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The Novel Human Protein Arginine *N*-Methyltransferase PRMT6 Is a Nuclear Enzyme Displaying Unique Substrate Specificity*

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Protein arginine methylation is a prevalent posttranslational modification in eukaryotic cells that has been implicated in signal transduction, the metabolism of nascent pre-RNA, and the transcriptional activation processes. In searching the human genome for protein arginine *N*-methyltransferase (PRMT) family members, a novel gene has been found on chromosome 1 that encodes for an apparent methyltransferase, PRMT6. The polypeptide chain of PRMT6 is 41.9 kDa consisting of a catalytic core sequence common to other PRMT enzymes. Expressed as a glutathione *S*-transferase fusion protein, PRMT6 demonstrates type I PRMT activity, capable of forming both ω-N^G-monomethylarginine and asymmetric ω-N^G,N^G-dimethylarginine derivatives on the recombinant glycine- and arginine-rich substrate in a processive manner with a specific activity of 144 pmol methyl groups transferred min^{-1} mg^{-1} enzyme. A comparison of substrate specificity reveals that PRMT6 is functionally distinct from two previously characterized type I enzymes, PRMT1 and PRMT4. In addition, PRMT6 displays automethylation activity; it is the first PRMT to do so. This novel human PRMT, which resides solely in the nucleus when fused to the green fluorescent protein, joins a family of enzymes with diverse functions within cells.

The family of protein arginine *N*-methyltransferases (PRMTs)[1] catalyze the sequential transfer of a methyl group from AdoMet to the side chain nitrogens of arginine residues within proteins to form methylated arginine derivatives and *S*-adenosyl-L-homocysteine (1). To date, two distinct PRMT activities have been found in mammalian cells. Type I PRMT activity is defined by the formation of asymmetric ω-N^G,N^G-dimethylarginine residues, whereas type II activity is defined by the formation of symmetric ω-N^G,N'^G-dimethylarginine residues. The methylation of arginine residues has been implicated in the regulation of signal transduction (2–4), transcription (5), RNA transport (6, 7), and possibly splicing (8).

Within recent years the number of PRMT family members has been increasing (9). Currently, known type I enzymes include the catalytic chain of PRMT1 (10, 11) and its yeast homologue arginine methyltransferase 1 (also known as heterogeneous nuclear ribonucleoprotein methyltransferase 1) (12, 13), the zinc finger-containing enzyme PRMT3 (14, 15), and the coactivator-associated arginine methyltransferase CARM1/PRMT4 (5). Several endogenous type I substrates have been determined (3, 4, 16–25). The majority of type I arginine methylation occurs within glycine- and arginine-rich (GAR) domains (1, 10, 14, 26, 27). The only type II PRMT identified to date is the Janus kinase-binding protein JBP1/PRMT5 (28–30). Its homologue in budding yeast is Hsl7 (histone synthetic lethal 7) (31) and Skb1 in fission yeast (32, 33). Known substrates to contain symmetric ω-N^G,N'^G-dimethylarginine are myelin basic protein (34, 35) and small ribonucleoproteins D1 and D3 (8, 36).

The crystal structure of the rat PRMT3 catalytic core (amino acids 201–528) in complex with *S*-adenosyl-L-homocysteine determined at 2.0 Å resolution represents the first solved structure of a PRMT (37). Shortly thereafter, the crystal structure of RMT1 (amino acids 22–348) was determined at 2.9 Å resolution (38). These structures reflect a striking structural conservation of the PRMT catalytic core (37, 38). In addition to the sequences of the conserved AdoMet-dependent methyltransferase motifs I, post-I, II, and III, the suggested site for arginine binding contains several amino acids that appear to be conserved throughout the PRMT family (37, 38). These proposed active site residues might aid in recognizing novel PRMTs.

The recent sequencing of the entire human genome and its public availability has provided a first glimpse at all of the genes coding for a variety of protein families (39, 40). In searching the human genome for PRMT family members, a novel gene coding for what appears to be the sixth known human PRMT enzyme was identified. A recombinantly expressed form of this new PRMT, referred to now as PRMT6, exhibits type I PRMT activity and can methylate itself. PRMT6 does not recognize the same substrates as PRMT4/CARM1 and displays limited substrate overlap with PRMT1. As a GFP fusion protein, PRMT6 resides in the nucleus of HeLa cells along with PRMT1, PRMT2, and PRMT4, whereas PRMT3 and PRMT5 GFP fusions localize exclusively to the cytoplasm.

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[1] The abbreviations used are: PRMT, protein arginine *N*-methyltransferase; [^{3}H]AdoMet, *S*-adenosyl-L-[*methyl*-^{3}H]methionine; CARM1, coactivator-associated arginine methyltransferase 1; JBP1, Janus kinase-binding protein; GST, glutathione *S*-transferase; GAR, glycine- and arginine-rich; AdOx, adenosine dialdehyde; PCR, polymerase chain reaction; GFP, green fluorescent protein; PBS, phosphate-buffered saline.

