Yeast Ribosomal Protein L12 Is a Substrate of Protein-arginine Methyltransferase 2*

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Type III protein-arginine methyltransferase from the yeast Saccharomyces cerevisiae (RMT2) was expressed in Escherichia coli and purified to apparent homogeneity. The cytosolic, ribosomal, and ribosome salt wash fractions from yeast cells lacking RMT2 were used as substrates for the recombinant RMT2. Using S-adenosyl-L-methionine as co-substrate, RMT2 methylated a protein in the ribosome salt wash fraction. The same protein in the ribosomal fraction was also methylated by RMT2 after pretreating the sample with endonuclease. Amino acid analysis affirmed that the labeling products were δ-N-monomethylarginines. The methylated protein from the ribosomal or the ribosome salt wash fraction was isolated by two-dimensional gel electrophoresis and identified as ribosomal protein L12 by mass spectrometry. Using synthetic peptides, recombinant L12, and its deletion mutant as substrates, we pinpointed Arg[^14] on ribosomal protein L12 as the methyl acceptor. L12 was isolated from wild type yeast cells that have been grown in the presence of S-adenosyl-L-[[^3H]methyl]methionine and subjected to amino acid analysis. The results indicate that L12 contains δ-N-monomethylarginines.

Proteins can have a large variety of post-translational modifications, including the N-methylation of the arginine side chains. The process involves the transfer of methyl group from S-adenosyl-L-methionine (AdoMet) to the guanidino nitrogen atoms of arginine with protein-arginine N-methyltransferases (PRMTs) (1). There are at least three types of PRMTs (2). The type I PRMTs mediate the formation of ω-N[^4]-monomethylarginine and asymmetric ω-N[^4]-N[^2]-dimethylarginine. The type II enzymes have the symmetric ω-N[^4]-N[^2]-dimethylarginine in addition to the ω-N[^4]-monomethylarginine as products (see Ref. 1 for a review). Recently, a type III PRMT was discovered in yeast (3). This enzyme catalyzes the monomethylation of the δ-guanidino nitrogen of arginine side chains.

At least five complete genes have been reported for the type I PRMTs. They are the PRMT1 from rat and human, the protein-arginine methyltransferase 1 from Saccharomyces cerevisiae (RMT1), the PRMT3 from rat, and the mouse coactivator-associated arginine methyltransferase 1/PRMT4. The genes of human PRMT1 (4), rat PRMT1 (5), PRMT3 (6), and coactivator-associated arginine methyltransferase 1/PRMT4 (7) were isolated in two-hybrid screening experiments by interacting with type I interferon receptor, the immediate early protein, rat PRMT1, and the hormone receptor coactivator, respectively. The yeast RMT1 was identified independently by homology searches of the yeast genomic data base (1) or genetic screening of proteins that interact with Npl3p, a poly(A)^+ RNA-binding protein (8). These enzymes methylate proteins with an Arg-Gly-Gly-rich (9) or Arg-Xaa-Arg-rich (10) region. In vitro experiments have shown that heterogeneous nuclear ribonucleoprotein A1 (5), fibrillarin (6), histone H3 (7), Npl3p (8), and poly(A)-binding protein II (10) can be methylated by these type I PRMTs. However, only Npl3p has been shown to be the in vivo substrate of RMT1 (8).

A putative human type II PRMT, Jak-binding protein 1/PRMT5 (11, 12), and its yeast homologue (Hal7p) (13) were recently identified. Jak-binding protein 1/PRMT5 was found interacting with Janus kinase 2 (Jak2) (11) and the nonstructural protein 3 (NS3) of hepatitis C virus (12). It has been shown that Jak-binding protein 1/PRMT5 can utilize myelin basic protein, histones H2A and H4, and interestingly, a glutathione S-transferase fibrillarin-glycine- and arginine-rich domain fusion protein (GST-GAR) as substrates. GST-GAR has been routinely used in the assay of type I PRMTs (2, 6).

In addition to the PRMTs mentioned above, there are two other clones that code for arginine methyltransferases. The human PRMT2 (HRMT1L1) has 60% amino acid sequence similarity with the yeast RMT1 protein (14).However, the recombinant GST-PRMT2 fusion protein does not show any enzymatic activity with GST-GAR, myelin basic protein, or cytosolic extracts from a yeast rmt1 deletion strain as substrates (1). Lately, the heterogeneous nuclear ribonucleoprotein E1B-AP5 has been suggested as the in vivo substrate of PRMT2 (15).

