Characterization of a *Trypanosoma brucei* RNA cap (guanine N-7) methyltransferase

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ABSTRACT

The m⁷GpppN cap structure of eukaryotic mRNA is formed by the sequential action of RNA triphosphatase, guanylyltransferase, and (guanine N-7) methyltransferase. In trypanosomatid protozoa, the m⁷GpppN is further modified by seven methylation steps within the first four transcribed nucleosides to form the cap 4 structure. The RNA triphosphatase and guanylyltransferase components have been characterized in *Trypanosoma brucei*. Here we describe the identification and characterization of a *T. brucei* (guanine N-7) methyltransferase (TbCmt1). Sequence alignment of the 324-amino acid TbCmt1 with the corresponding enzymes from human (Hcm1), fungal (Abd1), and microsporidian (Ecm1) revealed the presence of conserved residues known to be essential for methyltransferase activity. Purified recombinant TbCmt1 catalyzes the transfer of a methyl group from S-adenosylmethionine to the N-7 position of the cap guanine in GpppN-terminated RNA to form the m⁷GpppN cap. TbCmt1 also methylates GpppG and GpppA but not GTP or dGTP. Mutational analysis of individual residues of TbCmt1 that were predicted—on the basis of the crystal structure of Ecm1—to be located at or near the active site identified six conserved residues in the putative AdoMet- or cap-binding pocket that caused significant reductions in TbCmt1 methyltransferase activity. We also report the identification of a second *T. brucei* RNA (guanine N-7) cap methyltransferase (named TbCgm1). The 1050-amino acid TbCgm1 consists of a C-terminal (guanine N-7) methyltransferase domain, which is homologous with TbCmt1, and an N-terminal guanylyltransferase domain, which contains signature motifs found in the nucleotidyl transferase superfamily.

Keywords: mRNA capping; m⁷G methyltransferase; *Trypanosoma brucei*

INTRODUCTION

The earliest event in the modification of the 5' terminus of eukaryotic mRNA is the formation of the m⁷GpppN cap structure, which is required for mRNA stability and efficient translation. Capping entails three enzymatic reactions in which the 5'-triphosphate terminus of the nascent RNA is cleaved to a diphosphate by RNA triphosphatase, capped with GMP by RNA guanylyltransferase, and methylated at the N-7 position of the cap guanosine by RNA (guanine N-7) methyltransferase (hereafter referred to as m⁷G methyltransferase) (Shuman 2000). Consistent with the fundamental role of the cap in eukaryotic gene expression, all three activities are essential for viability in budding yeast (Schwer and Shuman 1994; Schwer et al. 2001). Studies in yeast and mammals suggest that the specificity of capping pol II transcripts is achieved by the direct interaction of

capping enzyme components with the elongating form of pol II (Cho et al. 1997; McCracken et al. 1997; Yue et al. 1997; Pillutla et al. 1998; Ho and Shuman 1999).

In kinetoplastid protozoa, the mechanism by which the mRNA acquires a cap is different from other eukaryotes. The cap is formed on the spliced leader transcript (SL RNA), then transferred to the 5' end upstream of the start site of individual precursor mRNAs by *trans*-splicing to form mature monocistronic mRNAs (Agabian 1990; Adams et al. 1996; Liang et al. 2003). The SL RNA cap is unique among eukaryotes in that the first four nucleotides following the cap are hypermethylated and is referred to as cap 4. Cap 4 consists of a standard m⁷GpppN with 2'-O methylations on the first four ribose sugars (A_mA_mC_mU_m), and additional base methylations on the first adenine (m^{6,6}A) and the fourth uracil (m³U) (Bangs et al. 1992; Mair et al. 2000). Analysis of *Trypanosoma brucei* SL RNA biosynthesis using cap methyltransferase inhibitors suggests that the cap is formed co-transcriptionally and that the methylation steps that lead to cap 4 are essential for the *trans*-splicing reaction (Ullu and Tschudi 1991; McNally and Agabian 1992; Mair et al. 2000). It has been suggested that cap methyltransferases are promising drug targets

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against kinetoplastid parasites, because the mechanism of cap formation and the structure of the cap are fundamentally different from those of the host (Tschudi and Ullu 2002). However, only the RNA triphosphatase and the RNA guanylyltransferase have been characterized biochemically (Silva et al. 1998; Ho and Shuman 2001).

T. brucei RNA triphosphatase (TbCet1) is a 253-amino acid monomeric protein that catalyzes the removal of the γ phosphate from triphosphate-terminated RNA in the presence of divalent cations (Ho and Shuman 2001). TbCet1 is mechanistically similar to the RNA triphosphatases found in fungi, microsporidia, and other protozoa but is unrelated to the cysteine phosphatase-type RNA triphosphatases found in metazoans and plants. The *T. brucei* guanylyltransferase (TbCe1) catalyzes the transfer of GMP from GTP to the 5'-diphosphate terminus of the RNA to form GpppN (Silva et al. 1998). The primary structure and mechanism of TbCe1 resemble those of RNA guanylyltransferases from other eukaryotes, except that TbCe1 contains an N-terminal extension of 250 amino acids of unknown function that is dispensable for guanylyltransferase activity. Neither TbCet1 nor TbCe1 appear to possess any cap methyltransferase activity.

For synthesis of the m⁷GpppN cap, kinetoplastids must encode a separate m⁷G methyltransferase. We searched the *T. brucei* proteome for a polypeptide resembling human and yeast m⁷G methyltransferases, and identified two candidate m⁷G methyltransferases: a 324-amino acid polypeptide, which we refer to as TbCmt1 (*T. brucei* m⁷G cap methyltransferase 1), and a 1050-amino acid polypeptide, which we refer to as TbCgm1 (*T. brucei* cap guanylyltransferase-methyltransferase 1). Here we show that recombinant TbCmt1 has intrinsic m⁷G methyltransferase activity; it catalyzes the transfer of a methyl group from S-adenosylmethionine (AdoMet) to a cap-terminated RNA (GpppRNA) to form methylated capped RNA (m⁷GpppRNA) and S-adenosylhomocysteine (AdoHcy). Mechanistic conservation among the m⁷G methyltransferases is suggested by structure-based mutational analysis of the putative AdoMet- and cap-binding pockets in TbCmt1, with reference to the crystal structures of the microsporidian parasite *Encephalitozoon cuniculi* m⁷G methyltransferase (Fabrega et al. 2004; Hausmann et al. 2005).

