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The divergent eukaryote *Trichomonas vaginalis* has an m^7G cap methyltransferase capable of a single N2 methylation

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ABSTRACT

Eukaryotic RNAs typically contain 5′ cap structures that have been primarily studied in yeast and metazoa. The only known RNA cap structure in unicellular protists is the unusual Cap4 on *Trypanosoma brucei* mRNAs. We have found that *T. vaginalis* mRNAs are protected by a 5′ cap structure, however, contrary to that typical for eukaryotes, *T. vaginalis* spliceosomal snRNAs lack a cap and may contain 5′ monophophates. The distinctive 2,2,7-trimethylguanosine (TMG) cap structure usually found on snRNAs and snoRNAs is produced by hypermethylation of an m^7G cap catalyzed by the enzyme trimethylguanosine synthase (Tgs). Here, we biochemically characterize the single *T. vaginalis* Tgs (TvTgs) encoded in its genome and demonstrate that TvTgs exhibits substrate specificity and amino acid requirements typical of an RNA cap-specific, m^7G-dependent N2 methyltransferase. However, recombinant TvTgs is capable of catalysing only a single round of N2 methylation forming a 2,7-dimethylguanosine cap (DMG) as observed previously for *Giardia lamblia*. In contrast, recombinant *Entamoeba histolytica* and *Trypanosoma brucei* Tgs are capable of catalysing the formation of a TMG cap. These data suggest the presence of RNAs with a distinctive 5′ DMG cap in *Trichomonas* and *Giardia* lineages that are absent in other protist lineages.

INTRODUCTION

Trichomonas vaginalis, an anaerobic protist that infects the urogenital tract of humans, is a divergent, deep-branching eukaryote (1,2). Transcriptional and post-transcriptional gene regulation in this and other unicellular protists is poorly understood. In *T. vaginalis*, transcription of protein coding genes is typically initiated only 5 to 20 nt upstream of the AUG translation initiator codon resulting in mRNAs with unusually short 5′ untranslated regions (UTRs) (3–6). *Trichomonas vaginalis* mRNAs possess 5′ cap structures and have 3′ poly(A) tails (7). Only few *T. vaginalis* pre-mRNAs are predicted to undergo splicing to remove a single intron (6,8). Recently, we have characterized *T. vaginalis* spliceosomal small nuclear RNAs (snRNAs) predicted to mediate pre-mRNA splicing. Although conserved in structure relative to other eukaryotic snRNAs, all five examined *T. vaginalis* snRNAs were found to lack a 5′ cap structure and appear to instead have 5′-monophosphate termini (7).

snRNAs, small nucleolar (sno) RNAs (9,10), telomerase RNA (11) and mRNAs that undergo trans-splicing in nematodes (12) typically contain a distinctive hypermethylated 5′-cap structure composed of a 2,2,7-trimethylguanosine (TMG) (13). TMG caps are formed by post-transcriptional dimethylation of monomethyl m^7G caps (14). In contrast, the 5′ ends of mRNAs are not subject to hypermethylation and retain an m^7G cap. The biological significance of hypermethylated cap structures remains unclear. In humans, m^7G cap snRNAs are first exported to the cytoplasm, Sm proteins then bind, followed by a two-step cap methylation to produce the

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mature snRNA TMG cap (15). The return of these snRNAs to the nucleus, required for their participation in pre-mRNA splicing, is dependent on the presence of both a TMG cap structure and Sm protein assembly (16). Alternatively, some snoRNAs are not exported to cytoplasm (17), and their caps become trimethylated in the nucleolus, mediated by specific snoRNA structural motifs (18,19). The human RNA cap hypermethylase is consistently found in both nuclear Cajal bodies and in the cytoplasm of mammalian cells (20,21). Contrary to the situation observed in humans, the yeast RNA cap hypermethylase resides exclusively in the nucleolus, indicating that yeast snRNA and snoRNA hypermethylation occur in this compartment (22). In *Trypanosoma cruzi*, it has been recently demonstrated that a functional Tgs1p localizes throughout the nucleoplasm and in spots outside the nucleolus (23)

Trimethylguanosine synthase (Tgs) is the enzyme responsible for converting an m^7G RNA cap to a TMG cap. *Saccharomyces cerevisiae* Tgs (ScTgs) is essential for hypermethylation of snRNAs and snoRNAs (22). Genetic depletion of ScTgs1p produces a cold-sensitive splicing defect that correlates with retention of the m^7G-capped U1 snRNA in the nucleolus (22). Homology modelling and mutagenesis studies of ScTgs1 have identified residues required for the formation of the m^7G-binding site and catalysis (24). Recombinant Tgs homologues from *Schizosaccharomyces pombe* (SpTgs) and the divergent protist *Giardia lamblia* Tgs2 (GlTgs) have likewise been biochemically characterized recently (25,26,27). These studies have shown that Tgs catalyses guanosine N2 methylation, requires *S*-adenosylmethionine (SAM)) as a methyl donor, has substrate specificity for m^7G nucleotides, and does not require any RNA or protein partners for catalysis.

Here, we have biochemically characterized a *T. vaginalis* Tgs (TvTgs) that is encoded by a single gene in the genome of this parasite. Contrary to that observed for SpTgs, but similar to that found for GlTgs (25), this *T. vaginalis* RNA cap hypermethylase catalyses only a single round of methylation on a m^7G cap to produce a 2,7-dimethylguanosine (DMG) instead of a TMG cap. In contrast, the predicted TMG cap was formed using recombinant Tgs from two other unicellular eukaryotes *Entamoeba histolytica* (EhTgs) and *Trypanosoma brucei* (TbTgs). Moreover, the end products of catalysis by TgS from the four different unicellular eukaryotes were the same whether an m^7G cap or a m^7G RNA transcript was used as substrate. The lack of 5′-end cap structures on *T. vaginalis* splicesomal snRNAs (7), typical substrates for Tgs, and the unusual methylation properties of both TvTgs and GlTgs suggest that an unidentified subset of RNAs with DMG cap structures are present in these divergent eukaryotes.

MATERIALS AND METHODS

Recombinant TvTgs expression and purification

The open reading frames encoding homologues of the *T. vaginalis* Tgs (TvTgs; GenBank™ accession number EAY18619), *G. lamblia* Tgs2 (GlTgs; GenBank™ accession number XP_001704513), *E. histolytica* Tgs (EhTgs; GenBank™ accession number XP_651698), *T. brucei* Tgs (TbTgs; GenBank™ accession number Tb11.02.5090) and *S. pombe* Tgs (SpTgs; GenBank™ accession number BAA13836) were cloned into the *Escherichia coli* expression vector pET200D (Invitrogen). Induction of expression and purification of the N-terminal His-tagged Tgs were carried out as described (25). Protein purity and concentration were evaluated by SDS–PAGE and densitometry using BSA standards and employing ImageJ software.

