Specificity and Mechanism of RNA Cap Guanine-N2 Methyltransferase (Tgs1)*

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The 2,2,7-trimethylguanosine (TMG) cap structure is characteristic of certain eukaryotic small nuclear and small nucleolar RNAs. Prior studies have suggested that cap trimethylation might be contingent on cis-acting elements in the RNA substrate, protein components of a ribonucleoprotein complex, or intracellular localization of the RNA substrate. However, the enzymatic requirements for TMG cap formation remain obscure because TMG synthesis has not been reconstituted in vitro from defined components. Tgs1 is a conserved eukaryotic protein that was initially identified as being required for RNA cap trimethylation in vivo in budding yeast. Here we show that purified recombinant fission yeast Tgs1 catalyzes methyl transfer from S-adenosylmethionine (AdoMet) to m7GTP and m7GDP. Tgs1 also methylates the cap analog mGppA but is unreactive with GTP, GDP, GpppA, m2,2,7GTP, m2,2,7GDP, ATP, CTP, UTP, and ITP. The products of methyl transfer to m7GTP and m7GDP formed under conditions of excess methyl acceptor are 2,7-dimethyl GTP and 2,7-dimethyl GDP, respectively. Under conditions of limiting methyl acceptor, the initial m2,7-GDP product is converted to m2,2,7-GDP in the presence of excess AdoMet. We conclude that Tgs1 is guanine-specific, that N7 methylation must precede N2 methylation, that Tgs1 acts via a distributive mechanism, and that the chemical steps of TMG synthesis do not require input from RNA or protein cofactors.

Many small noncoding eukaryotic RNAs contain a distinctive hypermodified 2,2,7-trimethylguanosine (TMG)1 cap structure (1, 2). TMG caps are also found on nematode mRNAs that undergo trans-splicing of a 5′-capped leader sequence (3). It is generally assumed that TMG caps are formed by post-transcriptional methylation of mG caps, but there is, as far as we know, no definitive evidence for an obligate relationship between prior N7 methylation and subsequent N2 methylation. Many studies, usually entailing RNA microinjections in am-

The abbreviations used are: TMG, 2,2,7-trimethylguanosine; AdoMet, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine; snRNP, small nuclear ribonucleoprotein; snRNA, small nuclear RNA; snoRNA, small nuclear RNA; PEI, polyethylamine; DTT, dithiothre-

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snRNAs were shown to be trimethylated in the cytoplasm in a reaction that depended on an RNA element to which Sm protein components of the snRNP bind (4). Trimethylation of snoRNAs occurs in the nucleus and depends on RNA structural motifs specific to the snoRNAs (5, 6). An analysis of snRNA trimethylation in an in vitro system derived from human cytosol indicated that: (i) AdoMet is the methyl donor in the reaction; (ii) snRNA pre-assembled with Sm proteins into a snRNP particle is an effective methyl acceptor; (iii) the Sm binding site in the RNA is required for trimethylation in vitro; and (iv) free snRNA does not serve as a methyl acceptor (7).

A major step forward in defining the genetic pathway of TMG cap formation was made when Bordonné and colleagues (8) identified the yeast Tgs1 protein in an interaction screen using a yeast Sm protein as bait. A tgs1Δ deletion mutant was viable even though the snRNAs and snoRNAs in the tgs1Δ strain lack TMG caps, as gauged by precipitation with anti-TMG antibody (8). The presence of a putative AdoMet binding motif in the Tgs1 polypeptide, mutation of which affected TMG formation in vivo (8, 9), suggested that Tgs1 might be involved directly in TMG formation. Although Tgs1 has consequently been dubbed a trimethylguanosine synthase, there is no evidence as yet that this protein is itself the catalyst of guanosine hypermethylation and, if so, what its specificity, mechanism, and requirements for RNA or proteins partners might be. To address these issues, we produced and characterized the Tgs1 ortholog for the fission yeast Schizosaccharomyces pombe.