Recently, Zobel-Thropp et al. (5) identified the presence of δ-N-monomethylarginine in the yeast cell lysate. Furthermore, Niewmierzycka and Clarke (16) demonstrated that the gene product of YDR465c, RMT2, is responsible for the formation of
the δ-N-monomethylarginines. RMT2 is a protein of 412 amino acid residues, including the initiator methionine. It shares 23 and 22% sequence identity with yeast RMT1 and the rat PRMT1, respectively. Noticeably, the protein contains the sequence motif (GXXGXX) conserved in S-adenosyl-l-methionine-dependent methyltransferases (16). However, the substrate(s) for RMT2 has not been identified in either in vivo or in vitro labeling experiments.

We report here that robust protein L12 from the YDR465c disruption mutant (ΔRMT2) can be specifically labeled by recombinant RMT2 heterologously expressed in Escherichia coli. We affirm that the reaction product is δ-N-monomethylarginine. We demonstrate with synthetic peptides, recombinant L12, and its mutant that Arg67 on ribosomal protein L12 is the methyl acceptor of RMT2. Furthermore, L12 isolated from wild type yeast strain contains δ-N-monomethylarginine.

EXPERIMENTAL PROCEDURES

Materials—Yeast strain SEY6210 (MATa, ura3–52, leu2–3, leu2–112, his3Δ200, trp1–200, trp1–280, ura2–350, suc2–29, GAL), open reading frames (ORFs) (YBR034c and YDR465c), and genomic DNA from S. cerevisiae strain YDR465c (obtained from the Yeast ORF collection AL). The expression vector pET-15b and the His-Ribd resin were purchased from Novagen (Madison, WI). The yeast integrating plasmids pRS403 and pRS404 and the QuikChange site-directed mutagenesis kit were obtained from Stratagene (La Jolla, CA). The Fast Q chromatographic medium, the prepacked Mono Q and Superdex 75 columns, the Immobiline DryStrips, Pharmalytes, and the IPGphor electrophoresis system were obtained from Amersham Biosciences. Precast Novex Tris/ glycine gels were products of Invitrogen (Carlsbad, CA). S-Adenosyl-l-[methyl-3H]methionine (1′H)AdoMet (75 Ci/mmol, 0.55 mCi/ml) and Enlightening enhancer were purchased from PerkinElmer Life Sciences. The AA511 column (0.46 × 12 cm) prepacked with sulfonated polystyrene divinylbenzene copolymer was obtained from Pierce. Synthetic peptides were custom made by SynPep (Dublin, CA). They were extensively against buffer V before storage at −80°C.

Expression and Purification of RMT1 and RMT2—Yeast ORFs encoding YBR034c and YDR465c were used as templates in PCR experiments. Primers employed in the cloning experiments are listed in Table 1. YBR034c was amplified with PCR using primers 1 and 2. The product was restricted with NdeI and Xhol and then inserted into the pET-15b vector and transformed into E. coli BL21(DE3) for protein expression. The recombinant RMT1 has a His6 tag at the N terminus that is purified on a Ni2+ affinity column according to the manufacturer’s instructions. The recombinant proteins in this study (His-RMT1, His-RMT2, and RMT2) have the tendency to form aggregates. All buffers used in the chromatographic steps contain 10% glycerol for protein stabilization. Soluble proteins (15 mg/ml for His-RMT1, 2–5 mg/ml for His-RMT2, 7–9 mg/ml for RMT2) were separated from precipitates by centrifugation and stored at −80°C in the elution buffer containing 1% imidazole.

YDR465c contains an NdeI site near the C terminus of the coding sequence. PCR was carried out with primers 3 and 4 to eliminate this NdeI restriction site. The product was then combined with primer 5 in another PCR experiment to obtain the complete coding sequence of RMT2. The PCR product was restricted with NdeI and Xhol and then inserted into pET-15b vector. The resulting plasmid is designated as pET-RMT2. Transformation into bacterial cells, expression of the Histagged protein (His-RMT2), and purification with metal affinity column were carried out as outlined above. Samples from the affinity column were further separated from low molecular weight contaminants on a gel filtration column eluted with buffer I (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 6 mM β-mercaptoethanol, and 10% glycerol).

