Tgs1 is the enzyme responsible for converting 7-methylguanosine RNA caps to the 2,2,7-trimethylguanosine cap structures of small nuclear and small nucleolar RNAs. Whereas budding yeast Saccharomyces cerevisiae and fission yeast Schizosaccharomyces pombe encode a single Tgs1 protein, the primitive eukaryote Giardia lamblia encodes two paralogs, Tgs1 and Tgs2. Here we show that purified Tgs2 is a monomorphic enzyme that catalyzes methyl transfer from AdoMet ($K_m$ of 6 $\mu$M) to m2GDP ($K_m$ of 65 $\mu$M; $k_{cat}$ of 14 min$^{-1}$) to form m3GDP. Tgs2 also methylates m1GTP ($K_m$ of 30 $\mu$M; $k_{cat}$ of 13 min$^{-1}$) and m1GpppA ($K_m$ of 7 $\mu$M; $k_{cat}$ of 14 min$^{-1}$) but is unreactive with GDP, GTP, GpppA, ATP, CTP, or UTP. We find that the conserved residues Asp-68, Glu-91, and Trp-143 are essential for Tgs2 methyltransferase activity in vitro. The m2,7-GDP product formed by Tgs2 can be converted to m2,2,7-GDP by S. pombe Tgs1 in the presence of excess AdoMet. However, Giardia Tgs2 itself is apparently unable to add a second methyl group at guanine-N2. This result implies that 2,2,7-trimethylguanosine caps in Giardia are either synthesized by Tgs1 alone or by the sequential action of Tgs1 and Tgs2. The specificity of Tgs2 raises the prospect that some Giardia mRNAs might contain dimethylguanosine caps.

Many small noncoding eukaryotic RNAs contain a hypermodified 2,2,7-trimethylguanosine (TMG)$^2$ 5’-cap structure (1, 2). TMG caps are also found on nematode mRNAs generated via trans-splicing (3). TMG cap formation in Saccharomyces cerevisiae depends on the Tgs1 protein (4). The presence of a putative AdoMet binding motif in the Tgs1 polypeptide, the mutation of which affects TMG formation (4, 5), suggests that Tgs1 might be directly involved in TMG formation. Biochemical studies of Schizosaccharomyces pombe Tgs1 showed that it is indeed a catalytic of TMG synthesis (6). Methylation of guanine-N2 by S. pombe Tgs1 in vitro is strictly dependent on the prior methylation of guanine-N7, indicating that TMG caps are formed by post-transcriptional methylation of standard m7G caps (6). Guanine-N2 methylation by S. pombe Tgs1 in vitro requires no RNA component and no protein cofactor (6). Although early models suggested that the TMG synthase reaction might require cis-acting RNA signals or the assembly of specific ribonucleoprotein structures (7–10), the recent work on S. pombe Tgs1 instates a more conservative model in which ribonucleoprotein components might simply target Tgs1 to a particular subset of cellular RNAs that already have an m7G cap.

Given the ubiquity of TMG caps in eukaryotic species, it is surprising that an S. cerevisiae tgs1 deletion mutant is viable, even though the small nuclear RNAs and small nucleolar RNAs in the tgs1Δ strain lack TMG caps (4). Genetic analysis indicates that Tgs1 is also nonessential for growth of S. pombe (11). In contrast, TMG synthesis is essential in Drosophila, where mutations in the putative Tgs1 active site cause lethality at the early pupal stage of development that correlates with depletion of TMG-containing RNAs (11).

The protozoan parasite Giardia lamblia is posited to occupy a deeply branching position in eukaryotic phylogeny. Analysis of the Giardia genome is providing important insights to the early origins of RNA processing mechanisms that are regarded as uniquely eukaryotic (12). Although there had been some debate whether Giardia mRNAs even have a 5’-cap structure (13, 14), recent studies show that Giardia does possess the enzymatic machinery for m7G cap synthesis (15), caps the 5’ ends of its mRNAs, and exploits the m7G cap for enhanced translation of a reporter mRNA in vivo (15–17).

