Cap analog substrates reveal three clades of cap guanine-N2 methyltransferases with distinct methyl acceptor specificities

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ABSTRACT

The Tgs proteins are structurally homologous AdoMet-dependent eukaryal enzymes that methylate the N2 atom of 7-methylguanosine nucleotides. They have an imputed role in the synthesis of the 2,2,7-trimethylguanosine (TMG) RNA cap. Here we exploit a collection of cap-like substrates to probe the repertoire of three exemplary Tgs enzymes, from mammalian, protozoan, and viral sources, respectively. We find that human Tgs (hTgs1) is a bona fide TMG synthase adept at two separable transmethylation steps: (1) conversion of m⁷G to m^{2,7}G, and (2) conversion of m^{2,7}G to m^{2,2,7}G. hTgs1 is unable to methylate G or m²G, signifying that both steps require an m⁷G cap. hTgs1 utilizes a broad range of m⁷G nucleotides, including mono-, di-, tri-, and tetraphosphate derivatives as well as cap dinucleotides with triphosphate or tetraphosphate bridges. In contrast, *Giardia lamblia* Tgs (GlaTgs2) exemplifies a different clade of guanine-N2 methyltransferase that synthesizes only a dimethylguanosine (DMG) cap structure and cannot per se convert DMG to TMG under any conditions tested. Methylation of benzyl⁷G and ethyl⁷G nucleotides by hTgs1 and GlaTgs2 underscored the importance of guanine N7 alkylation in providing a key π -cation interaction in the methyl acceptor site. Mimivirus Tgs (MimiTgs) shares with the *Giardia* homolog the ability to catalyze only a single round of methyl addition at guanine-N2, but is distinguished by its capacity for guanine-N2 methylation in the absence of prior N7 methylation. The relaxed cap specificity of MimiTgs is revealed at alkaline pH. Our findings highlight both stark and subtle differences in acceptor specificity and reaction outcomes among Tgs family members.

Keywords: *Giardia*; cap hypermethylation; dimethylguanosine; mimivirus

INTRODUCTION

A 2,2,7-trimethylguanosine (TMG) cap is found on non-coding eukaryal RNAs such as small nuclear (sn) and small nucleolar (sno) RNAs and telomerase RNA (Busch et al. 1982; Seto et al. 1999). The enzyme Tgs1 is required for TMG capping in budding and fission yeasts (Mouaikel et al. 2002; Hausmann et al. 2007; Franke et al. 2008; Gallardo et al. 2008), *Drosophila* (Komonyi et al. 2005), and human somatic cells (Lemm et al. 2006). Tgs homologs are distributed widely among primitive and higher eukarya. Initial biochemical characterization of *Schizosaccharomyces pombe* Tgs1 (SpoTgs1) indicated that it catalyzes two

successive methyltransfer reactions from AdoMet to the N2 atom of guanosine via a distributive mechanism (Hausmann and Shuman 2005a). SpoTgs1 activity in vitro was strictly dependent on guanine-N7 methylation, signifying that its activity in vivo is likely confined to RNAs that already have an m⁷G cap. Cap-specific guanine-N2 methyltransferase activity has since been reported for human Tgs1 (Hausmann et al. 2008) and several protozoan Tgs homologs, including those of *Giardia lamblia*, *Trichomonas vaginalis*, *Entamoeba histolytica*, and *Trypanosoma brucei* (Hausmann and Shuman 2005b; Simoes-Barbosa et al. 2008). A recently characterized Tgs homolog encoded by mimivirus is the first example of a viral cap-specific guanine-N2 methyltransferase (Benarroch et al. 2009).

The fungal, protozoal, mammalian, and viral Tgs enzymes comprise a distinct branch of the AdoMet-dependent methyltransferase superfamily. Mutational studies of the fungal, *Giardia*, human, *Trichomonas*, and mimivirus proteins

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highlighted a constellation of conserved essential amino acid side chains that were surmised to form the binding pockets for the AdoMet donor and m^7G acceptor (Mouaikel et al. 2003; Hausmann et al. 2007, 2008; Simoes-Barbosa et al. 2008; Benarroch et al. 2009). The recent crystal structure of human Tgs1 (hTgs1) in a ternary complex with m^7GTP and AdoHcy (Monecke et al. 2009) confirmed the substrate interactions inferred from mutational studies and modeling exercises, including an essential tryptophan that forms a π -cation stack on the m^7G base (Supplemental Fig. S1). Most notably, the structure revealed an extensive network of hTgs1 contacts to the triphosphate moiety of m^7GTP that presumably contribute to its cap specificity (Supplemental Fig. S1; Monecke et al. 2009).