RESULTS

Identification of a *T. brucei* m⁷G methyltransferase

We searched for candidate *T. brucei* cap methyltransferase enzymes by querying the Sanger Database for proteins related to known RNA (guanine N-7) cap methyltransferases. We identified an open reading frame on *T. brucei* chromosome 10 with extensive homology with the catalytic domain of human (Hcm1, blast score 199) and *Saccharomyces cerevisiae* (Abd1, blast score 152) m⁷G methyltrans-

ferases. The alignment of the primary structures of TbCmt1, Abd1, Hcm1, and *E. cuniculi* m⁷G methyltransferases (Ecm1) is shown in Figure 1. TbCmt1 displays conservation at 76 of 324 positions among these enzymes and contains a well-characterized AdoMet binding motif (70-VLDLASGRGGD LGK-83). Additionally, residues that contact AdoMet and the cap, as observed in the co-crystal structures of Ecm1 complexed with AdoMet, AdoHcy, and the m⁷GpppA cap analog are present in TbCmt1 (Fabrega et al. 2004). All 12 residues that were found to be critical for m⁷G methyltransferase activity in Ecm1 are present in TbCmt1 (indicated by "0" in Fig. 1), leading to the prediction that this gene product encodes a m⁷G cap methyltransferase component of the *T. brucei* capping apparatus (Mao et al. 1995, 1996; Wang and Shuman 1997; Fabrega et al. 2004; Hausmann et al. 2005).

FIGURE 1. Sequence similarity among the protozoan, the fungal, and the mammalian RNA m⁷G methyltransferases. The amino acid sequences of m⁷G methyltransferases encoded by *T. brucei* (TbCmt1: Sanger Institute TV10.v4.004), *T. cruzi* (TcCMT1: XP_811819.1), and *L. major* (LmCmt1: CAJ08963.1) are aligned to the sequences of *H. sapiens* (Hcm1), *S. cerevisiae* (Abd1), and *E. cuniculi* (Ecm1) m⁷G methyltransferases. Gaps in the alignment are indicated by dashes. Side-chain identity in all proteins is highlighted by shaded boxes. Ecm1 residues that interact with AdoMet and the cap analog are indicated below as "a" and "c," respectively. Conserved residues in Ecm1 that were found by mutational analysis to be essential for function are indicated by "0." TbCmt1 residues that were mutated to alanine in this study are denoted by arrows.

To determine if other kinetoplastid protozoa encode m⁷G methyltransferases, we performed a reciprocal search using the TbCmt1 sequence and found TbCmt1 homologs in *Trypanosoma cruzi* (TcCmt1: 66% identity and 80% similarity) and *Leishmania major* (LmCmt1: 45% identity and 58% similarity) from the National Center for Biotechnology Information database (Fig. 1). The *T. cruzi* (313 amino acids) and *L. major* (323 amino acids) proteins are similar in size to the *T. brucei* protein. The kinetoplastid proteins are slightly larger than the “minimal” cap methyltransferase Ecm1 (298 amino acids) but are smaller than Abd1 (549 amino acids) and Hcm1 (520 amino acids) (Pillutla et al. 1998; Saha et al. 1999; Hausmann et al. 2002). Note that kinetoplastid m⁷G methyltransferases lack a counterpart to the N-terminal ~150–amino acid segment present in the budding yeast and human enzymes. These N-terminal segments were dispensable for their methyltransferase activities in vitro and in vivo (Mao et al. 1996; Saha et al. 1999).

Expression of TbCmt1 in bacteria

To evaluate the m⁷G methyltransferase activity, the recombinant TbCmt1 protein was produced in bacteria. The *TbCMT1* coding sequence was amplified from *T. brucei* genomic DNA and inserted into an inducible T7 RNA polymerase-based pET vector such that 10 tandem histidines (His-tag) were fused N-terminal to the TbCmt1 protein. The pET-*TbCMT1* expression plasmid was introduced into *Escherichia coli* BL21(DE3)Gold, a strain that contains the T7 RNA polymerase gene under the control of a lacUV5 promoter. A 39-kDa protein corresponding to His-tagged TbCmt1 was detectable by SDS-PAGE in soluble extracts of IPTG-induced bacteria (Fig. 2A, lane L). The TbCmt1 protein was purified from soluble extract by nickel-agarose, followed by phosphocellulose chromatography. The recombinant protein adsorbed to the phosphocellulose and was recovered in a 0.2 M NaCl fraction following step elution (Fig. 2B). The phosphocellulose preparation was virtually homogeneous with respect to the 39-kDa protein.

Characterization of TbCmt1 activity

RNA m⁷G cap methyltransferases catalyze the transfer of a methyl group from AdoMet to GpppRNA to form m⁷GpppRNA and AdoHcy. To determine if TbCmt1 is capable of catalyzing this reaction, purified TbCmt1 was incubated with ³²P-GpppRNA₅₃ (bold type indicates the labeled phosphorus) in the presence and the absence of AdoMet. The substrate for this assay, a 53-mer ³²P-GpppRNA₅₃ was prepared from a triphosphate-terminated runoff transcript synthesized in vitro by T7 RNA polymerase, then cap-labeled with [α-³²P]GTP using the mammalian capping enzyme (see Materials and Methods). The

FIGURE 2. Expression and purification of TbCmt1. The protein compositions of the recombinant TbCmt1 protein at sequential stages of purification were analyzed by SDS-PAGE. (A) Ni-NTA Agarose fractions: Soluble lysate of IPTG-induced BL21(DE3)Gold/pET-*TbCMT1* (lane L), Ni-NTA agarose flowthrough (FT), and indicated imidazole eluates. (B) Phosphocellulose fractions: Aliquots of the 0.2-M imidazole eluate from Ni-Agarose chromatography (Ni), phosphocellulose flowthrough (FT), and indicated NaCl eluates. The gels were fixed and stained with Coomassie blue dye. The positions and sizes (in kilodaltons) of the coelectrophoresed marker proteins are shown to the left. The position of TbCmt1 is indicated by an arrow to the right.

