Review

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Flavivirus RNA methylation

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The 5’ end of eukaryotic mRNA contains the type-1 (m7GpppNm) or type-2 (m7GpppNmNm) cap structure. Many viruses have evolved various mechanisms to develop their own capping enzymes (e.g. flavivirus and coronavirus) or to ‘steal’ caps from host mRNAs (e.g. influenza virus). Other viruses have developed ‘cap-mimicking’ mechanisms by attaching a peptide to the 5’ end of viral RNA (e.g. picornavirus and calcivirus) or by having a complex 5’ RNA structure (internal ribosome entry site) for translation initiation (e.g. picornavirus, pestivirus and hepacivirus). Here we review the diverse viral RNA capping mechanisms. Using flavivirus as a model, we summarize how a single methyltransferase catalyses two distinct N-7 and 2’-O methylations of viral RNA cap in a sequential manner. For antiviral development, a structural feature unique to the flavivirus methyltransferase was successfully used to design selective inhibitors that block viral methyltransferase without affecting host methyltransferases. Functionally, capping is essential for prevention of triphosphate-triggered innate immune activation; N-7 methylation is critical for enhancement of viral translation; and 2’-O methylation is important for subversion of innate immune response during viral infection. Flaviviruses defective in 2’-O methyltransferase are replicative, but their viral RNAs lack 2’-O methylation and are recognized and eliminated by the host immune response. Such mutant viruses could be rationally designed as live attenuated vaccines. This concept has recently been proved with Japanese encephalitis virus and dengue virus. The findings obtained with flavivirus should be applicable to other RNA viruses.

Introduction

The 5’ ends of almost all eukaryotic cellular mRNAs possess a cap structure, which consists of an N-7 methylguanosine (m7G) moiety linked to the first nucleotide of the nascent mRNA via a 5’-5’ inverted triphosphate bridge (Fig. 1a). The cap structure plays important roles in mRNA splicing, intracellular RNA transport, RNA stability and turnover, and recognition by eukaryotic translation initiation factor 4E (eIF4E) for efficient translation (Banerjee, 1980; Furuichi & Shatkin, 2000; Ghosh & Lima, 2010). The cap structure of mRNA is co-transcriptionally formed through three sequential enzymic reactions in the nucleus (Shuman, 2001) (Fig. 1b): (i) an RNA triphosphatase (RTPase) removes the γ-phosphate from the 5’-triphosphate end of the nascent RNA to generate 5’-diphosphate RNA (pppN-RNA→ppN-RNA); (ii) an RNA guanylyltransferase (GTase) transfers the GMP moiety from GTP to ppN-RNA to yield the cap core structure (GTP + ppN-RNA→GpppN-RNA); and (iii) an RNA guanine-methyltransferase (N-7 MTase) methylates the guanine at the N-7 position to produce a type-0 cap structure (m7GpppN-RNA). The type-0 cap is only found in metazoan and lower eukaryotes. In higher eukaryotes, m7GpppN-RNA is further methylated at the ribose 2’-O position of the nascent mRNA by a ribose 2’-O methyltransferase (2’-O MTase) to form mainly type-1 (m7GpppNm) and also type-2 (m7GpppNmNm) cap structures. Both 2’-O MTase and N-7 MTase use S-adenosyl-L-methionine (SAM) as the methyl donor and generate S-adenosyl-L-homocysteine (SAH) as a by-product (Fig. 1c).

Most viruses carry the same type-1 cap structure as cellular mRNA. Since many RNA viruses replicate in the cytoplasm, they do not have access to the host capping machinery located in the nucleus. Therefore, these viruses have evolved to encode their own capping apparatus, many of which are different from the canonical capping pathway of host mRNA as described above. The function of each element of viral RNA cap (m7GpppN) has been determined: (i) the guanosine cap core structure protects the 5’ triphosphates from activating the host innate immune response (Pichlmair et al., 2006); (ii) the N-7 methylation is essential for viral replication through enhancement of viral RNA translation (Ray et al., 2006); and (iii) the 2’-O methylation functions to evade the host immune response by mimicking cellular mRNA (Daffis et al., 2010; Züst et al., 2011). The essential
functions of viral RNA cap and methylation, together with distinct mechanisms between host RNA cap formation and viral RNA cap formation (which allows design of inhibitors that selectively block the viral RNA cap formation without affecting the host RNA cap formation), have opened new opportunities for vaccine and antiviral development.

Here we review the current understanding of diverse viral RNA capping mechanisms. We summarize the recent progress in flavivirus RNA methylation and its use as a target for antiviral and vaccine development. The capping of viral mRNA from DNA viruses (such as vaccinia virus) is not described here. Interested readers are encouraged to read a broader review on the capping mechanisms of viral mRNA (Decroly et al., 2012).

**Distinct capping pathways of RNA viruses**

**Flavivirus**

Viruses from the genus *Flavivirus* within the family *Flaviviridae* contain a single-strand, plus-sense RNA genome of approximately 11 kb. Flavivirus replicates in the cytoplasm without entering the nucleus, and encodes its own capping enzymes. The cap formation of flavivirus RNA follows the conventional pathway of eukaryotic mRNA cap formation, through sequential enzymatic actions of RTPase, GTase and MTase (Fig. 2a). Flavivirus RTPase has been mapped to the C-terminal domain of non-structural protein 3 (NS3) (Li et al., 1999; Wengler & Wengler, 1991). Three studies suggest that the GTase may reside in the MTase domain of NS5 (Bollati et al., 2009; Egloff et al., 2007b; Issur et al., 2009); however, more studies are needed to clearly demonstrate the full activity of GTase of flavivirus NS5. The N-7 and 2′-O methyl groups have been located to the N-terminal domain of NS5 (Egloff et al., 2002a; Ray et al., 2006; Zhou et al., 2007).

