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Protein Arginine Methyltransferase I: Substrate Specificity and Role in hnRNP Assembly

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Abstract Prmt1, the major protein arginine methyltransferase in mammalian cells, has been implicated in signal transduction, transcriptional control, and protein trafficking. In the present study, mouse embryonic stem cells homozygous for an essentially null mutation in the *Prmt1* gene were used to examine Prmt1 activity and substrate specificity, which by several criteria appeared to be highly specific. First, other methyltransferases did not substitute for the loss of Prmt1 activity. Second, almost all proteins modified by recombinant Prmt1 in vitro were authentic substrates, i.e., proteins rendered hypomethylated by *Prmt1* gene disruption. Finally, Prmt1 did not modify the substrates of other methyltransferases from cells treated with methyltransferase inhibitors. Recombinant proteins corresponding to two splice-variants, $Prmt1^{353}$ and $Prmt1^{371}$, methylated different, proteins in vitro, providing the first evidence for functional differences between the two isoforms. However, the differences in substrate specificity were lost by the addition of an N-terminal His_6 tag. Loss of Prmt1 activity (and hypomethylation of hnRNPs) has no obvious effect on the formation or composition of hnRNP complexes. Finally, methylation of the most abundant Prmt1 substrates appeared to be extensive and constitutive throughout the cell cycle, suggesting the modification does not modulate protein function under normal growth conditions. J. Cell. Biochem. 87: 394–407, 2002.

Key words: post-translational modification; protein methylation; gene regulation

Methylation of protein arginine residues is one of many post-translational modifications that increase the structural diversity of proteins and potentially modulate protein functions [for reviews see Gary and Clarke, 1998; McBride and Silver, 2001]. Type I protein-arginine *N*-methyltransferases (e.g., protein methylase I, EC2.1.1.23) transfer methyl groups form S-adenosylmethionine (AdoMet) to guanidino nitrogens of protein arginine, forming N^G-monomethylarginine and asymmetric N^G,N^G-dimethylarginine. The major substrates of Type I methyltransferases appear to be nuclear and nucleolar nucleic acid binding proteins. Sixty five percent of the nuclear dimethylarginine is incorporated into heteronuclear RNA binding proteins, hnRNPs [Liu and Dreyfuss, 1995], that are involved in pre-mRNA processing and nucleocytoplasmic transport [Boffa et al., 1977]. Other extensively methylated proteins, fibrillarin [Christensen and Fuxa, 1988] and nucleolin [Lischwe et al., 1982, 1985a], are implicated in pre-rRNA processing and ribosome biogenesis [Ghisolfi et al., 1990; Kass et al., 1990; Baserga et al., 1991; Tollervey et al., 1991].

Genes encoding four distinct Type I arginine *N*-methyltransferases have been cloned from eucaryotic sources. PRMT1 has been cloned from rat [Lin et al., 1996], human [Scott et al., 1998], and mouse [Pawlak et al., 2000], is ubiquitously expressed in mammalian tissues, and accounts for more than 85% of Type I enzymatic activity [Pawlak et al., 2000; Tang et al., 2000a].

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Disruption of the murine *Prmt1* gene results in an early embryonic lethal phentoype, consisitent with a fundamental and nonredundant function [Pawlak et al., 2000]. However, ES cell lines deficient in the enzyme are viable, although the content of asymmetric dimethylarginine is reduced by more than 50% [Pawlak et al., 2000]. Other mammalian Type I enzymes, PRMT3 [Tang et al., 1998] and coactivator-associated arginine methyltransferase (CARM1/PRMT4) [Chen et al., 1999], both display properties distinct from PRMT1. RMT1, the only known yeast Type I methyltransferase, was cloned based on its similarity to PRMT1 [Gary et al., 1996], and independently, by a genetic screen for mutations that interact with Npl3p, an hnRNP-like protein [Henry and Silver, 1996]. Deletion of the yeast *RMT* gene reduced the levels of asymmetric dimethylarginine by 85% without affecting cell viability [Gary et al., 1996].

Type I enzymes have been implicated in a number of processes including signal transduction [Lin et al., 1996; Abramovich et al., 1997; Lim et al., 1998; Altschuler et al., 1999], transcriptional regulation [Tang et al., 2000b; Mowen et al., 2001; Strahl et al., 2001; Wang et al., 2001] and protein trafficking [Shen et al., 1998; Yun and Fu, 2000]. However, the biochemical and biological functions of arginine methylation have not been determined. Prmt1 preferentially methylates RGG box motifs, a common feature of RNA binding proteins [Liu and Dreyfuss, 1995], suggesting a mechanism to modulate protein–protein and protein–RNA interactions. Levels of protein methylarginine may change in response to extracellular stimuli under circumstances in which biological responses are also suppressed by methyltransferase inhibitors. These include NGF-induced neurite outgrowth in PC12 cells [Cimato et al., 1997], and mitogenic responses of LPS-treated B cells [Kim and Yang, 1991].

Interactions between PRMT1 and potential signaling proteins have emerged from yeast two-hybrid screens. For example, TIS21 (BTG2) and BTG1 interact with PRMT1 and and appear to modulate its enzymatic activity in vitro [Lin et al., 1996; Lim et al., 1998]. TIS21 and BTG1 both belong to a family of mitogen-induced proteins implicated in negative regulation of the cell cycle. PRMT1 binds to the cytoplasmic domain of the interferon α,β receptor, and appears to influence interferon signaling [Abramovich et al., 1997; Altschuler et al., 1999].

Modification of cellular proteins by arginine methylation also appears to play a role in transcription. Methylation of the Stat1 transcription factor enhances its function by suppressing interactions with an inhibitory protein, PIAS1 [Mowen et al., 2001]. Interleukin enhancer binding factor 3 (ILF3) binds PRMT1 and inhibits the methyltransferase activity of the enzyme [Tang et al., 2000b]. CARM1/PRMT4 associates with p160 coactivators and serves as a secondary coactivator of nuclear hormone receptors [Chen et al., 1999]. PRMT1 also has coactivator activity [Wang et al., 2001]. Transcriptional coactivation may stem from the ability of CARM1/PRMT4 and PRMT1 to methylate histones H3 and H4, respectively, thereby enhancing histone acetylation [Schurter et al., 2001; Strahl et al., 2001; Wang et al., 2001].

While arginine methylation may influence the function of a variety of proteins, the significance of the modification as a mechanism for regulating protein functions has not been resolved. A major problem concerns the possibility that arginine methylation may be both constitutive and irreversible. Well-characterized substrates, fibrillarin and nucleolin exist largely in a fully methlyated state [Lischwe et al., 1985a,b]. Similarly, we previously showed that most substrates of the Prmt1 enzyme appear to be fully methylated in normal cells but accumulate in a hypomethylated state following disruption of the *Prmt1* gene. This analysis did not exclude the possibility that specific substrates are hypomethylated in wild-type cells or that the methylation status of individual proteins may change in response to different physiological conditions. Hypomethylated proteins may also exist only transiently, e.g., during a specific step in a biochemical process. However, this latter possibility would require an arginine demethylase, an activity that has not yet been identified in mammalian cells [Gary and Clarke, 1998; McBride and Silver, 2001].