All of the recombinant Tgs enzymes studied to date are able to convert simple substrates such as m^7G nucleotides or m^7G cap dinucleotides to their respective 2,7-dimethylguanosine (DMG) derivatives, as gauged by transfer of a tritiated methyl group from [3H -CH₃]-AdoMet to an m^7G nucleotide acceptor (Hausmann and Shuman 2005a; Hausmann et al. 2008). This result signifies that there is no inherent requirement for an RNA polynucleotide for cap binding or catalysis. The capacity of SpoTgs1 and hTgs1 to convert a DMG cap to a TMG cap was surmised from a pulse-chase experiment, whereby the 3H -labeled $m^{2,7}G$ nucleotide formed during a pulse reaction could be chased into a 3H -labeled $m^{2,2,7}G$ product in the presence of excess cold AdoMet and fresh Tgs enzyme (Hausmann and Shuman 2005a; Hausmann et al. 2008). Thus, SpoTgs1 and hTgs1 are plausible single agents for TMG capping in *S. pombe* and human cells, respectively. In contrast, the *G. lamblia* Tgs (GlaTgs2), *T. vaginalis* Tgs (TvTgs), and mimivirus Tgs (MimiTgs) enzymes were unable to perform the second methylation reaction (DMG to TMG) when tested using the same pulse-chase procedures (Hausmann and Shuman 2005b; Simoes-Barbosa et al. 2008; Benarroch et al. 2009). This disparity in reaction outcomes between, for example, SpoTgs1 and GlaTgs2, was not an artifact of reliance on m^7G nucleotides as methyl acceptors. When similar experiments were performed using $m^7[\alpha^{32}P]GpppN$ cap-labeled RNA as the methyl acceptor, it was again noted that the Tgs proteins fell into two functional classes: (1) those that generated only a DMG-capped RNA product (GlaTgs2 and TvTgs) that could be subsequently converted to TMG during a chase reaction catalyzed by SpoTgs1; and (2) those that synthesized per se a TMG-capped product (SpoTgs1, *Entamoeba* and *Trypanosoma* Tgs) (Simoes-Barbosa et al. 2008).

To better understand and classify the substrate specificities of the Tgs enzymes implicated in cap hypermethylation, it is critical to be able to assay the first and second guanine-N2 methylation reactions separately, so that detection of DMG conversion to TMG is not contingent on prior DMG formation in the same reaction mixture. Here, we exploited a collection of differentially methylated guanine nucleotides and cap-like dinucleotides to achieve this aim. We report

that hTgs1 performs the second methylation reaction (DMG to TMG) and that both guanine-N2 methylation steps are strictly dependent on prior guanine-N7 methylation. In contrast, GlaTgs2 and MimiTgs cannot add a second methyl group to an N2-monomethylated guanine nucleotide under any conditions tested, signifying that these enzymes exemplify a distinct subfamily of DMG-only synthases. We proceeded to evaluate the effects of the cap 5'-phosphates, guanine-N7 substituent, and cap-proximal nucleoside on methyltransferase activity. The findings emphasize the importance of guanine N7 alkylation, rather than a specific requirement for an N7 methyl group, in sustaining the π -cation interaction in the methyl acceptor site. The two obligate DMG synthases are distinguished by their stringency of cap specificity. Whereas GlaTgs2 is strictly dependent on guanine-N7 methylation under all conditions tested, we find that MimiTgs can convert G to m^2G with good yield at alkaline pH.

RESULTS

New insights to the methyl acceptor specificity of human Tgs1

We surveyed a collection of cap-like guanine nucleotides for their ability to serve as methyl acceptors for Tgs enzymes, by incubating the proteins with 100 μM [3H -CH₃]-AdoMet and 1 mM guanine nucleotide substrate for 60 min at 37°C. The reaction products were then analyzed by polyethyleneimine (PEI)-cellulose thin layer chromatography (TLC), and the 3H -labeled material was visualized by autoradiography. Note that the ionic strength of the TLC buffer was varied for each set of substrates assayed in parallel in order to attain adequate separation of the guanine nucleotide substrates and 3H -labeled guanine nucleotide products from each other (to provide convincing evidence that different substrates yielded different products) and from the 3H -AdoMet donor. In general, the methyl-labeled products migrated immediately ahead of the unlabeled guanine nucleotide substrates, which were visualized by UV illumination of the chromatogram.

An exemplary product analysis in Figure 1 shows that 1 μg of hTgs1 sufficed to transfer nearly all of the input 3H -CH₃ from 0.1 mM AdoMet to 1 mM m^7GpppG to form the DMG cap product $m^{2,7}GpppG$ (Fig. 1B). In contrast, hTgs1 was unreactive with the unmethylated dinucleotide GpppG (Fig. 1B). Similarly, hTgs1 catalyzed methyl transfer to the mononucleotide m^7GTP to yield $m^{2,7}GTP$ (Fig. 1A). (Note that the minor labeled product migrating just below AdoMet corresponds to $m^{2,7}GDP$ formed by reaction with m^7GDP in the m^7GTP preparation) (Hausmann and Shuman 2005a). hTgs1 was unreactive with unmethylated GTP (Fig. 1A). These results underscore the requirement for an N7 methyl group for the first guanine-N2 methylation reaction of the two-step TMG synthetic pathway.

FIGURE 1. Methyl acceptor specificity of human Tgs1. Reaction mixtures (10 μ L) containing 50 mM Tris-HCl (pH 8.0), 5 mM DTT, 50 mM NaCl, 100 μ M [3 H-CH $_3$]AdoMet, 1 mM guanine nucleotide methyl acceptor as specified, and 1 μ g of hTgs1(631–853) were incubated for 60 min at 37°C. Aliquots (6 μ L) were spotted on PEI-cellulose TLC plates, which were developed either with 0.2 M (NH $_4$) $_2$ SO $_4$ (A,D) or 0.05 M (NH $_4$) $_2$ SO $_4$ (B,C,E). A control reaction mixture containing enzyme but no guanine nucleotide was included in panel B (lane –). The chromatograms were treated with Enhance, and 3 H-labeled material was visualized by autoradiography. (Arrowheads) Positions of unlabeled markers corresponding to methyltransferase reaction products.

