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Analysis of Flavivirus NS5 Methyltransferase Cap Binding

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Summary

The flavivirus NS5 N-terminal RNA methyltransferase (MTase) enzyme is responsible for methylating the viral RNA cap structure. To increase our understanding of the mechanism of viral RNA cap binding we performed a detailed structural and biochemical characterization of guanosine cap binding pocket of the dengue (DEN) and yellow fever (YF) virus MTase enzymes. We solved an improved 2.1 Å resolution crystal structure of DEN2 MTase and new 1.5 Å resolution crystal structures of the YF virus MTase domain in apo form and new 1.45 Å structure in complex with GTP and RNA cap analog. Our structures clarify the previously reported DEN MTase structure, suggest novel protein–cap interactions, and provide a detailed view of guanine specificity. Furthermore, the structures of the DEN and YF proteins are essentially identical, indicating a large degree of structural conservation amongst the flavivirus MTases. GTP analog competition assays and mutagenesis analysis, performed to analyze the biochemical characteristics of cap binding, determined that the major interaction points are (i) guanine ring via π – π stacking with Phe24, N1 hydrogen interaction with the Leu19 backbone carbonyl via a water bridge, and C2 amine interaction with Leu16 and Leu19 backbone carbonyls, (ii) ribose 2' hydroxyl interaction with Lys13 and Asn17, and (iii) α -phosphate interactions with Lys28 and Ser215. Based on our mutational and analog studies, the guanine ring and α -phosphate interactions provide most of the energy for cap binding, while the combination of the water bridge between the guanine N1 and Leu19 carbonyl and the hydrogen bonds between the C2 amine and Leu16/Leu19 carbonyl groups provide for specific guanine recognition. A detailed model of how the flavivirus MTase protein binds RNA cap structures is presented.

Keywords

Flavivirus; NS5 Methyltransferase; RNA cap binding; Guanine recognition; K_d determination

Introduction

Flavivirus genomes consist of a 5' capped 10.7–11.0 kilobase positive-strand RNA that lacks a poly(A) tail¹. Viral proteins are translated directly from the genomic RNA in a cap-dependent manner to produce a single polyprotein that is co-translationally cleaved into 10 mature

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proteins. Insect-borne flaviviruses require the 5' RNA cap structure for efficient translation of the viral polyprotein. The endogenous mRNA capping machinery is located in the nucleus, while flavivirus RNA replicates in endoplasmic reticulum derived replication compartments in the cytoplasm. Because flaviviruses do not have access to the cellular capping machinery, they have evolved their own capping enzymes to form a cap structure that can be recognized by the cellular translation machinery. Flavivirus genomic RNA is modified at the 5' end by a cap-1 structure (me⁷-GpppA-me²) generated by the virus encoded nucleotide triphosphatase (NS3), 2'-O-nucleoside methyltransferase (NS5), guanine-N7-methyltransferase (NS5), and as yet unidentified guanylyltransferase ^{2; 3; 4; 5; 6; 7; 8}. The structure of the dengue, West Nile, Murray Valley encephalitis (MVEV), and Mebean virus NS5 N-terminal 2'-O- / Guanine N7-methyltransferase domain (from now on referred to as MTase) have been solved ^{5; 6; 9; 10; 11; 12; 13; 14}. The MTase protein binds the guanosine nucleoside and phosphates of the viral RNA cap structure and sequentially transfers a methyl group from S-adenosyl methionine first to the N7 position of the guanosine cap and then to the 2' hydroxyl position on the ribose of the first viral genomic RNA nucleotide to form the cap-1 structure ^{8; 9}. Cap-1 modified mRNAs are specifically recognized by the translation initiation factor eIF4E in combination with eIF4A and eIF4G to form the eIF4F cap binding complex ¹⁵. Ribosome-mediated translation cannot occur on many mRNA species without specific recognition of me⁷-GpppN by eIF4E, and it has been suggested that efficient ribosome recruitment is enhanced by the 2'-O-methylation of cap 1 modified mRNAs ^{16; 17}. Mutation of flavivirus MTase residues that interact with the guanosine base of the viral RNA cap is lethal to viral replication ^{8; 18; 19}, indicating that MTase guanosine binding is essential to viral growth. The essential nature of this cap-binding pocket for RNA replication makes it an ideal target for drug development, and a thorough understanding of how the MTase recognizes and binds cap structures is necessary to facilitate rational drug design efforts.

Limited analysis of flavivirus MTase cap binding has been performed ^{5; 20}, but a thorough description of the protein and cap components of binding is lacking. To increase our understanding of how the flavivirus MTase binds RNA cap structures, we have performed a detailed analysis of the guanosine cap binding characteristics of the dengue (DEN) and yellow fever (YF) MTase enzymes. We have solved the three dimensional structure of the YF MTase domain in the apo form at 1.5 Å resolution and complexed with GTP and cap analog at 1.45 Å resolution. We used this high-resolution structural information to guide our assessment of the critical structural components of guanosine binding using solution-based competition assays with GTP analogs and mutational analysis. We show the relative contributions of both known amino acid/cap interactions as well as several novel interactions not previously described. We also evaluated the functional components of GTP necessary for strong interaction with the cap binding site and determined the detailed mechanism for cap guanine recognition by flavivirus MTases. The results of these experiments expand our knowledge of the cap binding site in flavivirus MTase enzymes, and provide a foundation for the rational development of potent and specific antivirals active against flaviviruses. Furthermore, as many of the amino acids involved in MTase activity appear to be conserved across the entire flavivirus genus, antivirals designed to bind to this site may possess significant broad-spectrum activity against multiple flaviviruses.

