

Ribosomal protein S2 is a substrate for mammalian PRMT3 (protein arginine methyltransferase 3)

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PRMT3 (protein arginine methyltransferase 3) is one of four type I arginine methyltransferases that catalyse the formation of asymmetric dimethylarginine. PRMT3 is unique in that its N-terminus harbours a C₂H₂ zinc-finger domain that is proposed to confer substrate specificity. In addition, PRMT3 is the only type I enzyme that is restricted to the cytoplasm. Known *in vitro* substrates for PRMT3 include GST–GAR (a glutathione S-transferase fusion protein containing the glycine- and arginine-rich N-terminal region of fibrillarlin), Sam68 (Src-associated substrate during mitosis 68 kDa) and PABP-N1 [poly(A)-binding protein-N1; PABP2]. Here we report the identification of an *in vivo* substrate for mammalian PRMT3. We found that FLAG-tagged PRMT3 can ‘pull down’ a protein with a molecular mass of 30 kDa from HeLa cell extracts. MS identified this PRMT3-

interacting protein as rpS2 (ribosomal protein S2). *In vitro* studies showed that the zinc-finger domain of PRMT3 is necessary and sufficient for binding to rpS2. In addition, rpS2 is methylated by PRMT3 *in vitro* and is also methylated in cell lines. Deletion analysis of the rpS2 amino acid sequence identified a N-terminal Arg–Gly repeat as the methylation site. Furthermore, both PRMT3 and rpS2 co-sediment with free ribosomal subunits. These studies implicate PRMT3 in ribosomal function and in the regulation of protein synthesis.

Key words: arginine methylation, protein arginine N-methyltransferase 3 (PRMT3), ribosome, ribosomal protein S2 (rpS2), zinc finger.

INTRODUCTION

Arginine methylation is a stable post-translational modification that is found on both nuclear and cytoplasmic proteins [1,2]. This modification is catalysed by a family of enzymes called the PRMTs (protein arginine methyltransferases), of which there are currently seven known members (PRMT1–PRMT7) [3]. A subset of these enzymes (PRMTs 1, 3, 4 and 6) catalyse the formation of aDMA (asymmetric ω -N^G,N^G-dimethylarginine) residues and are classified as type I enzymes [4–6]. The type II enzyme PRMT5 catalyses the formation of symmetric ω -N^G,N^G-dimethylarginine residues [7]. Both enzyme types generate ω -N^G-monomethylarginine intermediates. PRMTs target a wide array of different proteins for post-translational modification. The majority of aDMA residues occur within GAR (glycine- and arginine-rich) domains [2]. Proteins with methylated GAR domains include Sam68 (Src-associated substrate during mitosis 68 kDa), hnRNP K (heterogeneous nuclear ribonucleoprotein K), hnRNP U, ILF3 (interleukin enhancer binding factor 3) and FUS {fusion (involved in t(12;16) in malignant liposarcoma)} [8–10]. Structural information has demonstrated that the substrate-binding surfaces of Hmt1p, PRMT1 and PRMT3, although not identical, are very similar, thus facilitating the recognition of related GAR-like motifs [11–13].

Attempts to elucidate the biological roles of arginine methylation have included the identification and characterization of specific PRMT substrates [9,10,14], and the targeted disruption of the *Prmt1* and *Prmt4* genes in mice [15,16], the *HMT1/RMT1* gene in budding yeast [17], and the *prmt3* gene in fission

yeast [18]. These studies and others have revealed that the biological consequences of arginine methylation are likely to involve the regulation of protein–protein interactions [19,20] and possibly protein–RNA interactions [21]. The impact that arginine methylation has on molecular interactions leads in turn to the modulation of a range of cellular processes, including transcription [22,23], protein subcellular localization [10,17] and splicing [24].

PRMT3 was identified as a PRMT1-binding protein in a yeast two-hybrid screen [5]. However, gel filtration analysis of Rat1 cells demonstrated that PRMT3 occurs as a monomer and is not complexed *in vivo* with PRMT1 [5]. In addition, it is unlikely that PRMT1 and PRMT3 occur in a complex, due to the fact that PRMT1 is a predominantly nuclear protein, whereas PRMT3 is cytoplasmic. Indeed, PRMT3 is the only type I arginine methyltransferase that does not display a nuclear localization [5,6]. Another unique property of PRMT3 is that it harbours a zinc-finger domain at its N-terminus. It has been proposed that this domain may play a role in the regulation of PRMT3 activity or in the recognition of PRMT3 substrates [5,25]. Deletion analysis studies demonstrated that PRMT3 lacking the zinc-finger domain is still active *in vitro*; however, such an enzyme loses its ability to methylate substrate when presented with a complex mixture of hypomethylated proteins isolated from Rat1 cells [25]. This suggests that the zinc finger is the substrate recognition module of PRMT3.

