The Predominant Protein-arginine Methyltransferase from Saccharomyces cerevisiae*

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We have identified the major enzymatic activity responsible for the S-adenosyl-L-methionine-dependent methylation of arginine residues (EC 2.1.1.23) in proteins of the yeast Saccharomyces cerevisiae. The RMT1 (protein-arginine methyltransferase), formerly ODPL, gene product encodes a 348-residue polypeptide of 39.8 kDa that catalyzes both the \( \text{N}^\text{G} \)-mono- and \( \text{N}^\text{G} \text{,N}^\text{G} \)-asymmetric dimethylation of arginine residues in a variety of endogenous yeast peptides. A yeast strain in which the chromosomal RMT1 gene was disrupted is viable, but the level of \( \text{N}^\text{G} \text{,N}^\text{G} \)-[\( ^3\text{H} \)dimethyl]arginine residues detected in intact cells incubated with S-adenosyl-L-[methyl-\( ^3\text{H} \)]methionine is reduced to less than 15% of the levels found in the parent strain, while the \( \text{N}^\text{G} \text{,N}^\text{G} \)-[\( ^3\text{H} \)monomethyl]arginine content is reduced to less than 30%. We show that soluble extract from parent cells, but not from mutant rmt1 cells, catalyzes the in vitro methylation of endogenous polypeptides of 55, 43, 38, 34, and 30 kDa. The hypomethylated form of these five polypeptides, as well as that of several others, can be mono- and asymmetrically dimethylated by incubating the mutant rmt1 extract with a purified, bacteriaially produced, glutathione S-transferase-RMT1 fusion protein and S-adenosyl-L-[methyl-\( ^3\text{H} \)]methionine. This glutathione S-transferase-RMT1 fusion protein is also able to methylate a number of mammalian polypeptides including histones, recombinant heterogeneous ribonucleoprotein A1, cytochrome c, and myoglobin, but cannot methylate myelin basic protein. RMT1 appears to be a yeast homolog of a recently characterized mammalian protein-arginine methyltransferase whose activity may be modulated by mitotic stimulation of cells.

Evidence for the posttranslational methylation of arginine residues in proteins was first provided by the presence of radioactive species chromatographing at positions near that of arginine in acid hydrolysates of isolated calf thymus nuclei incubated with S-adenosyl-L-[methyl-\( ^{14}\text{C} \)]methionine (Paik and Kim, 1967). The methylated species were later determined to be arginine derivatives that had been mono- and dimethylated on their guanidino group (Paik and Kim, 1968; Nakajima et al., 1971). S-Adenosyl-L-methionine-dependent methyltransferase activities that catalyze these reactions have now been characterized in a number of eukaryotic tissues and organisms.

When the partially purified protein-arginine methyltransferase activity from calf brain was incubated with histones as a methyl-accepting substrate, the products of the reaction, after acid hydrolysis, were determined to be \( \text{N}^\text{G} \)-monomethylarginine, \( \text{N}^\text{G} \text{,N}^\text{G} \)-dimethylarginine (asymmetric), and \( \text{N}^\text{G} \text{,N}^\text{G} \)-dimethylarginine (symmetric) (Lee et al., 1977) (see Fig. 1). However, the substrate specificity of the enzyme at each stage of the purification suggested that two distinct methyltransferases are responsible for the creation of the three types of methylated species (Miyake and Kakimoto, 1973; Lee et al., 1977). In support of this conclusion, two distinct protein methylases have been partially purified from calf brain. One enzyme activity specifically monomethylates myelin basic protein (Ghosh et al., 1988) (Fig. 1) on arginine residue 107 (Baldwin and Carnegie, 1971; Bostroff and Eylar, 1971). The second activity was initially described as a histone-specific methyltransferase (Ghosh et al., 1988), although it is now known to be much more efficient for substrates such as the heterogeneous ribonucleoprotein A1 (hnRNP A1) and catalyzes the mono- and asymmetric dimethylation of arginine residues (Fig. 1). The site of arginine methylation in hnRNP A1 is within a domain that has been designated GAR for glycine and arginine-rich and contains multiple repeats of a consensus arginine methylation site RG (Rajpurkar et al., 1994a). Several other potential substrates for this enzyme also contain a similar GAR domain (Najbauer et al., 1993). Members of this family of methyl-accepting substrates include nucleolin and fibrillarin proteins that contain 10–12 residues of asymmetric dimethylarginine (Lischwe et al., 1985a; Lischwe et al., 1985b).

These proteins, like hnRNP A1, are involved in the processing of pre-RNAs.

A number of purifications of a histone hnRNP A1-specific mammalian arginine methyltransferase activity have been reported (Ghosh et al., 1988; Rawal et al., 1994; Liu and Dreyfuss, 1995). However, the polypeptide composition of this enzyme is still not established. Similarly, no genes encoding for a protein-arginine methyltransferase have been identified to date. Two purifications of the enzyme activity resulted in preparations that demonstrated multiple polypeptide species present after SDS-gel electrophoresis. Ghosh et al. (1988) identified two polypeptides (110 and 75 kDa) associated with a histone-specific arginine methyltransferase from calf brain. The purest fraction obtained from HeLa cells by Liu and Dreyfuss (1995) still had eight polypeptide bands present, with two prominent species of 100 and 45 kDa. A single 110-kDa polypeptide by SDS-gel electrophoresis was identified by Rawal et al. (1994)

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‡The abbreviations used are: hnRNP, heterogeneous ribonucleoprotein; AdoMet, S-adenosyl-L-methionine; [\( ^3\text{H} \)AdoMet, S-adenosyl-L-[methyl-\( ^3\text{H} \)]methionine; GST, glutathione S-transferase; GAR, glycine and arginine-rich region; kbp, kilobase pair(s); bp, base pair(s); LB, Luria Bertani; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; SCD, synthetic complete medium with dextrose; YPD, yeast peptone and dextrose medium.
after a four-step purification from rat liver. However, only a small amount of material was analyzed by electrophoresis so that additional polypeptides of lower molecular mass may have escaped detection.