In the present study, we examined Prmt1 activity and substrate specificity in wild-type and Prmt1-deficient cells. Recombinant Prmt1 enzymes were used to identify hypomethylated substrates induced either by disruption of the *Prmt1* gene or by treatment with the methyltransferase inhibitor, adenosine dialdehyde (AdOx). The methylation status of individual substrates was assessed under normal

growth conditions and at different stages of the cell cycle. Methylation of cellular proteins in vitro by recombinant Prmt1 appeared to be limited to authentic substrates, i.e., proteins rendered hypomethylated by disruption of the *Prmt1* gene. Moreover, methylation of the most abundant Prmt1 substrates in vivo appeared to be extensive and constitutive. Thus while methylation of cellular proteins by Prmt1 is highly specific and selective, the modification does not appear to play a dynamic role in regulating protein function under normal growth conditions.

MATERIALS AND METHODS

Cell Culture and Protein Extracts

ES cells were maintained on 0.1% gelatinized tissue culture plates in high glucose Dulbecco Modified Eagle's Medium (Invitrogen, Carlsbad, CA) supplemented with 15% preselected fetal bovine serum (Hyclone; heat inactivated at 55°C for 30 min), 0.1 mM 2-mercaptoethanol, 100 mM nonessential amino acids (Invitrogen), and 1,000 U/ml of leukemia inhibitory factor (ESGRO, Invitrogen). Cultures were trypsinized every 2 days and were replated at a 1:3 ratio on feeder layers of gamma-irradiated MEF's. To prepare cell extracts, the cells were passaged at least three times without feeder layers.

For whole cell extracts, cells at 75% of confluency were washed twice and scraped in PBS, pelleted, and resuspended in 1 ml/10 cm plate of reaction buffer (50 mM Tris-HCl pH 7.6, 0.1 mM EDTA, 0.1 mM DTT, and 1 mM PMSF). All steps were performed at 4°C. Alternatively 10^7 HB101 colorectal carcinoma cells fractionated via centrifugal elutriation were resuspended in 300 μl of the same buffer. Cell suspensions were sonicated on ice with two 15 s bursts using a microtip sonicator (model XL2015, Heat Systems, Farmingdale, NY) at a setting of 4.0, clarified by centrifugation at 10,000*g* for 30 min, and the supernatants were flash frozen in an ethanol/dry ice bath. Protein concentrations were determined by modified Lowry assay (DC Protein Assay, BioRad, Hercules, CA) using bovine serum albumin as a standard.

Expression of Recombinant Proteins

Prmt1^{353} and Prmt1^{371} cDNAs were subcloned into the *Eco*R1 sites of pVL1392 and pAcHLT-B baculovirus transfer vectors (BD PharMingen, San Diego, CA) to express native and His_6-tagged proteins, respectively. Two micrograms of each recombinant transfer vector was cotransfected with 0.5 μg of linearized AcNPV Baculovirus DNA (BD PharMingen) into 2×10^6 *Sf9* cells. Methods for the construction and propagation of recombinant baculovirus vectors, protein expression, and purification of His_6-tagged Prmt1 proteins were as described in Pharmingen baculovirus system manual. Protein expression was monitored by Western blot analysis [Pawlak et al., 2000] by using a polyclonal anti-rat PRMT1 antibody. The purified enzymes were concentrated to 1 mg/ml using Centricon 10 (Amicon) spin cartridges, dialyzed against two, 1 L changes of reaction buffer containing 50% glycerol and stored at −80°C.

Methylatransferase Assays

Methyltransferase activities of recombinant Prmt1 enzymes were assayed at 36°C in reactions containing 50 μM S-adenosyl-L-[*methyl*-^{3}H]methionine (5,000 dpm/pmol, Amersham Pharmacia Biotech, Piscataway, NJ), 3 μg of purified enzyme, and either 120 μg of histone, or 60 μg of cell extract in 150 μl of reaction buffer. At each time point, 20 μl aliquots were withdrawn and the samples were mixed with an equal volume of 2 × SDS sample buffer and heated to 100°C for 5 min to stop the reactions. The samples were diluted with 1 ml of PBS and protein [^{3}H]-*methyl* was determined via a filter-binding assay [Liu and Dreyfuss, 1995].

In vitro methylation of whole cell extracts [20 μg of either wild-type, Prmt1$^{-/-}$, (AdOx)-treated (20 μM for 48 h), or elutrated cells as a source of substrates] contained 50 μM S-adenosyl-L-[*methyl*-^{3}H]methionine (5,000 dpm/pmol, Amersham-Pharmacia), and either 1 μg of purified Prmt1 enzymes or 10 μg of lysates from baculovirus-infected insect cells or 10 μg of elutrated cell lysates in a final volume of 50 μl. Methylation reactions were performed for 30 min at 36°C, and the methylated proteins were detected by fluorography following 12% SDS–PAGE. Fluorographs were exposed at −80°C for 1 week.

For in vivo methylation of cellular proteins, one 75% confluent, 150 mm diameter plate of either wild type, or Prmt1$^{-/-}$ mutant ES cells was incubated with cycloheximide (100 μg/ml), and chloramphenicol (40 μg/ml) in ES cell

medium for 30 min. Subsequently, the medium was replaced with methionine-free ES cell medium supplemented with 10 μCi/ml of L-[*methyl*-^{3}H]methionine (Amersham Pharmacia Biotech), and cells were incubated for additional 3 h in the presence of the same concentrations of protein synthesis inhibitors. The cells were washed twice, scraped into PBS, pelleted, resuspended in 1 ml of buffer A (10 mM Tris-Cl pH 7.6, 100 mM NaCl, 2.5 mM $MgCl_2$) containing 0.5% Triton X-100, 10 μg leupeptin, 10 μg antipain, 10 μg pepstatin A, 10 μg chymostatin, and 10 mM DTT, and homogenized with 15 strokes in a dounce homogenizer. After centrifugation at 3,000*g* for 15 min, the nuclear pellet was washed twice with 0.5 ml buffer A, resuspended in 300 μl of the same buffer, and sonicated as described above. Aliquots of the supernatant and pellet fractions containing 25 μg of protein were mixed with an equal volume of 2 × SDS sample buffer, boiled for 5 min, and fractionated on a 12% SDS–PAGE. Gels were fluorographed and exposed at −80°C for 2 weeks. Inhibition of protein synthesis was assessed in parallel experiments in which the cells were labeled with 10 μCi/ml of L-[^{35}S]methionine (Amersham Pharmacia).

Purification of hnRNP Complexes

hnRNP complexes were immunoprecipitated from wild-type and Prmt1$^{-/-}$ cells labeled with L-[^{35}S]methionine (20 μCi/ml in ES cell medium containing 10% of normal methionine levels for 20 h). 4F4 anti-hnRNP C monoclonal antibody [Choi and Dreyfuss, 1984] treated with RNasin (Promega, Madison, WI) and bound to 25 μl of protein A-agarose was incubated with nuclear extracts for 10 min at 4°C with gentle rocking. The beads were washed five times with 1 ml of buffer A and the bound material was eluted into 50 μl of 2D-gel sample buffer. Isoelectric focusing was performed using Immobiline DryStrip Gels (pH 3–10 NL, 70 mm), IPG buffer pH 3–10 NL and IPGphor IEF system (Amersham-Pharmacia). Electrophoresis involved 10% SDS–PAGE (1.5 mm gel thickness) using Mini-Protean II apparatus (BioRad). Gels were fluorographed, dried, and exposed at −80°C for 5 days.