**Alphavirus**

The RNA cap formation of alphavirus, a group of plus-strand RNA viruses within the family *Togaviridae*, follows an unconventional mechanism (Fig. 2b). As exemplified by Semliki Forest virus (SFV), the viral RNA capping takes place in the cytoplasm. Viral nsP2 functions as RTPase cleaving the β-γ phosphate bond at the 5′ end of viral RNA to form ppN-RNA (Vasiljeva et al., 2000). nsP1, possessing N-7 MTase and GTase activities, catalyses the transfer of a methyl group from SAM to the N-7 position of GTP to yield m7GTP before formation of the covalent m7GMP-enzyme (m7GMP-nsP1) complex. Type-0 cap m7GpppN-RNA is formed by transferring m7GMP to ppN-RNA (Ahola & Kääriäinen, 1995; Ahola et al., 1997). The type-0 cap of alphavirus RNA is not methylated at the ribose 2′-O position of the first nucleotide of

*Fig. 1.* Canonical mechanism of mRNA cap formation. (a) The mRNA cap structure. The cap structure consists of an N-7 methylguanosine, a triphosphate bridge and one or two 2′-O methyl nucleotide(s). The N-7 and 2′-O methyl groups are indicated in blue. (b) Canonical mechanism of mRNA cap formation. Four enzymes required for cap formation are indicated: 5′ RTPase, GTase, N-7 MTase and 2′-O MTase. Phosphates are coloured to indicate their origins. The red phosphate is derived from GTP during the GTase reaction. (c) Structures of methyl donor SAM and by-product SAH of methylation reaction. The transferred methyl group of SAM is coloured in blue.
RNA; instead, a methyl group is transferred to the exocyclic N2 position of the cap structure in SFV (van Duijn et al., 1986); the function of this methylation remains unclear. Since alphavirus RNA does not have 2'-O-methylation, the N2 methylation or an unidentified mechanism may play a role in subverting host innate immune response during viral infection.

**Coronavirus**

Members from the family *Coronaviridae* produce mRNAs with a type-1 cap structure (Lai et al., 1982; Lai & Stohlman, 1981). It has been predicted that nsp13 has an RTPase activity (Ivanov et al., 2004; Ivanov & Ziebuhr, 2004), but direct evidence of nsp13 in performing the

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**Fig. 2.** Distinct pathways for various viral RNA cap formation. (a) Flavivirus RNA cap formation. Flavivirus follows the canonical RNA cap formation except that a single MTase domain catalyses both N-7 and 2'-O methylations in a sequential manner. (b) Alphavirus cap formation (exemplified by SFV). Alphavirus nsP1 possesses both N-7 MTase and GTase activities; nsP1 catalyses N-7 methylation of GTP to generate m7GTP. The m7GMP moiety of m7GTP is then transferred to ppN-RNA, resulting in m7Gppp-N-RNA. The m7Gp-ppN-RNA of alphavirus is not further methylated at the ribose 2'-O position of the first nucleotide of RNA. (c) Rhabdovirus cap formation (exemplified by VSV). GTP is first hydrolysed to GDP by an unidentified NTPase (indicated by '?'). The 5'-monophosphorylated viral mRNA is transferred to the GDP by a PRNTase, generating GpppA-RNA. The RNA cap is then subjected to 2'-O and N-7 methylations in a sequential manner by the L protein of VSV. (d) Influenza virus cap formation. Influenza virus polymerase contains three subunits: PA, PB1 and PB2. The PB2 subunit binds to the 5'-end of capped cellular mRNA; the PA subunit (containing an EndoN activity) cleaves the 5'-capped RNA fragment from the cellular mRNA; the cleaved short capped mRNA is used as a primer to synthesize nascent viral mRNA by PB1. RdRp, RNA-dependent RNA polymerase.
RTPase activity remains to be demonstrated. The GTase activity for coronavirus remains elusive. The N-7 MTase of severe acute respiratory syndrome coronavirus was mapped to nsp14 (Chen et al., 2009). Using nsp10 as a co-factor, nsp16 acts as a 2′-O MTase; the nsp10 activates nsp16 2′-O MTase activity by facilitating nsp16 to bind m7GpppA-RNA substrate and the SAM donor (Bouvet et al., 2010; Chen et al., 2011; Decroly et al., 2011). Notably, the nsp10/nsp16 can only methylate m7GpppA-RNA, not m7GpppG-RNA (Bouvet et al., 2010), suggesting a specific recognition between the enzyme and RNA substrate during the 2′-O methylation reaction. Specific recognition between MTase and RNA substrate has also been observed in flavivirus (see below) and Sendai virus, a member of the paramyxovirus subfamily (Ogino et al., 2005).

**Rhabdovirus**

Viruses from family *Rhabdoviridae* and other families from the order *Mononegavirales* form their RNA cap through a mechanism that is completely different from the cellular mRNA capping pathway. The cap formation of mRNA from these viruses is best characterized for vesicular stomatitis virus (VSV; from the family *Rhabdoviridae*) (Ogino & Banerjee, 2011). During the VSV mRNA cap formation (Fig. 2c), GTP is hydrolysed to GDP by an as-yet-unknown NTase. The 5′-monophosphorylated viral mRNA with the conserved vesiculoviral mRNA-start sequence is transferred to the GDP by a polynucleotidyltransferase (PRNTase), generating the Gpp-pA-RNA cap structure (Ogino & Banerjee, 2007; Ogino et al., 2010). Next, the RNA cap is sequentially methylated at the ribose-2′-O position and the guanine-N-7 position, resulting in m7GpppAm-RNA (Li et al., 2005, 2006; Rahmeh et al., 2009). Both the PRNTase and MTase (including both 2′-O and N-7 methylations) activities have been mapped to the L protein of VSV. Compared with the canonical pathway, the cap formation of VSV RNA exhibits three unique features. (i) The VSV capping enzyme transfers p-RNA to GDP, rather than GMP to pp-RNA. (ii) The PRNTase covalently binds to monophosphorylated mRNA to form the enzyme–pNP-RNA intermediate. Such covalent linkage involves a conserved His residue within an ‘HR (His-Arg)’ motif of PRNTase; in contrast, the conventional GTase uses a conserved lysine to covalently link to GMP during the enzyme–GMP intermediate formation. (iii) 2′-O methylation of the VSV mRNA cap structure precedes N-7 methylation.

