A Methyltransferase-independent Function for Rmt3 in Ribosomal Subunit Homeostasis*

Received for publication, November 19, 2008, and in revised form, April 8, 2009 Published, JBC Papers in Press, April 9, 2009, DOI 10.1074/jbc.M109.004812

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Schizosaccharomyces pombe Rmt3 is a member of the protein-arginine methyltransferase (PRMT) family and is the homolog of human PRMT3. We previously characterized Rmt3 as a ribosomal protein methyltransferase based on the identification of the 40 S Rps2 (ribosomal protein S2) as a substrate of Rmt3. RMT3-null cells produce nonmethylated Rps2 and show mis-regulation of the 40 S/60 S ribosomal subunit ratio due to a small subunit deficit. For this study, we have generated a series of RMT3 alleles that express various amino acid substitutions to characterize the functional domains of Rmt3 in Rps2 binding, Rps2 arginine methylation, and small ribosomal subunit production. Notably, catalytically inactive versions of Rmt3 restored the ribosomal subunit imbalance detected in RMT3-null cells. Consistent with a methyltransferase-independent function for Rmt3 in small ribosomal subunit production, the expression of an Rps2 variant in which the identified methyl-arginine residues were substituted with lysines showed normal levels of 40 S subunit. Importantly, substitutions within the zinc finger domain of Rmt3 that abolished Rps2 binding did not rescue the 40 S ribosomal subunit deficit of RMT3-null cells. Our findings suggest that the Rmt3-Rps2 interaction, rather than Rps2 methylation, is important for the function of Rmt3 in the regulation of small ribosomal subunit production.

Protein arginine methylation is a posttranslational modification catalyzed by a family of enzymes known as protein-arginine methyltransferases (PRMTs). Although protein-arginine methyltransferase activity has never been demonstrated in prokaryotic organisms, genes encoding PRMTs have been identified in a variety of unicellular and multicellular eukaryotes (1, 2). In humans, 10 PRMTs have so far been identified (3). Most PRMTs are divided in two major classes, depending on the type of dimethylarginine they produce. Whereas both type I and II PRMTs use S-adenosyl-L-methionine as a cofactor for the monomethylation of specific arginines within substrate proteins, type I and type II enzymes can also produce asymmetric N\(^{\text{rho}}\),N\(^{\text{rho}}\)-dimethylarginine and symmetric N\(^{\text{rho}}\),N\(^{\text{rho}}\)-dimethylarginine, respectively (1). Interestingly, protein arginine methylation is often found within arginine-glycine (RG)-rich regions of nucleic acid-binding proteins (4).

The functional role of PRMTs is likely to be mediated by the modification of substrate proteins. Accordingly, proteins involved in specific steps of gene expression, including transcription (5, 6), splicing (7), polyadenylation (8, 9), mRNA export (10), and translation (11–13), are modified by arginine methylation. Methylation of specific arginine residues within the N-terminal tails of nucleosomal histones is also important for gene regulation and chromatin remodeling (14, 15), thereby influencing biological processes, such as cell fate determination (16) and oncogenesis (17). As yet, however, the biological role of most PRMTs remains poorly understood.

The ribosome is the macromolecular complex responsible for protein synthesis in all living cells. In eukaryotes, the 80 S ribosome is composed of a small (40 S) and a large (60 S) ribosomal subunit; the 40 S subunit is composed of the 18 S rRNA and roughly 30 ribosomal proteins, whereas the 60 S subunit contains three rRNAs (25, 5.8 S, and 5 S) and ~50 ribosomal proteins. Eukaryotic ribosomal proteins are substrates of arginine methylation (18–21). Proteomic approaches corroborate these findings and indicate that ribosomal proteins contain a variety of covalent modifications, including phosphorylation, acetylation, ubiquitination, methylation of lysines and arginines, and neddylation (22–25). Notably, asymmetric dimethylarginine is the predominant methylated amino acid in both the eukaryotic 40 and 60 S ribosomal subunits (26). Although evidence for the methylation of ribosomal proteins was demonstrated more than 30 years ago, mechanisms by which this modification regulates the synthesis and/or the function of ribosomes remain elusive.

