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Rapid Communication

Separate molecules of West Nile virus methyltransferase can independently catalyze the N7 and 2′-*O* methylations of viral RNA cap

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ABSTRACT

West Nile virus methyltransferase catalyzes N7 and 2′-*O* methylations of the viral RNA cap (GpppA-RNA→m^{7}GpppAm-RNA). The two methylation events are independent, as evidenced by efficient N7 methylation of GpppA-RNA→m^{7}GpppA-RNA and GpppAm-RNA→m^{7}GpppAm-RNA, and by the 2′-*O* methylation of GpppA-RNA→GpppAm-RNA and m^{7}GpppA-RNA→m^{7}GpppAm-RNA. However, the 2′-*O* methylation activity prefers substrate m^{7}GpppA-RNA to GpppA-RNA, thereby determining the dominant methylation pathway as GpppA-RNA→m^{7}GpppA-RNA→m^{7}GpppAm-RNA. Mutant enzymes with different methylation defects can *trans* complement one another *in vitro*. Furthermore, sequential treatment of GpppA-RNA with distinct methyltransferase mutants generates fully methylated m^{7}GpppAm-RNA, demonstrating that separate molecules of the enzyme can independently catalyze the two cap methylations *in vitro*.

Introduction

Many members of the genus *Flavivirus* are significant human pathogens, including West Nile virus (WNV), yellow fever virus (YFV), dengue virus (DENV), Japanese encephalitis virus, and tick-borne encephalitis virus (Lindenbach and Rice, 2001). The plus-strand RNA genome of flavivirus contains a 5′-terminal cap 1 structure (m^{7}GpppAm) (Cleaves and Dubin, 1979; Wengler and Gross, 1978). Cap formation on eukaryotic mRNA entails four enzymatic reactions, in which the 5′-triphosphate of the nascent RNA is cleaved to a diphosphate by an RNA triphosphatase, capped with GMP by an RNA guanylyltransferase, methylated at the N7 position of the cap guanosine by an RNA guanine-methyltransferase (N7 MTase), and methylated at the ribose 2′-OH positions of the first and/or second nucleotides of RNA by a nucleoside 2′-*O* MTase (Furuichi and Shatkin, 2000; Shuman, 2001). Since host mRNA capping occurs in the nucleus, viruses that replicate in the cytoplasm, such as flaviviruses, encode their own capping apparatus. For flaviviruses, the RNA triphosphatase and MTase are respectively located in the C-terminus of NS3 (Bartelma and Padmanabhan, 2002; Wengler and Wengler, 1993) and the N-terminus of NS5 (Egloff et al., 2002; Ray et al., 2006), whereas the location of the guanylyltransferase remains elusive.

We recently found that a single MTase of flavivirus performs both the N7 and 2′-*O* methylations of the viral RNA cap (Ray et al., 2006; Zhou et al., 2007). In WNV, the two cap methylations require distinct RNA elements within the 5′-terminal stem-loop of the genomic RNA (Dong et al., 2007). Incubation of unmethylated GpppA-RNA substrate with flavivirus MTase in the presence SAM at high pH (pH 9–10) sequentially generated products GpppA-RNA→m^{7}GpppA-RNA→m^{7}GpppAm-RNA. These results indicate that (i) the two methylation events are sequential; and (ii) 2′-*O* methylation may be dependent on prior N7 methylation. Optimization of assay conditions showed that the N7 and 2′-*O* methylations require different pH, pH 7 and 9–10, respectively (Zhou et al., 2007). At pH 7, the N7 methylation is optimal, but no 2′-*O* methylation can occur. At pH 9–10, 2′-*O* methylation is optimal, and N7 methylation occurs at about 30–50% of the optimal activity (measured at pH 7.0). The distinct assay conditions could be used to separate the two methylation events. Specifically, the N7 methylation can be measured by conversion of GpppA-RNA→m^{7}GpppA-RNA at pH 7; no 2′-*O* methylation would occur under this condition. The 2′-*O* methylation can be monitored by conversion of m^{7}GpppA-RNA→m^{7}GpppAm-RNA; no N7 methylation would occur under this condition, because the substrate has already been methylated at the guanine N7 position. These assays have allowed us to dissect mutational effects of the MTase on the two methylation events (Dong et al., 2008; Zhou et al., 2007).

