Methylation of Ribosomal Protein S10 by Protein-arginine Methytransferase 5 Regulates Ribosome Biogenesis

Received for publication, January 14, 2010, and in revised form, February 11, 2010 Published, JBC Papers in Press, February 16, 2010, DOI 10.1074/jbc.M110.103911

Jinqi Ren, Yaqing Wang, Yuheng Liang, Yongqing Zhang, Shilai Bao, and Zhiheng Xu

From the Institute of Genetics and Developmental Biology, The Key Laboratory of Molecular and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China

Methylation of ribosomal protein S10 by protein-arginine methyltransferase 5 (PRMT5) is a novel substrate of an oncoprotein, protein-arginine methyltransferase 5 (PRMT5). We show that PRMT5 interacts with RPS10 and catalyzes its methylation at the Arg158 and Arg160 residues. The methylation of RPS10 at Arg158 and Arg160 plays a role in the proper assembly of ribosomes, protein synthesis, and optimal cell proliferation. The RPS10-R158K/R160K mutant is not efficiently assembled into ribosomes and is unstable and prone to degradation by the proteasomal pathway. In nucleoli, RPS10 interacts with nucleophosmin/B23 and is predominantly concentrated in the granular component region, which is required for ribosome assembly. The RPS10 methylation mutant interacts weakly with nucleophosmin/B23 and fails to concentrate in the granular component region. Our results suggest that PRMT5 is likely to regulate cell proliferation through the methylation of ribosome proteins, and thus reveal a novel mechanism for PRMT5 in tumorigenesis.

Ribosomes are complex macromolecular machines composed of rRNAs and ribosomal proteins and are responsible for the synthesis of polypeptide chains. Ribosome biogenesis is thus a critical process inextricably linked to cell growth and proliferation. The production of mature ribosomes competent to translate mRNAs is a highly coordinated multistep process (1). Post-translational modifications of ribosomal proteins, including phosphorylation, methylation, and acetylation, affect their characteristics and ribosome biogenesis. Increased phosphorylation of ribosomal protein S6, for example, has been shown to result in enhanced translation efficiency of specific mRNAs (2).

Methylation is considered to be one of the most common forms of post-translational modification of ribosomal proteins (3). It was shown in 1979 that 11 ribosomal proteins are likely to be methylated in HeLa cells (4). Lhoest et al. (5) estimated from in vivo labeling experiments that the yeast 60 S subunit contains about 10 methylation groups, whereas the 40 S subunit has 2–4 methylation groups. The pattern of ribosomal protein methylation appears to be similar in various eubacteria, but there is a higher occurrence of arginine methylation in higher eukaryotes (3). Ribosomal protein S2 was the first arginine methylation substrate found for protein-arginine methyltransferase 3 (PRMT3) (6, 7). Yeast cells lacking PRMT3 showed accumulation of free 60 S ribosomal subunits, resulting in an imbalance in the 40 S:60 S free subunits ratio (8). However, arginine methylation of other ribosomal proteins, especially in higher eukaryotes, and its biofunctions remain to be elucidated.

Protein-arginine methyltransferases are enzymes that catalyze the transfer of a methyl group from S-adenosylmethionine to arginine (9, 10). PRMTs are classified into two groups. Although type I PRMTs catalyze the formation of monomethylarginine and asymmetric dimethylarginine (aDMA), type II enzymes catalyze monomethylarginine and symmetric dimethylarginine (sDMA). Recently, accumulating evidence has revealed that arginine methylation has important roles in many cellular processes, including RNA processing, transcriptional regulation, signal transduction, DNA repair, and protein-protein interactions (9, 10). In humans, the PRMT family has been shown to contain around 11 methyltransferases, designated as PRMT1–11 based on their primary sequence and substrate specificity (11).

PRMT5 or Skb1Hs, a type II arginine methyltransferase was originally cloned as a human homolog of Shk1 kinase binding 1 (Skb1) in fission yeast (12). It was later reported to interact with a number of complexes and to be involved in a variety of cellular processes. PRMT5 methylates histones H2A, H3, and H4 to regulate gene expression (13–15), Sm proteins B-B', D1, and D3 to promote their transfer to the SMN complex and enhance small nuclear ribonucleoprotein biogenesis (16–18), and SPT5 to regulate its interaction with RNA polymerase II thus controlling transcriptional elongation (19).

The biological functions of PRMT5 have been identified in recent years. We have shown in fission yeast that Skb1 plays an important role in regulating cell growth and polarity under hyperosmotic stress (20). We also found that the homolog of PRMT5 in plants, SKB1, plays a role in methylation of histone S.
PRMT5, RPS10 Methylation, and Ribosome Assembly

H4R3 and is involved in the control of flowering time in Arabidopsis (21). In mammalian cells, overexpression of PRMT5 induces hyperproliferation, whereas knockdown of PRMT5 inhibits cell growth and proliferation (22–24). By forming complexes with hSWI/SNF chromatin-remodeling proteins BRG and BRM, PRMT5 can facilitate ATP-dependent chromatin remodeling and is required for muscle differentiation and myogenesis (25, 26). More importantly, PRMT5 protein levels are elevated in gastric cancer and various human lymphoid cancer cells and mantle cell lymphoma clinical samples (23, 27). PRMT5 is also associated with tumorigenesis by suppressing cells and mantle cell lymphoma clinical samples (23, 27). PRMT5 is also associated with tumorigenesis by suppressing the transcription of the RB family and ST7 (suppressor of tumorigenicity 7) in leukemia and lymphoma cells (23, 28).

Arginine methylation of p53 by PRMT5 also affects the target gene specificity of p53, and PRMT5 depletion triggers p53-dependent apoptosis (29). Loss of E-cadherin is a hallmark of epithelial-mesenchymal transition. A recent report demonstrated that PRMT5 knockdown results in elevated E-cadherin expression, further indicating that PRMT5 is an oncoprotein (30).