Methyltransferase activity assays and Thin-Layer Chromatography (TLC) analysis

With the use of nucleotide substrates, our standard methyltransferase reaction (10–20 µl) was performed at 37°C for 40 min in 50 mM Tris–HCl pH 7.5, 5 mM DTT, 1.25–5 µM [*methyl*-^{3}H]AdoMet, up to 10 mM of nucleotide substrate, using variable amounts of recombinant enzyme. Except when otherwise indicated, m^7GDP was the nucleotide substrate used. To measure substrate specificity, we utilized 5 µM [*methyl*-^{3}H]AdoMet, 0.5 mM of the specified nucleotide and 3.5 µg recombinant TvTgs. For the inhibition assay using the end product of AdoMet-dependent methyltransferases *S*-adenosyl-L-homocysteine (AdoHcy), 2.5 µM [*methyl*-^{3}H]AdoMet, 2 mM m^7GDP and 0.35 µg of recombinant TvTgs was used with an incubation time of 20 min. To determine optimal pH, we used 1.25 µM [*methyl*-^{3}H]AdoMet, 0.5 mM m^7GDP and 7.0 µg recombinant TvTgs and adjusted the pH using 50 mM Tris–acetate (pH 7.0 and below) or 50 mM Tris–HCl (pH 7.5 and above). When evaluating enzyme concentration and time required to reach saturation, 5 µM [*methyl*-^{3}H]AdoMet, 1 mM m^7GDP substrate and either 0.875 µg, 1.75 µg or 3.5 µg recombinant TvTgs were utilized in a reaction incubated for 60 min, with 10-min time points taken for analysis. TvTgs affinities for the nucleotide substrates m^7GDP, m^7GTP and m^7GpppA were measured by increasing concentration of each substrate. For the inhibition assay by GTP, reactions were done independently with one of the three methylated nucleotide substrates at 0.5 mM in the presence of increasing amounts of the inhibitor GTP. Aliquots of reactions, in triplicate, were then spotted on DEAE-cellulose filters (Whatman) and washed five times for 4 min each with 20-mM ammonium bicarbonate. Filters were air-dried and radioactivity was measured by liquid scintillation counting. Background was adjusted by subtracting the value obtained from a reaction without enzyme. As indicated aliquots of the reactions were also spotted on a PEI-cellulose TLC plates (Merck) and developed with 50 mM ammonium sulphate in one dimension (1D). TLC plates were treated with EN3HANCE (Perkin Elmer) and exposed to X-ray films (Biomax, Kodak). Relative migration of the methylated products were compared to products methylated by *G. lamblia* Tgs2 and *S. pombe* Tgs1 (25,14). Sodium periodate oxidation sensitivity was evaluated as previously described (25). The two sequential N2-methylation reaction protocol of Hausmann and

Shuman (25) was used to compare TvTgs, *S. pombe* Tgs1 and *G. lamblia* Tgs2.

For evaluation of methyltransferase activity by Tgs using a m^7G RNA substrate, T7 RNA polymerase promoter (sequence underlined below) was incorporated into a PCR product containing 352 bp of *T. vaginalis* β-tubulin (GenBank™ accession number XM_001582993) using TubF (GTC TCG GCA CAC TCC TTC TC) and TubR_T7 (TAA TAC GAC TCA CTA TAG GGA GAC GTG GGA ATG GAA CAA G) oligonucleotides. The gel-eluted PCR product was used as template for T7 RNA transcription (AmpliScribe T7-Flash Transcription™, Epicentre), purified by ProbeQuantTM G-50 Micro Column (GE Healthcare) and capped using Vaccinia Virus capping enzyme (Ambion) in the presence of [α-^{32}P]GTP. The ^{32}P-labelled m^7G cap RNA was then purified from unincorporated [α-^{32}P]GTP by three consecutive ProbeQuantTM G-50 Micro Column (GE Healthcare) leaving no detectable traces of unincorporated [α-^{32}P]GTP. Our standard methyltransferase reaction (10–20 µl) was performed at 37°C for 40 min in 50 mM Tris–HCl pH 7.5, 5 mM DTT, with 2 fmol of ^{32}P-labelled m^7G cap RNA substrate and 0.5 mM AdoMet and 1 µg of recombinant Tgs. The RNA was subsequently purified by phenol/chloroform extraction and ethanol precipitation and the modified cap structure was released by digestion with Tobbaco acid pyrophosphatase (TAP) (Epicentre).

To measure substrate specificity using the m^7G RNA substrate, increasing concentrations of AdoMet in a range of 0–3.2 mM, 0–0.78 mM and 0–5 mM were used for reactions analysing TvTgs, GlTgs and SpTgs, respectively. To calculate affinity of Tgs enzymes, reactions were analysed by 1D-TLC, as described above, and spots were quantified by liquid scintillation counting. For better analysis of the end products of reactions performed by GlTgs2, TvTgs, EhTgs and TbTgs, two-dimensional (2D) TLC was used. In addition, part of the reaction was further incubated with 1 µg of SpTgs at 37°C for 40 min, to drive the formation of a TMG. Plain cellulose TLC plates (Merck) were loaded with 1 µg of each one of the monophosphate ribonucleotide (AMP, CMP, GMP and UMP), and 400–1000 c.p.m. of the TAP-digested RNAs. Individual reactions were analysed on 10 × 10-cm TLC plates that were developed using either solvents A and B or A and C in a two-dimensional TLC system as described (28), and exposed to X-ray films (Biomax, Kodak). The composition of the solvents was: solvent A, isobutyric acid/concentrated ammonia/water [66/1/33 (v/v/v)]; solvent B, phosphate buffer/NH_4 sulphate/*n*-propanol [100/60/2 (v/w/v)]; solvent C, isopropanol/concentrated HCl/water [68/18/14 (v/v/v)].

Sucrose gradient sedimentation

Recombinant TvTgs was analysed by zonal velocity sedimentation in a sucrose gradient (29) and the molecular mass of the active methyltransferase activity was determined by assaying fractions using a m^7GDP substrate, as described above. Fifty micrograms of TvTgs was mixed with 50 mM Tris–HCl pH 7.5; 0.2 M NaCl; 1 mM EDTA, 2 mM DTT; 2% sucrose and 50 µg of the following protein standards: soy bean trypsin inhibitor (20 kDa), BSA (66 kDa) and catalase (248 kDa). The mix was loaded on the top of a 4–14% sucrose step gradient and centrifuged at 38 000 *g* for 16 h. Fractions (21 × 0.5 ml each) were collected from top to bottom of the gradient and analysed by SDS–PAGE and assessed for methyltransferase activity.

Structure modelling and mutagenesis

TvTgs sequence was compared with other Tgs sequences in the SWISSPROT database using the FASTA3 program (http://www.ebi.ac.uk/fasta33/). Secondary structure prediction was carried out by PROFSEC. The best templates for Tgs model construction were determined by threading methods, using Bio-Info Meta Server (http://bioinfo.pl/meta/) (30). In this site, the online services PDB-Blast (rscore 9e-46), FFAS03 (rscore -48.9), INUB (rscore 177.98) and 3DPSSM (rscore 3.7e-08) indicated that the *E. coli* HemK, a (N5)-glutamine methyltransferase (1T43) (31) was the best template for homology modelling. Using this template, ten models were constructed using MODELLER (32,33), and the PROSA II (34) was used to select the model with the most favourable packing and solvent exposure characteristics. After superposition of atomic coordinates, an energy minimization was done using Gromos96, a force field that predicts the dependence of a molecular conformation on the type of environment. This program calculates the relative binding constants by evaluating free energy differences between various molecular complexes using thermodynamic integration, perturbation and extrapolation. The software predicts energetic and structural changes caused by amino acid modifications using six subsequent rounds, minimizing backbone and side chains (3000 steps of steepest descent). Procheck (34) was used for additional analysis of stereochemical quality. Low PROSA II scores and high Procheck G-factors characterize high-quality models. Finally, Ramachandran plot and rmsd values were considered to validate the model. Based on the resulting structural model of TvTgs and the recent characterization of *S. cerevisiae*, *S. pombe* and *G. lamblia* Tgs (25,14,24,27), the following amino acid substitutions were made: S99I, S99R, D145E, D145R, S191R, P193A, W194A. Mutagenesis was introduced by PCR as described (36).