**Influenza virus**

Influenza virus and some other single-strand, negative-sense RNA viruses from families *Orthomyxoviridae*, * Arenaviridae* and *Bunyaviridae* have evolved to steal cap structure from host mRNA to generate their viral RNA cap. This mechanism, known as cap snatching, was first discovered in influenza virus (Bouloy et al., 1978). The cap snatching during influenza virus replication is performed by viral polymerase which consists of three subunits (PA, PB1 and PB2) (Fig. 2d). Subunit PB2 binds to the 5′ end of capped cellular mRNA (Fechter et al., 2003; Guilligay et al., 2008); the cellular mRNA is cleaved at 10–13 nt downstream from the cap structure by the viral endonuclease (EndoN) located at the N terminus of the PA subunit (Dias et al., 2009; Yuan et al., 2009); the released short capped mRNA is used as a primer by the viral polymerase to synthesize nascent viral mRNA. The sequence, length and structure of the 5′ capped mRNA vary from one virus to the other.

**Picornavirus and calicivirus**

Viruses from the families *Picornaviridae* and *Caliciviridae* (both are single-stranded positive-sense RNA viruses) use a protein instead of a cap structure to initiate viral RNA transcription and to protect RNA from degradation. This phenomenon was first discovered in picornavirus (Flanegan et al., 1977; Lee et al., 1977) and later found in calicivirus (Schaffer et al., 1980). The 5′ end of viral RNA is covalently attached to a small basic protein VPg (viral protein genomelinked); the 5′ VPg-pU-pU-RNA structure is formed between a phosphate from viral RNA and a conserved Tyr residue of VPg. During viral replication, the tyrosine hydroxyl group from VPg functions as a ‘protein-primer’ to produce VPg-linked polyU; the VPg-linked polyU then serves as a primer to synthesize RNA by viral polymerase (3DPol) (Paul et al., 1998). Several crystal structures of 3DPol in complex with VPg have been solved (Chen et al., 2013a; Ferrer-Orta et al., 2006; Gruez et al., 2008); these structures suggest that 3DPol from different picornaviruses may perform VPg uridylation through distinct mechanisms. For viral translation, poliovirus utilizes an internal ribosome entry site (IRES) instead of a cap for translation initiation; therefore, VPg is not required for establishing the initial infection of poliovirus (Fitzgerald & Semler, 2009). In contrast, calicivirus VPg protein interacts directly with the cap-binding protein of the ribosome, eIF4E. This interaction is essential for viral translation; therefore, calicivirus VPg acts as a ‘cap substitute’ during initiation of translation of viral mRNA (Goodfellow et al., 2005).

**Hepacivirus and pestivirus**

The 5′ ends of hepacivirus and pestivirus (two genera from the family *Flaviviridae*) genomic RNA do not carry a cap structure. Instead, they contain IRES to initiate viral translation in a cap-independent manner. The hepatitis C virus (HCV) IRES directly recruits the ribosome 40S subunit (ribosomal P-site) to the translation initiator codon of the genome without mRNA scanning; such IRES-mediated translation does not require eukaryotic initiation factors eIF1, 1A, 4A, 4B and 4E (López-Lstra et al., 2005). Compared with HCV, the IRES from picornavirus differs significantly in both structure and functionality (Niepmann, 2013). The picornavirus IRES does not directly attract the ribosomal 40S subunit, but rather does so through the high-affinity eIF-4G-binding site (Hellen & Sarnow, 2001).
Because the 5' termini of both HCV and bovine viral diarrhea virus (a pestivirus) genomes contain an unprotected triphosphate, these RNAs are prone to trigger innate immune response. Consequently, these viruses have also evolved distinct mechanisms to limit and evade the antiviral response (Garaigorta & Chisari, 2009; Guidotti & Chisari, 2001).

Flavivirus 5' RNA cap methylation

Sequential N-7 and 2'-O methylation of RNA cap

The current understanding of flavivirus RNA cap methylation has been mainly derived from studies of West Nile virus (WNV) and dengue virus (DENV) MTases. The flavivirus genome encodes a single MTase domain, located at the N-terminal one-third of the NS5 protein, which methylates guanosine N-7 and ribose 2'-O positions of the viral RNA cap. The C-terminal two-thirds of the NS5 protein contains an RNA-dependent RNA polymerase (RdRp) activity. The flaviviral MTase domain performs the two cap methylations in a sequential manner: N-7 methylation followed by 2'-O methylation, i.e. GpppA-RNA → m7GpppA-RNA → m7GpppAm-RNA (Ray et al., 2006). The sequential cap methylation have been shown in several flaviviruses such as WNV, DENV and yellow fever virus (YFV) (Zhou et al., 2007).