YDR465c was PCR-amplified with primers 5 and 6. The product was inserted into the NdeI and SalI sites of the pBACEvector and transformed into Escherichia coli DH5α cells for protein expression at 30°C as described (17). The recombinant protein (RMT2) thus expressed does not have a His tag.

Cells containing RMT2 were lysed in buffer II (40 mM Tris-HCl, pH 8.0, 100 mM NaCl, 6 mM β-mercaptoethanol, and 10% glycerol), and the debris was removed by ultracentrifugation in a Beckman Type 70 Ti rotor at 50,000 rpm for 2.5 h. The supernatant was loaded directly onto a Fast Q anion exchanger equilibrated in buffer II. The column was eluted with an NaCl gradient, and RMT2 came off of the column at ~0.4 mM NaCl. Fractions containing RMT2 were collected and diluted an with equal volume of buffer II. The purified protein was loaded onto a Mono Q column (1.0 × 10 cm) and eluted with a NaCl concentration gradient. The purity of the RMT2 preparation was examined by SDS-PAGE, and the molecular mass of the recombinant protein was confirmed by electrospray ionization-mass spectrometry (18).

Creation of Yeast Strains Lacking RMT1 (ΔRMT1) and RMT2 (ΔRMT2)—Arabidopsis thaliana (pBA-L12) was used as a template for DNA fragment in which TRP1 is flanked by the 5′ and 3′ sequences of RMT1 was obtained by PCR amplification of a plasmid encoding the TRP1 gene (pRS404). The primers used for amplification contain the flanking sequences of TRP1 (underlined) and either the 5′ (primer 7) or 3′ (primer 8) sequence of RMT1 (Table I). The amplified DNA fragment was transformed into the haploid strain SEY6210 to disrupt the genomic RMT1 by homologous recombination. Transformants with disrupted RMT1 gene (ΔRMT1) were screened and verified by PCR.

The yeast strain lacking RMT2 (ΔRMT2) was obtained similarly. A DNA fragment in which HIS3 is flanked by the 5′ and 3′ sequences of RMT2 was PCR-amplified from pRS403 with hybrid primers containing the flanking sequences of HIS3 (underlined) and either the 5′ (primer 9) or 3′ (primer 10) sequence of RMT2 (Table I). The amplified DNA fragment was transformed into the haploid strain SEY6210 for homologous recombination. Transformants with disrupted RMT2 gene (ΔRMT1) were screened and verified by PCR.

Expression and Purification of Yeast Ribosomal Protein L12 and Its Mutant—YDR415W, the ORF encoding yeast ribosomal protein L12B, was obtained by amplifying genomic DNA from S. cerevisiae strain S288C with primers 11 and 12 (Table I). The PCR product was restricted with NdeI and SalI and then inserted into the pBACEvector (pBA-L12) for protein expression as outlined above. The recombinant protein L12 with an Arg67 to lysine substitution was generated with the QuikChange site-directed mutagenesis kit according to the manufacturer’s instructions. The pBA-L12 vector was used as template, and primer 13 (Table I) was used as the mutation primer. Cell transformation and protein expression were carried out as outlined above.

Riboosomal protein L12 and its mutant were expressed as inclusion bodies. DH5α cells containing recombinant proteins were lysed in buffer III (50 mM sodium phosphate, pH 7.2, 6 mM β-mercaptoethanol, and 0.25 mM sucrose) at a concentration of 75 A260 units/ml. After cell lysis, the debris was removed by centrifugation in a Sorvall SS34 rotor at 10,000 rpm for 20 min. The recombinant proteins were then precipitated in buffer IV (0.5 M Tris, pH 8.5, 6 mM guanidinium chloride, and 1 mM EDTA) and separated from cell debris and organelles by centrifugation in a Beckman Type 70.1 Ti rotor at 50,000 rpm for 3 h. The recombinant proteins were then aliquoted and stored at −80°C until use.

Fractionation of Yeast Extracts—Yeast strains were grown with constant shaking at 30°C in YPD medium (1% w/v) yeast extract, 2% (w/v) bactopeptone, and 2% (w/v) glucose) until A600 was 2. Cells were collected by centrifugation and washed three times with buffer V (50 mM sodium phosphate, pH 7.0, 1 mM EDTA and 1 mM EGTA). The wet pastes were used immediately or stored at −80°C.