RESULTS

TvTgs is an AdoMet- and m^7G-dependent methyltransferase with strict substrate specificity

The *T. vaginalis* genome database (http://www.trichdb.org) was searched for putative Tgs genes and only a single gene (accession # XP_001579605) was found. This gene, which we call TvTgs, encodes a 32-kDa protein with 36% identity and 51% similarity to *S. pombe* Tgs (SpTgs), 34% identity and 46% similarity to *S. cerevisiae* Tgs (ScTgs) and 26% identity and 41% similarity to *G. lamblia* Tgs2 (GlTgs). For comparison, GlTgs shares 27% identity and 48% similarity to SpTgs, and 21% identity and 41% similarity to ScTgs. TvTgs contains the majority of the amino acids known to be necessary for AdoMet

dependent methyltransferase activity of ScTgs and GlTgs (25,14,27) as well as those essential for ScTgs1 substrate binding (24) (Supplementary Figure 1). The TvTgs transcript was detected in *T. vaginalis* mRNA by reverse transcription and PCR (data not shown).

To evaluate substrate specificity, the protein-coding region of the TvTgs gene was cloned with a histidine-tag (His-tag) at its N-terminus and subsequently expressed and purified from *E. coli* using nickel chromatography. Tgs transfers a *methyl*-^{3}H group from AdoMet to a nucleotide substrate producing an anionic methylated nucleotide that can be adsorbed to a DEAE filter and separated from the cationic AdoMet substrate. TvTgs was assayed for methyltransferase activity in the presence of [*methyl*-^{3}H]AdoMet and a variety of potential nucleotide substrates (Figure 1A). We found that TvTgs can use m^{7}GDP or m^{7}GTP as a substrate but not non-methylated nucleotides, including GTP. Similarly, a guanine nucleotide cap analogue can only serve as a substrate if it has undergone prior N7 methylation, while nonmethylated or m^{2,2,7}G cap analogues do not serve as substrates. Thus, TvTgs nucleotide substrate dependence matches that described for other Tgs (25,14). Additional analyses showed that TvTgs exhibits a bell-shaped pH dependence with optimal activity at pH 7.5 and highly reduced activity at pH 9.0 and pH 5.5 (data not shown). Methylation by TvTgs was also shown to increase with increasing enzyme concentration and time, and reactions reached saturation after 20–25 min of incubation with product formation reaching a stable plateau (data not shown). These findings mirror those previously reported for *S. pombe* and *G. lamblia* Tgs (25,14), consistent with the TvTgs gene encoding a functional Tgs.

A hallmark of AdoMet-dependent methyltransferases is the formation of AdoHcy resulting from donation of the methyl group from AdoMet to its acceptor nucleotide.

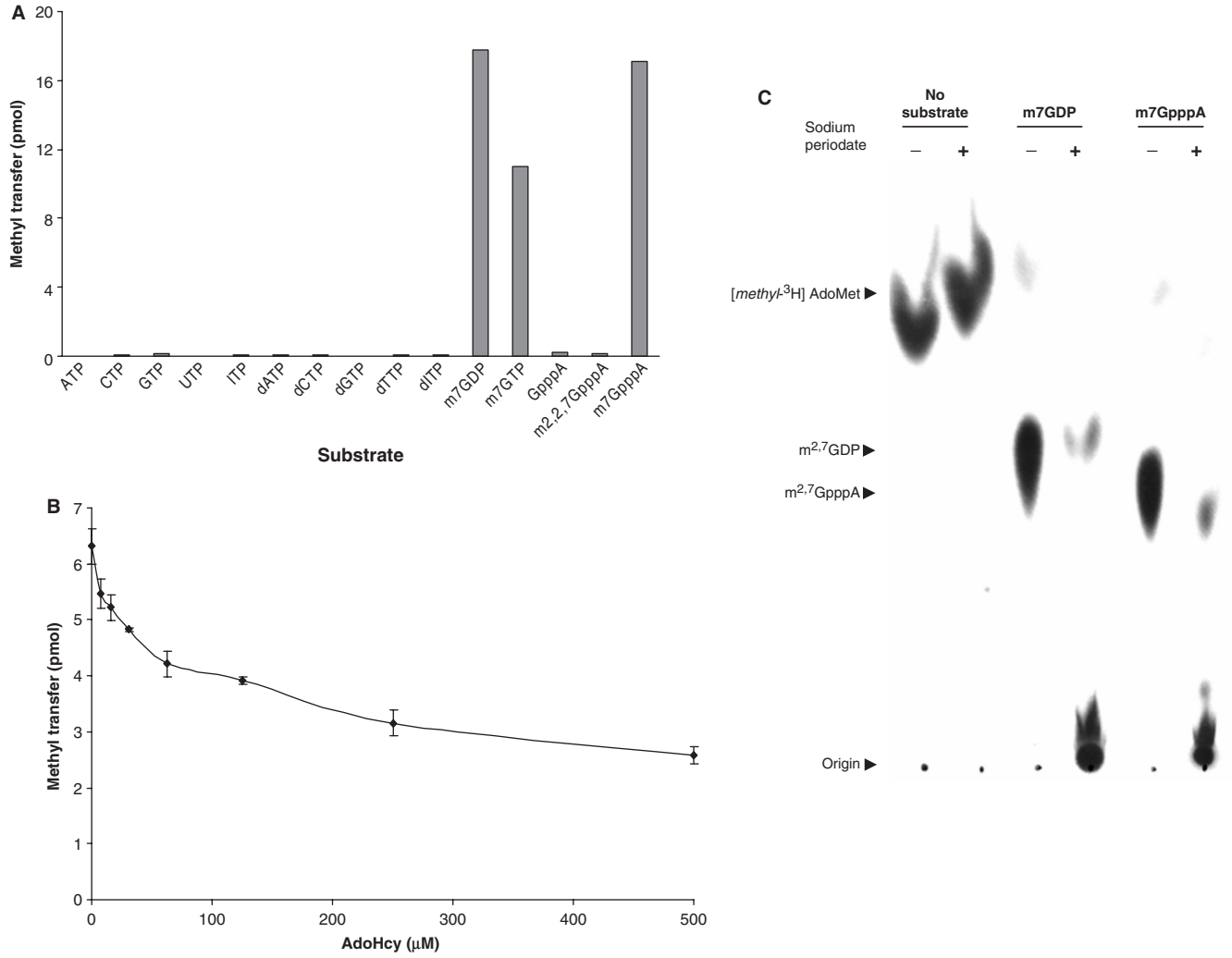

Figure 1. TvTgs is an RNA cap specific guanine N2 methyltransferase. **(A)** Nucleotide substrate specificity of TvTgs and **(B)** inhibition of TvTgs by AdoHcy. Reactions using [*methyl*-^{3}H]AdoMet as the methyl donor were conducted as described in 'Materials and methods' section. Methyltransferase activity was subsequently quantified by measuring ^{3}H incorporation into the reaction products using scintillation counting. **(C)** Sodium periodate sensitivity of the TvTgs methylated nucleotide product. Methylation reactions mediated by TvTgs using either m^{7}GDP or m^{7}GpppA as the nucleotide substrate (see 'Materials and methods' section) were further subjected to either 100 mM sodium periodate (+) or water (–). Products were spotted on DEAE-cellulose and subjected to TLC analyses. Arrows mark origin, substrate and products.