reaction products were digested to cap dinucleotides with nuclease P1 and analyzed by polyethyleneimine-cellulose (TLC) plate (Fig. 3). In the absence of nuclease P1 digestion, all ³²P-labeled material remained at the origin in polynucleotide form (Fig. 3, lane 1). Treatment of the reaction products with nuclease P1 resulted in liberation of a labeled species that co-migrated with m⁷GpppG, generated in a parallel reaction mixture containing purified yeast Abd1 (Fig. 3, lanes 3,5). A trace amount (~10%) of methylation was observed in the absence of AdoMet (Fig. 3, lane 4), suggesting that some AdoMet may have been bound to the enzyme and co-purified during the TbCmt1 preparation. One of the products, AdoHcy (500 μM), was inhibitory in a reaction containing 50 μM AdoMet (Fig. 3, lane 6). We further verified that methylation occurs at the terminal guanosine nucleoside by digesting the reaction products with nucleotide pyrophosphatase (NPPase), a nuclease that cleaves between the γ and the β phosphates in the capped structure. Both the TbCmt1 and Abd1 reaction products liberated m⁷Gp, which migrated near the top of the TLC (Fig. 3, lanes 7–9). These results indicate that TbCmt1 catalyzes methylation at the N-7 position of the terminal cap guanosine.

The amount of m⁷GpppG formed during a 30-min reaction in the presence of 50 μM AdoMet was proportional to

FIGURE 3. Analysis of the methyltransferase reaction product. Methyltransferase reaction mixture (10 μL) contained 100 fmol of ³²P-GpppRNA₅₃ with the following components: 2 pmol Abd1 + 50 μM AdoMet (lanes 3,8), 2 pmol TbCmt1 (lane 4), 2 pmol TbCmt1 + 50 μM AdoMet (lanes 5,9), 2 pmol TbCmt1 + 50 μM AdoMet and 500 μM AdoHcy (lane 6). Control reactions in the absence of enzyme are indicated (lanes 1,2,7). After incubation at 27°C for 30 min, samples were incubated with either 140 ng of nuclease P1 (lanes 2–6) or 0.1 unit of nucleotide pyrophosphatase (NPPase) (lanes 7–9) for 60 min at 37°C. The digests were then spotted on polyethyleneimine-cellulose TLC plates and developed with 0.45 M (NH₄)₂SO₄. A PhosphorImager scan of the chromatogram is shown. The chromatographic origin (ori) and positions of m⁷Gp, m⁷GpppG, GMP, and GpppG are noted.

the amount of added TbCmt1 (Fig. 4A). At saturating enzyme, input ³²P-GpppRNA₅₃ substrate was quantitatively methylated to form m⁷GpppRNA. Kinetic analysis of methyl transfer indicated that the reaction was completed within 120 min at 27°C. The yield of methylated product varied with AdoMet concentration. Half-maximal activity was observed at ~1 μM AdoMet and plateau at 10 μM (data not shown). TbCmt1 formed ~70 fmol of methylated capped ends per 50 fmol of enzyme in 30 min. This value corresponds to a turnover number of 0.047 min⁻¹, which is similar to the turnover number for recombinant Hcm1 (0.037 min⁻¹) (Saha et al. 1999). The activity was measured at temperatures ranging from 22° to 65°C. The extent of m⁷GpppG formation was optimal at 27°C, whereas activity was ~50% of the optimum value at either 22° or 37°C, and the enzyme was completely inactive at 65°C. Methylation was optimal in 50 mM Tris-HCl buffer between pH 8.0 and 8.5 and declined sharply at and below neutral pH (Fig. 4D). The standard methyltransferase reaction mixture contained 10 mM NaCl from the enzyme preparation. The effect of increasing ionic strength on methyltransferase activity was measured by supplementing the reactions with NaCl. Activity in the presence of 60 mM NaCl was reduced to ~25% of that of 10 mM NaCl and was virtually null at 125 mM NaCl (data not shown).

Structure-based mutagenesis of TbCmt1

Crystal structures of Ecm1 have been solved complexed with AdoMet, AdoHcy, m⁷GpppG, and more recently with aza-AdoMet (Fabrega et al. 2004; Hausmann et al. 2005). The active site of Ecm1 is composed of two separate ligand-binding pockets: one for AdoMet and one for the GpppN cap. Fabrega and colleagues (Fabrega et al. 2004) suggest that cap guanine N-7 methylation is accomplished predominantly by optimally orienting the AdoMet and GpppN cap in close proximity, facilitating the methyl transfer reaction. Alanine mutations of the residues that contact the AdoMet ribose (Ecm1: Asp94), methionine (Ecm1: Lys54, Asp78), or adenine (Ecm1: Ile95, Asp122, Ser123, Tyr124), as well as cap ribose (Ecm1: Tyr284, Asn50) and cap guanine (Ecm1: Phe141, Tyr284) determined that they were all critical for Ecm1 m⁷G methyltransferase function.

FIGURE 4. Cap methylation activity of recombinant TbCmt1. (A) Protein titration. Methyltransferase reaction mixtures (10 μL) containing 100 fmol of ³²P-GpppRNA₅₃, 50 μM AdoMet, and TbCmt1 as specified were incubated for 30 min at 27°C. The extent of m⁷GpppG formed was plotted as a function of input protein. (B) Kinetics. Reaction mixtures (10 μL) contained 100 fmol of ³²P-GpppRNA₅₃, 50 μM AdoMet, and 2 pmol TbCmt1. The reaction was initiated by enzyme addition and was incubated at the indicated temperature. Aliquots (2 μL) were withdrawn at the times indicated, and the reaction was quenched immediately by addition of sodium acetate to pH 5.5 and heating for 5 min at 80°C. Products were then digested with nuclease P1. The extent of m⁷GpppG formed is plotted as a function of incubation time. (C) Inhibition by AdoHcy. Reaction mixtures (10 μL) contained 100 fmol of ³²P-GpppRNA₅₃, 50 μM AdoMet, 2 pmol TbCmt1, and AdoHcy as indicated. The yield of m⁷GpppG is plotted as a function of AdoHcy concentration. (D) pH dependence. Reaction mixtures (10 μL) containing 50 mM Tris buffer (either Tris-acetate at pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.8, and 7.0 or Tris-HCl at pH 7.5, 8.0, 8.5, and 9.0), 5 mM dithiothreitol, 100 fmol of ³²P-GpppRNA₅₃, 50 μM AdoMet, and 2 pmol TbCmt1 were incubated for 30 min at 27°C. The extent of m⁷GpppG formed is plotted as a function of pH.