Cells from 4 liters of culture were suspended in 50 ml of buffer V in the presence of protease inhibitors (1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml of aprotinin, leupeptin, and pepstatin A). Cell breakage was induced by passing the suspension through a Microfluidizer (Avestin, Ottawa, Canada) at 25,000 p.s.i. 7–9 times. Cell debris was removed by centrifugation in a Beckman Type 70 Ti rotor at 20,000 rpm for 30 min. The supernatant was collected and layered on top of a 10% sucrose cushion, and centrifugation was carried out in a Type 70 Ti rotor at 50,000 rpm for 4 h. The supernatant was designated as the cytosolic fraction (C) and stored at −80°C after dialysis against buffer V. The crude ribosomal pellet was resuspended overnight at 4°C in buffer VI (50 mM sodium phosphate, pH 7.0, 20 mM MgCl2 and 0.5 mM NH4Cl). Insoluble particulate was removed by spinning in a Type 70 Ti rotor at 20,000 rpm for 30 min. The supernatant was then spun down in a 10% sucrose cushion as mentioned above and layered on top of a 20% sucrose cushion (SS15 wash). The pellet ribosome (R) was resuspended in buffer V. Both fractions were dialyzed extensively against buffer V before storage at −80°C.

Protein-arginine Methyltransferase Assay—The cytosolic (C), R, and S fractions from SEY6210, ΔRMT1, and ΔRMT2 strains were used as methyl acceptors in the assays. The reactions were carried out in buffer V at a final volume of 40 μl. The reaction mixture contained 340 μg of substrate(s) in the assays. The reactions were carried out in buffer V at a final volume of 40 μl. The reaction mixture contained 340 μg of

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"RMT2 Methylates Yeast Ribosomal Protein L12"

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protein, 5 μg of methyltransferase, and 0.59 μM (1.65 μCi) of [3H]AdoMet. Incubation was carried out at 30 °C for 30 min. Trichloroacetic acid or SDS-PAGE sample buffer was added to stop the methyltransferase reaction.

For the methylation of synthetic peptides, a mixture of 4 μg of methyltransferase, 2.5 nmol of N-terminal biotinyl peptides, and 0.39 μM (1.38 μCi) [3H]AdoMet was prepared in 50 μl of 0.1 M sodium phosphate, pH 7.2. Reactions were carried out at 30 °C for 1 h. N-terminal biotinyl peptides were captured on streptavidin-agarose beads and washed extensively with buffer VII (10 mM sodium phosphate, pH 7.2, 150 mM NaCl, 0.1% (v/v) Nonidet P-40, and 0.1% (w/v) bovine serum albumin) to remove the unincorporated [3H]AdoMet prior to scintillation counting. The supernatant was then passed through a filtration unit equipped with a 10-kDa cut-off membrane, and the filtrate was dried in vacuo to completion. The peptides thus collected were hydrolyzed and analyzed as outlined above.

**Amino Acid Analysis of Proteins Methylated in Vivo**—The SEY6210 and ARMT2 yeast cells were labeled in vivo with [3H]AdoMet for 60 min as described (16). The cells were harvested and resuspended in 50 μl of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 2 μg/ml of leupeptin and pepstatin) and then lysed by vortexing with acid-washed glass beads (420–600 μm, Sigma). The lysate was collected, and the glass beads were washed repeatedly with buffer VIII (50 mM Tris-HCl, pH 7.4, 50 mM KCl, and 5 mM Mg(OAc)2). These fractions were combined and cleared by centrifugation at 12,000 × g for 15 min. The ribosomes in the combined fractions were pelleted by spinning at 427,300 × g for 2 h. The ribosomes were then resuspended in 150 μl of buffer IX (50 mM Tris-HCl, pH 7.4, 500 mM KCl, and 5 mM Mg(OAc)2) and 1 mM DTT. Two volumes of glacial acetic acid were added, and the magnesium ion concentration was raised to 0.1 M for RNA precipitation. The RNA was removed by centrifugation, and the proteins in the supernatant were dialyzed overnight at 4 °C against 66.7% acetic acid. Proteins in the sample were then dried down in vacuo, separated by SDS-PAGE, and visualized by Coomassie Blue dye staining and fluorography. Recombinant L12 was loaded on the same gel to facilitate the localization of the corresponding in vivo labeled protein. L12 is one of the two major methylated ribosomal proteins on the fluorograph (data not shown). The in vivo methylated L12 was excised from the gel and eluted overnight by incubation in 100 μl of 0.3 M (NH4)HCO3, and 0.5% SDS. The eluted protein was then dried in vacuo and hydrolyzed for amino acid analysis as described above.

