West Nile Virus Methyltransferase Catalyzes Two Methyllations of the Viral RNA Cap through a Substrate-Repositioning Mechanism

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Flaviviruses encode a single methyltransferase domain that sequentially catalyzes two methyllations of the viral RNA cap, GpppA-RNA→m7GpppA-RNA→m7GpppAm-RNA, by using S-adenosyl-L-methionine (SAM) as a methyl donor. Crystal structures of flavivirus methyltransferases exhibit distinct binding sites for SAM, GTP, and RNA molecules. Biochemical analysis of West Nile virus methyltransferase shows that the single SAM-binding site donates methyl groups to both the N7 and 2'-O positions of the viral RNA cap, the GTP-binding pocket functions only during the 2'-O methylation, and two distinct sets of amino acids in the RNA-binding site are required for the N7 and 2'-O methylations. These results demonstrate that flavivirus methyltransferase catalyzes two cap methylations through a substrate-repositioning mechanism. In this mechanism, guanine N7 of substrate GpppA-RNA is first positioned to SAM to generate m7GpppA-RNA, after which the m7G moiety is repositioned to the GTP-binding pocket to register the 2'-OH of the adenosine with SAM, generating m7GpppAm-RNA. Because N7 cap methylation is essential for viral replication, inhibitors designed to block the pocket identified for the N7 cap methylation could be developed for flavivirus therapy.

Eukaryotic mRNAs contain a 5′ cap structure that is essential for RNA splicing, export, stability, and translation (12). In general, RNA capping consists of four steps. (i) The 5′-triphosphate end of the nascent RNA transcript is hydrolyzed to a 5′-diphosphate by an RNA triphosphatase; (ii) the GMP moiety of GTP is transferred to the 5′-diphosphate of RNA by a RNA guanylyltransferase; (iii) the N7 position of guanine is methylated by an RNA guanine-methyltransferase (N7 MTase), yielding a cap 0 (m7GpppNm) and cap 2 (m7GpppNmNm) structures, respectively (12). S-Adenosyl-L-methionine (SAM) is the methyl donor for both the N7 and 2′-O positions of the viral RNA cap, the GTP-binding pocket functions only during the 2′-O methylation, and two distinct sets of amino acids in the RNA-binding site are required for the N7 and 2′-O methylations. These results demonstrate that flavivirus methyltransferase catalyzes two cap methylations through a substrate-repositioning mechanism. In this mechanism, guanine N7 of substrate GpppA-RNA is first positioned to SAM to generate m7GpppA-RNA, after which the m7G moiety is repositioned to the GTP-binding pocket to register the 2′-OH of the adenosine with SAM, generating m7GpppAm-RNA. Because N7 cap methylation is essential for viral replication, inhibitors designed to block the pocket identified for the N7 cap methylation could be developed for flavivirus therapy.

For flavivirus cap formation, viral NS3 functions as an RNA triphosphatase (37) while NS5 has dual functions as both an N7 MTase and a 2′-O MTase (9, 28); the identity of the guanylyltransferase, however, remains elusive. The flavivirus MTase resides in the N-terminal one-third of NS5, and the C-terminal two-thirds of NS5 contains an RNA-dependent RNA polymerase. Compared with cellular MTases and many viral MTases, flavivirus MTase is unique in that a single MTase domain catalyzes two methylation events, in the order GpppA→m7GpppA→m7GpppAm (28, 39); additionally, both cap methylations are dependent on the viral RNA sequence (8). Despite the two distinct methylation activities, the crystal structure of flavivirus methyltransferase exhibits only a single binding site for SAM as the methyl donor (4, 9, 20, 39). The structural information, together with the RNA footprinting results suggesting that the first three and four nucleotides of the viral RNA are, respectively, protected by WNV NS5 during the N7 and 2′-O methylations (8), suggests that the substrate GpppA-RNA translocates on the enzyme sur-
face so as to accept a methyl group from SAM during each of the two methylation events.

The flavivirus NS5 protein has several features in common with the L protein from viruses in the order Mononegavirales. Both proteins contain the MTase and RNA-dependent RNA polymerase domains; mutations of the predicted SAM-binding site of VSV L protein are defective in both N7 and 2'-O cap methylations, indicating that the two methylation reactions share a SAM-binding site (18); the recombinant L protein of Sendai virus catalyzes the N7 cap methylation in a viral sequence-dependent manner (25). However, major differences in cap formation exist between the two distant virus groups. In VSV, cap I of the viral mRNA is formed through transfer of a GDP moiety from GTP to the 5' monophosphate of the acceptor RNA (1), with subsequent methylations at 2'-O and guanine N7 positions of the cap (22, 36). Remarkably, it was recently discovered that the L protein of VSV functions as a guanylyltransferase through a mechanism distinct from that of cellular and other known viral counterparts (24).

The goal of this study was to experimentally demonstrate a molecular repositioning mechanism for flavivirus RNA cap methylation by using WNV as a model. The results elucidate the two distinct positions where viral RNA binds to the WNV MTase during each of the two methylation events. We also show that N7 methylation is essential for viral replication. Sinelfungin (SIN), a SAM analogue that inhibits viral MTase, suppresses WNV replication. Collectively, the results suggest that compounds targeting the pocket required for the N7 methylation can potentially be designed for flavivirus therapy.