We and others have previously characterized the PRMT3 enzyme as a ribosomal protein methyltransferase (11, 13). PRMT3 modifies the RG-rich region of the 40 S Rps2 (ribosomal protein S2) via asymmetric dimethylation of as yet unidentified arginine residues. Deletion of the genomic copy of the RMT3 gene (homolog of human PRMT3) in fission yeast results in the expression of unmethylated Rps2 and a 40 S ribosomal subunit deficit that appears to be caused by defects beyond pre-rRNA processing (11). Recently, mice with a targeted disruption of the PRMT3 gene that results in a 10-fold reduction in PRMT3 expression were generated (27). It was found that PRMT3-deficient embryos have reduced levels of Rps2 methylation and are small in size. Such a phenotype is similar to Drosophila Minute mutants that are often attributable to ribosomal proteins insufficiency and that have a wide panorama of phenotypes, including slow development and small body size (28, 29).
The mechanism by which the absence of Rmt3 expression results in a 40 S ribosomal subunit deficit and whether this reduction is directly linked to Rps2 methylation are unknown. In this study, we identified substitutions in fission yeast Rmt3 that prevent Rps2 methylation but not binding to Rps2. Unexpectedly, such catalytically inactive alleles of RMT3 fully rescued the ribosomal subunit imbalance of RMT3-null cells. Consistently, a methylation-deficient allele of RPS2, in which the identified methylarginines were substituted with lysine residues, was functional and showed no reduction in small ribosomal subunit levels. Notably, expression of Rmt3 variants that were impaired in binding Rps2 showed a small ribosomal subunit deficit similar to RMT3-null cells. Our findings reveal that the association between Rps2 and Rmt3, but not Rps2 methylation, is important for the function of Rmt3 in ribosomal subunit homeostasis.

**MATERIALS AND METHODS**

**Yeast Strains and Growth Conditions**—Strain genotypes are listed in Table 1. Cells were grown at 30 °C in yeast extract medium with amino acid supplements and Edinburgh minimal medium containing appropriate amino acid supplements. *Schizosaccharomyces pombe* cells were transformed with plasmids and PCR products by the lithium acetate method. The pJK210 and pJK148 vectors, respectively (30).

**Plasmid Constructs**—The cDNA carrying RPS2-FLAG plus upstream (RPS2) and downstream (NMT1) regulatory sequences was excised from pFB98 (31) using PstI-EcoRI restriction enzymes. The pJK-EcoRI fragment was subsequently cloned into PstI-EcoRI-digested pJK148 and pJK210, creating plasmids pFB48 and pFB227, respectively. Construction of the of the arginine-to-alanine (Arg → Ala) and arginine-to-lysine (Arg → Lys) alleles of RPS2 were carried out by simultaneous site-directed mutagenesis of Arg5, Arg11, Arg14, Arg17, Arg19, Arg21, Arg23, and Arg24 using 136-nucleotide complementary oligonucleotides that changed these arginine codons to either alanine or lysine. The cDNA carrying wild-type RMT3 plus upstream (RMT3) and downstream (NMT1) regulatory sequences was cloned into pJK148. Construction of the different RMT3 alleles was by site-directed mutagenesis. The construct that expresses glutathione S-transferase (GST) fused to wild-type Rmt3 was previously described (11). All Rmt3 variants were subcloned into EcoRI-Xhol-digested pGEX-4T-2. All of the constructs used in this study were verified by automated sequencing at the University of Calgary DNA Core Facility.

**Protein Analysis**—For the Rmt3 copurification experiments shown in Fig. 2, 50 ml of midlog phase cells grown in Edinburgh minimal medium were used. Cells were lysed with a Fastprep FP120 (Thermo Electro Corp.) using 0.5-mm glass beads in ice-cold polysome buffer (20 mM Tris–HCl, pH 7.5, 10 mM MgCl2, 1 mM dithiothreitol, 50 mM KCl) containing a mixture of protease and RNase inhibitors. Clarified lysates were normalized for total protein concentration, and 1 mg of total proteins were subjected to immunoprecipitation using agarose-conjugated anti-FLAG M2 (Sigma). Immunopurified proteins were competitively eluted from the beads in polysome buffer supplemented with 200 ng/ml FLAG peptide. Eluted proteins were separated on 12% SDS-PAGE, transferred to nitrocellulose membranes, and analyzed by immunoblotting using a rabbit polyclonal antibody specific to *S. pombe* Rmt3 (8) and a mouse monoclonal antibody specific to the FLAG epitope (Sigma). Membranes were then probed with goat anti-rabbit and anti-mouse secondary antibodies conjugated to AlexaFluor 680 (Molecular Probes) and IRdye 800 (Rockland Immunocographics), respectively. Linear detection of the proteins was performed and quantified using an Odyssey infrared imaging system (LI-COR). The percentage Rps2 binding for the different variants of Rmt3 was established as follows: (signal ratio of copurified Rmt3 over purified Rps2-FLAG)/(signal ratio of input Rmt3 over input Rps2). The corresponding values from this calculation were normalized to wild-type Rmt3, which was arbitrarily set to 100%. Purification of FLAG-tagged Rps2 for the identification of methylated arginines by mass spectrometry was as described above except that 250 ml of yeast cultures were used. Eluted proteins were also trichloroacetic acid-precipitated before being subjected to 14% SDS-PAGE and visualized by Coomassie Blue staining.

