Trypanosoma brucei Encodes a Bifunctional Capping Enzyme Essential for Cap 4 Formation on the Spliced Leader RNA*

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The 5′ end of kinetoplastid mRNA possesses a hypermethylated cap 4 structure, which is derived from standard m7GpppN (cap 0) with additional methylations at seven sites within the first four nucleosides on the spliced leader RNA. In addition to TbCe1 guanylyltransferase and TbCmt1 (guanine N-7) methyltransferase, Trypanosoma brucei encodes a second cap 0 forming enzyme. TbCgm1 (T. brucei cap guanylyltransferase-methyltransferase) is a novel bifunctional capping enzyme consisting of an amino-terminal guanylyltransferase domain and a carboxyl-terminal methyltransferase domain. Recombinant TbCgm1 transfers the GMP to spliced leader RNA (SL RNA) via a covalent enzyme-GMP intermediate, and methylates the guanine N-7 position of the GpppN-terminated RNA to form cap 0 structure. The two domains can function autonomously in vitro. TbCGM1 is essential for parasite growth. Silencing of TbCGM1 by RNA interference increased the abundance of uncapped SL RNA and lead to accumulation of hypomethylated SL RNA. In contrast, silencing of TbCe1 and TbCMT1 did not affect parasite growth or SL RNA capping. We conclude that TbCgm1 specifically cap SL RNA, and cap 0 is a prerequisite for subsequent methylation events leading to the formation of mature SL RNA.

The 5′ cap is an essential feature of eukaryotic mRNAs and snRNAs, and is required for RNA stability and efficient translation (1). The cap 0 (m7GpppN) is formed by sequential action of three enzymatic activities. The 5′ triphosphate of the nascent RNA is hydrolyzed to a diphosphate by RNA triphosphatase, the diphosphate end is capped with GMP by guanylyltransferase, and the GpppN cap is methylated at the N-7 position by (guanine N-7) methyltransferase (2). Whereas the three-step capping reaction is universal to all eukaryotes, organization of the capping enzyme to the site of transcription is likely to resemble that of yeast, with separate triphosphatase (TbCet1), guanylyltransferase (TbCe1), and methyltransferase (TbCmt1) components. TbCet1 is a metal-dependent phosphohydrolase that catalyzes the removal of the terminal phosphate from triphosphate-terminated RNA (29). TbCe1 is mechanistically related to other cellular guanylyltransferases except that it contains an amino-terminal extension of 250 amino acids of unknown function (30). TbCmt1 catalyzes the guanine N-7 methylation on a GpppN-terminated RNA (31). In addition, two separate 2′-O-nucleoside methyltransferases implicated in SL RNA cap 4 methylation have been identified and characterized (32–35).

We recently reported the identification of a second candidate T. brucei capping enzyme, which we named TbCgm1 (31). The

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§The abbreviations used are: snRNA, small nuclear RNA; AdoMet, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine; DTT, dithiothreitol; pol II, polymerase II; SL RNA, spliced leader RNA; RNAi, RNA interference; nt, nucleotide(s).
primary structure of TbCgm1 suggests that the enzyme consists of a guanylyltransferase domain and a methyltransferase domain (Fig. 1A). The amino-terminal portion of TbCgm1 contains the defining sequence motifs of the covalent nucleotidyltransferase superfamily (I, III, IIIa, IV, V, and VI) involved in GTP binding and catalysis, except that the 139-amino acid interval between motifs I and III of TbCgm1 is slightly longer than that of other cellular guanylyltransferases. Motif I (127KADGTR132) contains the presumptive active site lysine to which GMP becomes covalently linked via a phosphoamide bond (8, 36). Residues that are essential in Saccharomyces cerevisiae guanylyltransferase Ceg1 are conserved in TbCgm1, as well as those residues that make direct contacts with the GTP substrate, as deduced from the Chlorella virus guanylyltransferase-GTP cocrystal structure (8, 37, 38). The carboxyl-terminal portion of TbCgm1 contains an AdoMet binding motif (785VADLCGRGG794), along with a number of key residues that make direct contacts with the GpppA cap as shown in the crystal structure of E. cuniculi methyltransferase (39).

Here, we show that purified recombinant TbCgm1 is a bifunctional capping enzyme with an amino-terminal guanylyltransferase (amino acids: 1–567) domain and a carboxyl-terminal (guanine N-7) methyltransferase domain (amino acids 717–1050). Each domain can function autonomously in vitro. TbCgm1 is essential for parasite growth. RNAi-mediated down-regulation of TbCGM1 shows reduced levels of cap 4 methylation on SL RNA. In contrast, down-regulation of TbCE1 and TbCMT1 were not essential for viability and did not affect SL RNA capping. Together, these results demonstrate that the bifunctional TbCgm1 is responsible for m7GpppN formation on the SL RNA.