Results

Structures of MTase and Complexes

The YF MTase domain has a compact globular fold where one face of the protein is involved in ligand binding as judged by strong electron density for a bound Ado-Hcy coproduct that co-purified with the protein and the binding site for GTP (Figure 1a). The structure is highly homologous to those of the dengue fever and West Nile virus proteins ^{5; 8; 11}, with structural

differences being limited to small changes in the conformation of loop residues, as shown by the maximum-likelihood based multiple structure superpositioning²¹ (Figure 1b). Crystal soaking experiments with GTP and GpppA resulted in clear positive difference density for these ligands in a surface exposed pocket located ~15 Å away from the Ado-Hcy coproduct across one face of the protein (Figure 1a). Definitive density was observed for the guanine and ribose groups while phosphate density was generally limited to the α and β phosphate groups, except for a very interesting stacked conformation of the GpppA cap analog as discussed below. Altogether, there are surprisingly few nucleotide specific interactions in the MTase-GTP complex and it is difficult to discern obvious reasons for GTP specificity in the binding pocket by inspection of the structure.

The dominant structural interaction between the bound GTP and the protein is a π - π stacking interaction with Phe24 that positions the nucleotide base moiety on the surface of the YF MTase and locks the ribose into a shallow hydrated binding pocket (Figure 2). This stacking interaction results in a small shift in the position of the α -helix composed of residues 24–34 upon ligand binding (Figure 1b). The exocyclic C2 amine of the guanine forms a pair of hydrogen bonds with the backbone carbonyls of Leu16 and Leu19 in a loop of the protein at the outer edge of the GTP binding pocket. In addition, all the ligand-bound structures show a well-ordered water molecule that is hydrogen bonded between the N1 nitrogen on the guanine ring and the backbone amide of Leu19 (labeled “W” in Figure 2). This water appears to be an integral part of the nucleotide bound complex because there is no electron density evidence whatsoever for it in the apo structure. There is a potential interaction between the exocyclic O6 atom and Lys21 that could provide a guanine specific recognition point, but the lysine sidechain is in fairly weak density and thus this does not appear to be a particularly strong interaction, consistent with our binding data from mutagenesis experiments.

The ribose moiety is involved in several hydrogen bonds to protein residues and bound waters (Figure 2). The outside of this pocket is composed of three consecutive serine residues (150–152) that are all involved in ribose-triphosphate interactions. Ser150 forms a hydrogen bond with the β phosphate group, the backbone carbonyl of Ser151 helps to position Lys13 for interactions with the 2' and 3' ribose hydroxyls, and the backbone carbonyl of Ser152 is hydrogen bonded to the sidechain nitrogen of Asn17, positioning the Asn17 sidechain oxygen for a hydrogen bond with the 2' hydroxyl. The GTP phosphate groups extend out over the surface of the protein toward the Ado-Hcy binding site and the α and β phosphate groups are generally well ordered while little density is observed for the terminal γ phosphate.

The structure of the GpppA cap analog in the citrate crystal form is intriguing in that there is clear electron density for the entire GpppA molecule in an internally stacked conformation where the adenosine is packed onto the solvent exposed face of the bound guanosine (Figure 2C). A similar conformation was recently reported in DEN MTase crystals soaked with various cap analogs¹⁰. In contrast, the low ionic strength MPD crystal form only shows density for the guanine ring, ribose, and α - β phosphates while the adenosine is disordered (Figure 2F) similar to what was observed with MVEV MTase complexed with m⁷GpppA¹². In both cases the stacked orientation was not observed when the ionic strength of the crystallization buffer was low (Figure 2F and reference¹²) whereas the stacked orientation is only observed when the ionic strength is very high (Figure 2C and reference²⁰). The stacked interaction is likely stabilized by the high ionic conditions, reducing electrostatic repulsion within the triphosphate moiety that is now folded back on itself, although we do not see any well-defined electron density for ordered sodium counterions. Because this stacked conformation has not been observed in less than 1.6 molar sulfate or citrate, the biological significance of the stacked conformation is unclear.

Last, we see crystallographic evidence for a second triphosphate binding site in the MPD crystal form complexes with GTP and GpppA. In these structures there is significant density for a di- and tri-phosphate moiety in a new site near Arg27 and Arg87 that is located above the bound Ado-Hcy coproduct (Figure 1a). This is the same site in which a sulfate ion was previously observed in the DEN MTase structure^{5; 11} as well as in our DEN structure, and has been suggested act as a channel for RNA entry into the MTase^{13; 20}. In examining the electron density maps, there is no evidence of a specific orientation of the attached but unresolved ribose and guanine base in this binding site.