The fact that Giardia encodes two homologs of the cap-binding translation initiation factor elf4E (17) suggests that Giardia is not an exception to the general reliance on the cap structure for optimal gene expression in eukaryotes. However, characterization of the Giardia elf4E proteins revealed that one paralog, elf4E2, binds to m7G caps, whereas the other paralog, elf4E1, binds to m2,2,7-G caps (17). The existence of a specific cap-binding protein is consistent with an early study identifying several small RNAs in Giardia that reacted with antibody to the TMG cap (18). Because transfected TMG-capped mRNAs are not translated in Giardia (17), it is inferred that elf4E1 and TMG-capped RNAs are involved in RNA transactions unrelated to bulk mRNA translation.

The apparent complexity of cap function in Giardia is highlighted by our finding of two Tgs-like proteins in this primitive organism. One of the Tgs paralogs, which we name Tgs1, is a 300-amino-acid polypeptide that is 32% identical to S. pombe Tgs1 over a 174-amino acid segment of sequence similarity that is shown in Fig. 1. The second paralog, Tgs2, is a 258-amino acid polypeptide that is 25% identical to S. pombe Tgs1 over an 80-amino acid segment of sequence similarity that is shown in Fig. 1. Tgs2 is a 2,2,7-trimethylguanosine-specific enzyme that catalyzes addition of a single methyl group to form a 2,7-dimethylguanosine cap.
blia genomic DNA with primers that introduced an NdeI site at the start codon and a BglII site 3' of the stop codon. The PCR product was digested with NdeI and BglII and inserted into pET16b. The resulting pET-His10Tgs2 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 g/ml kanamycin and 50 g/ml chloramphenicol until the *A*$_{600}$ reached 0.6. The culture was adjusted to 2% ethanol and 0.2 mM isopropyl-1-thio-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, and 10% glycerol). Cell lysis was achieved by the addition of lysozyme to 100 g/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-nitrilotriacetic acid-agarose resin (Qiagen) that had been 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 compositions of the column fractions were monitored by SDS-PAGE. The recombinant His$_{10}$-Tgs2 polypeptide was recovered predominantly in the 250 mM imidazole fractions. The 250 mM imidazole eluate was dialyzed against a buffer containing 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM DTT, 1 mM EDTA, and 10% glycerol and then stored at 80 °C. The protein concentration was determined with the Bio-Rad dye reagent using bovine serum albumin as the standard. The single alanine mutations D68A, E91A, and W143A were introduced into the *TGS2* gene by the PCR-based two-stage overlap extension method (32). The mutated genes were inserted into the pET16b. The inserts were sequenced completely to exclude the acquisition of unwanted mutations during amplification and cloning. The D68A, E91A, and W143A proteins were produced in *E. coli* and isolated from soluble bacterial extracts as described above for wild-type Tgs2.

**Methyltransferase Assay**—Reaction mixtures (20 µl) containing 2.5 mM m7GTP, 50 µM [methyl-3H]AdoMet, and wild-type (WT) or mutant Tgs2 as specified were incubated for 15 min at 37 °C. The extent of methyl transfer is plotted as a function of input enzyme.

**FIGURE 1.** Two Tgs1-like paralogs in *G. lamblia*. The amino acid sequence of *G. lamblia* (Gla) Tgs2 from residues 17 to 190 is aligned to the sequence of its paralog GlaTgs1 and to the sequences of homologous Tgs1 polypeptides encoded by *S. pombe* (Spo), *S. cerevisiae* (Sce), and *Homo sapiens* (Hsa). Gaps in the alignment are indicated by dashes. Positions of identity/similarity in all five proteins are indicated by dots. The peptide motifs proposed to comprise the binding sites for the methyl donor and acceptor are highlighted in shaded boxes. Residues within the motifs that were subjected to alanine substitution in Tgs2 are indicated by vertical bars.