For the evaluation of the level of Rps2 methylation catalyzed by the different Rmt3 substitutions, total cell extracts prepared in polysome lysis buffer were separated on 12% SDS-PAGE,
transferred to nitrocellulose membrane, and visualized by immunoblotting using rabbit polyclonal antibodies specific to Rmt3 and Rps2 (32) as well as using a methylarginine-specific polyclonal antibody (33). Linear detection of the proteins was performed and quantified using an Odyssey infrared imaging system (LI-COR) as described above.

In Vitro Methylation Assays—Purification of GST and GST-Rmt3 fusion proteins expressed in Escherichia coli was as previously described (11). The substrate for the in vitro methylation assays was unmethylated Rps2 that was immunopurified from extracts of RMT3-null as described above except that 20 mM EDTA and 1 M NaCl were added to the purification buffer to reduce the level of coprecipitating proteins. In vitro methylation activity assays were performed as 30 μL reactions in 50 mM sodium phosphate, pH 8.0, 150 mM NaCl, 2 mM EDTA. Reactions were incubated at 30 °C for 3 h and terminated by the addition of 1 volume of 2× SDS-PAGE sample buffer and subsequent incubation at 95 °C for 5 min. The samples were resolved on 10% SDS-PAGE followed by Coomassie Blue staining and fluorography (Enhance; PerkinElmer Life Sciences).

Ribosome Profiles—Ribosome profiles and the relative ratios of free 40 and 60 S ribosomal subunits were as previously described (11). Sucrose gradients were fractionated by upward displacement with 55% (w/w) sucrose using a gradient fractionator (Brandel Inc.) connected to a UA-6 UV monitor (Teledyne Isco) for continuous measurement of the absorbance at 254 nm.

Mass Spectrometry—Mass spectrometry analysis of Rps2 was carried out at the Southern Alberta Mass Spectrometry Centre for Proteomics of the University of Calgary. Peptide separation was achieved with a Zorbax 300 SB-C18 analytical column (75–μm inner diameter × 150 mm, 3.5 μm, 100 Å; Agilent Technologies) using an Agilent 1100 nano-LC system. A 2-pmol digest was loaded onto an enrichment column (Zorbax 300 SB-C18; 5 μm, 5 × 0.3 mm) for 5 min using an Agilent binary pump with a flow rate of 10 μL/min. The analytical column was equilibrated for 5 min with 97% mobile phase A (0.2% formic acid in water), and peptides were eluted using a linear gradient from 3 to 90% mobile phase B (0.2% formic acid, 10% water in acetonitrile) over 50 min with a constant flow rate of 0.3 μL/min. The column was washed for 10 min with 90% B and re-equilibrated with 3% B for 15 min prior to subsequent sample loading. The analytical column was connected online to a QSTAR XL quadrupole time-of-flight mass spectrometer equipped with a nano-electrospray ion source (AB/MDS-Sciei, Toronto, Canada) fitted with an 8-μm nanospray tip (New Objective). The ion spray voltage was set at 1500 V, and the interface heater temperature was 150 °C. Precursor ion scanning experiments were carried out as previously described (34) for methylated peptide detection, and time-of-flight mass spectrometry (MS) experiments were performed in positive ion mode over an m/z range of 400–1500 for charged state identification.

RESULTS

S. pombe Rmt3 is a cytosolic type I arginine methyltransferase that harbors a C2H2 zinc finger domain, N-terminal conserved regions 1 and 2, conserved methyltransferase motifs, and a poorly characterized C-terminal domain (Fig. 1) (1, 11). To determine whether the methyltransferase activity of Rmt3 is...
required for small ribosomal subunit production, variants of Rmt3 that can bind but not methylate Rps2 were needed. We therefore generated a series of RMT3 alleles that express single and double amino acid substitutions to characterize the functional domains of Rmt3 in Rps2 binding, Rps2 arginine methylation, and ribosomal subunit homeostasis. Substitutions were introduced at evolutionarily conserved residues within various domains of S. pombe Rmt3 (Fig. 1). Substitutions of critical cysteine and histidine residues of the zinc finger (Cys63 with His76 and Cys80 with His81) were introduced because the substrate specificity and/or the enzymatic activity of Rmt3 are likely to be regulated by the zinc finger (13, 35). To create a catalytically inactive Rmt3 variant, a conserved glutamic acid residue (Glu338) shown to be required for PRMT1 catalysis (36) was modified. Alignment of Rmt3 sequences from diverse organisms revealed the presence of two conserved stretches of amino acids in the N-terminal region that were called conserved regions 1 and 2 (CR1 and -2) (Fig. 1) (1). Amino acid substitutions at evolutionarily conserved residues within the CR1 (Asn104 and Ile106) and CR2 (Tyr130) motifs were thus introduced to begin to characterize the functional roles of these conserved regions. A cysteine residue (Cys873) specific to fission yeast Rmt3 was also deleted, and a tryptophan (Trp488) residue within the conserved C-terminal THW motif of Rmt3 was modified (Fig. 1). The THW motif is found in most PRMTs and is predicted to form a loop structure near the active site, as determined by x-ray crystallography (37). To prevent overexpression, the different RMT3 alleles were chromosomally integrated as single copy into RMT3-null cells and expressed from their endogenous promoter.

