GST-GAR—GST-GAR is a recombinant protein arginine methyltransferase substrate containing the first 148 amino acids of the human fibrillarin protein, fused in-frame to GST. The amino-terminal region of fibrillarin contains 14 arginine residues, the majority of which are present in “RGG” consensus methylation sites (30–32). GST-GAR is not methylated in *E. coli*, which lacks protein arginine methyltransferase activity (4).

GST fusions of the asymmetrically methylating (type I) protein arginine N-methyltransferases from *S. cerevisiae* (Rmt1) and rat (PRMT1) catalyze the methylation of GST-GAR (Fig. 2A). Recombinant GST-PRMT3 also catalyzes methylation of GST-GAR. Both monomethylated arginine and asymmetrically dimethylated arginine are present after acid hydrolysis of the GST-PRMT3 reaction product mixture (Fig. 2B). A similar result is observed when GST-Rmt1 and GST-PRMT1 methylation products are analyzed (7, 17). The 3 H-labeled amino acids elute just ahead of unlabeled standards, due to their increased molecular weight and slightly altered pI values (33, 34). Although enzymatically active, it is clear that GST-PRMT3 is much less active than is GST-PRMT1 when GST-GAR is used as sub-

strate (Fig. 2A). By examining the reactions as a function of time and enzyme concentrations (Fig. 3), we determined that the specific activity of GST-PRMT3 for the GST-GAR substrate is 0.8% that observed for GST-PRMT1.

An amino-terminal truncated version of PRMT3, GST-PRMT3-(184–528), was used to determine whether the PRMT3 amino-terminal extension, not present in Rmt1 or PRMT1, may play a regulatory role in PRMT3 enzymatic activity. The ability of GST-PRMT3-(184–528) to methylate GST-GAR is reduced when compared with the activity of the GST-PRMT3 (Fig. 2A). GST-PRMT3-(184–528) has approximately 3-fold lower specific activity relative to GST-PRMT3, based on quantitation of the radioactive methylarginine peak areas in Fig. 2, B and C, when normalized for the amounts of recombinant enzyme present.

PRMT1 and PRMT3 Associate in Yeast Two-hybrid Interaction Analysis—The recovery of PRMT1 and PRMT3 cDNAs from the yeast two-hybrid screening experiment, using PRMT1 as bait, suggested that PRMT1 forms both homo-oligomers with itself and hetero-oligomers with PRMT3. To test whether PRMT3 can form homo-oligomers, two bait plasmids, pBTM117-PRMT3-(90–528) and pBTM117-PRMT3-(214–528), in which regions of PRMT3 were fused to the LexA DNA binding domain, were constructed. PRMT3-(90–528) contains a transcription activation domain (Fig. 4C) and cannot, therefore, be used as bait in a yeast two-hybrid analysis. The combination of pBTM117-PRMT3-(214–528), which does not by itself activate transcription (Fig. 4C), and pGAD425-PRMT3-(90–528) (a fusion protein of the GAL4 activation domain and PRMT3-(90–528)) can activate β -galactosidase expression (Fig. 4A). Therefore, PRMT3 has the potential to form homo-oligomers.

An amino-terminal truncation of PRMT3, PRMT3-(90–528), was initially identified by yeast two-hybrid analysis as a protein interacting with PRMT1. To determine whether full-length PRMT3 can interact with PRMT1, pPC86-PRMT3-(90–528) and pPC86-PRMT3 (in which the respective PRMT3 sequences are fused to the GAL4 activation domain) were each transformed into yeast in combination with pLexA(L)-PRMT1.