Thus, we tested whether AdoHcy is a product of this reaction by poisoning the enzyme with increasing amounts of AdoHcy. This was found to severely inhibit *T. vaginalis* methyltransferase with a half maximal (50%) inhibitory concentration value (or IC_{50}) of ~250 μM (Figure 1B), providing additional evidence that TvTgs is an AdoMet-dependent methyltransferase. To determine whether this enzyme is a specific guanine N2 and not a ribose O2′or O3′ methyltransferase, as predicted for a TgS (24), we evaluated the resistance of TvTgs methylated products to sodium periodate oxidation (Figure 1C). It has been shown that methylation at exocyclic N2 atom of m^7G leaves the ribose O2′ or O3′ sensitive to oxidation, whereas ribose methylation renders these sites resistant to oxidation. When the ribose site is available for oxidation, the opened-ring 2′,3′-dialdehyde forms a covalent Schiff base adduct that binds PEI-cellulose at the origin and does not migrate during TLC analysis, allowing the oxidation state of the m^7G ribose to be determined (25). As shown by TLC analyses, addition of 100 mM sodium periodate to reactions pre-incubated with m^7GDP or m^7GpppG, recombinant TvTgs and [*methyl*-^{3}H]AdoMet resulted in retention of the labelled product at the origin (Figure 1C). This was also observed using m^7GTP as the substrate (data not shown). The sensitivity of these TvTgs products to periodate oxidation indicates that ribose hydroxyls are not methylated, but instead that the N2 atom of m^7G is the methylation site. These results are in agreement with the lack of the catalytic signature (the tetrad KDKE) present in 2′-*O*-ribose methyltransferases (37), as observed in our structure model (see below), further confirming that this enzyme is a homologue of other characterized eukaryotic Tgs.

Nucleotide substrate preference of TvTgs

The substrate preference of TvTgs for transfer of [*methyl*-^{3}H]AdoMet was assessed. The methyl transfer activity of TvTgs was found to display a hyperbolic dependence on an m^7G substrate (Figure 2A). K_m values were calculated using m^7GDP, m^7GTP and m^7GpppG substrates and found to be 250 +/– 30.7 μM, 1.59 +/– 0.205 mM and 2.23 +/– 0.327 mM, respectively. These results indicate that TvTgs has a higher affinity for m^7GDP. The observed specificity of TvTgs for these nucleotides substrates is similar to *S. pombe* Tgs but significantly lower than that reported for the *G. lamblia* enzyme (25,14). m^7G-dependent methyltransferases bind to non-methylated nucleotides with reduced affinity without promoting N2 methylation (14). Thus we tested whether GTP inhibits TvTgs binding and methylation of the three m^7G substrates and compared inhibition levels (Figure 2B). Under our assay conditions, we observed that the higher the K_m value, the higher the inhibition by GTP. This result indicates a greater TvTgs affinity for m^7GDP than m^7GTP and/or m^7GpppG. Contrary to *G. lamblia* Tgs2 (25), a γ-phosphate substrate (m^7GTP) and a 5′-nucleoside substrate (m^7GpppG) decreases TvTgs activity by 6- and 9-fold, respectively, as suggested by their relative K_m values.

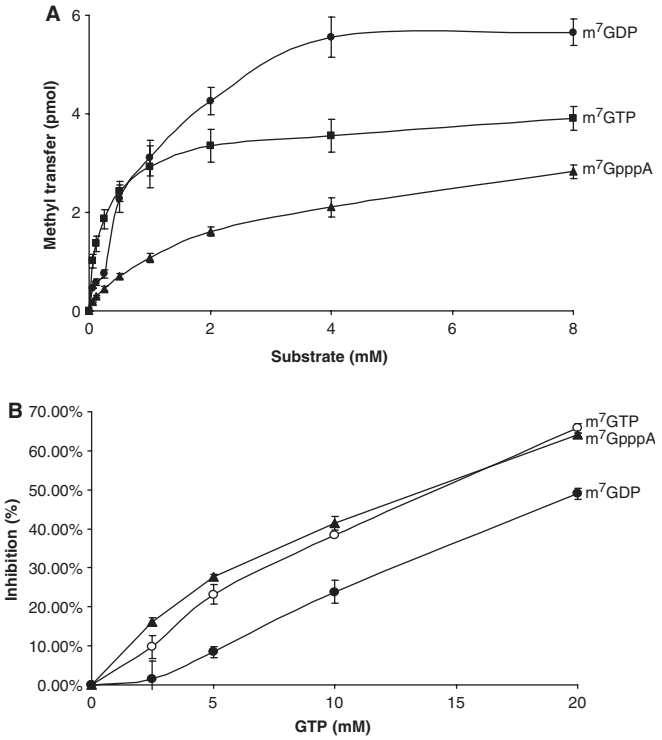

Figure 2. Nucleotide preference of TvTgs. **(A)** The relative affinity of TvTgs for m^7GDP, m^7GTP and m^7GpppA, indicated by the K_m values, was measured as described in 'Materials and methods' section. **(B)** Inhibition of TvTgs activity by increasing amounts of GTP using equivalent concentrations of the three substrates (indicated on the right) was determined.

Structure-function analysis of TvTgs

Comparing structural modelling and mutagenesis data from *S. cerevisiae* and *G. lamblia* Tgs (25,24,27) and *E. coli* HemK (1T43), a (N5)-glutamine methyltransferase (31), to that predicted for TvTgs, showed secondary structural similarity and conservation of critical amino acids (Figure 3A). Using *E. coli* HemK (1T43) as a model, a three-dimensional structure of TvTgs can be predicted including the identification of putative catalytic and donor sites (Figure 3A, bottom). The 280-amino-acid TvTgs is predicted to contain two structural domains: a four-helix N-terminal bundle (residues 1–64), and a C-terminal catalytic domain with a seven-stranded β-sheet (residues 65–280), characteristic of methyltransferases. Also as predicted a β-hairpin, which interacts with the C-terminal domain via hydrogen bonds and a salt bridge, connects the two domains (Figure 3A, top).