The Zinc Finger Motif of Rmt3 Is Critical for Binding Rps2—Fission yeast and human Rmt3 directly interact with Rps2 (11, 13). A copurification assay was thus used to define regions of Rmt3 necessary for Rps2 association. The RMT3 alleles described above were expressed in RMT3-null cells that also expressed a functional FLAG-tagged version of Rps2 (31). Using the FLAG epitope as an affinity tag, FLAG immunoprecipitates were prepared to isolate complexes between Rmt3 and Rps2. Eluted proteins were then separated by SDS-PAGE and analyzed for the ability of the different Rmt3 variants to copurify with Rps2 by Western blotting. As can be seen in Fig. 2A, wild-type Rmt3 was recovered in FLAG immunoprecipitates (lane 2) but not from extracts of control cells that expressed an untagged version of Rps2 (lane 1), demonstrating the specificity of our copurification assay. The level of Rmt3 binding to Rps2 was quantified (see “Materials and Methods”) for the different Rmt3 variants and normalized to wild-type Rmt3 (Fig. 2B). Alterations within the carboxyl-terminal region of Rmt3 (C475A and W488A) did not perturb the ability of Rmt3 to bind Rps2 (Fig. 2B). These results indicate that the zinc finger domain is the primary determinant of Rmt3 required for the interaction with Rps2 in fission yeast.

Identification of Catalytically Inactive Versions of Rmt3—We have previously demonstrated that Rps2 is modified by asymmetric dimethylation of arginine in an Rmt3-dependent fashion (11). To define functional domains of Rmt3 required for arginine methylation of Rps2, we determined the levels of Rps2 methylation catalyzed by the different Rmt3 variants shown in Fig. 1. A methylarginine-specific antibody (33) was used to assess the status of Rps2 methylation. As can be seen in Fig. 3A, this antibody recognized arginine methylated Rps2 from extracts of wild-type cells (lane 1) but not unmethylated Rps2 from extracts of RMT3-null cells (lane 2). Longer exposures of membranes blotted with the methylarginine-specific antibody did not show additional Rmt3-dependent substrates (data not shown). To confirm that the protein recognized by the methy-
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larginine-specific antibody was Rps2, an RPS2-null strain that expressed a GFP-tagged version of Rps2 was used. As expected, only the GFP-tagged version of Rps2 was detected when an Rps2-specific antibody was used to analyze extracts from this strain (Fig. 3A, lane 3, top). An equivalent size shift was seen when the methylarginine-specific antibody was used to analyze the same extract from the strain expressing rpS2-GFP (Fig. 3A, lane 3, bottom). These results clearly establish that the 28 kDa band detected by the methylarginine-specific antibody in extracts of wild-type (lane 1) but not RMT3-null cells (lane 2) is endogenous Rps2.

The methylarginine-specific antibody was thus used to assess the ability of the different Rmt3 variants to catalyze arginine methylation of Rps2. Extracts of yeast that expressed RMT3 alleles with substitutions of critical cysteine and histidine residues within the zinc finger contained mostly unmethylated Rps2 (Fig. 3, B and C), consistent with the inability of the Rmt3 zinc finger mutants to bind Rps2 (Fig. 2). Variant N104D within the CR1 motif of Rmt3 that showed a 40% reduction in Rps2 binding (Fig. 2) resulted in roughly 35% of arginine methylated Rps2 (Fig. 3, B and C). Importantly, variants of Rmt3 that bound Rps2 but that did not catalyze its methylation were identified. First, substitution E338Q near motif II of the Rmt3 methyltransferase domain (Fig. 1) completely abolished Rps2 methylation (Fig. 3, B and C), consistent with an analogous substitution in rat PRMT1 (36). Substitution W488A within the conserved THW loop also resulted in a catalytically inactive version of Rmt3 (Fig. 3, B and C). Extracts prepared from cells that expressed the I106S, Y130A, and the deletion of cysteine 475 within S. pombe Rmt3 showed Rps2 methylation levels that corresponded to 1.3-, 0.6-, and 0.8-fold, respectively, relative to wild-type Rmt3 (Fig. 3C).