In this study, we identified RPS10 as a novel substrate for PRMT5. PRMT5 interacts with RPS10 and methylates it on both Arg158 and Arg160 residues. RPS10 is essential for cell proliferation and methylation. Furthermore, we provide evidence here that PRMT5-mediated RPS10 methylation plays a role in the interaction between RPS10 and B23, the localization of RPS10 in the granular component (GC) region of the nucleus and in the assembly of RPS10 into ribosomes.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection

HEK293 and U2OS cells were cultured in Dulbecco’s modified Eagle’s medium, whereas the hepatocellular carcinoma cell line Bel7402 was grown in RPMI-1640, supplemented with 10% fetal bovine serum. Cell transfection was performed using Lipofectamine 2000 (Invitrogen).

Antibodies and Chemicals

β-Tubulin, FLAG antibody, and FLAG-agarose beads were purchased from Sigma; Lamin B and MG132 from Calbiochem; rabbit SYM11 polyclonal antibody from Upstate Biotechnology Inc.; Myc tag monoclonal antibody, clone PL14 from MBL Ltd.; mouse GFP (clone B-2) and GAPDH antibody from Santa Cruz. RPS10 antisera was raised in mice using purified human His-tagged RPS10 as the immunizing antigen. To prepare the His-tagged RPS10 protein, pET-30a-RPS10 was transformed into Escherichia coli, induced by 0.4 mM isopropyl thio-β-D-galactopyranoside at 25 °C overnight, and purified by nickel-nitrilotriacetic acid-agarose (Qiagen).

Plasmids

The RPS10 gene was amplified by PCR from human cDNA library using primers 5′-CGGGATCCCTAGGATGTGAGTGCTAG-3′ and 5′-CCGCTGAGGCGCATGAGGCAATG-3′, and inserted in EcoRV and BamHI sites of pcDNA3.1/Myc-His(−)B (Invitrogen) and pET-30a (+) (Novagen). Glutathione S-transferase (GST) fusion RPS10 plasmid was subcloned in pGEX-4T-2 (Amersham Biosciences) by PCR and inserted in the BamHI/Sall sites (primers used: 5′-CGGGAATCCACCATGTTGAGTGGCATAG-3′ and 5′-CGGCTCGAGGTTCGAGGCTACG-3′).

GAR deletion of the GST fusion RPS10 (AGAR) plasmid was constructed by PCR using the following primers: 5′-CGGGATCCACCATGTTGAGTGGCATAG-3′ and 5′-CCGCTGAGGCGCATGAGGCAATG-3′, and inserted into the EcoRI/Sall site of pGEX-4T-2. Mutation constructs of GST fusion RPS10 plasmid were constructed by annealing different synthesized oligos and inserting into the EcoRI/Sall site of pGEX-4T-2; R158K, 5′-CATCCAGGTAGGATGACGCAC-3′ and 5′-CGGCTCGAGGTTCGAGGCTACG-3′; R160K, 5′-CCGCTGAGGCGCATGAGGCAATG-3′ and 5′-CGGCTCGAGGTTCGAGGCGCATGAGGCAATG-3′; R158/160K, 5′-CCGCTGAGGCGCATGAGGCAATG-3′ and 5′-CGGCTCGAGGTTCGAGGCTACG-3′; and 5′-CGGCTCGAGGTTCGAGGCTACG-3′.

Purification of GST and GST-RPS10 Fusion Proteins

GST, GST-RPS10 wild type, and different mutants were expressed in E. coli BL21(DE3) and purified as described previously (44). GST fusion proteins were eluted from the beads with elution buffer (10 mM Tris-HCl, 20 mM reduced glutathione,
pH 8.0). The result of purification is shown in supplemental Fig. S1.

**RNA Interference Vector and siRNA-resistant Construction**

RNA interference plasmids were prepared as previously described (48). For **PRMT5** siRNA, 5′-GAGAAGATCTG-CAGAAGACTC-3′ and 5′-GCTGACCTCCCATCTAATC-3′ were selected and designed as shRNAs in pNeoU6 vector. For **RPS10**, 5′-GAGCTGGCCAGACAAGAT-3′ and 5′-GAGTTGTTGAGGAAA-3′ were selected and named siRPS10-A and siRPS10-B. The oligonucleotides were annealed and cloned into RNAi-Ready pSIREN-DNR-DsRed (BD Biosciences). siRNA-resistant **RPS10** constructs were generated by site-directed mutagenesis of pcDNA3.1 **RPS10-Myc** using the QuikChange mutagenesis kit (Stratagene).

**In Vitro Methylation Assay with GST Fusion Proteins**

**In vitro** methylation reactions were performed in 40 μl of methylation buffer (25 mM Tris, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaCl, pH 8.5) containing 3 μg of substrate and FLAG-PRMT5 immunopurified from transfected HEK293 cell extract. All methylation reactions were carried out in the presence of 0.42 μM [3H]S-adenosyl-l-methionine (GE Healthcare). The reaction was performed at 30 °C for 1 h, separated on SDS-PAGE, treated with En’hance™ (PerkinElmer Life Sciences), and exposed to film overnight.

**Immunoprecipitation, Western Immunoblotting, and In Vitro Binding Assay**

Immunoprecipitation, Western immunoblotting, and **in vitro** binding assay were performed as previously described (49, 50).

**Nucleus/Cytosol Isolation**

To isolation the cytosol and nuclear components, the cells were washed in ice-cold phosphate-buffered saline 24 h after transfection and collected by centrifugation in a microcentrifuge at 5,000 × g for 2 min; and the supernatant was kept as the cytoplasmic extract. The nuclear pellet was washed in ice-cold phosphate-buffered saline and precipitated with 10% trichloroacetic acid, washed with ice-cold acetone, and vacuum-dried. Total protein content was measured using the Bradford assay, and proteins were separated by SDS-PAGE and exposed to film overnight.