**MATERIALS AND METHODS**

**WNV mutant MTases.** Mutagenesis was performed with a pET28(a) expression vector containing the WNV MTase domain, representing the N-terminal 300 amino acids of NS5 (28). Specific mutations were engineered into the MTase expression plasmid with a QuikChange II XL site-directed mutagenesis kit (Stratagene). The complete sequence of each mutant MTase was validated by DNA sequencing. All MTases contained an N-terminal His tag and were expressed and purified through an Ni-nitrilotriacetic acid column (28). The protein (>90% purity) was quantified by Bradford assay (Bio-Rad) and verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**Methylation assays.** The 5'-end-labeled substrates GpppA, GpppAm, and m'GpppAm-gRNA, representing the first 190 nucleotides of the WNV genome (the asterisk indicates that the following phosphate is 32P labeled), were prepared with a vaccinia virus capping enzyme by following the manufacturer's protocol (Epiprect). The labeled RNAs were purified through two Sephadex G-25 spin columns (GE Healthcare), extracted with phenol-chloroform, and precipitated with ethanol. We previously showed that the optimal pH values for the N7 and 2'-O methylations are 7 and 10, respectively (39). The high-pH requirement for 2'-O methylation activity allowed us to selectively perform the N7 methylation of GpppA RNA to m'GpppAmRNA without subsequent 2'-O methylation by incubating the reaction mixture in a neutral-pH buffer. The 2'-O methylation of m'GpppAmRNA could be efficiently accomplished by incubating the reaction mixture in a neutral-pH buffer. The 2'-O methylation by incubation in a neutral-pH buffer. The 2'-O methylation of m'GpppAmRNA could be efficiently accomplished by incubating the reaction mixture in a neutral-pH buffer. The 2'-O methylation by incubation in a neutral-pH buffer.

**Gel mobility shift assays.** Gel mobility shift assays were performed with [α-32P]GTP-labeled m'GpppA RNA (containing the 5'-terminal 190 nucleotides of the WNV genome) as a probe. Reactions were performed in a 20-μl volume containing 50 mM glycine, pH 10.0, 5% glycerol, 2 mM DTT, 3 μM RNA probe, 100 ng yeast RNA, and the indicated amounts of wild-type (WT) or mutant MTase. The reaction mixtures were incubated at 22°C for 10 min and resolved by 5% native PAGE (16–18 mm gel). Gel electrophoresis was performed in Tris-borate-EDTA buffer at 4°C and 200 V for 2.5 h. After electrophoresis, the gels were stained and analyzed by autoradiography.

**Construction of mutant full-length cDNA of WNV.** A full-length cDNA clone of WNV, plasmid pFLWNV (33), was partially digested with the double-cutter enzyme KpnI (at nucleotide positions 5,340 and 7,762; GenBank accession no. AF404756) and completely digested with the unique enzyme XbaI (site located at the 3' end of the genome). The DNA fragment spanning the region from nucleotide 5,340 to the 3' end of the genome was ligated into the pCDNA3.1 (+) vector predegenerated with KpnI and XbaI, resulting in a shuttle vector. This shuttle vector was used for mutagenesis of MTase through a QuikChange II XL site-directed mutagenesis kit. The engineered mutations without any undesigned change in the shuttle vector were verified by DNA sequencing. The mutated shuttle vector was then cut and pasted into pFLWNV with the unique enzymes BsiWI (nucleotide position 5,780) and XbaI, resulting in mutant pFLWNV plasmids.

**In vitro transcription, RNA transfection, immunofluorescence assay (IFA), and specific infectivity assay.** The assay protocols used were described previously (27, 32). Briefly, RNAs were transcribed from full-length cDNA plasmids and then electroporated into BHK cells. Viral protein synthesis in transfected cells was monitored by IFA with WNV-immune mouse ascites fluid (American Type Culture Collection) and goat anti-mouse immunoglobulin G conjugated with Texas Red as the primary and secondary antibodies, respectively. For specific infectivity assays, the transfected cells were adjusted to 104 and 105 PFU/ml of culture medium. One milliliter of each of a series of 1:10 dilutions of the transfected cells was seeded onto nearly confluent Vero cells in six-well plates. The first layer of agar was added at 7 h post cell seeding (26). Unless indicated otherwise, a second layer of agar containing neutral red was added after incubation of the plates for 2 days. Plaques were counted at 16 to 20 h after addition of the second agar layer. The specific infectivity was calculated as the number of PFU per microgram of transfected RNA.

**Plaque assay and viral genome sequencing.** Viral titers of culture fluids from BHK cells at day 5 posttransfection (p.t.) were quantified through a double-layer plaques assay (27). The stabilities of mutant viruses were examined by continuous passaging of the viruses in Vero cells for four passages (3 days per passage), followed by comparison of the plaque morphologies of the passaged and unpassaged viruses. For each recovered virus, viral RNA was extracted with an RNeasy kit (Qiagen), the NS5 gene was amplified by reverse transcription-PCR, and the complete MTase domain of the PCR product was sequenced.

**Modeling of the WNV MTase complexed with N7 and 2'-O RNA substrates.** To model the WNV MTase in complex with a 2'-O RNA substrate (m'GpppAm-gRNA), the crystal structure of the VP9 2'-O MTase in complex with an RNA substrate, m'GpppA3G (PDB entry 1AVNo (14), was superimposed onto the structure of the WNV MTase (PDB entry 2BY0) (39) with the CCP4 suite (15). The position of GA was retained for further modeling. To position the m'Gppp moiety of the cap structure, the crystal structure of the DENV-2 MTase in complex with GpppA and SAH (PDB entry 2PSL) (10) was superimposed onto that of the WNV MTase. The coordinates of the resulting Gppp replaced the m'Gppp from the VP9 MTase. With the Turbo