The majority of these residues are present in TbCmt1 and are conserved among other m⁷G methyltransferases (Fig. 1).

If TbCmt1 is a true member of the m⁷G methyltransferase family, then removal of side chains that make contacts to the substrates should elicit a significant loss in activity. To illuminate the structure–function relationship for TbCmt1, 10 residues in TbCmt1 were selected for alanine-mutagenesis. These residues were selected based on the active site structure of Ecm1 and their conservation in m⁷G methyltransferase family members (denoted by arrows in Fig. 1). These include residues in TbCmt1 that may comprise the putative AdoMet-binding pocket (Asp80, Lys83, Asp144) and cap-binding pocket (Phe171, His174, Tyr175, Tyr246, Glu258). Alanine substitutions were also introduced at nonconservative residues (Val241, Val242). The D80A, K83A, D144A, F171A, H174A, Y175A, V241A, V242A, Y246A, and E258A mutations were introduced into the *TbCMT1* gene, and the TbCmt1-Ala proteins were expressed as His₁₀-tagged derivatives in *E. coli* in parallel with wild-type TbCmt1. In every case, the 39-kDa TbCmt1 protein was the predominant polypeptide. The recombinant mutant TbCmt1 proteins were purified from soluble bacterial extracts by nickel-agarose and phosphocellulose chromatography as described for wild-type TbCmt1. SDS-PAGE analysis of the polypeptide compositions of the phosphocellulose protein preparations revealed that a similar extent of purification was achieved for each mutant as for the wild-type protein (Fig. 5A).

Effect of alanine mutations on TbCmt1 m⁷G methyltransferase activity

Cap methyltransferase activity of the wild-type and mutant TbCmt1 proteins was assayed in the presence of 50 μM AdoMet and 10 nM cap-terminated RNA. The specific

activities of the TbCmt1-Ala mutants were quantified from protein titrations in the linear range of enzyme dependency (Fig. 5B). The D80A, K83A, and E258A mutants were unable to support methylation at levels of input protein sufficient to convert all the input substrate to the methylated form by wild-type TbCmt1. We calculated that the specific activities of D80A, K83A, and E258A mutants were <0.1% of the wild-type activity. Severe mutational effects were also observed for D144A (5% of wild-type activity), Y175A (3% of wild-type activity), and Y246A (2% of wild-type activity). In contrast, the F171, H174A, V241A, and V242A mutants displayed near-wild-type activities and thus were not essential for the activity of TbCmt1. With the exception of the F171A mutant, these results are in agreement with the mutational analyses reported for Ecm1 and Abd1 (Mao et al. 1996; Wang and Shuman 1997; Fabrega et al. 2004; Hausmann et al. 2005). It is thus likely that TbCmt1 adopts a similar active site as that of Ecm1.

Methyl acceptor specificity

To determine the minimum substrate for TbCmt1 methyltransferase activity, we tested whether nucleotides and cap analogs can serve as methyl acceptors for TbCmt1. Recombinant TbCmt1 was incubated with GpppA and GpppG, and various nucleotides in the presence of [¹⁴C-CH₃]AdoMet, and the individual products were resolved by TLC. The positions of unlabeled standards were identified by UV shadowing. Incubation of increasing amounts of GpppA with 50 μM [¹⁴C-CH₃]AdoMet and TbCmt1 resulted in the formation of [¹⁴C-CH₃]-labeled product that co-migrated with the m⁷GpppA standard (Fig. 6A). Approximately 90% of input label was converted to the m⁷GpppA product in the presence of 4 mM GpppA. TbCmt1 also catalyzed near-quantitative

FIGURE 5. Effect of alanine mutations on m⁷G methyltransferase activity of TbCmt1. (A) Protein purification. Aliquots (3 μg) of phosphocellulose fractions of wild-type (WT) TbCmt1 and the indicated Ala-mutants were analyzed by SDS-PAGE. Proteins were visualized by staining with Coomassie blue. The positions and sizes (in kilodaltons) of marker proteins are indicated to the left. (B) Methylation of capped RNA. Reaction mixtures (10 μL) contained 100 fmol of ³²P-GpppRNA₅₃, 50 μM AdoMet, and wild-type or mutant enzymes as specified. The extent of m⁷GpppG formed is plotted as a function of input enzyme.

FIGURE 6. Methylation of cap analog by TbCmt1. (A) GpppA titration. Reaction mixtures (10 μ L) containing 50 mM Tris-HCl (pH 8.0), 5 mM dithiothreitol, 50 μ M [¹⁴C-CH₃]AdoMet, 8 pmol TbCmt1, and increasing concentration of GpppA (0.004, 0.04, 0.4, and 4 mM, proceeding from left to right in the titration series) were incubated for 30 min at 27°C. A control reaction lacking GpppA is indicated by (-). An aliquot (2 μ L) of the mixture was applied to a PEI-cellulose TLC plate, which was developed with 0.4 M LiCl and visualized by PhosphorImager. (B) Methyl-acceptor specificity. Reaction mixtures (10 μ L) containing 50 μ M [¹⁴C-CH₃]AdoMet, 8 pmol TbCmt1, and either 4 mM GTP, GpppG, GpppA or no methyl-acceptor as indicated by (-). Reaction products were analyzed by TLC and visualized by PhosphorImager. The positions of GTP, GpppA, and GpppG were located by UV illumination as indicated. (C) Mutational analysis. Reaction mixtures (10 μ L) contained 50 μ M [¹⁴C-CH₃]AdoMet, 4 mM GpppA and 8 pmol of wild-type or mutant TbCmt1. Reaction products were analyzed by PEI-cellulose TLC. The extent of [¹⁴C-CH₃] m⁷GpppA formation, quantitated by PhosphorImager, is plotted for each enzyme.

methyltransfer to a GpppG acceptor to form m⁷GpppG (Fig. 6B). In contrast, GTP was a poor methyl acceptor even at the highest concentration sufficient to methylate cap analogs (4 mM). Neither UTP, CTP, ATP, nor dGTP was detectably methylated under these conditions (data not shown). The TbCmt1 reaction was identical in all respects to methylation of GpppA and GpppG cap analogs by the Abd1 enzyme as analyzed in parallel reactions (data not shown). These results suggest that TbCmt1 has a higher affinity to the cap dinucleotides than GTP, and that the RNA component is not strictly essential for catalysis.