Catalysis by hTgs1 of the second methylation step was evinced by the efficient transfer of 3 H-CH $_3$ from AdoMet to either the DMG cap dinucleotide $m^{2,7}$ GpppG to form a TMG cap product $m^{2,2,7}$ GpppG (Fig. 1B) or to the DMG mononucleotide $m^{2,7}$ GTP to form $m^{2,2,7}$ GTP (Fig. 1A). hTgs1 titration experiments indicated that the methyltransferase specific activity with either cap dinucleotide- or guanosine mononucleotide-based methyl acceptors (1 mM each) was similar for the DMG synthase and TMG synthase steps (Supplemental Fig. S2A,B). hTgs1 effected no label transfer from AdoMet to $m^{2,2,7}$ GpppG (Fig. 1B) or $m^{2,2,7}$ GTP (Fig. 1A), signifying that a TMG cap is the final product of this enzyme's activity.

By titrating the cap dinucleotide substrates m^7 GpppG and $m^{2,7}$ GpppG, we derived K_m values for the first and second methylation steps of 134 μ M m^7 GpppG and 135 μ M $m^{2,7}$ GpppG, respectively (Table 1). The k_{cat} values for the first (0.73 min $^{-1}$) and second (0.80 min $^{-1}$) methylation steps were also similar. Thus, the catalytic efficiencies (k_{cat}/K_m) of the DMG and TMG synthase reactions of hTgs1

with a cap dinucleotide methyl acceptor were virtually the same.

An important mechanistic question is whether the requirement for the guanine-N7 methyl group is maintained during the second catalytic step, it being conceivable that monomethylation at guanine-N2 allows for effective addition of a second N2 methyl group, whether or not N7 is methylated. We approached the issue by testing m^2 G nucleotides as methyl acceptors. The instructive findings were that 1 μ g of hTgs1 displayed no detectable label transfer from 0.1 mM AdoMet to either 1 mM m^2 GpppG (Fig. 1C) or m^2 GTP (Fig. 1A) under the same conditions that allowed for nearly complete label transfer to the control substrates m^7 GpppG and m^7 GTP. We conclude that hTgs1 enforces m^7 G cap specificity at both methyl transfer steps. Because the guanine-N7 moiety makes only a single van der Waals contact to protein in the hTgs1 structure (to the Gly770 C α atom) (Supplemental Fig. S1), we surmise that its contribution to the methyltransferase reaction derives from the positive charge it imparts to the

TABLE 1. Cap dinucleotide methyl acceptors for human Tgs1

Nucleotide	K_m (μM)	k_{cat} (min^{-1})
m ⁷ GpppG	134 ± 18	0.73 ± 0.02
m ⁷ GpppA	105 ± 19	0.71 ± 0.11
m ⁷ GpppC	121 ± 14	0.84 ± 0.15
m ⁷ Gppp(dG)	87 ± 28	0.77 ± 0.08
Ethyl ⁷ GpppG	576 ± 56	0.076 ± 0.016
Benzyl ⁷ GpppG	319 ± 32	0.50 ± 0.04
m ^{2,7} GpppG	135 ± 24	0.80 ± 0.06

Substrate titrations were performed as follows, except for ethyl⁷GpppG. Reaction mixtures (10 μL) containing 50 mM Tris-HCl (pH 8.0), 5 mM DTT, 50 mM NaCl, 100 μM [³H-CH₃]AdoMet, 0.5 μg of hTgs1, and varying concentrations of the indicated nucleotides (in twofold decrements from 500 μM to either 31 or 16 μM) were incubated for 15 or 30 min at 37°C. The reaction mixtures for titrations of ethyl⁷GpppG (in twofold decrements from 1000 to 31 μM) contained 2 μg of hTgs1 and were incubated for 60 min. Aliquots (6 μL) of the mixtures were spotted on PEI-cellulose TLC plates, which were developed in 0.05 M (NH₄)₂SO₄. The extents of methyl transfer were quantified and plotted as a function of methyl acceptor concentration. K_m and k_{cat} were obtained by nonlinear regression curve fitting of the data for each experiment to the Michaelis-Menten equation in Prism. The values shown are averages of three independent substrate titration experiments ± SEM, except for m⁷Gppp(dG) and m^{2,7}GpppG, which are averages of four independent experiments ± SEM.

purine ring, resulting in π -cation stacking of m⁷G on the Trp766 side chain (Supplemental Fig. S1), which is essential for hTgs1 activity (Hausmann et al. 2008).

To probe further the role of the guanine-N7 alkyl group, we tested N7-ethyl and N7-benzyl derivatives of GpppG and GTP for their ability to serve as methyl acceptors at 1 mM concentration. hTgs1 was adept at efficient label transfer from AdoMet to benzyl⁷GTP to form a product with distinctive TLC migration that we presume is benzyl⁷m²GTP (Fig. 1A). The enzyme also methylated the cap dinucleotide benzyl⁷GpppG (Fig. 1B). In contrast, the extents of methyl transfer to ethyl⁷GTP and ethyl⁷GpppG were conspicuously lower (Fig. 1A,B). A protein titration experiment indicated that the specific activity in methylating the ethyl⁷GpppG substrate was about one-tenth the activity with m⁷GpppG (Supplemental Fig. S2C). It appears that hTgs1 methyltransferase activity does not correlate with the size of the N7 substituent, insofar as the larger benzyl moiety is superior to the smaller ethyl group (Supplemental Fig. S2C). This trend was confirmed by titrations of the N7 alkylated cap dinucleotides. The N7-ethyl substitution increased K_m to 576 μM and elicited a significant (10-fold) decrement in k_{cat} (Table 1). The N7-benzyl group also increased K_m (to 319 μM) but had little impact on k_{cat} (Table 1). Manual superposition of an isolated benzyl group over the N⁷-CH₃ of the m⁷GTP in the hTgs1 crystal structure indicated that the benzyl moiety could be accommodated without gross steric hindrance, such that the benzene ring packs against Pro769, Phe670, and Ser671 (model not shown).