Establishment of fluorescence polarization assay for GTP binding

GTP binding has been used previously as a surrogate for guanosine cap binding⁷ because the guanosine cap structure on cellular mRNAs and flavivirus RNA resembles an inverted GTP. Previous analysis of GTP/cap binding has utilized a UV crosslinking assay with radiolabeled GTP to study binding, followed by PAGE and autoradiography. To study the binding of GTP, and by extension the guanosine cap structure, to the MTase enzyme, we have established an *in vitro* fluorescence polarization (FP) based binding assay. This assay allows for sensitive, homogenous, and real-time determination of GTP binding to purified MTase protein. FP assays have been successfully used to analyze GTP binding to a number of proteins^{22; 23}. We first tested the binding affinity of the DEN, WNV, and YF MTase proteins for GTP-Bodipy using binding saturation curves. Using this approach, we obtained similar K_d values for GTP-Bodipy bound to each protein (DEN = 72 ± 2 nM; YF = 124 ± 6 nM; WNV = 115 ± 6 nM (Figure 3A)). To confirm that GTP-Bodipy has a similar K_d to that of GTP we measured the change of FP as a function of DEN protein concentration in the presence of various concentrations of GTP (GTP-Bodipy K_d = 126 ± 15 nM; GTP K_i = 119 ± 25 nM (Figure 3B)). These data demonstrate that GTP-Bodipy and GTP bind with similar affinity, thus validating our assay. DEN, WNV, and YF MTase proteins also have very similar K_d values for GTP-Bodipy binding (data not shown). To establish the specificity of guanine cap binding, we attempted to determine K_d values for MTase ATP-Bodipy binding. DEN MTase bound ATP-Bodipy very poorly as compared to GTP-Bodipy ($K_d > 13$ μ M (Figure 3C)), but we were not able to determine an exact K_d for ATP-Bodipy because we could not get the protein concentration in our experiments high enough to saturate binding. Finally, we determined the K_i of GTP and ATP for binding to the DEN MTase using a GTP-Bodipy displacement assay (Figure 3D). 10 nM GTP-Bodipy was complexed with 500 nM DEN MTase protein (~75% fractional saturation), increasing amounts of competitor was added, FP signal was detected, and the resulting K_i value calculated. We determined the K_i of GTP to be 40 ± 10 nM, and the K_i of ATP to be 350 ± 39 μ M. Because the MTases appear to be nearly identical in terms of structural and GTP-Bodipy binding characteristics and DEN MTase was the most soluble during purification, we chose to perform the remaining biochemical characterization of guanosine cap binding only with the DEN MTase.

Mutation of residues involved in DEN MTase-GTP binding suggests novel interactions

To test the contribution of conserved residues suggested to be involved in cap binding (Figure 4), we mutated individual residues and assessed the effect the mutations had on the affinity of the protein for GTP-Bodipy (Table 2). Of the mutants tested, the most significant effects were observed with the Phe24Ala, Lys28Ala, and Ser215Ala mutants. Interestingly, mutation of Phe24 to Tyr had almost no effect on GTP-Bodipy binding. This result was surprising because the Phe24 is invariant in all known flaviviral MTase enzymes, suggesting that the conservation of Phe24 is due to factors other than guanine binding. Mutation of Lys28 or Ser215 to Ala also resulted in decreased binding (Table 2), indicating that two contacts, one strong (Lys28) and one weak (Ser215), are made with the α -phosphate as evidenced by the structure. Ser150 was previously suggested to interact strongly with the α -phosphate^{5;13}, but our structural data indicate that an interaction with the β -phosphate is more structurally favorable (Figure 2B).

Mutation of Ser150 to Ala resulted in a very modest decrease in binding, suggesting that this interaction makes only a minor contribution to binding. Interestingly, we observed that Arg213 was in a position to potentially interact with the GTP γ -phosphate, though the $2F_o - F_c$ density maps showed weak density for the residue and the γ -phosphate. Mutating Arg213 to Ala resulted in a modest 2.5 fold reduction in binding affinity, indicating that an interaction between this residue and the γ -phosphate is possible, but not very strong. The γ -phosphate was disordered in the YF GTP and GpppA $2F_o - F_c$ density maps and the β -phosphate was in weak density, indicating significant flexibility of these phosphates and weaker interactions with the protein as compared to the α -phosphate.

Lys21 is a charge-conserved residue located near α -helix 24–34 in a position near the cap guanosine. The $2F_o - F_c$ density map shows minimal density for the sidechain of Lys21. However, one of the sidechain rotamers can be positioned within 3 Å of the C6 carbonyl group on the guanosine ring structure. To test if the Lys21 sidechain contributes to binding of the guanine ring, we mutated Lys21 from a basic residue to acidic glutamic acid. The Lys21Glu mutation reduced the affinity by 3.2 fold. However, when we changed Lys21 to Ala, we observed only a minor increase in GTP affinity as compared to wildtype protein, suggesting that the positively charged lysine may be slightly inhibitory to cap binding. Therefore, the Lys21 sidechain does not seem to contribute to guanosine binding, and the effect seen with the Lys21Glu mutant is likely due to repulsion between the C6 amine and the Lys21 amine groups or alteration of the peptide backbone position.

We also attempted to test the contribution of residues that appear to interact with the ribose 2' and 3' hydroxyl groups, Lys13 and Asn17. Neither of these mutants expressed in BL21 *E. coli* despite repeated attempts. DEN Asn17 was mutated and expressed previously⁵, so our inability to purify the proteins may be due to the strain of DEN virus MTase used (16681 vs New Guinea) or the difference in the length of the C-terminal domain between the two constructs. We also attempted to purify a Lys28Ala/Ser215Ala double mutant to determine if binding was affected more than with the individual mutants, but the protein was completely insoluble and could not be purified.