**SAM-binding assays.** A UV-cross-linking assay was used to compare the SAM-binding affinities of the WT and mutant MTases. A 15-μl reaction mixture contained 5 μg of MTase and 10 μCi of methyl-32P-SAM (82 Ci/mmole; GE Healthcare) in 50 mM Tris-HCl, pH 7.0, and 2 mM DTT. The reaction mixtures were incubated at room temperature for 10 min and irradiated for 30 min on ice with a 254-nm UV lamp held at a distance of 4 cm. After adding 3 μl 6× SDS-PAGE loading buffer, the UV-cross-linked products were heated at 95°C for 3 min and separated by 10% SDS-PAGE. For assaying SAM inhibition of MTase binding to the reaction mixtures. After electrophoresis, the SDS-PAGE was fixed with 5% glacial acetic acid, 5% isopropanol alcohol, and 90% water at room temperature for 20 min; rinsed in a continuous flow of tap water for >15 min to remove acetic acid residue; treated with AutoFluor (National Diagnostics) for 30 min/mm of gel thickness; dried; and exposed to film at ~80°C for 3 weeks. The density of H-SAM-MTase bands was quantified with a densitometer (Molecular Dynamics).
GpppAGU substrate, the m7Gppp is deleted, and the remaining Gppp moiety was retained for further modeling with the WNV MTase. To place the remaining RNA nucleotides of the m7GpppAGU sequence on the surfaces of the MTases, we used distinct assay conditions to separate N7 and 2′-O methylations. Specifically, N7 methylation was performed by conversion of GpppA-RNA at pH 7.0; 2′-O methylation was performed by conversion of GpppA-RNA at pH 10. We prepared and assayed for viral RNA cap methylations. Since N7 and 2′-O methylations require different pHs, 7.0 and 10, respectively (39), we used distinct assay conditions to separate the two methylation events. Specifically, N7 methylation was performed by conversion of GpppA-RNA→m7GpppA-RNA at pH 7.0; 2′-O methylation was performed by conversion of m7GpppA-RNA→m7GpppAm-RNA. These assays were used throughout this study to estimate the mutational effects of individual amino acids on the two methylation reactions. Using these assays, we showed that mutations at the SAM-binding site impaired both the N7 and 2′-O methylations (Fig. 2A and B). However, each mutation exhibited different levels of reduction in the two methylation events (Fig. 2C). For example, mutant S56A reduced the N7 and 2′-O methylation activities to 25% and 5% of the WT MTase levels, respectively, while mutant H110A showed 10% and 65% of the WT N7 and 2′-O methylation activities. Nevertheless, the results demonstrate that the single SAM-binding site donates a methyl group to both the N7 and 2′-O positions. Since single amino acid mutations within the SAM-binding pocket affected the N7 and 2′-O methylations to different degrees, the SAM molecule may require a slightly different binding conformation in the SAM-binding pocket during the two methylation reactions.

Mutations within the SAM-binding site impair both the N7 and 2′-O methylations. We performed a systematic mutagenesis to define the functions of the SAM-, GTP-, and RNA-binding sites. Figure 1B illustrates the interactions between the SAH and the WNV MTase. A side chain oxygen of D131 forms a hydrogen bond with the amine of the adenine moiety, the side chains of I147 and K105 sandwich the adenine base, a side chain nitrogen of H110 interacts with the 2′-OH of the ribose of SAH through a hydrogen bond, a side chain oxygen of E111 forms a hydrogen bond with the 3′-OH of the ribose, the D146 side chain interacts with the amine group of SAH, and the side chain of S56 forms two hydrogen bonds with the carbonyl groups of SAH. Sequence alignments showed that, except for K105, all of the amino acids listed here are conserved among the MTases of WNV, DENV, and YFV (data not shown).

A panel of mutants, each of which contained an Ala substitution of one amino acid involved in SAM binding, was prepared and assayed for viral RNA cap methylations. Since N7 and 2′-O methylations require different pHs, 7.0 and 10, respectively (39), we used distinct assay conditions to separate the two methylation events. Specifically, N7 methylation was performed by conversion of GpppA-RNA→m7GpppA-RNA at pH 7.0; 2′-O methylation was performed by conversion of m7GpppA-RNA→m7GpppAm-RNA. These assays were used throughout this study to estimate the mutational effects of individual amino acids on the two methylation reactions. Using these assays, we showed that mutations at the SAM-binding site impaired both the N7 and 2′-O methylations (Fig. 2A and B). However, each mutation exhibited different levels of reduction in the two methylation events (Fig. 2C). For example, mutant S56A reduced the N7 and 2′-O methylation activities to 25% and 5% of the WT MTase levels, respectively, while mutant H110A showed 10% and 65% of the WT N7 and 2′-O methylation activities. Nevertheless, the results demonstrate that the single SAM-binding site donates a methyl group to both the N7 and 2′-O positions. Since single amino acid mutations within the SAM-binding pocket affected the N7 and 2′-O methylations to different degrees, the SAM molecule may require a slightly different binding conformation in the SAM-binding pocket during the two methylation reactions.

Point mutations within the SAM-binding site do not knock out SAM binding. A SAM-binding assay was performed to establish whether the reduced methylation efficiency of the

FIG. 1. Three SAM, GTP, and RNA molecule-binding sites conserved in flavivirus MTases. (A) The three structural elements conserved in flaviviruses are shown on the surface of WNV MTase, which is shown as an electrostatic-potential map with positive charges in blue, negative charges in red, and neutral in white. The depiction of the SAM- or SAH-binding pocket is based on the cocystal structure of the WNV MTase with SAH (39). The GTP-binding site of the WNV MTase was modeled by structural alignment with the DENV-2 GTP-SAH-MTase tertiary complex (9) with PyMOL. The putative RNA-binding site is indicated by the extended patch of positively charged residues. (B) Amino acids involved in interactions with the SAH or SAM molecule are shown in a ball-and-stick representation, with atom colors as follows: carbon, yellow; oxygen, red; nitrogen, blue; sulfur, green. (C) Residues interacting with the GMP moiety of GTP are indicated. Hydrogen bonds are indicated by dotted lines.