The effects of mutations on TbCmt1's ability to transfer a methyl group from AdoMet to the GpppA acceptor were measured under the reaction conditions that convert 90% of input AdoMet to m⁷GpppA by wild-type TbCmt1 (Fig. 6C). All six mutations that were essential for methylation of capped RNA also elicited severe defects in m⁷GpppA formation. The activities of the D80A, Y175A, and E258A mutants were <1% of the wild type, whereas the Y246A mutant formed trace amounts of methylated product (4% of wild-type level). The K83A and D144A mutations had a less drastic effect on GpppA methylation than on methylation of cap-terminated RNA (~20% that of the wild-type value). The F171A, H174A, V241A, and V242A mutants had modest (within twofold) or no effect on m⁷GpppA formation. These results further support the conclusion that these essential residues comprise the TbCmt1 active site.

DISCUSSION

Kinetoplastid protozoan mRNAs acquire their 5' caps via *trans*-splicing of an SL RNA sequence containing the hypermethylated cap 4 structure (Bangs et al. 1992; Mair et al.

2000). While the RNA triphosphatase (TbCet1) and guanylyltransferase (TbCe1) have been characterized (Silva et al. 1998; Ho and Shuman 2001), none of the cap-methylating enzymes have been identified in kinetoplastid protozoa. Here we report the first identification of a cap RNA methyltransferase in *T. brucei*. Recombinant TbCmt1 transfers a methyl group from AdoMet to the N-7 position of the GpppN cap to form the conventional m⁷GpppN structure present in all eukaryotes. The catalytic properties of the trypanosome enzyme are similar to other cellular m⁷G methyltransferases with respect to AdoMet-dependency and product inhibition by AdoHcy. TbCmt1 also methylates cap analogs. However, GTP was not an effective methyl acceptor for TbCmt1. Thus, the trypanosome enzyme is capable of discriminating cap analogs from GTP, ensuring that methylation takes place only on capped substrates.

We can make reasonable predictions about the roles of TbCmt1 residues by comparison with Ecm1 (Fabrega et al. 2004; Hausmann et al. 2005). Based on structural homology, TbCmt1 Asp80, Lys83, and Asp144 likely coordinate AdoMet in the active site, as alanine substitutions of any of these residues either abolished or diminished the enzyme's ability to methylate GpppA or cap-terminated RNA. TbCmt1 Lys83 may interact with Asp80, which in turn may make a water-mediated contact to the AdoMet amine as in Ecm1 (Fabrega et al. 2004; Hausmann et al. 2005). Alanine substitution of the corresponding Asp and Lys in Ecm1 failed to support growth in an *ABD1*-deficient yeast strain. TbCmt1 Asp144 is the putative equivalent of Asp122 in Ecm1 and is thought to form a hydrogen bond to the adenine N6 atom of AdoMet. Ecm1 Asp122 is not strictly essential for catalysis but elicits a temperature sensitive growth defect in yeast (Fabrega et al. 2004). Based on

these results, we predict that Asp80, Lys83, and Asp144 participate in forming the AdoMet binding pocket in TbCmt1.

Of the five TbCmt1 mutations introduced in the putative cap guanine binding pocket, replacement of Tyr175, Tyr246, and Glu258 by alanine eliminated methyltransferase activity. With reference to the Ecm1 crystal structure complexed with AdoMet or m⁷GpppG, Tyr175 (equivalent of Ecm1 Tyr145) may bridge the two substrates in the active site by making a water-mediated contact with the cap guanine O6 atom as well as van der Waals contacts with AdoMet (Fabrega et al. 2004). Hausmann et al. (2005) showed that Ecm1 Tyr145 can be replaced either by phenylalanine or by leucine, suggesting that the dominant role of tyrosine at this position is to provide aliphatic contacts with AdoMet. Consistent with our finding that Tyr175 is essential for the TbCmt1 activity, the corresponding tyrosines in fungal (Abd1: Tyr254), metazoan (Hcm1: Tyr289), and viral (vaccinia: Tyr683) m⁷G methyltransferases were indispensable for their activities (Mao and Shuman 1996; Mao et al. 1996; Saha et al. 1999, 2003). TbCmt1 Glu258 (Ecm1: Glu225) may form hydrogen bonds with the N1 atom and may also contact the exocyclic 2-NH₂ of the guanine. The hydroxyl of Tyr246 (Ecm1: Tyr212) may assist this interaction by forming a hydrogen bond to the Glu225 carboxylate. Ecm1 Y212A and E225A mutants both elicit temperature-sensitive phenotypes in yeast strains lacking the *ABD1* gene (Fabrega et al. 2004), consistent with the requirement for each residue in TbCmt1. Alanine substitution at the conserved His174 did not affect TbCmt1 activity. While the Ecm1 structure suggests that the corresponding His144 acts as a hydrogen donor to the guanine O6 and, thereby, provides additional guanine specificity, this residue does not appear to have a significant effect on catalysis. In support of this view, Ecm1 His144 was dispensable for methyltransferase activity (Hausmann et al. 2005). However, Phe171 was dispensable for TbCmt1 activity, whereas the equivalent Phe141 of Ecm1 is absolutely essential for methyltransferase activity in vitro and the F141A mutant failed to complement the *ABD1*-deficient yeast strain (Hausmann et al. 2005). In the Ecm1-cap structure, Phe141 makes multiple van der Waals contacts with the cap guanine and ribose (Fabrega et al. 2004). It is conceivable that an alternate phenylalanine or other aliphatic residues in TbCmt1 may contribute to this essential interaction. Further mutagenesis studies should clarify this apparent discrepancy.