Crystal structures of flavivirus MTases (Assenberg et al., 2007; Egloff et al., 2002; Mastrangelo et al., 2007; Zhou et al., 2007) exhibit distinct binding sites for *S*-adenosyl-L-homocycteine (SAH), GTP, and

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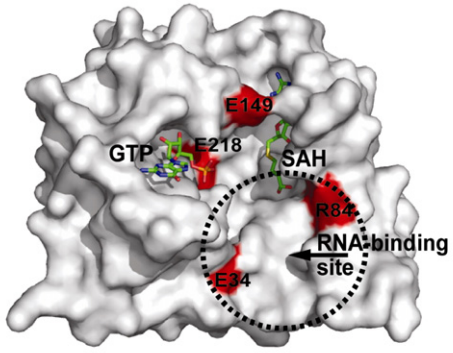
RNA (Fig. 1A). The binding site for SAH, the byproduct from *S*-adenosyl-L-methionine (SAM) after transfer of its methyl group, was assumed to be the binding site for the methyl donor SAM. The RNA cap is bound at the GTP site, as evidenced by co-crystal structures of MTase complexed with cap analogues (Assenberg et al., 2007; Egloff et al., 2002, 2007). The RNA-binding site was proposed to interact with the 5′-terminal region of RNA (Zhou et al., 2007). Despite the single known binding site for SAM, flavivirus MTase performs two distinct methylation reactions. Therefore, the substrate GpppA-RNA must be repositioned so as to accept the N7 and 2′-OH methyl groups from SAM. In the repositioning model, guanine N7 of GpppA-RNA is first positioned next to SAM to generate m^7GpppA-RNA, after which the m^7G moiety is repositioned to the GTP-binding pocket so as to precisely register the 2′-OH of the adenosine to the SAM molecule, resulting in m^7GpppAm-RNA (Zhou et al., 2007). The molecular repositioning model was supported by mutagenesis results showing that two distinct sets of amino acids on the surface of the WNV MTase are required for the two methylation reactions (Dong et al., 2008). One key question looming is what determines the sequential N7 and 2′-*O* methylations of the flavivirus RNA cap.

The goal of this study is to analyze the relationship between the N7 and 2′-*O* methylations during WNV cap formation. We report, for the first time, that N7 methylation could efficiently occur using substrates that are either 2′-*O* unmethylated (GpppA-RNA) or 2′-*O* methylated (GpppAm-RNA). In contrast, 2′-*O* methylation prefers a substrate that is N7 methylated (m^7GpppA-RNA) to a substrate that is N7 unmethylated (GpppA-RNA). The results suggest that the preference for the substrate with prior N7 methylation during 2′-methylation is the determinant for sequential methylations of flavivirus RNA cap.



B

MTase	Methylation activity: N7 (GpppA-RNA→ m7GpppA-RNA)	Methylation activity: 2'-O (m7GpppA-RNA →m7GpppAm-RNA)	Viral RNA replication	Reference
E34A	72%	10%	+	Dong et al, 2008
R84A	4%	100%	-	
E149A	2%	69%	-	
E218A	76%	0%	+	Zhou et al, 2007
WT	100%	100%	+	

Fig. 1. Mutant MTases of WNV used in this study. (A) Surface representation of the WNV MTase structure. Amino acids E34, R84, E149, and E218 are indicated in red. The SAH molecule is depicted based on the co-crystal structure of the WNV MTase (Zhou et al., 2007). The GTP molecule was modeled through alignment of the WNV MTase structure with the DENV-2 GTP-SAH-MTase tertiary complex structure (Egloff et al., 2002), using PyMOL. The putative RNA-binding site is also indicated. (B) Summary of N7 and 2′-*O* methylation activities of mutant MTases. For each mutant MTase, the N7 and 2′-*O* methylation activities were respectively calculated from TLC analysis of G*pppA-RNA→m^7G*pppA-RNA and m^7G*pppA-RNA→m^7G*pppAm-RNA reactions. All RNAs used in this study represented the 5′-terminal 190 nucleotides of the WNV genome. Symbol "*" indicates that the following phosphate is ^{32}P-labeled. The WT methylation activity was set as 100% for each of the two methylations. The effects of individual mutations on viral replication in the context of genome-length RNA are indicated. Symbols "+" and "−" indicate that genome-length RNA containing the MTase mutation are replicative (indicated by plaque formation) and non-replication, respectively.