Distinct RNA elements required for flavivirus N-7 and 2'-O methylations

Compared with cellular mRNA cap methylations, flavivirus MTase catalyses its cap methylations with two unique features. First, cellular N-7 and 2'-O methylations are catalysed by two separate MTase proteins, while flavivirus N-7 and 2'-O methylations are performed by a single MTase protein. Second, cellular MTases catalyse mRNA cap methylation in an RNA sequence-independent manner, while flavivirus MTase catalyses its cap methylations in a viral sequence/structure-dependent manner. Specifically, flavivirus N-7 methylation requires the 5'-terminal stem-loop structures of viral genomic RNA (especially the second and third nucleotides; Fig. 3a); the sequence of the stem region is not important. The 2'-O methylation requires the first two nucleotides of the genomic RNA and a minimal RNA length of 20 nt (Dong et al., 2007, 2010a). It should be noted that the first two nucleotides of the flavivirus genome (GpppAG) are absolutely conserved (Wengler & Wengler, 1981) and the 5' UTR of the flavivirus genome forms a conserved stem-loop structure (Brinton & Dispoto, 1988). Furthermore, footprinting studies using GpppA-RNA and m7GpppA-RNA showed that the 5' termini of RNA selectively interact with the recombinant NS5 MTase. Specifically, the first three and four nucleotides of the viral RNA substrates were bound by the enzyme during the N-7 and 2'-O methylations, respectively; in addition, nucleotides 18–21 of the WNV genomic RNA were bound by recombinant MTase (Dong et al., 2007, 2008d). Since flavivirus methylation requires viral RNA sequence and structure, studies using only cap analogues or non-viral RNA sequence should be avoided, especially when performing biochemical analysis.

Substrate preference determines the sequential order of flavivirus N-7 and 2'-O methylations

What dictates the sequential order of N-7 and 2'-O methylations? Flavivirus MTase catalyses the two methylations at different optimal pHs in vitro: pH 7.0 for N-7 methylation and pH 9.0 for 2'-O methylation (Fig. 3b; Zhou et al., 2007). Therefore, performing methylation assays in buffers with different pH allows analysis of one type of methylation without being complicated by the other type of methylation. kinetic analysis showed that WNV MTase performs N-7 methylation at a comparable efficiency when using substrates GpppA-RNA and GpppAm-RNA (i.e. GpppA-RNA → m7GpppA-RNA and GpppAm-RNA → m7GpppAm-RNA) (Dong et al., 2008a). In contrast, the 2'-O methylation prefers substrate mGpppA-RNA to substrate GpppA-RNA (i.e. the reaction of m7GpppA-RNA → m7GpppAm-RNA is far more efficient than the reaction of GpppA-RNA → GpppAm-RNA). In agreement with the latter result, cap analogue mGpppA binds to flavivirus MTase with a higher affinity than cap analogue GpppA (S. P. Lim, unpublished result). Collectively, the preference of 2'-O methylation for a substrate with the N-7 position pre-methylated, mGpppA-RNA, is the underlying driving force for the sequential order of N-7 and 2'-O methylations. However, whether these in vitro results reflect the in vivo activity during viral replication remains to be determined.

Flavivirus MTase structure

Crystal structures of MTases from many flaviviruses have been solved, including DENV-2 (Egloff et al., 2002b), WNV (Zhou et al., 2007), Murray Valley encephalitis virus (MVEV; Assenberg et al., 2007), Meaban virus (Mastrangelo et al., 2007), YFV (Geiss et al., 2009), Modoc virus (Jansson et al., 2009) and Wesselsborn virus (Bollati et al., 2009). All flavivirus MTases exhibit a conserved structure consisting of three motifs: (i) an N-terminal domain; (ii) a core domain with a typical structure of SAM-dependent MTase; and (iii) a C-terminal domain (Fig. 4a). The core domain contains a conserved SAM-binding pocket, a GTP-binding pocket and an RNA-binding site (Fig. 4b). The K-D-K-E tetrad (Fig. 4c) forms the active site that is conserved among all 2'-O MTases from various organisms. For flavivirus MTase, mutagenesis studies showed that the Asp residue of the K-D-K-E tetrad is essential for both methylation activities; the other three residues of the K-D-K-E tetrad are essential for 2'-O methylation, but not for N-7 methylation (Dong et al., 2010a; Ray et al., 2006).

SAM-binding pocket. Flavivirus MTase has a single SAM-binding site (Fig. 4b), indicating that the SAM molecules bind to this pocket and donate the methyl groups during both methylation reactions. In agreement with the structure result, mutations of the SAM-binding pocket of
WNV and DENV MTase impaired both N-7 and the 2′-O methylations. Sinefungin, a SAM analogue, inhibited both N-7 and 2′-O MTase activities of WNV and DENV (Chung et al., 2010; Dong et al., 2008b; Kroschewski et al., 2008). These results indicate that flavivirus MTase uses a single methyl donor site to methylate two chemically distinct reactions. An interesting observation is that recombinant MTases of various flaviviruses were always co-purified with SAM or SAH, raising the question about whether the binding of SAM or SAH contributes to the structural integrity of the enzyme.

**GTP-binding pocket.** A GTP-binding pocket (Fig. 4b) was first identified when the crystals of DENV-2 MTase were soaked with GTP (Egloff et al., 2002b). The aromatic ring of a Phe residue at position 24 or 25 (the numbering varies...
among different flaviviruses) of the MTase forms a stacking interaction with the guanosine moiety of GTP. Two distinct functions have been proposed for the GTP-binding pocket. First, the GTP pocket binds the guanosine moiety of GTP during the GTase reaction. This hypothesis was initially proposed based on the crystal structure of DENV MTase in complex with cap analogue GpppA (Egloff et al., 2007a), and was later supported by some experimental evidence (Bollati et al., 2009; Issur et al., 2009). Second, the GTP pocket binds the N-7 methylated guanosine (m7G) cap during the 2'-O methylation reaction. This function is indicated by three lines of evidence. (i) Crystal structures of flavivirus MTase in complex with GTP or cap analogue GpppA showed that the guanosine moiety stacks with residue Phe24/25 and extends the phosphate groups towards the SAM-binding pocket where the 2'-O methylation occurs (Assenberg et al., 2007; Egloff et al., 2002b). (ii) Mutations of the GTP-binding pocket selectively affected 2'-O methylation, not N-7 methylation (Dong et al., 2008b; 2010a). (iii) Addition of GTP to the methylation assay selectively inhibited 2'-O methylation activity, suggesting that exogenous GTP competitively binds to the GTP pocket to suppress 2'-O methylation. Similarly, ribavirin (a guanosine analogue) 5'-triphosphate also selectively inhibits DENV-2 2'-O MTase activity; the co-crystal structure showed that ribavirin 5'-triphosphate binds to the GTP pocket (Benarroch et al., 2004). The latter result indicates that, besides multiple known antiviral mechanisms (Leyssen et al., 2005), ribavirin may also inhibit flavivirus replication through blockage of flaviviral 2'-O methylation.