Major cap-protein Interactions

We performed a series of GTP-Bodipy displacement assays similar to Figure 3D with GTP analogs to determine which components of GTP are important in binding to the MTase. K_i values for each analog are shown in Table 3. We observed that GTP was the most effective competitor of GTP-Bodipy binding with a K_i of 40 nM, as compared to the K_d of GTP-Bodipy (72 nM). Our finding that the K_i of GTP is comparable to the K_d for GTP-Bodipy confirms that GTP-Bodipy is binding to the MTase in a similar manner to GTP. The slight difference between the K_d of GTP-Bodipy and the K_i of GTP is likely due to weak steric hindrance caused by the Bodipy group as compared to no Bodipy group with GTP. Once we had a baseline for binding, we tested the contribution of each of the phosphates to binding. Removing the γ -phosphate (GDP) or γ - and β -phosphates (GMP) increased the K_i by 3 and 29 fold, respectively. Removing all three phosphates (guanosine) almost completely abolished the ability to compete with GTP-Bodipy binding (6400 fold reduced). Therefore, while the γ - and β -phosphates do provide some energy to guanosine cap binding, the α -phosphate interaction with Lys28 and Ser215 appear to be critical to tight cap binding. Interestingly, GpppG and GpppA had slightly higher K_i s than GTP, which may be due to a slight steric clash with the second guanine or adenine with the MTase protein that would not be present in with GTP.

We then tested the contribution of the ribose 2' and 3' hydroxyl groups to binding. dGTP (lacking the 2' OH) displayed a K_i of 610 nM, which represents a 15 fold decrease in binding as compared to GTP. Removing both hydroxyl groups (ddGTP) resulted in a 770 nM K_i , indicating only a weak contribution from the 3' hydroxyl.

The purine ring structure of the guanosine cap is a major component of cap binding. To understand what aspects of the guanine ring are important for guanine binding and specificity, we performed competition experiments with guanosine triphosphate analogs modified at different positions on the guanine ring (Table 3). Adenosine triphosphate (ATP), which is identical to GTP except for the purine N1, C2, and C6 prosthetic groups, was unable to effectively compete with GTP-Bodipy ($K_i = 350 \mu\text{M}$). Therefore the groups at N1, C2, and/or C6 positions are primary determinants for the specificity of binding guanine as compared to adenine. We first examined the contribution of the C2 position by using GTP analogs with substitutions at the C2 position, which was previously suggested to be the primary determinant for guanine specificity^{5; 11}. Inosine triphosphate (ITP) removes the C2 amine group from GTP, which represents an intermediate between ATP and GTP. ITP was able to compete well for binding (3 fold reduction), indicating a minor contribution of the C2 amine to binding. We then tested xanthine triphosphate (XTP), which replaces the GTP C2 amine with a carbonyl group. XTP was a slightly weaker competitor than ITP (12 fold reduced compared to 3 fold reduced). Therefore, alteration of the C2 amine on the guanine ring structure appears to weakly contribute individually to guanine recognition by the flavivirus MTase but is not the only determinant for binding. We then tested the ability of guanine N1 to act as a hydrogen bond donor to the water that resolved in the YF structures. We used 6-methylthio-GTP (6-MT GTP), which replaces the C6 amine with a thiomethyl group and results in a C6-N1 double bond that removes the N1 hydrogen atom, to test the contribution of the N1 position to binding. 6-MT GTP competed only slightly worse than ITP (230 nM vs 120 nM), but was much better than ATP (350 μM), indicating that the N1 position is a necessary but not sufficient component of guanine recognition.

Discussion

In this study we performed a detailed biochemical and structural analysis of the guanosine cap binding characteristics of the DEN and YF virus NS5 MTase domains. We present new high-resolution structures of the YF MTase domain complexed with GTP and cap analog, a higher resolution DEN MTase structure, and define many of the critical protein and GTP components of guanosine cap binding. Based on the combination of our structural and biochemical analysis, we present a detailed scheme for the binding of the guanosine triphosphate portion of the cap structure to the flavivirus MTase enzyme (Figure 5). The relative binding affinities for most of the critical interactions observed in the structures are shown. Our data provides the first highly detailed description of the correlates of guanosine cap binding by the flavivirus MTase enzymes. Our studies have indicated a much higher affinity for guanosine cap binding than had previously been recognized, identified several new protein-cap interactions, and demonstrate a detailed mechanism of guanine recognition.

Using our new fluorescence polarization based assay, we determined that the K_d of the flavivirus MTase for GTP-Bodipy is approximately 100 nM, and that the K_i for both GTP and cap analog is in a similar range. The K_d value of the flavivirus MTase domain for cap analog is somewhat higher than the 2 nM cap-binding affinity of the cellular cap-binding eIF4E protein for cap analog as determined by stopped-flow fluorescence titration experiments²⁴. However, eIF4E stacks the cap guanine ring between two tryptophane residues, which likely provides more stabilization than the single phenylalanine residue in the flavivirus MTase and accounts for the increased affinity observed with eIF4E. In light of the high affinity of eIF4E for guanosine cap, the 100 nM K_d value determined using our assay seems more reasonable than the 58 μM K_d that was previously determined⁵.

We performed a comprehensive mutational analysis of the DEN MTase enzyme to determine the contribution of individual amino acids to guanosine cap binding. Figure 4 shows residues within a 10 Å sphere of the cap binding pocket. Conserved residues within hydrogen bonding

distance of GTP in our crystal structures noted and were tested for their contribution to GTP interaction. Phe24 and Lys28 have been previously suggested as major interaction points^{5; 20}. By far the most dramatic effect was seen with the Phe24Ala mutant, indicating that π - π interaction between the guanosine base and the phenylalanine side chain is essential for binding. However, mutation of Phe24 to Tyr did not significantly alter binding. The Phe residue is invariant across the flavivirus family, even though a single uracil to adenine transversion mutation in the second position of the codon would change the residue from Phe to Tyr. This suggests that the Phe residue is functionally conserved, but there may be an additional role for the residue outside of cap binding that requires the residue to remain phenylalanine rather than a tyrosine. Experiments mutating Phe24 to Tyr in infectious virus assays may shed light on why this residue is highly conserved.