**Identification of the Methylated Protein**—Overlaying the fluorograph with two-dimensional polyacrylamide gel identified the methylated protein. The protein was excised from the gel with a pipette tip. In gel digestion was performed essentially as described by Li et al. (21) with minor modifications. The gel plug was washed extensively with destaining solution (10% acetic acid and 50% methanol in water). It was then incubated in the digestion buffer (0.05 M Tris-HCl, pH 8.0, and 20 mM CaCl2) for 10 min. The gel plug was removed, and the gel plug was dehydrated by adding acetonitrile followed by vacuum centrifugation. Trypsin solution (0.0375 mg/ml in digestion buffer) was then added to restore the gel plug to its original volume. Digestion was carried out at 25 °C overnight. Resulting peptides in the gel plug were extracted sequentially with 60% acetonitrile in digestion buffer, 60% acetonitrile in water with 0.1% trifluoroacetic acid and acetonitrile. The solutions were combined, and the acetonitrile was removed by vacuum centrifugation. The solution was further cleaned up with a ZipTip pipette tip before MALDI-TOF analysis.

**Peptide mass mapping** was performed on a Bruker (Brunner-Daltonics, Bremen, Germany) REFLEX III time-of-flight mass spectrometer equipped with the SCOUT source and delayed extraction. Detection was set in positive ion reflection mode with each mass determination performed at the m/z value of 100 spectra. Samples for mass spectrometry were prepared using the solution-phase nitrocellulose method (22) with α-cyano-4-hydroxycinnamic acid as matrix. Angiotensin II (DVIYIK) was used as an external calibration standards. Peptide masses obtained were searched against the database of the M.92200 genome.
polyacrylamide gel, and the tritium-labeled proteins were visualized by fluorography. The fluorographs represent a 37-h exposure.

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abeled by His-RMT2 differs from that methylated by His-RMT1; His-RMT1 methylated predominantly two proteins of 40 and 10 kDa in the ribosome salt wash fraction from ΔRMT2 (data not shown).

RNase pretreatment of yeast and mammalian cell extracts can affect the in vitro methylation pattern of type I PRMTs (26). To test whether a similar phenomenon can be observed for RMT2, we pretreated the fractionated yeast extracts with endonuclease (Benzonase; Sigma) at 30 °C for 30 min prior to the methylation assay. The proteins were separated on SDS-PAGE, and the isotopically labeled proteins were visualized by fluorography. Fractions from ΔRMT1 were also treated with endonuclease and methylated with His-RMT1 as controls. As reported by Frankel and Clarke (26), the intensity of some of the protein bands methylated by His-RMT1 was either enhanced or diminished upon endonuclease treatment (data not shown). As for the protein band labeled by His-RMT2 in the ribosome salt wash fraction of ΔRMT2, we observed a slight increase in intensity upon endonuclease treatment (Fig. 1C). In addition, a protein at approximately the same molecular weight was methylated in the ribosomal fraction of the ΔRMT2 extracts.

**Analyses of the RMT2 Methylation Products**—Proteins from the methylation assay were precipitated with trichloroacetic acid and collected by centrifugation. The pellet was hydrolyzed with HCl, and the amino acids were analyzed on a cation exchanger. Part of the chromatogram of the amino acids from the ribosome salt wash fraction of ΔRMT2 methylated with His-RMT2 is shown in Fig. 2A. The radioisotopes were eluted between ω-N\(^2\)G-dimethylarginine (ADMA) and ω-N\(^2\)G-monomethylarginine (MMA). As a control, hydrolysate from the ribosome salt wash fraction of ΔRMT1 methylated with His-RMT1 was analyzed, and the chromatogram is presented in Fig. 2B. The retention times of the radioisotope peaks coincide with those of the ADMA and the MMA standards. The results suggest strongly that RMT2 is not a type I arginine methyltransferase. Note also that the scale of the y axis in Fig. 2B is 5-fold larger than that of Fig. 2A. With our assay conditions, RMT1 has a much higher enzymatic activity than that of RMT2.