RNA-binding site. Electrostatic potential analysis of flavivirus MTase structures identified a putative RNA-binding site (Fig. 4b) that is mainly formed by a number of conserved, positively charged residues. Mutagenesis studies showed that distinct sets of amino acids within the putative RNA-binding site are required for N-7 and 2'-O methylations. The exact positioning of RNA substrate on the RNA-binding site remains to be determined during the two methylation reactions. Co-crystal structures of the enzyme in a complex of m7GpppA-RNA or GpppA-RNA are critical to reveal this information. It should be noted that the crystal structure of DENV-3 MTase in complex with a 5'-capped RNA (GpppAGAACCGUG) was solved (Yap et al., 2010). The structure shows that two RNA molecules are encircled by four MTase monomers around a twofold non-crystallography symmetry axis; only two of the four monomers make direct contact with the 5' end of RNA. This structure may represent the product of guanylation of the viral genome prior to the subsequent methylation events that require repositioning of the RNA substrate to reach the methyl-donor sites.

A molecule reposition model for flavivirus RNA cap methylation

How could flavivirus MTase with one methyl donor (SAM-binding pocket) catalyse two distinct N-7 and 2'-O methylation reactions? A molecule reposition model (Dong et al., 2008b) was proposed to consolidate the structure and mutagenesis results discussed above. As shown in Fig. 5, during N-7 methylation, the guanine N-7 of GpppA-RNA substrate is first positioned next to the methyl group of SAM to generate m7GpppA-RNA. Upon N-7 methylation, the RNA substrate is repositioned on the enzyme surface so that the m7G moiety of the m7GpppA-RNA binds to the GTP pocket. In this way, the 2'-OH of the adenosine ribose of m7GpppA-RNA registers next to SAM for 2'-O methylation, generating m7GpppAm-RNA.

It is not known whether the reposition model is performed by one or two MTase molecules during the two methylation reactions. If one MTase molecule catalyses two reactions, repositioning of the RNA substrate should coordinate with a replenishment of SAH with SAM, in preparation for the second methylation (Fig. 5a). If two MTase molecules are involved, m7GpppA-RNA has to dissociate from the first MTase after the N-7 methylation, and then bind to a second MTase to perform the 2'-O methylation (Fig. 5b). The two-molecule model is supported by the result that two distinct mutant MTases (one defective in N-7 methylation and another defective in 2'-O methylation) could trans complement with each other to complete the GpppA-RNA→m7GpppAm-RNA double methylation reaction (Dong et al., 2008a). Structure studies of MVEV MTase also support the two-molecule model (Assenberg et al., 2007). This RNA–enzyme dissociation-and-association process is similar to the capping process of reovirus and bluetongue viruses, in which the N-7 and 2'-O methylation are sequentially performed by two separate MTase domains within a large polyprotein (Reinisch et al., 2000; Sutton et al., 2007). However, it should be noted that the one-molecule model should be more energeticallyfavoured than the two-molecule model because it avoids the RNA–protein dissociation-and-association events. For both one- and two-molecule models, repositioning of m7GpppA-RNA on the original enzyme (one-molecule model) or dissociation from the original enzyme (two-molecule model) may be regulated by the release of by-product SAH after the N-7 methylation.

Flavivirus internal RNA methylation

Flavivirus NS5 is recently found to harbour an internal RNA methylation activity (Dong et al., 2012). Recombinant full-length NS5 or the MTase domain of DENV can methylate viral RNA without a 5' cap structure. Mutagenesis analysis showed that the MTase K-D-K-E tetrad serves as the active site for internal methylation. In contrast to cap methylations which require specific viral RNA sequence/structure, flavivirus internal methylation does not require viral RNA sequence or structure. Non-viral RNA or synthetic polyA (but not polyG, polyC or polyU) can also be methylated. RNAs with all adenosines pre-methylated to 2'-O methyladenosines (Am) are not active substrates for internal methylation, indicating that the
internal methylation occurs at the 2′-OH position of adenosine. Mass spectroscopic analysis further demonstrated that the internal methylation product is 2′-O methylenadenosine. Importantly, mass spectroscopic study showed that genomic RNA purified from the WT DENV particles contained internal 2′-O methylenadenosines, whereas genomic RNA purified from the 2′-O methylation defective virus (containing an E217A mutation of the MTase K-D-K-E tetrad) did not have internal 2′-O methylenadenosines. It should be noted that the internal methylation activity of flavivirus MTase is much lower than cap methylation activity. Interestingly, flavivirus MTase can methylate host tRNA and ribosome rRNA in vitro, suggesting that viral MTase may modulate host RNA in the infected cells. Internal RNA 2′-O methylation is found in cellular splicesomal small nuclear RNAs (snRNAs) and rRNAs of cells; such modification regulates mRNA splicing, translation or decay, through interplay with RNA-binding proteins and microRNAs. The biological function of flavivirus internal methylation and the significance of its ability to methylate host RNA remain to be determined.

**Genetic and physical interactions between flavivirus MTase and RdRp domains**

A revertant analysis was performed using a lethal WNV (containing a D146S mutation of the K-D-K-E tetrad) that was completely defective in N-7 methylation (Zhang et al., 2008). Sequencing of the revertant viruses revealed two adaptive mutations, K61Q in MTase and W751R in RdRp. Analysis of recombinant WNVs showed that the D146S substitution alone was lethal for viral replication; the
compensatory mutations rescued viral replication. Biochemical analysis showed that a low level of N-7 methylation of the D146S MTase is essential for the recovery of revertant viruses; the K61Q mutation facilitates viral replication through improvement of N-7 methylation; the RdRp W751R mutation improves viral replication through enhancement of polymerase activity. Collectively, the results raise the possibility that mutation(s) within RdRp that enhances the polymerase activity could potentially rescue the replication defect that is caused by an inhibitor (when such a compound is identified in the future; see below) of N-7 methylation.