EXPERIMENTAL PROCEDURES

TbCGM1 Expression Plasmids—The TbCGM1 gene (accession number XP_840738.1) was PCR amplified from total T. brucei brucei genomic DNA (a gift of Laurie Read, SUNY at Buffalo) and cloned into BamHI and XhoI sites of pET28-His-Smt3 vector (a gift of Chris Lima, Sloan-Kettering Institute) to fuse the 1050-amino acid TbCgm1 polypeptide in-frame to the amino-terminal His-Smt3 tag to obtain pET-HisSmt3-TbCGM1. The carboxyl-terminal truncation mutant, TbCGM1-(1–567), was constructed by PCR amplification using sense primer that introduced an NdEl site at a start codon and an antisense primer that introduced an XhoI site immediately downstream of the new stop codon at Glu668. TbCGM1-(717–1050) was constructed by PCR amplification using a sense primer that introduced a translation start codon at Leu716 with an NdEl site at the new start codon. The PCR products were digested with NdEl and XhoI, and then inserted into pET16b to obtain pET-TbCGM1-(1–567) and pET-TbCGM1-(717–1050), respectively.

Expression and Purification of Recombinant TbCgm1—pET-HisSmt3-TbCGM1 was transformed into Escherichia coli BL21(ROS2). A 1-liter culture amplified from a single transformant colony was grown at 37 °C in LB medium containing 60 μg/ml kanamycin and 100 μg/ml chloramphenicol until the A600 reached 0.4. The culture was adjusted to 2% ethanol and incubated at 17 °C for 18 h. Cells were harvested by centrifugation and stored at ~80 °C. Thawed bacteria were resuspended in 50 ml of Buffer A (50 mM Tris-HCl, pH 7.5, 0.25 mM NaCl, 10% sucrose). Lysozyme and Triton X-100 were added to final concentrations of 50 μg/ml and 0.1%, respectively. The lysates were sonicated to reduce viscosity, and insoluble material was removed by centrifugation for 45 min at 14,000 x g in a Beckman T14-50 rotor. The soluble lysate was applied to 1.5-ml columns of nickel-nitritriacetic acid-agarose (Qiagen) equilibrated with Buffer A containing 0.1% Triton X-100. The column was washed with 15 ml of the same buffer and eluted stepwise with 3 ml of Buffer B (50 mM Tris-HCl, pH 8, 0.25 mM NaCl, 10% glycerol, 0.05% Triton X-100) containing 0, 0.005, 0.05, 0.1, 0.2, 0.5, and 1 M imidazole. The recombinant His-Smt3-TbCgm1 was recovered in the 0.2 M imidazole eluate (0.5 mg of proteins per 1-liter culture). Two hundred micrograms of His-Smt3-TbCgm1 polypeptide was incubated with 60 μg of His-tagged ULP1 protease on ice for 1 h to cleave the NH2-terminal His-Smt3 tag. Sample was then diluted to 0.05 M imidazole in Buffer B and applied to 0.5 ml of nickel-agarose equilibrated with Buffer B. The native TbCgm1 protein was recovered in a flow-through fraction and concentrated to 0.17 mg/ml. All enzyme fractions were stored at ~80 °C and thawed on ice just prior to use. Protein concentrations were determined using the Bio-Rad dye-binding assay with bovine serum albumin as a standard.

TbCgm1-(1–567) and TbCgm1-(717–1050) proteins were expressed in 500-ml cultures of E. coli BL21(DE3) in LB medium containing 100 μg/ml ampicillin at 37 °C until the A600 reached 0.4. The cultures were adjusted to 0.4 M isopropyl β-D-thiogalactoside and 2% ethanol, and incubated for 18 h at 17 °C. Cells were harvested by centrifugation and stored at ~80 °C. Thawed bacteria were resuspended in 20 ml of Buffer A. Soluble lysates were prepared as described for full-length TbCgm1 and then applied to 1-ml columns of nickel-nitritriacetic acid-agarose equilibrated with Buffer A containing 0.1% Triton X-100. Columns were washed with 10 ml of Buffer A containing 0.1% Triton X-100 and eluted stepwise with Buffer B containing 0, 0.05, 0.1, 0.2, 0.5, and 1 M imidazole. Recombinant proteins retained on the column and were recovered predominantly in the 0.2 M imidazole eluate (1.6 mg of TbCgm1-(1–567) and 3.3 mg of TbCgm1-(717–1050) per 500 ml of culture).

Guanylyltransferase Assay—Standard reaction mixtures (10 μl) containing 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 2 mM MgCl2, 20 μM [α-32P]GTP, and enzyme were incubated at 30 °C for 15 min. The reactions were quenched with SDS loading buffer and the products were resolved by 10% SDS-PAGE. Enzyme-32P/GMP adduct was visualized by autoradiography of the dried gel and quantified by scanning the gel with a Storm 860 PhosphorImager.