**Experimental Procedures**

**Recombinant Tgs1**—The open reading frame encoding Tgs1 was amplified from an S. pombe cDNA library by PCR and inserted into pET28-His10-Smt3. The pET-His10-Smt3-Tgs1 plasmid was transformed into Escherichia coli BL21CodonPlus(DE3). A 500-ml culture amplified from a single transformant was grown at 37 °C in Luria-Bertani medium containing 50 μg/ml kanamycin and 50 μg/ml chloramphenicol until the A600 reached 0.6. The culture was adjusted to 2% ethanol and 0.2 mM isopropyl-1-thio-β-D-galactopyranoside and then incubated at 17 °C for 20 h with constant shaking. Cells were harvested by centrifugation, and the pellet was stored at −80 °C. All subsequent procedures were performed at 4 °C. thawed bacteria were resuspended in 25 ml of buffer A (50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 10% glycerol). Cell lysis was achieved by the addition of lysosome to 0.4 mg/ml. The lysate was sonicated to reduce viscosity, and insoluble material was removed by centrifugation. The soluble extract was applied to a 1-ml column of nickel-nitrioltriacetic acid-agarose resin (Qiagen) equilibrated with buffer A. The column was washed with 10 ml of the same buffer and then eluted stepwise with 2-ml aliquots of buffer A containing 50, 100, 250, and 500 mM imidazole. The polypeptide containing fractions were monitored on SDS-PAGE. The recombinant His6-Smt3-Tgs1 polypeptide was recovered predominantly in the 250 mM imidazole fractions. The 250 mM imidazole eluate was dialyzed against buffer containing 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM DTT, 1 mM EDTA, 10% glycerol and then stored at −80 °C. The protein concentration determined by SDS-PAGE analysis of serial dilutions of the Tgs1 preparation and a BSA standard. The gel was stained with Coomassie Blue, and the staining intensities of the His6-Smt3-Tgs1 and BSA polypeptides were quantified with a FujiFilm FLA-5000 digital imaging and analysis system. Tgs1 concentration was calculated by interploation to the BSA standard curve.

**Methyltransferase Assay**—Reaction mixtures (20 μl) containing 50 mM Tris-HCl, pH 8.0, 5 mM DTT, 50 μM [3H-CH3]AdoMet, 5 mM m7GTP or m7GDP, and enzyme were incubated for 30 min at 37 °C. The mixtures were spotted on DEAE-cellulose filters (25 mm, Whatman DE81), which were washed three times batchwise with 20 mM ammonium bicarbonate (modified from Ref. 10). The filters were dried, and the radioactivity adsorbed to the filter was quantified by liquid scintillation counting.

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Materials—[3H-CH₃]AdoMet was purchased from PerkinElmer Life Sciences. m²GTP, m²GDP, ATP, GTP, GDP, UTP, CTP, ITF, AdoMet, and AdoHcy were purchased from Sigma. m²GpppA and GpppA were purchased from New England Biolabs. 3'-O-MeGTP was from Amer sham. m²²,⁷GTP and m²²,⁷GDP were obtained from Dr. Richard Davis (University of Colorado Health Sciences Center).

RESULTS

Recombinant Tgs1 Is an AdoMet-dependent Methyltransferase—The 239-amino acid S. pombe Tgs1 protein was produced in E. coli as a His₁₀-Smt3-tagged fusion and purified from a soluble bacterial extract by adsorption to nickel-agarose and elution with imidazole. SDS-PAGE revealed two predominant polypeptides, a larger species corresponding to His₁₀Smt3-Tgs1 and a smaller polypeptide corresponding to the His₁₀-Smt3 tag (Fig. 1A). The methyltransferase activity of Tgs1 was demonstrated by incubating the protein with 50 μM [3H-CH₃]AdoMet and 5 mM m⁷GTP at 37 °C, which resulted in label transfer from AdoMet to m⁷GTP to form an anionic methylated nucleotide product that was adsorbed to a DEAE filter and thereby separated from the cationic AdoMet substrate. The extent of methylation increased with time and was proportional to input enzyme (Fig. 1B). Methyl transfer displayed a bell-shaped pH dependence with optimal activity at pH 8.0 in Tris-HCl buffer (data not shown); activity was 60% of the optimum at pH 9.5 (Tris-HCl) and 25% of the optimum at pH 5.5 (Tris acetate).

The recombinant protein was subjected to zonal velocity sedimentation in a 15–30% glycerol gradient (Fig. 2). Marker proteins catalase (native size 248 kDa), BSA (66 kDa), and cytochrome c (12 kDa) were included as internal standards. His₁₀Smt3-Tgs1 (calculated to be a 41-kDa polypeptide) sedimented as a discrete peak (fraction 17) between BSA and cytochrome c and was clearly resolved from the free His₁₀-Smt3 tag (fraction 19). The methyltransferase activity profile paralleled the abundance of the tagged Tgs1 polypeptide and peaked at fraction 17. We surmise from these results that the methyltransferase activity is intrinsic to Tgs1 and that the tagged enzyme is a monomer in solution.