FRODO graphic program (31), the GA5 sequence was replaced with AGUAGU, the authentic WNV 5′-terminal sequence, and the N7 methyl group was generated. The resulting WNV MTase-m7GpppAGUAGU complex was subjected to energy minimization with CNS 1.0 (5).

To model the WNV MTase in complex with an N7 RNA substrate (GpppAGU), the crystal structure of the Ecm1 MTase-m7GpppG-SAH complex (PDB entry 1R11) (11) was superimposed onto that of the WNV MTase. The methyl group of the m7Gppp was deleted, and the remaining Gppp moiety was retained for further modeling with the WNV MTase. To place the remaining RNA nucleotides of the GpppAGU substrate, the m7G of the m7GpppAGU substrate from the 2′-O modeling was superimposed onto the cap Gppp from the Ecm1 complex. With the graphic program TURBO FRODO (5), the orientations and positions of AGU were manually adjusted and docked into the RNA-binding cleft of the WNV MTase. The resulting WNV MTase-GpppAGU complex was subjected to energy minimization with CNS 1.0 (5).

RESULTS

The binding sites for the SAM/SAH, GTP, and RNA molecules are conserved among flavivirus MTases. Crystal structures of MTases have been reported for four flaviviruses, including DENV-2 (PDB code 1L9K) (9), WNV (PDB code 2OY0) (39), Meaban virus (PDB code 2OXT) (20), and Murray Valley encephalitis virus (MVEV; PDB code 2PXC) (4). Comparison of these MTases revealed three conserved structural elements, i.e., binding sites for SAM/SAH, GTP, and RNA. Figure 1A shows the three binding sites on the surface of WNV MTase. The SAM/SAH-binding pocket is depicted on the basis of the cocystal structure of the WNV MTase with SAH (39). The GTP-binding site of WNV MTase was modeled through structural alignment with the DENV-2 GTP-SAH-MTase tertiary complex (PDB code 2PID) (9). The putative RNA-binding site is indicated by the extended patch of positively charged residues; similar positively charged patches occur at the corresponding location on the surfaces of the other flavivirus MTases (4, 9, 20).

Mutations within the SAM-binding site impair both the N7 and 2′-O methylations. We performed a systematic mutagenesis to define the functions of the SAM-, GTP-, and RNA-binding sites. Figure 1B illustrates the interactions between the SAH and the WNV MTase. A side chain oxygen of D131 forms a hydrogen bond with the amine of the adenine moiety, the side chains of I147 and K105 sandwich the adenine base, a side chain nitrogen of H110 interacts with the 2′-OH of the ribose of SAH through a hydrogen bond, a side chain oxygen of E111 forms a hydrogen bond with the 3′-OH of the ribose, the D146 side chain interacts with the amine group of SAH, and the side chain of S56 forms two hydrogen bonds with the carbonyl groups of SAH. Sequence alignments showed that, except for K105, all of the amino acids listed here are conserved among the MTases of WNV, DENV, and YFV (data not shown).

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Point mutations within the SAM-binding site do not knock out SAM binding. A SAM-binding assay was performed to establish whether the reduced methylation efficiency of the
SAM-binding mutants was due to the reduction of SAM binding. Equal amounts of WT and mutant MTases were UV cross-linked to the 3H-labeled SAM and analyzed by SDS-PAGE (Fig. 2D). The molar ratio of MTase and 3H-SAM was kept at about 1:1 and 8 to 9/20 M in the cross-linking reaction mixtures. Quantification of the 3H-labeled SAM-MTase complexes showed that, among the SAM-binding mutants, only the D131A mutation significantly reduced the SAM-binding activity, whereas other mutations had only a minor or no effect on SAM binding. In a negative control, no 3H-labeled MTase complex was observed if the reaction mixture was not exposed to UV light (see Fig. 3B). The results indicate that a single amino acid change within the SAM-binding pocket is not sufficient to block SAM-MTase complex formation.

Interestingly, although the D131A mutant reduced SAM binding to 10% of the WT level, the mutant retained relatively high N7 activity (79% of the WT activity). In contrast, mutants S56A, K105A, H110A, and E111A maintained high SAM-binding activities (85 to 124% of the WT level; Fig. 2D) but their N7 methylation activities were relatively low (<40% of the WT level; Fig. 2C). Similar results were obtained for the 2'-O methylation activities of the SAM-binding mutants when D131A was compared with other mutants. These results indicate that SAM binding may not directly correlate with the N7 and 2'-O methylation activities of the enzyme. Since the product molecule SAH copurifies with the protein (as evidenced by crystallized protein containing the SAH molecule [9, 39]), the copurified SAH is expected to inhibit the methylation activities of the enzyme. Therefore, kinetic analysis of the mutant enzymes was not pursued.