To test this concept, we used an affinity purification approach, coupled with MS, to identify the 40 S rpS2 (ribosomal protein S2) as a zinc-finger-dependent substrate of PRMT3. Importantly,

Abbreviations used: aDMA, asymmetric ω -N^G,N^G-dimethylarginine; [³H]AdoMet, S-adenosyl-L-[methyl-³H]methionine; CARM1, co-activator-associated arginine methyltransferase 1; DMEM, Dulbecco's modified Eagle's medium; GAR, glycine- and arginine-rich; GFP, green fluorescent protein; GST, glutathione S-transferase; GST–GAR, GST fusion protein containing the N-terminal GAR region of fibrillarlin; hnRNP, heterogeneous nuclear ribonucleoprotein; MALDI-TOF, matrix-assisted laser desorption ionization–time-of-flight; PABP1, poly(A)-binding protein 1; PRMT, protein arginine N-methyltransferase; PRMT3*, zinc-finger mutant of PRMT3 in which the two conserved cysteine residues in the C₂H₂ zinc-finger motif are replaced with alanine residues (C₂H₂ → A₂H₂); rpS2, ribosomal protein S2; Sam68, Src-associated substrate during mitosis 68 kDa.

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it was reported recently that this same enzyme/substrate pair (PRMT3/rpS2) exists in fission yeast [18], and that disruption of the *prmt3* gene in this organism results in an imbalance in the 40 S/60 S free subunit ratio. Thus, taken together, these results demonstrate a highly conserved role for arginine methylation in ribosomal assembly and protein biosynthesis.

EXPERIMENTAL

Antibodies and plasmids

pGEX-CARM1 (where CARM1 is co-activator-associated arginine methyltransferase 1) was a gift from Michael Stallcup (University of Southern California, Los Angeles, CA, U.S.A.) [23]. pGEX-GAR and pGEX-PRMT1 were a gift from Steve Clarke (UCLA, Los Angeles, CA, U.S.A.) [25]. The generation of pGEX-PABP1 [where PABP1 is poly(A)-binding protein 1] and pGEX-PRMT6 has been described previously [6,9]. pGEX6P1-PRMT3 was generated by PCR using FirstChoice™ PCR-ready mouse kidney cDNA (Ambion, Austin, TX, U.S.A.) and the primer pair 5'-TGCCAATTGCTGGAGATGGCGGATGACGCCGGTGCA-3' and 5'-TAAGTCGACTTATGTGGTAGCCACAGCTGG-3'. All GST (glutathione S-transferase)-rpS2 fusion proteins were subcloned in pGEX6P1 (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.); the subcloned regions are detailed in Figure 4. For GFP (green fluorescent protein) fusion constructs, the pGEX6P1-PRMT3 construct was cut and the PRMT3 insert was subcloned into pEGFP-C1 (Clontech, Palo Alto, CA, U.S.A.). For the FLAG-tagged expression vector, FLAG-PRMT3 was subcloned into the pTRACER vector (Invitrogen, Carlsbad, CA, U.S.A.). Site-directed mutagenesis was performed on full-length PRMT3 in pTRACER-FLAG-PRMT3 and pGEX6P1-PRMT3 plasmid DNA by PCR. To generate the C₂H₂ → A₂H₂ PRMT3 mutants, the following primer pair was used: 5'-GAGGAAACGTTTT**AGCCTGTAAGTTGGAGGCTCAATTTAATATT**-3' and 5'-AATATTAAT**TGAGCCTCCA**ACTTACAGGCTGAAAACGTTTCCTC-3' (bold underlined residues are the mutated sites). The anti-PRMT3 antibody was raised in rabbits against GST-PRMT3(zf), which encodes amino acids 1–183 of mouse PRMT3. The anti-rpS2 antibody was raised in rabbits against GST-rpS2, which encodes amino acids 1–293 of mouse rpS2. The anti-GFP antibody was obtained from Molecular Probes Inc. (Eugene, OR, U.S.A.), the anti-FLAG antibody M2 from Sigma-Aldrich (St. Louis, MO, U.S.A.), and the anti-rpS6 antibody from Cell Signaling Technology, Inc. (Beverly, MA, U.S.A.).

FLAG-tag pull-down and *in vitro* methylation assay

HeLa cells were transfected with 10 µg of plasmid DNA of FLAG-PABP1, FLAG-PRMT3 or FLAG-PRMT3* [zinc-finger mutant of PRMT3 in which the two conserved cysteine residues in the C₂H₂ zinc-finger motif were replaced with alanine residues (C₂H₂ → A₂H₂)] using 30 µl of Lipofectamine™2000 (Invitrogen) in serum-free DMEM (Dulbecco's modified Eagle's medium). At 24 h after transfection, cells were washed with PBS and cell extracts were prepared using mild lysis buffer (150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10 mM Tris/HCl, pH 7.5). FLAG-tagged proteins were immunoprecipitated using anti-FLAG®-M2-agarose beads (Sigma-Aldrich). After 3 h of incubation at 4°C, anti-FLAG-M2-agarose beads were washed with mild lysis buffer and resuspended in protein loading buffer. Immunoprecipitated proteins were separated by SDS/PAGE and visualized by Sypro® Ruby (Bio-Rad, Hercules, CA, U.S.A.) staining. For the *in vitro* methylation assay, FLAG-tagged proteins bound to anti-FLAG-M2-agarose beads were washed with PBS

and resuspended in PBS to final volume of 28 µl. Then 2 µl of [³H]AdoMet (*S*-adenosyl-L-[methyl-³H]methionine) (79 Ci/mmol from a 7.5 µM stock solution; Perkin-Elmer, Boston, MA, U.S.A.) was added and samples were incubated at 30°C for 1.5 h, with gentle shaking every 10 min. Methylated proteins were separated by SDS/PAGE, and visualized by fluorography.