Substrate Specificity and Product Analysis—Various nucleotides were tested as methyl acceptors at 5 mM concentration (Fig. 3). Tgs1 readily methylated m²GTP, m²GDP, and m²GpppA but not GTP, dGTP, dGDP, 3'-O-MeGTP, GDP, GpppA, ITP, XTP, ATP, CTP, or UTP. Thus, Tgs1 is a guanine-specific methyltransferase that requires prior methylation at N7 of the purine ring. The trimethylated nucleotides m²²,⁷GTP and m²²,⁷GDP were not methylated by Tgs1 (Fig. 3), suggesting that the methylation reaction targeted the exocyclic amine of the guanine base.

The product of the Tgs1-catalyzed reaction of 5 mM m⁷GTP with 5.4 μM [3H-CH₃]AdoMet, the specified nucleotide (5 mM) (or no nucleotide where indicated), and 0.25 μg of Tgs1 were incubated for 60 min at 37 °C. The extent of methyl transfer to yield DE81-absorbable material is shown.
of the \([^{3}H-CH_{3}]\text{AdoMet}\) was consumed, and the label transferred to two products: (i) a major species peaking at fraction 6 that migrated between the m7GTP and m2,2,7GTP standards; and (ii) a second species peaking at fraction 11, one fraction ahead of the m7GDP standard (Fig. 4, upper panel). We surmise that the major reaction product is 2,7-dimethyl GTP and the minor product is likely to be 2,7-dimethyl GDP, formed by reaction of Tgs1 with the contaminating m7GDP nucleotide present in the commercial m7GTP preparation, which was visible on the UV-shadowed chromatogram.

We also analyzed the product of Tgs1 reaction with 5 mM m7GDP with 5.4 \(\mu\text{M} \ [^{3}H-CH_{3}]\text{AdoMet}\) by PEI-cellulose TLC developed with 0.1 M ammonium sulfate (Fig. 4B). Most of the radioactivity in the no enzyme control comigrated with AdoMet in fractions 11–12 (Fig. 4B, lower panel). In the presence of Tgs1, the \([^{3}H-CH_{3}]\text{AdoMet}\) was depleted and the label transferred to a single product peaking at fraction 6, between the m7GDP and m2,2,7GDP standards (Fig. 4B, upper panel). For-mation of only one 3H-labeled product migrating between m7GDP and m2,2,7GDP was confirmed by autoradiography of a duplicate TLC plate (not shown). We surmise that the reaction product is 2,7-dimethyl GDP.

We exploited the TLC assay to gauge the extent of methyl transfer from 50 \(\mu\text{M} \ [^{3}H-CH_{3}]\text{AdoMet}\) to m7GDP as a function of m7GDP concentration (Fig. 5A). From a double-reciprocal plot of the data, we calculated a \(K_{m}\) value of 0.57 mM m7GDP and a \(k_{cat}\) value of 2 min\(^{-1}\). Methylation of m7GDP by Tgs1 displayed a hyperbolic dependence on AdoMet concentration; from a double-reciprocal plot of the data, we calculated a \(K_{m}\) value of 8 \(\mu\text{M} \) AdoMet (not shown). Methylation of 5 mM m7GTP in the presence of 50 \(\mu\text{M} \ [^{3}H-CH_{3}]\text{AdoMet}\) was inhibited in a concentration-dependent fashion by AdoHcy; the apparent IC\(_{50}\) for AdoHcy was 50 \(\mu\text{M} \) (data not shown). Thus, Tgs1 has similar...
affinity for its substrate AdoMet and its product AdoHcy. Addition of up to 20 mM GDP had only a modest (40%) inhibitory effect on methylation of 1 mM m7GDP by Tgs1 (Fig. 5B). From the inhibition profile, we estimate that Tgs1 binds GDP with one-thirtieth the affinity that it binds m7GDP. TLC analysis confirmed that no 3H-labeled m2,7GDP was formed by Tgs1 in the presence of 20 mM GDP (not shown).