FIG. 1. **Amino acid sequence alignment of the human PRMT family.** The catalytic core regions common to all PRMT family members are shown. Amino acids *boxed* in *black* match the primary structure of PRMT6. Signature methyltransferase motifs are *boxed*, and amino acids are highlighted in *gray*. The accession numbers for the protein sequences used in this alignment are as follows: AAF62893 for PRMT1-*v*2 (361 amino acids); AAH00727 for PRMT2 (433 amino acids); AAC39837 for PRMT3 (512 amino acids); NT_011176.3 contains a gene comprised of 16 exons on chromosome 19 that codes for PRMT4 (608 amino acids), the apparent human homologue to the mouse CARM1, AF117887; AAF04502 for PRMT5 (637 amino acids); and AY043278 for PRMT6 (375 amino acids).

EXPERIMENTAL PROCEDURES

Isolation of the Full-length PRMT6 Transcript—To obtain the full-length sequence of PRMT6, we used the reported partial human kidney expressed sequence tag BC002729 (GenBank™) to engineer primers for 5′-rapid amplification of cDNA ends. The first primer sequence was 5′-GGA CCG AAA CGT CCG AGT AGC ATC G-3′, and the second "nested" primer sequence was 5′-TGG TCC CGT TCC CGC TTA GTC CTC CG-3′. We used kidney RACE-Ready cDNA® (Ambion) to isolate the 5′ coding region of PRMT6. Full-length PRMT6 was amplified by the PCR from human kidney cDNA using the following oligonucleotide set: 5′-CAT GGA TCC ATG TCG CAG CCC AAG AAA AGA AAG C-3′ and 5′-TGA GAA TTC TCA GTC CTC CAT GGC AAA GTC-3′. The resulting PCR fragment was digested with *Bam*HI and *Eco*RI and subcloned in-frame into the *Bam*HI and *Eco*RI sites of pGEX-6P1 (Amersham Biosciences, Inc.).

Northern Blot Analysis—A human poly(A)$^+$ RNA blot (Origene Technologies, Inc.) was probed with DNA corresponding to the open reading frames of PRMT1 and PRMT6, which were liberated from the appropriate GST fusion vectors by restriction enzyme digestion. DNA probes were labeled with [^{32}P]dCTP using a Prime-it II® kit (Stratagene, Inc.). Northern blot analysis was performed following standard procedures described in Ref. 41. Probed blots were exposed overnight at −80 °C.

Preparation of the GST-PRMT6 Fusion Protein—GST-PRMT6 was overexpressed in *Escherichia coli* DH5α cells (Invitrogen) by induction with a final concentration of 0.4 mM isopropyl-β-D-thiogalactopyranoside. Washed cells were resuspended in 2 ml of phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4) and 100 μM phenylmethylsulfonyl fluoride/g of cells and subsequently 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 for 40 min at 23,000 × *g* at 4 °C. The GST fusion protein was then batch-purified from extracts by binding to glutathione-Sepharose 4B beads (Amersham Biosciences, Inc.) and washed in PBS per the manufacturer's instructions in the presence of 100 μM phenylmethylsulfonyl fluoride. The purified proteins were eluted from the beads with 30 mM glutathione, 50 mM Tris-HCl, pH 7.5, 120 mM NaCl, and 2% glycerol.

Subcloning of GFP Fusion Vectors Harboring the PRMTs—pGEX-PRMT1 (13) was digested with *Bam*HI and *Sal*I, and the resulting 1.1-kb fragment was cloned into the *Bgl*II and *Sal*I sites of pEGFP-C1 (CLONTECH, Inc.). The PRMT2 gene was PCR-amplified from human expressed sequence tags T77642 and T75034 to yield a single 1.3-kb

TABLE I
Sequence identity between all PRMT family members

The percentage of identity in the conserved catalytic core shared between all PRMT family members is listed. Each pairwise comparison was performed with sequence alignments from Fig. 1 using the BLAST 2 sequence program (46).

	PRMT catalytic core sequence identity					
	PRMT1	PRMT2	PRMT3	PRMT4	PRMT5	PRMT6
			%			
PRMT1	100	34	51	35	18	34
PRMT2	34	100	37	35	23	38
PRMT3	51	37	100	32	17	34
PRMT4	35	35	32	100	29	36
PRMT5	18	23	17	29	100	28
PRMT6	34	38	34	36	28	100