Cap Methyltransferase Assay—Triphosphate-terminated poly(A) was synthesized (40) and then converted to 32P-capped poly(A) (m7GpppA terminated poly(A); boldface indicates radiolabeled phosphate) as described previously (41). The length of cap-labeled poly(A) was between 150 and 250 nt. Standard reaction mixtures (10 μl) containing 50 mM Tris acetate (pH 6), 2 mM DTT, 20 μM AdoMet, 67 fmol of 32P-capped poly(A), and either TbCgm1 or TbCgm1-(717–1050) were incubated for 30 min at 30 °C. The reaction mixtures were...
adjusted to 100 mM sodium acetate (pH 5.5) and incubated with 100 ng of nuclease P1 for 60 min at 37 °C. Aliquots (3 μl) were spotted onto a PEI cellulose thin-layer chromatography (TLC) plate, which was developed with 0.45 mM ammonium sulfate. The extent of methylation (mGpppA/[mGpppA + GpppA]) was quantified by scanning the TLC plate with a phosphorimagerr.

**Results**

**TbCgm1 is a Bifunctional Capping Enzyme with Guanylyltransferase and Methyltransferase Activities**—To determine whether TbCgm1 has an intrinsic capping activity, we expressed the recombinant protein in *E. coli* as an N-terminal His-Smt3-tagged fusion protein to facilitate solubility and purification. The fusion protein was purified from soluble bacterial lysate by adsorption to nickel-agarose and elution with 0.2 M imidazole (Fig. 1B, lanes 1 and 2). The native TbCgm1 protein was obtained by cleaving the fusion protein with His-tagged ULP1 protease, followed by removal of the His-smt3 tag by a second round of nickel-agarose chromatography (Fig. 1B, lane 3). TbCgm1 was further purified away from a ~65-kDa bacteria contaminant (Fig. 1B, asterisk) by glycerol gradient sedimentation (see below).

The guanylyltransferase reaction entails two sequential nucleotidyl transfer steps. In the first step, nucleophilic attack on the α-phosphate of GTP results in formation of a covalent EpG intermediate and liberation of PPi. The guanylyltransferase activity of TbCgm1 was evinced by label transfer from [α-32P]GTP to the TbCgm1 polypeptide to form a SDS-stable nucleotidyl-protein adduct that migrated as an 116-kDa species (Fig. 1C, lane 3). Note that three other polypeptides in the range of 60–100 kDa were labeled with GMP to a lesser extent, which suggested that these were proteolytic fragments of TbCgm1. We conclude that TbCgm1 is a covalent nucleotidyltransferase.

The methyltransferase activity of TbCgm1 was assayed by conversion of [33P]cap-labeled poly(A) to methylated cap-labeled poly(A) in the presence of AdoMet. Digestion by nuclease P1 liberated a labeled species that co-migrated with m7GpppA, generated in a parallel reaction mixture containing purified yeast Abd1 (Fig. 1D, lanes 2 and 5, respectively). The activity was dependent on AdoMet and the inclusion of AdoHcy was inhibitory to the reaction (Fig. 1D, lane 4). We further verified that methylation occurs at the terminal guanosine nucleoside by digesting the reaction products with phosphomimager.
with nucleotide pyrophosphatase, a nuclease that cleaves between the γ and β phosphates in the capped structure. Both the TbCgm1 and Abd1 reaction products liberated m7Gp (data not shown). We conclude that TbCgm1 catalyzes methylation at the N-7 position of the terminal cap guanosine to form cap 0.

The native size of TbCgm1 was gauged by sedimentation through a 15–30% glycerol gradient and the fractions were assayed for enzyme-GMP formation and methyltransferase activities. The guanylyltransferase and methyltransferase activities co-sedimented as a 6.7 S peak (Fig. 1E). Based on the predicted molecular weight, we conclude that TbCgm1 is a monomeric protein in solution.

The Amino-terminal Domain of TbCgm1 Can Function Autonomously as Guanylyltransferase—To evaluate whether the amino-terminal portion of TbCgm1 constituted an autonomous functional guanylyltransferase domain, we expressed the TbCgm1 segment from residues 1–567 as a His-tagged fusion protein (Fig. 2A). The choice of residue 567 as a domain breakpoint was based on the location of motif VI in TbCgm1, which is situated at residues 491–500 in TbCgm1 (Fig. 1A). In most cellular guanylyltransferases, motif VI is positioned at ~50 amino acids upstream of the carboxyl-terminal end (20, 21). The iso-propyl β-D-thiogalactose-induced bacteria accumulated substantial amounts of a soluble 65-kDa polypeptide corresponding to His-tagged TbCgm1-(1–567), which adsorbed to nickel-agarose and was eluted at 0.1–0.2 M imidazole (Fig. 2A). TbCgm1-(1–567) reacted with [α-32P]GTP to form a radiolabeled enzyme-GMP (EpG) adduct (Fig. 2B). The 0.2 M imidazole fraction of TbCgm1-(1–567) was used to characterize the guanylyltransferase activity.