Protein identification by peptide mass mapping

The protein band at approx. 30 kDa from the FLAG-tag pull-down SDS/PAGE gel was excised and the protein digested in-gel with trypsin using a modified Rosenfeld procedure [26,27]. The tryptic digests were analysed on a Voyager-DE PRO MALDI-TOF (matrix-assisted laser desorption ionization-time-of-flight) mass spectrometer (Applied Biosystems, Foster City, CA, U.S.A.) as described previously [28]. The digest was desalted using a µC18 ZipTip (Millipore, Bedford, MA, U.S.A.) and spotted on the MALDI target in two spots. Automated mass spectral acquisition and peak procession of the 15 most intense peptide ions was performed using instrument software. A human + rodent subset of the NCBI (National Center for Biotechnology Information) non-redundant protein database was queried using the search engine MS-Fit 3.2.1, in the Protein Prospector suite. The protein represented by the highest number of peptide masses matched is reported with a mass tolerance of 30 p.p.m.

Preparation of GST fusion proteins

GST fusion proteins were overexpressed in *Escherichia coli* BL21 cells (Stratagene, La Jolla, CA, U.S.A.) by induction with a final concentration of 0.2 mM isopropyl β-D-thiogalactopyranoside. Washed cells were resuspended in 2 ml of PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.4) and 100 µM PMSF per g of cells, and subsequently broken by four 30-s sonicator pulses (30% duty; 0.5 s on/off) on ice with a Sonic Dismembrator (Fisher Scientific, Pittsburg, PA, U.S.A.). The resulting lysate was centrifuged for 10 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 Pharmacia Biotech) and washed in PBS as described by the manufacturer in the presence of 100 µM PMSF. The purified proteins were eluted from the beads with 30 mM glutathione, 50 mM Tris/HCl (pH 7.5) and 120 mM NaCl.

In vitro methylation assay with GST fusion proteins

In vitro methylation reactions were performed in a final volume of 30 µl of PBS (pH 7.4). The reaction contained 0.5–1.0 µg of substrate and 1 µg of recombinant PRMT1, PRMT3 or PRMT6. All methylation reactions were carried out in the presence of 0.42 µM [³H]AdoMet (79 Ci/mmol from a 7.5 µM stock solution; Perkin-Elmer). The reaction was incubated at 30°C for 1 h and then subjected to fluorography by separation on SDS/PAGE (12% gel), transferred to a PVDF membrane, treated with En³hance™ (Perkin-Elmer) and exposed to film overnight.

Transient transfection and *in vivo* methylation assay

Cells were labelled using a previously described *in vivo* methylation assay [29]. Briefly, for analysis of *in vivo* methylation of rpS2 in HeLa cells, GFP fusion constructs were transiently transfected into cells using Lipofectamine™2000 (Invitrogen) in serum-free DMEM containing non-essential amino acid solution. At 1 day after transfection, cells were rinsed with PBS to remove serum, and then incubated with 5 ml of methionine-free DMEM containing 10% (v/v) dialysed fetal bovine serum, 10 µCi/ml L-[methyl-³H]methionine (1 mCi/ml; Perkin-Elmer), 200 µg/ml

cycloheximide and 80 $\mu\text{g/ml}$ chloramphenicol for 3 h. Cells were then lysed in a mild lysis buffer and immunoprecipitations were performed with anti-GFP antibodies. Immunoprecipitates were analysed by autoradiography after SDS/PAGE and transfer to a PVDF membrane. To gauge the expression levels of GFP fusion proteins, a Western blot with an anti-GFP antibody was performed using the same membrane.

GST pull-down assay

GST, GST-PRMT3(zf), GST-PRMT3 and GST-PRMT3* ($\text{C}_2\text{H}_2 \rightarrow \text{A}_2\text{H}_2$) were expressed in *E. coli* and purified on glutathione-Sepharose™ 4B beads as described above. However, the recombinant protein was not eluted from the beads. HeLa cells were transiently transfected with pFLAG-rpS2 and were lysed 48 h later in 1 ml of mild lysis buffer. Samples of 1 μg of each fusion protein bound to beads were incubated with FLAG-rpS2-expressing HeLa cell extract (derived from a 10 cm plate) for 2 h. Beads with their associated proteins were washed three times with mild lysis buffer. Bound proteins were separated by SDS/PAGE and transferred to PVDF membranes.