ScTgs belongs to a large family of Rossmann-fold AdoMet-dependent methyltransferases (24,38). Amino acids in motif I conserved among different Tgs, are involved in AdoMet binding, including glycine residues which are also present in *E. coli* HemK (31) (Supplementary Figure 1) and 2′-*O*-ribose methyltransferases (37). Catalytic amino acids within the nine Rossmann-fold AdoMet-dependent methyltransferase motifs can vary among different types of methyltransferase. For example, 2′-*O*-ribose methyltransferases have the catalytic tetrad KDKE distributed within motifs X, IV, VI and VIII (37).

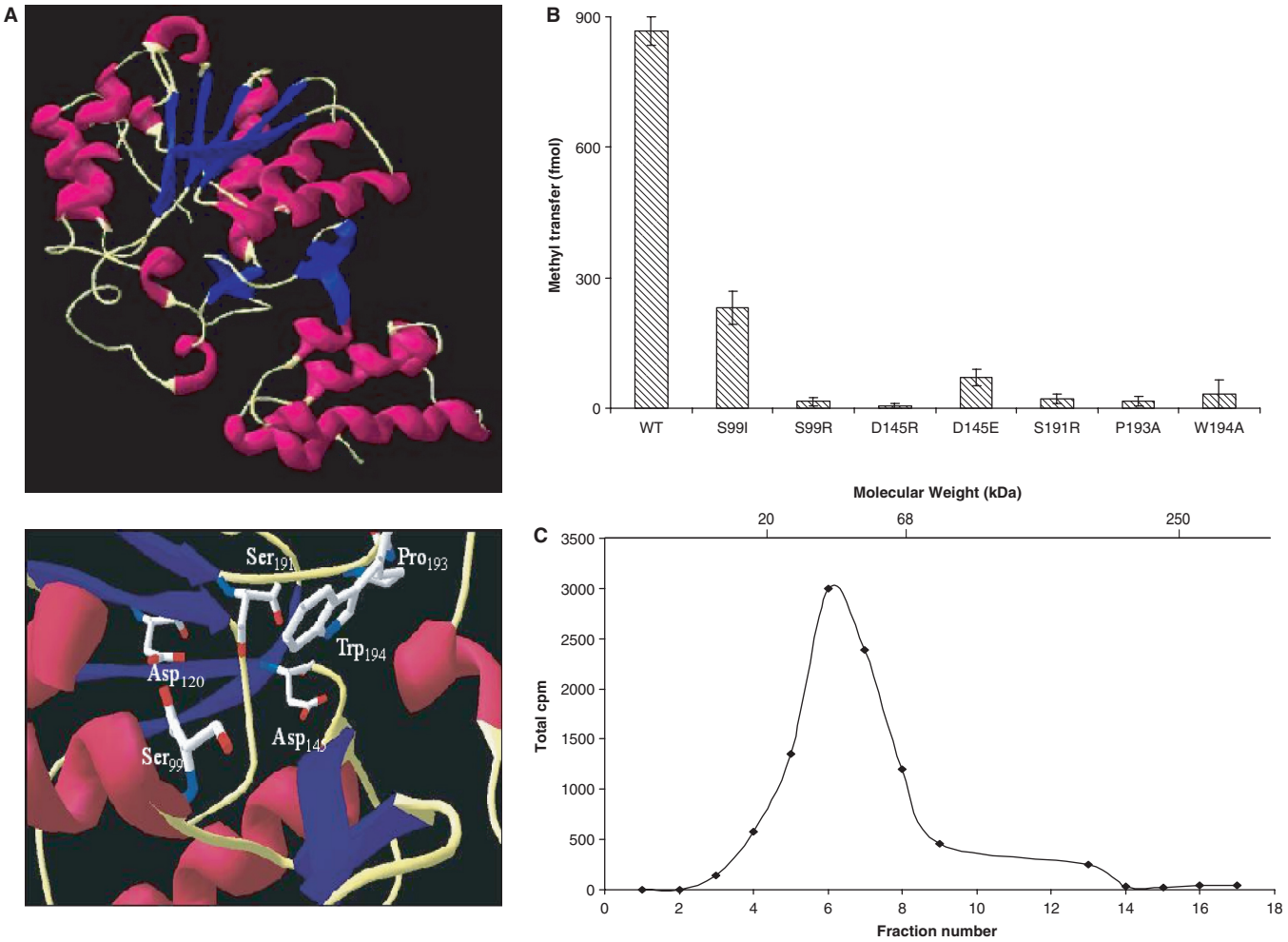

Figure 3. Structure–function analysis of recombinant TvTgs. **(A)** A ribbon representation of the predicted TvTgs structure (top) and predicted catalytic and donor sites (bottom) indicated by homology modelling are shown. Conserved amino acids known to be indispensable for *S. cerevisiae* TvTgs activity (22,24) are presented (bottom). **(B)** Methyltransferase activity was measured using saturating amounts of m^7GDP (10 mM) as substrate and equivalent amounts of wild-type (WT) and mutant TvTgs proteins as indicated. **(C)** Zonal velocity sedimentation analysis of TvTgs. Recombinant TvTgs and internal molecular weight standards (20-kDa soybean trypsin inhibitor, 66-kDa bovine serum albumin and 250-kDa catalase) were fractionated on a sucrose gradient. Aliquots of each fraction were then tested for methyltransferase activity, using [*methyl*-^{3}H]AdoMet as the methyl donor. The peaks for separation of the protein standards, as determined using SDS–PAGE, are indicated at the top.

This tetrad is not observed in any described Tgs, including TvTgs (Supplementary Figure 1). Specific amino acids necessary for catalysis of ScTgs were recently determined (22,24). Many are in motif IV and are conserved among Tgs homologues. Based on our structure model, all of these except TvTgs S99 (ScTgs I83) are conserved in TvTgs (Supplementary Figure 1; Figure 3A, bottom). These include two aspartate residues involved in both water-mediated coordination of the methionine moiety of AdoMet and binding to the 2′- and 3′-OH groups of the ribose (ScTgs D103 and D126; TvTgs D128 and D145), and serine and proline catalytic residues (ScTgs S175 and P177; TvTgs S191 and P193) (Figure 3A, bottom). A tryptophan important for stabilization of the target base (ScTgs W75) was reported to be invariant in all eukaryotic homologues of Tgs except *Plasmodium*; however, it is not required for ScTgs activity (24). Like *Plasmodium*, TvTgs and GlTgs also contain a neutral non-polar amino acid substitution at this position (Supplementary Figure 1), consistent with ScTgs W75 not being necessary for catalysis, binding or correct folding of the protein (24).

To test whether these conserved amino acids are required for TvTgs activity, residues were individually mutated, and mutant proteins were subsequently expressed and purified from *E. coli*. In each case, mutation resulted in either loss of or significantly diminished activity (Figure 3B). TvTsg mutants D145R, D145E, S191R, P193A and W194A were found to be inert in methyl transfer catalysis, consistent with these residues working cooperatively for substrate binding and catalysis (24). A conserved amino acid found in the m^7G-binding pocket, shown to be required for yeast Tgs activity, ScTgs I83, (24) is absent in TvTgs. Instead a polar residue, TvTgs S99, has replaced the equivalent isoleucine or valine found in all other reported eukaryotic Tgs homologues.