Characterization of TbCgm1-GMP Complex Formation—The amount of EpG formed in the presence of 20 μM [α-32P]GTP and 2 mM MgCl2 was proportional to the amount of input TbCgm1-(1–567) (Fig. 2C). Under this condition, the extent of EpG formation is linear over 30 min, and reached plateau thereafter (data not shown). TbCgm1-(1–567) reacted specifically with GTP. Inclusion of other rNTPs or dNTPs at 100-fold excess over [α-32P]GTP did not inhibit 32P-EpG formation (data not shown). Activity increased as a function of GTP concentration and reached saturation near 40 μM [GTP] (Fig. 2D). Half-saturation was achieved at 5 μM GTP. The yield of EpG was proportional to the magnesium concentration from 0.1 to 2 mM and declined at higher concentrations (Fig. 2E). Manganese was a more effective cofactor than magnesium at concentrations below 0.5 mM but was progressively less at higher concentrations. Calcium, copper, and zinc did not support the activity at 2 mM concentration (data not shown).

FIGURE 1. Purification and capping activities of TbCgm1. A, the 1050-amino acid TbCgm1 polypeptide is illustrated with the amino-terminal guanylyltransferase-(1–567) and the carboxyl-terminal methyltransferase-(717–1050) domains. Positions of nucleotidyltransferase motifs (I, III, IIIa, IV, V, and VI) and AdoMet binding motif are denoted by shaded and solid boxes, respectively. B, purification of TbCgm1. Aliquots of the bacterial soluble lysate (lane 1), the nickel-agarose eluate fraction (lane 2), and the second nickel-agarose fraction upon removal of His-Smt3 tag (lane 3) were analyzed by SDS-PAGE. A Coomassie Blue-stained gel is shown. The values to the left are molecular sizes in kDa. An asterisk shows a position of ~65-kDa bacteria contaminant copurified with TbCgm1 preparation. C, guanylyltransferase activity. Aliquot (1 μl) of the fractions indicated in B were assayed for enzyme-GMP complex formation in the presence of 10 nM [α-32P]GTP and 2 mM MgCl2. The reaction products were resolved by SDS-PAGE. An autoradiograph of the gel is shown. D, methyltransferase activity. Fifty nanograms of TbCgm1 (without His-Smt3 tag) was incubated with 67 fmol of cap-labeled poly(A) in a reaction mixture (10 μl) containing 20 μM AdoMet (lane 1), 2 mM AdoHcy (lanes 3), or 20 μM AdoMet plus 2 mM AdoHcy (lanes 4). Control reactions with no enzyme (lane 1) or 10 ng of yeast Abd1 plus 20 μM AdoMet (lane 5) are indicated. Products were digested with nuclease P1 and resolved on a TLC plate, developed with 0.45 M (NH4)2SO4. A phosphorimager scan of the chromatogram is shown. The chromatographic origin (ori), positions of m7GpppA and GpppA are noted. E, sedimentation analysis. Aliquots (500 μg) of TbCgm1 (without His-Smt3 tag) was mixed with marker proteins (catalase, bovine serum albumin, cytochrome c) and was applied to a 5–ml 15–30% glycerol gradient containing 50 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 5 mM DTT, and 0.05% Triton X-100. The gradient was centrifuged at 50,000 X g for 19 h at 4 °C in a Beckman SW50 rotor. Fractions (~0.2 ml) were collected from the bottom of the tube (fraction 1). Aliquots of the gradient fractions indicated were assayed for guanylyltransferase (closed circle) and cap methyltransferase (open circle) activities. The peaks of the marker proteins, catalase (11.3 S), bovine serum albumin (BSA) (4.3 S), and cyto c (1.9 S), are indicated.
Characterization of (Guanine-N-7)-methyltransferase Activity—To demonstrate that the carboxyl-terminal segment of TbCgm1 has an intrinsic cap methyltransferase activity, truncated proteins TbCgm1-(568–1050), TbCgm1-(630–1050), and TbCgm1-(717–1050) were expressed as His-tagged fusion proteins in E. coli. TbCgm1-(568–1050) and TbCgm1-(630–1050) were largely insoluble in bacteria (data not shown). Whereas 42-kDa TbCgm1-(717–1050) was soluble and this protein was purified from the lysate by one-step nickel-agarose affinity chromatography (Fig. 3 A). The 0.2 M imidazole fraction of TbCgm1-(717–1050) was incubated with 32P-labeled GpppA-terminated poly(A) RNA in the presence of AdoMet. Treatment of the reaction product with nuclease P1 resulted in the liberation of labeled species that co-migrated with m7GpppA (Fig. 3 B). As expected, TbCgm1-(717–1050) was incapable of forming a covalent adduct with [α-32P]GTP (data not shown). We conclude that cap methyltransferase activity resides on the carboxyl-terminal domain of TbCgm1.