Rmt3 variants were also examined for in vitro methyltransferase activity using recombinant versions that were purified as GST fusion proteins expressed in E. coli. In vitro methylation assays were performed in the presence of 3H-labeled S-adenosyl-L-methionine using GST fusions and unmethylated Rps2 that was immunopurified from RMT3-null cells. Consistent with our previous results (11), GST fused to wild-type Rmt3 specifically methylated Rps2 in vitro (Fig. 4, lane 2). In contrast, Rps2 did not incorporate radiolabeled S-adenosyl-L-methionine when incubated with GST alone (Fig. 4, lane 1). Consistent with the analysis of in vivo Rps2 methylation by Western blot (Fig. 3B), W488A and E338Q substitutions showed no methyltransferase activity in vitro (Fig. 4, lanes 7 and 10, respectively). Rmt3 variants containing substitutions at conserved cysteines and histidines within the zinc finger domain showed some levels of Rps2 methylation in vitro (Fig. 4, lanes 5 and 6). These

recognizes methylated Rps2. Shown is Western blot analysis of total cell lysates prepared from wild-type cells (lane 1), RMT3-null cells (lane 2), and RPS2-null cells that were previously transformed with a plasmid expressing Rps2-GFP (lane 3). Antibodies for Western blotting (WB) were Rps2-specific and methylarginine-specific (mRG) polyclonal antibodies (top and bottom, respectively), B, Western blot analysis of total cell lysates prepared from RMT3-null cells (lane 2) that expressed various alleles of RMT3 (lanes 1 and 3–10). Antibodies for Western blotting were Rmt3-specific, methylarginine-specific, and Rps2-specific rabbit polyclonal antibodies (top, middle, and bottom, respectively). C, percentage of Rps2 methylation as determined from at least three independent experiments as shown in B. WT, wild type.
results suggest that the zinc finger domain of Rmt3 is not required for catalytic activity in vitro but is important to guide the methyltransferase activity of Rmt3 to Rps2 in vivo. Variant N104D also showed reduced Rps2 methylation in vitro (Fig. 4, lane 4), consistent with the analysis of Rps2 methylation from cell extracts using the methylarginine-specific antibody (Fig. 3B). In vitro methylation assays using recombinant versions of the Rmt3 variants C475Δ, Y130A, and N104S showed activity comparable with that of the wild type (Fig. 4).

Taken together, our results using two independent methylation assays indicate that glutamic acid 338 within the conserved methyltransferase domain as well as tryptophan 488 from the THW loop are both critical for Rmt3-dependent Rps2 methylation but not for Rps2 binding.

**The Methyltransferase Activity of Rmt3 Is Not Required for Small Ribosomal Subunit Production—Rmt3-null cells have reduced levels of small ribosomal subunit that lead to the accumulation of the large subunit (11, 31). To test whether the ribosomal subunit imbalance detected in RMT3-null cells is dependent on the methyltransferase activity of Rmt3, we used a functional complementation assay to assess the ability of the various RMT3 alleles to restore the altered ribosome profile of RMT3-null cells. Extracts from RMT3-null cells expressing different RMT3 alleles were subjected to ultracentrifugation through sucrose gradients and fractionated to monitor rRNA levels. Consistent with our earlier studies (11, 31), the free 60 S:40 S ribosomal subunit ratio in RMT3-null cells was significantly greater compared with wild-type cells (Fig. 5, A and G). As a control, expression of wild-type Rmt3 in RMT3-null cells fully restored the ribosomal subunit imbalance (Fig. 5B). Similarly, the catalytically inactive Rmt3 variants E338Q and W488A showed a free 60 S:40 S ratio similar to that of wild-type Rmt3 (Fig. 5, E and F, respectively, and G). Conversely, variants with substitutions at conserved cysteine and histidine residues within the zinc finger domain of Rmt3 did not restore the 40 S ribosomal subunit deficit of RMT3-null cells (Fig. 5, C and D), resulting in a free 60 S:40 S ratio similar to that of RMT3-null cells (Fig. 5G). Extracts prepared from cells expressing Rmt3 variants I106S, Y130A, N104D, and C475D demonstrated free 60 S:40 S ratios similar to that of wild-type Rmt3. We conclude that the function Rmt3 in maintaining proper 40 S ribosomal subunit levels does not require its methyltransferase activity. Our results suggest that the Rmt3-Rps2 interaction but not Rps2 methylation is important for the function of Rmt3 in small ribosomal subunit production.