**SIN inhibits WNV through suppression of cap methylations.**

SIN is a SAM analogue. The methyl group that is donated in SAM is replaced by an amino group in SIN. We evaluated the effects of SIN on WNV cap methylation and on viral replication. In methylation assays, SIN inhibited both N7 and 2'-O methylations in a dose-responsive manner, with a 50% inhibitory concentration (the concentration of SIN required to inhibit 50% of the enzyme activity) of approximately 14 M for both methylations (Fig. 3A). To determine whether SIN inhibits the methylation reactions through competitive binding to the SAM-binding site of the MTase, we examined the ability of SIN to compete against 3H-labeled SAM-MTase complex formation (Fig. 3B). Increasing amounts of SIN led to decreasing amounts of 3H-SAM-MTase complex formation; almost no 3H-SAM-MTase complex was detected at 10 and 20 M SIN. As a negative control, a reaction mixture containing 3H-

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**FIG. 2.** Analysis of the SAM-binding site. (A) Effects of mutations of the SAM-binding site on N7 methylation of WNV RNA cap. The substrate G*pppA-RNA, representing the first 190 nucleotides of the WNV genome (the asterisk indicates that the following phosphate was 32P labeled), was incubated with WT or mutant MTase in the presence of SAM. The MTase variants are indicated at the top. Since the reactions were performed in N7 buffer (pH 7.0), which supports N7 methylation but not 2'-O methylation, G*pppA-RNA was converted to m7G*pppA-RNA without further 2'-O methylation (39). The reaction mixtures were digested with nuclease P1 to release m7G*pppA (product) or G*pppA (residual substrate) and then analyzed on a TLC plate. The methylation efficiencies of mutant MTases were compared with that of the WT MTase (set at 100%) and are indicated below the TLC results. (B) Effects of mutations of the SAM-binding site on 2'-O methylation. The experiments were performed as described for panel A, except that the substrate m7G*pppA-RNA was incubated in 2'-O buffer (pH 10, which supports 2'-O methylation), resulting in m7G*pppAm-RNA. 32P-labeled markers, G*pppA, and m7G*pppA are indicated at the top of panel A. The position of the origin and the migration positions of the G*pppA, m7G*pppA, and m7G*pppAm molecules are shown to the left of the TLC images. (C) Summary of methylation results from panels A and B. Averages of three independent experiments are presented. (D) SAM-binding activities of mutant MTases. The UV-cross-linked 3H-SAM-MTase complex was analyzed by SDS-PAGE and quantified by fluorography (top). The relative cross-linking efficiencies of the WT and mutant MTases are indicated below the autoradiograph. Five micrograms of each indicated MTase was separated by SDS-PAGE and stained with Coomassie blue (bottom).
SAM and MTase (in the absence of SIN) without UV cross-linking did not yield any detectable 3H-SAM-MTase complex (Fig. 3B).

Consistent with the above biochemical results, viral titer reduction assays showed that SIN could suppress WNV replication, with a 50% effective concentration (EC50; the concentration required to inhibit 50% of the viral titer) of approximately 27 μM (Fig. 3C). A 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide-based cell viability assay showed no cytotoxicity when cells were treated with SIN at up to 167 μM; however, cytotoxicity was detected at concentrations of ≥500 μM; the 50% cytotoxicity concentration (CC50; the concentration of SIN required to reduce cell viability by 50%) was about 4.5 mM (Fig. 3C). Therefore, the therapeutic index (CC50/EC50) of SIN against WNV in cell culture is around 167. Overall, the results indicate that SIN inhibits WNV via suppression of viral MTase through direct binding to the SAM-binding site.

**Mutations within the GTP-binding site specifically reduce 2'-O methylation.** A GTP-binding pocket was initially found when crystals of the DENV-2 MTase were soaked with GTP; this GTP-binding site was proposed to interact with the cap structure so as to position the 2'-OH of the adenosine during 2'-O methylation (9). Structural comparison showed that the WNV and DENV-2 MTases are superimposable, allowing us to model the GMP on the WNV MTase. As depicted in Fig. 1C, F24 of the MTase stacks with the guanine base, a side chain oxygen of N17 forms a hydrogen bond with the 2'-OH of the ribose, the side chain of K13 interacts with both 2'-OH and 3'-OH groups through two hydrogen bonds, and the γ-phosphate of GTP forms hydrogen bonds with the side chains of R28 and S150. Sequence alignment indicated that these residues are conserved among the WNV, DENV, and YFV MTases (data not shown).

Mutant MTases containing an Ala substitution within the GTP-binding pocket were prepared and assayed for their methylation activities (Fig. 4). For N7 methylation, only the F24A substitution produced a substantial effect (15% of the WT activity); all other mutations had very minor effects (>77% of the WT activity). In contrast, all of the mutants...
showed a reduction in 2'-O cap methylation (7 to 62% of the WT activity). These results demonstrate that, except for F24, residues within the GTP-binding pocket specifically function during 2'-O methylation rather than during N7 methylation.

Cap methylation requires a stacking interaction between the bases of RNA cap and an aromatic residue. Since RNA cap recognition often requires stacking between the base of the cap and aromatic rings from a protein (15, 16, 21), we examined whether other aromatic residues could substitute for F24 in the WT MTase (Fig. 4). As expected, the F24W and F24Y mutants exhibited 94 to 100% and 61 to 90% of the WT N7 and 2'-O methylation activities, respectively. Compared with the non-aromatic F24A mutant, the aromatic F24W and F24Y mutants exhibited an improvement in both methylations. These results demonstrate that the stacking interaction between the base of the cap and an aromatic residue at position 24 facilitates the methylation activities.

GTP and GpppA specifically inhibit 2'-O, but not N7, methylation activity. Mutagenesis results showed that the GTP-binding pocket functions only at the step of 2'-O methylation (Fig. 4). On the basis of this result, we hypothesized that addition of GTP to the methylation reaction mixture would cause the GTP-binding pocket of the MTase to become occupied, leading to specific inhibition of 2'-O methylation. As expected, GTP inhibited 2'-O methylation in a dose-responsive manner (Fig. 5A). In contrast, the N7 methylation was not suppressed by GTP up to 1 mM, the highest concentration tested. As a control, we found that ATP did not inhibit 2'-O methylation until the concentration reached 1 mM, which suppressed the 2'-O methylation by only 20% (data not shown).