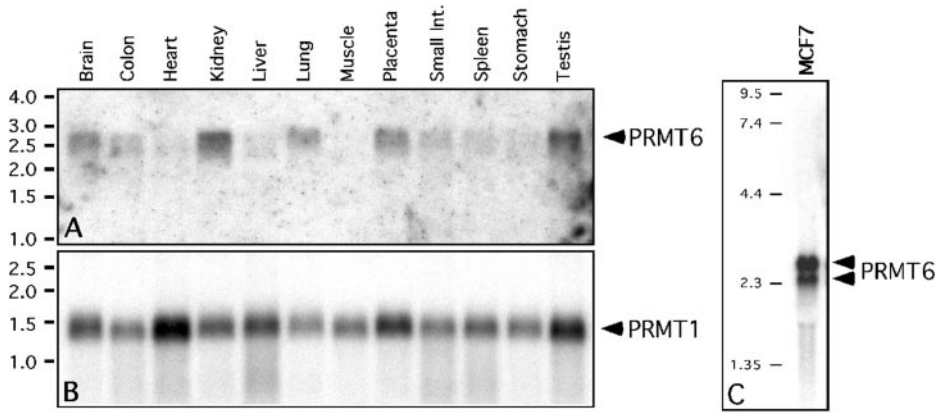

FIG. 2. **Northern analysis comparing the expression of PRMT6 and PRMT1 mRNA in different tissues.** The expression levels of PRMT6 (*A*) and PRMT1 (*B*) in various human tissues were examined by hybridization to a multiple tissue Northern blot. *C*, Northern analysis of MCF7 cell RNA, subjected to extended agarose separation, reveals that there are two PRMT6 transcripts that run as a doublet at 2.4 and 2.6 kb. The probes were generated through the restriction enzyme liberation of the respective full open reading frame from GST fusion constructs. Overnight exposures are depicted. The positions of RNA size markers are shown.

product with the primer set: 5′-GAG CCT AAG GGA TCC ATG GCA ACA TCA GGT-3′ and 5′-C CAA ATA AAG CAT GAA TTC TCA TCT CCA G-3′. The PRMT2 PCR product was digested with *Bam*HI and *Eco*RI and cloned into pGEX-2T (Amersham Biosciences, Inc.) to make pGEX-PRMT2. The coding region of PRMT2 was amplified by PCR using the oligonucleotide set: 5′-CGT GGA TCC GCA ACA TCA GGT GAC TGT CCC-3′ and 5′-CTA GAA TTC AAC TGT CAT CTC CAG-3′. pGEX-PRMT2 was used as a template, and the amplified band of 1.3 kb was digested with *Bam*HI and *Eco*RI and cloned into the *Bgl*II and *Eco*RI sites of pEGFP-C1. pGEX-PRMT3 (15) was digested with *Bam*HI and *Sac*I, and the resulting 1.6-kb insert was cloned into the *Bgl*II and *Sac*I sites of pEGFP-C1. pGEX-PRMT4 (a gift from Michael Stallcup at the University of Southern California) was used as a template to amplify the coding region of PRMT4 by PCR using the primer set: 5′-TGA GAT CTC ACC ATG GCA GCG GCG GCA GCG ACG GC-3′ and 5′-AGT AAG CTT ACT CCC ATA GTG CAT GGT GTT-3′. The amplified band of 1.8 kb was digested with *Bgl*II and *Hin*dIII and cloned into the *Bgl*II and *Hin*dIII sites of pEGFP-N1 (CLONTECH, Inc.). Using the restriction enzymes *Bam*HI and *Apa*I, PRMT5 was shuttled from the pTKB175 vector (a gift from Sidney Pestka at the Robert Wood Johnson Medical School) to the SuperLinker® vector (pSL301) (Invitrogen). pSL301-PRMT5 was digested with *Bam*HI and *Sal*I, and the resulting 2.2-kb fragment was cloned into the *Bgl*II and *Sal*I sites of pEGFP-C3 (CLONTECH, Inc.). pGEX-PRMT6 (see above) was digested with *Bam*HI and *Eco*RI, and the resulting 1.3-kb fragment was cloned into the *Bgl*II and *Eco*RI sites of pEGFP-C1.

Protein Concentration Determination—A modification of the Lowry procedure was used to determine protein concentrations of GST fusions and AdOx-treated RAT1 cell extracts following precipitation with 1.0 ml of 10% (w/v) trichloroacetic acid (43). A stock solution of bovine serum albumin was used as a protein standard.

In Vitro Methylation of Substrates—GST-GAR has been described previously (1). GST-Npl3 was a gift from Pam Silver and was described previously (38). GST-PABPf harbors 100 amino acids of the PABP1 protein, and the expression construct was generated by PCR from a HeLa cell cDNA template using the following oligonucleotide set: 5′-TGC GGA TCC GCA AGT GTA CGA GCT GTT CCC-3′ and 5′-TAA GAA TTC TTA ACG CTG TGT TGA CAT GAC TCG-3′. The resulting PCR fragment was cloned into the *Bam*HI and *Eco*RI sites of pGex-6P-1 (Amersham Biosciences, Inc.). GST fusion proteins were prepared as described above. Recombinant forms of these protein substrates often undergo partial proteolysis during the course of their purification and consequently appear as either a smear or multiple bands on SDS-PAGE gels. The preparation of AdOx-treated and untreated RAT1 extracts and their subsequent pretreatment with bovine pancreatic RNase A (10 mg/ml; Sigma) has been described previously (15).

All methylation reactions were carried out in the presence of *S*-adenosyl-L-[*methyl*-^{3}H]methionine ([^{3}H]AdoMet; 79 Ci/mmol from a 12.6 μM stock solution in dilute HCl/ethanol 9:1, pH 2.0–2.5; Amersham Biosciences, Inc.) and PBS (described above). Additional information pertaining to reaction conditions is described in each of the figure legends.