**Polysome Isolation and Polysome Profiling**

**Polysome Isolation**—48 h after transfection, cells were treated with 100 μg/ml of CHX for 15 min, and then washed with ice-cold phosphate-buffered saline (containing 100 μg/ml of CHX). The cell extract was prepared using 500 μl of polysome lysis buffer (1.0% Triton X-100, 20 mM Tris, pH 7.5, 50 mM KCl, 10 mM MgCl₂, 1 mM dithiothreitol, 0.5 unit/μl of RNase inhibitor, 100 μg/ml of CHX, 0.2 mg/ml of heparin and protease inhibitors). Following a 10-min incubation on ice, cellular debris was discarded after centrifugation at 14,000 × g for 15 min at 4 °C. The supernatants were centrifuged for 1 h at 40,000 × g at 4 °C in a Beckman SW41Ti rotor. Pellets were resuspended in polysome buffer and separated by SDS-PAGE and analyzed by Western blotting. The ratio of RPS10 in polysome/total cell lysate was analyzed with the Image J 1.36b software (rsb.info.nih.gov/ij).

**Polysome Profiling**—Cells were treated with CHX and lysed as described above. The supernatant was layered over a 5–45% (w/v) sucrose density gradient in ribosome lysis buffer and ultracentrifuged in a swinging bucket MLA-55 rotor at 40,000 rpm for 15 min at 4 °C in a Beckman SW41Ti rotor. Pellets were resuspended in polysome buffer and separated by SDS-PAGE and analyzed by Western blotting. The ratio of RPS10 in polysome/total cell lysate was analyzed with the Image J 1.36b software (rsb.info.nih.gov/ij).

**Measurement of Protein Synthesis**

Cells were incubated in methionine-cysteine-free Dulbecco’s modified Eagle’s medium for 30 min before labeling. In **vivo** metabolic labeling was performed by adding Trans35S-label at a final concentration of 10 μCi/ml containing labeled l-methionine. After 90 min, the cells were washed with ice-cold phosphate-buffered saline and precipitated with 10% trichloroacetic acid, washed with ice-cold acetone, and vacuum-dried. Total protein content was measured using the Bradford assay, and proteins were separated by SDS-PAGE and exposed to film.

**Image Acquisition and Analyses**

24 h after transfection, cells were treated with 0% formaldehyde solution in phosphate-buffered saline. Confocal images were captured on a Leica TCS SP5 confocal microscope. Immunofluorescent images were scanned with a 1042 × 1024 frame size, 4 × zoom, and <1.0 μm optical thickness. Detector gain and amplifier offset were adjusted to ensure all signals were appropriately displayed within the linear range. Exposure time was set so that the brightest intensity reached 80% of the saturation intensity.
PRMT5, RPS10 Methylation, and Ribosome Assembly

38,000 × g for 3 h at 4 °C. Total layers containing the ribosome 80 S, 60 S large subunit and 40 S small were collected. The gradients were next fractionated by upward displacement using a Biologic Duo-flow (Bio-Rad) apparatus connected to a UV detector for continuous absorbance measurements at 254 nm.

RESULTS

RPS10 Is a Substrate of PRMT5—During our study of the leucine-rich repeat kinase 2 (LRRK2)-associated complex, we purified a group of endogenous LRRK2-interacting proteins. Within this group, we found several proteins that belong to a previously reported complex, methylome, including PRMT5, MEP50 (methylosome protein 50, a scaffold for methylosome) (31), and several substrates for PRMT5 such as histone 2A and histone 4. This suggests that the LRRK2-associated complex may play a role in protein methylation. Because the glycine- and arginine-rich motif (GAR motif) is a conserved methylation site for a subset of arginine methyltransferases, we searched for other LRRK2-associated proteins that contain the GAR motif and found RPS10.

RPS10 is a member of the ribosome 40 S subunit complex. We hypothesized that RPS10 is a substrate of PRMT5 based on the following evidence. First, mass spectrometry results for 40 S human ribosome indicate that RPS10 is methylation modified (32); second, a similar mass spectrometry analysis of rat brain extract uncovered an RPS10 C-terminal peptide with a 56-Da mass larger than predicted (33); third, 87.1% of anti-Sm sera (which recognizes sDMA in Sm) from patients with systemic lupus erythematosus, has antibody activity against RPS10 (34). Finally, Y12, a specific symmetric dimethylation antibody, recognizes the sDMA of Sm and cross-reacts with RPS10 (34).

To test our hypothesis that RPS10 is a novel substrate of PRMT5, we investigated first whether RPS10 and PRMT5 interact with each other using reciprocal immunoprecipitation assay. In transfected cells, where different tagged PRMT5 and RPS10 proteins were co-expressed and subsequently immunoprecipitated, a specific complex between RPS10 and PRMT5 was detected (Fig. 1, A and B). To evaluate whether endogenous PRMT5 and RPS10 interact, lysates of HEK293 cells were subjected to co-immunoprecipitation and Western blotting with PRMT5 and RPS10 antisera. Analysis of the immunoprecipitated immunocomplexes were probed separately for the presence of GFP-PRMT5 (A). In reciprocal co-expression/imunoprecipitation (IP) experiments, cell lysates were subjected to immunoprecipitation with anti-Myc antibody and immunocomplexes were probed for the presence of Myc-RPS10 (B). C, endogenous PRMT5 and RPS10 interact with each other. HEK293 cell lysates were subjected to immunoprecipitation with anti-RPS10 or IgG antibodies as indicated. The immunocomplexes were probed separately for PRMT5 and RPS10. D, PRMT5 interacts with RPS10 in vitro. GST and GST-RPS10 fusion protein for binding assays were expressed, purified, and incubated with FLAG-PRMT5 protein purified from HEK293 cell lysates with FLAG beads. GST, GST-RS10, and FLAG-PRMT5 were detected with GST and FLAG antiserum separately.