A polar amino acid substitution at this position is also seen in GlTgs (GlTgs Y44). Interestingly, mutation of TvTgs S99 and subsequent analysis of the mutant protein demonstrated it is critical for TvTgs activity, as S99R lost methyl transfer activity and S99I had highly reduced activity (Figure 3B).

Finally, we determined the molecular mass of the active, recombinant form of TvTgs, by zonal velocity sedimentation in a 2–14% sucrose gradient using native protein markers soybean trypsin inhibitor (20 kDa), bovine serum albumin (66 kDa) and catalase (250 kDa) as internal standards. The majority of His-tagged TvTgs sedimented in fractions 4–8 between the 20- and 66-kDa markers (data not shown). Fractions were tested for methyltransferase activity (Figure 3C) and the peak of activity was found in a fraction corresponding to a molecular mass of ~35.1 kDa, in agreement with the predicted mass (~36 kDa) of His-tagged TvTgs (Figure 3C). Thus, the active form of TvTgs appears to be monomeric, as previously described for other Tgs (25,14).

TvTgs and GlTgs are unique dimethylguanosine synthases

Synthesis of TMG *in vitro* occurs in two steps via a distributive mechanism. Using m^7G as a substrate for methylation, first DMG is formed, which must then dissociate and compete with m^7G for rebinding the enzyme to allow a second methylation forming TMG. To complete the reaction, it is necessary to add a large molar excess of AdoMet after the first methylation reaction reaches saturation (14). The recent observation that under these conditions *G. lamblia* Tgs2 can catalyse the addition of only one methyl group to m^7G to form DMG (25) led us to evaluate whether TvTgs can produce both DMG and TMG. To this end, we expressed and purified His-tagged TvTgs, *S. pombe Tgs* and *G. lamblia* Tgs2 and compared the ability of the three enzymes to form DMG and TMG. Following a first round of methylation using 5 μM [*methyl*-^{3}H]AdoMet and 0.5 μg of one of the three recombinant Tgs, the reaction was supplemented with 3.2 mM of unlabelled [*methyl*]AdoMet and 1 μg of the respective enzyme and the incubation was continued. Equal amounts of both reactions, using the three enzymes, were then subjected to PEI-cellulose TLC analyses. TvTgs was found capable of catalyzing only one round of methylation, similar to *G. lamblia* Tgs, and in contrast to *S. pombe* Tgs, which is capable of two consecutive rounds of methylation (Figure 4A). We then determined whether the DMG formed by TvTgs catalysis could act as a substrate for additional methylation, upon addition of *S. pombe* Tgs. After its formation by incubation with TvTgs, *S. pombe* Tgs was added during the second incubation in the presence of excess unlabeled [*methyl*]AdoMet. Under these conditions, TMG is formed (Figure 4B). Conversely, replacing *S. pombe* Tgs with TvTgs during the second round of incubation after formation of DMG by the former enzyme did not support TMG formation. Together, these data strongly indicate that TvTgs is not a trimethylguanosine synthase, as typically found in other eukaryotes, but is instead a dimethylguanosine synthase.

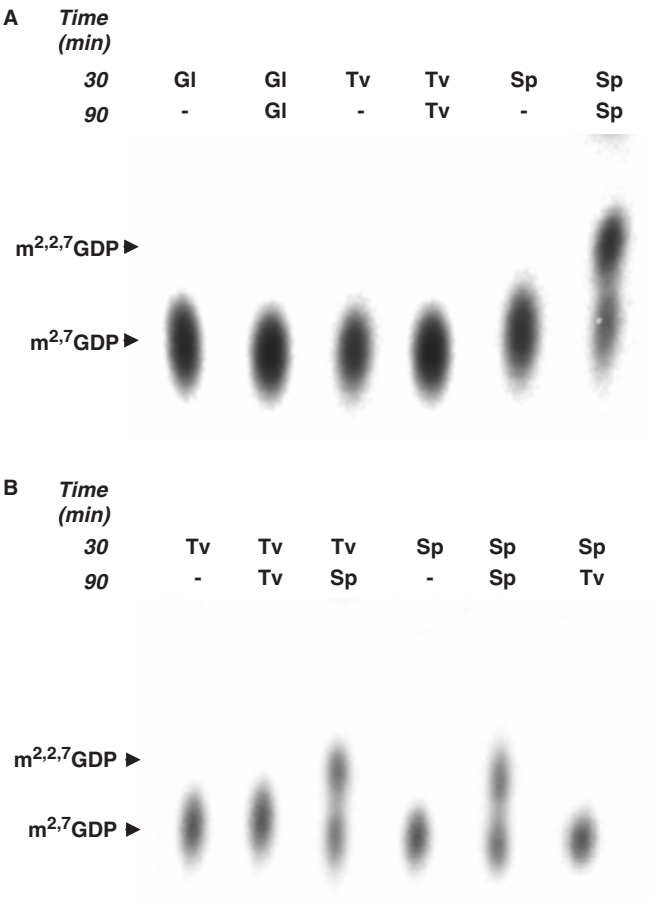

Figure 4. Comparison of the distributive mechanism of RNA cap methyltransferase activity by *S. pombe Tgs*, *G. lamblia* Tgs2 and TvTgs. **(A)** Methyltransferase reactions (see 'Materials and methods' section) were first conducted for 30 min with 5 μM [*methyl*-^{3}H] AdoMet, 0.1 mM m^7GDP and 0.5 μg of the recombinant Tgs enzyme listed at the 30-min time. The reaction was then supplemented with 3.2 mM of unlabelled [*methyl*]AdoMet and 1 μg of the respective recombinant enzyme followed by incubation for an additional 60 min at 37°C, listed at the 90-min time. Equivalent amounts of the first and second reactions were then subjected to TLC analyses as described in Figure 1 legend and in 'Materials and methods' section. In this experiment, TvTgs (Tv), GlTgs (Gl) and SpTgs (Sp) were compared side by side. **(B)** Methyltransferase reactions, conducted as described in (A) above, contained either TvTgs (Tv) or SpTgs (Sp) during the first 30-min incubation, as indicated at the 30-min time. TvTgs reactions were then supplemented with either TvTgs or SpTgs, as indicated for the additional 60-min incubation step (90 min). SpTgs reactions were also supplemented with either SpTgs or TvTgs, as indicated for the additional 60-min incubation step (90 min). Reaction products were analysed using TLC as described above. Methylated products are indicated and marked by arrows.