Alternatively hnRNP complexes were purified by glycerol-gradient velocity centrifugation. Cells were harvested from one 15 cm plate as described above and resuspended in 5 ml of ice cold Buffer I (10 mM Tris-Cl pH 8.0, 3 mM $CaCl_2$, 2 mM MgAcetate, 0.1 mM EDTA, 0.1% Triton X-100, 1 mM DTT, and 0.32 M Sucrose). Cells were homogenized with 15 strokes in a Dounce homogenizer; an equal volume of Buffer II (10 mM Tris-Cl pH 8.0, 5 mM MgAcetate, 0.1 mM EDTA, 1 mM DTT, and 2 M Sucrose) was added, and the nuclei were centrifuged (30,000*g* for 45 min at 4°C) through an equal volume of Buffer II. The pelleted nuclei were washed twice with ice-cold STM buffer (20 mM Tris-Cl pH 8.0, 90 mM NaCl, 1 mM $MgCl_2$) and resuspended in 1 ml/5×10^7 nuclei of STM buffer containing 0.5 mM PMSF and sonicated as described above. The samples were then incubated at 37°C for 5 min, chilled on ice and centrifuged at 1,000*g* for 10 min at room temperature. The resulting supernatants were layered on top of 15–30% glycerol gradient containing STM buffer, 0.5 mM PMSF, and 1 μl/ml RNasin (Promega) and centrifuged at 25,000 rpm for 18 h. The gradients were fractionated into sixteen 1 ml fractions while monitoring UV absorbance at 254 nm. Proteins in each fraction were precipitated with 3 ml of absolute ethanol, resuspended in 2 × SDS sample buffer, resolved on 12% SDS–PAGE, and stained with Coomassie blue. Alternatively, to analyze hnRNP proteins methylated in vivo, hnRNP complexes were isolated from ES cells labeled with L-[*methyl*-^{3}H]methionine as described above and the resulting protein gels were fluorographed and exposed at −80°C for 2 weeks.

Analysis of Protein Dimethylarginine

Whole-cell and nuclear extracts and glycerol gradient fractions corresponding to 40S hnRNP complexes were isolated as described above. Asymmetric dimethylarginine content in each sample was analyzed via HPLC as described previously [Pawlak et al., 2000].

RESULTS

In Vitro Methylation of Proteins Extracted From Prmt1$^{-/-}$ Cells

Alternative splicing of the *Prmt1* gene generates coding sequences for two enzymes of 353 and 371 amino acids, designated Prmt1^{353} and Prmt1^{371}, respectively. Both enzymes were expressed using baculovirus vectors (Fig. 1A,B) either in native form or as 6 ×His-tagged proteins. Following Ni^{2+} affinity chromatography, the His_6-Prmt1 proteins were tested for methyltransferase activity in vitro, using

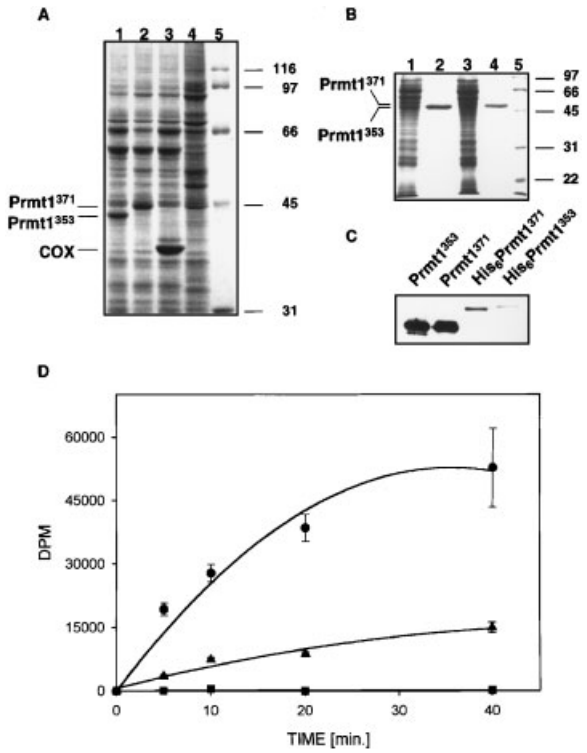

Fig. 1. Expression and activity of recombinant Prmt1 enzymes. **A**: Lysates from insect cells infected with Prmt1 Baculovirus expression vectors were fractionated by SDS–PAGE and stained with coomassie blue. Lysates were from cells expressing Prmt1^{353} (**lane 1**), Prmt1^{371} (**lane 2**), a control protein (**lane 3**), or from uninfected cells (**lane 4**), and were compared to molecular weight standards (**lane 5**). **B**: SDS–PAGE analysis of Baculovirus expressed His$_6$-tagged Prmt11 isoforms. Samples include lysates from cells expressing His$_6$-Prmt1^{353} (**lane 1**) and His$_6$-Prmt1^{371} (**lane 3**), purified His$_6$-Prmt1^{353} (**lane 2**), and His$_6$-Prmt1^{371} (**lane 4**), and molecular weight standards (**lane 5**). **C**: Western blot analysis of native and His$_6$-tagged Prmt11 enzymes. **D**: Methyltransferase activity of purified His$_6$-tagged Prmt11 isoforms, showing in vitro methylation of a histone substrate by His$_6$-Prmt1^{353} (circles), His$_6$-Prmt1^{371} (triangles), or a control protein, XylE (squares).

^{3}H-S-adenosyl methionine (^{3}H-AdoMet) as a methyl donor and histone as a substrate (Fig. 1C). Prmt1^{353} consistently displayed higher specific activity (4 pmoles CH_3 incorporated/min/μg enzyme) than Prmt1^{371} (0.8 pmole/min/μg enzyme) and transferred about four times more CH_3- into histone (1.6 nmoles/mg) in a 40 min reaction. Similar results were obtained with reactions using unpurified native Prmt1 enzymes present in insect cell lysates (not shown).

Previous studies demonstrated that Prmt1 substrates in *Prmt1*$^{-/-}$ ES cells are hypomethylated and can be methylated in vitro by using extracts from wild-type ES cells as a source of enzyme [Pawlak et al., 2000]. By contrast, proteins from wild-type cells displayed little methyl-acceptor activity, suggesting that potential substrates in wild-type cells exist largely in a fully methylated state. Similarly, wild-type cell lysates incorporated only low levels of label (530 and 400 pmoles/mg cell protein) when incubated with a large excess (1 μg) of purified His$_6$-Prmt1 enzymes (Fig. 2A). By contrast, the methyl acceptor activity of *Prmt1*$^{-/-}$ extracts was 14- to 16-fold higher (8.5 and 7.3 nmoles/mg cell protein) with the Prmt1^{353} and Prmt1^{371} isoforms, respectively (Fig. 2B). The reaction rates (V_i) of Prmt1^{353} and Prmt1^{371} were also much higher when assayed against *Prmt1*$^{-/-}$ cell proteins than with the histone substrate (6.0 and 2.9 pmoles/min, respectively). This is despite the fact that the individual substrates represent a small proportion of the protein in whole-cell lysates.