To test our hypothesis that RPS10 is a novel substrate of PRMT5, we investigated first whether RPS10 and PRMT5 interact with each other using reciprocal immunoprecipitation assay. In transfected cells, where different tagged PRMT5 and RPS10 proteins were co-expressed and subsequently immunoprecipitated, a specific complex between RPS10 and PRMT5 was detected (Fig. 1, A and B). To evaluate whether endogenous PRMT5 and RPS10 interact, lysates of HEK293 cells were subjected to co-immunoprecipitation and Western blotting with PRMT5 and RPS10 antisera. Analysis of the immunoprecipitated immunocomplexes revealed clear interactions between the endogenous proteins (Fig. 1C). To further characterize the interaction between PRMT5 and RPS10, we incubated purified GST and GST-fused RPS10 with FLAG-PRMT5 purified from HEK293 cell lysates. Interactions between GST-RPS10 and FLAG-PRMT5 were observed (Fig. 1D).

After recognizing that RPS10 interacts with PRMT5 under physiological conditions, we next examined whether RPS10 is a substrate of PRMT5. By incubating immunopurified FLAG-PRMT5 with GST-RPS10 and [methyl-3H]S-adenosylmethionine, we found that PRMT5 can methylate GST-RPS10 but not GST (Fig. 2B). RPS10 contains several potential glycine-arginine-rich (GAR) motifs (Arg-Gly-Gly-Phe (RGGF) and Arg-Gly (RG)) at its C-terminal end (Fig. 2A). To determine whether this region in RPS10 is the methylation site or not, we constructed a C-terminal GAR motif deletion mutant (ΔGAR). The removal of GAR motifs resulted in the loss of methylation modification by PRMT5 (Fig. 2B). This indicates that PRMT5 methylates RPS10 within its C-terminal GAR motifs.

There are three arginine residues in the GAR motifs at the RPS10 C terminus, namely Arg158, Arg159, and Arg160. Mass spectrometry results show there is a 56-Da increase in the molecular mass of RPS10 (33), suggesting the existence of two dimethylated arginines. To determine the exact location of the methylation sites, we mutated the potential amino acids into lysine residues, either alone or in combination. The resulting R158K and R160K mutants of RPS10 showed weaker methylation by PRMT5 and methylation of the R158K/R160K mutant was totally lost (Fig. 2C). Arg158 and Arg160 of RPS10 are therefore likely to be the sites of methylation by PRMT5.

To investigate whether PRMT5 is required for the methylation of RPS10 and whether Arg158 and Arg160 of RPS10 are the arginine methylation sites in vivo, we used a symmetric dimethylarginine-specific antibody, SYM11, to detect the methylated arginine residues in immunopurified RPS10 and RPS10-R158K/R160K proteins. As shown in Fig. 2D, the SYM11 antibody clearly detected methylation in WT RPS10, but not in RPS10 (R158K/R160K) immunopurified from transfected 293 cells. We then knocked down the expression of PRMT5 in U2OS cells with different shRNA and found that the levels of methylation of endogenous RPS10 reduced significantly.

3 J. Ren, Y. Wang, Y. Liang, Y. Zhang, S. Bao, and Z. Xu, manuscript in preparation.
PRMT5, RPS10 Methylation, and Ribosome Assembly

Methylation of RPS10 by PRMT5 Is Required for Normal Cell Proliferation—Because a group of ribosomal proteins have been reported to regulate cell cycle and cell proliferation (35, 36), we investigated the physiological role of RPS10 in cell proliferation. We generated two different shRNA-expressing constructs to knock down the expression of endogenous RPS10 in Bel7402 cells. As shown in Fig. 3, A–C, endogenous RPS10 protein levels were reduced significantly 2 days after transfection, and cells transfected with siRPS10 constructs grew much slower than cells transfected with control siRNA. By day 5, cells transfected with control siRNA, but not those transfected with siRPS10 constructs, formed colonies (Fig. 3A). Similar results were obtained for the other cell lines, including U2OS and HEK293 (data not shown). This indicates that RPS10 is required for sustained cell proliferation.

To examine whether methylation of RPS10 plays a role in proliferation, we compared the rescue effects of WT RPS10 and the RPS10 methylation mutant in RPS10 knock-down cells. First, we designed both WT and methylation mutant siRNA-
resistant RPS10 (SR-RPS10) by changing three nucleotides in RPS10 without altering the amino acid sequence (Fig. 3D). Bel7402 cells were co-transfected with siRPS10 together with either the siRNA-resistant WT or methylation mutant of RPS10. As shown in Fig. 3E, expression of the WT SR-RPS10 in the RPS10 knock-down cells restored cell proliferation more effectively than the methylation mutant SR-RPS10. Western blotting of total cell lysates revealed that RPS10 protein levels were down-regulated in siRPS10-transfected cells and expression of siRNA-resistant RPS10s (WT or methylation mutant) restored the total RPS10 levels similar to those of the control (Fig. 3D). Thus, we speculate that methylation of RPS10 plays a role in the appropriate cell proliferation. To further support this conclusion, we were able to establish permanent cell lines with SR-RPS10, but not with SR-RPS10-R158K/R160K, in cells expressing RPS10 shRNA (supplemental Fig. S2 and data not shown).