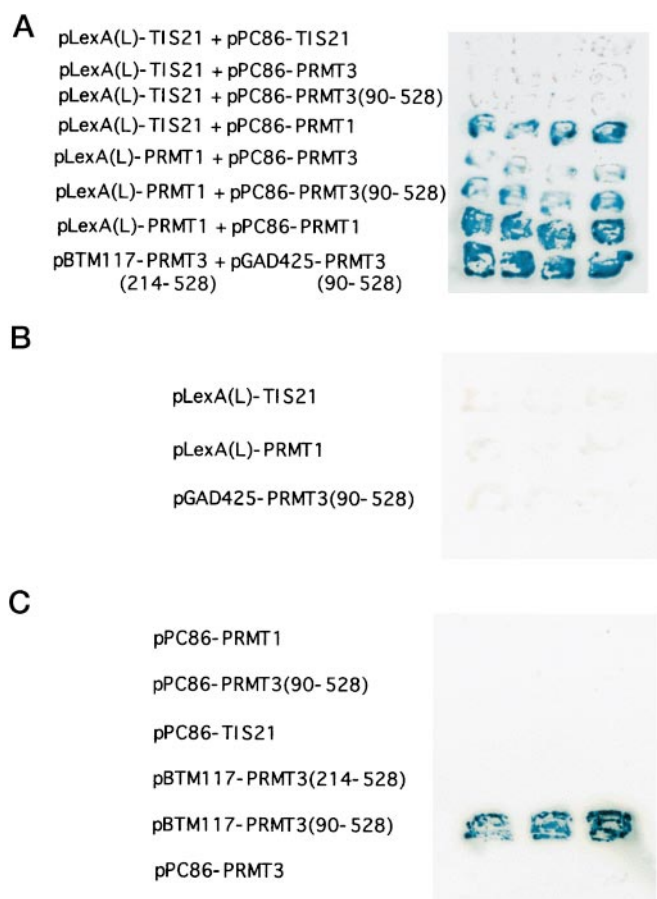


FIG. 4. Yeast two-hybrid analyses of interactions between PRMT1, PRMT3, and TIS21. pLexA(L)-TIS21 and pLexA(L)-PRMT1 are two-hybrid bait plasmids that express TIS21 and PRMT1 fusion proteins linked to the LexA transcription factor DNA binding domain. PBMT117-PRMT3-(90–528) and pBTM117-PRMT3-(214–528) are bait plasmids that express fusion proteins between the LexA transcription factor DNA binding domain and amino-terminal truncations of PRMT3. The pPC86 and pGAD425 prey plasmids express fusions of the GAL4 transcription factor activation domain with the indicated regions of PRMT1 or PRMT3. The choice of plasmids used in the various two-hybrid analyses in A was determined by the genetic requirements necessary for the stable maintenance of the bait and prey plasmids. Yeast strain L40 was transformed with plasmids encoding the LexA DNA binding domain fusion proteins or the GAL4 transcription activation domain fusion proteins alone (B and C), or in different combinations of bait and prey plasmids (A). The transformants were patched onto appropriate media: SC-ura⁻lys⁻trp⁻ (C), SC-ura⁻lys⁻leu⁻ (B), and SC-ura⁻lys⁻leu⁻trp⁻ (A), and assayed for β -galactosidase activity using a yeast colony filter assay. Blue color develops when the fusion proteins in yeast can activate the expression of the *lacZ* gene.

Both combinations activate LexA-responsive *lacZ* (Fig. 4A) and *HIS3* gene expression. Therefore the full-length PRMT3 protein can interact with PRMT1 in yeast two-hybrid analysis.

PRMT1 was originally isolated from a yeast two-hybrid screen with the TIS21 immediate-early gene product as the bait (7). To determine whether TIS21 can also interact with PRMT3, the bait plasmid pLexA(L)-TIS21 was transformed into yeast in combination with the prey plasmids pPC86-PRMT3 or pPC86-PRMT3(90–528). The interaction between TIS21 and PRMT1 was used as a positive control and the lack of interaction between TIS21 and TIS21 as a negative control (7). TIS21 interacts only with PRMT1 and does not interact with PRMT3 or its amino-terminal truncated derivative (Fig. 4A).

Gel Filtration Chromatography of RAT1 Cell Extracts Fails to Demonstrate Interactions between PRMT1 and PRMT3—To investigate whether PRMT1 and PRMT3 directly associate in

mammalian cells and whether PRMT3 can form homo-oligomers *in vivo*, a protein extract from RAT1 embryo fibroblast cells was fractionated on a Sephacryl S300HR gel filtration column. Elution of PRMT proteins was determined by Western analysis and by enzyme activity assay.

PRMT3 antigen elutes from the Sephacryl column at the approximate position of a 37-kDa globular polypeptide (Fig. 5A), somewhat smaller than its calculated molecular mass of 59.4 kDa (Fig. 1) and its estimated size of 60 kDa in SDS-PAGE immunoblotting experiments with RAT1 extract (data not shown). However, PRMT3 antigen eluted as a 64-kDa species when using a Superdex S200 (Amersham Pharmacia Biotech) column (data not shown). Endogenous PRMT3 is, therefore, probably present in RAT1 cells as a monomer. PRMT1, with a predicted molecular mass of 40.5 kDa (7), elutes in a broad peak ranging between 200 and 440 kDa (Fig. 5). PRMT1 and PRMT3 do not, in RAT1 cells, form hetero-oligomers that can survive cell disruption and gel filtration.

GST-GAR methylation could only be detected where PRMT1 elutes (Fig. 5, A and C). A lower level of PRMT3 protein and a significantly lower specific activity of the PRMT3 enzyme for GST-GAR probably account for the lack of detectable enzyme activity eluting with PRMT3.