Co-immunoprecipitation experiments

NIH 3T3 cells were lysed in 1 ml of mild lysis buffer and PRMT3 or rpS2 was immunoprecipitated using appropriate antisera; as a control, pre-immunization serum from the corresponding immunized rabbits was used. Immunoprecipitated proteins were separated by SDS/PAGE and transferred on to PVDF membranes. To visualize the interaction between PRMT3 and rpS2 *in vivo*, Western blot analysis was performed using anti-PRMT3 and anti-rpS2 sera.

Polysome profiling

NIH 3T3 cells at 90% confluency were incubated for 15 min at 37 °C in 10% (v/v) fetal bovine serum/DMEM supplemented with 0.1 mg/ml cycloheximide. After that, cells were washed with ice-cold PBS (containing 0.1 mg/ml cycloheximide). Next, a cell extract was prepared using 500 μl of polysome lysis buffer (1.0% Triton X-100, 20 mM Tris/HCl, pH 7.5, 50 mM KCl, 10 mM MgCl_2 , 1 mM dithiothreitol, 0.5 unit/ μl RNase inhibitor, 0.1 mg/ml cycloheximide, 0.2 mg/ml heparin and protease inhibitors). Following a 10 min incubation on ice, cellular debris was sedimented by a centrifugation at 14000 rev./min for 15 min at 4 °C. Extracts containing a total of 20 A_{260} units were layered on to 5–45% (w/v) sucrose gradients prepared in polysome lysis buffer and centrifuged for 3 h at 39000 rev./min at 4 °C in a Beckman SW41Ti rotor. The gradients were then fractionated by upward displacement with Fluorinert using a gradient fractionator (Model 640; Instrument Specialties Co., Lincoln, NE, U.S.A.) connected to a UV monitor for continuous measurement of the absorbance at 254 nm. A total of 18 fractions of 0.6 ml each were collected, and proteins were precipitated with trichloroacetic acid (20%), washed with ice-cold acetone, and vacuum-dried. Precipitated proteins were separated by SDS/PAGE and transferred to a PVDF membrane.

RESULTS AND DISCUSSION

The zinc-finger domain of PRMT3 is required for recognition of a 30 kDa substrate

The zinc-finger domain of PRMT3 is required for the enzyme to methylate substrates in hypomethylated Rat1 cell extracts [25], suggesting that this domain is the substrate-recognition module of the enzyme. Starting from this premise, we decided to identify

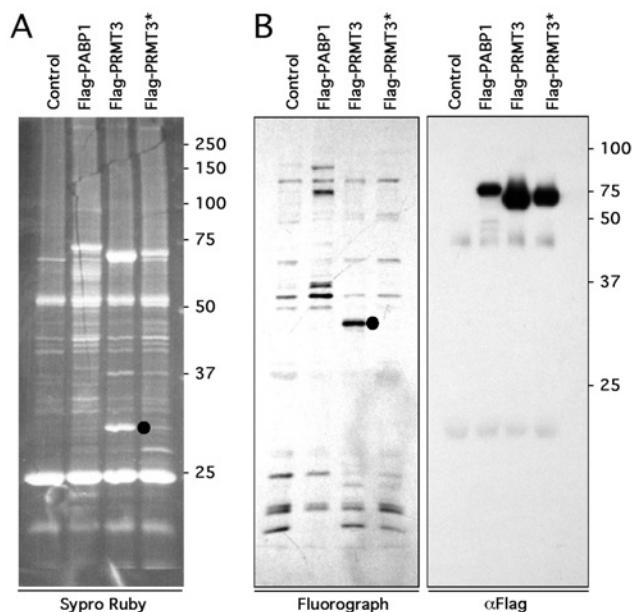


Figure 1 FLAG-tagged PRMT3 interacts with rpS2 in a zinc-finger-dependent fashion

HeLa cells transiently expressing FLAG-PABP1, FLAG-PRMT3 or FLAG-PRMT3* ($\text{C}_2\text{H}_2 \rightarrow \text{A}_2\text{H}_2$) fusion proteins were lysed and the prepared cell lysates were incubated with anti-FLAG-M2-agarose beads. After extensive washing, the immunoprecipitated proteins were separated by SDS/PAGE. Molecular masses are indicated in kDa. **(A)** Proteins were visualized by a Sypro® Ruby staining. The filled circle (●) on the gel indicates the protein band that was cut off the gel and analysed by MS. **(B)** Proteins bound to anti-FLAG-M2-agarose beads were incubated with [^3H]AdoMet and separated by SDS/PAGE, and the methylated proteins were visualized by fluorography (left panel). The filled circle (●) on the gel indicates the position of a potential PRMT3 substrate. A duplicate blot harbouring proteins immunoprecipitated with anti-FLAG-M2-agarose beads was subjected to Western blot analysis with anti-FLAG-M2 antibody (αFlag) (right panel).