Results

Distinct mutant MTases

During the course of study of flavivirus MTase, we have identified a panel of WNV mutant MTases in which several single Ala-substitutions could each cause a defect in either N7 or 2′-*O* methylation. Fig. 1 shows four such mutants: E34A and E218A were competent in N7 methylation, but defective in 2′-*O* methylation; whereas R84A and E149A were defective in N7 methylation, but competent in 2′-*O* methylation. The N7 and 2′-*O* methylation activities of these mutant MTases (Fig. 1B) were previously quantified through thin-layer chromatography (TLC) analysis of the GpppA-RNA→m^7GpppA-RNA and m^7GpppA-RNA→m^7GpppAm-RNA reactions, respectively. On the crystal structure of the WNV MTase (Fig. 1A), E34 and R84 are within the RNA-binding site; E149 is adjacent to SAH molecule, and is above the RNA-binding site; and E218 is within the active site of 2′-*O* methylation (K61-D146-K182-E218 tetrad), and is located between the GTP- and SAH-binding sites. These four mutants, together with the wild-type (WT) MTase, were used in this study to analyze the relationship between the two methylation events.

The 2′-O methylation is independent of the N7 methylation, but it does prefer a substrate with prior N7 methylation

To examine whether 2′-*O* methylation is dependent on prior N7 methylation, we performed a time-course study of the methylation, using two N7-defective mutants (R84A and E149A) and the WT MTase on the substrates G*pppA-RNA and m^7G*pppA-RNA (representing the first 190 nucleotides of the WNV genome; "*" indicates that the following phosphate is ^{32}P-labeled). The reactions were performed at pH 9 buffer, which is optimal for 2′-*O* methylation and supports about 50% of the optimal N7 methylation activity (Zhou et al., 2007). Vaccinia virus VP39, a well-characterized 2′-*O* MTase, was included as a positive control. The methylation reactions were treated with nuclease P1 (to release cap structures) and intestinal alkaline phosphatase (to remove terminal phosphate). The reaction mixtures were then analyzed on a 20% polyacrylamide denaturing gel (Fig. 2A). We chose high percentage gel, rather than TLC, to improve the resolution of the detection method (i.e., to clearly separate four different cap structures: G*pppA, m^7G*pppA, G*pppAm, and m^7G*pppAm). Similar to VP39, mutants R84A and E149A could each perform 2′-*O* methylation on both G*pppA-RNA and m^7G*pppA-RNA substrates, demonstrating that the 2′-*O* methylation is independent of the N7 methylation. However, the 2′-*O* methylation was more efficient when the m^7G*pppA-RNA substrate, rather than the G*pppA-RNA substrate, was used; this difference was more dramatic for the control VP39 than those for the R84A and E149A mutants (Fig. 2B). For the WT MTase, the conversion of G*pppA-RNA→m^7G*pppA-RNA→m^7G*pppAm-RNA was detected; however, small amount of G*pppAm-RNA product was also detected on the gel. Collectively, the results demonstrate that the 2′-*O* methylation is not absolutely dependent on the N7 methylation, but it does prefer a substrate with prior N7 methylation.

Two mutant MTases with different methylation defects, but not mutants with the same methylation defect, can complement one another in trans

We examined whether two mutant enzymes with different methylation defects can complement one another, converting the unmethylated G*pppA-RNA to the fully methylated m^7G*pppAm-RNA product. As shown in Fig. 3A, E149A alone (lane 2) converted G*pppA-RNA to G*pppAm-RNA, but almost no other products, confirming that the E149A MTase is competent in 2'-O methylation, but defective in N7 methylation. In contrast, E34A (lane 3) or E218A