Ser215 appears to coordinate the cap α -phosphate in combination with Lys28. The combination of Lys28 and Ser215 interacting with the α -phosphate appears to provide a great deal of stability to the bound cap, as the α -phosphate is well resolved in our structures whereas the β and γ phosphates are not. Mutational and analog competition analyses support this observation, as the Lys28Ala mutation binds GTP-Bodipy weakly and guanosine is a very poor competitor. We also observe a minor effect on binding when we mutate Arg213, even though there is only very weak electron density for the residue. This residue appears structurally poised to influence γ -phosphate binding. Arg213 is also in a region that could be involved in interaction with the 5' adenosine ring structure of the first viral nucleotide. The position of the viral RNA or 5' RNA adenosine has not been observed as of this report, but we have performed preliminary computer modeling of a capped viral RNA into the YF MTase structure and observed that the molecular distances are possible for this interaction. Additionally, if the 5' adenosine base is stabilized between Arg213 and Arg56, this would position the ribose 2' hydroxyl near AdoMet, increasing the efficiency and specificity of the 2'-O-methyltransferase reaction.

We observed electron density in the positively charged region across from the cap binding site in structures soaked with GTP (Figure 1A) or cap analog (data not shown). We were able to model a triphosphate from GTP into the resolved density, although density for the ribose and guanine on the GTP was not observed. This density could represent a binding site for the triphosphate between the first and second nucleotides in the genomic RNA, with the adenosine residue bound somewhere on the face of the MTase between the AdoHcy and GTP binding sites. Consistent with this, the Arg213/Arg56 pocket at the back of this face could provide hydrogen bonding interactions with the adenosine base. Alternatively, this second phosphate binding site could be involved in binding the cap guanosine residue for the guanine N7 methylation reaction because we can fairly easily place the guanosine N7 atom within a few ångströms of the Ado-Hcy sulfur after modeling a triphosphate into the observed density. As the methylation of the flavivirus cap occurs sequentially with the guanine N7 being methylated before the ribose 2' hydroxyl, the observed triphosphate density could indicate the path for the first methylation event.

The α -phosphate is a major interaction point between the protein and cap structure. One possibility for the strength of this α -phosphate: Lys28: Ser215 interaction is that it may represent the as yet unidentified guanylyltransferase activity. Egloff et al. previously showed that α -³²P GTP can be covalently crosslinked to DEN MTase in a Mg^{2+} independent manner^{5; 20}, which could represent the protein-GMP adduct. Such a role may explain the strength of interaction that is observed between the α -phosphate and the protein, whereas the β - and γ -phosphates appear more weakly associated with the protein. Additionally, in high ionic strength crystallization conditions (1.6M sodium citrate (Figure 1) or 0.4M ammonium sulfate/1.2 M lithium sulfate²⁰) the cap analog appears to fold back on itself, leaving the α -phosphate exposed for nucleophilic attack or adduct formation. This orientation has been suggested to be involved in guanylyltransferase activity²⁰, and Lys28 appears to be well positioned to act as

a nucleophile. Non-UV based α - ^{32}P -GTP labeling experiments examining the formation of DEN MTase-GMP adducts using established guanylyltransferase assays²⁵ did not detect adduct formation with either the wildtype or K28A DEN MTase proteins (Aaron Shatkin, personal communication). Additionally, the stacked orientation mentioned above was not observed in our lower strength ionic MPD conditions with the same crystal packing lattice (Figure 2) or that of the MVEV MTase¹², raising questions about the physiological relevance of this stacked conformation. Further studies are needed to determine if the stacked conformation indeed plays a role in flavivirus guanylyltransferase activity.

The tight cap binding by MTase ($K_d \sim 100$ nM) may represent a mechanism to sequester the RNA cap structure from cellular eIF4E or ribosomes until the positive-strand genomic RNA has become long enough to prevent polyprotein translation from interfering with continued RNA replication. This model would require the guanosine cap to be released from its binding site either by a directed release mechanism (i.e. conformational change) or by folding of an RNA structure that triggers the release of the cap from the MTase. Flavivirus MTase specifically binds GTP (or cap), but binds the structurally similar ATP very poorly. It was initially postulated that the mechanism for guanine specificity lay in the interaction of the guanine C2 amine group with the backbone carbonyls of Leu16 and Leu19⁵. While this interaction does provide a small contribution to binding, our analog studies show that the C2 moiety is not the sole factor in guanine specificity. The observation that ITP, which lacks the C2 amine group but possess a hydrogen bond capable hydrogen at N1, can strongly compete with GTP-Bodipy binding indicates that there is at least a second contact between the guanine ring and the MTase. Were the C2 amine position the only selective interaction point, ITP would bind as weakly as ATP. We originally hypothesized that the Lys21 sidechain could interact with the C6 carbonyl group on guanine. However, our mutational analysis indicated that the Lys21 sidechain does not play a role in the observed specificity. Surprisingly, a structurally conserved water molecule is found between the guanine N1 hydrogen and the Leu19 backbone carbonyl in each of our GTP/cap soaked structures derived from completely different crystallization conditions. The presence of this bridging water molecule, which is within hydrogen bonding distance of the guanine N1 hydrogen and the Leu19 backbone carbonyl oxygen, was not observed in previously published lower resolution MTase-GTP structures^{5; 9; 10; 11; 12; 13; 14}, but is present in a 1.8 Å Den MTase-cap analog structure (PDB Code 2P41)¹⁰. The presence of the water molecule in three different crystallization conditions with two different flavivirus MTase proteins suggests that the coordinated water is not a crystallization artifact and appears to represent an important interaction. The combination of the N1 water-bridge and C2 amine interactions with MTase backbone interactions appear to contribute almost equal amounts of binding energy based on our competition data, and the combination of the two interactions appear to confer specific recognition of guanine largely independent of side-chain composition. These two interactions increase our understanding of guanine binding by flavivirus MTase proteins. This recognition mode is considerably different from that observed in other RNA cap binding proteins (vaccinia VP39, cellular CBP20 and eIF4E) which bind guanosine caps between two aromatic sidechains^{24; 26; 27}. Water-mediated nucleoside recognition has been reported previously with calf purine-nucleoside phosphorylase²⁸, although in that case the water interaction was observed between the C6 carbonyl and an Asn sidechain.