In the linear range of enzyme dependence, the full-length TbCgm1 formed 11 fmol of methylated capped ends per nanogram of enzyme in 30 min (Fig. 3 C). This value corresponds to a turnover number of 0.043 min⁻¹, which is similar to the turnover number for TbCmt1 (0.047 min⁻¹) (31). In contrast, the specific activity of TbCgm1-(717–1050) was ~70-fold lower than the full-length TbCgm1. Thus, further characterization of methyltransferase activity was conducted using the full-length protein. Cap methylation depended on inclusion of AdoMet in the reaction mixture (Fig. 3 D). From a double-reciprocal plot of the data, we calculated a \( K_m \) of 75 nM AdoMet. AdoHcy product was a weak inhibitor for the TbCgm1 methyltransferase activity. Fifty percent inhibition was achieved at 0.5 mM AdoHcy in the presence of 20 \( \mu \)M AdoMet (Fig. 3 E). TbCgm1 has much higher affinity for AdoMet substrate than AdoHcy product. The methyltransferase activity was also inhibited in a concentration-dependent manner by sinefungin, which was a 1000-fold more potent inhibitor than AdoHcy; 50% inhibition was achieved at \( \sim 0.5 \) \( \mu \)M sinefungin in the presence of 20 \( \mu \)M AdoMet (Fig. 3 E). TbCgm1 had a bell-shaped pH profile with optimal activity at slightly acidic pH (pH 5.5–6.5) and the activity declined sharply at alkaline pH (data not shown). Previously we showed that TbCmt1 and yeast Abd1 were capable of methylating cap dinucleotide analogues (31). However, neither the full-length TbCgm1 nor TbCgm1-(717–1050) transferred the methyl group from 14C-AdoMet to P. pi.
Generation of Conditional RNAi Knockdowns of T. brucei Capping Enzymes—To assess the in vivo role of TbCgm1 in T. brucei and determine which enzyme is responsible for capping SL RNA, we silenced the expression of TbCGM1, TbCE1, and TbCMT1 by RNA interference. A 300–500-bp coding sequence from each gene was inserted between the two opposing tetracycline-regulated T7 promoters. The linearized plasmid was transfected into T. brucei procyclic strain 29.13, which harbors integrated genes encoding T7 RNA polymerase and tetracycline repressor protein. After phleomycin selection and cloning by limiting dilution, stably transfected cell lines were induced by tetracycline to synthesize double-stranded RNA encoded by the DNA insert (45). The guanylyltransferase assay is a highly sensitive assay in which the EpG complex can be detected directly from T. brucei whole cell extracts (30). In the uninduced cells, the 67-kDa TbCe1 and 116-kDa TbCgm1 react with [α-32P]GTP to form covalent enzyme-GMP complexes (Fig. 4A, top panel, lane –). Upon induction with tetracycline, TbCGM1 RNAi-induced cells showed significant reduction in levels of [32P]GMP-TbCgm1, while retaining the [32P]GMP-TbCe1 activity. After 3 days of induction, less than 1% of TbCgm1 activity was detected compared with the uninduced cells (Fig. 4A, bottom panel). The decreased growth rate observed upon tetracycline induction was specific to cells harboring the TbCGM1 RNAi construct, as tetracycline addition had no effect on growth of the parental strain (data not shown). In contrast, silencing of TbCE1 or

GpppA (Fig. 3F) or GpppG (data not shown), suggesting that an RNA chain is required for activity.
Tatambrui SL RNA Capping Enzyme

FIGURE 4. Generation of conditional RNAi knockdown of T. brucei capping enzymes. Top panel: A, down-regulation of TbCGM1 by RNAi. Guanylyltransferase assay of total protein from TbCGM1 RNAi uninduced (−) and induced with tetracycline for the number of days indicated. Reaction mixture containing 12.5 μg of total protein, 12.5 mM MgCl₂, and 4 μM [α-³²P]GTP were incubated at 30 °C for 30 min. The enzyme-GMP complex was resolved on a 10% SDS-PAGE and visualized by phosphorimager. Control reactions with recombinant TbCe1 and TbCgm1 (without His-Smt3 tag) are shown in the lanes marked rTbCe1 and rTbCgm1, respectively. Positions of TbCgm1 and TbCe1 are indicated on the right. B, down-regulation of TbCE1 by RNAi. Total protein from TbCE1 uninduced (−) or induced with tetracycline for the number of days indicated were assayed for guanylyltransferase activities as described in A. C, down-regulation of TbCMT1 by RNAi. Western blot analysis of total protein from TbCMT1 RNAi uninduced (−) or induced with tetracycline for the number of days indicated. Protein (25 μg) was separated by 10% SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with anti-TbCmt1 serum. Bottom panel, growth curve of T. brucei cells carrying the TbCGM1 (left), TbCE1 (middle), and TbCMT1 (right) RNAi constructs. The growth of uninduced cells (open circle) was compared with cells induced for double-stranded RNA production with tetracycline (closed circle). Growth curves were obtained by plotting the product of cell density (cell/ml) and total dilution factors.

TbCMT1 did not show any growth defect up to 8 days post-induction (Fig. 4, B and C, bottom panel). These results indicate that TbCmt1 is essential for parasite survival. The fact that neither TbCe1 nor TbCmt1 were able to rescue the growth defect of TbCGM1 knockdown suggests that TbCE1 and TbCMT1 activities do not function in the same pathway as TbCGM1 in vivo.