Mutant lysates also showed higher levels of methylation by the XylE control than wild-type cell lysates. This could reflect the presence of

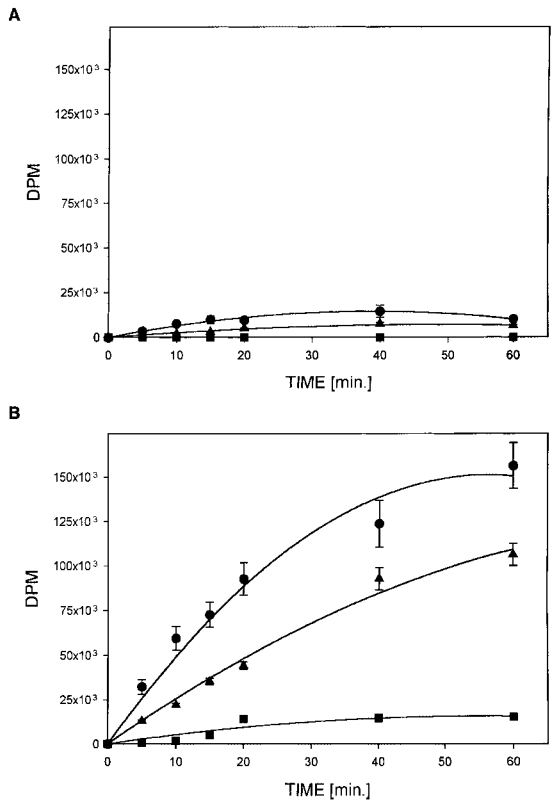

Fig. 2. In vitro methylation of wild-type and Prmt1-deficient ES cell lysates. Twenty micrograms of either wild-type (**A**) or *Prmt1*$^{-/-}$ (**B**) cell extracts were labeled in methyltransferase reactions containing 1 μg of purified His$_6$-Prmt1^{353} (circles), His$_6$-Prmt1^{371} (triangles), or a control protein, XylE (squares).

residual Prmt1 in *Prmt1*$^{-/-}$ cells, which was previously estimated at about 1% of wild-type levels. Alternatively, the hypomethylated substrates may be aberrantly methylated at a low level in vitro by other cellular methyltransferases.

Substrate Specificity of Prmt1 Isoforms

Figure 2 suggests that most potential substrates in wild-type cells are blocked by prior methylation but are substantially hypomethylated in *Prmt1*$^{-/-}$ cells. However, this analysis does not exclude the possibility that individual substrates are differentially hypomethylated in wild-type cells. To examine individual Prmt1 substrates, cell extracts from both wild-type and *Prmt1*$^{-/-}$ ES cells were labeled in vitro using either purified recombinant His_6-Prmt1 proteins or unpurified native enzymes, and the reaction products were analyzed by polyacrylamide gel electrophoresis (Fig. 3). Consistent with the hypothesis that most substrates in wild type are fully methylated, individual proteins were not labeled by Prmt1 (lanes 7–10), with the exception of two lightly labeled polypeptides of 34.5 and 36 kDa (lane 8). By contrast, 10 proteins from *Prmt1*$^{-/-}$ cell extracts were heavily methylated in vitro, and a number of other proteins were labeled to a lesser extent (lanes 11–14). While the His_6-Prmt1 isozymes modified similar sets of proteins of 32, 34.5, 36, 38, 39, 43, 65, 79, and 115 kDa (lanes 13 and 14), unpurified native Prmt1 enzymes expressed in insect cells had different substrate specificities (lanes 11 and 12). Thus, Prmt1^{353} predominantly methylated proteins of 29, 31, 32, and 72 kDa, whereas, substrates of Prmt1^{371} had apparent molecular weights of 31, 32, 34.5, 35, 38, 39, 40, 44, 65, 72, and 79 kDa. The differences in methylation patterns were not due to extraneous methyltransferases in the insect cell lysates, since lysates either from uninfected cells (lanes 3 and 4) or from cells infected with a baculovirus vector expressing an irrelevant protein (lanes 5 and 6) lacked detectable methyltransferase activity. Moreover, differences between the native enzymes did not result from interactions with insect proteins, as unpurified His_6-tagged Prmt1 enzymes in cell lysates had the same, identical specificity as the purified enzymes (Fig. 4). Therefore, it appears that each native Prmt1 isoenzyme has a distinct specificity for different substrates, which is lost by the addition of an N-terminal His_6 tag.

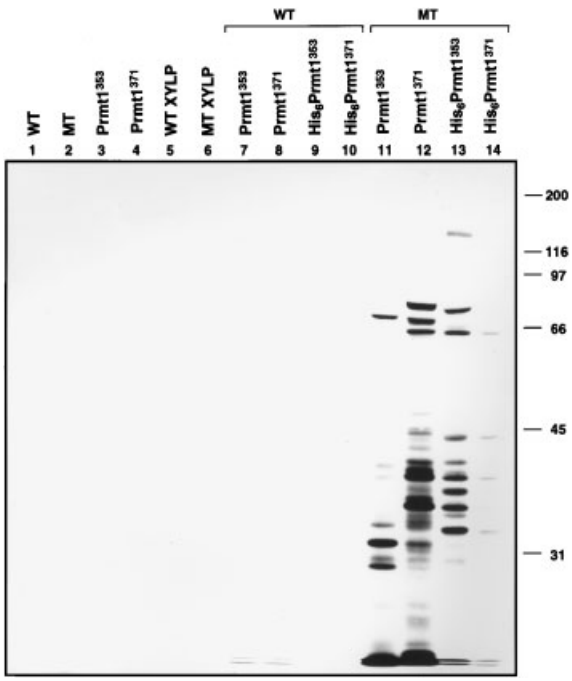

Fig. 3. In vitro methylation of cellular proteins from wild-type and Prmt1-deficient cells. Twenty micrograms of lysates from wild-type (**lanes 7–10**) or *Prmt1*$^{-/-}$ (**lanes 11–14**) cells were incubated with either 10 μg of insect cell lysates expressing native Prmt1 isoforms (lanes 7, 8, 11, 12) or 1 μg of purified His_6-tagged Prmt1 isoforms in the presence of ^{3}H-AdoMet. Control reactions consisted of cell lysates alone (**lanes 1** and **2**), insect cell lysates expressing recombinant Prmt1 isoforms alone (**lanes 3** and **4**), and wild-type or mutant lysates incubated with a control protein, XylE (**lanes 5** and **6**). Reaction products were fractionated by SDS–PAGE and visualized by fluorography.

Comparison of In Vivo and In Vitro Methylation Products in Wild-Type and *Prmt1*$^{-/-}$ ES Cells

The specificity of Prmt1 enzymes is illustrated by their failure to methylate proteins from wild-type cells, even in the presence of a large excess of enzyme. Since proteins from wild-type cells are not gratuitously methylated, proteins methylated in mutant cell extracts appear to be physiological substrates. Nevertheless, it is possible that the activity or specificity of the enzyme in vivo may differ from that of the purified enzyme in vitro, particularly since cellular Prmt1 activity appears to co-purify with large protein complexes. To examine the substrate specificity of endogenous methyltransferases in vivo, wild-type and *Prmt1*$^{-/-}$ ES cells were metabolically labeled with L-[*methyl*-^{3}H]methionine, which provides [^{3}H]CH_3- groups to AdoMet, and labeled nuclear and cytoplasmic proteins were fractionated by SDS–PAGE and detected by fluorography [Liu and Dreyfuss, 1995].