Whereas the m⁷G base is buried deep within a hydrophobic pocket of hTgs1, the triphosphate bridge projects outward to the enzyme's surface, on which the γ phosphate of m⁷GTP is exposed (Supplemental Fig. S3). It is not yet clear whether or how the N nucleoside of the m⁷GpppN cap substrate might interact with hTgs1 on its surface adjacent to the γ phosphate. In order to probe the influence (if any) of the N nucleoside on guanine-N2 methylation, we tested a variety of m⁷GpppN cap analogs (at 1 mM concentration) that differed with respect to the base or sugar at the N position. First, we noted that hTgs1 efficiently methylated m⁷Gppp(dG) and m⁷GpppG_m dinucleotides containing 2'-deoxy and 2'-OCH₃ sugar modifications, respectively (Fig. 1B). Second, hTgs1 generated similar high yields of dimethylated products with m⁷GpppG, m⁷GpppA, and m⁷GpppC acceptors (Fig. 1C); note that the three m^{2,7}GpppN products migrated differently during TLC according to the identity of the N base. Protein titration experiments revealed similar specific activities for the various m⁷GpppN substrates (Supplemental Fig. S2), suggesting that hTgs1 does not strongly discriminate the base (Supplemental Fig. S2A) or sugar (Supplemental Fig. S2D) of the nucleoside adjacent to the cap. Substrate titrations confirmed that hTgs1 is relatively indifferent to the cap-flanking nucleoside. Changing the N ribonucleoside to A or C or substituting dG for G had little effect on K_m or k_{cat} (Table 1).

In light of the interactions of hTgs1 with the m⁷G triphosphate bridge (Supplemental Fig. S1), we tested a series of m⁷G nucleotides that differed in the number of 5'-phosphate groups. hTgs1 efficiently methylated m⁷GMP, m⁷GDP, and m⁷GTP to yield dimethylated products m^{2,7}GMP, m^{2,7}GDP, and m^{2,7}GTP that were well resolved by PEI-cellulose TLC according to their relative negative charge (Fig. 1E). hTgs1 also efficiently methylated the tetraphosphate derivative m⁷G(5')pppp to generate a DMG product m^{2,7}G(5')pppp (Fig. 1D). No label transfer was detected to the unmethylated substrates GMP, GDP, GTP, or G(5')pppp (Fig. 1D,E), again indicative of stringent cap specificity. Protein titration experiments revealed similar specific activities for the m⁷G nucleoside mono-, di-, tri-, and tetraphosphates (Supplemental Fig. S2E,F). These experiments implicated m⁷GMP as a minimal effective substrate for guanine-N2 methylation. By titrating this minimal substrate, we derived a K_m of 109 ± 21 μM m⁷GMP and a k_{cat} of 0.73 ± 0.08 min⁻¹ (data not shown).

Having seen that hTgs1 can accommodate an extra phosphate in the context of a mononucleotide acceptor for the DMG synthase reaction, we proceeded to test whether the enzyme could also tolerate a tetraphosphate bridge in the context of a cap dinucleotide. We found that hTgs1 efficiently catalyzed methylation of m⁷GppppG to yield m^{2,7}GppppG and of m^{2,7}GppppG to yield m^{2,2,7}GppppG (Fig. 1D; Supplemental Fig. S2F). Thus, hTgs1 is not constrained by a longer phosphoanhydride bridge for either the first or second methylation reactions of the TMG synthesis pathway.

Giardia Tgs2 is a DMG synthase only

Giardia Tgs2 catalyzed label transfer from AdoMet to the cap dinucleotide m^7 GpppG and the mononucleotides m^7 GTP and m^7 GDP to form 3 H-labeled dimethylated products that were resolved from AdoMet by PEI-cellulose TLC (Fig. 2A,B). GlTgs2 formed no labeled product when reacted with any of the corresponding dimethylated $m^{2,7}$ G nucleotides: $m^{2,7}$ GpppG, $m^{2,7}$ GTP, or $m^{2,7}$ GDP (Fig. 2A,B). Thus, GlTgs2 is apparently unable to transfer a second methyl group to $m^{2,7}$ G, a property that sharply distinguishes the *Giardia* enzyme from its human homolog hTgs1 (vide supra). GlTgs2 failed to methylate GTP (Fig. 2A) or GpppG (data not shown), verifying its reliance on an N7-methylated cap. The possibility that the N7-methyl group might somehow mask the capacity for a second guanine-N2 methylation reaction by GlTgs2 was ruled out by the finding that m^2 GTP was also unreactive (Fig. 2A). An N7 alkyl group, rather than a methyl moiety per se, was critical for GlTgs2 activity, insofar as the enzyme was as effective in label transfer from AdoMet to the N7-substituted cap analogs benzyl⁷GpppG and ethyl⁷GpppG as to the standard m^7 GpppG cap (Fig. 2C). GlTgs2 readily methylated tetraphosphate cap analogs m^7 G(5')pppp and m^7 GppppG, but was unreactive with G(5')pppp or $m^{2,7}$ GppppG (Supplemental Fig. S4A).