Electrophoresis and Fluorography of Methylation Reactions—After the assay, 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) was added to the reaction, heated at 100 °C for 5 min, and separated on slab gels prepared from 8.0% (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 (42) at a constant current of 35 mA for ~4 h (42). Following electrophoresis, the 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^{3}HANCE (PerkinElmer Life Sciences) per the manufacturer's instructions. Gels were dried *in vacuo*, and radioactivity was visualized by fluorography (gels were exposed to film at −80 °C for the times indicated in the figure legends).

Amino Acid Analysis of Methylated GST-GAR—Methylation reactions were quenched by the addition of 30 μl of 25% (w/v) trichloroacetic acid to precipitate proteins in 6 × 50-mm glass vials. Precipitated proteins were centrifuged at 4000 × *g* for 40 min at 25 °C. The supernatant was discarded, and the pellets were washed once with an equal volume of cold acetone (−20 °C). After an additional centrifugation for 20 min, the acetone was discarded, and the pellets were allowed to dry. Acid hydrolysis was carried out on the dried pellet in a Waters Pico-Tag vapor-phase apparatus containing 200 μl of 6 N HCl for 20 h *in vacuo* at 110 °C. The hydrolyzed samples were resuspended in 100 μl of water mixed with 1 μmol each of ω-N^G-monomethylarginine (Sigma product M7033; acetate salt) and asymmetric ω-N^G,N^G-dimethylarginine (Sigma product D4268; hydrochloride) as standards. Hydrolyzed amino acids and standards were loaded onto a Beckman AA-15 sulfonated polystyrene cation exchange column (0.9 cm × 11 cm) that was pre-equilibrated with Na^+ citrate buffer (0.35 M in Na^+, pH 5.27) at 55 °C and regenerated with 0.2 N NaOH. Approximately 1 ml/min column fractions were collected for analysis. ^{3}H radioactivity was detected by adding 200 μl from each fraction to 400 μl of water, mixing with 5 ml of fluor, and counting on a scintillation counter. Unlabeled methylargin-

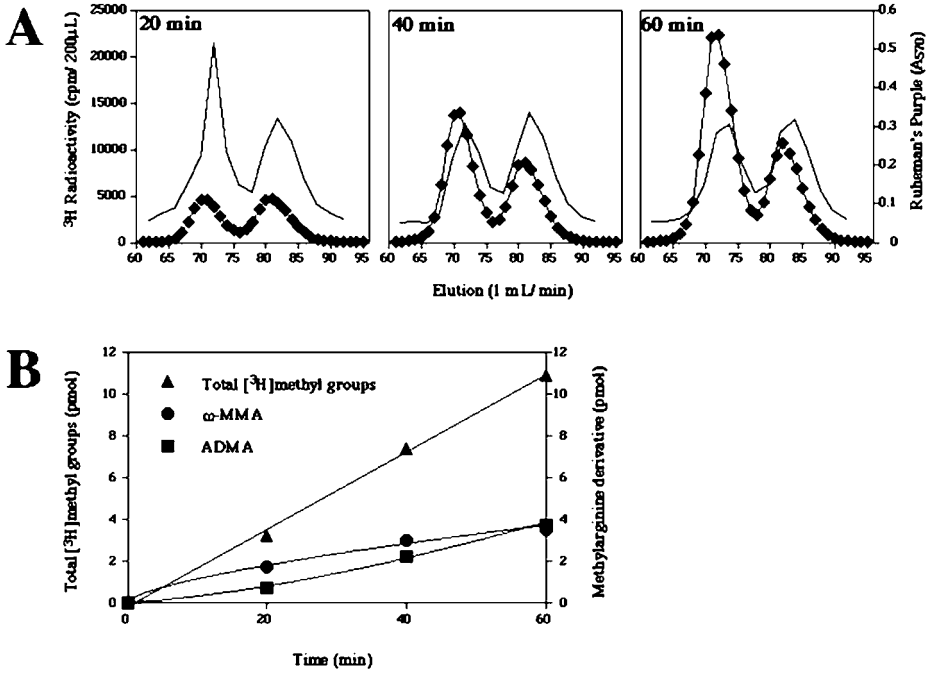

FIG. 3. **PRMT6 is a processive type I protein arginine *N*-methyltransferase.** Purified GST-PRMT6 (2 μg) was incubated with 5 μg of GST-GAR (1) in the presence of 1.0 μM [^{3}H]AdoMet for 1 h at 37 °C in a final reaction volume of 30 μl of PBS. Subsequent amino acid analysis was performed as described under "Experimental Procedures." *A*, methylation reactions were conducted for 20, 40, and 60 min. Unlabeled amino acid standards reacted with ninhydrin to form Ruheman's Purple elute from the cation exchange column first with asymmetric ω-N^G,N^G-dimethylarginine followed by ω-N^G-monomethylarginine. The *diamonds* indicate radioactivity, and Ruheman's Purple is shown as a *solid line*. *B*, the total radioactivity as well as an integration of each radioactive peak was quantitated to calculate the total pmol methyl groups transferred and the amount of asymmetric ω-N^G,N^G-dimethylarginine (*ADMA*) and ω-N^G-monomethylarginine (ω-*MMA*) formed at each time point.

ine standards were detected by analyzing 100 μl of every other fraction by a ninhydrin method described previously (44).