Since co-crystal structures showed that cap analogues also bind to the GTP pocket in the DENV-2 (10) and MVEV (4) MTases, we examined the effect of GpppA on the methylation reactions (Fig. 5B). Similar to the GTP results, GpppA inhibited the 2'-O methylation, but not the N7 methylation; however, the concentrations required to suppress the 2'-O methylation are higher for GpppA than that for GTP. These functional data are in agreement with the previous finding that binding affinity for DENV-2 MTase decreases in the order GTP > GpppA > ATP (9). Nevertheless, the selective suppression of 2'-O methylation by GTP and GpppA again suggests that the GTP-binding site is specifically important during 2'-O cap methylation.

Distinct amino acids within the putative RNA-binding site are required for the N7 and 2'-O methylations. To identify the amino acids that are important for interaction with RNA dur-
ing cap methylations, we selected 20 amino acids on the surface of the WNV MTase molecule for Ala-scanning mutagenesis (Fig. 6A). The rationale for the selection of residues K21, R37, K41, R44, K45, R57, R84, and R213 is that they form a continuous positively charged patch on the surface of the WNV structure. These basic residues could bind to the negatively charged phosphate backbone of the RNA during methylation reactions. Importantly, the positively charged surface is close to the SAM-binding pocket. Electropotential analyses of flavivirus MTase structures showed that this positively charged patch is conserved, and no alternative RNA-binding path on the protein surface could be found (4, 9, 20, 39). Besides the basic
residues, we have also selected a number of amino acids such as aromatic (W87, F133, Y220, and Y254), acidic (E34 and E149), and other (L16, H42, V55, Q114, L184, and T216) amino acids. The aromatic residues may undergo stacking interactions with the bases of the RNA chain. Amino acids adjacent to the putative RNA-binding site were also selected, because these residues could interact with RNA substrates during the methylation reactions.

Mutants with Ala substitutions of the above 20 residues were prepared and assayed for two methylation activities. A summary of the methylation results of three to five independent experiments is presented in Fig. 6B and C. For N7 methylation, mutations R37A, R57A, R84A, W87A, and E149A (red residues, left half of Fig. 6C) decreased the activity to <20% of the WT enzyme activity; mutations V55A, R213A, and Y220A (green residues, right half of Fig. 6C) suppressed N7 methylation to 20 to 60% of the WT activity. Interestingly, none of these residues critical for N7 methylation is located near the putative cap-binding pocket; instead, they are all clustered near the SAM-binding pocket and within the putative RNA-binding site. For 2′-O methylation, mutations E34A, R37A, V55A, R57A, W87A, R213A, and Y220A reduced the activity to <20% of the WT enzyme activity (red residues, right half of Fig. 6C); mutations L16A, L184A, and Y254A reduced the 2′-O activity to 20 to 60% of the WT activity (green residues, right half of Fig. 6C).

The effects of individual mutations on the two methylation events could be categorized into four groups. The first group contains residues critical for both methylation activities; these are R37, R57, and W87. Mutations of any of these residues reduced both methylation activities to <20% of the WT activity level. The second group contains residues selectively important for the N7 methylation activity; these are R84 and E149. Mutations of these amino acids dramatically suppressed the N7 methylation but had no or a minor effect on the 2′-O methylation activity. The third group contains amino acids selectively important for the 2′-O methylation activity; these are L16, E34, L184, and Y254. Mutations of these residues substantially suppressed the 2′-O methylation but not the N7 methylation. The fourth group contains residues not important for either methylation activity; these are K21, K41, H42, R44, K45, Q114, F133, and T216. Overall, the results demonstrate that certain residues within or near the putative RNA-binding site are important for both methyllations; however, distinct sets of amino acids are required for the N7 and 2′-O methyllations. On the MTase surface, residues critical for the 2′-O methylation are more dispersed than those important for the N7 methylation; furthermore, a larger number of residues are critically involved in the 2′-O methylation than in the N7 methylation (compare the two parts of Fig. 6C). These results suggest that the RNA substrate binds to different positions on the enzyme surface during the two methylation reactions.

A single amino acid change within the RNA-binding site is not sufficient to decrease RNA-MTase complex formation. To test whether the reduction in methylation activities of the RNA-binding mutants was due to the decreased RNA binding, we directly compared the RNA-protein complex formation between the WT and mutant MTases by using a gel mobility shift assay (Fig. 6D). The R37A and R57A mutants were selected because substitutions of these residues resulted in the reduction of both N7 and 2′-O methylations (Fig. 6B). Incubation of 32P-labeled m7G(PP)pppA-RNA (representing the first 190 nucleotides of the WNV genome) with increasing amounts of WT MTase sequentially revealed multiple RNA-protein complexes. No difference in RNA-binding affinity was observed when WT and mutant (R37A and R57A) MTases were used (Fig. 6D, right half). Since RNA-MTase binding could involve multiple contact sites, the weakening of one binding interaction with R37 or R57 (due to Ala substitution) may not be sufficient to block the RNA-protein interaction. So, we prepared a double-mutant R37A/R57A MTase (Fig. 6D, left half). Methylation assays showed that the R37A/R57A mutations completely abolished both the N7 and 2′-O methylations (data not shown). In the gel mobility shift assay, slightly more R37A/R57A mutant protein was required than the WT or the single amino acid mutant MTase for the formation of equivalent amounts of RNA-protein complexes (Fig. 6D). Similar binding results were obtained when unmethylated G(PP)pppA-RNA was used as a probe in the gel mobility shift assay (data not shown). These results suggest that the complexes are formed through multiple contact sites; a single mutation within the RNA-binding site is not sufficient to abolish complex formation; nevertheless, mutation of a single residue may disrupt transient local RNA interactions, resulting in reduced methylation activities.