Mimivirus Tgs is a DMG synthase with less stringent requirements for N7 methylation

Surprising findings emerged when we surveyed the substrate specificity of mimivirus Tgs under the same reaction conditions used to assay the human and *Giardia* enzymes. MimiTgs catalyzed label transfer from AdoMet to 1 mM m^7 GTP to form the 3 H-labeled dimethylated $m^{2,7}$ GTP, but

formed no methylated product when presented with either 1 mM $m^{2,7}$ GTP or m^2 GTP (Fig. 3A, left panel), signifying that MimiTgs, like *Giardia* Tgs2, performs only a single step of guanine-N2 methylation. The distinctive and unexpected property of MimiTgs was that it catalyzed methyl transfer to GTP to generate, in high yield, a product migrating just ahead of the chromatographic origin, at the same position as unlabeled m^2 GTP (highlighted by the oval in Fig. 3A, left panel). The apparent capacity of MimiTgs for guanine-N2 methylation in the absence of N7 methylation contrasted with our initial finding that MimiTgs methyltransferase activity was cap-specific (Benarroch et al. 2009). A potential explanation for this disparity was that, in the initial study, activity was measured in reaction mixtures containing 50 mM Tris-acetate buffer at pH 6.0, whereas all of the present experiments were performed in 50 mM Tris-HCl at pH 8.0. Indeed, when MimiTgs activity was assayed in parallel at pH 6.0, little or no methyl transfer was detected from AdoMet to GTP, though the enzyme catalyzed near quantitative label transfer to m^7 GTP under the same conditions (Fig. 3A, right panel).

These findings were fortified by parallel assays with other guanine nucleotides. MimiTgs catalyzed high-yield methylation of m^7 GDP and m^7 GpppG at either pH 8.0 or 6.0 (Fig. 3B), while no methylation of $m^{2,7}$ GDP was seen at either pH. Activity at pH 6.0 was contingent on N7 alkylation with either a methyl, ethyl, or benzyl group (Fig. 3B,C). MimiTgs also methylated tetraphosphate cap analogs m^7 Gppppp and m^7 GppppG at pH 6.0, while showing little or no activity with G(5')pppp or $m^{2,7}$ GppppG (Supplemental Fig. S4B). In contrast, at pH 8.0 we readily detected methyl transfer to GDP and GpppG (highlighted by ovals in Fig. 3B, left panel). We also noted a lower extent of label transfer to the cap dinucleotides $m^{2,7}$ GpppG and $m^{2,2,7}$ GpppG at pH 8.0 (also highlighted by ovals in Fig. 3B,

FIGURE 2. Methyl acceptor specificity of *Giardia* Tgs2. Reaction mixtures (10 μ L) containing 50 mM Tris-HCl (pH 8.0), 5 mM DTT, 50 mM NaCl, 100 μ M [3 H-CH₃]AdoMet, 1 mM guanine nucleotide methyl acceptor as specified, and 0.5 μ g of GlTgs2 were incubated for 60 min at 37°C. Aliquots (6 μ L) were spotted on PEI-cellulose TLC plates, which were developed either with 0.2 M (NH₄)₂SO₄ (A) or 0.05 M (NH₄)₂SO₄ (B,C). 3 H-labeled material was visualized by autoradiography.

FIGURE 3. Mimivirus Tgs catalyzes a single methyl addition at cap guanine-N2 and has relaxed cap specificity at alkaline pH. Reaction mixtures (10 μ L) containing either 50 mM Tris-HCl (pH 8.0) or 50 mM Tris-acetate (pH 6.0) as indicated; 5 mM DTT, 50 mM NaCl, 100 μ M [3 H-CH₃]AdoMet, 1 mM methyl acceptor as specified; and 1 μ g (A,B) or 0.5 μ g (C) of MimiTgs were incubated for 60 min at 37°C. Aliquots (6 μ L) were spotted on PEI-cellulose TLC plates, which were developed with 0.2 M (NH₄)₂SO₄ (A) or 0.05 M (NH₄)₂SO₄ (B,C). 3 H-labeled material was visualized by autoradiography.

left panel), but not at pH 6.0. Because no such methylation occurred when MimiTgs was presented with the corresponding DMG and TMG mononucleotides $m^{2,7}$ GDP and $m^{2,2,7}$ GDP or $m^{2,7}$ GTP and $m^{2,2,7}$ GTP (Fig. 3A,B), we surmised that the enzyme had added a methyl group at the N2 position of the cap-flanking guanosine, thereby forming $m^{2,7}$ Gppp(m^2 G) and $m^{2,2,7}$ Gppp(m^2 G) products, respectively.

The relaxed cap-dependence of MimiTgs activity as a function of increasing pH was confirmed with either 1 mM GDP (Fig. 4A) or 1 mM GTP (Fig. 4B) as the methyl acceptor. Quantification of results from three independent experiments showed a sharp increase in the yield of m^2 G product beginning at pH 7.5 and continuing to pH 9.0

(Supplemental Fig. S5). (Higher pH values were not tested). In contrast, MimiTgs catalyzed high-yield methylation m^7 GDP and m^7 GTP at all pH values over the same pH range (Supplemental Fig. S6).

The acceptor specificity of cap-independent methylation by MimiTgs at pH 9.0 was evaluated in the experiment shown in Figure 4C. The enzyme reacted with 1 mM GTP to yield a single labeled product (m^2 GTP), but was unreactive with ATP, ITP, or UTP. That MimiTgs failed to react with ITP (which lacks an N2 atom but is otherwise identical to GTP) provides further evidence that the guanine-N2 atom is the target of cap-independent methyl transfer.