TbCGM1 Is Required for Cap 4 Methylation—To evaluate the consequence of TbCGM1 knockdown, we first examined the effect on SL RNA cap 4 formation. Previously we showed that a 48-kDa TbCom1 cap 2 methyltransferase, which modifies position 2 on the SL RNA, requires m7GpppN cap at the 5′-end (34). Thus, we predict that absence of m7GpppN, due to the silencing of capping enzyme components, would prevent hypermethylation of the SL RNA. Primer extension analysis were performed using total RNA extracted from RNAi cells and ³²P-labeled primers complementary to the SL RNA intron. As previously shown (46, 47), endogenous SL RNAs are predominantly hypermethylated at the first 4 nt, resulting in termination of primer extension at position +5 (Fig. 5, WT). Treatment with sinefungin inhibits cap methylations and results in longer primer extension product, which terminates at the +1 position (Fig. 5A, sin). RNA derived from TbCGM1 RNAi cells shows an increased level of hypomethylated SL RNA over a period of RNAi induction (Fig. 5). The amount of cap 4 SL RNA was reduced to ~60% at 4 days post-RNAi induction. No detectable changes in primer extension products were observed in TbCE1- and TbCMT1-RNAi-induced samples up to 4 days post-induction. These results demonstrate that silencing of TbCgm1 accumulates hypomethylated SL RNA, presumably due to a lack of m7GpppN.

TbCGM1 Knockdown Results in Accumulation of Uncapped SL RNA—To specifically assay for a capping defect, we used immobilized antibody against m2,2,7-G to detect capped and uncapped RNA produced in vivo. The anti-m2,2,7-G antibody recognizes both m2,2,7-GpppN and m7GpppN cap with high affinity, but not GpppN (48). Total RNA derived from RNAi-induced and uninduced cells were immunoprecipitated with anti-m2,2,7-G antibody linked to agarose beads, and the bound (capped) and unbound (uncapped) products were analyzed by primer extension. In the uninduced cells, as well as TbCE1 and TbCMT1 RNAi- induced cells, the majority of the SL RNA was hypermethylated, and was present in the bound fraction (Fig. 6 top panel, and data not shown). In contrast, the hypomethylated SL RNA accumulated in TbCGM1-RNAi-induced cells was found exclusively in the unbound fraction, implying that SL RNA lacks m7GpppN at the 5′ end. We also note that hypomethylated SL RNA present in sinefungin-treated cells did not bind to anti-m2,2,7-G antibody, presumably due to a lack of guanine N-7 methylation to form cap 0.
T. brucei SL RNA Capping Enzyme

**FIGURE 5.** Increase in the abundance of hypomethylated SL RNA upon TbCGM1 disruption in T. brucei. A, total RNA prepared from wild-type (WT), TbCGM1-, TbCE1-, and TbCMT1-RNAi cells after 2 and 4 days of tetracycline induction was subjected to primer extension analysis using radiolabeled oligonucleotides complementary to SL RNA. Extension products (SL hypo and hyper-methylated) were resolved on 20% denaturing PAGE and visualized by phosphorimager. Sinefungin-treated RNA (sin) was isolated 6 h after addition of 2 μg/ml sinefungin to the culture. B, quantification of the percent cap 4 formation, represented as the amount of SL hyper/SL hyper + SL hypo, over a period of 4 days post-induction. The data shown represent the average of two separate primer extension analysis.

**FIGURE 6.** Hypomethylated SL RNA accumulated by depletion of TbCGM1 lacks m7GpppN. Total RNA isolated from TbCGM1-, TbCE1-, and TbCMT1-RNAi cells after 4 days of tetracycline induction (+ Tet) were immunoprecipitated using anti-m2,2,7G-agarose beads (“Experimental Procedures”). Control reactions were performed using RNA isolated from uninduced TbCGM1-RNAi cells (− Tet) and sinefungin-treated cells. Aliquots of input (I), bound (B), and unbound (U) fractions were subjected to primer extension using 32P-labeled SL, U2, and 5S specific primers. The extension products were separated on 20% denaturing PAGE and visualized by phosphorimager. 5S RNA do not possess cap structure and serve as a negative control.

Level of Capped U2 snRNA Is Not Affected by Depletion of TbCgm1, TbCe1, or TbCmt1—Trypanosome U2 snRNA appears to be synthesized by pol III and possesses an m2,2,7G cap structure (27, 28). To address whether TbCgm1, TbCe1, or TbCmt1 depletion affects capping of snRNA, the level of capped and uncapped U2 snRNA was detected by immuno-precipitation using anti-m2,2,7G antibody, followed by primer extension (Fig. 6, middle panel). In uninduced cells, U2 snRNA was predominantly capped, as shown by retention in the bound fractions (Fig. 6 and data not shown for TbCE1 and TbCMT1). Whereas trace amounts of uncapped U2 snRNA were detected in TbCE1 and TbCGM1 RNAi-induced cells, the majority of U2 snRNA appears to be capped. We conclude that a single knockdown of TbCGM1, TbCE1, or TbCMT1 does not significantly reduce the level of capped U2 snRNA in vivo. We also note that sinefungin treatment, which likely inhibits m2,2,7G methylation, did not accumulate uncapped U2 snRNA.