We explored a yeast genetic complementation approach to address whether the trypanosome m⁷G methyltransferase can function in yeast cells. We transformed a plasmid containing *TbCMT1* under a constitutive yeast *TPI* promoter into an *S. cerevisiae* strain with a deletion of the chromosomal copy of *ABD1* (Mao et al. 1995). We found that expression of *TbCMT1* on either *CEN* or 2- μ plasmid did not sustain growth in the *Abd1* deletion strain (data not shown). A plausible explanation for the failure of *TbCMT1* to function in yeast could be that the trypanosome protein

fails to interact with the yeast RNA polymerase II. In fungi, plants, and metazoans, capping enzyme components interact with the carboxyl-terminated domain (CTD) of the large subunit of RNA polymerase II, which is composed of YSPTSPS heptapeptide repeats. The phosphorylated CTD acts as a landing pad and targets the capping apparatus to the transcription elongation complex (Cho et al. 1997; McCracken et al. 1997; Ho and Shuman 1999; Fabrega et al. 2003). It is not clear how the trypanosome capping apparatus is recruited to sites of transcription as the large subunit of *T. brucei* RNA polymerase II lacks the conventional CTD element, although it does appear to be phosphorylated (Evers et al. 1989; Smith et al. 1989; Chapman and Agabian 1994).

A further search of the NCBI database using the TbCmt1 sequence uncovered a second m⁷G methyltransferase ORF encoded in all three kinetoplastid protozoa (Fig. 7). The 1050-amino acid *T. brucei* protein (designated TbCgm1) is significantly larger than TbCmt1, and homology with the m⁷G methyltransferases is restricted to the C-terminal 350-amino acid segment (Fig. 7A). The most significant difference between TbCmt1 and TbCgm1 is that TbCgm1 contains motifs characteristic of RNA guanylyltransferases (Fig. 7B). The N-terminal 650-amino acid segment of TbCgm1 has all six conserved nucleotidyl transferase motifs (I, III, IIIa, IV, V, and VI) involved in GTP-binding and catalysis in the standard order and spacing, except that the 139-amino acid interval between motifs I and III is slightly longer than that of known RNA guanylyltransferases (Hakansson et al. 1997; Wang et al. 1997). Motif I (127-KADGTR-132) contains a conserved lysine residue to which GMP becomes covalently linked via a phosphoamide bond to the ϵ -amino group of the enzyme (Schwer and Shuman 1994; Wang et al. 1997). All of the amino acid residues that are essential for the function of the *S. cerevisiae* RNA guanylyltransferase Ceg1 are conserved in TbCgm1, as are the residues that make direct contacts with the GTP substrate in the *Chlorella* virus and *Candida albicans* RNA guanylyltransferases (Schwer and Shuman 1994; Hakansson et al. 1997; Wang et al. 1997; Hakansson and Wigley 1998; Fabrega et al. 2003). These similarities strongly suggest that TbCgm1 possesses RNA guanylyltransferase activity in addition to the putative m⁷G methyltransferase activity.

It is noteworthy that two distinct RNA guanylyltransferase polypeptides have been detected in *T. brucei* and the related parasite *Crithidia fasciculata*. Silva et al. (1998) showed that 68-kDa and ~116-kDa polypeptides reacted with GTP to form covalent GMP catalytic intermediates in *T. brucei* whole-cell extract, as gauged by SDS-PAGE analysis. While the 68-kDa polypeptide was purified, cloned, and characterized as TbCe1, the identity of the 116-kDa polypeptide remained in question. Based on the calculated molecular weight of the TbCgm1 polypeptide, we predict that the 115-kDa protein-GMP complex corresponds to the *TbCGM1* gene product.

A.

TbCgm1	697	-----VDSASPESTETPERVPLHLQGVPAVLQVEQVNA--HYACKTELSTGKNRSILRHNNWIKGVLISTSVSYLR	
TcCgm1	689	GRERVLDVMPVVNTQKPN-----GMIGEPHMLVEKINS--HYACKTRELLSGKNRSVLRKFNWVKNVLSNAVSAYK	
LmCgm1	680	-----TAPETATLERK--RDAPLVVSRPHSSEATQQTSTRHYSTVAKELAN--ERSDLRRFNNWVKSVLTTMAAAIR	a a
TbCgm1	771	SNNKGEFDNDGMVVDLCSCRGGLDHWRAHQPKLLFMTDCCLEAVAEAAARYSITKGLSIVKVPDHKNPPGIRAQFCV	
TcCgm1	761	SRSBK--DSECGELAVADVCCGRGGDLFKWRAHNPRLFMVDSCLAEVAAAGRYSVSKGLSLRIVPQDKSSVGVIAFYTV	
LmCgm1	750	RALKP-----PAKLHVLDCCGRGGLLKQWQHHPAPLFMTDASVECVAAEAAARYSTSEGGSVKVK--GKQK--GFPAPFAV	a a
TbCgm1	851	LDVFEKGLSLYTKLEEFLLKQCHDGGKLDVVSCQFSIHGYCSNEBRVRVLSAVSSTLKGSGGIFIGTTVSDTELLRRLRQY	
TcCgm1	840	CDVFEAGAFIVKLEDFNQLKGRRLDIVSCQFSIHGYCSSTEQKSCFLRAISKALRPGGVFFGTTVSDVELLRQVAEH	
LmCgm1	824	HDAFHAASGLREDLLK-----RG--PFQLASCQFSMHYGCSSQESMRYFVKATADSLVPHGRFVGTTVSDVELLYRAKEH	aaa ac c
TbCgm1	931	GTFPGNGIYTVRFPPTDAVPND-----SFGVEYSVSFESSVSEMPYLVPNWRVNLCSAYNLQLVESFGFVEYGD	
TcCgm1	920	GPVFGNSLSYVRFPSETPPD-----SFGVQYFISVESVSSELPEYLVPWNRVNLCSAYNLQLVESFGFVEYGD	
LmCgm1	897	GAEFGNDVYGVRFGEAFALQSANFEPAALSFQVPAATVERVSQDMEYTVVPWDAFVALCAEHLKLVLEDNFHIFYG	c c c
TbCgm1	1001	MHYNLSALGQELRDASMNNGRRSDG--HLRLRLSPDEAAAGLFRFTLVFKI	1050
TcCgm1	990	MHYNLSPIGQKLRDAVLRRGGKRDSDG--HALQLSSEEREAAALFRFTLVFKI	1050
LmCgm1	977	QHKDTEAGKAMTLEQR--KRHNDGDVDCPLSPSEQAVALGLVLFVFEKTKAKQSSCGTAER	1037

B.