In light of these results, we retested the ability of hTgs1 and GlaTgs2 to methylate GTP or GDP over the same pH range and found no detectable cap-independent activity even at pH 9.0 (data not shown). Taken together, the distinctive biochemical properties of MimiTgs—DMG synthase only, but relaxed cap-dependence at alkaline pH—define the viral enzyme as a third functional clade within the Tgs family of guanine-N2 methyltransferases.

The K_m and k_{cat} values of MimiTgs for m^7 GDP and GDP were derived from substrate titration experiments at pH 9.0 (Table 2). The salient findings were that the guanine-N7 methyl moiety is a key determinant of substrate binding, eliciting a 175-fold increase in affinity for m^7 GDP (K_m of 5 μ M) compared with GDP (K_m of 874 μ M). In contrast, the N7 methyl group has only a 6.6-fold impact on k_{cat} . The salutary effects of

the N7 methyl (comprising a 1100-fold increase in catalytic efficiency for m^7 GDP versus GDP) are likely because of the positive charge imparted to the guanine base.

DISCUSSION

The present study sharpens the distinctions between cap-specific guanine-N2 methyltransferases, exemplified by human Tgs1, that catalyze two sequential methylation steps to yield a TMG cap, and a functionally separate clade of enzymes—including *Giardia* Tgs2 and mimivirus Tgs—that can only perform the first step of DMG cap synthesis in vitro. Our findings implicate hTgs1 as sufficient for catalysis of TMG capping in human cells. In contrast, the properties

FIGURE 4. Guanidine-N2 methylation without N7 methylation. (A,B) Reaction mixtures (10 μ L) containing 50 mM Tris buffer (either Tris-acetate pH 5.0–6.5 or Tris-HCl pH 7.0–9.0), 5 mM DTT, 50 mM NaCl, 100 μ M [3 H-CH $_3$]AdoMet, 1 mM GDP (A) or 1 mM GTP (B), and 1 μ g of MimiTgs were incubated for 60 min at 37°C. Aliquots (6 μ L) were spotted on PEI-cellulose TLC plates, which were developed either with 0.2 M (NH $_4$) $_2$ SO $_4$ (A) or 0.4 M (NH $_4$) $_2$ SO $_4$ (B). (C) Reaction mixtures (10 μ L) containing 50 mM Tris-HCl (pH 9.0), 5 mM DTT, 100 μ M [3 H-CH $_3$]AdoMet, 1 mM GTP, ATP, ITP, or UTP as specified, and 1 μ g of MimiTgs were incubated for 60 min at 37°C. Aliquots (6 μ L) were spotted on PEI-cellulose TLC plates, which were developed with 0.4 M (NH $_4$) $_2$ SO $_4$. 3 H-labeled material was visualized by autoradiography.

of GlaTgs2 and MimiTgs suggest that: (1) DMG-capped RNAs might exist naturally in protozoa (mimivirus being a pathogen of amoebae); and (2) if the DMG-only synthases function in TMG capping, they do so with help from other proteins, for example, a separate cap guanine-N2 methyltransferase enzyme that specifically converts DMG to TMG or a regulatory protein or subunit that enables GlaTgs2 or MimiTgs to catalyze the second guanine-N2 methylation step.

By using synthetic DMG cap analogs, we were able to study the second hTgs1 cap guanine-N2 methylation reaction in isolation and thereby compare the specificity determinants and kinetic parameters for the TMG synthase reaction of hTgs1 to those of the preceding DMG synthase step. The key findings were that: (1) both guanine-N2 methylation reactions were dependent on prior guanine-N7 methylation; and (2) the catalytic efficiencies of the DMG and TMG synthase steps were similar for a cap dinucleotide substrate. These results reinforce the initial inferences that hTgs1 and SpoTgs1 generate TMG caps *in vitro* via a distributive mechanism (Hausmann and Shuman 2005a; Hausmann et al. 2008), entailing dissociation of the m 2,7 G and AdoHcy products of the first step prior to binding of m 2,7 G and AdoMet substrates for the second step.

From the structure of the hTgs1-m 2,7 GTP-AdoHcy ternary complex (Monecke et al. 2009), we can safely surmise that the N2 methyl group must change position during the transition from first step product, where it will be situated “in-line” to the AdoHcy S8 leaving atom, to second step substrate, whence it must rotate about the C 2 –N 2 bond to free up space for the methyl group of the incoming AdoMet donor. In a distributive mechanism, the newly bound m 2,7 G could be accommodated directly and specifically in the second step substrate-binding mode by either or both of the following mechanisms: (1) provision of a vicinal docking site for the N2-methyl, oriented away from the

AdoMet methyl, that steers the reactive N2 orbital for in-line attack; or (2) adoption of an ordered bi–bi kinetic mechanism in which AdoMet binds first and thereby precludes binding of m 2,7 G in the first-step product mode. Note that a processive mechanism for TMG capping would involve retention of the m 2,7 G first step product in the cap-binding pocket as the AdoHcy is released from the donor site, followed by rotation of the N 2 –CH $_3$, and then binding of fresh AdoMet donor for the second transmethylation step. If the processive mechanism applied to hTgs1, the reaction with m 2,7 G nucleotides (in 10-fold molar excess over AdoMet) ought to yield predominantly TMG products, not the DMG products that are observed.