We have recently described the cDNA cloning and analysis of a mammalian gene product designated PRMT1 for protein-arginine methyltransferase (Lin et al., 1996). The 40.5-kDa protein encoded by this rat cDNA was found to interact, in a yeast two-hybrid screen (Fields and Song, 1989), with the murine primary response immediate early gene product TGF1 (Fletcher et al., 1991; Varnum et al., 1994) and its family member the mouse antiproliferative gene product BTG1 (Rouault et al., 1992). A search of the sequence data base revealed that the mammalian PRMT1 gene product was similar in sequence to the yeast Saccharomyces cerevisiae ODP1 gene product. ODP1 has been identified as a partial open reading frame downstream of the PDX3 gene (Loubbardi et al., 1995), and its complete sequence was determined with the yeast chromosome II genomic sequencing effort (Feldmann et al., 1994). No function for ODP1 has been suggested. Using the combined biochemical and genetic approaches that can be taken in yeast, we were interested in exploring whether this gene product harbored activity in yeast have been unsuccessful (Liu and Dreyfuss, 1995). Nevertheless, a search of the GenBank data base with the consensus arginine methylation sequence FGGRGGF has revealed numerous potential substrates for an enzyme of similar activity in yeast (Najbauer et al., 1993), including the nucleolin homolog NSR1, the fibrillarin homolog NOP1, as well as the SSB1, GAR1, and NPL3 proteins.

In this paper we provide evidence that the ODP1 gene product is, in fact, a protein-arginine methyltransferase that we now designate RMT1 (protein arginine methyltransferase). This enzyme can catalyze both the mono- and asymmetric dimethylation of the guanido nitrogens of arginine residues present in a number of yeast polypeptides. We have constructed a yeast strain in which the RMT1 gene has been disrupted. The mutant cells are viable, but analysis of in vivo and in vitro methylated proteins from this strain demonstrated a dramatic decrease in the levels of mono- and asymmetrically dimethylated arginine residues present. The RMT1 gene product is therefore required for the majority of the mono- and asymmetric dimethylation of arginine residues in yeast. We also created and purified an N-terminal fusion of RMT1 with glutathione S-transferase. Incubation of the fusion protein with the methyl donor S-adenosyl-L-methionine results in the mono- and asymmetric dimethylation of the hypomethylated substrate proteins from the rmt1 soluble extract. These results demonstrate that the RMT1 gene product is a protein-arginine methyltransferase that plays a major role in modifying proteins containing a GAR domain, many of which interact with RNA. The methylation of arginine residues within these proteins may modulate this interaction, and may regulate other activities as well.

**EXPERIMENTAL PROCEDURES**

Cloning and Chromosomal Disruption of the Yeast Protein-arginine Methyltransferase Gene RMT1—The RMT1 gene (formerly ODP1) was PCR amplified (Scharf, 1990) from genomic DNA isolated (see below) from S. cerevisiae strain YJ43 (MATa, leu2-3, leu2-112, ura3-52, trp1-289, his4-580) (McEwen et al., 1986) using the single-stranded DNA primers RMT1-N1 (5'-AAAGAAAACAATAAGAGAACGCC) and RMT1-C1 (5'-CTCTGGACCAAAACAAAGCCTTTAAATC). These primers overlap the ATG initiation codon and the TAA termination codon, respectively (underlined). The resulting 1.1-kbp PCR product was purified from a 1% agarose gel using GeneClean II (BioIabs 101) and digested with NdeI and HindIII sites that were engineered into primers RMT1-N1 and RMT1-C1, respectively (bold). This fragment was then ligated into a similarly digested and purified pT7-7 vector (Tabor and Richardson, 1985) to create pJG-RMT1, whose insert contains the entire coding region of the gene (Fig. 2).

Plasmid pJ-G-RMT1 was digested at the unique BstXI site in the middle of the coding region (Fig. 2), and the 3' overhang was removed by subsequent treatment with T4 DNA polymerase. A LEU2 disruption cassette was obtained from plasmid Ydp-LEU2 (Barben et al., 1991) by digestion with BamHI, and the resulting 5' overhang of the cassette was filled in by treatment with T4 DNA polymerase. The blunt-ended LEU2 cassette was then purified from a 1% agarose gel as described above and ligated into the BstXI-linearized pJ-G-RMT1 to create the disruption vector pJ-G-RMT1::LEU2. The orientation of the insertion was confirmed to be in the direction indicated in Fig. 2 by restriction digests with NdeI/HindIII, NdeI/EcoRV, and SspI.

Replacement of the wild-type RMT1 chromosomal locus with the disruption construct was accomplished in the strain CH9100-2 (MATa, prc1-407, prb1-1122, pep4-3, leu1, trp1, ura3-52, ydl273w::URA3) (Hrycyna and Clarke, 1993) using the single-stranded DNA disruption plasmid pJ-G-RMT1::LEU2 (10 μg) was digested with NdeI and HindIII, and the entire mixture was used to transform CH9100-2 cells by the lithium acetate method (Rose et al., 1990). The transformed cells were then selected by plating onto leucine-deficient SCD plates (Rose et al., 1990). Positives were screened on selective plates twice. Genomic DNA was isolated from cells remaining after the three screens. The replacement of the wild-type RMT1 locus by the LEU2 disrupted version was confirmed by PCR analysis using primers RMT1-N2 (5'-TTGTTACCTTTAATTACACACAGAA-CAAGG) and RMT1-C2 (5'-CATATGCTGATGATACAGGAC) (Fig. 2). The wild-type loci produces a PCR product of 700 bp, but the disrupted locus gives a 2.4-kbp product. All putative positives tested from the auxotrophic screen produced only the 2.4-kbp product upon PCR analysis. The new rmt1 strain, in which the genomic copy of RMT1 has been disrupted by a LEU2 cassette transcribed in the same direction, is designated JDG9100-2 (MATa, prc1-407, prb1-1122, pep4-3, leu1, trp1, ura3-52, ydl273w::URA3, rmt1::LEU2).