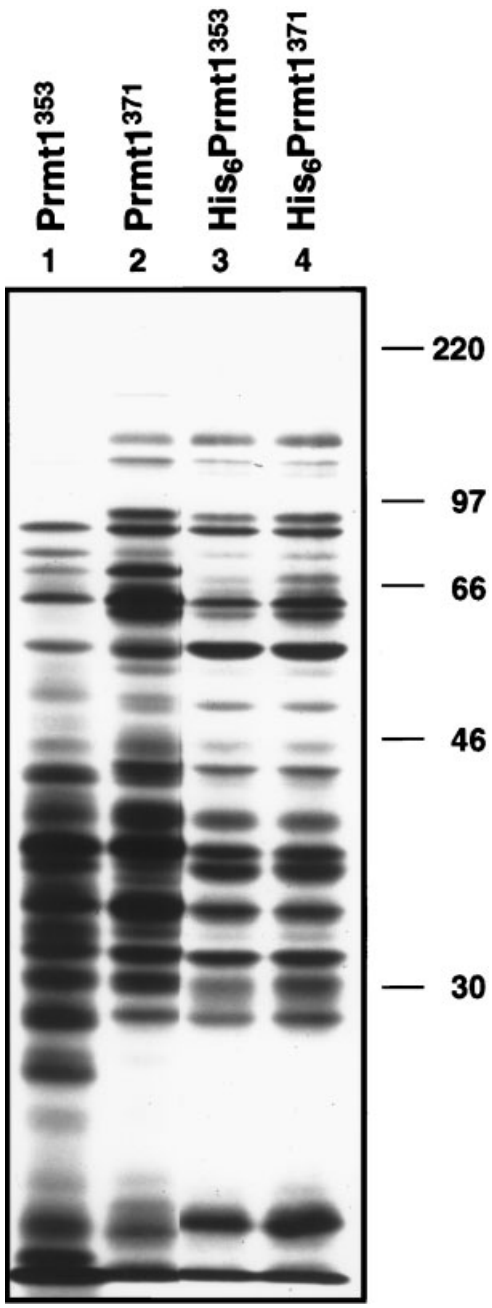

Fig. 4. Substrate specificity of native and His_6-tagged Prmt1 isoforms. Twenty micrograms of *Prmt1*$^{-/-}$ cell lysates were incubated with 10 μg of lysates from insect cells expressing either native (**lanes 1** and **2**) or His_6-tagged (**lanes 3** and **4**) Prmt1 isoforms in the presence of ^{3}H-AdoMet. Reaction products were resolved by SDS–PAGE and visualized by fluorography.

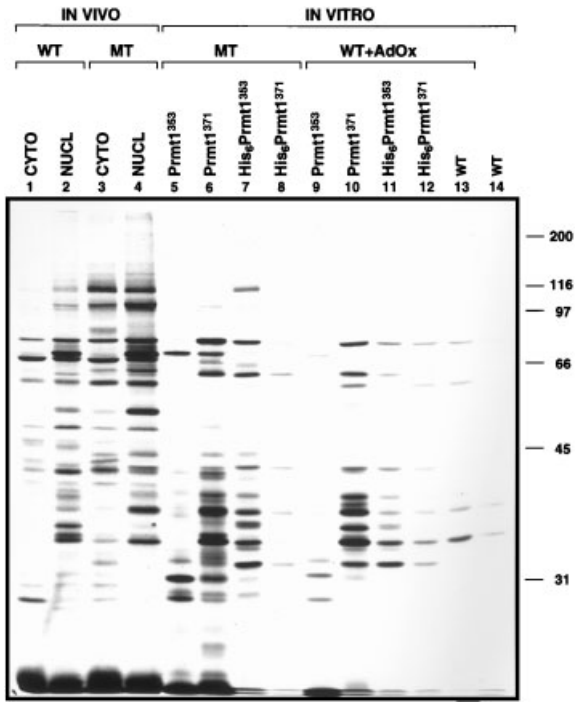

Fig. 5. In vivo and in vitro methylation of proteins in wild type, *Prmt1*$^{-/-}$ and adenosine dialdehyde (AdOx)-treated cells. Methyltransferase substrates in wild-type (**lanes 1** and **2**) and mutant (**lanes 3** and **4**) cells were labeled in vivo and were separated into cytoplasmic (CYTO) and nuclear (NUCL) fractions. In vitro methylation reactions used 20 μg of lysates from either *Prmt1*$^{-/-}$ (**lanes 5–8**) or AdOx-treated wild-type (**lanes 9–12**) cells together with either 10 μg of insect cell lysates expressing native Prmt1 isoforms (lanes 5, 6, 9, 10) or 1 μg of purified His_6-tagged Prmt11 isoforms (lanes 7, 8, 11, 12). **Lanes 13** and **14** correspond to reactions containing AdOx-treated and untreated wild-type cell lysates without additional Prmt1 enzymes.

The cells were also treated with cyclohexamide, which in separate experiments completely blocked the translational incorporation of methionine into proteins (data not shown). As shown in Figure 5, the majority of substrates labeled in vitro (lanes 5–8) were also methylated in vivo (lanes 1–4). Interestingly, proteins in mutant cells (lanes 3–4) were labeled as well as those in wild-type cells (lanes 1–2). This probably reflects the balance between levels of enzyme and available substrate in the two cell types. Thus, while mutant cells express only residual Prmt1 activity, they also contain large pools of hypomethylated substrates. By contrast, the existing methylated substrates in wild-type cells can not be radiolabeled, and the production of newly synthesized (i.e., unmethylated) proteins is limited in cyclohexamide-treated cells.

The specificity of recombinant Prmt1 enzymes was also examined by using hypomethylated substrates from ES cells treated for 24 h with 20 μM AdOx. AdOx inhibits all cellular methyltransferases that use AdoMed as a methyl donor, increasing the levels of their hypomethylated substrates [Li et al., 1998; Najbauer and Aswad, 1990]. Recombinant Prmt1 enzymes

modified similar proteins in vitro regardless of whether AdOx-treated or *Prmt1*$^{-/-}$ cell extracts were used as a source of substrates (Fig. 5, compare lanes 5–8 and 9–12).

The greatest number and variety of methylated proteins were observed in cells labeled in vivo with [CH_3-^{3}H]methionine. For example, a number of high molecular weight proteins (lanes 1–4) and a prominently labeled 55 kDa protein (lanes 2–4) were not labeled in vitro by Prmt1. These proteins are presumably modified by methyltransferases other than Prmt1. Similarly, methylation of a 60 kDa polypeptide (Fig. 5, lanes 10–13) did not require the addition of Prmt1 enzyme (lane 13). A few proteins from mutant cell extracts (e.g., the 32 kDa band in lanes 5, 6, 9) were methylated to a greater extent in vitro than in vivo or in wild-type AdOx-treated cell extracts. These may represent relatively stable, methylated proteins. Most of the prominent bands labeled in vivo appear to represent Prmt1 substrates, as previously reported [Najbauer and Aswad, 1990]. This may reflect the relative abundance of the proteins, specific activities of the enzymes, and turnover rates of the substrates and/or methyl groups. Moreover, products of carboxymethylation are base labile and are not expected to survive heating in alkaline SDS loading buffer.