	I	III	IIIa	IV	V	VI
Chv	KTDGIR -38- SIFDGLC -8- AFVLFDVAVVSG -59- DGLII -14- LFKLKPGETHTID -49- WKYIQRSDK					
Sce	KTDGIR -51- TLLDGLV -12- RYLMFDCIAING -66- DGLIF -15- LLKWKPEQENTVD -110- WEMLRFRDDK					
Spo	KSDGIR -48- TLLDGLV -11- RYLVFDCIACDG -67- DGLIF -14- LLKWKPEKMENTID -76- WRFLRFRDDK					
Cal	KTDGIR -48- TLLDGLV -11- RYVIFDALAIHG -68- DGLIY -14- LLKWKPEAENTVD -79- WEMLRFRDDK					
Cel	KADGMR -37- TLVDTEVI -12- RMLIYDIMRFNS -68- DGLIF -14- VLKWKPPSHNSVD -66- WKFMRETRDK					
Mus	KADGTR -40- TLLDGLV -10- RYLIYDIKFNNA -68- DGLIF -13- LLKWKPPSLNSVD -66- WVFMRQRIDK					
Pfa	KTDGVR -38- TLLDGLV -16- VYLIYDGLYIQR -218- DGLIF -14- LFKWKPLNLNTVD -83- WIAQKIRFDK					
TbCel	KADGIR -54- FLLDAELS -8- TLYIYDFIYFWG -54- DGLIF -13- LKWKPVHLCTVD -60- WIFRNARNDK					
TbCgm1	KADGTR -139- FVVDALM -10- LLGCFDVFYVI -89- DGFIF -14- QLKWKWPSMLSVD -68- WSIERLRNDK					
TcCgm1	KSDGVR -134- FAVDGEEM -13- IYSCFDVFRYTP -89- DGFIF -14- QLKWKWPSMLSVD -68- WSIERLRNDK					
LmCgm1	KSDGIR -137- FVLDALM -15- VLGVDLFAYAG -80- DGLIF -14- QLKWKWQHLISID -68- WYIQLRDPDK					

FIGURE 7. *Trypanosoma* and *Leishmania* encode a second m⁷G methyltransferase with an associated guanylyltransferase domain. (A) m⁷G methyltransferase domains. Amino acid sequence alignment of the C-terminal methyltransferase domains of *T. brucei* Cgm1 (TbCgm1, accession no. XP_824771), *T. cruzi* Cgm1 (TcCgm1, XP_820423), and *L. major* Cgm1 (LmCgm1, CAJ03800.1). Side-chain identity and similarity in all proteins are indicated by asterisks and dots, respectively. Equivalent residues that interact with AdoMet and the cap analog in the Ecm1 co-crystal structure are indicated below the alignment as "a" and "c," respectively. (B) Conserved sequence elements in mRNA guanylyltransferases. Alignment of six colinear guanylyltransferase motifs (I, III, IIIa, IV, V, and VI) from *Chlorella* virus PBCV-1 (ChV), *S. cerevisiae* (Sce), *Schizosaccharomyces pombe* (Spo), *C. albicans* (Cal), *Caenorhabditis elegans* (Cel), *Plasmodium falciparum* (Pfa), *T. brucei* Cel (TbCel), *T. brucei* Cgm1 (TbCgm1), *T. cruzi* (TcCgm1), and *L. major* (LmCgm1). A number of amino acid residues separating the motifs are indicated.

To examine whether TbCgm1 indeed possesses m⁷G cap methyltransferase activity, the C-terminal TbCgm1 methyltransferase domain, TbCgm1 (717–1050), was expressed in bacteria and purified. Preliminary experiments indicate that TbCgm1 (717–1050) is capable of transferring a methyl group from AdoMet to a GpppN cap terminated poly(A) to form an m⁷GpppA structure (Y. Takagi, M.P. Hall, and C.K. Ho, unpubl.).

We speculate that a bifunctional TbCgm1 guanylyltransferase-m⁷G methyltransferase and TbCet1 triphosphatase are likely to act together to form the cap 0 structure. TbCet1 may also function together with TbCel1 guanylyltransferase and TbCmt1 m⁷G methyltransferase, unless TbCel1 has an intrinsic 5'-processing activity that can act as a triphosphatase (Silva et al. 1998). Alternatively, an additional RNA triphosphatase enzyme may be encoded by *T. brucei*. However, we have been unable to identify a homolog that resembles either *TbCET1* or a cysteine phosphatase-type RNA triphosphatase from the trypanosome genome.

The identification of *TbCMT1* and *TbCGM1* raises the interesting question of why two separate m⁷GpppN cap-forming activities are encoded in trypanosomes. One plausible explanation is that two separate sets of capping enzymes act on different RNA substrates. Tschudi and Ullu (2002) proposed that m⁷GpppN formation on SL RNA and snRNA utilizes fundamentally different mechanisms. In most eukaryotes, a single set of capping enzymes is sufficient to cap mRNA and snRNA as they are both transcribed by pol II and the cap is added co-transcriptionally to all pol II transcripts. In trypanosomes, while the SL RNA is transcribed by pol II and capped co-transcriptionally, the trimethylguanosine-capped U2, U3, and U4 snRNAs appear to be transcribed by pol III (Tschudi et al. 1986; Mottram et al. 1989; Fantoni et al. 1994; Nakaar et al. 1994). It is not clear whether U snRNAs are capped co-transcriptionally, as other pol III transcripts, such as tRNA, 5S RNA, and 7SL RNA, lack a cap structure. We speculate that one set of capping enzymes is responsible for SL RNA capping and another set is designated for snRNA capping. It is also conceivable that additional capping enzymes may be required for capping polycistronic pre-mRNAs. Alternatively, both sets of capping enzymes may act on SL RNA and regulate the level of *trans*-splicing-competent capped SL RNA. Further biochemical and genetic characterization of the *T. brucei* capping apparatus

should clarify the role of individual capping enzyme components in trypanosome cap biosynthesis.