substrates that interacted with the PRMT3 zinc-finger domain. For this purpose we generated a FLAG-tagged form of full-length PRMT3 (FLAG-PRMT3). To assay for domain-dependent interactions, a zinc-finger mutant of this construct was generated (FLAG-PRMT3*) by replacing the two conserved cysteine residues in the C_2H_2 zinc-finger motif with alanine residues ($\text{C}_2\text{H}_2 \rightarrow \text{A}_2\text{H}_2$). HeLa cells were then transiently transfected with FLAG-tagged PRMT3, PRMT3* or PABP1 (as an additional control). Cell lysates expressing the FLAG-tagged proteins were immunoprecipitated with anti-FLAG-M2-agarose resin, and the associated proteins were separated by SDS/PAGE. The gel was stained with Sypro® Ruby to visualize the immunoprecipitated proteins (Figure 1A). A single protein of ≈ 30 kDa was seen to interact with wild-type PRMT3, but not with the zinc-finger mutant. This band did not co-immunoprecipitate with FLAG-PABP1 or untransfected cells.

To establish whether the ≈ 30 kDa protein is not only a zinc-finger-interacting protein, but also a potential substrate for PRMT3, we repeated the immunoprecipitation experiment and added [^3H]AdoMet to the anti-FLAG-M2-agarose resin. After a 90 min reaction, the immunoprecipitated complexes were separated by SDS/PAGE and transferred to a PVDF membrane. The membrane was subjected to fluorography to visualize the methylated proteins (Figure 1B, left panel). The same membrane was then probed with an anti-FLAG antibody to demonstrate roughly equal levels of expression of the FLAG-tagged proteins (Figure 1B, right panel). The ≈ 30 kDa protein was methylated in this assay, suggesting that this protein is both a binding partner and a substrate of PRMT3.

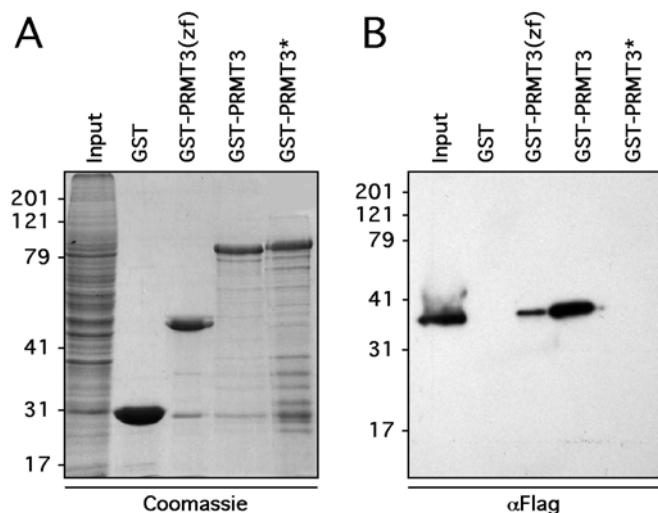


Figure 2 The zinc-finger domain of PRMT3 is sufficient for an interaction with rpS2

GST, GST-PRMT3(zf), GST-PRMT3 and GST-PRMT3* (C₂H₂ → A₂H₂) fusion proteins were expressed in *E. coli* and bound to glutathione-Sepharose™ 4B beads. These bound GST fusion proteins were incubated with HeLa cell extracts containing expressed FLAG-rpS2 and a pull-down experiment was performed. Molecular masses are indicated in kDa. (A) Coomassie Blue-stained gel demonstrating equal loading of GST fusion proteins. (B) The efficiency of the pull-down experiment was assessed by Western blot analysis with anti-FLAG-M2 antibody (αFlag).

To identify the ≈ 30 kDa protein, the band was excised from the Sypro® Ruby-stained gel and digested with trypsin. The resulting peptides were eluted from the gel piece and analysed by MS. The experimental peptide mass list was queried against the NCBI non-redundant protein database using the MS-Fit search engine, which identified the protein as 40 S rpS2. Seven different peptides matched rpS2, providing 29% coverage of the protein sequence. The calculated molecular mass of rpS2 is 31 kDa, in agreement with the molecular mass of the gel band. In addition, rpS2 harbours a GAR motif at its N-terminal end, marking it a strong candidate for methylation by a type I arginine methyltransferase.

The zinc finger of PRMT3 is necessary and sufficient for the interaction with rpS2

The fact that rpS2 associated with PRMT3 but not PRMT3* indicates that the zinc finger is necessary for the interaction between the two proteins. To test whether the zinc finger can act alone, and is sufficient to mediate an interaction, we generated GST fusion proteins of PRMT3, PRMT3* and the PRMT3 zinc-finger alone. In addition, a FLAG-tagged rpS2 expression construct was engineered and HeLa cells were transiently transfected with this vector. Cell lysates harbouring FLAG-rpS2 were used for pull-down assays with an immobilized GST fusion protein panel. Like FLAG-PRMT3 (Figure 1), GST-PRMT3 also requires an intact zinc finger for an interaction with rpS2 (Figure 2). In addition, the isolated zinc finger of PRMT3 was able to pull down rpS2, demonstrating that this is the only region of PRMT3 that is essential for the interaction.