Different models have been proposed for intra-molecular interactions between flavivirus MTase and RdRp domains in the context of full-length NS5. The discrepancy is due to different methods used to map the interaction, including in silico docking in combination with revertant mapping (Malet et al., 2007), small-angle X-ray scattering (Bussetta and Choi, 2012) and crystallography (Lu & Gong, 2013). Specifically, in silico docking and reverse genetic analyses using WNV NS5 suggest a cooperative model in which the MTase domain is located in front of the RdRp domain; once newly synthesized RNA exits from the ‘RNA exit tunnel’ of RdRp (located in the front of RdRp), the 5’ end of RNA is ready for the capping process (Malet et al., 2007). The small-angle X-ray scattering study using DENV NS5 suggests an elongated side-by-side model in which the MTase domain is located next to the finger subdomain of RdRp (Bussetta & Choi, 2012). The crystal structure of full-length NS5 of Japanese encephalitis virus (JEV) shows that the MTase domain is attached to the backside of the RdRp through key hydrophobic interactions, shielding the top-right rim of the ‘NTP entry channel’ (Lu & Gong, 2013). Biochemical and mutagenesis analyses are needed to define the relevance of these structural conformations during distinct steps of viral RNA synthesis and cap modification.

Targeting flavivirus MTase and GTase for antiviral development

N-7 MTase is essential for flavivirus replication

The function of cap methylation has been extensively studied using genome-length RNA and replicon RNA of WNV (Dong et al., 2008c) and DENV (Dong et al., 2010a; Kroschewski et al., 2008). Compared with a WNV replicon without a 5’ cap (i.e. pppA-RNA), RNA with a 5’ GpppA, m7GpppA or m7GpppAm cap increased the translation level by approximately 16-, 25- and 25-fold, respectively. These results suggest that (i) the guanine cap is essential for translation; (ii) N-7 cap methylation enhances translation efficiency; and (iii) 2’-O methylation does not contribute to viral translation. Mutagenesis analysis showed that mutations that ablated N-7 MTase activity were lethal for flavivirus replication; in contrast, mutations that abolished 2’-O MTase activity did not ablate viral replication in Vero cells (defective in IFN production). These results have provided genetic evidence that N-7 MTase is a potential antiviral target.

Rational design of selective inhibitor of flavivirus MTase

The core domain of SAM-dependent MTase is conserved among host and viral enzymes. It is therefore a challenge to design inhibitors that selectively suppress flavivirus MTase without affecting host MTases. A close analysis of flavivirus MTase structure uncovered a hydrophobic cavity located next to the SAM-binding pocket (Fig. 6a) (Dong et al., 2010b). This cavity is conserved among all flavivirus MTases, and is not found in various host MTases. Chemical modification of SAH with substituents that extend into the identified cavity generated inhibitors (exemplified by compound 10; Fig. 6b) that showed improved and selective activity against DENV MTase, but not against related human enzymes (Fig. 6c) (Lim et al., 2011). The crystal structure of DENV MTase in complex with an SAH derivative (compound 10) confirmed that its N6-substituent bound in this cavity (Fig. 6d) and induced conformational changes in residues lining the pocket (Fig. 6e). These results demonstrate that targeting structural features unique to viral MTase can generate selective inhibitors without affecting host MTases. Further studies are needed to improve the cell permeability of these compounds and to show antiviral activity in cell culture.

A panel of nucleoside analogues containing thymidine base were recently reported to have selective inhibition against WNV MTase (Chen et al., 2013b). Although the potency was low [with Ki (the dissociation constant of an enzyme-inhibitor complex) values of 24.2 μM and 3.9 μM for N-7 and 2’-O methylations, respectively, for the most potent compound GRL-002], the compounds did not inhibit human RNA MTase even at 300 μM. Molecular modelling suggests that these compounds could potentially bind to the SAM-binding pocket with specific interactions with the MTase residues. For further development, the potency of these compounds needs to be significantly improved.

Inhibition of flavivirus GTase (and 2’-O MTase) through targeting the GTP pocket of MTase domain

Since the GTP pocket is essential in binding substrate GTP during the GTase reaction, Geiss et al. (2011) developed an assay to screen for inhibitors that can displace GTP from the DENV MTase domain. After screening 235 456 compounds, they identified a class of compounds that could compete against GTP binding to the GTP pocket, with IC50 values of single-digit micromolar concentration. The compounds showed double-digit micromolar EC50 values in DENV-2 replicon cells, with an undesirable therapeutic index (TI) of <10 (Stahla-Beek et al., 2012). Interestingly, the compounds specifically inhibited flaviviruses Kunjin virus and YFV, but not alphavirus Sindbis virus, in viral titre reduction assays. Although the potency remains to be improved, these compounds represent a reasonable chemistry starting point for further development. As mentioned above, since the GTP pocket participates in 2’-O methylation, it remains to be differentiated how much antiviral activity of the compounds was contributed
by blocking the 2′-O methylation and by blocking the GTase activity.