PRMT1 and PRMT3 Reside in Distinct Subcellular Compartments in RAT1 Cells—To localize endogenous PRMT1, RAT1 cells were cultured in chamber slides and stained with anti-PRMT1 or pre-immune serum after fixation and permeabilization by PFA and Triton X-100 (Fig. 6). PRMT1-specific staining appears predominantly in the nucleus, although the cell cytoplasm is faintly stained. To confirm that the staining is specific for PRMT1, anti-PRMT1 antiserum was immunodepleted by GST-PRMT1 or GST-PRMT3. PRMT1 reactivity was depleted by GST-PRMT1 but not by GST-PRMT3(90–528) (data not shown). To confirm this subcellular localization result, we also performed immunofluorescence studies with RAT1 cells fixed and permeabilized by methanol fixation at -20°C . These results also demonstrated that PRMT1 is found predominantly nuclear, with some PRMT1 antigen present in the cytoplasm (data not shown).

PRMT3 was immunolocalized primarily to the cytoplasm with anti-PRMT3 antiserum, using either PFA/Triton X-100 fixation (Fig. 7) or methanol fixation (data not shown). PRMT3 staining could be depleted by GST-PRMT3(90–528) but not by GST-PRMT1 (data not shown). In summary, PRMT1 is predominantly nuclear, whereas PRMT3 is predominantly cytoplasmic.

Tissue Distribution of PRMT1 and PRMT3—We compared the expression of PRMT1 and PRMT3 mRNAs in common preparations of rat tissues. PRMT3 distribution closely resembles PRMT1 distribution (Fig. 8). Small variations in PRMT1 and PRMT3 expression are observable, however. For example, PRMT1:PRMT3 ratios are reversed for heart and small intestine.

PRMT3, Like PRMT1, Is Constitutively Expressed in Cells—We performed Northern analyses with total RNA from rat PC12 pheochromocytoma cells to determine whether PRMT3 message is constitutively expressed or is induced by ligand stimulation. Like PRMT1, PRMT3 mRNA is constitutively expressed in PC12 cells (Fig. 9). Forskolin induction of the *c-fos* gene illustrates induction of a primary response gene. The PRMT1 and PRMT3 probes each detect both messages; the PRMT1 probe shows slight cross-hybridization with PRMT3 message and vice versa (Fig. 8). The PRMT3 probe also identifies a third, higher molecular weight message, whose identity is unknown.

GST-PRMT1 and GST-PRMT3 Methylate Distinct Substrates in Hypomethylated Yeast Cell Extracts—A soluble protein extract from yeast lacking *RM1*, the major *S. cerevisiae*

FIG. 5. PRMT1 and PRMT3 proteins present in RAT1 cell extract are separated by gel filtration chromatography. RAT1 cell-soluble extract (2.2 ml, 8.8 mg of protein) was loaded onto a Sephacryl S300HR (Amersham Pharmacia Biotech) gel filtration column (63-cm column height $\times$ 2.6-cm inner diameter, 334-ml bed volume). The column was equilibrated and eluted in 25 mM Tris-HCl, 1 mM sodium EDTA, and 1 mM sodium EGTA, pH 7.5, at 4 °C, at a flow rate of 22 ml/h, and 3-ml fractions were collected. The main section of A shows that the total protein elution profile as absorbance at 280 nm (filled circles). Protein arginine *N*-methyltransferase activity was determined by incubating 21 μ l of every other column fraction with 0.7 μ g of GST-GAR and 0.92 μ M (2.2 μ Ci) [3 H]AdoMet in a final volume of 30 μ l at 37 °C for 30 min. The reaction mixtures were then subjected to SDS-PAGE and fluorographic analysis (C), and GST-GAR methylation was quantitated by densitometry (A, open triangles). Western analysis of 40- μ l samples (B) was used to determine the elution positions of PRMT1 (A, closed squares) and PRMT3 (A, open squares). The relative concentrations of each protein were determined by densitometry. Arrows (A) indicate the column fraction containing the peak activity of each size marker enzyme, assayed as described in the Worthington enzyme manual (ferritin was quantitated by absorbance at 400 nm). The inset in A shows the peak elution positions of the protein standards, plotted against the logarithm of their molecular mass. The peaks of PRMT1 activity and PRMT3 protein are in **bold type**. Their native molecular masses (317 and 37 kDa, respectively) were approximated using the equation of the best curve fit. B shows Western blots of column fractions, using anti-PRMT1 (1:10,000 dilution) and anti-PRMT3 (1:5,000 dilution) antisera. C shows the protein arginine *N*-methyltransferase activity of column fractions, using GST-GAR as substrate.