Formation of hnRNP Complexes Does Not Require Prmt1 Activity

In principle, the methylation of arginines within RGG box domains of hnRNPs could affect protein–protein interactions and cooperative RNA binding [Cobianchi et al., 1988; Rajpurohit et al., 1994]. Therefore, we investigated whether the lack of Prmt1 activity in *Prmt1*$^{-/-}$ cells affected the composition of hnRNP complexes. Nuclear components from the mutant and wild-type cells were extracted following sonication and mild RNase digestion and were fractionated by velocity sedimentation. As shown in Figure 6, samples from both mutant and wild-type cells produced similar symmetrical peaks of optical density corresponding to the position (arrow) of 40S hnRNP particles [Beyer et al., 1977]. When the fractionated gradients were analyzed by SDS–PAGE, peak RNA-containing fractions were enriched for major hnRNP complex proteins, migrating in the 30–66 kDa molecular weight range, (Fig. 6, bottom). The identity of individual proteins corresponding to hnRNPs A1, A2, B1, B2, C1, C2, L, and U was confirmed by analyzing tryptic digests of gel-purified proteins by mass spectrometry and by Western blot analysis (data not shown).

The gradient fractions were also compared to hnRNP complexes isolated from wild-type and mutant cells labeled in vivo with L-[*methyl*-^{3}H]-methionine (Fig. 6). Proteins present in peak RNA-containing fractions were methylated to a higher extent in mutant cells than in wild-type cells. The predominant labeled proteins associated with complexes isolated from wild-type cells were 34.5 and 36 kDa in size, while those from mutant cells were 34.5, 38, 40, and 65 kDa. These proteins co-migrated with the major substrates modified by Prmt1 both in vivo and in vitro (Fig. 5), and based on size, they appeared to be type A, B, and L hnRNPs.

HnRNP complexes from wild-type and *Prmt1*$^{-/-}$ ES cells were also analyzed by 2D-gel electrophoresis (Fig. 7). The samples were prepared by labeling cells with ^{35}S-methionine, followed by immunoprecipitation of hnRNP complexes with the 4F4 anti-hnRNP C monoclonal antibody. Since hnRNP C is not methylated, this antibody was selected to reduce the possibility that the epitope would be affected by the loss of Prmt1 activity which could result in the under-recovery of hypomethylated complexes from mutant cells. While a number of other hnRNP proteins are methylated by Prmt1, the composition of hnRNP complexes, at least those containing hnRNP C, appeared to be unaffected by the loss of Prmt1 activity.

The presence of some methylated hnRNPs in mutant cells raised the possibility that these proteins are selectively incorporated into hnRNP complexes. If methylation is required for complex formation, then hnRNP complexes in wild-type and mutant cells should be methylated to a similar extent. To test this possibility, hnRNP complexes isolated from both *Prmt1*$^{-/-}$ and wild-type cells were analyzed for asymmetric dimethylarginine content following acid hydrolysis and HPLC (Fig. 8). Prmt1, the major mammalian Type I arginine methyltransferase (specific for RGG substrates), is responsible for over half of the asymmetric N^G,N^G-dimethylarginine in cells, whereas, other methyltransferases generate symmetric dimethylarginine. All of the arginine methyltransferases, including Prmt1, also produce monomethylarginine. As shown in Figure 8, the levels of N^G,N^G-dimethylarginine incorporated

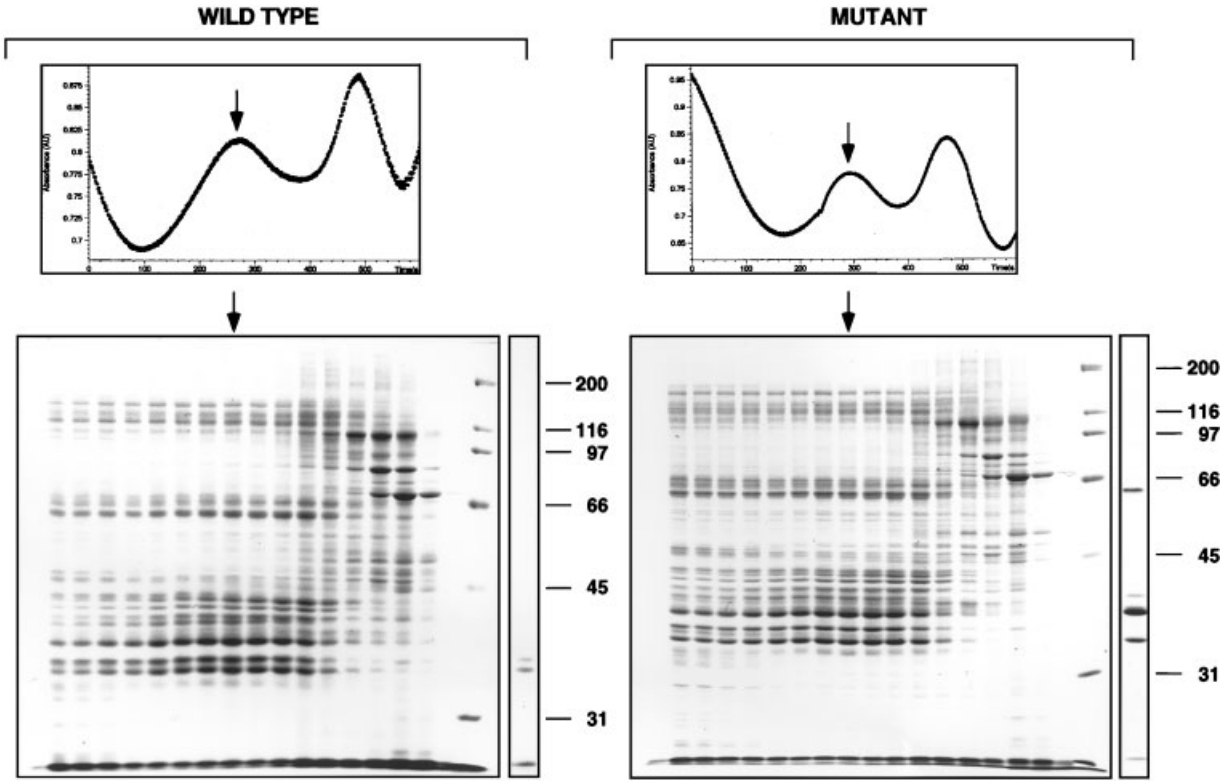

Fig. 6. Sucrose gradient analysis of 40S hnRNP particles from wild-type and *Prmt1*$^{-/-}$ ES cells. Nuclear extracts were sedimented on 15–30% sucrose gradients for 18 h at 25,000 rpm and were analyzed as described in Materials and Methods. In the upper panels, the distribution of RNA in the gradients was determined by measuring the absorbance at 254 nm as the gradients were fractionated. In the lower panels, the proteins in each fraction were analyzed by SDS–PAGE and stained with coomassie blue. The positions of 40S hnRNP complexes are indicated by vertical arrows. The direction of sedimentation was from right to left. Proteins in each peak fraction that could be labeled in vivo are shown in separate lanes to the right of the stained gels.

into proteins associated with hnRNP complexes was reduced by 70% in *Prmt1*$^{-/-}$ mutant cells. For comparison, the N^G,N^G-dimethylarginine content of whole-cell and nuclear extracts from *Prmt1*$^{-/-}$ cells was reduced by 50 and 60%, respectively. This indicates that hnRNP complexes are enriched for Prmt1 substrates and that hypomethylated hnRNP proteins are capable of forming structurally normal hnRNP complexes.