The results of the experiments described here further our understanding of the architecture of the MTase guanosine cap-binding site. The flavivirus MTase is a new and potentially valuable antiviral target to develop drugs against flavivirus replication. Drugs that interfere with influenza virus RNA cap binding have been previously described²⁹, indicating that viral cap binding is a viable drug target. Our increased understanding of how the flavivirus MTase binds RNA cap structures will aid in the rational development of highly potent and specific drugs for the treatment of flavivirus infections.

Materials and Methods

Production of recombinant flavivirus MTase proteins

Recombinant NS5 MTase domains from DEN (strain 16681, AA 1-267), YF (strain 17D, AA 1-268), and WNV (strain NY1999, AA1-268) were cloned into an inducible T7 expression plasmid that contains a carboxy-terminal 6-histidine tag. YF Protein was produced in BL21 (DE3) Codon Plus *E. coli* cells (Novagen) and DEN and WNV protein was produced in BL21 (DE3) pLysS cells. 750 ml cultures were induced with 400 μ M IPTG overnight at 22°C and the bacterial pellets were collected and stored at -20°C in his-tag lysis buffer³⁰. The frozen pellets were thawed and incubated with additional lysozyme for 1 hr on ice, lysed by sonication, and the lysate was clarified by centrifugation at 18K RPM in a SS-24 rotor. The histidine-tagged protein was purified from clarified lysates using a nickel-loaded chelating sepharose column on an AKTA Purifier FPLC system. The eluted proteins were further purified over a HiTrap-S cation exchange column (Amersham), concentrated using 10K Amicon Ultra concentrators (Millipore), and buffer exchanged into 200 mM NaCl, 20 mM Tris pH 7.5, 0.02% sodium azide and 5mM Tris(2-Carboxyethyl) phosphine Hydrochloride (TCEP-HCl) on a Superdex 200 gel filtration column (Amersham). The isolated proteins were >99% pure as estimated from SDS-PAGE and Coomassie Blue staining. Purified proteins for use in crystallization studies were concentrated using 10K Amicon Ultra concentrators to approximately 400 μ M and the concentrations were determined by the absorbance at 280nm using extinction coefficients obtained from the ExPASy web site (<http://www.expasy.ch/tools/protparam.html>). Purified protein intended for crystallization studies was stored at 4°C and used within 1 week of preparation. Purified protein for use in biochemical studies was supplemented with TCEP-HCl and concentrated to 300 μ M, then brought to 20% glycerol and stored at -20°C until use.

Crystallization of DEN and YF proteins

Yellow Fever virus NS5 MTase crystals were grown by hanging drop vapor diffusion at 16°C using ~10 mg/ml protein in two different conditions yielding distinct crystal morphologies with identical crystal lattices. The “tapered-rod” morphology grew in ~5 days with a precipitant/well solution containing 25% (v/v) MPD, 0.1 M Tris pH 8.5, 2 mM DTT and 0.02% (w/v) sodium azide. The “bow-tie” shaped crystals grew in ~10 days with a precipitant/well solution containing 1.6 M sodium citrate pH 6.5. To obtain the ligand-bound structures, crystals were soaked for 2 to 6 hours at 4°C in mother liquor solutions containing ~3 mM GTP or GpppA. The tapered-rod crystals were flash frozen in liquid nitrogen with a final MPD concentration of 25% (v/v), and the bow-tie crystals were briefly dunked in a solution containing 1.6M sodium citrate (pH 6.5) before being frozen. Dengue virus MTase crystals were obtained using a smaller protein construct than that used for the previous structure^{5; 11} by deleting the last 31 amino acids that were disordered in that structure (residues 1-267 vs. 1-298). Optimal crystallization conditions were found by screening around the original conditions and resulted in crystals that diffracted to 2.2 Å resolution.

Structure determination

Diffraction data were collected at the MBC beamline 4.2.2 at the Advanced Light Source (Berkeley, CA). Reflections were integrated, merged, and scaled using d*TREK³¹, as summarized in Table 1. The initial structure solutions were obtained by using molecular replacement using the program CNS³² with the Dengue NS5 methyltransferase structure (PDB code 1R6A) as the search model. Manual model rebuilding was performed using O³³ and refined with the CNS package using the MLI target. Refinement parameters for GTP, GpppA, and S-adenosyl-homocysteine were obtained from the HIC-up database³⁴ and Dundee PRODRG2 server (Schüttelkopf and van Aalten, 2004). Figures were generated with Pymol Molecular Graphics System (www.pymol.org)³⁵.