RESULTS

Identification of a Novel PRMT Gene—The PRMT family of enzymes exhibits amino acid conservation within the characteristic methyltransferase motifs I, post-I, II, and III (45), and in other portions of the polypeptide chain. A search of the publicly available human genome sequence (39) using the gapped BLAST method (46) provided by the National Center for Biotechnology Information for sequences that match known PRMT sequences identified a novel locus (AK001421) cited as coding for the 316-amino acid hypothetical protein FLJ10559. A GST fusion protein of this hypothetical protein was generated and found to display no arginine methyltransferase activity when assayed on a number of PRMT1 and PRMT4 substrates (data not shown). Upon further scrutiny of the DNA sequence, we identified similarities between a region upstream of the sequence encoding the predicted initiator methionine and those encoding the common N terminus of the PRMT family of proteins, suggesting that a portion of the N-terminal end of the predicted protein was missing. To obtain the complete open reading frame we performed 5′-rapid amplification of cDNA ends on human kidney cDNA and identified additional 5′ sequence. The newly obtained open reading frame codes for a 375-amino acid protein, now referred to as PRMT6. The recombinant form of PRMT6 is an active enzyme (see below). It has a calculated polypeptide molecular mass of 41.9 kDa and is 46% identical to a putative PRMT in *Arabidopsis thaliana* (BAB01859) as its closest homologue.

The alignment of the common sequences shared by all human PRMT family members is shown in Fig. 1. PRMT6 shares with other PRMTs conserved amino acid sequences within the AdoMet-dependent methyltransferase motifs I, post-I, II, and III, as well as amino acids outside of the these regions with some notable exceptions (45). Table I lists the percentage of identity shared between each catalytic core portion of all PRMTs. PRMT6 is most similar to PRMT2 in the catalytic core region, sharing 38% sequence identity; however, it does not contain an N-terminal Src homology 3 domain found in the PRMT2 sequence. Much like PRMT1, PRMT6 is comprised solely of the PRMT catalytic core without any additional domains.

PRMT6 Expression Pattern—Northern analysis of PRMT6 reveals that it is highly expressed in kidney and testes (Fig. 2*A*). When compared, the expression levels of PRMT1 and PRMT6 display some degree of tissue specificity. In addition, at least 80 expressed sequence tags from a variety of different tissues have been identified for PRMT6, and a virtual Northern blot (*www.ncbi.nlm.nih.gov/SAGE/sagevn.cgi*) generated from a serial analysis of gene expression tag at the 3′ end of the PRMT6 cDNA sequence also suggests a broad expression pattern for this transcript. The hybridized band in the PRMT6 Northern blot was more diffuse than that displayed by PRMT1 (Fig. 2, *A* and *B*), suggesting that PRMT6 may run as two transcripts of similar size. Indeed, when we performed Northern analysis on an RNA sample subjected to an extended separation, we do see two bands (2.4 and 2.6 kb) for PRMT6 (Fig. 2*C*). A comparison of human PRMT6 expressed sequence tags revealed no alternative splicing events, implying that the two transcripts may arise from different lengths of 5′- or 3′-untranslated regions.

PRMT6 Is a Type I Protein Arginine N-Methyltransferase—Several PRMTs have been shown to catalyze type I methylation of arginine residues within proteins; these enzymes include PRMT1 (10), PRMT3 (14), and PRMT4 (48). The only known type II PRMT enzyme is PRMT5 (30). To determine the type of activity of PRMT6, GST-PRMT6 was assayed for methyltransferase activity using GST-GAR as a substrate. The fusion protein is able to catalyze the formation of both ω-N^G-monomethylarginine and asymmetric ω-N^G,N^G-dimethylarginine, thus substantiating that PRMT6 is a type I enzyme, as shown in Fig. 3*A*. We find a specific activity of 144 ± 11 pmol methyl groups transferred min^{-1} mg^{-1} PRMT6 (Fig. 3*B*). The total incorporation is linear during the 60-min assay. As expected, we observe an initial accumulation of the monomethyl species followed by a greater rate of accumulation of the final dimethylarginine product (Fig. 3*B*). Because the amount of available RG sites on the GST-GAR substrate (1800 pmol) is much larger than the total accumulation of methylarginine seen at 60 min (10.9 pmol), it appears that this reaction is processive. Otherwise, we would expect predominantly ω-N^G-monomethylarginine to form within this time period because