Prmt1 Activity and Substrate Methylation are not Regulated During the Cell Cycle

While most Prmt1 substrates in wild-type cells appear to be constitutively and completely methylated, the methylation status of individual substrates may change in response to specific physiological conditions. For example, the content of N^G, N^G-dimethylarginine in cellular proteins and arginine methyltransferase activity has been reported to vary with the cell cycle [Lee and Paik, 1972; Chang et al., 1978]. Therefore, two types of experiments were performed to assess whether Prmt1 activity is modulated in a cell cycle-dependent manner.

First, human colon cancer cells (HCT116) were fractionated according to their cell cycle stage by centrifugal elutriation (Fig. 9A), and extracts from each fraction were tested for methyl-acceptor activity in vitro. Except for a single 49 kDa methyl-accepting protein, proteins from HCT116 cells had negligible methyl-acceptor activity (Fig. 9B), with levels comparable to those of wild-type ES cells (Fig. 3). The extent of protein hypomethylation was slightly higher in G1 and G2 phase cells. However, the degree of hypomethylation was far less than in Prmt1$^{-/-}$ cells (Fig. 9B, lane MT) and may result from increased protein synthesis during these phases of the cell cycle. While the 49 kDa protein was not labeled in the absence of recombinant Prmt1 (data not shown), the protein may be a gratuitous substrate, since a similar protein was not labeled in extracts from

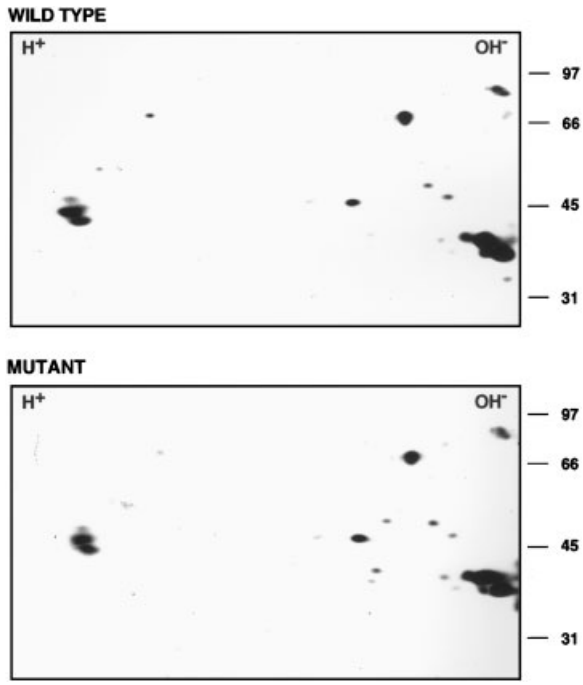

Fig. 7. Protein composition of hnRNP complexes from wild-type and *Prmt1*$^{-/-}$ ES cells. hnRNP complexes were immunopurified with a monoclonal antibody (4F4) to hnRNP C1/C2 from nuclei of [^{35}S]-methionine-labeled ES cells. Immunoprecipitated proteins were separated by 2D-gel electrophoresis and detected by fluorography.

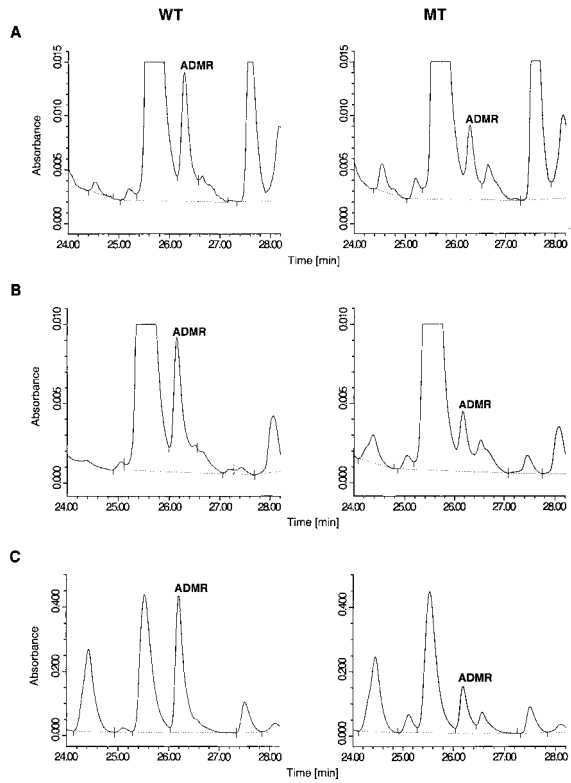

Fig. 8. Asymmetric NG,NG-dimethylarginine (ADMR) content in nuclear and 40S hnRNP fractions from Prmt1-deficient cells. Proteins from whole cell lysates (**A**), nuclei (**B**), or 40S hnRNP particles (**C**) were prepared from wild-type (WT) and *Prmt1*$^{-/-}$ (MT) ES cells and were subjected to acid hydrolysis and analyzed for ADMR content by high-pressure liquid chromatography.

Prmt1$^{-/-}$ cells (Fig. 9B, lane MT). In any event, no protein appeared to be specifically or substantially hypomethylated during any stage of the cell cycle.

Second, extracts from elutriated HC116 cells were tested for Prmt1 enzyme activity, using *Prmt1*$^{-/-}$ cell extracts as a source of hypomethylated substrates. As shown in Figure 9C, all elutriated cell fractions expressed Prmt1 activity and methylated proteins similar in size to those modified by recombinant Prmt1. Methyltransferase activity was highest during G1 and declined slightly, but reproducibly, through S, G2, and M. The variation in enzyme activity was not uniform, with methylation of 39 and 65 kDa substrates showing somewhat greater levels of decline.

DISCUSSION

We previously characterized a recessive, embryonic lethal mutation in the murine *Prmt1* gene [Pawlak et al., 2000]. Although the enzyme is required for early post-implantation development, Prmt1-deficient ES cell lines could be established from preimplantation embryos. Other arginine methyltransferases were unable to substitute for the loss of Prmt1 in mutant cells, and consequently, substrates of the enzyme accumulated in a hypomethylated state. In contrast, the major Prmt1 substrates in normal cells appeared to be extensively and constitutively methylated, both under normal growth conditions and at different stages of the cell cycle. These results suggest that methylation by Prmt1 does not play a dynamic role in regulating protein function under normal growth conditions.

A number of mammalian protein arginine methyltransferases have been characterized. As with earlier immunodepletion experiments, our genetic studies suggest that Prmt1 is responsible for most of the arginine methyltransferase activity in mammalian cells, at least when assayed on RGG-containing substrates. However, levels of asymmetric dimethylarginine were reduced by just over 50% in Prmt1-deficient cells suggesting that other type I enzymes make significant contributions to the total asymmetric dimethylarginine content of