**FIGURE 2.** Methyltransferase activity of recombinant Tgs2. A, aliquots (3 µg) of the dialyzed nickel-agarose fractions of wild-type (WT) Tgs2 and the D68A, E91A, and W143A mutants were analyzed by SDS-PAGE. The Coomassie Blue-stained gel is shown. The positions and sizes (kDa) of marker polypeptides are indicated on the left. B, methyltransferase reaction mixtures (20 µl) containing 2.5 mM m7GTP, 50 µM [methyl-3H]AdoMet, and wild-type (WT) or mutant Tgs2 as specified were incubated for 15 min at 37 °C. The extent of methyl transfer is plotted as a function of input enzyme.
**RESULTS**

Tgs2 Is an AdoMet and m⁷G-dependent Methyltransferase—The *Giardia* Tgs2 protein was produced in *E. coli* as a His$_{10}$ fusion and purified from a soluble bacterial extract by adsorption to nickel-agarose and elution with imidazole (Fig. 2). The methyltransferase activity of Tgs2 was demonstrated by incubating increasing amounts of the protein with 50 μM [methyl-$^3$H]AdoMet and 2.5 mM m⁷GDP at 37 °C, which, at saturating enzyme, resulted in 93% label transfer from AdoMet to the methyl-C of m⁷GDP (Fig. 2) to form a $^3$H-labeled product that was separated from the labeled AdoMet by PEI-cellulose TLC in 0.1 M ammonium sulfate (Fig. 3). The methyltransferase activity of Tgs2 was inhibited in a concentration-dependent fashion by the methylation of 2.5 mM m⁷GDP in the presence of 50 μM [methyl-$^3$H]AdoMet and 2.5 mM m⁷GDP at 37 °C, which, at saturating enzyme, resulted in 93% label transfer from AdoMet to the methyl-C of m⁷GDP (Fig. 2) to form a $^3$H-labeled product that was separated from the labeled AdoMet by PEI-cellulose TLC in 0.1 M ammonium sulfate (Fig. 3). The methyltransferase activity of Tgs2 was inhibited in a concentration-dependent fashion by the methyltransferase reaction products m²,7GDP (panel A), m²,7GTP, and m²,7GpppA (panel B) and m²,7GpppA (panel C) are denoted by arrowheads at the right of the chromatograms.

**Glycerol Gradient Sedimentation**—An aliquot (40 μg) of the nickellagarose preparation of Tgs2 was mixed with catalase (45 μg), bovine serum albumin (45 μg), and cytochrome c (45 μg). The mixture was applied to a 4.8-ml 15–30% glycerol gradient containing 50 mM Tris-HCl (pH 8.0), 0.2 M NaCl, 1 mM EDTA, and 2 mM DTT. The gradient was centrifuged for 18 h at 37 °C in a Beckman SW50 rotor at 50,000 rpm. Fractions (~0.19 ml) were collected from the bottom of the tube.

**Materials**—[methyl-$^3$H]AdoMet was purchased from New England Nuclear. m²,GTP, m²,GDP, AdoMet, AdoHcy, sinefungin, and sodium periodate were purchased from Sigma. m²,GpppA and GpppA were purchased from New England Biolabs.

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The cap dinucleotide m7GpppA to form a single product, presumed to be m^2,7GpppA, that was resolved from AdoMet by PEI-cellulose TLC in 0.05 M ammonium sulfate (Fig. 3B). This product migrated immediately ahead of the input m^7GpppA substrate, which was visualized by UV illumination of the chromatogram (not shown). No novel product was formed in the presence of the unmethylated cap dinucleotide GpppA (Fig. 3B). Simultaneous TLC analysis of the reaction product formed with m^7GTP revealed a major species (m^{2,7}GTP) that barely migrated off the origin in 0.05 M ammonium sulfate plus a minor contaminant (m^{2,7}GDP) that migrated slower than m^{2,7}GpppA (Fig. 3B). Tgs2 formed no new labeled product when reacted with 2.5 mM GTP, ATP, CTP, or UTP (Fig. 3B). Collectively, these results highlight the requirement for prior N7 methylation of the guanine nucleotide substrate, which can be either a cap dinucleotide, a mononucleoside triphosphate, or a mononucleoside diphosphate.

The extent of methyl transfer by Tgs2 in the presence of 50 μM AdoMet displayed a hyperbolic dependence on m^7GDP (Fig. 6A) or m^7GTP (Fig. 6B) concentration. From double-reciprocal plots of the data, we calculated a K_m for m^7GDP of 65 μM with a k_cat of 14 min^-1 and a K_m for m^7GTP of 30 μM with a k_cat of 13 min^-1. The cap dinucleotide m^7GpppA was a more avid methyl acceptor than either m^7GDP or m^7GTP (Fig. 6C); we calculated a K_m of 7 μM m^7GpppA and a k_cat of 14 min^-1. These experiments show that, whereas the affinity of Tgs2 for the m^7G nucleotide methyl acceptor is enhanced 2-fold by the γ-phosphate of m^7GTP and an additional 4-fold by the 5’-nucleoside of the cap analog, the methyltransferase activity is intrinsic to Tgs2 and that the enzyme is a monomer in solution.