The inability of TvTgs or GlTgs to convert m^7G to TMG could be attributed to the lack of an appropriate substrate. Therefore, instead of using nucleotide substrates, we prepared transcripts that have a [^{32}P]m^7G cap structure using the Vaccinia Virus capping enzyme (VVC). The m^7G cap RNA substrate was then incubated with the Tgs, and products were analysed by TLC following RNA purification and tobacco acid pyrophosphatase (TAP) treatment to release the guanosine cap. As predicted, we observed that RNA that is not treated with VVC (to generate m^7G) does not serve as a substrate for TvTgs,

GlTgs or SpTgs (data not shown). In contrast the m^7G-capped RNA substrate was utilized by all three Tgs with higher affinity than mononucleotides, as suggested with the K_m values of 54.8 +/– 4.3 μM, 9.4 +/– 0.8 nM and 34.5 +/– 15.4 nM calculated for TvTgs, GlTgs and SpTgs, respectively (data not shown). To further examine the cap structure formed when incubating an m^7G-capped RNA substrate with TvTgs, we subjected the end products of these assays to 2D-TLC analyses, using two different solvents in the second dimension (Figure 5A and B). In addition to testing TvTgs, we included Tgs from other unicellular parasites, *G. lamblia* (GlTgs), *E. histolytica* (EhTgs) and *T. brucei* (TbTgs) to compare TgS specificity among unicellular eukaryotes (Figure 5). The use of the higher avidity, ^{32}P-labelled mRNA substrate in these assays improved detection of Tgs activity and allowed us to determine whether di- and trimethylguanosines end products were formed by comparing their migration with mono, di and trimethylguanosine standards (Figure 5A and B, columns 1, 3 and 5). A 1000-fold molar excess of the methyl-donor substrate AdoMet relative to the ^{32}P-labelled m^7G RNA acceptor substrate was used to drive the methyltransferase reaction to completion. Reaction products were then either directly analysed using 2D-TLC following TAP treatment to remove the cap structure (Figure 5A and B, columns 2 and 3) or were also further incubated with SpTgs to drive formation of a TMG prior to TAP treatment and 2D-TLC analyses (Figure 5A and B, columns 4 and 5). Our results clearly demonstrate that TvTgs and GlTgs are capable of catalysing the formation of a DMG, but not a TMG cap structure. Furthermore, these DMG caps can be converted to TMG upon subsequent incubation with SpTgs, confirming its identity as a *bona fide* DMG. Interestingly, we found that the ability to form only a DMG cap is limited to *G. lamblia* and *T. vaginalis* as both *E. histolytica* and *T. brucei* Tgs can convert a m^7G capped RNA to a TMG capped RNA as observed for *S. pombe* and thought to be standard for eukaryotic Tgs. The ability of TvTgs to promote dimethylation of m^7G cap RNAs and our previous observation that *T. vaginalis* spliceosomal snRNA are not endogenous substrates for TvTgs (7) suggests that *T. vaginalis* may contain a novel subset of DMG capped RNAs.

DISCUSSION

We have identified and characterized a homologue of Tgs, an RNA cap-specific m^7G-dependent N2 methyltransferase, in the divergent eukaryotic parasite *T.* vaginalis. This RNA methyltransferase, called TvTgs, can utilize a variety of m^7G substrates, with different affinities, to form a $m^{2,7}G$ cap and displays a strict dependence on prior methylation of guanine N7, a unique property of Tgs. Substrate affinity, inhibition by AdoHcy and sensitivity to sodium periodate oxidation indicate that TvTgs is a N2 guanine methyltransferase. As previously shown for GlTgs and SpTgs, recombinant TvTgs can methylate m^7G nucleotides to form a DMG cap in the absence of any RNA or protein co-factor. Both TvTgs and GlTgs also convert the m^7G cap on an RNA substrate to a DMG cap, having a significantly higher affinity for this substrate relative to nucleotide substrates.

The predicted structure and key amino acids in the active site that are required for activity of yeast and *G. lamblia* Tgs (25,24,27) are conserved in TvTgs. Mutagenesis of selected residues results in a substantial decrease or loss of enzyme activity, similar to that observed for *S. cerevisae* Tgs (ScTgs). A significant difference observed in the predicted TvTgs m^7G-binding pocket is the presence of a serine (TvTgs S99) replacing an isoleucine (ScTgs I83). Except for GlTgs which also contain a polar residue at this position (GlTgs Y44), the corresponding residue in all previously characterized Tgs is either an isoleucine or valine (24). All three of these residues are short-chain amino acids; however, serine (TvTgs S99) and tyrosine (GlTgs Y44) are polar whereas isoleucine (ScTgs I83) and valine (SpTgs V59) are non-polar. In *T. vaginalis*, replacement of this serine residue with an isoleucine in TvTgs resulted in greatly reduced activity. In *S. cerevisiae*, replacement of ScTgs I83 to a polar arginine did not rescue cold-strain phenotype and did not lead to formation of $m^{2,2,7}G$ cap sn(o)RNAs in the yeast mutant (26).

Recombinant SpTgs is an RNA methyltransferase capable of transferring *in vitro* two methyl groups to N2 of a guanosine previously methylated at the N7 position (25,24,27). Despite similarity in predicted structure and conserved catalytic residues, recombinant TvTgs is limited to a single round of N2 methylation forming a 2,7-dimethylguanosine as end product. 2D-TLC analyses using high avidity and ^{32}P-labelled m^7G cap RNA substrate indicate the complete absence of a trimethylated guanosine as an end product for either TvTgs or GlTgs. It is notable that *T. vaginalis* and *G. lamblia* Tgs are similar in this regard and divergent from all other characterized eukaryotic Tgs, including *E. histolytica* and *T. brucei* Tgs analysed here, which catalyse the formation of an $m^{2,2,7}G$ cap (TMG).

Our data do not preclude the presence of additional *in vivo* factors that might drive TvTgs and GlTgs to a second round of methylation or additional Tgs genes missing from the *T. vaginalis* genome database (6) that are capable of two rounds of methylation. Indeed, there are two Tgs genes in *G. lamblia*, only one of which (Tgs2) has been characterized as a dimethylguanosine synthase (25) while the other (Tgs1) remains uncharacterized. *Giardia* has been reported to have snRNAs with trimethylguanosine (TMG) caps (39), consistent with the uncharacterized *G. lamblia* Tgs1 being the trimethylguanosine synthase responsible for the formation of TMG cap structures.

We have recently demonstrated that *T. vaginalis* snRNAs are uncapped and contain free phosphates at their 5′ ends (7). Other small RNAs in *T. vaginalis*, such as intronic snoRNAs, also appear to lack TMG caps (our unpublished data). The lack of a classical Tgs in *T. vaginalis* is consistent with the lack of snRNAs with TMG caps. This, nevertheless, begs the question of what RNA population(s) is the target of TvTgs in *T. vaginalis*. What is the consequence of these RNAs containing