Isolation of Genomic DNA from S. cerevisiae—We used a modified method of the procedure described by Hoffman and Winston (1987). Yeast were grown in YPD (Rose et al., 1990) at 30°C to an A600 nm of 3–4. 10–20 A600 nm units were then pelleted into a 1.5-mL microcentrifuge tube by centrifugation at 13,600 × g for 5 min at 25°C. The pelleted cells were washed with water and resuspended in 500 μL of a 50 mM Tris-HCl solution containing 20 mM sodium EDTA and 1% (w/v) SDS at pH 7.5. Baked glass beads (425–600 μm, Sigma) (0.6 g) were
then added and the suspension was vortexed for 1 min and then cooled at 0°C for 1 min; this process of vortexing and cooling was repeated five times. The lysed cells were then incubated at 70°C for 10 min before the addition of 1 ml of 100 mM tris-HCl, pH 7.5, and the supernatant was removed to a fresh tube, and two volumes of ethanol were added. The DNA was pelleted by centrifugation as just described and then washed once with 70% ethanol. The final DNA pellets were resuspended in 820 μl of 10 mM Tris-HCl solution containing 1 mM sodium EDTA at pH 7.0 containing 0.67 mM PMSF. To each of the mixtures, 0.2 g of baked ice-cold zirconium beads were added and the suspension was vortexed for 1 min and then cooled at 0°C for 1 min; this process of vortexing and cooling was repeated seven times. An aliquot containing 10 μg of protein from each extract was then mixed with an equal volume of 25% (w/v) trichloroacetic acid in a 6 × 50-mm glass vial and incubated at 25°C for 10 min before pelleting the precipitated material at 4,000 × g for 10 min at 4°C. The pellets were then washed twice with 1 ml of water and 1 ml of 12 M HCl at 20°C, dried, and then acid-hydrolyzed with 200 μl of 12 N HCl at 110°C for 20 h in a Waters Pico-Tag vapor-phase apparatus. The hydrolyzed samples were then resuspended in 50 μl of water, and 25 μl of each sample was analyzed by cation exchange chromatography.

FIG. 2. A schematic diagram of the wild-type RMT1, formerly ODP1, gene in S. cerevisiae, and of the constructed chromosomal insertion mutant. The lower portion represents a PCR product containing the complete coding region of RMT1. The initiator codon ATG is within the NdeI site and the HindIII site is 1 bp downstream of the termination codon TAA. Relevant restriction enzyme sites and the locations of conserved methyltransferase regions I, II, III, and post III (Kagan and Clarke, 1994; Kagan and Clarke, 1995) are included in the figure. The NdeI and HindIII sites were engineered into the 1.1-kbp fragment using primers RMT1-N1 and RMT1-C1, respectively, as described under “Experimental Procedures.” Arrowheads indicate the location of the 25-bp single-stranded DNA primers that were used for PCR analysis. The upper portion of the figure displays the insertional cassette from the plasmid YEp-LEU2 (Berben et al., 1991); the LEU2 coding region is represented by the filled arrow. The insertion of the LEU2 cassette into the unique BstXI site of RMT1 is described under “Experimental Procedures.”

protein concentration determination—A modification of the Lowry procedure (Bailey, 1967) was used to determine the concentration of protein after precipitation with 1 ml of 10% (w/v) trichloroacetic acid. Bovine serum albumin was used as a standard.

fractionation of polypeptides by SDS-Gel Electrophoresis—Samples were analyzed by mixing them 1:1 with SDS-containing sample buffer (Hrycyna et al., 1994), heating to 100°C for 5 min, and then loading onto slab gels (1.5 mm thick, 30 cm long, and 20 cm wide) containing 0.75% (w/v) acrylamide and 0.1% (v/v) bisacrylamide matrix was utilized with a constant current of 35 mA. The gels were then washed with buffer (25 mM Tris-HCl, 1 mM sodium EDTA, 3 mM sodium EGTA at pH 7.5) and centrifuged as described above. The resulting pellets (2.6–3.0 g, wet weight) were resuspended in 2 ml of buffer/g (wet weight) of cells, and 40 μl of a 10 mM Tris-HCl solution containing 1 mM sodium EDTA at pH 7.0 containing 5 μg of DNase-free RNase.

Isolation of Soluble Proteins from S. cerevisiae—Cells (500 ml) of both the parent (CH9100-2) and the mutant (JG9100-2) strains were grown to early log phase (OD600 nm = 0.6–0.8) in YPD media at 30°C. Five cultures (500 ml) of each strain were harvested by centrifugation at 1,600 × g for 6 min at 25°C. The pelleted cells were then washed twice with 10 vol of water and 150 μl of sterile 5 M potassium acetate and then disrupted by sonication with a microtip (Heat Systems-Ultrasonics, Inc.) for 20 min at 25°C, dried, and then acid-hydrolyzed with 200 μl of 12 N HCl at 110°C for 20 h in a Waters Pico-Tag vapor-phase apparatus. The hydrolyzed samples were then resuspended in 50 μl of water, and 25 μl of each sample was analyzed by cation exchange chromatography.