TABLE 2. N7 methyl effect on mimivirus Tgs

Nucleotide	K_m (μ M)	k_{cat} (min $^{-1}$)
GDP	874 $\pm$ 92	0.85 $\pm$ 0.11
m 2,7 GDP	5 $\pm$ 0.5	5.6 $\pm$ 1

GDP methylation reaction mixtures (10 μ L) containing 50 mM Tris-HCl (pH 9.0), 5 mM DTT, 50 mM NaCl, 50 μ M [3 H-CH $_3$]AdoMet, 0.15 μ g of MimiTgs, and varying concentrations of GDP (in twofold decrements from 4 mM to 125 μ M) were incubated for 30 min at 37°C. m 2,7 GDP methylation reaction mixtures (10 μ L) containing 50 mM Tris-HCl (pH 9.0), 5 mM DTT, 50 mM NaCl, 50 μ M [3 H-CH $_3$]AdoMet, 0.03 μ g of MimiTgs, and varying concentrations of m 2,7 GDP (in twofold decrements from 50 μ M to 3.1 μ M) were incubated for 5 min at 37°C. The reactions were quenched by adding AdoHcy to 2 mM final concentration. Aliquots (6 μ L) were spotted on PEI-cellulose TLC plates, which were developed in either 0.05 M (NH $_4$) $_2$ SO $_4$ (m 2,7 GDP substrate) or 0.2 M (NH $_4$) $_2$ SO $_4$ (GDP substrate). The extents of methyl transfer were quantified and plotted as a function of methyl acceptor concentration. K_m and k_{cat} were obtained by nonlinear regression curve fitting of the data for each experiment to the Michaelis–Menten equation in Prism. The values shown are averages of three independent titration experiments $\pm$ SEM.

The available hTgs1 crystal structure captures an inhibitor complex of the first methylation reaction. Absent a true substrate or product ternary complex for either step, we cannot surmise the exact position or contacts of an N2 methyl group bound as the second step substrate, although it does appear that the human Tgs1 structure has room to accommodate an N2 methyl facing away from the bound AdoHcy within a hydrophobic pocket lined by Ser763, Pro764, Trp766, and Phe804. A parsimonious explanation for the failure of *Giardia* Tgs2 and MimiTgs to perform the second methylation reaction is that they cannot accommodate the guanine-N2 methyl in an orientation compatible with AdoMet binding to the donor site. Consistent with this model, we observed that the extent of methyl transfer from 100 μ M [$^3\text{H-CH}_3$]-AdoMet to 100 μ M $m^7\text{GTP}$ by either GlaTgs2 or MimiTgs was undiminished by inclusion of up to 1 mM $m^{2,7}\text{GTP}$ in the reaction mixtures (data not shown). The lack of inhibition by a 10-fold excess of the DMG product suggests that DMG does not rebinding to the enzyme in the presence of AdoMet to compete with the $m^7\text{G}$ substrate. It is noteworthy that the Phe804 side chain in the putative N2-methyl pocket of hTgs1 is conserved in *Schizosaccharomyces*, *Entamoeba*, and *Trypanosoma* Tgs1 proteins (each of which has biochemically documented TMG synthase activity), but is substituted by a lysine in *Giardia* Tgs2 and MimiTgs, which are DMG-only synthases. A simple speculation is that the hydrophobic-to-charged substitution might disrupt the putative $m^{2,7}\text{G}$ substrate docking site for the second transmethylation reaction. However, this cannot be the only means to limit action to a single step, insofar as the *Trichomonas vaginalis* Tgs (a DMG-only synthase) (Simoes-Barbosa et al. 2008) retains a phenylalanine in the position corresponding to Phe804 in human Tgs1. Needless to say, a fuller appreciation of the basis for the different repertoires of the cap guanine-N2 methyltransferase clades depends on obtaining an atomic structure of one or several of the DMG-only synthases.

Here, we used a collection of alkylated guanosine nucleotides to probe the methyl acceptor specificity of the DMG synthase reaction. The relative indifference of hTgs1 to the N nucleoside base or sugar of a $m^7\text{GpppN}$ dinucleotide substrate jibes with the putative exposure of this moiety above the enzyme's surface. An especially instructive finding was that the cap specificity of the hTgs1 and GlaTgs2 DMG synthase reactions is enforced by guanine-N7 alkylation, rather than a specific requirement for a methyl group. Thus, it appears that productive hTgs1 binding to the methyl acceptor is driven in large part by a π -cation interaction with the tryptophan side chain in the Tgs active site. In this respect, Tgs enzymes echo the primacy of π -cation interactions for cap recognition by vaccinia virus cap-specific nucleoside-2'-O-methyltransferase (Hu et al. 1999, 2002) and the cap-binding translation initiation factor eIF4E (Niedzwiecka et al. 2002).

Mimivirus Tgs differs from the other enzymes studied here with its relaxed stringency of cap dependence at alkaline pH, whereby it methylates guanine nucleotides that lack the defining N7-methyl group of the RNA cap. The unmethylated GDP substrate has significantly lower affinity for MimiTgs than $m^7\text{GDP}$, presumably reflecting the loss of the stabilizing cationic component of the π stack of guanosine and tryptophan in the methyl acceptor pocket. It is not immediately clear why the cap-independent methyltransferase of MimiTgs is unmasked at alkaline pH. It is possible that increased pH caused deprotonation of an amino acid on the enzyme, which then enables productive binding to unmethylated guanine. Alternatively, the switch might be related to deprotonation of the guanine-N1 atom ($pK_a \sim 9.4$), which might favor the enolate tautomer and allow productive binding without an N7 alkyl group.

MATERIALS AND METHODS

Materials

[$^3\text{H-CH}_3$]AdoMet and Enhance were purchased from Perkin Elmer Life Sciences. AdoMet, GTP, GDP, ATP, ITP, and UTP were purchased from Sigma. Protein concentrations were determined by using the Bio-Rad dye reagent with bovine serum albumin (BSA) as the standard.