**Functions of 2′-O methylation in evasion of innate immune response**

Although 2′-O methylation was discovered more than 35 years ago, the function has been elusive until recently. We and others have demonstrated that the 2′-O methylation is required to escape the IFN-mediated innate immune response in host cells. Infection with 2′-O MTase mutant mouse hepatitis virus (MHV; a coronavirus) resulted in increased levels of IFN production in cell culture; mechanistically, the 2′-O MTase mutant MHV was recognized by the cytosolic innate immune receptor MDA5, leading to the upregulation of IFN-β expression (Züst et al., 2011). In contrast, cells infected by WT or mutant 2′-O MTase WNV produced equivalent levels of IFN, indicating that the WNV RNA without 2′-O methylation does not potentiate IFN production (Daffis et al., 2010). However, the 2′-O MTase-defective mutants of WNV, MHV and vaccinia virus (an enveloped DNA virus belonging to the poxvirus family) were shown to be more sensitive to antiviral inhibition by IFIT1 (IFN-induced proteins with tetra-tricopeptide repeat) than the WT viruses. Using IFIT1 knockout mice, Szeretter et al. (2012) showed that WT WNV could largely evade the antiviral effect of IFIT1, whereas the 2′-O MTase mutant WNV was restricted in vivo by IFIT1-dependent and -independent mechanisms in different cell types. More recently, Kimura et al. (2013) reported that IFIT1 inhibited JEV through binding to the 5′ capped RNA without 2′-O methylation. The binding of IFIT1 to 2′-O-unmethylated capped RNA impairs the loading of eukaryotic translation initiation factors to the 2′-O-unmethylated RNA template, leading to selective inhibition of viral RNA translation (Habjan et al., 2013). Collectively, methylation studies of members from different virus families have provided an evolutionary explanation for the 2′-O methylation of eukaryotic mRNA: to function as a molecular pattern recognition signal to distinguish self from non-self RNA during viral infection. Many viruses that replicate in the cytoplasm have evolved their own RNA-modifying enzymes to mimic cellular mRNA to evade host innate immune suppression.

**2′-O MTase mutant flaviviruses as a vaccine approach**

The discovery that the 2′-O methylation of flavivirus RNA cap is an immune evasion strategy to hide from the host cell’s innate immune recognition provides a new way to
attenuate viruses by targeting the MTase. A cytoplasmic virus defective in 2'-O methylation is replicative; but its viral RNA lacks 2'-O methylation, and is recognized and subsequently eliminated by the host immune response. Such mutant viruses could be rationally designed as a live attenuated vaccine. Using JEV and DENV, we recently showed that 2'-O MTase-defective viruses could be rationally designed as a new approach for live attenuated flavivirus vaccine development. For both viruses, mutants carrying a Glu→Ala substitution in the K-D-K-E tetrad (E218A and E217A for JEV and DENV-2, respectively) were attenuated in mice and in monkeys, while being sufficiently immunogenic to generate a protective immune response (Li et al., 2013; Züst et al., 2013).

**JEV**

2'-O MTase E218A mutant JEV was attenuated in 129 mice (Fig. 7a). Its virulence was restored in the A129 mice (defective in type I IFN receptor) (Fig. 7b). In BALB/c mice, inoculation (intraperitoneal, i.p.) of 4 × 10⁷ p.f.u. WT JEV killed the mice, whereas neither morbidity nor mortality was observed in the mice inoculated with an equal amount of 2'-O MTase mutant virus (Li et al., 2013). Compared with the WT JEV, the neurovirulence of mutant JEV was attenuated by about 24-fold, as indicated by the LD₉₀ values: 7.9 p.f.u. for the WT and 188.4 p.f.u. for the mutant virus. Mice immunized with 1 × 10⁶ p.f.u. mutant virus were fully protected from challenge with 1 × 10⁶ p.f.u. WT SA14 or SX06 JEV strains. Neutralizing antibodies and specific T-cells were induced in the immunized mice (Li et al., 2013). In cell culture, the mutant JEV was more sensitive than the WT virus to IFN inhibition (Fig. 7c).

**DENV-2**

Similar to JEV, 2'-O MTase E217A mutant DENV-2 was more sensitive to IFN suppression than the WT virus (Fig. 8a). Rhesus monkeys immunized with 1 × 10⁸, 1 × 10⁹ or 1 × 10¹⁰ p.f.u. of a 2'-O MTase mutant DENV-2 produced neutralizing antibody titres that were in the range of other live attenuated vaccine candidates currently under development (Fig. 8b). After challenging the animals with 1 × 10⁹ p.f.u. WT DENV-2, all monkeys across the three immunization groups were fully protected with no detectable viraemia. The results indicate that as little as 1 × 10⁸ p.f.u. and one dose of immunization were fully protective (Züst et al., 2013). These results are encouraging, but will have to be repeated by extending the time between immunization and challenge to address how long the protection lasts. An immunization schedule that requires only one dose would be an optimal solution for travellers and to reduce cost in endemic countries. Interestingly, mosquitoes feeding on blood containing the mutant DENV-2 were not infected, whereas mosquitoes feeding on blood containing the WT virus were infected and thus able to transmit it (Züst et al., 2013).

There is no established correlate of protection for DENV vaccine development. The vaccine efficacy study with CYD dengue vaccine in Thailand has confirmed that the neutralization antibody titres are not necessarily predictive for protection. Despite equal neutralizing titres against all four serotypes of DENV in vaccinated individuals, the efficacy was only 9.2% for DENV-2 compared with 100% protection for DENV-4 (Sabchareon et al., 2012). The structural proteins prM and E primarily induce a B-cell response and antibodies, while non-structural proteins induce potent T-cell responses. E-protein-specific antibodies