Construction, Expression, and Purification of a GST-RMT1 Fusion Protein—The RMT1 coding region was amplified by PCR using the plasmid pG-RMT1 as a template and the primers RMT1-N3 5’-CAAGATGATGACAAGACAGCCGTGAAA and RMT1-C3 5’-CAGAGTATATCCTCTTATGCTAATAAAG. Primer RMT1-N3 has the ATG initiation codon and a BamHI site (bold) inserted for in-frame linkage into the GST expression vector pGEX-2T (Pharmacia Biotech Inc.). RMT1-C3 incorporates an engineered EcoRI site (bold) just downstream from the RMT1 termination codon (underlined) for cloning purposes. The 1.1-kbp PCR product was digested with BamHI and EcoRI, purified from a 1% agarose gel using Genedean II, and ligated into the pGEX-2T vector, which had been similarly digested and isolated to generate the fusion construct pGEX-RMT1, which contains the Schizosaccharomyces japonicum glutathione S-transferase gene in frame with the entire coding region of the RMT1. This plasmid was propagated in the Escherichia coli host strain DH5α (Life Technologies Inc.) to express the fusion protein. The bacteria were grown at 37°C in Luria Bertani medium (Sambrook et al., 1989) with 100 μg/ml ampicillin until the A600 nm reached 0.5–0.6. Isopropyl-1-thio-β-D-galactopyranoside was then added to a final concentration of 1 mM, and the culture was incubated for an additional 5 h at 37°C to induce the expression of the fusion protein. The bacteria were harvested by centrifugation (5,000 × g for 10 min at 4°C), washed twice with phosphate-buffered saline (10 mM diosub sodium phosphate, 1.8 mM monobasic potassium phosphate, 140 mM NaCl, 2.7 mM KCl at pH 7.4), resuspended in extraction buffer (phosphate-buffered saline, 5% glycogen, 1 mM sodium EDTA, 1 mM dithiothreitol, 40 mg/ml leupeptin, 40 μg/ml aprotinin, 20 μg/ml pepstatin, 1 mM PMSF, and 0.5% (w/v) Triton X-100), and disrupted by sonication with a microtip (Heat Systems-Ultrasonics, Inc.) using a 15-s continuous pulse and then cooling to 0°C in 1 min; this process of sonication and cooling was repeated four times. Cell debris was removed by centrifugation (16,000 × g for 20 min at 4°C). The supernatant was concentrated and purified by affinity chromatography with glutathione-Sepharose 4B (Pharmacia) beads according to the manufacturer’s instructions. An identical procedure was used to create the mammalian PRMT1 fusion protein, except primers corresponding to the RMT1 gene were used.

Growth of RAT1 Cells and Preparation of Soluble Extracts—RAT1 fibroblast cells were grown to confluence in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% fetal bovine serum (Genuine Bioproducts, Inc.). Prior to harvesting, cells were washed once with phosphate-buffered saline (10 mM diosub sodium phosphate, 1.8
Fig. 3. An alignment of the conserved methyltransferase regions from the S. cerevisiae RMT1, the rat protein-arginine methyltransferase PRMT1, and the E. coli L1 methyltransferase. The yeast RMT1 (PIR 548989) shares an overall 45% identity with its mammalian counterpart PRMT1 (Lin et al., 1996). The E. coli L11 protein methyltransferase (GenBank U18997, cf. GenBank Z26847) is the highest scoring protein found using a BLAST search with the RMT1 sequence and shares an 11% identity with RMT1. However, when methyltransferase regions I, II, and III (Kagan and Clarke, 1994) for the three proteins are aligned, the sequences are highly conserved as is the inter-region spacing (not shown).

RESULTS

The S. cerevisiae ODP1 Gene Encodes a Protein with Sequence Similarities to the Mammalian Protein-arginine Methyltransferase PRMT1—We have recently identified a rat cDNA that encodes the catalytic subunit of a mammalian protein-arginine methyltransferase (Lin et al., 1996). A search of the non-redundant protein data base (PDB = SwissProt + PIR + SPUPdate + GenPept + GPUpdate) using the BLAST method (NCBI) (Altschul et al., 1990) revealed that the S. cerevisiae gene previously designated ODP1 (GB Z35903) encodes a protein whose sequence shares a 45% identity with the rat protein-arginine methyltransferase. The ODP1 gene was originally described as a partial open-reading frame adjacent to the PDX3 gene (Loubbardi et al., 1995) with no assigned function. Because of these sequence similarities to the mammalian enzyme, we were interested in determining whether the ODP1 gene product is also a protein-arginine methyltransferase. The yeast gene encodes a 39.8-kDa polypeptide of 348 residues with a calculated pI of 5.3. Several of the regions of sequence similarity between the yeast ODP1 and the rat PRMT1 proteins have been identified previously as conserved regions among a large number of methyltransferases (Kagan and Clarke, 1994, 1995) (Fig. 3). Regions I, II, III, and post region I have been shown to be involved in recognizing the methyl-donating cofactor S-adenosyl-L-methionine (Schlueckebier et al., 1995).

The ODP1/RMT1 Gene is Required for Protein Methylation—To determine whether the ODP1/RMT1 gene is in fact the gene for a protein methyltransferase, we created a S. cerevisiae strain in which this gene is disrupted by the insertion of a LEU2 cassette into its unique BstXI site, as described under “Experimental Procedures” (Fig. 2). The LEU2 cassette inserts an additional 1.6 kbp into the RMT1 gene, and the disrupted gene is predicted to create a non-functional protein because the insertion would cause an early translational termination 5 amino acid residues downstream from the insertion site. Analysis of the mutant cells demonstrated no obvious growth defects when compared to the parent cells. A light microscopic analysis of the two strains revealed no gross morphological abnormalities.

We then examined the endogenous protein methylation patterns in the crude soluble fraction from both strains by incubating these extracts with [3H]AdoMet and then separating the polypeptides by SDS gel electrophoresis (Fig. 4A). At least five polypeptides (55, 41, 38, 34, and 30 kDa) that are methylated in the lane containing parent RMT1 soluble extract show little or no methylation in the mutant RMT1 soluble extract lane. Therefore ODP1/RMT1 is required for a protein methyltransferase activity in yeast.

To characterize the stability of the [3H]methylated residues, we compared the total radioactivity present with the base-dependent volatile radioactivity in parallel gel slices. The pattern of total radioactive methyl group incorporation present in each slice (Fig. 4B) closely matches the data obtained from the fluorograph, as expected (Fig. 4A). The major methylated polypeptides at 55, 41, and 36 kDa from the endogenously methylated parent soluble extract are again absent when extract obtained from mutant rmt1 cells was used (Fig. 4B). The peak of radioactivity at 97 kDa and the broad signal from 20–29 kDa are found in both the parent and the mutant extracts and presumably represent methylation reactions not dependent upon the ODP1/RMT1 gene product. Gel slices from lanes parallel to those shown in Fig. 4B were then analyzed after base treatment using a vapor phase diffusion assay (Fig. 4C) that detects [3H]methyl groups in either ester linkages or linkages to the guanidino groups of arginine residues (Paik and Kim, 1980; Najbauer et al., 1991; Hrycyna et al., 1994). The results obtained from the analysis of the parent cell extract indicate that the major 55-, 41-, and 36-kDa substrates for the RMT1-dependent methyltransferase also represent the majority of the base-labile, methylatable species present. To distinguish volatile [3H]methylamine (derived from [3H]methylarginine residues) from [3H]methanol (derived from [3H]methyl ester residues), we neutralized the base-treated gel slices with HCI prior to the determination of volatility. We found that the base-labile radioactivity derived from the 55-, 41-, and 36-kDa polypeptides in the parent extract are not observed under these conditions (data not shown), suggesting that the product of the base treatment was, in fact, [3H]methylamine as the methyl-ammonium cation would not be expected to be volatile.