WNVs containing single amino acid mutations within the SAM-, GTP-, or RNA-binding site are defective or lethal in viral replication. To examine the biological relevance of the above biochemical results, we introduced Ala substitutions of selected residues into an infectious cDNA of WNV. One (S56), two (K13 and F24), and three (R37, W87, and E149) residues were selected for alteration for the SAM-, GTP-, and RNA-binding site, respectively. The nucleotide changes in the mutant full-length RNAs are underlined in Fig. 7A. Initially, we used specific infectivity (the number of PFU that were generated upon transfection of 1 μg of full-length RNA) to measure the effects of the mutations on viral replication (Fig. 7A). The results showed that only the WT, K13A mutant, and S54A mutant RNAs generated 3.0 × 10^4, 1.5 × 10^5, and 5.3 × 10^5 PFU/μg RNA, respectively. In contrast, the F24A, R37A, W87A, and E149A mutant RNAs did not yield any PFU under the assay conditions used (day 3 to 4 p.t.). Next, an IFA was performed to monitor viral protein synthesis in the transfected cells (Fig. 7B). IFA-positive cells transfected with WT RNA appeared earlier than did those transfected with mutant RNAs. Among the mutant RNAs, only K13A, F24A, and S56A RNA transfections exhibited IFA-positive cells; transfection with other mutant RNAs did not yield positive cells up to 72 h p.t., the latest time point tested.

Viral titers in the culture fluid from the transfected cells at day 5 p.t. were quantified. Only the WT, K13A, F24A, and S56A RNAs produced viruses; no detectable virus was observed in cultures transfected with the R37A, W87A, or E149A mutant RNA. Among the replication-competent mutant RNAs, the K13A and F24A RNAs yielded homogeneous plaques with smaller sizes than the WT (Fig. 7C); continuous culturing of these mutant viruses (a total of 12 days in Vero cells) did not change the small-plaque phenotype; sequencing of the complete MTase domain of the viral RNAs recovered the engineered mutations without any secondary changes.
These results suggest that the K13A and F24A mutant viruses are stable and attenuated in replication. For S56A RNA, viruses with heterogeneous plaque morphology were recovered from the transfected cells on day 5 p.t. (Fig. 7C); sequencing of the RNA extracted from the viral pools showed a mixture of WT and mutated nucleotides; continuous culturing of the S56A virus (9 days in Vero cells) revealed large plaques, with the engineered mutations having reverted to the WT sequence (data not shown), suggesting that the S56A mutant virus is not stable. The higher stability of the K13A and F24A viruses than of the S56A virus is likely due to three-nucleotide versus one-nucleotide changes between the two groups of RNAs (Fig. 7A).

In addition to the above mutants, we prepared three more
For the SAM-bindingsite, mutation of residues that interact with molecular repositioning model for flavivirus cap methylations. Structured comparisons among flavivirus MTases revealed three conserved functional elements: SAM-, GTP-, and putative RNA-binding sites of the WNV MTase attenuate or abolish viral replication. The effect of an individual mutation on viral replication correlated with the mutation's effect on N7 methylation. For example, mutation K13A, for which the N7 and 2'-O methylation activities were, respectively, 77% and 7% of the WT activities, yielded a stable mutant virus. In contrast, mutation E149A, for which the N7 and 2'-O methylation activities were, respectively, about 2% and 69% of the WT activities, did not yield any virus in the specific infectivity assay and in culture fluid. Finally, it should be noted that the above results could not exclude the possibility that cellular 2'-O MTase may methylate the viral RNA cap when the viral 2'-O MTase is defective. However, this possibility is remote because the host capping machinery resides in the nucleus whereas WNV RNA replicates in the cytoplasm.

**DISCUSSION**

We recently elucidated the general mechanism by which a single MTase domain of flavivirus methylates guanine N7 on the cap and ribose 2'-OH on the first transcribed nucleotide (8, 28). This flaviviral mechanism contrasts with cellular and many viral mRNA cap methylations which require two separate MTase proteins or domains for the N7 and 2'-O methylation reactions (29, 34, 35). Crystal structures of four flavivirus MTases consistently showed a single SAH-binding site (4, 9, 20, 39). Therefore, the SAM molecule, binding in the same binding pocket as does SAH, donates methyl groups to both the N7 and 2'-O positions during flavivirus RNA cap methylation. Since the two methylation reactions have different methyl acceptors, the 5' terminus of substrate GpppA-RNA needs to be repositioned between the two methylation events. The present report represents the first study to experimentally examine the molecular repositioning model. Using a structure-based mutagenesis approach, we demonstrate that substrates GpppA-RNA and m7GpppA-RNA require two distinct subsets of amino acids on the surface of WNV MTase for the N7 and 2'-O methylation reactions to occur (Fig. 8A and Table 1).