To confirm the identity of the \(^3\)H-labeled residue, the radioisotopic peak from Fig. 2A was collected and base-hydrolyzed. After acidification and dilution with water, the radioisotopes were mixed with δ-N-monomethylornithine and rechromatographed on the same column. The radioisotopes co-eluted with the δ-N-monomethylornithine (Fig. 2C). Therefore, RMT2 is a δ-N-monomethylarginine transferase as suggested by Niewmięrzycka and Clarke (16).

**Identification of in Vitro Methylated Protein**—The ΔRMT2 ribosome salt wash fraction was incubated with His-RMT2 and \(^{3}\)HAdoMet. The proteins were separated on a linear pH 3–10 Immobiline DryStrip in the first dimension, followed by SDS-PAGE on a 15% homogeneous gel in the second dimension. The silver-stained pattern of 170 kDa in the ribosome salt wash fraction from ΔRMT2 is shown in Fig. 3. The fluorograph of the same gel is presented in Fig. 3A. The fluorograph identified the methylated protein as S19 (accession number gi 6324027) and S20 (accession number gi 6323717). Other proteins that were located near to L12 (Fig. 4) were identified as ribosomal homolog (accession number gi 6321772). Interestingly, in our gel system, S19 has

![Fig. 2. Amino acid analysis of methylated proteins. A, the S fraction of ΔRMT2 was incubated with His-RMT2 and \(^{3}\)HAdoMet. The proteins were precipitated with trichloroacetic acid and then hydrolyzed with HCl. The released amino acids in 0.2 n sodium citrate, pH 2.0, were mixed with MMA and asymmetric ADMA as internal standards and then analyzed on an AA511 column that had been equilibrated in 0.35 n sodium citrate, pH 5.28, and 60 °C. The column was eluted at 0.5 ml/min, and fractions were collected for scintillation counting (A——A) and ninhydrin assay (——). B, the S fraction of ΔRMT1 was incubated with His-RMT1 and \(^{3}\)HAdoMet. The resulting proteins were hydrolyzed and analyzed similarly. C, the radioactive peak from A was collected, and NaOH was added to give a final concentration of 2 M. The mixture was incubated at 55 °C for 24 h and then acidified with one-fifth volume of 12 n HCl. The mixture was then diluted 10-fold with water, and δ-N-monomethylornithine was added as internal standard. The sample was then analyzed on an AA511 column as outlined for A. using the MASCOT program (available on the World Wide Web at www.matrix-science.com). The masses of nine peptides covering 99 residues can be matched with those deduced from the amino acid sequence of yeast ribosomal protein L12 (Fig. 5; accession number gi 6320625). The deviations between the experimental results and theoretical calculations are 47–130 ppm. The most abundant ion (m/z 1240.8) on the spectrum was generated from a peptide with nonspecific proteolytic cleavage covering residues 61–71 (QLKIQRNQRQAAA) of L12. The partial sequence of this peptide was deduced from a postsource decay spectrum (data not shown) and affirms that the protein spot represents ribosomal protein L12.

Careful examination of the L12 protein spot revealed that there is a protein located right next to it on the basic side of the gel. We identified this protein as the mitochondrial HMG-1 homolog (accession number gi 6323717). Other proteins that were located near to L12 (Fig. 4) were identified as ribosomal proteins S19 (accession number gi 6324027) and S20 (accession number gi 6321772). Interestingly, in our gel system, S19 has
Whether the slower migration rate of S20 is due to post-translational modification or other factors remains to be investigated. Yeast ribosomal protein L12 (27) is also known as YL23 and L15 (28, 29). It contains dimethyllysine(s), trimethyllysines(s) (30), and methylarginine(s) (31). It can complex with rRNA in the presence of 0.5M LiCl (32). Incubating the ribosome with 0.5 M NH4Cl generated the ribosome salt wash fraction in this study. This incubation step has been routinely used to generate prokaryotic ribosome and usually does not result in removing ribosomal proteins (33). L12 and a group of acidic proteins can be removed from the ribosome by incubating the macromolecular complex in 1M ammonium salt and 50% ethanol (34, 35).

We compared the protein profiles of the ribosomal and ribosome salt wash fractions from wild type and ΔRMT2 yeast strains of our preparations. We loaded equal amounts of protein (85 μg) on each gel, and the proteins were visualized by silver staining. The regions covering L12 and the surrounding proteins (mitochondrial HMG-1 homolog, S19, and S20) are presented in Fig. 4, B–E. Apparently, L12 and the HMG-1 homolog are more abundant in the ribosome salt wash fraction of ΔRMT2 than that of the SEY6210. In our gel system, we cannot detect the acidic proteins (P1B, P2A, and P2B; pI values from 3.8 to 3.9) that normally associate with L12. We speculate that L12 binds less tightly with the ribosome in the ΔRMT2 cells.