**Identification of Rmt3-dependent Arginine Methylated Residues of Rps2—**The aforementioned results suggest that arginine methylation of Rps2 is not required for producing or maintaining proper levels of small ribosomal subunits. To test this directly, we first identified the methylarginine residues of Rps2 using MS. Rps2 was affinity-purified from extracts of cells that expressed a FLAG-tagged version of Rps2 in the presence or absence of Rmt3. Eluates of the FLAG purification were analyzed by SDS-PAGE and visualized by Coomassie Blue staining. Consistent with the results presented in Figs. 2 and 3, Rps2 migrated slightly faster after purification from extracts of RMT3-null cells (Fig. 6A, lane 2) as compared with Rps2-FLAG from wild-type cells (Fig. 6A, lane 1). The Coomassie Blue-stained bands corresponding to Rps2 were excised, subjected to tryptic digestion, and analyzed by LC-MS/MS that confirmed the identity of *S. pombe* Rps2 (data not shown). The LC-MS/MS procedure was followed by a precursor ion scan experiment that allows the detection of asymmetric and symmetric dimethylarginine-containing peptides (34). Whereas no ions were detected after precursor ion scanning for the Rps2 prepared from RMT3-null cells, *m/z* 485 and 488 ions were detected in the precursor ion scan of Rps2 prepared from wild-type cells (Fig. 6B). Inspection of the MS data revealed that the *m/z* 485 ion had a charge state of +6 and a mass of 2905.72 atomic mass units, whereas the *m/z* 488 ion had charge state of +5 and a mass of 2437.45 atomic mass units (Fig. 6, B and C). With instrument recalibration and reacquisition of an LC-MS data set, the mass accuracy of the measurement was sufficient to unambiguously map these two peptides to the N-terminal arginine-glycine (RG)-rich region of Rps2 (Fig. 6C). Furthermore, the appearance of satellite peaks — 45 atomic mass units from *m/z* 485 and 488 (Fig. 6B) is consistent with the loss of dimethylamine and thus the presence of asymmetric dimethylation. These results are consistent with our earlier findings (11). Notably, no other signals were detected by precursor ion scanning, suggesting that methylated arginines are restricted to the RG-rich region of Rps2. Since trypsin very inefficiently cleaves after methylarginines, the two methylated peptides had a number of missed cleavages (Fig. 6C). Accordingly, peptides corresponding to the RG-rich region of Rps2 were not detected from extracts of RMT3-null cells, since trypsin digestion yielded peptides (2–4 amino acids) that are too small to be detected (34), confirming the absence of Rps2 arginine methylation in RMT3-null cells. These results indicate that Rmt3 catalyzes the methylation of 8 arginine residues within the RG-rich region of fission yeast Rps2.

**Normal Ribosomal Subunit Levels in Cells That Express a Methylation-deficient Version of Rps2—**The normal levels of 40 S ribosomal subunit in catalytically inactive mutants of RMT3 suggest that arginine methylation of Rps2 has little impact on
FIGURE 5. Catalytically inactive alleles of RMT3 restore the ribosomal subunit imbalance detected in RMT3-null cells. Shown is sucrose gradient analysis of total extracts prepared from RMT3-null cells (A) that expressed wild-type (B), C63S/H76A (C), C60S/H81A (D), E338Q (E), and W488A (F) alleles of RMT3. G, quantification of the 60 S/40 S free ribosomal subunit ratio for the different RMT3 alleles as determined from at least three independent experiments. Asterisks above bars indicate statistically significant increase relative to wild-type Rmt3 (p < 0.0001), as determined by Student’s t tests.
ribosome biosynthesis. To further examine the significance of arginine methylation within the RG-rich domain of Rps2, all eight methylated arginines identified by mass spectrometry (Fig. 6) were substituted to alanine (Arg → Ala) or lysine (Arg → Lys) residues. An alanine residue has a small and uncharged side chain, whereas both lysine and arginine are long

FIGURE 6. Identification of methylarginine residues of fission yeast Rps2 by MS. A, large scale purification of fission yeast Rps2. Coomassie Blue staining of FLAG immunoprecipitates prepared from extracts of strain FBY154 (lane 1) and FBY157 (lane 2). The arrowheads indicate the position of Rps2-FLAG. Molecular mass markers are indicated on the right in kDa. B, MS spectrum of two asymmetrically dimethylated peptides from S. pombe Rps2. Asterisks and diamonds mark those peaks in the MS spectrum that have been correlated with dimethylarginine modifications, as a result of signal generation in the ion precursor scan at m/z 46. The peptide sequence is shown beside each dimethylated peptide, as verified by accurate mass measurement. C, arginine-methylated residues found in S. pombe Rps2. The positions of the two different methylated peptides are in amino acids. MC, missed cleavages. IP, immunoprecipitation.
chain amino acids with a positive charge under physiological conditions. Importantly, lysine residues are not targets for PRMTs (38). The mutated alleles of RPS2 as well as wild-type RPS2 were expressed from their endogenous promoters and chromosomally integrated into a conditional strain in which the genomic copy of RPS2 is deleted and plasmid-borne RPS2 is expressed from the inducible/repressible NMT1⁺ promoter (32). Because expression from the NMT1⁺ promoter is strongly repressed following thiamine addition (32), the RPS2 conditional strain was unable to grow when thiamine was supplemented (Fig. 7A). In contrast, wild-type RPS2 fully complemented the conditional strain in thiamine-supplemented conditions (Fig. 7A). Similarly, the Arg → Lys version of Rps2 complemented the RPS2 conditional strain, whereas the Rps2 Arg → Ala showed some growth defects (Fig. 7A). Accordingly, the protein level of the Arg → Ala version of Rps2 was found to be reduced 5-fold relative to wild-type Rps2 (Fig. 7B; compare lanes 2 and 3).