Recombinant rpS2 is methylated *in vitro* by PRMT3

rpS2 has an extensive GAR motif at its N-terminal end. In general, a GAR motif is recognized by a subset of type I arginine methyltransferases that includes PRMTs 1, 3 and 6. To confirm that rpS2 can be methylated by PRMT3, and possibly also by other PRMTs, *in vitro* methylation assays were performed using GST-

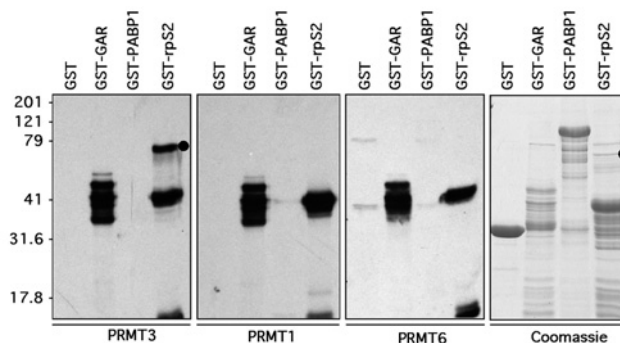


Figure 3 Recombinant rpS2 is methylated *in vitro*

Purified GST, GST-GAR, GST-PABP1 and GST-rpS2 were incubated separately with recombinant PRMT1, PRMT3 or PRMT6 in the presence of [³H]AdoMet. Methylated proteins were separated by SDS/PAGE and visualized by fluorography. A duplicate gel was stained with Coomassie Blue to demonstrate equal loading of substrates. The filled circle (●) on the gels indicates the position of the full-length GST-rpS2 fusion protein that is methylated. Molecular masses are indicated in kDa.

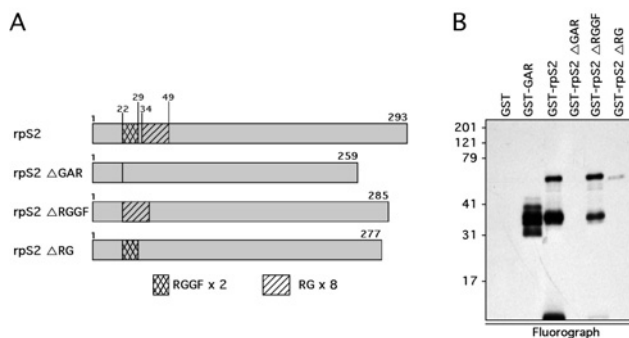


Figure 4 Deletion analysis of rpS2

(A) Structures of rpS2 deletion mutants. (B) The deletion mutants of rpS2 were expressed as GST fusion proteins. Purified GST-rpS2 deletion mutants were incubated with recombinant PRMT3 in the presence of [³H]AdoMet. Methylated proteins were separated by SDS/PAGE and visualized by fluorography. Molecular masses are indicated in kDa.

rpS2, GST-GAR (GST fused to the GAR motif of fibrillarin) and the CARM1 substrate GST-PABP1f. A degradation product of GST-rpS2 was methylated *in vitro* by PRMT1, PRMT3 and PRMT6 (Figure 3). GST-rpS2 is very susceptible to degradation, and only a small fraction of full-length fusion protein was seen in the Coomassie Blue-stained gel (Figure 3, right panel). Interestingly, only PRMT3 was capable of methylating the full-length recombinant GST-rpS2 (Figure 3, left panel), suggesting that there is a unique relationship between PRMT3 and rpS2. Instability of the yeast rpS2 fusion protein was also recently observed by Bachand and Silver [18].

Mapping the site of rpS2 methylation

The N-terminal region of rpS2 is glycine/arginine-rich, pointing to this area as the domain of arginine methylation. There are two distinct motifs in this region: an Arg-Gly-Gly-Phe (RGGF) sequence that is repeated twice, and an Arg-Gly (RG) sequence that is repeated eight times. Type I arginine methyltransferases have been reported to methylate RGGF motifs in fibrillarin and nucleolin (reviewed in [2]), as well as direct RG repeats in Sam68 [10] and other proteins [30]. To establish which of these motifs in rpS2 is methylated by PRMT3, we disrupted each of these repeat types, independently and together, within the context of the full-length GST fusion protein (Figure 4A). The removal of both repeat