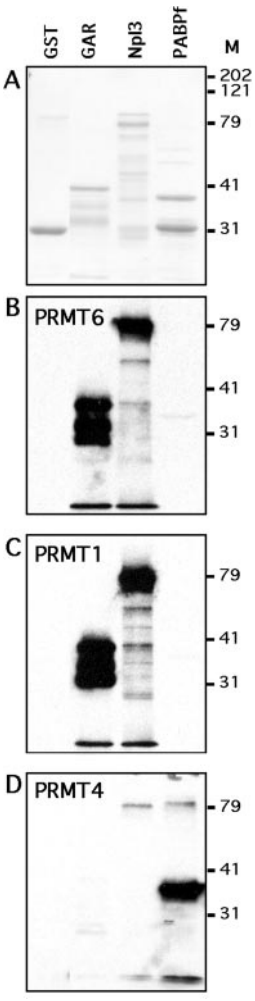

FIG. 4. **A demonstration of PRMT6 *in vitro* substrate specificities.** Recombinant PRMT6 (1 μg), PRMT1 (1 μg), and PRMT4 (1 μg) arginine methyltransferases were incubated with GST (1 μg), GST-GAR (1 μg), GST-Npl3 (1 μg), and GST-PABPf (1 μg) *in vitro* in the presence of 0.5 μM [^{3}H]AdoMet for 30 min at 37 °C in a final volume of 30 μl of PBS. *A*, for quantification purposes the set of methylation substrates were separated by SDS-PAGE and stained with Coomassie. *B*, methylation of substrate set with recombinant PRMT6. *C*, methylation of substrate set with recombinant PRMT1. *D*, methylation of substrate set with recombinant PRMT4. In *B–D*, the methylated proteins were visualized by fluorography (approximately a 12-h exposure) following separation by SDS-PAGE. The molecular mass markers are shown on the *right* in kDa.

the enzyme would catalyze a single methylation reaction and then release its protein substrate.

PRMT6 Substrate Specificity—Two distinct classes of *in vitro* protein substrates have been described for protein arginine *N*-methyltransferases: those that are methylated by PRMT1 (and PRMT3) and those that are methylated by PRMT4 (5). As a new member of the PRMT family, PRMT6 was tested for its substrate specificity using as methyl acceptors the known PRMT1 substrates GST-GAR and the yeast protein Npl3, as well as a PRMT4 substrate, PABPf, which is a 100-amino acid region of PABP1 that was identified in a screen for PRMT4 substrates.[2] PRMT6 preferentially methylated the recombinant forms of GAR and Npl3 substrates, thus displaying substrate specificity similar to that of PRMT1 (Fig. 4).

To identify the subtleties in PRMT6 substrate specificity, we also tested the ability of this enzyme to methylate proteins in a RAT1 cell extract prepared from cells either treated or untreated with AdOx (Fig. 5). AdOx treatment of mammalian cells results in an increase in type I methyl acceptors by inhibiting the breakdown of *S*-adenosyl-L-homocysteine, the product inhibitor of AdoMet-dependent methylation reactions (15, 26). In comparing *panels A* and *B* of Fig. 5, we observe a substantial increase in methyl acceptors upon AdOx treatment of RAT1 cells for all three enzymes. To our surprise, the substrate specificity differences between PRMT1, PRMT4, and PRMT6 are more pronounced in untreated cells as compared with AdOx-treated cells. RNase treatment of the cell extracts also substantially alters the observed methylation patterns for all three enzymes in untreated cells as compared with AdOx-treated cells, although each enzyme demonstrates higher activity in hypomethylated cell extracts.

In AdOx-treated RAT1 cell extracts, we found that PRMT4 and PRMT6 also methylate some of the same polypeptides recognized by PRMT1 (Fig. 5*B*). Nevertheless, one of the most heavily methylated substrates observed for PRMT6 in AdOx-treated cells (Fig. 5*B*, *lanes 10* and *11*), indicated by a single asterisk, does not appear to be a substrate for either PRMT1 (*lanes 4* and *5*) or PRMT4 (*lanes 7* and *8*), demonstrating at least one unique methyl acceptor in cell extracts for PRMT6. This polypeptide appears to be a less significant substrate in untreated cell extracts (Fig. 5*A*, *lanes 5* and *6*), suggesting that it may represent an endogenous substrate. We also observed an additional methylated polypeptide in all PRMT6-containing lanes in Fig. 5, indicated by *two asterisks*, which is present even in incubations lacking cell extract. This band corresponds to the full-length GST-PRMT6 enzyme with a calculated molecular mass of 69 kDa. This polypeptide was excised from the SDS-PAGE gel, acid-hydrolyzed, and subjected to cation exchange chromatography as described under "Experimental Procedures." We found that it contained both ω-N^G-monomethylarginine and asymmetric ω-N^G,N^G-dimethylarginine amino acid derivatives (data not shown). These results indicate that GST-PRMT6 is capable of methylating itself as well as other protein substrates. To confirm that the methylation occurred on the PRMT6 portion of the fusion protein and not on the GST moiety, we cleaved GST-PRMT6 after automethylation with PreScission Protease™ (Amersham Biosciences, Inc.). The tritium label associated with PRMT6 and not GST (data not shown). Automethylation of PRMT6 is not seen in Fig. 4*B* because of the relatively short exposure time.