Fluorescence Polarization Assays

Fluorescence polarization (FP) assays were performed with purified MTase protein and GTP-Bodipy γ -phosphate labeled fluorescent analog (Invitrogen Catalog #G22183) as the ligand. All FP experiments were performed in 50 μ l volumes in opaque black 384 well microtiter plates (Corning # 3573). Reactions were carried out in 50 mM Tris pH 7.5, 0.01% NP-40, and 10 nM GTP-Bodipy. To perform binding saturation experiments, purified proteins were diluted as 10X concentrates using gel filtration buffer in a 1.5 fold dilution series, and 5 μ l diluted protein was added to each well containing 45 μ l of the reaction mixture. Plates were incubated at room temperature for 5 minutes and FP and total fluorescence signals were detected using a Victor V multimode plate reader with fluorescence polarization capability (Perkin Elmer). To perform analog competition studies, 11 nM GTP-Bodipy was incubated with 550 nM wildtype DEN MTase protein (~70–80% fractional saturation as determined by binding saturation curves performed with each experiment) in the reaction buffer described above in a volume of 45 μ l. 5 μ l of diluted competitor was added to the wells (diluting GTP-Bodipy to 10 nM and DEN WT to 500 nM), incubated for 5 minutes at room temperature, and FP and total fluorescence signal was detected. All FP experiments were performed three times in duplicate. Nucleotide analogs used in competition studies were purchased from Jena Bioscience (Jena, Germany).

Accession Codes

Coordinates and structure factors have been deposited in the Protein Data Bank with accession numbers 3EVA, 3EVB, 3EVC, 3EVD, 3EVE, 3EVF, and 3EVG.

Data analysis

GTP-Bodipy binding affinity for MTase protein was directly determined by fitting the fluorescence polarization data to equation 1, where $f.p_{obs}$ is fluorescence polarization observed, $f.p_i$ is fluorescence polarization initial, $\Delta f.p.$ is change in fluorescence polarization or

$$f.p_{obs} = f.p_i + \Delta f.p. (f.b.) \quad \text{eq. 1}$$

fluorescence polarization max minus fluorescence polarization initial and $f.b.$ is fraction bound. Fraction bound ($f.b.$) is equal to equation 2, where P_t is the total protein

$$f.b. = \frac{P_t}{P_t + K_d} \quad \text{eq. 2}$$

concentration and K_d is the dissociation constant. The affinity of GTP for DEN MTase was determined using a competition based assay and measuring the affinity of GTP-Bodipy in the presence of multiple concentrations GTP. These data were globally fit using Prism (www.graphpad.com) to equation 1 where $f.b.$ is equal to equation 3, where P-GTP is GTP bound protein.

$$f.b. = \frac{(P_t - P \bullet GTP)}{(P_t - P \bullet GTP)_t + K_d} \quad \text{eq. 3}$$

P-GTP is equal to equation equation 4, where GTP_t is total GTP added and

$$P \bullet GTP = \frac{(K_d^{GTP} + P_i + GTP_i) + \sqrt{-(K_d^{GTP} + P_i + GTP_i)^2 - 4 * P_i * GTP_i}}{2} \quad \text{eq. 4}$$

K_d^{GTP} is the disassociation constant for the GTP. Other inhibition assays were fit using Kalidagraph software package (Synergy Software) and Microsoft Excel with K_i and K_d equations based on ³⁶.

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Figure 1. Structural conservation of flavivirus NS5 MTase domain

a) Surface representation of the yellow fever virus NS5 MTase domain showing $2F_o - F_c$ electron density maps contoured at 1.9σ for the Ado-Hcy coproduct that co-purified with the protein (lower left), the soaked in GTP molecule (right), and the secondary phosphate binding site (upper left). b) Maximum likelihood multiple structure superposition of the dengue virus (magenta) and yellow fever virus (yellow) MTase domains. The Ado-Hcy coproduct and the phenylalanine residue in the GTP binding site are shown in sticks. Structural differences are largely limited to small movements of the helix adjacent to the GTP binding site (•).

Figure 2. Structural details of the cap binding site and ligand complexes in the citrate (a–c) and MPD (d–f) crystal forms

$2F_o - F_c$ maps contoured at 1.8σ are shown for the apo (a,d) and the bound GTP ligand (b,e) structures. Composite 2500K simulated-anneal omit maps are shown for the non-methylated GpppA cap analog in the citrate crystal form (c) and for the N7 methylated GpppA cap analog in the MPD crystal form (f). The view in panel c is from the top of the GpppA binding site to show the stacking interactions involving Phe 24 and the guanosine and adenosine from the cap analog. “W” denotes the bridging water molecule. Residues described in the text are labeled in panels a and e.

Figure 3. Determination of Dengue, West Nile, and Yellow fever virus MTase GTP-Bodipy binding characteristics

A) Determination of K_d for GTP-B binding to Dengue, Yellow Fever, and West Nile virus MTase proteins. B) Determination of GTP K_d as a function of GTP-Bodipy displacement C) Comparison of DEN MTase K_d for GTP-Bodipy and ATP-Bodipy. Curve fits and K_d values were determined with the KaleidaGraph software package as described in the Data Analysis section. D) Determination of GTP and ATP K_i values. GTP-Bodipy displacement assays were performed as described in the text.