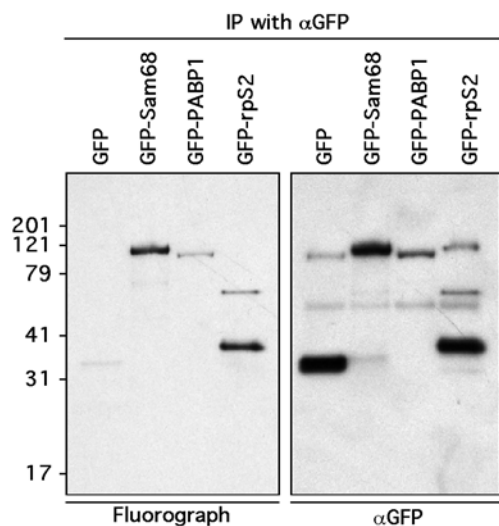


Figure 5 rpS2 is methylated *in vivo*

HeLa cells transiently expressing GFP, GFP-Sam68, GFP-PABP1 or GFP-rpS2 were incubated with chloramphenicol, cycloheximide and L-[methyl-³H]methionine for 3 h. Next, cell lysates were prepared and the GFP-fusion proteins were immunoprecipitated (IP) with anti-GFP antibody (α GFP). *In vivo* methylation was visualized by fluorography (left panel). Expression of GFP and GFP-fusion proteins was analysed by Western blot with an anti-GFP antibody (right panel). Molecular masses are indicated in kDa.

types (Δ GAR) resulted in the loss of rpS2 *in vitro* methylation. Removal of the two RGGF motifs had very little effect on the ability of PRMT3 to methylate rpS2. However, removal of the eight RG repeats resulted in a dramatic loss of the ability of rpS2 to act as a methyl-acceptor (Figure 4B). The RG repeats are therefore the sites of *in vitro* arginine methylation in rpS2, and probably also the site of *in vivo* methylation. These data are supported by the observation that the N-terminal portion of *Schizosaccharomyces pombe* rpS2, which like mammalian rpS2 is methylated by PRMT3 [18], does not contain an RGGF motif, but does harbour RG and RGG repeats.

Full-length rpS2 is methylated *in vivo*

To determine whether rpS2 is methylated in cells, we used an *in vivo* methylation assay described by Liu and Dreyfuss [29]. The total methylated protein pool is labelled by incubating cells with L-[methyl-³H]methionine in the presence of the protein synthesis inhibitor cycloheximide. HeLa cells were transiently transfected with GFP, GFP-PABP1, GFP-Sam68 or GFP-rpS2, and the methylated proteins labelled as described in the Experimental section. After immunoprecipitation with anti-GFP antibodies, the proteins were separated by SDS/PAGE, transferred to a PVDF membrane and subjected to fluorography (Figure 5, left panel). GFP-PABP1 (a substrate for CARM1) and GFP-Sam68 (a substrate for PRMT1) served as positive controls for *in vivo* methylation [9]. GFP alone was a negative control to confirm that translation was indeed inhibited. The GFP fusion proteins of PABP1, Sam68 and rpS2 were methylated in HeLa cells, whereas GFP alone remained largely unmodified, indicating that cellular rpS2 (fused to GFP) is methylated *in vivo*. The same membrane was then subjected to Western analysis using an anti-GFP antibody to confirm roughly equal loading (Figure 5, right panel). Here again we see the propensity of an rpS2 fusion protein to be degraded, and like the *in vitro* scenario, both full-length rpS2 and its degradation product are methylated. In a number of studies, arginine methylation has been implicated in the regulation