Uncapped SL RNA Can Be Guanylated by Recombinant TbCgm1—If the silencing of TbCgm1 results in accumulation of uncapped SL RNA, the 5′ terminus should be available for guanylation by addition of an exogenous capping enzyme. To evaluate the direct effect of TbCgm1 depletion on SL RNA capping, total RNA extracted from uninduced and RNAi-induced cells were incubated with recombinant TbCgm1 in the presence of [α-32P]GTP and AdoMet. Samples were separated on a polyacrylamide gel and the amount of capping-competent RNA was detected by radioactivity transfer of [α32P]GMP. A strong signal that migrates at ~140-nt, corresponding to the size of SL RNA, was specifically detected in TbCGM1 RNAi cells after 2 and 4 days of tetracycline induction (Fig. 7A). We verified that the ~140-nt radiolabeled species is indeed SL RNA by purifying the radiolabeled RNA from the gel and subjecting it to RNase H digestion using DNA oligonucleotides complementary to either SL exon, exon-intron junction or intron regions (Fig. 7B). The sizes of RNA species generated by RNase H treatment agree with the distance between the 5′ end of the SL RNA and target SL oligos. Because T. brucei U2 snRNA (145 nt) and 5S RNA (120 nt) migrate at similar positions, we examined whether these RNAs were also capped by TbCgm1. Antisense U2 and 5S oligos did not hybridize to the ~140-nt RNA, as evidenced by lack of digestion product by RNase H. Moreover, the labeled SL RNA was resistant to alkaline phosphatase and protease K treatment, but was sensitive to nucleotide pyrophosphatase and RNase digestion, confirming that [α-32P]GMP is attached as a cap on the 5′ end of the SL RNA (data not shown). No substantial increase of the ~140-nt-labeled species was detected from TbCE1 and TbCMT1 RNAi-induced cells (Fig. 7A), indicating that the majority of the SL RNA was pre-guanylated in vivo. In addition to the SL RNA, TbCgm1 also capped RNA species that migrated above the 1.3-kb marker and series of short RNAs ranging between 65 and 100 nt. We did not pursue characterization of these RNAs because they were present in both uninduced and induced cells. Similarly, when recombinant mammalian capping enzyme (Mccl) was substituted for TbCgm1, we detected an increase in labeling of the ~140-nt species from RNA isolated from TbCGM1 RNAi-induced cells, but not from TbCE1 or TbCMT1 cells (data not shown). Taken together, we conclude that TbCgm1 is responsible for m7GpppN formation on the SL RNA.

**DISCUSSION**

Kinetoplastid protozoa have a number of distinctive features of gene expression including RNA editing, trans-splicing, poly-
We demonstrated that TbCgm1 is indeed a bifunctional capping enzyme. This was accomplished by purifying the recombinant TbCgm1 protein from bacteria and assaying for guanylyltransferase and methyltransferase activities. TbCgm1 is a monomer in solution and reacts with GTP to form a covalent enzyme-GMP intermediate, and transfers GMP to the uncapped SL RNA isolated from *T. brucei*. In respect to divergent cation requirements and pyrophosphate inhibition, the mechanism of enzyme-GMP formation by TbCgm1 appears to be similar to TbCegt1 and other cellular cap guanylyltransferases.

Further biochemical analysis of TbCgm1 and TbCe1 guanylyltransferase activity is necessary to evaluate whether two enzymes have different RNA substrate specificity.

TbCgm1 also catalyzes a transfer of the methyl group from AdoMet to the N-7 position of the GpppN cap to form cap 0. The guanylyltransferase and methyltransferase activities are functionally autonomous, as individual domains have respective activities. The size of the TbCgm1 methyltransferase domain (333 amino acids) is similar in size to TbCmt1, as well as to catalytic domains of human (Hcm1) and yeast (Abd1) guanine N-7 methyltransferases (31, 49, 50). However, unlike TbCmt1 and Abd1, cap analogues were not effective methyl acceptors for TbCgm1, suggesting that the RNA chain is required for methyltransferase activity (31). In addition, we note that AdoHcy was a weak inhibitor for TbCgm1 methyltransferase activity, compared with TbCmt1 or *E. cuniculi* cap methyltransferase (31, 51), requiring greater than 50-fold excess of AdoHcy over AdoMet to achieve 50% inhibition. This result is in agreement with previous findings that inclusion of 0.5 mM AdoHcy in permeabilized *T. brucei* did not completely inhibit guanine N-7 methylation on a newly synthesized SL RNA (47). In contrast, sinefungin was an effective inhibitor for TbCgm1 methyltransferase activity, 1000-fold more potent than AdoHcy. The stronger inhibition of TbCgm1 methyltransferase activity by sinefungin over AdoHcy is similar to that seen in Abd1 and vaccinia virus cap methyltransferase (52, 53).