Figure 4. Conservation of binding site residues amongst flavivirus family members

Black background denotes non-similar substitutions, and the shaded rectangles bring attention to residues where increased variability is observed. Numbering for YF is noted and is used throughout the manuscript to describe all MTase residues. Residues with side chains within hydrogen bonding distance of GTP are marked (*). Residues with backbone carbonyl groups interacting with the conserved water and the guanine ring are marked (@).

Figure 5. Flatland model for interactions between flavivirus MTase cap-binding pocket and the GTP cap structure

A line structure of GTP is surrounded by MTase residues is presented, with types of interactions and relative strengths indicated within the figure. Numbering for the residues is representative of the YF MTase.

Table 1
Summary of crystallographic and structure refinement data

Virus source	YF	YF	YF	YF	YF	YF	YF-	Dengue
Ligand	Apo	Apo	GTP	GTP	GpppA	Me7GpppA	Apo	
Crystal form	Na ⁺ citrate	MPD	Na ⁺ citrate	MPD	Na ⁺ citrate	MPD		
PDB Code	3EVA	3EVB	3EVC	3EVD	3EVE	3EVF	3EVG	
Space Group								
Unit Cell (Å)	$P6_5$ $a=b=105.3$ $c=51.3$	$P6_5$ $a=b=105.1$ $c=51.5$	$P6_5$ $a=b=104.5$ $c=51.7$	$P6_5$ $a=b=105.0$ $c=51.9$	$P6_5$ $a=b=104.4$ $c=51.8$	$P6_5$ $a=b=104.8$ $c=51.8$	$P3_121$ $a=b=112.6$ $c=55.7$	
X-Ray Source	ALS 4.4.2	ALS 4.4.2	ALS 4.4.2	ALS 4.4.2	ALS 4.4.2	ALS 4.4.2	Cu-Kα	
Reflections								
Resolution Limits (Å) ¹	45.6 – 1.50 (1.55–1.50)	45.5 – 1.85 (1.92–1.85)	45 – 1.60 (1.66–1.60)	45 – 1.50 (1.55–1.50)	45 – 1.70 (1.76–1.70)	45 – 1.4 (2.59–2.50)5	28 – 2.20 (2.28–2.20)	
Total Collected	514371	172084	470497	567524	274988	607812	136702	
Unique	50591	27875	42150	52306	35604	57555	20448	
Redundancy	10.2 (9.9)	6.2 (6.0)	11.2 (11.1)	10.8 (10.1)	7.7 (7.6)	10.6 (8.4)	6.7 (6.3)	
I/σ	19.0 (6.7)	8.7 (2.4)	14.0 (4.3)	18.4 (4.9)	10.0 (2.9)	18.8 (4.1)	12.7 (3.6)	
Completeness (%)	97.1 (94.8)	100 (100)	98.9 (97.5)	100 (100)	100 (100)	99.9 (100)	97.8 (95.1)	
R _{sym} (%)	5.6 (28.8)	10.1 (45.6)	7.1 (44.2)	4.4 (42.7)	8.5 (46.2)	5.2 (39.2)	9.9 (43.9)	
Refinement								
Resolution Range (Å)	30 – 1.50	30 – 1.85	30 – 1.60	30 – 1.50	30 – 1.70	30 – 1.45	28 – 2.2	
R-work (%)	20.3	20.4	20.3	21.9	20.7	20.7	24.0	
R-free (%) ²	21.6	23.1	22.3	23.7	22.8	22.7	25.3	
Ramachandran statistics ³								
Favored	215	215	215	217	215	218	195	
Allowed	14	14	14	12	14	11	19	
Generous	0	0	0	0	0	0	2	
Disfavored	0	0	0	0	0	0	1	

¹Data in parentheses are for the highest resolution shell.

²R-free was computed using 10% of the data assigned randomly and model bias was removed with a 1500K slowcool simulated annealing step.

³Ramachandran statistics do not include 23 glycine,⁷ proline and 2 end residues for YF MTase.

Table 2

Mutation of DEN cap-binding pocket residues affects its affinity for GTP-Bodipy.

Mutants	K _d	Standard Error	Fold Affinity Reduction
WT DEN	70 nM	5 nM	-
LEU 19 MET	50 nM	4 nM	<1
LYS 21 ALA	35 nM	1 nM	<1
LYS 21 GLU	250 nM	9 nM	3.2
PHE 24 ALA	5.3 μM	0.9 μM	69
PHE 24 TYR	105 nM	12 nM	1.4
LYS 28 ALA	870 nM	52 nM	11.3
SER 150 ALA	150 nM	8 nM	2
ARG 213 ALA	190 nM	10 nM	2.5
SER 215 ALA	220 nM	30 nM	2.9

Table 3
GTP Analog: GTP-Bodipy competition analysis with DEN MTase

K_i	Standard Error	Fold Affinity Reduction	Purine Ring Structure
0nM	10nM	0
0nM	30nM	3	
0nM	70nM	29	
0μM	8uM	6400	
0nM	70nM	15	
0nM	70nM	19	
0uM	39uM	8750
0nM	30nM	3
0nM	110nM	12

K _i	Standard Error	Fold Affinity Reduction	Purine Ring Structure
0nM	90nM	6	<chem>Nc1nc2c(ncn2S)c3ncn3</chem>
0nM	20nM	2.6	
0nM	20nM	4.1
			<chem>Nc1nc2c(ncn2S)c3nc(=O)[nH]3</chem>

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