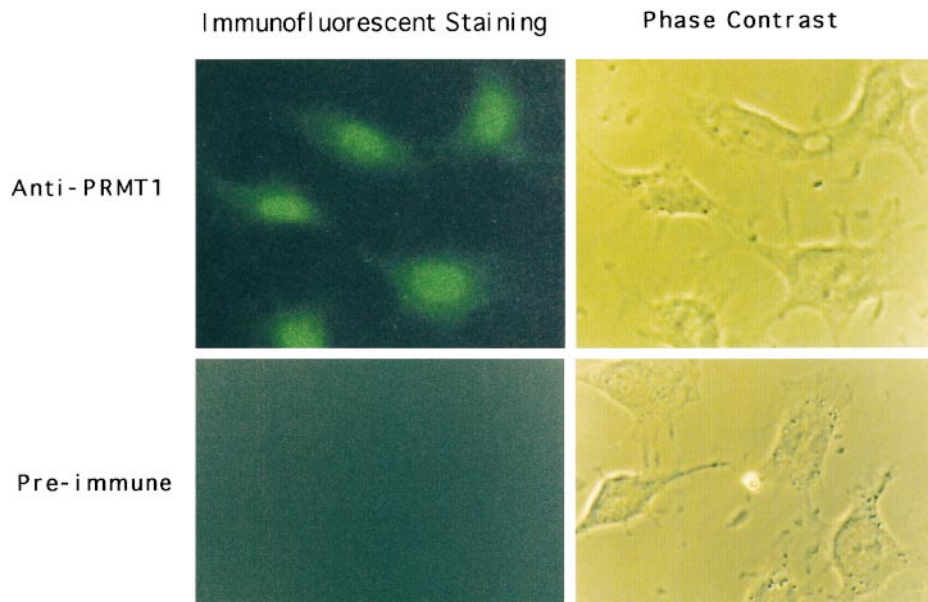
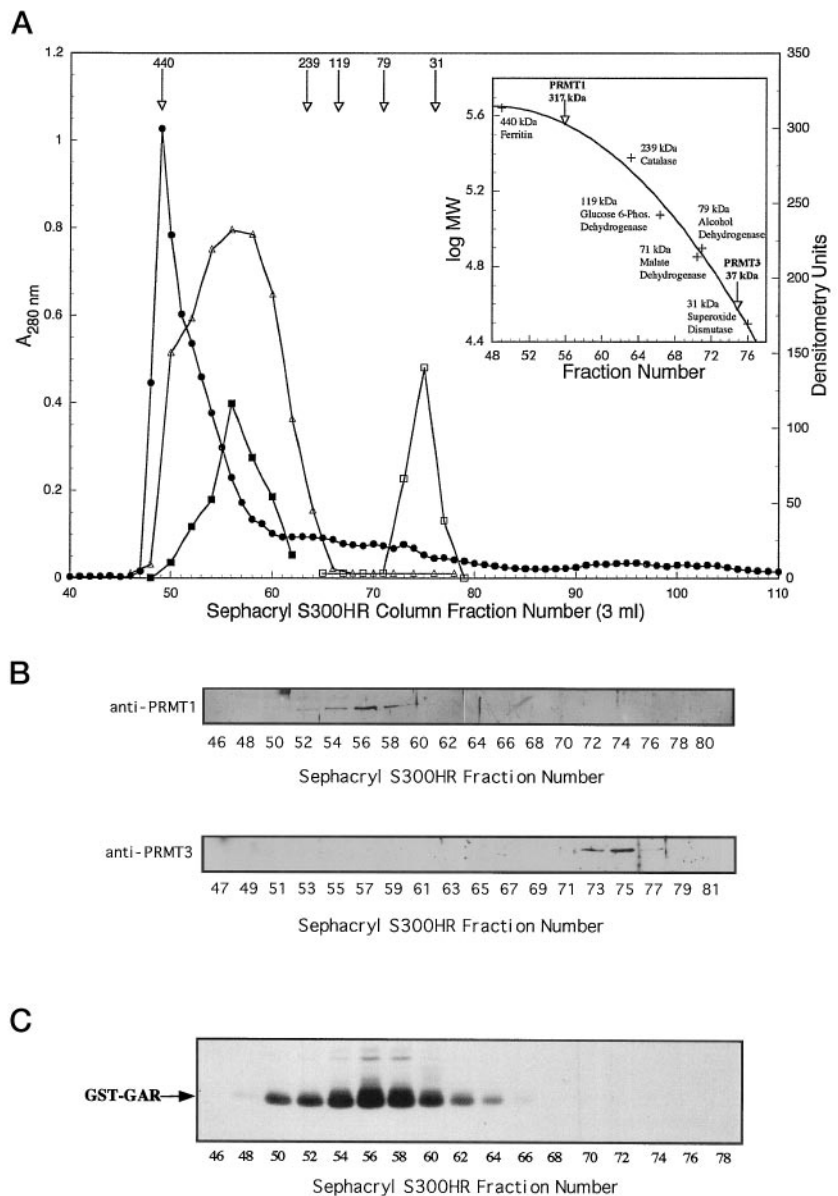


FIG. 6. Immunofluorescence localization of PRMT1 in RAT1 cells. RAT1 cells were fixed with 2% PFA and then permeabilized with 0.1% Triton X-100 in 2% PFA. The fixed and permeabilized cells were stained and photographed as described under "Experimental Procedures" using anti-PRMT1 and pre-immune serum at a 1:350 dilution.