The results shown in Fig. 4 suggest that the yeast ODP1/RMT1 gene product is required for a large fraction of the total protein methylation as well as the predominant fraction of potential protein-arginine methyltransferation reactions in vitro, since most of the base-labile methyl linkages observed are dependent upon the presence of RMT1. The remaining base-labile, volatile radioactivity seen at 42 kDa in the mutant extract in Fig. 4C may represent the C-terminal leucine methyl ester in the catalytic subunit of protein phosphatase 2A (Xie and Clarke, 1993, 1994). Similarly, the radioactivity peak in the 22-kDa region may represent the C-terminal isoprenylcysteine methyl esters in small G-proteins or the formation of methyl esters on tRNA molecules (Hrycyna et al., 1994).

The ODP1/RMT1 Gene Product is Necessary for the in Vivo Mono- and Asymmetric Dimethylation of Arginyl Residues in S. cerevisiae—To directly demonstrate the chemi-
The results of Figs. 5 and 6 provide conclusive evidence for a protein-arginine methyltransferase activity that is dependent on the RMT1 gene product. Furthermore, this activity represents the major arginine methyltransferase activity present in intact yeast cells. The analysis of in vivo methylated cells indicates that the RMT1 gene product is required for about 89% of the asymmetrically dimethylated arginine residues and about 66% of monomethylated arginine residues (Fig. 5, B and D). In vitro, the RMT1 gene product is responsible for 83% of the asymmetrically dimethylated arginine residues, but does not seem to be required for the production of monomethylarginine (Fig. 6, B and D). These results indicate the presence of at least one RMT1-independent protein-arginine methyltransferase in yeast.

**Methylation of Hypomethylated Yeast Substrates Using a GST-RMT1 Fusion Protein**—The absence of protein-arginine methyltransferase activity in the yeast rmt1 mutant allowed us to use the soluble fraction from these cells as a source of hypomethylated protein substrates for this enzyme. We prepared an N-terminal GST fusion construct in which the RMT1 gene is translated in frame with the 26-kDa GST polypeptide and expressed the fusion protein in bacteria. When the soluble fraction from the rmt1 mutant strain was incubated with the purified GST-RMT1 fusion protein and [3H]AdoMet, a large number of methylated products were present that were not observed in the control reactions lacking the GST-RMT1 fusion protein (Fig. 7) or containing rmt1 soluble extract and purified GST itself (data not shown). The major methylated species

Fig. 4. **Loss of in vitro protein methylation in soluble extract from the yeast rmt1 mutant strain.** In panel A, cytosolic fractions containing parent extract (269 μg of protein) or mutant extract (338 μg of protein) (see “Experimental Procedures”) were incubated in 0.8 μM [3H]AdoMet (2.2 μCi) and a buffer of 25 mM Tris-HCl, 1 mM sodium EDTA, and 1 mM sodium EGTA at pH 7.5 in a final volume of 34 μl. After 30 min at 30 °C, the reactions were stopped by adding an equal volume of 2 × SDS-gel electrophoresis sample buffer (Haryya et al., 1994). The samples were then separated by SDS-gel electrophoresis (10% acrylamide) and the gel fluorographed as described under “Experimental Procedures.” These hydrolysates were then analyzed by amino acid analysis using high-resolution cation-exchange chromatography (Lee et al., 1977) under conditions where N6-monomethylarginine, N6,N8-dimethylarginine (asymmetric), and N8,N6-dimethylarginine (symmetric) are resolved from each other. The results from experiments where intact yeast cells were incubated with [3H]AdoMet (Fig. 5, B and D) show that a disruption of the RMT1 gene reduces the peak of asymmetrically [3H]dimethylarginine by greater than 85% compared to the level in the parent cells, with a smaller reduction in the amount of [3H]monomethylarginine formed. We observed little or no change in the earlier eluting [3H]methylated hydrolytic products between the parent and mutant substrates (Fig. 5, A and C). Similar results were obtained when we analyzed the acid-hydrolyzed products of in vitro, endogenously methylated parent and mutant soluble extracts (Fig. 6). Incubation of the parent extract with [3H]AdoMet produced equal levels of both mono- and asymmetrically dimethylated arginine residues (Fig. 6B), whereas only monomethylarginine was produced in the mutant extract (Fig. 6D). No changes were observed in the amounts of the previously eluted methylated species in the parent and mutant extracts (Fig. 6, A and C).
The yeast RMT1 fusion protein is a protein-arginine methyltransferase and has a broad substrate specificity for purified exogenous proteins. The studies described above demonstrate that RMT1 is required for a protein-arginine methyltransferase activity in S. cerevisiae, but do not directly demonstrate that the product of the RMT1 gene encodes the catalytic activity. We thus tested the ability of the GST-RMT1 fusion protein to methylate the peptide R1 (GGFGGRGGFG-amide) (a gift from D. Aswad, University of California, Irvine), which contains a consensus arginine methylation site (Najbauer et al., 1993). This fibrillarin/nucleolin-related peptide inhibits the methylation of endogenous protein-arginine methyltransferase substrates in PC12 cells and can be methylated by a partially purified bovine brain protein-arginine methyltransferase (Najbauer et al., 1993). This peptide is also an efficient substrate for the purified GST-RMT1 fusion protein; this result shows that the RMT1 gene product is itself an arginine methyltransferase, rather than an activator of a separate yeast catalytic activity. GST-RMT1 only monomethylates the arginine residue in the R1 peptide, with an initial velocity of 105 pmol/min/mg of fusion protein (data not shown). We propose that the lack of a proper tertiary structure in the 10-residue peptide is the cause for its specific monomethylation. Because this peptide contains the RGG-consensus sequence found in the mono- and asymmetric arginine methylation sites in fibrillarin (Lischwe et al., 1985b), nucleolin (Lischwe et al., 1985a), and hnRNP A1 (Rajpurohit et al., 1994a), we chose to test whether specific mammalian proteins include polypeptides of 57, 55, 42, 38, 35, 33, 30, 29, 27, 26, 20, and 13 kDAs and a minor methylated species at 66 kDa. These results directly show that the RMT1 gene product is necessary for a yeast protein-arginine methyltransferase activity.