**Synthesis of Trimethylguanosine by a Distributive Mechanism**—The absence of 2,2,7-trimethylguanosine products in the experiments presented above implies that either: (i) Tgs1 is not responsible for the second methylation reaction; or (ii) Tgs1 does catalyze the second methylation reaction, but we are precluded from detecting it because the enzyme acts distributively, *i.e.* the labeled m2,7GTP or m2,7GDP product dissociates after a single round of catalysis and must compete with a >1,000-fold molar excess of unlabeled m2GTP or m2GDP for rebinding to Tgs1. To address these issues, we analyzed the products of methylation reactions containing low concentrations of the methyl acceptor. As outlined in Fig. 6, we incubated Tgs1 with 50 μM m7GDP and 100 μM [3H-CH3]AdoMet for 60 min and then supplemented the reaction mixture with 1 mM cold AdoMet and allowed the reaction to proceed. Aliquots were taken at 30-min intervals; the products were analyzed by TLC and the 3H-methylated nucleotides visualized by autoradiography. The instructive finding was that the majority of the m2,7GDP formed during the pulse-labeling phase was subsequently converted to m2,7GDP during the chase phase in the presence of excess cold AdoMet (Fig. 6). These results show that Tgs1 is a *bona fide* trimethylguanosine synthase capable of catalyzing serial methylation reactions at the exocyclic amino nitrogen of m7G via a distributive mechanism.

**DISCUSSION**

The experiments presented here show that Tgs1, a protein implicated genetically in TMG cap formation (8), is indeed a catalyst of TMG synthesis. A key finding is that methylation of guanine N2 by Tgs1 *in vitro* is strictly dependent on prior methylation of guanine N7. This requirement is unique to Tgs1, *i.e.* no such requirement applies to two other RNA guanine-N2 methyltransferases, Trm1 and Trm-G10 (11, 12), and it suffices to explain why Tgs1 is cap-specific. Previous *in vivo* studies of Tgs1 had not illuminated this specificity, because the genetic analysis is complicated by the fact that whereas Tgs1 is not required for growth of *S. cerevisiae* (8), the upstream enzyme (Abd1) that catalyzes cap guanine-N7 methylation is essential for growth (13). Moreover, the reliance on antibodies to characterize the state of the cap in mutant yeast strains (8) places limits on what can be measured, *i.e.* anti-TMG antibodies might not be of use in determining whether snRNAs synthesized at restrictive temperature in an *abd1-ts* strain are methylated at N2 in the absence of methylation at N7.

Our results argue against Tgs1 acting processively to add two methyl groups to the guanine N2 following a single event of m7GDP binding to the active site. In a processive mechanism, the AdoHcy product of the first methylation step would have to dissociate and the N-CH3 rotate about the C2–N2 bond to the purine ring in order to clear the transferred methyl group out of the active site to make room for an incoming AdoMet donor for the second methylation step. Our findings favor a distributive mechanism in which the AdoHcy and m2,7GDP products dissociate from Tgs1 after the first methylation step, and the second methylation step entails rebinding of m2,7GDP from solution.

The finding here that guanine-N2 methylation of the cap by Tgs1 *in vitro* requires no RNA component and no protein cofactor forces reevaluation of the prevailing models that TMG synthesis depends strictly on cis-acting RNA signals or the assembly of specific RNP structures. Rather, our results instate a more conservative model in which RNP components might simply target Tgs1 to a particular subset of cellular RNAs that already have an m7G cap. This view of TMG formation, in which Tgs1 macromolecular interactions dictate access but not catalysis, mirrors the problem of enzymatic specificity *versus* cellular targeting encountered during the formation of the m7G cap itself. To wit, m7G capping *in vivo* is targeted specifically to cellular transcripts synthesized by RNA polymerase II, but the enzymes that perform the three capping reactions, starting from a triphosphate-terminated RNA, have no intrinsic ability to discriminate one RNA substrate from another as long as it has the requisite number of 5′-phosphates. Indeed, cap guanine-N7 methyltransferase is capable of methylating free guanine nucleotides (14) and thus does not even require an RNA moiety to achieve its specificity in catalysis (15). The capping apparatus is targeted to nascent RNAs by virtue of direct binding of the component enzymes to the RNA polymerase II transcription elongation complex (16).

In conclusion, the present biochemical characterization of Tgs1 as an autonomous cap-specific guanine-N2 methyltransferase should stimulate efforts to dissect substrate selectivity determinants in a defined reconstituted system and to probe the structural basis for catalysis of TMG formation.

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