Methylation of RPS10 Plays a Role in the Efficient Assembly of RPS10 into Ribosomes—Because methylation of RPS10 is important for cell proliferation, we went on to investigate the underlying mechanism by examining the assembly of RPS10 into ribosomes. WT RPS10, or the RPS10 methylation mutant, was transfected into HEK293 cells and ribosomes were isolated by ultracentrifugation. As shown in Fig. 4A, translating polysomes were pooled in the ultracentrifugation pellet. Endogenous RPS10 was present, as expected, in all the ribosomal fractions including the total cell lysate and supernatant and polysome fractions, whereas GFP and GAPDH were detected only in total cell lysates and supernatant fractions. The FLAG-tagged WT RPS10 protein was present in the free cytoplasmic fractions as well as polysomes indicating that it can be assembled into ribosomes properly.

To determine whether the RPS10 methylation mutant was assembled into ribosomes as efficiently as WT RPS10, we analyzed the fraction of exogenous RPS10 associated with polysomes using ImageJ software. The percentage of polysome-associated exogenous RPS10 was calculated as RPS10-FLAG in polysomes/endogenous RPS10 in polysomes and normalized with RPS10-FLAG in total cell lysates/endogenous RPS10 in total cell lysates (Fig. 4B). The fraction of the RPS10-R158K/R160K mutant associated with ribosomes was less than that of WT RPS10 (Fig. 4B), indicating that the RPS10 methylation mutant was assembled into ribosomes less efficiently.

Rmt3, the homolog of PRMT3 in humans, has been characterized as a methyltransferase for ribosomal protein Rps2 (21, 42). RMT3-null cells produce nonmethylated Rps2 and show deregulation of the 40 S:60 S ribosomal subunit ratio (21). Because the RPS10 methylation mutant is less efficient in assembling into ribosomes, we analyzed ribosome profiles in sucrose gradients, including the levels of free 40 S and 60 S ribosomal subunits and 80 S monosomes, to determine whether ribosome biosynthesis is affected. As was the case when yeast Prmt3 was disrupted, expression of RPS10-R158K/R160K in cells when endogenous RPS10 was knocked down by shRNA led to perturbation of the 40 S:60 S ribosomal subunit ratio. The A_{254} 60 S:40 S ratio for cells expressing wild-type RPS10 was 2.01; the A_{254} ratio from three independent experiments increased by about 19.5% to 2.40 in cells expressing mutant RPS10 (Fig. 4C, arrow in peak). This suggests that the methylation mutation of RPS10 may lead to misregulation of the 40 S:60 S ribosomal subunit ratio.

To examine whether the ribosomal subunit imbalance seen in cells expressing mutant RPS10 affects global protein synthesis in cells, total protein was labeled with [35S]methionine and separated on an SDS-PAGE gel. As shown in Fig. 4, D and E, the levels of most newly synthesized proteins from cells expressing mutant RPS10 were lower than those from cells expressing WT...
RPS10 (right panel, comparing those bands labeled by arrows in lane 4 with lane 3), however, there was not much difference in the total protein levels (left panel, compare lanes 1 and 2). We noticed that only one band corresponding to the size of over-expressed DsRed showed no apparent change (dashed arrow). Our data suggests that biogenesis of new proteins is slower in cells expressing mutant RPS10 compared with those expressing WT RPS10.

The RPS10 Methylation Mutant Is Unstable Compared with WT RPS10—Ribosomal proteins are expressed at high levels and different unassembled ribosomal proteins have been shown to be degraded in the nucleolus (37). We noticed that exogenously expressed RPS10-R158K/R160K was consistently present at lower levels than WT RPS10 (supplemental Figs. 2 and 4C). This led us to investigate the relationship between methylation of RPS10 and its stability. WT RPS10 and different amounts of RPS10-R158K/R160K were co-transfected with EGFP expression vector into 293 cells. As shown in Fig. 5A, the expression level of WT RPS10 was much higher than that of the mutant. The expression of the mutant was lower even though 3 times more of the mutant was transfected and with a higher transfection efficiency as indicated by EGFP (Fig. 5A). To test whether rapid degradation could account for the low expression level of the mutant RPS10 protein, CHX (an inhibitor of de novo protein synthesis) pulse-chase experiments were performed. As shown in Fig. 5B, levels of RPS10 declined in the presence of CHX. However, we found that the RPS10-R158K/R160K mutant was much more unstable (the half-life of RPS10-R158K/R160K was ~5 h, whereas that of WT RPS10 was around 8 h as analyzed with ImageJ software). As CHX treatment has been reported to affect ribosome assembly and stability of subunits, we used [35S]methionine pulse-chase analysis of quantitative immunoprecipitations of WT RPS10 and the RPS10-R158K/R160K mutant with PRMT5 as a control. As shown in Fig. 5D, the RPS10-R158K/R160K mutant was apparently less stable than WT RPS10.

Many ribosomal proteins are degraded in a proteasome-dependent manner (37). To determine whether RPS10 is also degraded through the proteasomal pathway, we analyzed the effect of the proteasome inhibitor, MG132, on the degradation of RPS10. As shown in Fig. 5C, both WT RPS10 and RPS10-R158K/R160K accumulated rapidly in the presence of MG132. To further examine whether or not RPS10 expression is subject to regulation by ubiquitination, WT RPS10 and its methylation mutant were co-transfected into HEK293 cells together with Myc-tagged ubiquitin. RPS10 was immunoprecipitated and the ubiquitination level was analyzed. We detected a high molecular weight smear above RPS10 with its intensity significantly enhanced by MG132 treatment (Fig. 5E, lane 2 versus lane 5). Ubiquitination of the RPS10 methylation mutant was much more apparent than for WT RPS10, especially in the presence of MG132 (Fig. 5E, lane 5 versus lane 6). Together, our results indicate that the RPS10 methylation mutant is less stable and is prone to degradation by the proteasomal pathway.