The yeast polypeptides methylated when incubated with the purified GST-RMT1 fusion protein (Fig. 7, lane 1) are mono- and asymmetrically dimethylated (Fig. 8A). The yeast GST-RMT1 fusion protein was incubated with [3H]AdoMet in the presence or absence of rmt1 mutant extract. The proteins were then precipitated and acid-hydrolyzed to quantitate the amount of [3H]mono- and asymmetrically dimethylated arginine residues formed (Fig. 8, A and C). In the absence of rmt1 extract, only a small background of methylated arginine residues was detected (Fig. 8, compare A with C).

We also compared the ability of the yeast GST-RMT1 and the mammalian GST-PRMT1 fusion proteins to cause the methylation of the hypomethylated substrates present in the yeast rmt1 soluble extract. The purified GST fusion protein containing the rat PRMT1 sequence was able to efficiently methylate only a 55-kDa species, while the corresponding yeast GST fusion protein promoted the methylation of 10 or more polypeptide species (Fig. 7, compare lanes 1 and 3). Thus the rat GST fusion enzyme appears to have a quite restricted substrate specificity for yeast hypomethylated proteins when compared to the yeast GST fusion protein. This narrow specificity demonstrated by the rat fusion protein also reflects the activity of the native rat enzyme, since the 55-kDa species is also the major methylated polypeptide when a soluble extract of RAT1 cells is used to methylate rmt1 mutant cytosol. We do find, however, two additional minor methylated polypeptides at 34 and 24 kDa when rmt1 extract is incubated with RAT1 extract and [3H]AdoMet (Fig. 7). We determined that the purified GST-PRMT1 fusion protein specifically mono- and asymmetrically dimethylates the 55-kDa yeast substrate (Fig. 8, B and D).

Yeast RMT1 Fusion Protein is a Protein-arginine Methyltransferase and Has a Broad Substrate Specificity for Purified Exogenous Proteins—The studies described above demonstrate that RMT1 is required for a protein-arginine methyltransferase activity in S. cerevisiae, but do not directly demonstrate that the product of the RMT1 gene encodes the catalytic activity. We thus tested the ability of the GST-RMT1 fusion protein to methylate the peptide R1 (GGFGGRGGFG-amide) (a gift from D. Aswad, University of California, Irvine), which contains a consensus arginine methylation site (Najbauer et al., 1993). This fibrillarin/nucleolin-related peptide inhibits the methylation of endogenous protein-arginine methyltransferase substrates in PC12 cells and can be methylated by a partially purified bovine brain protein-arginine methyltransferase (Najbauer et al., 1993). This peptide is also an efficient substrate for the purified GST-RMT1 fusion protein; this result shows that the RMT1 gene product is itself an arginine methyltransferase, rather than an activator of a separate yeast catalytic activity. GST-RMT1 only monomethylates the arginine residue in the R1 peptide, with an initial velocity of 105 pmol/min/mg of fusion protein (data not shown). We propose that the lack of a proper tertiary structure in the 10-residue peptide is the cause for its specific monomethylation. Because this peptide contains the RGG-consensus sequence found in the mono- and asymmetric arginine methylation sites in fibrillarin (Lischwe et al., 1985b), nucleolin (Lischwe et al., 1985a), and hnRNP A1 (Rajpurohit et al., 1994a), we chose to test whether specific mammalian proteins...
**DISCUSSION**

Although a number of proteins have been identified that contain methylated arginine residues and protein-arginine methyltransferase activities have been characterized in a variety of eukaryotic cells, the functional significance of this type of protein modification is not well understood (Kim et al., 1990; Lischwe, 1990; Clarke, 1993). For myelin basic protein, the could serve as substrates for the yeast protein-arginine methyltransferase fusion protein. Several substrates have been used for both purification and differentiation of the two types of arginine methyltransferases (Lee et al., 1977; Farooqui et al., 1985; Ghosh et al., 1988; Rawal et al., 1994).

We found that the purified GST-RMT1 fusion protein is able to methylate crude histones, recombinant hnRNP A1 (a gift from A. Kainer and A. Mayeda, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), to a lesser extent cytochrome c and myoglobin (Fig. 9A). On the other hand, GST-RMT1 did not appear to methylate myelin basic protein (Fig. 9A). We found that hnRNP A1 is mono- and asymmetrically dimethylated on arginine residues by the GST-RMT1 fusion protein (Fig. 9B). We also determined that the chemical identity of the methylated residues in crude histones is mono- and asymmetric diamethylarginine (data not shown).

Sensitivity of the Yeast and Mammalian Protein-arginine Methyltransferases to Inhibitors—We compared the yeast and mammalian protein-arginine methyltransferase fusion proteins for their ability to be inhibited by AdoMet analogs that have been used to study cellular methylation reactions (Kujubu et al., 1993; Law et al., 1992). We incubated each of the fusion proteins with 5'-methylthioadenosine and S-adenosyl-L-homocysteine using rmt1 soluble extract as a source of methyl-accepting polypeptides. We found that neither enzyme was inhibited by 220 μM MTA (data not shown). However, S-adenosyl-L-homocysteine was found to be an inhibitor of both enzymes (Fig. 10, A and B). Using this assay, it appears that the yeast fusion protein is at least 20-fold less sensitive to S-adenosyl-L-homocysteine than the mammalian fusion protein. We estimate that the concentration needed to inhibit half of the activity of the yeast fusion enzyme is 250 μM, while that of the mammalian fusion enzyme is at least an order of magnitude lower.