We next examined ribosome profiles from cells that expressed the different RPS2 alleles to assess the functional importance of Rps2 arginine methylation in ribosome biogenesis. The different strains were grown in thiamine-containing medium for 12 h to deplete plasmid-expressed Rps2, and cell extracts were analyzed by velocity sedimentation using sucrose gradients. At this point, plasmid-expressed Rps2 was undetectable by immunoblotting (data not shown), and this resulted in the reduction of free 40 S subunits and polysomes as well as the accumulation of free 60 S subunits (Fig. 7C, control panel). Consistent with the growth assays (Fig. 7A), wild-type Rps2 and Rps2 Arg → Lys maintained normal ribosome profiles after repression of plasmid-borne RPS2 expression (Fig. 7C). Notably, extracts prepared from the conditional strain that expressed Rps2 Arg → Lys did not

FIGURE 7. Arginine methylation is not required for the function of Rps2 in ribosome biogenesis. A, 10-fold serial dilutions of strains FBY136 (control), FBY217 (wild type; WT), FBY218 (Arg → Ala), and FBY219 (Arg → Lys) were spotted onto Edinburgh minimal medium plates with (right) or without (left) thiamine. B, Western blot analysis of total cell lysates prepared from strain FBY136 (lane 1), FBY217 (lane 2), FBY218 (lane 3), and FBY219 (lane 4). Antibodies for Western blotting were a rabbit polyclonal anti-Rmt3 (top) and a mouse monoclonal anti-FLAG (bottom). C, sucrose gradient analysis of total extracts prepared from strains FBY136 (control), FBY217 (wild type), FBY218 (Arg → Ala), and FBY219 (Arg → Lys).
show a deficit in free 40 S subunit levels. In contrast, Rps2 Arg → Ala resulted in the accumulation of free 60 S ribosomal subunit due to a 40 S ribosomal subunit deficit (Fig. 7C). Together, these results suggest that the overall positive charge of the RG-rich region, rather than arginine methylation, is significant for the role of Rps2 in ribosome biosynthesis.

DISCUSSION

Arginine Methylation of Rps2 Is Not Required for Ribosome Biogenesis—Depletion of Rps2 leads to the complete inhibition of 18 S rRNA synthesis and 40 S subunit production in fission yeast (Fig. 7) (32), which is probably the cause of lethality in Rps2-null cells. We have previously reported that the deletion of rmt3 in fission yeast leads to unmethylated Rps2 and causes a 40 S ribosomal subunit deficit (11, 31), which suggested that Rps2 methylation by Rmt3 regulates ribosome biosynthesis. We show here, however, that the function of Rps2 in ribosome biogenesis is not modulated by arginine methylation. This conclusion is supported by two independent observations: (i) catalytically inactive alleles of RMT3 that result in non-methylated Rps2 produce normal levels of small ribosomal subunit, and (ii) expression of a methylarginine-deficient version of Rps2 does not lead to a 40 S ribosomal subunit deficit. Importantly, these results are consistent with studies that addressed the functional role of ribosomal protein lysine methylation in yeast (39–42). These studies demonstrate that deletion of genes encoding specific SET domain lysine methyltransferases does not perturb ribosome biogenesis. It is possible, however, that the absence of a single type of modification is not sufficient to perturb ribosome synthesis because posttranslational modifications of ribosomal proteins may act cooperatively, as was recently reported for rRNA modifications (43). Arginine methylation of specific ribosomal proteins may also function in aspects of translation other than ribosome assembly, such as translational control and/or fidelity. Accordingly, phosphorylation of an evolutionarily conserved serine residue in budding yeast Rps2 was recently shown to be important for translation accuracy (44). Combinations of ribosomal protein modifications may also regulate the translation of specific cellular transcripts, similarly to how posttranslational modifications of nucleosomal histones regulate transcription (45). This is supported by evidence for ribosome heterogeneity (46) as well as data indicating that IRES-bound ribosomes contain a different methylation pattern than native ribosomes (47). Given that arginine methylation of Rps2 is evolutionarily conserved (11, 13), it is likely that this modification is functionally significant. Future studies will therefore be required to determine whether the absence of Rps2 methylation leads to underlying defects in translation or in an extraribosomal function.