The Subcellular Localization of PRMT6—One way to differentiate the functions of the growing number of PRMTs that may exist within a cell at any given time is by their localization. PRMT1, although implicated as having cytosolic roles (24, 49), appears to be localized and to function in the nucleus (3, 10, 14, 50, 51). A FLAG (peptide epitope DYKDDDDK) epitope-tagged form of PRMT2 has recently been shown to localize to the nucleus (52). PRMT3 and PRMT5, on the other hand, have been shown to be predominantly localized in the cytoplasm (14, 29). PRMT4 and PRMT6 have yet to be localized to a specific region of an intact cell. In an attempt to obtain a comparative overview of the subcellular localization of the PRMTs as a whole, we made GFP fusion constructs of all the described arginine methyltransferase enzymes and studied their cellular localization by confocal microscopy as shown in Fig. 6. GFP fusion proteins of PRMT6 display a strong nuclear localization, as does PRMT4. PRMT1 and PRMT2 GFP fusions appear to be largely localized to the nucleus, but significant fluorescence is observed in the cytosol. The fluorescence signals from PRMT3 and PRMT5 GFP fusions are excluded from the nucleus. The concordance of these results for the GFP fusions of PRMT1, PRMT2, PRMT3, and PRMT5 with previous determinations of the native proteins described above support the conclusion that

[2] Mark T. Bedford, unpublished data.

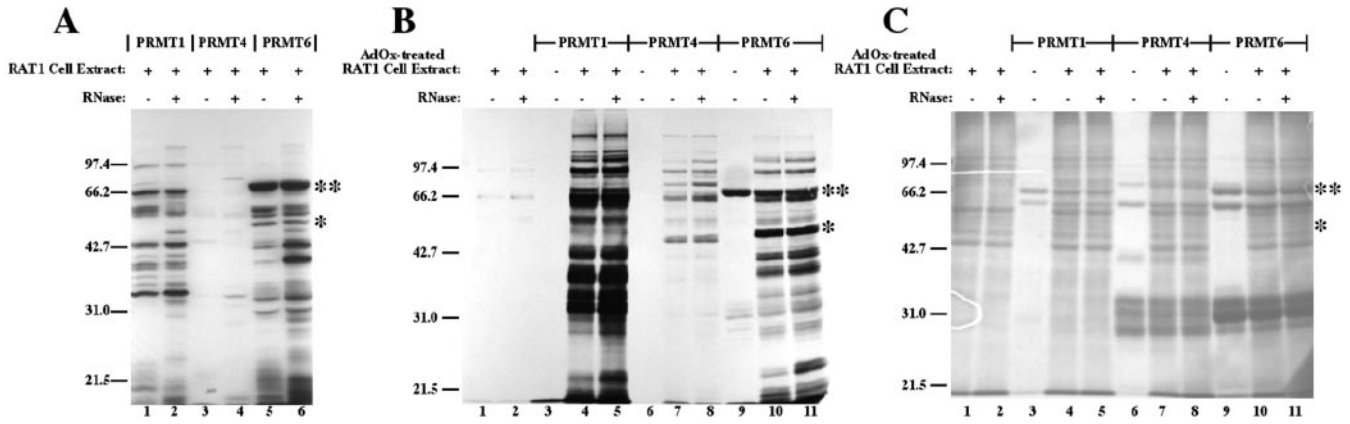

FIG. 5. **PRMT1, PRMT4, and PRMT6 exhibit differential substrate specificities in RAT1 cell extracts.** RAT1 cell extracts (10 μg) with or without RNase A treatment were incubated with either 5 μg of GST-PRMT1, 12 μg of GST-PRMT4, or 10 μg of GST-PRMT6 in the presence of 0.5 μM [^{3}H]AdoMet for 60 min at 37 °C in a final volume of 50 μl of PBS. *A*, reactions were run on SDS-PAGE and fluorographed for a 5-day exposure. *B*, the same assay was performed as in *A*, but the RAT1 cell extracts were prepared from cells treated with AdOx as described previously (15). *C*, the corresponding Coomassie-stained gel is shown. A *single asterisk* marks the position of a unique PRMT6 substrate. *Two asterisks* mark the position of GST-PRMT6, which is automethylated. The molecular mass markers are shown on the *left* in kDa.