**FIG. 6.** RMT1 is required for the asymmetric dimethylation of arginine residues in cellular extracts. Panels A and C show the fraction of asymmetrically dimethylated arginine (DMA) and monomethylated arginine (MMA) formed during in vitro methylation reactions containing parent (RMT1) or mutant (rmt1) yeast extract with a 1-h incubation at 30 °C with [3H]AdoMet. The reactions contained either 269 μg of CH9100-2 extract or 338 μg of J DG9100-2 extract, buffer (25 mM Tris-HCl, 1 mM sodium EDTA, and 1 mM sodium EGTA at pH 7.5), and 0.82 μM [3H]AdoMet (2.2 μCi) in a final volume of 34 μl. After 30 min, 15 μl of each reaction was removed and placed in a glass vial (6 × 50 mm) and the protein was precipitated by the addition of an equal volume of 25% (w/v) trichloroacetic acid. The mixture was incubated at 25 °C for 10 min before pelleting the precipitate at 4,000 rpm for 20 min at 25 °C. The protein pellet was washed with −20 °C acetone and dried before being acid hydrolyzed as described under "Experimental Procedures." The hydrolyzed pellet was resuspended in 50 μl of water, and 10 μl was loaded onto the sulfonated polystyrene column as described in Fig. 5. Panels B and D are enlargements of panels A and C, respectively, in the region where methylated arginines elute.

**FIG. 7.** The addition of the yeast GST-RMT1 or the mammalian GST-PRMT1 fusion protein complements the methylation deficiency in rmt1 extract. Reactions (50 μl) in lanes 1–3, contained 169 μg of rmt1 soluble extract protein, 0.7 μM [3H]AdoMet (2.75 μCi), buffer (25 mM Tris-HCl, 1 mM sodium EDTA, and 1 mM sodium EGTA at pH 7.5), and either 1.95 μg of GST-RMT1 protein, 2.0 μg of GST-PRMT1 protein (see "Experimental Procedures"), or no fusion protein, as the control. The reactions were incubated and analyzed as described in Fig. 4A. In lanes 4 and 5, reactions (50 μl) contained 59 μg of soluble extract protein from RAT1 cells (see "Experimental Procedures"), buffer, 0.7 μM [3H]AdoMet (2.75 μCi), with or without the addition of 169 μg of extract protein from the rmt1 mutant cells. The reactions were incubated and analyzed as described above. This panel shows the result of a 5-day exposure at −80 °C. Molecular mass markers are indicated by the arrows.
only known protein that contains a symmetrically dimethylated arginine residue (Kim et al., 1990), methylation has been suggested to enhance the compaction of the opposing plasma membranes in myelin (Amur et al., 1986; Young et al., 1987; Rawal et al., 1992). For the group of proteins containing asymmetrically dimethylated arginine residues, a common thread has emerged; many of these species interact with RNA (Lischwe, 1990; Najbauer et al., 1993; Liu and Dreyfuss, 1995). Asymmetric dimethylation of arginine residues in these proteins could cause RNA binding to shift from a specific to a nonspecific mode due to the loss of specific hydrogen bonds (Calnan et al., 1991). Consistent with this model, there is reduced nucleic acid binding of methylated hnRNPA1 compared to the unmethylated form (Rajpurohit et al., 1994b). It is thus possible that arginine methylation modulates the activity of hnRNPA1 in pre-mRNA splicing or the activities of nucleolin and fibrillarin in processing preribosomal RNA (Lischwe et al., 1989; Aris and Blobel, 1991). Asymmetric dimethylation of arginine residues has also been proposed as a means of modulating nuclear localization of these and other proteins. For example, only the high molecular weight forms of basic fibroblast growth factor that contain methylated arginine residues are specifically found in the nucleus (Burgess et al., 1991). Additional roles of arginine methylation in the heat shock response (Desrosiers and Tanguay, 1988; Wang et al., 1992) and in virus-induced cell transformation (Enouf et al., 1992) have been suggested as well.

No genes encoding a protein-arginine methyltransferase activity have been previously identified. In this work, we show that the yeast ODP1 gene product, now designated RMT1, is a protein-arginine methyltransferase that is similar to a rat gene product we have recently characterized (Lin et al., 1996). The RMT1 protein catalyzes the formation of N-G-monomethylarginine and N-G,N-G-dimethyl- (asymmetric) arginine residues on a number of endogenous yeast substrates and on exogenous mammalian proteins, including those containing a glycine and arginine-rich GAR domain.

In vivo analysis of methylated proteins in a rmt1 mutant strain indicates that the major activity responsible for asymmetrically dimethylation as well as monomethylating arginyl residues in S. cerevisiae is dependent upon a functional RMT1 gene product. Using soluble extracts from the parent and the rmt1 mutant yeast cells in in vitro assays, we confirmed that the predominant asymmetric dimethylarginine methyltransferase activity is dependent upon RMT1. However, the homogenization of the yeast cells appears to release an RMT1-independent monomethylarginine methyltransferase from a cellular compartment where the activity could not be detected in vivo. Direct evidence that RMT1 is a mono- and asymmetric dimethylarginine methyltransferase comes from the acid hydrolysis and amino acid analysis of in vitro reactions containing the GST-RMT1 fusion protein and recombinant hnRNPA1 methyl-accepting protein.

It should be noted that our results are in contrast to those of Liu and Dreyfuss (1995), where mammalian methylation was not observed in yeast extracts when recombinant hnRNPA1 was used as a substrate. One possible explanation for this difference is that the yeast strains used here are protease-deficient strains and that the methyltransferase activity may be highly susceptible to proteolysis when cells are disrupted for extract preparation.