**Characterization of the Reaction Product**—The primary structure similarity between Tgs2 and the yeast Tgs1 trimethylguanosine synthase enzymes engenders a prediction that the exocyclic N2 atom of m^7G is the methyl acceptor in the Tgs2-catalyzed reaction. Nonetheless, we considered the possibility that Tgs2 might transfer the methyl group to either the ribose or the base of the nucleotide substrate. If methyl transfer occurred at either the ribose O2’ or O3’ atoms of m^7GDP, then the resulting 2’-OCH_3 or 3’-OCH_3 products should be resistant to oxidation by periodate, whereas m^7G methylation at guanine-N2 would preserve the vicinal ribose hydroxyls and leave them sensitive to periodate oxidation. Fig. 7 shows that treatment of the 3H-labeled product of Tgs2-catalyzed methyl transfer from AdoMet to m^7GDP with sodium periodate caused the labeled nucleotide to be retained at the origin during PEI-cellulose TLC. The untreated product migrated at its usual position, slower than AdoMet and immediately ahead of the m^7GDP substrate. Retention at the origin is a consequence of oxidation of the ribose to a ring-opened 2’,3’-dialdehyde, which forms a covalent Schiff base adduct to the PEI at the site of application to the TLC plate. Control experiments showed that periodate treatment of unlabeled GTP quantitatively shifted the nucleotide to the origin, whereas periodate treatment of 3’-OCH_3 GTP had no effect on migration during TLC (not shown). We conclude that Tgs2 does not catalyze methylation of the ribose hydroxyls.

The labeled product formed by Tgs2 in reactions containing excess m^7GDP methyl acceptor comigrated during TLC with the m^{2,7}GDP product synthesized by S. pombe Tgs1 (data not shown). The absence of a 2,2,7-trimethylguanosine product of the Tgs2 reaction implies one of the following possibilities: (i) that Tgs2 is not able to perform the second methylation reaction at N2; or (ii) that Tgs2 does catalyze a second methylation reaction, but we are precluded from detecting it because the enzyme acts distributively, i.e. the labeled m^{2,7}GDP product dissociates after a single round of catalysis and must compete with a large molar excess of unlabeled m^7GDP for re-binding to Tgs2. Previous studies showed that S. pombe Tgs1 does catalyze sequential methylation reactions at N2 via a distributive mechanism (6).
To address this issue for the *Giardia* enzyme, we analyzed the products of a Tgs2 methylation reaction at equal concentrations of the methyl donor and acceptor. As outlined in Fig. 8, Tgs2 was incubated with 50 μM m7GDP and 50 μM \([methyl-3H]\)AdoMet for 30 min, at which time most of the label had been transferred to the substrate to form a single methylated product. The reaction mixture was then split and supplemented with 1 mM cold AdoMet and either *S. pombe* Tgs1 or fresh *Giardia* Tgs2. The reactions were continued for another 60 min, and the products were analyzed by TLC. The instructive finding was that about half of the m2,7GDP formed during the pulse-labeling phase was subsequently converted by *S. pombe* Tgs1 to the slightly more rapidly migrating trimethylated product m2,2,7GDP during the chase in the presence of excess cold AdoMet (Fig. 8). In contrast, adding more *Giardia* Tgs2 resulted in no change in the radiolabeled product distribution. These results indicate that Tgs2 is a dimethylguanosine synthase capable of catalyzing only one methyl addition reaction at the exocyclic amino nitrogen of m7G.