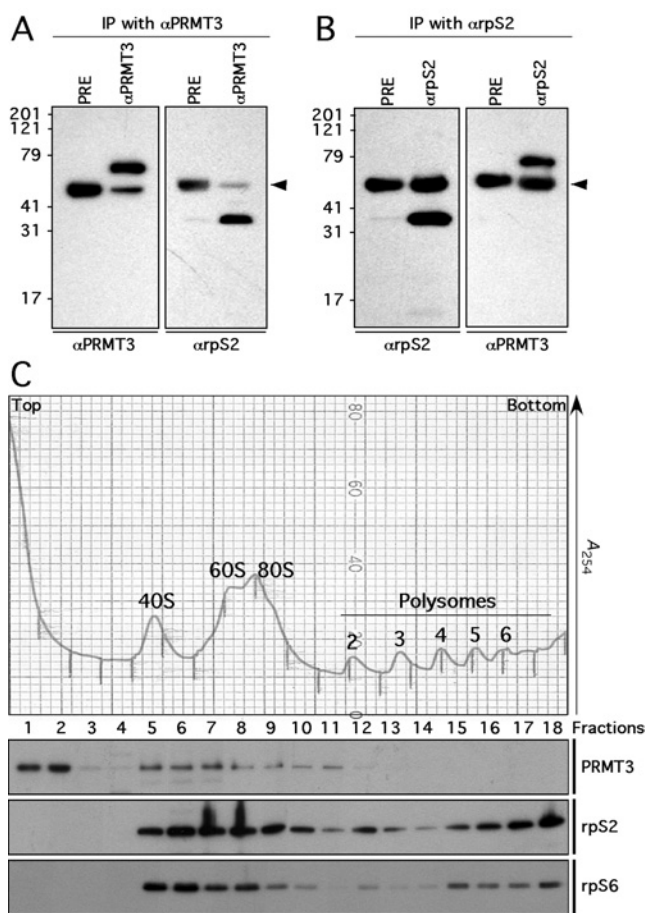


Figure 6 Endogenous rpS2 and PRMT3 interact

Antibodies were raised in rabbits against mouse rpS2 and mouse PRMT3. (A) Endogenous PRMT3 was immunoprecipitated (IP) with pre-immune (PRE) and anti-PRMT3 (α PRMT3) antisera using NIH 3T3 cell extracts. Immunoprecipitated proteins were separated by SDS/PAGE and transferred on to PVDF membranes. Western blot analysis was performed using anti-PRMT3 antibody and anti-rpS2 antiserum (α rpS2). (B) Similarly, endogenous rpS2 was immunoprecipitated with pre-immune and anti-rpS2 antisera using NIH 3T3 cell extracts, and Western blot analysis was performed using anti-rpS2 antiserum and anti-PRMT3 antibody. The arrowhead denotes the position of the IgG heavy chain band that is generated by the immunoprecipitation procedure. Molecular masses are indicated in kDa in (A) and (B). (C) Sucrose gradient fractionation of ribosomes isolated from NIH 3T3 cells. The polysome profile is depicted graphically (upper panel). The position of each collected fraction is marked on the trace. The positions of the free 40 S and 60 S ribosomal subunits, monosomes (80 S) and polysomes are shown. 'Top' denotes the 5% end of the sucrose gradient and 'Bottom' the 45% end. The fractions precipitated by trichloroacetic acid were separated by SDS/PAGE, transferred to a PVDF membrane and subjected to Western analysis with anti-PRMT3, anti-rpS2 and anti-rpS6 antibodies (lower three panels).

of protein subcellular localization [1]. We thus compared the localization of GFP-rpS2 and GFP-rpS2(Δ GAR), but no change in the subcellular localization of the GFP-rpS2(Δ GAR) variant was observed (results not shown). rpS2 is thus methylated in the cell; however, this methylation has no gross effect on its subcellular localization.

PRMT3 and rpS2 co-immunoprecipitate and co-fractionate

rpS2 was identified as a zinc-finger-dependent binding partner of ectopically expressed PRMT3 (Figure 1). To confirm that endogenous PRMT3 and rpS2 interact in the cell, we generated antibodies against the mouse proteins. Using an NIH 3T3 cell line, reciprocal co-immunoprecipitations were performed with these antibodies. Anti-PRMT3 antibodies immunoprecipitated

PRMT3 and rpS2 (Figure 6A), and likewise anti-rpS2 antibodies immunoprecipitated PRMT3 and rpS2 (Figure 6B). The PRMT3–rpS2 interaction seems to be fairly stable, judging from the strength of the co-immunoprecipitation (Figure 6) and pull-down experiments (Figures 1 and 2). It is thus possible that PRMT3 is a ribosome-associated protein. To determine this, we purified polysomes by sucrose gradient fractionation and performed Western analysis on the different fractions using anti-PRMT3 and anti-rpS2 antibodies (Figure 6C). PRMT3 was seen in the low-density fractions (fractions 1 and 2) and was also associated with the 40 S small ribosomal subunit, the 60 S large ribosomal subunit and 80 S monosomes (fractions 5–11), but was not present in the polysome-containing fractions (12–18). rpS2, like rpS6, co-fractionated with monosomes and polysomes. Thus over 50 % of PRMT3 was found floating free in the cytosol and the remainder was bound to monosomes.

Conclusions

The only type I arginine methyltransferase to be localized exclusively in the cytoplasm is PRMT3 [5,6]. Thus PRMT3 is likely to have a biological role in a cytosolic process and not as a transcriptional co-activator, as has been the reported role of other arginine methyltransferases (PRMT1 and CARM1) [14,16,23,31,32]. In the present study, in keeping with this assumption, we have identified rpS2 as a substrate of mammalian PRMT3. The fact that this enzyme/substrate pair is conserved in *S. pombe* underscores an important role for this modification. Indeed, yeast lacking *prmt3* display an imbalance in the cellular ratio of free 40 S and 60 S ribosomal subunits [18]. It has been demonstrated that growth factors can stimulate global protein synthesis by increasing the rates of translational initiation, translational elongation and ribosome biogenesis [33]. Growth-factor-regulated control of mRNA translation is mediated largely by the mTOR (mammalian target of rapamycin)/S6K1 (S6 kinase 1) pathway, which phosphorylates a component of the smaller 40 S ribosomal subunit, rpS6 [34,35]. With the finding that PRMT3 can modify rpS2, it is now possible that not only phosphorylation but also protein methylation play a key role in the regulation of protein synthesis.

It has long been known that there is prominent methylation of both lysine and arginine residues in the 40 S and 60 S subunit proteins of HeLa ribosomes [36]. Gross amino acid analysis of the ribosomal subunits revealed that aDMA is the predominant methylated species. Although eEF1A (eukaryotic elongation factor 1A) is methylated on lysine residues [37,38], until the recent findings that rpS2 is a substrate for PRMT3 in yeast [18] and mammalian cells (the present study), no methylated component of the 40 S or 60 S ribosomal subunits had been identified. The identification of PRMT3 as a ribosomal PRMT provides an entry point into understanding the biological role that this common post-translational modification plays in the control of protein synthesis.

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