FIG. 7. **Immunofluorescence localization of PRMT3 in RAT1 cells.** RAT1 cells were fixed with 2% PFA and then permeabilized with 0.1% Triton X-100 in 2% PFA. The fixed and permeabilized cells were stained and photographed as described under "Experimental Procedures," using anti-PRMT3 or pre-immune serum at a 1:200 dilution.

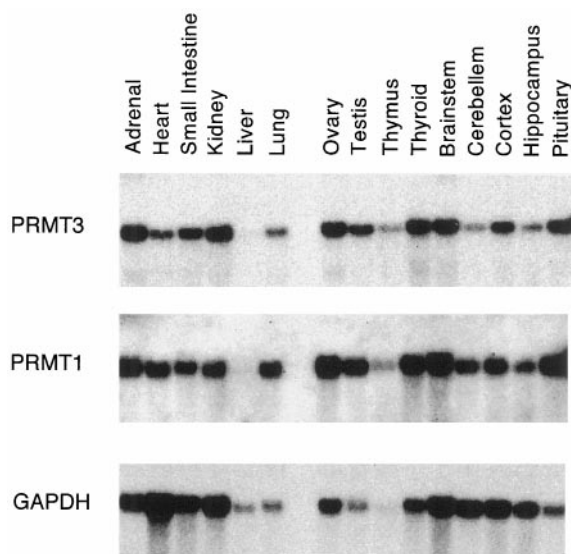
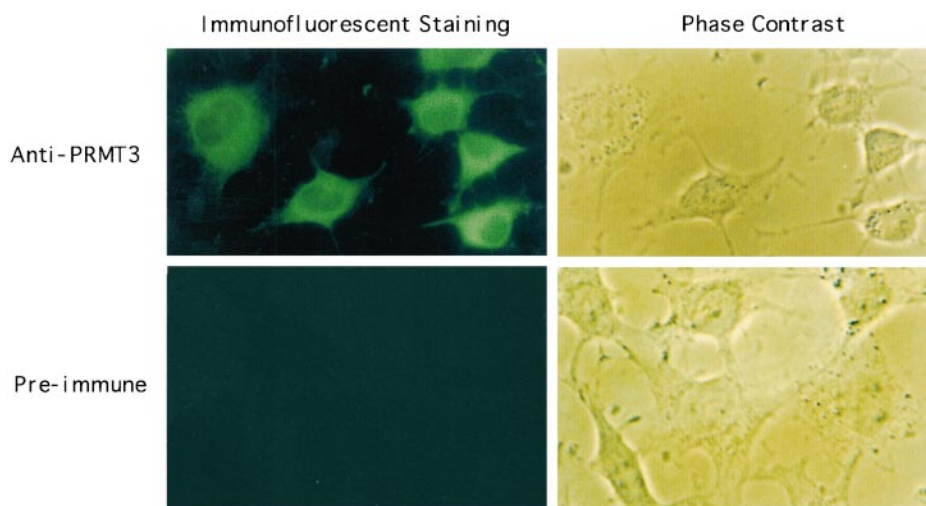


FIG. 8. **Expression of PRMT1 and PRMT3 in rat tissues.** Ten μ g of total RNA from each tissue was subjected to electrophoresis and analyzed for PRMT3 and PRMT1 mRNAs by Northern analysis. Hybridization with a GAPDH probe provides a standard for a constitutively expressed message.

protein arginine *N*-methyltransferase (7), was utilized as a collection of hypomethylated substrates to assess the enzymatic specificity of PRMT1 and PRMT3. When *rmt1* extract is incubated with GST-Rmt1 and radiolabeled [3 H]AdoMet, polypeptide species of 55, 38, 35, and 33 kDa are labeled (Fig. 10A). In similar reactions with GST-PRMT1, polypeptides of 55, 38, 35, and 26 kDa are methylated, with the predominant species at 55 and 26 kDa. In contrast to the several substrates methylated when GST-Rmt1 and GST-PRMT1 are incubated with the *rmt1* extract (Fig. 10A), only one 29-kDa polypeptide in the *rmt1* extract is methylated by GST-PRMT3 (Fig. 10B). In control reactions containing [3 H]AdoMet with *rmt1* extract alone or with GST-PRMT3 enzymes alone, no protein methylation is observed (Fig. 10B).

TIS21 associates with PRMT1 *in vitro* and qualitatively and quantitatively modulates PRMT1 activity (7). Methylation of a 26-kDa GST-PRMT1 substrate present in the *rmt1* yeast extract is suppressed when GST-TIS21 is included in the reaction (Fig. 10, compare lanes 2 and 3). In contrast, GST-TIS21 has no effect on GST-PRMT3 enzymatic activity (data not shown).