Cap analogs

Syntheses of dinucleotide cap analogs GpppG, $m^2\text{GpppG}$, $m^7\text{GpppG}$, $m^7\text{GpppA}$, $m^7\text{GpppC}$, $m^7\text{Gppp(dG)}$, $m^7\text{GpppGm}$, ethyl $^7\text{GpppG}$, benzyl $^7\text{GpppG}$, $m^{2,7}\text{GpppG}$, $m^{2,2,7}\text{GpppG}$, $m^{2,7}\text{GpppA}$, $m^7\text{GppppG}$, $m^{2,7}\text{GppppG}$, and $m^{2,2,7}\text{GppppG}$ were performed as described previously (Darzynkiewicz et al. 1990; Stepinski et al. 1995; Jankowska et al. 1996; Niedzwiecka et al. 2007). Mononucleotides $m^7\text{G(5')p}$, $m^{2,7}\text{G(5')p}$, $m^7\text{G(5')pp}$, $m^{2,7}\text{G(5')pp}$, $m^{2,2,7}\text{G(5')pp}$, $m^2\text{G(5')ppp}$, $m^7\text{G(5')ppp}$, $m^{2,7}\text{G(5')ppp}$, $m^{2,2,7}\text{G(5')ppp}$, ethyl $^7\text{G(5')ppp}$, benzyl $^7\text{G(5')ppp}$, G(5')pppp , and $m^7\text{G(5')pppp}$ were prepared according to the methods reported previously (Darzynkiewicz et al. 1985; Jankowska et al. 1993; Zuberek et al. 2004; Niedzwiecka et al. 2007).

Human Tgs1

Plasmid pET28b-hTgs1(631-853) encoding the C-terminal catalytic domain of hTgs1 (Hausmann et al. 2008) fused to an N-terminal His $_{10}$ Smt3 tag was transformed into *Escherichia coli* BL21(DE3)pRIL. A 4-L culture derived from a single transformant was grown at 37°C in Luria-Bertani medium containing 50 $\mu\text{g/mL}$ kanamycin and 125 $\mu\text{g/mL}$ chloramphenicol until the A_{600} reached 0.6. The culture were adjusted to 0.2 mM isopropyl β -D-thiogalactopyranoside and 2% ethanol and incubation was continued for 17 h at 17°C with constant shaking. The cells were harvested by centrifugation and stored at -80°C . All subsequent steps were performed at 4°C. Thawed bacteria were resuspended in 200 mL of buffer A (50 mM Tris-HCl, pH 8.0, 1 M NaCl, 10% glycerol). Lysozyme and Triton X-100 were added to final concentrations of 1 mg/mL and 0.1%, respectively. The lysate was sonicated to reduce viscosity, and insoluble material was

removed by centrifugation for 45 min at 16,000 rpm in a Sorvall SS34 rotor. Aliquots (50 mL) of the supernatant were mixed with 2 mL of Ni²⁺-nitrilotriacetic acid-agarose (Qiagen) that had been equilibrated with buffer A. Protein and resin were incubated for 30 min at 4°C and then poured into columns. The columns were washed with 100 mL of 50 mM imidazole in buffer A and then eluted with 500 mM imidazole in buffer A. The polypeptide compositions of the column fractions were monitored by SDS-PAGE. The peak 500 mM imidazole eluate fractions containing recombinant His₁₀Smt3-hTgs1(631-853) were combined (14 mg protein in 7 mL), supplemented with 10 µg of purified Ulp1 (a Smt3-specific protease) (Mosesso and Lima 2000), and then dialyzed overnight against 100 mM NaCl in buffer A. The dialysate was applied to mixed bed for 30 min with 1 mL of phosphocellulose that had been equilibrated with buffer A containing 100 mM NaCl. The resin was poured into a column, and the tag-free hTgs1(631-853) protein (7 mg) was recovered in the flow-through. The enzyme preparation was stored at -80°C.

Giardia Tgs2

GlaTgs2 was produced in *E. coli* as an N-terminal His₁₀ fusion and purified from a soluble bacterial lysate by Ni-agarose chromatography, as described previously (Hausmann and Shuman 2005b), except that the enzyme buffer contained 500 mM NaCl instead of 200 mM NaCl.

Mimivirus Tgs

MimTgs was produced in *E. coli* as a His₁₀Smt3 fusion and recovered from a soluble bacterial lysate by Ni-agarose chromatography as described previously (Benarroch et al. 2009). The tag was removed by treatment with Ulp1, and the tag-free MimTgs was recovered in the flow-through during a second round of Ni-agarose chromatography.

Methyltransferase assay

Reaction mixtures (10 µL) containing 50 mM Tris-HCl (pH 8.0) or Tris-Acetate (pH 6.0), 5 mM DTT, 50 mM NaCl, 100 µM [³H-CH₃]AdoMet, and guanine nucleotides and enzyme as specified were incubated at 37°C. Aliquots (6 µL) were withdrawn at the times specified and then spotted on polyethyleneimine cellulose TLC plates, which were developed with 0.05 M, 0.2 M, or 0.4 M (NH₄)₂SO₄ as specified. The dried TLC plates were sprayed with Enhance, and radiolabeled material was visualized by autoradiography after exposing the plates to X-ray film for 2 d at -80°C. Alternatively, the AdoMet- and ³H product-containing portions of the lanes were cut out, and the radioactivity in each was quantified by liquid scintillation counting.

SUPPLEMENTAL DATA

Supplemental material can be found at <http://www.rnajournal.org>.

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