Amino acid sequence analysis from a variety of species indicates that the N-terminal RG-rich domain of Rps2 is highly conserved (48), suggesting functional importance of this region. In addition to Rps2, several proteins involved in RNA-related functions contain an RG-rich domain and are often, if not always, substrates of arginine methylation (49, 50). RG-rich domains contribute to the RNA-binding (8, 51) and protein-protein interaction (52, 53) activities of specific proteins. In the case of Rps2, the biochemical role of the RG-rich domain remains elusive. Yet, our data suggest that the largely positive charge provided by the RG-rich region of Rps2, and not arginine methylation per se, is functionally important for maintaining proper levels of small ribosomal subunits (Fig. 7). Because expression of the Arg → Ala variant of Rps2 was reduced relative to wild-type Rps2 (Fig. 7B), we cannot exclude, however, the possibility that the observed defects in ribosome biosynthesis upon expression of Rps2 Arg → Ala are due to Rps2 deficiency.

A Methyltransferase-independent Function for Rmt3—In this study, we have identified substituted versions of Rmt3 that bind but do not catalyze arginine methylation of Rps2. Based on crystal structure studies, Glu<sup>338</sup> of S. pombe Rmt3 corresponds to the second of the two invariants glutamates that have been located within the active site of PRMT1 and PRMT3 (36, 37). Accordingly, arginine methylation of Rps2 was completely abolished in extracts of cells that expressed Rmt3 E338Q. Identical results were obtained after the substitution of the corresponding glutamate in rat PRMT1 (36), indicating that this conserved glutamic acid residue is likely to be critical for catalysis across the PRMT family. The other catalytically inactive variant of Rmt3 that showed Rps2 binding levels similar to wild type was W488A. Although this conserved tryptophan is distant from the methyltransferase domain in terms of primary structure, the crystal structure of a complex between PRMT1 and an RG-rich peptide indicates that this tryptophan is part of a loop (THW loop) proximal to the substrate peptide (36). To our knowledge, the predicted role of this loop structure in substrate recognition and/or catalysis had not been validated experimentally before our study.

Our results showed that catalytically inactive variants of Rmt3 restored the ribosomal subunit imbalance detected in RMT3-null cells (Fig. 5). These findings indicate that the role of Rmt3 in small ribosomal subunit production is independent of its methyltransferase activity. To our knowledge, this is the first demonstration of a methyltransferase-independent function for a PRMT. Our data also suggest that the Rps2 binding activity of Rmt3 is essential for the function of Rmt3 in ribosomal subunit homeostasis. This is supported by evidence that substitutions within the zinc finger domain of Rmt3 that abolished Rps2 binding (Fig. 2) did not rescue the 40 S ribosomal subunit deficit of RMT3-null cells (Fig. 5). Interestingly, examples of ribosome-modifying enzymes that function in ribosome biogenesis independently of their enzymatic activity have recently been reported (54–56). In the case of the RNA methyltransferase Bud23 (56), the authors propose that binding of Bud23 to pre-40 S in the nucleolus could function in small subunit assembly and that rRNA methylation by Bud23 may mark export-competent pre-40 S subunits to prevent rebinding by Bud23. Such a model is unlikely for Rmt3, because this protein localizes to the cytoplasm (11), whereas Bud23 is found in the nucleolus, where ribosome assembly takes place. We rather predict a model whereby the Rmt3-Rps2 interaction modulates the turnover and/or the nucleocytoplasmic trafficking of unassembled Rps2. A study recently demonstrated that eukaryotic cells ensure that ribosomal protein levels are never limiting for ribosome production by (i) the expression of ribosomal proteins at high levels beyond the requirement for ribosomal sub-
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unit production and (ii) the degradation of unassembled ribosomal proteins by the proteasome (57). According to such a model of constant synthesis and degradation of ribosomal proteins, Rmt3 could gauge the amount of Rps2 that is incorporated into pre-40 S subunits and thereby regulate small subunit production, since Rps2 is required for 40 S assembly (32). Alternatively, although little is known about degradation pathways of mature ribosomal subunits, the Rmt3-Rps2 interaction may control the recycling/turnover of mature 40 S subunits. Future studies will be required to establish how Rmt3 controls small subunit levels independent of its methyltransferase activity.

The expression of rRNAs and ribosomal proteins is tightly regulated in eukaryotic cells and depends on nutrient conditions, developmental stages, and environmental stresses (58–60). Accordingly, decreasing the level in any of several ribosomal proteins causes alteration in ribosome biogenesis and can lead to defects in cell size (61), organismal body size (61–63), and cell cycle progression (64–66). Conversely, haploinsufficiency for specific ribosomal protein genes has been linked to bone marrow failure syndromes (27). Given the recent findings that prmt3-deficient embryos exhibit Minute-like characteristics (27). Given the recent findings that link ribosomal subunit production to cell size control and tumorigenesis, it will be important to elucidate the mechanism by which the absence of the conserved methyltransferase Rmt3 leads to a small ribosomal subunit deficit.

Acknowledgments—We thank Ella Ng and Dr. David Schriemer at the Southern Alberta Mass Spectrometry Centre for help with the mass spectrometry analysis.

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JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 284•NUMBER 22•MAY 29, 2009

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