Methylation of RPS10 Is Required for Its Concentration in the GC Region of Nucleoli—Ribosome biogenesis takes place in the nucleolus, a specialized compartment within the nucleus. Each nucleolus contains three morphologically distinct components.

**FIGURE 5.** The RPS10 methylation mutant is less stable than WT RPS10. A, expression of RPS10 methylation mutant is much lower than that of WT RPS10. HEK293 cells were transfected with FLAG-tagged RPS10, or different amounts of RPS10-R158K/R160K, together with 1 μg of pCMS.EGFP. 36 h later, RPS10 expression was determined by Western blotting using anti-FLAG antibody. The blot was re-probed with anti-GFP antibody as a transfection efficiency control. B and C, RPS10 methylation mutant is more unstable and can be stabilized by the proteasome inhibitor. HEK293 cells were transfected with FLAG-tagged RPS10 or RPS10-R158K/R160K. 36 h later, cells were treated with either 100 μM cycloheximide (CHX) or 10 μM MG132 and cells were collected at different time points as indicated. RPS10 expression levels were determined by Western blotting using RPS10 antiserum. The blot was re-probed with β-tubulin as a loading control. D, HEK293 cells were transfected with either WT RPS10-FLAG or RPS10-R158K/R160K-FLAG together with FLAG-PRMT5. Pulse-chase experiments were done by labeling with [35S]methionine for 1 h and a subsequent chase with medium containing non-radioactive methionine for the indicated times before cells were lysed. WT and mutant RPS10 and PRMT5 proteins were immunoprecipitated with anti-FLAG beads. 35S-Labeled RPS10-FLAG was analyzed with a FLA-3000 phosphorimager, and FLAG-PRMT5 was used as a control. E, the RPS10 methylation mutant is prone to degradation by the proteasomal pathway. HEK293 cells were co-transfected with a control vector (lanes 1 and 4), RPS10-FLAG (lanes 2 and 3), or RPS10-R158K/R160K (lanes 5 and 6) together with Myc-tagged ubiquitin. 36 h after transfection, the cells were treated with MG132 (lanes 4–6) as indicated. FLAG-tagged RPS10 was immunoprecipitated using anti-FLAG beads, subjected to SDS-PAGE, and immunoblot (IB) analysis to detect the ubiquitylated RPS10 or RPS10-R158K/R160K with anti-Myc antiserum. The blot was re-probed with anti-FLAG antiserum to detect RPS10 and RPS10-R158K/R160K.
that reflect the process of ribosome production. Synthesis, modification, and initial cleavage of prerRNA take place within the fibrillar components and the surrounding dense fibrillar component, whereas later processing steps and assembly of ribosomes are performed in the GC region (38).

We determined the localization of RPS10 in the Bel7402 permanent cell line (which stably expresses RPS10-Myc) by immunostaining and expected it to localize in the nucleolus as other ribosomal proteins (39). RPS10-Myc was localized in nuclei where it appeared to accumulate primarily in structures corresponding to nucleoli (supplemental Fig. S3). A weaker and diffuse cytoplasmic staining was also noticed. The ring-shaped subcellular localization of RPS10 in the stable cell line corresponds to that of NPM/B23, a well known marker for the GC region of nucleoli, and was very similar to that of RPS10 fused to GFP in transiently transfected cells (supplemental Fig. S4 and data not shown). This led us to test whether RPS10 is concentrated in the GC region of nucleoli by co-transfecting B23 with either RPS10 or RPS10-R158K/R160K into U2OS cells. We found that 57% of WT RPS10 mainly co-localized with B23 suggesting that RPS10 was concentrated in the GC region (Fig. 6, A and E). To our surprise, co-localization of RPS10-R158K/R160K with B23 was not found in any cells. Instead, its signal intensified inside nucleolar areas other than the GC region (Fig. 6, B and E). To further define the location of RPS10, WT RPS10 and RPS10-R158K/R160K were co-transfected with Fibrillarin separately. As shown in Fig. 6, C and D, mutant RPS10, instead of WT RPS10, co-localized well with Fibrillarin. These results indicate that RPS10 is enriched in the GC region of nucleoli and that methylation of RPS10 is required for its concentration in the GC region of nucleoli.

As methylation of RPS10 is required for its concentration in the GC region of nucleoli and plays a
role in the assembly of RPS10 into ribosomes, we went on to investigate whether the distribution of RPS10 in the whole cell, especially its export to the cytosol, is affected by methylation. WT RPS10 and RPS10-R158K/R160K were transfected into HEK293 cells. Nuclear and cytoplasmic fractions were separated and analyzed 48 h later. Using Lamin B as a marker, Fig. 6F shows that more RPS10-R158K/R160K mutant is present in the nuclear fraction. Thus, our results suggest that methylation of RPS10 plays a role in its location in the GC region of nucleoli and may also play a role in its transport from the nucleus to the cytosol.

The RPS10 Methylation Mutant Shows Decreased Binding to B23—B23/NPM, also known as Numatrin1, plays a key role in the processing and/or assembly of ribosomes (40). The chaperone properties of B23 might be a key to this process by preventing the aggregation of proteins and selectively storing a subset of ribosomal proteins to facilitate ribosome biogenesis (41, 45). The co-localization of RPS10 with B23 led us to determine whether RPS10 interacts with B23. Reciprocal co-immunoprecipitation assays using lysates from cells transfected with tagged B23 and/or RPS10 were carried out and confirmed the specific interaction between RPS10 and B23 (Fig. 7, A and B). To evaluate whether endogenous RPS10 and B23 interact, lysates from HEK293 cells were subjected to co-immunoprecipitation and Western blotting with RPS10 and B23 antisera. Analysis of the immunoprecipitates revealed clear interaction between the endogenous proteins (Fig. 7C). To determine whether the interaction between B23 and RPS10 is direct or indirect, GST pull-down assays using B23 and RPS10 expressed and purified from E. coli were employed. As shown in Fig. 7D, GST-B23, but not GST, could pull-down His-tagged RPS10 suggesting that RPS10 interacts with B23 directly.