**SAM-binding site.** Structural comparisons among flavivirus MTases revealed three conserved functional elements: SAM-, GTP-, and putative RNA-binding sites (Fig. 1). Our biochemical analysis of each of these structural elements supports the molecular repositioning model for flavivirus cap methylations. For the SAM-binding site, mutation of residues that interact with SAM is detrimental to both the N7 and 2'-O methylations. However, each mutation affects the two methylation reactions to different degrees (Fig. 2). This is not surprising, given that the two methylation reactions employ distinct mechanisms (11, 13, 14). SAM-MTase binding experiments suggest that, except for mutant D131A, the decreased methylation activities of mutant MTases are not due to the loss of SAM binding (Fig. 2); instead, the decreased activities might be caused by a local conformational change in SAM. Overall, these results demonstrate that the single SAM-binding site donates methyl groups to both the N7 and 2'-O positions during viral RNA cap formation. **GTP-binding site.** Although cocystal structures of the DENV-2 and MVEV MTases showed that the GTP-binding pocket can accommodate GTP and cap analogues (4, 9, 10), no functional analysis was previously performed to ascertain how the GTP-binding pocket functions during flavivirus cap methylation. The present study showed that mutations within the GTP-binding site, except for F24, specifically decreased 2'-O, but not N7, methylation activities (Fig. 4). Moreover, addition of GTP and the cap analogue GpppA, each of which can occupy the GTP-binding site (as evidenced by the cocystal structures (9, 10), to the methylation reaction mixtures specifically inhibited 2'-O methylation (Fig. 5).**
Putative RNA-binding site. RNA-binding site mutagenesis experiments have mapped residues that are critical for distinguishing between the Gppp-RNA and m\(^7\)Gppp-RNA substrates during the two methylation reactions (Fig. 6). More residues are involved in the 2'-O methylation than are involved in the N7 methylation. On the surface of the WNV MTase, the residues critical for N7 methylation reside in the putative RNA-binding site and the area adjacent to the SAM-binding site. In contrast, the residues important for the 2'-O methylation are more dispersed, extending to the GTP-binding site. These functional results strongly argue that the RNA substrate binds to differently defined sites on the enzyme surface during the two methylation reactions.

A molecular repositioning model. Figure 8A summarizes the residues known to be important for cap methylations on the surface of WNV MTase. We previously showed that flavivirus MTases methylate the RNA cap in the order GpppA→m\(^7\)GpppA→m\(^7\)GpppAm (28, 39); the first three and four nucleotides of the viral RNA were, respectively, protected by the WNV NS5 protein during the N7 and 2'-O methylations in a footprinting experiment. Furthermore, distinct viral RNA sequences and structures are required for the two methylation reactions (8). On the basis of the RNA-SAH-MTase tertiary structures of the Ecml N7 MTase (11) and the VP39 2'-O MTase (14), we modeled the WNV RNA substrates onto the crystal structure of the WNV MTase-SAH binary complexes; no steric hindrance was observed between the WNV RNA and MTase (Fig. 8B). Collectively, these results have allowed us to define a molecular repositioning model for flavivirus cap methylations. Guanine N7 of the substrate GpppA-RNA is first positioned proximal to the methyl group of SAM to generate m\(^7\)GpppA-RNA, after which the m\(^7\)G moiety of m\(^7\)GpppA-RNA is repositioned to the GTP-binding pocket so as to register the 2'-OH of the adenosine with SAM, resulting in m\(^7\)GpppAm-RNA.

Although the present study has revealed snapshots of two distinct sets of amino acids distinctly required for the two cap methylation events, the results do not address the questions of how the RNA substrate is translocated and whether one or two MTase molecules are involved in the two methylation reac-
If a single MTase catalyzes both methylations, the SAH molecule should be replaced with a fresh SAM molecule after SAH dissociates from the SAH-bound MTase and reassociates with a new SAM molecule for 2'-O methylation. The latter scenario is reminiscent of the reovirus RNA cap formation process, in which the SAM-bound MTase for 2'-O methylation is released from the SAH-bound MTase and reassociates with a new SAM molecule. However, our recent experiments showed that two distinct mutant MTases (H. Dong and P.-Y. Shi, unpublished results) catalyze the N7 and 2'-O methylations of the flavivirus RNA cap through an RNA dissociation-and-reassociation process (Fig. 8C).

**Flavivirus MTase as a target for antiviral therapy.** A panel of recombinant WNVs was prepared to investigate the effects of the SAM-, GTP-, and putative RNA-binding sites on viral replication. Mutations within these binding sites attenuated or inactivated viral replication (Fig. 7). The negative effects of individual mutations on viral replication correlated with the remaining activities of N7 methylation. Along the same lines, our previous study of the MTase-conserved K61-D146-K182-E218 motif showed that mutation D146A, which completely knocked out 2'-O methylation but retained 42 to 76% of the WT N7 methylation activity, generated stable WNV in cell culture (39). These results strongly suggest that N7 methylation is essential for flavivirus replication and could therefore be targeted for antiviral therapy. As a proof of principle, we showed that SIN, a SAM analogue, inhibited WNV cap methylations, as well as viral replication in cell culture (Fig. 3). However, it should be noted that selection of resistant virus is required to demonstrate that the viral inhibition by SIN is due to its blockage of cap methylation. One potential problem of targeting the SAM-binding site for antiviral therapy is the lack of specificity. Since the geometry of the SAM-binding pocket is conserved among various MTases, inhibitors of SAM binding are likely to suppress both host and viral SAM-utilizing enzymes, resulting in toxicity. As mentioned above, we recently found that flavivirus cap methylation is viral RNA sequence specific (8), raising the possibility that inhibitors could be developed to specifically block viral RNA-MTase interactions without affecting host mRNA cap methylation.

In summary, our study has provided structural and biochemical evidence that flavivirus MTase catalyzes two cap methylations through a substrate-repositioning mechanism. Of the two methylation reactions, the N7 activity is the one essential for flavivirus replication. Therefore, the MTase regions identified as critical for N7 methylation in this study could be targeted for the development of drugs against emerging flaviviruses.

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VOL. 82, 2008 FLAVIVIRUS RNA CAP METHYLATION VIA REPOSITIONING MODEL 4307