We considered the hypothesis that the amino-terminal NAR extension of PRMT3 may regulate PRMT3 enzyme activity

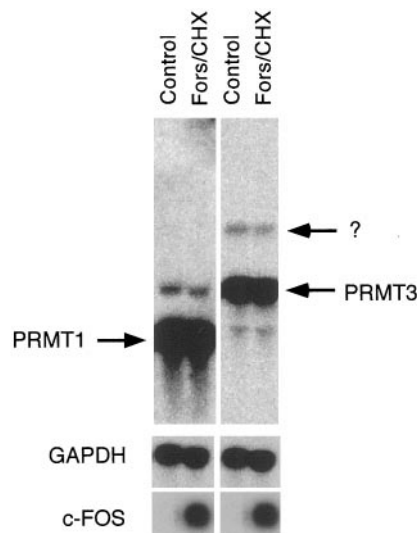


FIG. 9. **PRMT3 is constitutively expressed in cultured cells.** Rat PC12 pheochromocytoma cells were serum-starved in serum-free RPMI-1640 medium for 14 h. Half the plates were then stimulated with 50 μ M forskolin (*Fors*) in RPMI 1640 containing 10% fetal bovine serum and 10 μ g/ml cycloheximide (*CHX*) for 4 h. Total RNA (10 μ g) from each sample was subjected to electrophoresis and analyzed by Northern blot for PRMT1 and PRMT3 mRNAs. GAPDH was used to normalize the loading. The *c-fos* mRNA was used to show induction of immediate-early gene expression by forskolin and serum. Cycloheximide was present to stabilize the mRNAs of immediate-early/primary response genes (52). The arrow indicates an unidentified RNA present that hybridizes weakly with the PRMT3 probe.

and/or substrate specificity. To begin to determine the role of the amino-terminal domain of PRMT3, we used a GST-PRMT3-(184–528) construct that lacks the NAR domain but includes all of the conserved regions shared among methyltransferases. GST-PRMT3-(184–528) cannot methylate the 29-kDa polypeptide substrate in the yeast extract that is methylated by GST-PRMT3 (Fig. 10B; compare lanes 3 and 4). In contrast, GST-PRMT3-(184–528) fusion protein can methylate the GST-GAR substrate (Fig. 2, A and C).

DISCUSSION

Mammalian Cells Contain Multiple Type I Protein Arginine *N*-Methyltransferases—Type I protein arginine *N*-methyltransferases methylate protein arginine residues to form N^G, N^G -dimethylarginine residues (asymmetric). In contrast, type II protein arginine methyltransferases catalyze the formation of N^G, N^G -dimethylarginine residues (symmetric) (1–3). Type II protein arginine *N*-methyltransferase activity has not been

FIG. 10. GST-PRMT3 methylates substrates distinct from those of GST-Rmt1 or GST-PRMT1 in a hypomethylated *rmt1* yeast extract. A, soluble extract (190 μ g of protein) from the *rmt1* strain of *S. cerevisiae* was incubated with 0.75 μ M (3.3 μ Ci) [3 H]AdoMet and either 0.84 μ g of GST-Rmt1, 0.77 μ g of GST-PRMT1, 0.77 μ g of GST-PRMT1 together with 2.2 μ g of GST-TIS21 protein, 2.07 μ g of GST-PRMT3, or 10.6 μ g of GST-PRMT3-(184–528) in a final volume of 55 μ l containing 25 mM Tris-HCl, 1 mM sodium EDTA, 1 mM sodium EGTA at pH 7.5 for 40 min at 37 $^{\circ}$ C. The reaction mixtures were then subjected to SDS-PAGE and fluorography. B, *rmt1* yeast extract (190 μ g) was incubated with 1.65 μ Ci (0.46 μ M) of [3 H] AdoMet and 2.5 μ g of either GST-PRMT3 (lane 3) or GST-PRMT3-(184–528) (lane 4) in a final volume of 45 μ l in PBS for 40 min at 37 $^{\circ}$ C. Control reactions contained 1.65 μ Ci (0.46 μ M) of [3 H] AdoMet and 190 μ g of *rmt1* extract (lane 5), 2.5 μ g of GST-PRMT3 (lane 1) or 2.5 μ g of GST-PRMT3-(184–528) (lane 2). A is from a 2-day exposure; B is from an overnight (14 h) exposure. The arrows indicate the positions of polypeptide size standards.