Asp-68, Glu-91, and Trp-143 Are Essential for Tgs2 Methyltransferase Activity—Amino acid sequence comparisons of Tgs-like proteins and structural model building (5) led to the prediction of a canonical AdoMet binding site in *S. cerevisiae* Tgs1 composed of two peptide motifs highlighted in shaded boxes in Fig. 1 (corresponding to Tgs2 peptides 66VIDGTACVGG75 and 88VAIE91). A putative methyl acceptor site was predicted to reside within the conserved proline/glycine containing motif (140DPPWGGV146 in Tgs2; see Fig. 1). Bordonne and colleagues (4, 5) have shown that alanine mutations of *S. cerevisiae* Tgs1 at three conserved positions in these motifs (Asp-103, Asp-126, and Trp-178, corresponding to Asp-68, Glu-91, and Trp-143 in *Giardia* Tgs2) cause defects in TMG cap formation in vivo. To gauge the bio-
Giardia Cap Guanine-N2 Methyltransferase

chemical effects of such changes, we produced Giardia Tgs2 mutants D68A, E91A, and W143A in bacteria as His10 fusions and isolated them from soluble bacterial extracts by nickel-agarose chromatography (Fig. 2). We found that the Tgs2 mutants were inert in catalysis of methyl transfer from AdoMet to m7GDP, at a level of sensitivity of ≤1% of the wild-type specific activity (Fig. 2). These results verify that the methyltransferase activity is intrinsic to the recombinant Tgs2 protein. Based on the crystal structure of the cap guanine-N7 methyltransferase Ecm1 bound to AdoMet and on mutational analysis of that enzyme (19, 20), we suspect that essential Tgs2 residues Asp-68 and Glu-91 coordinate the methionine amine and adenosine ribose hydroxyls of AdoMet, respectively.

DISCUSSION

The experiments presented here show that Giardia Tgs2 is a mono-meric m’G-specific methyltransferase that catalyzes addition of one methyl group to the exocyclic guanine-N2 atom. The recombinant Giardia enzyme resembles the fission yeast Tgs1 protein in its requirement for prior methylation at guanine-N7 and its ability to methylate m’G mononucleotides and m’G cap dinucleotides in the absence of a RNA polynucleotide or a separate protein cofactor. Tgs2 has a Kₘ for AdoMet (6 μM) similar to that of S. pombe Tgs1 (9 μM) and, like the fission yeast enzyme, Tgs2 is inhibited by its reaction product AdoHcy. Tgs2 displays a higher affinity for m’GDP (Kₘ of 65 μM) than does S. pombe Tgs1 (Kₘ of 570 μM), and the turnover number of Tgs2 (14 min⁻¹) is higher than that of S. pombe Tgs1 (2 min⁻¹).

The most distinctive property of Giardia Tgs2 is that its activity is apparently limited to a single round of N2 methylation, resulting in the synthesis of a 2,7-dimethylguanosine product. In contrast, S. pombe Tgs1, the only other cap-specific N2 methyltransferase that has been characterized (6), is able to catalyze two sequential N2 methylations leading to TMG cap formation. tRNA-specific guanine-N2 methyltransferases also fall into two classes, depending on whether they catalyze either one methylation step to form 2-methylguanosine (21) or two sequential steps to generate 2,2-dimethylguanosine (22–25). The rRNA-specific guanine-N2 methyltransferase RsmC performs only one methylation step to generate 2-methylguanosine (26). Given the proposal that that members of the Tgs-like family are structurally homologous to RsmC (5) and the present biochemical characterization of Giardia Tgs2 as a 2,7-dimethylguanosine synthase, it is appropriate to sound a note of caution in attributing TMG cap synthetic roles to Tgs2 mutants.

but sensitive to pyrophosphatase (15), the structure of the blocking nucleoside is unknown. Also, whereas Giardia contains small RNAs that can be recovered using anti-TMG antibody (18), the structures of those caps have not been determined directly. The recent finding that Giardia has two eIF4Es with preferential affinity for m’G and m’2G caps, respectively (17), did not examine whether the TMG-specific eIF4E2 protein might bind as well or better to a 2,7-dimethylguanosine cap. Several independent studies have shown that 2,7-dimethyl- guanosine (DMG)-capped reporter mRNAs are translated better than standard m’G-capped transcripts in vitro, whereas TMG-capped reporter mRNAs are translated with low efficiency (29–31) or, in the case of Giardia RNA transfection experiments in vivo, not at all (17). In conclusion, the existence of cap-specific N2 methylating enzymes with TMG versus DMG synthase activities raises questions about the structural basis for the different reaction outcomes and the potential existence and function of DMG caps in eukaryotic RNA transactions.

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