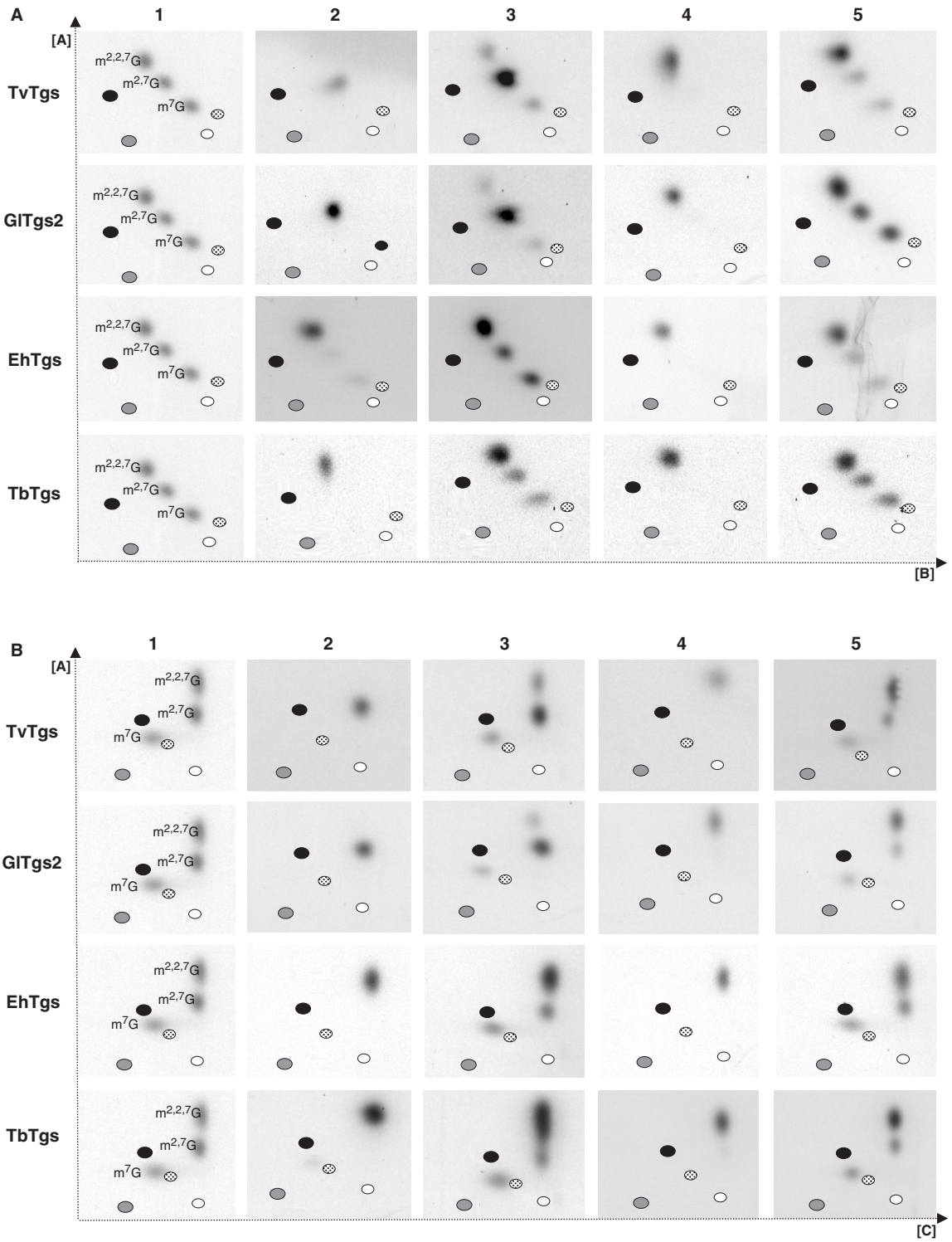

Figure 5. 2D-TLC analysis of cap structures formed using Tgs from four unicellular parasites. The first dimension was run using solvent A (28; see 'Materials and methods' section) and the second dimension used either solvent B or solvent C as shown in (**A**) or (**B**), respectively. ^{32}P-labelled m^{7}G cap RNA was the substrate for methyltransferase activity with recombinant Tgs from *T. vaginalis* (TvTgs), *G. lamblia* (GlTgs2), *E. histolytica* (EhTgs) and *T. brucei* (TbTgs) as indicated on the left. The guanosine capped end-products of these reactions were then immediately released by TAP treatment (columns 2 and 3) or were further incubated with *S. pombe* TgS (SpTgS) prior to release by TAP treatment (columns 4 and 5). Mono, di and trimethylguanosine standards were included as markers to reveal the relative migration of these cap structures (column 1). Black, grey, white and dotted ovals also indicate migration of the four monophosphate nucleotides—AMP, GMP, UMP and CMP, respectively. Samples were analysed in the absence (columns 2 and 4) or the presence of mono, di and trimethylguanosine standards (column 3 and 5) for clarity. In the latter case, the cap structure formed resulted in an increased intensity of the corresponding cap standard.

dimethylguanosine 5′ caps? What is the subcellular localization of these RNAs?

Sindbis and Semliki Forest eukaryotic viruses are reported to contain a significant fraction of 2,7-dimethylguanosine caps (40,41). In addition, $m^{2,7}G$ cap reporter mRNAs are translated with a higher efficiency than m^7G cap transcripts *in vitro*, whereas $m^{2,2,7}G$ cap transcripts are translated with very low efficiency (42–44). Recently, two eukaryotic translation initiation factor 4Es (eIF4E) were found in *G. lamblia* and shown to have preferential affinity for either m^7G or $m^{2,2,7}G$ caps. Knockdown of the eIF4E that specifically binds a m^7G cap blocked translation, whereas *Giardia* $m^{2,2,7}G$ cap mRNAs transfected into the parasite were not translated, consistent with *G. lamblia* mRNAs containing a 5′ m^7G cap (45). In light of our data and those demonstrating the presence of a dimethylguanosine synthase in *G. lamblia* (25) it would be interesting to determine whether either of the *G. lamblia* eIF4E proteins bind to $m^{2,7}G$ capped RNA and whether similar eIF4E proteins that bind $m^{2,7}G$ capped RNAs are present in *T. vaginalis*. Although both *G. lamblia* and *T. vaginalis* mRNAs were shown to be capped (7,26), their cap structure is not known.

The ability of the TvTgs studied here and the previously described *G. lamblia* Tgs2 (25) to catalyse only a single round of methylation is reminiscent of a subset of tRNA-specific N2 methyltransferases that execute only one cycle of methylation producing 2-methylguanosine and the rRNA-specific guanine-N2 methyltransferase, RsmC, which is also limited to a single round of methylation (47–51). tRNA-specific N2 methyltransferases are found in archaea and eukaryotes (51); whereas RsmC has only been reported in eubacteria (52,53). For example, the tRNA methyltransferase Trm-G10 from the archeae *Pyrococcus abyssi* exhibits a characteristic Rossmann fold and a SAM-dependent methyltransferase domain, both of which are found in archea and eukaryota and are absent in eubacteria (50). The Tgs gene family appears to be present exclusively in eukaryotes; however, an uncharacterized family of methyltransferases from archaea and Gram-positive bacteria exhibits significant similarity to ScTgs (24). The relationship of Tgs in divergent eukaryotes is not well resolved in evolutionary trees (24) making it difficult to trace their relatedness and evolutionary origin. Nonetheless, given the common features shared by TvTgs, *G. lamblia* Tgs2 and several eubacterial and archaeal methyltransferases, it is tempting to speculate that TvTgs and *G. lamblia* Tgs represent the basal state of eukaryotic Tgs (one capable of only a single round of methylation; a dimethylguanosine synthase) and that the acquisition of catalysing a second round of methylation, as observed in yeast, is a derived state. Alternatively, TvTgs and *G. lamblia* Tgs may have once been capable of generating an $m^{2,2,7}G$ cap independent of accessory factors, as observed for yeast Tgs, but have evolved to require additional cytosolic factors in the current state. An understanding of why purified Tgs from these basal unicellular eukaryotes acting as dimethylguanosine synthases and not trimethylguanosine synthasese awaits further analyses.

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