Upon nuclease digestion, a protein from the ribosomal fraction of ΔRMT2 was methylated (Fig. 1C). Further analyses with two-dimensional gel electrophoresis and mass spectrometry revealed that the methylated protein is also L12 (data not shown). Seemingly, rRNA and/or other ribosomal proteins protect the methylation site on L12. The same nuclease treatment of fractions from wild type yeast cells did not generate a new methylation site for His-RMT2 (data not shown). Probably, the endonuclease treatment of the wild type ribosome is insufficient in disrupting its structure and exposing the methylation site on L12. Niewmierzycka and Clarke (16) have shown that inhibition of protein synthesis prevents in vivo methylation of the RMT2 substrate. The data can be interpreted as methylation occurs either before protein folding or its assembly into the ribosome. The methylation site on L12—PRMTs usually methylate substrates containing glycine- and arginine-rich sequences in Arg-Gly-Gly or Arg-Xaa-Arg contexts (1, 10, 36). Recently, Mowen et
arginines. Two of them (Arg67 and Arg114) have adjacent glutamine residues, and an RDR sequence is located at residues Arg90 and Arg92), 109–122 (peptide 4, containing Arg117 and Arg118), 118–128 (peptide 5, containing Arg122, and 138–142 (peptide 6, containing Arg142) of L12 for in vitro methylation assays. All of these peptides have an N-terminal biotin group. A tyrosine residue was added to the C terminus of peptides 2, 3, 4, and 6 for quantification purpose. An additional leucine residue was added to the N-terminal of peptide 5, so that the penultimate aspartic acid would not convert into isoaspartic acid during peptide synthesis.

After the methylation reaction, the peptides were pulled down by streptavidin beads, and the tritium labels were detected by scintillation counting. Only peptide 2 was radioactively labeled (data not shown). We then combined peptides from 10 methylation assays for amino acid analysis. The data are presented in Fig. 6A and confirm that peptide 2 has a tritiated d-N-monomethylarginine. Peptide 4 was also submitted to amino acid analysis. The results show clearly that Arg114, although it has an adjacent glutamine, is not a methyl acceptor for RMT2 (Fig. 6A). This result also eliminates the possibility that the isotopes detected on peptide 2 were the consequence of arginine side chain tritium exchange. Therefore, using these synthetic peptides, we have shown that RMT2 methylates only Arg67 and none of the other seven arginines in L12.

To affirm that Arg67 on L12 is the methyl acceptor of RMT2, we expressed L12 and its R67K mutant in E. coli for in vitro assays. The total cell lysates from DH5α, cells expressing L12, and cells expressing its mutant are presented in Fig. 7 (lanes 1–3). The identity of yeast ribosomal protein L12 was confirmed by N-terminal sequencing after the overexpressed protein was electrophoretically transferred onto polyvinylidene difluoride membrane (data not shown). L12 is clearly a methyl acceptor in the presence of RMT2 and [3H]AdoMet (Fig. 7B, lane 4). The R67K mutant (Fig. 7B, lane 6) or the recombinant wild type L12 without RMT2 in the assay mixture (Fig. 7B, lane 5) was not labeled.

The methylated recombinant L12 was further subjected to amino acid analysis (Fig. 6B). The tritium labels have the same retention time on the ion exchange column as that of the hydrolysate from peptide 2 (Table II, Fig. 6A) or that of the fraction of ΔRMT2 that has been incubated with His-RMT2 and [3H]AdoMet (Fig. 2A). We collected the tritium labels from Fig. 6B and subjected them to base hydrolysis. Upon chromatography on the same column, the tritium labels co-migrated with d-N-monomethylornithine (data not shown). Therefore, after reacting with RMT2, the modified amino acid on the recombinant L12 is d-N-monomethylarginine.

In Vivo Methylation of Yeast Ribosomal Protein L12—Kruiswijk et al. (31) have reported the presence of methylated arginine and lysine residues on L12. Although they could not distinguish whether the arginine residue(s) was modified at the 0- or ω-position, they estimated that there was only 0.09–0.20 mol of methyl group/mol of modified ribosomal protein species.