We showed above that most of the WT RPS10 co-localizes with B23 in nucleoli and that the RPS10 methylation mutant fails to do so. We therefore investigated whether there are differences in the interaction between B23 and the WT and RPS10 methylation mutant using co-immunoprecipitation assays. It is interesting to note that interactions between RPS10-R158K/R160K and either overexpressed or endogenous B23 were weaker than those between WT RPS10 and either overexpressed or endogenous B23 (Fig. 7, E and F). Taken together, these results indicate that RPS10 is a novel B23-binding protein and the methylation mutation of RPS10 leads to decreased binding to B23 and it thus may explain why the RPS10 methylation mutant failed to co-localize with B23/NPM in the GC region of nucleoli.

DISCUSSION

RPS10 Is a Novel Substrate for PRMT5—Arginine methylation of ribosomal proteins is common in prokaryotes and eukaryotes. Although several methylated ribosomal proteins...
have previously been identified, the biological significance of this methylation is still poorly understood (43).

In this study, we found that RPS10 interacts with PRMT5 both in vivo and in vitro and it is a novel substrate for PRMT5. A knock-down experiment using shRNA indicated that PRMT5 played a role in the methylation of RPS10. Because the decrease of RPS10 methylation levels is not very significant despite efficient knockdown of PRMT5 (Fig. 2E), it is likely that RPS10 can be methylated by other PRMT family members as well. The methylation sites in RPS10 were identified as Arg\textsuperscript{158} and Arg\textsuperscript{160}.

Methylation of RPS10 Plays a Role in the Biogenesis of Ribosomes and Protein Synthesis—Ribosomal proteins are assembled in the nucleolus, especially in the GC region, to build the ribosome (38). They are then exported into the cytoplasm as ribosomal subunits. The role of ribosomal protein post-transcriptional modification in this process is poorly understood.

We found that WT RPS10 is enriched in the GC region of the nucleolus and that the methylation mutant failed to do so. This elucidates, at least in part, why the methylation mutant of RPS10 was not assembled into ribosomes as efficiently as WT RPS10. Methylation of RPS10 is likely to promote its interaction with B23 and its enrichment in the GC region. However, it is also possible that methylation of RPS10 induces the localization of RPS10 to the GC region and facilitates its interaction with B23 there.

It is interesting to note that methylation of RPS10 plays a role in the assembly of RPS10 into ribosomes. It is also important for ribosome biogenesis because mutation of RPS10 leads to misregulation of the 40 S:60 S ribosomal subunit ratio and suboptimal protein synthesis. This would explain why knocking down RPS10 expression with shRNA almost completely halted cell proliferation, which could then be rescued by WT RPS10 but to a lesser extent by the RPS10 methylation mutant.

It has been shown previously that unassembled ribosomal proteins are degraded quickly, whereas cytoplasmic pools of ribosome-associated ribosomal proteins are relatively stable (37). This may account for why the RPS10 methylation mutant, which was not assembled into ribosomes and exported to the cytoplasm efficiently, was less stable than WT RPS10 and degraded faster in a proteasome-dependent manner.

PRMT5, Methylation of RPS10, and Tumorigenesis—Both PRMT5 and ribosomal activity have been shown to be closely associated with cell proliferation and tumorigenesis (1, 22, 23). Like PRMT5, B23 is involved in many forms of tumors (40, 41). Our findings support a model in which PRMT5 may regulate cell growth via methylation of RPS10. Methylation of RPS10 by PRMT5 is likely to play a role in its interaction with B23, protein stability, localization to the GC region of the nucleolus, and efficient assembly into ribosomes.

It will be interesting to examine in the future whether PRMT5 controls cell proliferation through the regulation of ribosome assembly and protein synthesis. Accumulating evidence suggests that some proteins can be methylated by both type I and type II PRMTs. Thus, aDMA and sDMA methylation of the same protein may lead to different and possibly opposing biological consequences. It will therefore be intriguing to investigate whether other PRMT family members, in addition to PRMT5, play a role in the methylation, localization, and assembly of different ribosomal proteins and the regulation of cell proliferation.

PRMT5, Methylation of RPS10, and Systemic Lupus Erythematosus—Systemic lupus erythematosus (SLE) is characterized by the presence of circulating autoantibodies to cellular constituents. Among these, anti-Sm antibodies are found in more than 30% of patients with SLE and are used as a diagnostic marker for SLE (46). Our results indicate that RPS10 is dimethylated by PRMT5, and others have reported that anti-RPS10 antibodies were found in the sera of 11% of total SLE patients, and in 31% of active SLE patients. A high frequency of antibody activity against RPS10 (87.1%) has been found in anti-Sm sera from SLE patients. These patients tend to be in the active stage of SLE (34). More interestingly, sDMA of RG motifs in Sm and RPS10 are the major sites recognized. This raises a further question: what is the exact role of symmetrically dimethylated Sm and RPS10 in SLE? PRMT5 has previously been shown to produce sDMAs in the RG motifs of Sm proteins (17) and in RPS10 in this study. In addition to studying the relationship between PRMT5 and tumorigenesis, it will be intriguing to study in the future whether PRMT5 is also associated with SLE.

Acknowledgments—We thank Drs. L. Greene, J. Zhang, C. Yang, and X. Huang for comments and suggestions during the preparation of this manuscript.