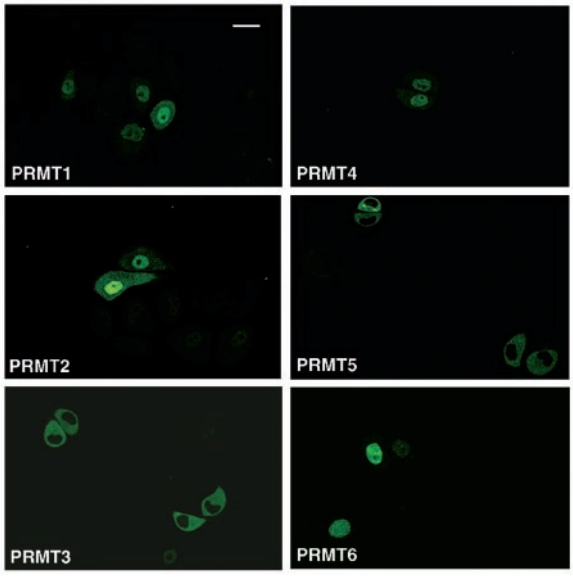

FIG. 6. **The intracellular localizations of GFP fusions of PRMT catalytic chains.** PRMT1, PRMT2, PRMT3, PRMT4, PRMT5, and PRMT6 cDNAs were cloned in-frame with GFP. The resulting constructs were transfected into HeLa cells, and protein localization was observed by confocal fluorescence microscopy. Each *panel* is marked with the identity of the GFP-PRMT fusion used for the transfection. All the PRMTs were fused to the C-terminal end of GFP, except for PRMT4, which was an N-terminal fusion. The *bar* represents 20 μm.

the localization of the GFP fusions of PRMT4 and PRMT6 observed here reflect the situation in intact cells for these proteins.

DISCUSSION

As the sixth member of the protein arginine *N*-methyltransferase family, PRMT6 shares both similarities and differences with its sibling enzymes. All of the PRMTs contain several regions of sequence conservation, including a pair of tyrosine residues in their respective N termini, AdoMet-dependent methyltransferase motifs I, post-I, II, and III, as well as the invariant "THW loop" (37, 38) within their respective C termini (Fig. 1). Similar to the majority of PRMTs, a recombinant form of PRMT6 demonstrates type I methyl transfer activity. As a GFP fusion protein, it localizes to the nucleus, similar to the state seen for PRMT4. However, PRMT6 is currently the only automethylating enzyme in the PRMT family.

Out of six potentially active mammalian PRMTs *in vivo*, five catalytic chains, PRMT1 (10), PRMT3 (14), PRMT4 (5), PRMT5 (28), and PRMT6, demonstrate methyl transfer activity *in vitro* as recombinant proteins. Because the majority of substrates that have been tested as methyl acceptors *in vitro* contain GAR domains (1, 3, 6, 10–12, 14, 15, 29, 53–55), it is difficult to distinguish substrate specificity within the PRMT family.

By compiling data on the sequence surrounding actual methylated residues, a consensus sequence can be generated for the type I PRMTs. Many known PRMT substrates fit the consensus (F/G)GGRGG(G/F), with only the underlined arginine and glycine residues found in all methylated sites (56). As more substrates are being identified for the different PRMTs, it is becoming clear that such a consensus may not accurately depict the enzyme substrate preference. We have shown that Sam68 can be methylated by PRMT1 on both RGRG repeats and PRG repeats (4). Analysis of the methylated sites in poly(A)-binding protein II revealed unique sites of methylation in RXR sequences and indicated that a glycine residue following an arginine is not necessary for a type I reaction (22). In addition, PRMT4 methylates histone H3, a substrate whose methylated arginine residues do not fit the consensus sequence (F/G)-GGRGG(G/F) (5, 48). PRMT6 appears to behave more like PRMT1 in this respect (Figs. 3 and 4).

The complexes in which PRMTs are found also aid in distinguishing their different cellular roles. PRMT1, which appears to be the dominant type I methyltransferase in mammalian cells (11), has been shown to interact with the tumor suppressor protein BTG1 (10), the anti-apoptotic protein TIS21 (10), the interleukin enhancer-binding factor 3 transcription factor (3), the intracytoplasmic domain of the IFNAR1 chain of the interferon-α,β receptor (49), the signal transducer and activator of transcription 1 (24), the transcription activation domain 2 of p160 (57), and the helicase domain of the hepatitis C virus nonstructural protein 3 (58), suggesting that it is involved in several cell signaling pathways. PRMT2 interacts with the methyl acceptor heterogeneous nuclear ribonucleoprotein E1B-AP5 through its Src homology 3 domain (52). PRMT4, like PRMT1, complexes to the activation domain 2 portion of p160 and potentiates transcription in reporter gene assays (5, 57). PRMT5, initially referred to as Skb1Hs (59), has been shown to form protein-protein interactions *in vitro* with the Jak kinases Jak1, Jak2, Jak3, and Tyk2 (28), the hepatitis C virus nonstructural 3 protein (29), and the membrane-bound chloride channel pICln (60). The polypeptides that copurify with immu-

noprecipitated forms of PRMT5 have not been identified (28) but may corroborate its known interactions with other proteins. PRMT3 and PRMT6 have not yet been shown to complex with other polypeptides, although it is plausible that the zinc finger domain of PRMT3 (15), much like the Src homology 3 domain of PRMT2 (52), may mediate its protein-protein interactions.

The features that make each PRMT family member unique, whether it is a particular domain, a distinct interaction, or a discrete substrate, will invariably shape our understanding of PRMT enzymatic function. Gene knockouts, such as RMT1 in yeast (6, 7, 12, 13) and PRMT1 in mouse (61), have been successful in demonstrating the role of methylation in mRNA trafficking and fetal development, respectively. Investigations of other PRMT gene knockouts will hopefully elucidate novel pathways involving arginine methylation.

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