To show that L12 is methylated in vivo, we grew wild type and ΔRMT2 yeast cells in the presence of [3H]AdoMet. L12 was then isolated electrophoretically and subjected to amino acid analysis. Tritium labels with retention time similar to that of d-N-
monomethylarginine were observed for the sample from the wild type yeast cells (Fig. 6C). This tritiated peak was absent from the L12 isolated from the ΔRMT2 strain (Fig. 6C). We also observed tritium labels that co-eluted with the trimethyllysine standard. Therefore, L12 is methylated at both arginine and lysine residues as suggested by Kruiswijk et al. (31).

Type I and II methyltransferases have been shown to utilize peptides with a glycine- and arginine-rich region as substrates (1). In the case of signal transducer and activator of transcription 1, a methyl acceptor for PRMT1, the modified arginine is followed by polar residues (IQNRQA). Whether RMT2 can modify other proteins and the substrate specificity of this enzyme remain to be elucidated.

A peptide that encompasses residues 61–71 of L12 (m/z 1240.8) was detected in the MALDI-TOF mass spectrum (Fig. 5). The methyl-accepting arginine, Arg67, is located in this fragment. Close scrutiny of the mass spectrum reveals an ion with an additional 14 daltons (m/z 1254.7; Fig. 5, inset). This ion possibly represents the methylated form of the peptide. Assuming the methylated and unmethylated forms of the peptide have the same ionization efficiency, ~1% of the peptide in the sample is methylated. This estimate is only slightly higher than that reported by Kruiswijk et al. (31).

Histone H3 (38) and H4 (39) are substrates of coactivator-associated arginine methyltransferase 1/PRMT4 and PRMT1, respectively. Amino acid analysis of histone H3 isolated from calf thymus revealed that only 3.7% of the molecules contain methylated arginine (38). N-terminal peptides with and without methylation at Arg67 have been recovered from tryptic digests of calf thymus histone H3 (39). Therefore, the level of in vivo protein-arginine-methyltransferase can be rather low and not stoichiometric.

We have detected an ion at m/z 727.5 (Fig. 5). The mass and the postsource decay spectrum of this ion match with those of a peptide covering residues 12–16 of L12 (YLYLK). We have also electroblotted L12 from a two-dimensional gel onto a membrane for sequence determination. The protein is N-terminally blocked. Therefore, the mature L12 does not start from residue 17 as reported on the Saccharomyces Genome Database (available on the World Wide Web at genome-www.standford.edu/Saccharomyces).

The yeast ribosomal protein L12 is immunologically and functionally equivalent to the bacterial protein L11 (35). The prokaryotic L11 is located in the GTPase center of the ribosome and is the target for a family of thiazole antibiotics (40). The structure of L11 from T. maritima complexed with RNA is known (41). L11 has a two-domain structure: the N-terminal domain and the RNA binding C-terminal domain. Upon alignment, Arg67 of L12 is located in the N-terminal domain and is not conserved among the L11 homologs from bacteria and yeast (41). However, yeast L12 has only 23% sequence identity with the T. maritima L11 and may not share similar structures. The consequence of Arg67 methylation on the structures and functions of L12 and the ribosome at large remains to be investigated.

In summary, we have heterologously expressed the yeast arginine methyltransferase RMT2 in E. coli. With [3H]AdoMet

Fig. 7. SDS-PAGE analyses of methylated recombinant L12. Total cell lysates from DH5α (lane 1), cells expressing L12 (lane 2), cells expressing the L12 R67K mutant (lane 3), purified L12 that has been incubated with [3H]AdoMet and His-RMT2 (lane 4), L12 that has been incubated with [3H]AdoMet alone (lane 5), and the L12 R67K mutant that has been incubated with [3H]AdoMet and His-RMT2 (lane 6) were analyzed on a 4–20% denaturing polyacrylamide gradient gel. The proteins were visualized with Coomassie Blue staining and presented in A. The same gel was subjected to fluorography, and the x-ray film that had been exposed for 18 h is presented in B.
as co-substrate, the purified recombinant protein can label yeast ribosomal protein L12. The enzymatic product is the \( N \)-monomethylarginine, and the methyl acceptor on L12 is the side chain of Arg67. We have proved that L12 from wild type yeast cells contains \( N \)-monomethylarginine. This modification is absent from yeast cells with RMT2 deletion. These results suggest that ribosomal protein L12 is the natural substrate of RMT2.

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