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**Fig. 7.** Enhanced sensitivity of 2'-O MTase (E218A) mutant JEV to IFN inhibition. (a, b) Groups of 3-week-old 129 mice (a) and A129 mice (defective in type I IFN receptor) (b) were i.p. inoculated with 5×10⁷ p.f.u. of WT or 2'-O MTase E218A mutant JEV. The mortality was monitored for up to 15 days. (c) Inhibition of WT and mutant MTase JEV by IFN-α. BHK-21 cells were infected with the WT or E218A mutant JEV (m.o.i. 5). The infected cells were then treated with 10, 100 or 500 U ml⁻¹ IFN-α. At 48 h post-infection (p.i.), culture fluids were quantified for virus yields by plaque assay. The IFN-α-mediated inhibition of virus production was presented. Values shown are means ± SD; *, P < 0.05; **, P < 0.005. This figure is modified from a previous study (Li et al., 2013).
are essential for protection because they bind viral particles to block infection of target cells. The CD8+ T-cell response in dengue patients is predominantly NS3- and NS5-specific (Duangchinda et al., 2010; Simmons et al., 2005), whereas CD4+ T helper cells are more diverse, with specificity to both structural and non-structural proteins (Rivino et al., 2013). The importance of both CD4+ and CD8+ T-cells for protection has been convincingly shown in mouse models, where it is possible to specifically deplete individual cell types and to dissect the role of CD4+ and CD8+ T-cells (Yauch et al., 2009, 2010). Moreover, in a human dengue challenge model, IFN-γ-producing CD8+ T-cells were associated with protection (Gunter et al., 2011). Furthermore, the CYD tetravalent formulation seems to have been protective for DENV-4 in the Thai trial (Sabchareon et al., 2012). In two independent cohorts, the CD4+ T-cell response to DENV-4 was highest and most long-lasting after the third dose in vaccinated individuals (Guy and Almond, 2008). Taken together, these findings are evidence for a contribution of both serotype-specific B- and T-cells to full protection and to the maintenance of immune memory after DENV infection. A tetravalent vaccine containing 2’-O MTase mutant DENV-1, -2, -3 and -4 covers the non-structural proteins of all four serotypes of DENV to generate T-cell responses specific for all four serotypes, which can potentially improve efficacy.

The possibility to introduce targeted mutations by reverse genetics enables generation of recombinant viruses based on the circulating virus strains or based on the geographical prevalence of viral genotypes. Reversion of mutations is a concern for using the recombinant 2’-O MTase mutant virus for vaccine development. So far, continuous passaging of the current 2’-O mutant JEV and DENV in cell culture or viruses recovered from the immunized mice and monkeys did not show any reversion. The risk of reversion can be minimized by introducing multiple amino acid changes in the vaccine candidates, such that, even if a particular amino acid reverts back to WT, additional attenuating amino acids will still be present in the virus genome. Deletions in the viral 3’UTR sequences may also be further incorporated to enhance the attenuated phenotype (Hanley et al., 2004). Besides minimizing the reversion risk on the virus side, the production of attenuated vaccines can also be made safer by introducing new technologies for quality control. In particular, next-generation sequencing technology can be very useful to make the manufacturing of attenuated viruses safer. During large-scale production, even a very small number of virus revertants could be detected with deep sequencing technology because of its higher sensitivity compared with PCR/sequencing techniques.

**Outlook**

Although significant progress has been made in understanding the flavivirus RNA cap formation during the past decade, many important questions remain elusive. (i) The identity of the GTase needs to be clearly demonstrated for flavivirus capping. Compared with GTases from other organisms, the currently reported GTase from flavivirus NS5 was very weak (Issur et al., 2009). In addition, the covalent linkage of GMP to flavivirus NS5 protein upon incubation with GTP is not specific: when incubated with 32P-labelled ATP, CTP or UTP, recombinant NS5 could also be 32P-labelled (H. Dong, unpublished results). It is likely that NS5 needs an unknown co-factor(s) to potentiate its GTase activity. (ii) A mechanism needs to be defined to coordinate the various enzymes required to form the type-1 cap structure. The NS3 RPTase and NS5...
GTase/MTase. How are these enzymes orchestrate RNA synthesis and cap formation? Capping of cellular mRNA is coupled to RNA synthesis through direct binding of the capping apparatus to the RNA polymerase II elongation complex (Cho et al., 1997; McCracken et al., 1997; Yue et al., 1997). It is also likely that flavivirus RNA capping and methylation are coupled to RNA synthesis. The recent crystal structure of full-length NS5 of JEV represents the first step to reveal the relative conformation between the MTase and RdRp domains (Lu & Gong, 2013). More biochemical and structural studies are needed to define the interaction between viral RNA synthesis and capping. (iii) The exact mechanism of flavivirus N-7 and 2'-O methylation remains to be illuminated at an atomic level. Since flavivirus MTase requires distinct viral RNA elements, it is challenging to obtain co-crystal structures of MTase in complex with RNA substrates. The structure information will greatly facilitate rational design of antiviral inhibitors as well as design of 2'-O MTase mutant viruses for vaccine development. (iv) It remains to be demonstrated whether the 5' end of minus-strand flaviviral RNA is capped and/or methylated. If not, how does the viral capping apparatus differentiate between the minus- and plus-strand RNAs?

For antiviral development, more effort should be made to inhibit flavivirus MTase. Besides targeting the hydrophobic pocket next to the SAM-binding site (Lim et al., 2011), the GTP-binding pocket could also be used for the design of an antiviral inhibitor. Although the GTP pocket functions at the step of 2'-O methylation (which is not essential for viral replication), its potential role in GTase (which should be essential for viral replication) justifies the effort to target this pocket. Since the crystallization procedure has been well established for flavivirus MTase, small-molecule fragment-based screening followed by co-crystallization could be pursued to design and synthesize inhibitors of this enzyme.

The mechanism by which 2'-O cap methylation evades host immune response remains to be further investigated. What are the differentiating viral elements that enhance IFN production in cells infected with MHV but not WNV 2'-O MTase (Daffis et al., 2010)? Is IFIT1 the only host restriction factor to sense RNA without 2'-O cap methylation? Can the prototype results generated with WNV 2'-O MTase be applied to other flaviviruses? The answers to these questions will greatly enhance our understanding of the biology of RNA methylation as well as allow us to apply the knowledge to develop medical intervention.

Acknowledgements

We thank our colleagues and collaborators for helpful discussions and support during the course of the work. We thank Tony Minson and reviewers for critical and constructive suggestions of the article. Work from P.Y.S and K.F.'s groups was partially supported by the TCR Council in Singapore.

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