This RMT1-dependent methylation is clearly not essential for viability since the rmt1 cells grow similarly to the parent cells in YPD. Assays with purified substrates, using the GST-RMT1 fusion protein, suggest that this enzyme has a broad specificity for methyl-accepting substrates. The yeast fusion protein is not only able to methylate mammalian histones and recombinant hnRNPA1, but also cytochrome c and myoglobin. The latter two proteins have been characterized as substrates for a Euglena gracilis protein-arginine methyltransferase.
In acid-hydrolyzed and analyzed by cation exchange chromatography as in Fig. 4 was incubated at 25°C for 10 min. Precipitated protein pellets were volume of 25% (w/v) trichloroacetic acid was added, and the mixture was incubated at 80°C. The samples were then loaded onto a 10% SDS-acrylamide gel and fluorographed. This panel represents a 7-day exposure at 80°C. The arrows indicate the positions of molecular mass standards described in Fig. 4.

Fig. 10. The yeast and mammalian arginine methyltransferase-GST fusions are differentially inhibited by S-adenosyl-L-homocysteine. rmt1 soluble extract (169 μg of protein) was incubated with 1.95 μg of GST-RMT1 protein or 0.78 μg of GST-PRMT1 protein, 0.93 μg or 1.16 μg [3H]AdoMet (2.2 μCi) respectively, the indicated concentrations of S-adenosyl-L-homocysteine (SAH) and buffer (25 mM Tris-HCl, pH 7.5, 1 mM sodium EDTA, and 1 mM sodium EGTA) in a final volume of 30 μl (panel A) and 24 μl (panel B). The reactions were performed at 30°C for 30 min and were stopped by the addition of an equal volume of 2 × SDS-gel electrophoresis sample buffer. The samples were then loaded onto a 10% SDS-acrylamide gel and fluorographed. Both panels represent 4-day exposures at ~80°C. The arrows indicate the positions of molecular mass standards described in Fig. 4.

Ghosh et al. (1988) partially purified two distinct protein-arginine methyltransferase activities from calf brain. One enzyme specifically methylated myelin basic protein (Ghosh et al., 1990), and the other was a histone arginine methyltransferase that was later shown to be more efficient toward hnRNP A1 (Rajpurohit et al., 1994a). Our substrate analysis of the yeast rmt1 enzyme suggests the functions of a single yeast protein-arginine methyltransferase may be distributed among a family of related enzymes in higher eukaryotes, as is the case for other enzymes responsible for the posttranslational protein modifications (e.g. cyclin-dependent protein kinases; Grana and Reddy, 1995). In fact, analysis of the expressed sequence tag data base indicates that there appear to be at least two related human cDNA sequences that are highly similar to the rat PRMT1/yeast RMT1 sequences.

Ghosh et al. (1988) partially purified two distinct protein-arginine methyltransferase activities from calf brain. One enzyme specifically methylated myelin basic protein (Ghosh et al., 1990), and the other was a histone arginine methyltransferase that was later shown to be more efficient toward hnRNP A1 (Rajpurohit et al., 1994a). Our substrate analysis of the yeast protein-arginine methyltransferase supports this idea of at least two distinct classes of arginine methyltransferases. The yeast protein-arginine methyltransferase specifically mono- and symmetrically dimethylates arginine residue 107 (Baldwin and Carnegie, 1971; Brostoff and Eylar, 1971), while the histone/hnRNP A1-specific enzyme mono- and asymmetrically dimethylates multiple arginine residues present in a GAR domain (Rajpurohit et al., 1992). The GST-RMT1 fusion protein methylated histones, hnRNP A1, cytochrome c, and myoglobin; substrates that can be modified to contain mono- or asymmetrically dimethylated arginine residues (Paik and Kim, 1969; Beyer et al., 1977; Karn et al., 1977; Farooqui et al., 1985). However, GST-RMT1 was not able to methylate myelin basic protein.

The potential in vivo yeast arginine methyltransferase substrates identified by Najbauer et al. (1993) have molecular sizes similar to those of the methylated polypeptides observed when
GSTM1 was incubated with hypomethylated rmt1 cytosol and [3H]AdoMet. For example, the NSR1 polypeptide has a predicted size of 45 kDa, but migrates as a 67-kDa polypeptide by SDS-gel electrophoresis. NSR1 is an essential gene in yeast, encoding a protein that not only binds nuclear localization sequences (Lee et al., 1991) but also is involved in the processing of pre-rRNA and is the homolog to the mammalian nucleolin (Kondo and Inouye, 1992; Kondo et al., 1992). Another potential RMT1 substrate is NPL3, whose gene was isolated from a mutant defective in localization of nuclear proteins and whose protein product migrates as a 55-kDa species (Bossie et al., 1992). The RNA and single-stranded DNA-binding protein SS81 also contains a GAR domain and migrates as a 45-kDa protein (Jong et al., 1987), while NPL1, the essential yeast fibrillarin homolog, migrates as a 38-kDa protein (Schimmang et al., 1989; Henriches et al., 1990). The GAR1 gene was identified by Southern analysis with a cDNA probe corresponding to the GAR domain of Xenopus fibrillarin. The encoded protein migrates at 24.5 kDa by SDS-gel electrophoresis (Girard et al., 1992). We plan to determine if these GAR domain-containing proteins interact with the GAR domain of NSR1 (Krainer and Akila Mayeda, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY) for supplying the recombinant GAR domain-containing protein NSR1 was incubated with hypomethylated 125I-hnRNP A1 sub-strate. We also thank Dr. Doug Black (UCLA, Los Angeles, CA) for supplying the recombinant hnRNP A1 sub-strate. We also thank Dr. Dana Aswad (University of California, Irvine) for supplying the R1 peptide and whether its protein product migrates as a 55-kDa species (Bossie et al., 1992; Liu and Dreyfuss, 1995). Furthermore, the ability of hnRNP A1 to preferentially promote splicing to more distal 5’-splice sites has been well characterized (Mayeda and Krainer, 1992; Mayeda et al., 1993) and provides an assay for determining the potential role of methylation in this context. The investigation into the identity of other GST-RMT1 substrates will also be of great importance in determining functions for arginine methylation.

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