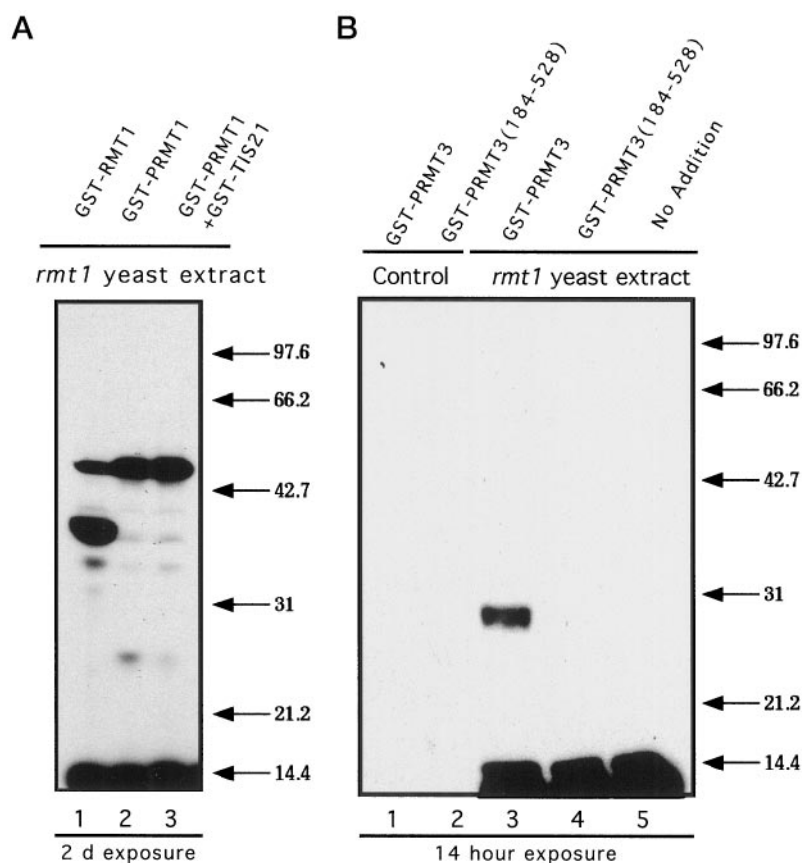


TABLE I
Comparison of two distinct type-I protein arginine N-methyltransferases

	Rat PRMT1	Rat PRMT3
Relative activity on GST-GAR	100%	~1%
Methylated arginine residue formed	Monomethylarginine and asymmetric dimethylarginine	Monomethylarginine and asymmetric dimethylarginine
Activity modulated by	TIS21 and BTG1 proteins	PRMT3 amino-terminal domain
Calculated molecular size of catalytic polypeptide	40.5 kDa	59.4 kDa
Native molecular size of enzyme estimated by Sephacryl S300 gel filtration	~317 kDa	~37 kDa
Predominant intracellular location	Nuclear	Cytoplasmic

purified to homogeneity, and the catalytic subunit has not been cloned. Both PRMT1, the catalytic subunit of a type I protein arginine methyltransferase from mammalian cells, and Rmt1, a type I protein arginine N-methyltransferase from *S. cerevisiae*, have recently been cloned and characterized (7, 15, 17, 18). Mutation of the *RMT1* gene of *S. cerevisiae* eliminates all detectable N^G,N^G -dimethylarginine residues present in proteins, suggesting that Rmt1 is the sole protein type I arginine N-methyltransferase present in this organism (17).²

Katsanis *et al.* (19) recently identified a human gene, *HRMT1L1* (PRMT2 in the GenBankTM data base), that is 33% identical in amino acid sequence with human *PRMT1*. Although we have not been able to demonstrate enzyme activity with a GST-HRMT1L1 fusion protein, the sequence similarities between HRMT1L1, PRMT1, and PRMT3 suggest that HRMT1L1 will also have enzyme activity on an appropriate substrate. Current data thus suggest that two mammalian genes, whose properties are summarized in Table I, encode demonstrated protein arginine N-methyltransferase catalytic subunits. EST data base searches suggest that these three genes may encompass the entire gene family.

PRMT1 and PRMT3 Have Distinct Enzymatic Properties—GST-PRMT1 and GST-PRMT3 both asymmetrically dimethylate arginine residues on GST-GAR. However, GST-PRMT3 has substantially lower activity (Figs. 2 and 3, Table I). Analysis of the protein arginine methyltransferase activity of RAT1 extracts suggests that the difference in specific activities of GST-PRMT1 and GST-PRMT3 is not a consequence of the GST fusion. Column fractions containing easily detectable levels of PRMT3 antigen have little or no detectable enzyme activity on GST-GAR, whereas fractions containing PRMT1 antigen have substantial enzyme activity (Fig. 5). In contrast, there exists at least one substrate, a 29-kDa protein present in hypomethylated yeast *rmt1* extracts, that can be methylated by GST-PRMT3 but not by GST-PRMT1 (Fig. 10).

We anticipated that a subset of the hypomethylated proteins present in adenosine dialdehyde-treated RAT1 cell extracts would serve as GST-PRMT3 substrates. Although numerous hypomethylated substrates are present, as demonstrated by GST-PRMT1 methylation (7), none could be detectably methylated by GST-PRMT3 (data not shown). There are a number of possible reasons for the failure to detect GST-PRMT3 substrates in RAT1 cell extracts. The GST-PRMT3 fusion protein may not accurately reflect the substrate specificity of the native

² J. D. Gary and S. Clarke, unpublished data.

enzyme. Alternatively, PRMT3 may need to be activated in some as yet unidentified fashion. PRMT3 substrates might be denatured during the preparation of hypomethylated RAT1 extract or only be available under specific conditions. In the latter case, it is possible that methyl-accepting sites would only be exposed to the PRMT3 enzyme when substrates have specific ligands bound or when segments of the polypeptide chain are partially unfolded, such as during or immediately after translation (35).