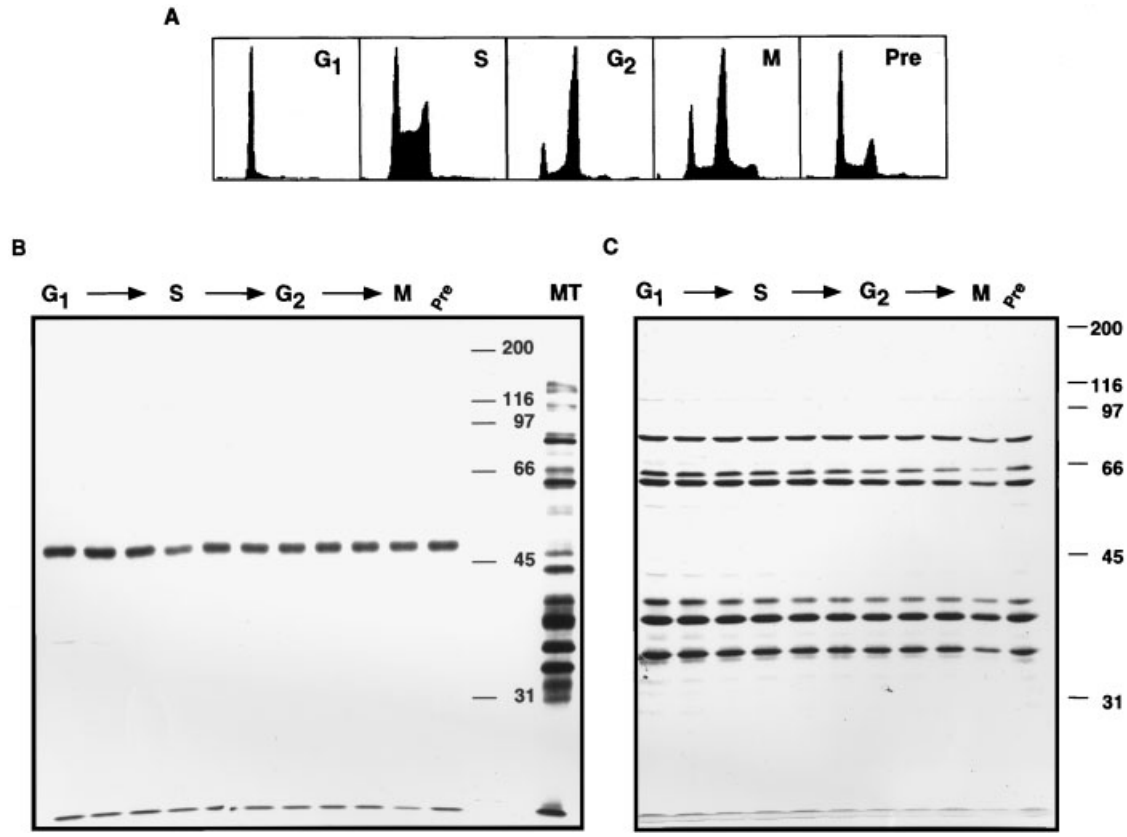

Fig. 9. Analysis of Prmt1 activity and substrate methylation during the cell cycle. **A**: HCT116 cells were fractionated by centrifugal elutriation and analyzed for DNA content by flow cytometry. Peak fractions from cells in the G1, S, G2, and M phases of the cell cycle are shown along with non elutriated (Pre) cells. **B**: Changes in the methylation status of Prmt1 substrates during the cell cycle were analyzed by in vitro methylation. Each reaction contained 20 μg of total cellular protein from each elutriated HCT116 fraction and 1 μg of His_6-$Prmt1^{353}$. Samples from peak fractions of G1, S, G2, and M phase cells and non-elutriated cells (Pre) are indicated. A control sample (MT) illustrates methylation of proteins from *Prmt1*$^{-/-}$ cells under the same conditions. **C**: Changes in methyltransferase activity during the cell cycle were assessed by testing extracts (5 μg) from elutriated HCT116 cells for their ability to methylate proteins from *Prmt1*$^{-/-}$ cells in vitro. Labeled proteins were fractionated by SDS–PAGE and were visualized by fluorography.

the cell. Such enzymes could include PRMT3 and HRMT1L1, Type I-related enzymes with little activity for RGG substrate.

Prmt1 substrates expressed in *Prmt1*$^{-/-}$ cells could be identified based on their capacity to be methylated in vitro by recombinant Prmt1 enzymes. Proteins of similar molecular weights were also metabolically labeled in vivo, as expected of physiological substrates. Nearly all proteins that could serve as substrates for in vitro methylation were hypomethylated to a much greater extent in *Prmt1*$^{-/-}$ cells than in wild-type cells. Moreover, even high levels of recombinant enzyme did not gratuitously modify most normal cellular proteins. While other methyltransferases did not significantly methylate Prmt1 substrates, neither did recombinant Prmt1 modify the substrates of other enzymes rendered hypomethylated by treating cells with methyltransferase inhibitors. Together, these results suggest that Prmt1 and other arginine methyltransferases display a high degree of substrate specificity.

Alternatively-spliced *Prmt1* transcripts encode proteins of 353 and 371 amino acids. Both proteins are phylogenetically conserved, suggesting that they have distinct functions, possibly related to substrate specificity or regulation of enzyme activity. Prmt1 proteins containing an amino-terminal His_6 sequence showed no differences in substrate specificity, as previously reported for human enzymes modified with an N-terminal GST tag [Gary and Clarke, 1998]. However, the native enzymes methylated different, but overlapping sets of proteins in Prmt1$^{-/-}$ cell extracts, providing the first evidence for functional differences between the $Prmt1^{353}$ and $Prmt1^{371}$ enzymes.

Modification of the N-terminus apparently abolishes substrate specificity conferred by the nearby alternative exon, while having little effect on enzyme activity.

HnRNP proteins are among the major substrates of Prmt1 [Liu and Dreyfuss, 1995]. hnRNP particles isolated from Prmt1 mutant cells were similar in size and protein composition to those of wild-type ES cells. The fact that hnRNPs co-sedimented with RNA suggest that nucleic acid binding was not severely altered, though dimethylarginine content was reduced by over 70%. The composition of hnRNP complexes immunopurified from *Prmt1*$^{-/-}$ cells also appeared unaffected, suggesting that arginine methylation by Prmt1 is not a prerequisite for hnRNP complex formation.

We also investigated whether Prmt1 activity or methylation status of Prmt1 substrates is regulated during the cell cycle. The levels of endogenous Prmt1 activity and hypomethylated substrates were highest during G1 phase and lowest during mitosis. However, the variation and extent of hypomethylation were quite small and may simply represent higher rates of protein synthesis in G1 phase of the cell cycle. Greater differences have been reported by other groups. For example, the content of N^G,N^G-dimethylarginine in HeLa ribosomal proteins apparently varies with the cell cycle [Chang et al., 1978], and extent of histone methylation, as well as Type I methyltransferase activity, change in a cyclic manner during hepatic tissue regeneration [Lee and Paik, 1972]. Some variation in the methylation status of cellular proteins may result from increased synthesis of Prmt1 substrates in proliferating cells [Celis et al., 1986; Leser and Martin, 1987; Minoo et al., 1989]. In addition, Type I enzymes other than Prmt1 may be responsible for dynamic changes in protein methylation.

While the methylation of all major Prmt1 substrates appears to be constitutive and extensive, the methylation status of individual proteins may change under other circumstances not examined in the present study. In addition, hypomethylated proteins of low abundance may be difficult to detect using whole-cell or partially fractionated lysates. However, the present study demonstrates that native Prmt1 isoforms have a high degree of substrate specificity and that this specificity can be exploited to detect changes in the methylation status of Prmt1 substrates, should these occur.

Prmt1$^{-/-}$ embryos die shortly after implantation, and Prmt1-deficient ES cells are defective in their ability to differentiate in vitro [Pawlak et al., 2000]. Thus, while arginine methylation by Prmt1 appears to be both constitutive and irreversible, Prmt1 activity is required for some protein functions. The availability of Prmt1-deficient cell lines provides a genetic system to characterize the enzyme, its substrates and metabolic processes they are involved in.

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