Physical and functional organization of the capping activities have diverged during eukaryotic evolution (3). Fungi and other lower eukaryotes have segregated the triphosphatase, guanylyltransferase, and methyltransferase functions to distinct gene products. In higher eukaryotes, the triphosphatase is fused to the guanylyltransferase in the same polypeptide with a separate methyltransferase protein. TbCgm1 is unique among the cellular capping enzymes in having the amino-terminal guanylyltransferase and methyltransferase protein. TbCgm1 is unique among the cellular capping enzymes in having the amino-terminal guanylyltransferase activity fused to the carboxyl-terminal methyltransferase domain. The linear order of guanylyltransferase and methyltransferase domains in TbCgm1 resembles vaccinia capping enzyme, in which the triphosphatase, guanylyltransferase, and methyltransferase active sites are arranged in sequential order within the D1 polypeptide (54, 55). Fusion between guanylyltransferase and methyltransferase not only ensures that both activities are tethered to the 5’ end of the target RNA, but it may favor equilibrium toward the forward reaction. The unmethylated cap (GpppN), product of the guanylyltransferase reaction, can be deguanylated to the diphosphate terminus by a reversible reaction. However, once methylated to form m’GpppN, reaction is irreversible (41). We speculate that TbCgm1 acts together with TbCegt1 tripos-
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TbCgm1 may also function together with TbCe1 and TbCmt1, which can explain why the triphosphatase is not fused to TbCgm1. Alternatively, the amino-terminal domain of TbCe1, which has homology to adenylate kinase, may possess 5'-processing activity that can act as a triphosphatase (30).

We showed that TbCGM1 is essential for normal trypanosome growth and participates in SL RNA capping. Uncapped SL RNA accumulated after induction of RNAi against TbCGM1, which can be guanylated in vitro by addition of recombinant TbCgm1 protein. Our results confirm that cap 0 is a pre-requisite for subsequent methylations on the SL RNA to form cap 4. This conclusion is consistent with our recent finding that TbCom1 cap 2 methyltransferase requires m7GpppN for binding and catalysis (34). Vaccinia VP39 also requires m7GpppN for efficient 2'-O-methylation on the first transcribed nucleoside on the mRNA to form cap 1 (56–58). We also note that SL RNA was predominantly uncapped from sifneugenin-treated cells. The effect of sifneugenin, previously attributed to inhibition of cap 4 methyltransferases, is likely due in part to inhibition of guanine N-7 methylation to form cap 0, which in turn precludes cap 4 biosynthesis. Although we have not directly examined the consequence of TbCGM1 knockdowns on other mRNA processing events, it is conceivable the cap may play an essential role in trans-splicing, transport, and/or translation efficiency, as cap 4 methylation facilitates binding of T. brucei cap binding protein (59).

In contrast to TbCGM1, RNAi-mediated knockdowns of TbCE1 and TbCMT1 exhibited no effects on cell growth and SL RNA capping, suggesting that TbCe1/TbCmt1 may function in a different capping pathway from TbCgm1. The U2 snRNA was predominantly capped in TbCgm1, as well as TbCe1- and TbCmt1-depleted cells. Possible explanations are (i) TbCgm1 and TbCe1/TbCmt1 are functionally redundant in capping snRNAs; (ii) newly synthesized U2 snRNA (upon RNAi induction) cannot be detected by our immunoprecipitation experiment due to the long half-life of pre-existing capped U2 snRNA. The fact that U2 RNA were predominantly capped in TbCgm1, as well as TbCe1- and TbCmt1-depleted cells. Possible explanations are (i) TbCgm1 and TbCe1/TbCmt1 are functionally redundant in capping snRNAs; (ii) newly synthesized U2 snRNA (upon RNAi induction) cannot be detected by our immunoprecipitation experiment due to the long half-life of pre-existing capped U2 snRNA. The fact that U2 RNA were predominantly capped in TbCgm1, as well as TbCe1- and TbCmt1-depleted cells. Possible explanations are (i) TbCgm1 and TbCe1/TbCmt1 are functionally redundant in capping snRNAs; (ii) newly synthesized U2 snRNA (upon RNAi induction) cannot be detected by our immunoprecipitation experiment due to the long half-life of pre-existing capped U2 snRNA. The fact that U2 RNA were predominantly capped in TbCgm1, as well as TbCe1- and TbCmt1-depleted cells. Possible explanations are (i) TbCgm1 and TbCe1/TbCmt1 are functionally redundant in capping snRNAs; (ii) newly synthesized U2 snRNA (upon RNAi induction) cannot be detected by our immunoprecipitation experiment due to the long half-life of pre-existing capped U2 snRNA.

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