MATERIALS AND METHODS

T7-based vector for expression of *T. brucei* RNA m⁷G cap methyltransferase

Preliminary sequence data for *TbCMT1* were obtained from the Sanger Institute *T. brucei* Genomes Web site (http://www.sanger.ac.uk/Projects/T_brucei). A DNA fragment containing the *TbCMT1* ORF on chromosome 10 was amplified by PCR from total *T. brucei* genomic DNA (a gift from Laurie Read, SUNY Buffalo) with the use of oligonucleotide primers designed to introduce an NdeI restriction site at the predicted translation start codon and a BamHI site 3' of the predicted stop codon. The ~1.0-kbp PCR product was digested with NdeI and BamHI and inserted into the T7 RNA polymerase-based expression vector pET16b (Novagen) to generate the plasmid pET-*TbCMT1*. The nucleotide sequence of the trypano-

some DNA insert was determined. The predicted amino acid sequence of the 324-amino acid TbCmt1 protein encoded by this plasmid is shown in Figure 1.

Expression and purification of *T. brucei* RNA m⁷G cap methyltransferase

The pET-TbCmt1 plasmid was transformed into *E. coli* BL21-Gold(DE3). A 2-L culture amplified from a single transformant was grown at 37°C in LB medium containing 0.1 mg/mL ampicillin until the OD₆₀₀ reached 0.4. The culture was adjusted to 0.4 mM IPTG and incubated at 17°C for 18 h. 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 100 mL of buffer A (50 mM Tris-HCl at pH 7.5, 10% sucrose) containing 0.25 M NaCl. Cell lysis was achieved by the addition of lysozyme and Triton X-100 to final concentrations of 100 µg/mL and 0.1%, respectively. The lysate was sonicated to reduce viscosity, and insoluble material was removed by centrifugation for 45 min at 14,000 rpm in a Beckman TA14-50 rotor. The soluble extract (450 mg of protein) was applied to a 5-mL column of Ni-NTA-agarose resin (Qiagen) that had been equilibrated with buffer A containing 0.1% Triton X-100. The column was washed with 50 mL of the same buffer and then eluted stepwise with 15-mL aliquots of buffer B (50 mM Tris-HCl at pH 8.0, 0.25 M NaCl, 10% glycerol) containing 0.05, 0.1, 0.2, 0.3, and 0.5 M imidazole. The protein composition of the column fractions was monitored by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The recombinant trypanosome protein was retained on the column and recovered in the 0.2 M imidazole eluate. This fraction was adjusted to 0.025 M NaCl by addition of 135 mL of buffer B, then applied to a 5-mL column of phosphocellulose pre-equilibrated in buffer B with 0.025 M NaCl. The column was washed with 50 mL of the same buffer and then eluted stepwise with 15 mL of buffer B containing 0.05, 0.1, 0.2, 0.3, 0.5, and 1.0 M NaCl. The wild-type and mutant trypanosome proteins were retained on the column and recovered predominantly in the 0.2 M NaCl fraction (~5 mg). The phosphocellulose preparation was concentrated by centrifugal ultrafiltration (Millipore), and the final protein concentrations were determined with the Bio-Rad dye reagent using bovine serum albumin as the standard.

Alanine mutants of TbCmt1

Alanine-substitution mutations were introduced into the *TbCMT1* gene by polymerase chain reaction using the two-stage overlap extension method. The residues targeted for amino acid substitution were Asp80, Lys83, Asp144, Phe171, His174, Tyr175, Val241, Val242, Tyr246, and Glu258. The pET-*TbCMT1* plasmid was the template for the first-stage amplifications. The DNA products of the second-stage amplification were digested with NdeI and BamHI and inserted into pET16b. The presence of the desired mutations was confirmed by DNA sequencing; the inserted restriction fragment was sequenced completely to exclude the acquisition of unwanted mutations during amplification and cloning. The His-tagged mutant proteins were purified from soluble bacterial lysates by nickel-agarose and phosphocellulose chromatography steps as described for the wild-type TbCmt1 protein. Protein concentration was determined by using the Bio-Rad dye reagent with bovine serum albumin as the standard.

Preparation of RNA substrates

Triphosphate-terminated RNA, pppRNA₅₃: 5'-ppp(GpApGpUpUp)₁₀GAG, was synthesized by in vitro transcription from plasmid pTZ18a (a gift of Paul Gollnick, SUNY Buffalo) (Elliott et al. 2001) using T7 RNA polymerase. The 53-mer pppRNA₅₃ was freed of protein and nucleotides by electrophoresis through a nondenaturing 8% polyacrylamide gel. The pppRNA₅₃ was localized by UV illumination, excised, and recovered by soaking the gel slice for 12 h in Tris-HCl (pH 8.0) and 1 mM EDTA. The ³²P-labeled capped RNA (GpppRNA₅₃) was prepared in a reaction mixture (200 µL) containing 50 mM Tris-HCl (pH 8.0), 5 mM dithiothreitol, 2.5 mM MgCl₂, 20 pmol of pppRNA₅₃, 0.33 µM [α-³²P]GTP, and 40 pmol of purified recombinant mammalian capping enzyme Mce1 (Ho et al. 1998). After incubation at 37°C for 20 min, the product was resolved by electrophoresis through a native 8% polyacrylamide gel. The ³²P-cap labeled GpppRNA₅₃ was located by autoradiography of the wet gel, eluted from an excised gel slice in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and precipitated by ethanol. Recovery of labeled RNA was assessed by scintillation counting. The molar concentration of cap-labeled RNA was calculated according to the specific activity of the input [α-³²P]GTP donor in the capping reaction.

Methyltransferase assay

Standard reaction mixtures (10 µL) containing 50 mM Tris-HCl (pH 8.0), 5 mM dithiothreitol, 100 fmol of ³²P-GpppRNA₅₃, and TbCmt1 as specified were incubated for 30 min at 27°C. The reaction mixtures were adjusted to 50 mM sodium acetate (pH 5.5) and samples were incubated with 140 ng of nuclease P1 (US Biologicals) or 0.1 unit of nucleotide pyrophosphatase (Sigma) for 60 min at 37°C. Aliquots (2 µL) were spotted onto a PEI cellulose thin-layer chromatography (TLC) plate (EMD Bioscience), which was developed with 0.45 M ammonium sulfate. The extent of methylation of the cap (m⁷GpppG / [m⁷GpppG + GpppG]) was quantitated by scanning the TLC plate with a Storm 860 PhosphorImager.

Materials

[¹⁴C-CH₃]AdoMet was purchased from GE Healthcare—Amersham Bioscience. AdoMet and AdoHcy were purchased from Sigma. Cap analogs GpppG, GpppA, m⁷GpppA and m⁷GpppA were purchased from Ambion.

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