REFERENCES
1. Ruggero, D., and Pandolfi, P. P. (2003) Nat. Rev. Cancer 3, 179–192
2. Ruvinsky, I., Sharon, N., Lerer, T., Cohen, H., Stolovich-Rain, M., Nir, T., Dor, Y., Zisman, P., and Meyuhas, O. (2005) Genes Dev. 19, 2199–2211
3. Polevoda, B., and Sherman, F. (2007) Mol. Microbiol. 65, 590–606
4. Scolnik, P. A., and Eliceiri, G. L. (1979) Eur. J. Biochem. 101, 93–101
5. Lhoest, J., Lobet, Y., Costers, E., and Colson, C. (1984) Eur. J. Biochem. 141, 585–590
6. Bachand, F., and Silver, P. A. (2004) EMBO J. 23, 2641–2650
7. Swiercz, R., Persson, M. D., and Bedford, M. T. (2005) Biochem. J. 386, 85–91
8. Bachand, F., Lackner, D. H., Bähler, J., and Silver, P. A. (2006) Mol. Cell Biol. 26, 1731–1742
9. Pahlisch, S., Zakaryan, R. P., and Gehring, H. (2006) Biochim. Biophys. Acta 1764, 1890–1903
10. Bedford, M. T., and Richard, S. (2005) Mol. Cell 18, 263–272
11. Bedford, M. T., and Clarke, S. G. (2009) Mol. Cell 33, 1–13
12. Gilbreh, M., Yang, P., Bartholomeusz, G., Pimental, R. A., Kansra, S., Gadiraju, R., and Marcus, S. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 14781–14786
13. Branscombe, T. L., Frankel, A. A., Lee, J. H., Cook, J. R., Yang, Z., Pestka, S., and Clarke, S. (2001) J. Biol. Chem. 276, 32971–32976
14. Pollack, B. P., Kotenko, S. V., He, W., Izotova, L. S., Barnoski, B. L., and Pestka, S. (1999) J. Biol. Chem. 274, 31531–31542
15. Ancelin, K., Lange, U. C., Hajkova, P., Schneider, R., Bannister, A. J., Kouzarides, T., and Surani, M. A. (2006) Nat. Cell Biol. 8, 623–630
16. Meister, G., Eggert, C., Büchner, D., Brahms, H., Kambach, C., and Fischer, U. (2001) Curr. Biol. 11, 1990–1994
17. Friesen, W. J., Paushkin, S., Wyce, A., Massenet, S., Pesiridis, G. S., Van Dunye, G., Rappsilber, J., Mann, M., and Dreyfuss, G. (2001) Mol. Cell. Biol. 21, 8289–8300
18. González, G. B., Tian, L., Ospina, J. K., Boisvert, F. M., Lamond, A. I., and Matera, A. G. (2007) J. Cell Biol. 178, 733–740
19. Kwak, Y. T., Guo, J., Prajapati, S., Park, K. J., Surabhi, R. M., Miller, B., Gehrig, P., and Gaynor, R. B. (2003) Mol. Cell 11, 1055–1066
20. Bao, S., Qyang, Y., Yang, P., Kim, H., Du, H., Bartholomeusz, G., Henkel, L., Pimental, R., Verde, F., and Marcus, S. (2001) *J. Biol. Chem.* **276**, 14549–14552

21. Wang, X., Zhang, Y., Ma, Q., Zhang, Z., Xue, Y., Bao, S., and Chong, K. (2007) *EMBO J.* **26**, 1934–1941

22. Pal, S., Vishwanath, S. N., Erdjument-Bromage, H., Tempst, P., and Sif, S. (2004) *Mol. Cell Biol.* **24**, 9630–9645

23. Pal, S., Baiocchi, R. A., Byrd, J. C., Grever, M. R., Jacob, S. T., and Sif, S. (2007) *EMBO J.* **26**, 3558–3569

24. Scoumanne, A., Zhang, J., and Chen, X. (2009) *Nucleic Acids Res.* **37**, 4965–4976

25. Dacwag, C. S., Ohkawa, Y., Pal, S., Sif, S., and Imbalzano, A. N. (2007) *Mol. Cell Biol.* **27**, 384–394

26. Dacwag, C. S., Bedford, M. T., Sif, S., and Imbalzano, A. N. (2009) *Mol. Cell Biol.* **28**, 6262–6277

27. Kim, J. M., Sohn, H. Y., Yoon, S. Y., Oh, J. H., Yang, J. O., Kim, J. H., Song, K. S., Rho, S. M., Yoo, H. S., Kim, Y. S., Kim, J. G., and Kim, N. S. (2005) *Clin. Cancer Res.* **11**, 473–482

28. Wang, L., Pal, S., and Sif, S. (2008) *Mol. Cell Biol.* **28**, 6262–6277

29. Louie, D. F., Resing, K. A., Lewis, T. S., and Ahn, N. G. (1996) *J. Biol. Chem.* **271**, 28189–28198

30. Xu, Z., Kukekov, N. V., and Greene, L. A. (2005) *Nucleic Acids Res.* **33**, 2251–2260

31. Xu, Z., Kukekov, N. V., and Greene, L. A. (2003) *EMBO J.* **22**, 252–261

32. Xu, Z., Maroney, A. C., Dobrzenski, P., Kukekov, N. V., and Greene, L. A. (2001) *Mol. Cell Biol.* **21**, 4713–4724

33. Friesen, W. J., Wyce, A., Ayyanathan, K., Yan, K. P., Langer, E. M., Longmore, G. D., and Rauscher, F. J., 3rd (2002) *J. Biol. Chem.* **277**, 8243–8247

34. Grisendi, S., Mecucci, C., Falini, B., and Pandolfi, P. P. (2006) *Nat. Rev. Cancer* **6**, 493–505

35. Lindström, M. S., and Zhang, Y. (2008) *J. Biol. Chem.* **283**, 15568–15576

36. Swierz, R., Cheng, D., Kim, D., and Bedford, M. T. (2007) *J. Biol. Chem.* **282**, 16917–16923