PRMT1 and PRMT3 Are Present in RAT1 Cells in Different Subcellular Compartments—Mammalian type I protein arginine methyltransferase enzyme activity has consistently been detected as a high molecular weight complex (4, 7, 20, 21). We consequently expected that PRMT3, the principal protein identified in a yeast two-hybrid screen for PRMT1-interacting proteins, would form hetero-oligomers with PRMT1. However, the PRMT1 and PRMT3 antigens are completely separated by gel filtration chromatography of RAT1 cell extracts. PRMT1 antigen is associated with the high molecular weight complex that demonstrates protein arginine methyltransferase activity. In contrast, PRMT3 protein chromatographs as an apparent monomer. The relatively low specific activity of PRMT3 on conventional type I protein arginine *N*-methyltransferase substrates explains why this isoform of the enzyme has not been detected in previous studies of protein arginine methyltransferase enzyme activity (1–3).

Since we isolated PRMT3 as a PRMT1-interacting protein in the yeast two-hybrid analysis, we were surprised to observe that PRMT1 is primarily nuclear and PRMT3 is primarily cytoplasmic in RAT1 cells. However, we investigated PRMT1 and PRMT3 localization in only one cell type, under a single physiological condition. If protein-protein interactions between PRMT1 and PRMT3 do occur in cells, it seems likely that these interactions are subject to regulatory events that may lead to altered subcellular distribution of at least a subpopulation of PRMT3 and/or PRMT1 molecules. Substantial precedent exists for ligand-induced alterations in subcellular distribution of enzymes (*e.g.* protein kinases) that result in altered protein-protein interactions. In this regard it is of interest that PRMT1 was also isolated as a protein that interacts with the cytoplasmic domain of the interferon- α , β receptor (15). This domain provides a docking site for many proteins involved in interferon signaling pathways, *e.g.* the JAK kinases and STAT transcription factors (16). It is, of course, possible that PRMT1 and PRMT3 do not form complexes in cells under any conditions and that the identification of PRMT3 by yeast two-hybrid analysis was a fortuitous artifact that resulted in the cloning of this second enzymatically active mammalian type I protein arginine *N*-methyltransferase.

Mammalian Type I Protein Arginine *N*-Methyltransferases Each Have a Potential Regulatory Region That May Influence Their Enzyme Activities—GST-PRMT1 methyltransferase activity is dramatically modulated by interaction with TIS21 protein; both quantitative activation and qualitative alterations in substrate specificity occur when GST-TIS21 is incubated with GST-PRMT1 (Ref. 7 and Fig. 10). In contrast, GST-TIS21 does not interact with GST-PRMT3 or modulate its methyltransferase activity. However, PRMT3 has an amino-terminal extension that is not shared with PRMT1. The tyrosine phosphorylation consensus sequence GLEFYGYIK of the PRMT3 NAR region is similar to the JAK kinase substrate site GPKGYIK present in STAT1 (29). Phosphorylation at this site is required for STAT1 dimerization and transport to the nucleus (29, 36). The PRMT3 amino-terminal region also contains a potential C2H2 zinc finger, a protein motif involved in both protein-protein (37–39) and protein-nucleic acid (40, 41) inter-

actions. The activity of amino-terminal truncated GST-PRMT3-(184–528) was reduced for GST-GAR (Fig. 2) and eliminated for the 29-kDa substrate present in *rmt1* yeast cell extract (Fig. 10), suggesting that the PRMT3 NAR region may play a regulatory role in PRMT3 enzymatic activity, subcellular localization, and/or protein-protein interactions.

HRMT1L1(PRMT2) also has an amino-terminal extension. Although this region of HRMT1L1 does not contain zinc finger or phosphorylation consensus sequences, it does contain a sequence that has 68% amino acid sequence similarity to the SRC SH3 domain. We suggest (i) that PRMT1, PRMT3, and HRMT1L1(PRMT2) each contains a catalytic methyltransferase domain and (ii) that each may be subject to distinct modes of protein-protein interaction and regulation of enzyme activity by interaction with TIS21 (PRMT1), by zinc finger-mediated interactions and/or modifications at the tyrosine phosphorylation site (PRMT3), and by SH3-mediated protein-protein interactions with proline-rich regions of regulatory molecules (HRMT1L1).

Protein arginine methylation extends across a wide range of eucaryotic organisms (1). Substrates for these enzymes play roles in a number of important biological functions. However, the biochemical and biological consequences of this post-translational modification are not well understood. With the isolation of distinct enzymes with alternative substrate specificities, cellular distributions, and regulatory interactions, it should now be possible to carry out the molecular and cellular studies that will elucidate the roles of these enzymes.

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