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Identification and Characterization of a Ribose 2′-O-Methyltransferase Encoded by the Ronivirus Branch of *Nidovirales*

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Running title: Identification of a 2′-O-Methyltransferase in a ronivirus

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Abstract

The order *Nidovirales* comprises four virus families: *Arteriviridae*, *Coronaviridae* (divided into two subfamilies *Coronavirinae* and *Torovirinae*), *Roniviridae* and recently recognized *Mesoniviridae*. RNA cap formation and methylation have been well studied for coronaviruses, focusing on identification and characterization of two cap methyltransferases (MTases), guanine-N7-MTase and ribose-2′-O-MTase. Although bioinformatic analyses suggest that the MTases may also be encoded by other large-sized nidoviruses such as toroviruses and roniviruses, no experimental evidences have been provided. In this study, we showed that a ronivirus, gill-associated nidovirus, encodes the 2′-O-MTase activity but we could not detect the activity in the homologous protein of a torovirus, equine torovirus, which is more closely related to coronaviruses. Roniviral 2′-O-MTase possesses similar and unique features in comparison with that of coronaviruses. It harbors a catalytic K-D-K-E tetrad and possibly a Rossman-like fold conserved among 2′-O-MTases and can only target the N7-methylated cap structure of adenylate-primed RNA substrates. However, roniviral 2′-O-MTase does not require a protein cofactor for activity stimulation as coronaviruses and shows distinct properties in several biochemical parameters such as reaction temperature and pH. Furthermore, the ronivirus 2′-O-MTase can be targeted by MTase inhibitors. These results extend current understanding on the RNA cap formation and methylation beyond coronaviruses in the order *Nidovirales*.

Introduction

The 5′-terminal cap structure, an N7-methylguanosine linked to the first transcribed nucleotide via a 5′-5′ triphosphate bridge, exists widely in many viral and eukaryotic cellular mRNAs, and plays a critical role in RNA stability and protein translation (Bouvet *et al.*, 2012; Furuichi & Shatkin, 2000; Ghosh & Lima, 2010). The cap-0 structure m7GpppN is formed by the sequential reactions catalyzed by RNA triphosphatase, guanylyltransferase and guanine-N7-methyltransferase (N7-MTase) (Bouvet *et al.*, 2012; Decroly *et al.*, 2012; Ghosh & Lima, 2010). In higher eukaryotes and most viruses, the cap-0 structure is further methylated at the ribose 2′-O position by ribose 2′-O methyltransferase (2′-O MTase) to form a cap-1 structure (m7GpppNm2) (Chen & Guo, 2016; Chen *et al.*, 2011; Decroly *et al.*, 2012). Recently, it has been reported that 2′-O methylation of viral mRNA cap is important for RNA viruses to evade host innate immune responses (Daffis *et al.*, 2010; Zust *et al.*, 2011).

The order *Nidovirales* is composed of the most complex positive-sense single-stranded RNA (ssRNA+) viruses with human and animal hosts, including the highly pathogenic severe acute respiratory syndrome (SARS) coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV). It consists of four virus families, including *Arteriviridae* (12.7-15.7-kb genomes; known as ″small-sized nidoviruses″), *Coronaviridae*, *Roniviridae* and the recently recognized *Mesoniviridae* (26.3-31.7 kb; the latter three families being referred to as ″large-sized nidoviruses″) (Lauber *et al.*, 2012). The *Coronaviridae* is further classified into two subfamilies, *Coronavirinae* (for coronaviruses) and *Torovirinae* (for toroviruses). The Nidoviruses are evolutionarily

related and share several common features, for example, synthesis of a set of subgenomic RNAs in the manner of discontinuous transcription and adoption of various RNA processing enzymes in genome replication *(Gorbalenya et al., 2006; Hussain et al*, 2005). Recently, the cap formation and methylation have been extensively studied for coronaviruses, focusing on two virally encoded S-adenosyl-L-methionine (SAM or AdoMet)-dependent methyltransferases: N7-MTase and 2′-O MTase. Our previous work has demonstrated that coronavirus non-structural protein (nsp) 14 functions as an N7-MTase (Chen *et al.*, 2009; Chen *et al.*, 2013) while other studies have shown that coronaviral nsp16 acts as a 2′-O-MTase with the help of the viral cofactor nsp10 (Bouvet *et al.*, 2010; Chen *et al.*, 2011; Decroly *et al.*, 2011; Decroly *et al.*, 2008; Wang *et al.*, 2015). However, there is no experimental evidence to show that any other nidoviruses (excepting coronaviruses) possess a functional methyltransferase.

According to bioinformatic analysis, all nidoviruses except arteriviruses encode a 2′-O-MTase domain at the C-terminal end of the large open reading frame 1b whereas all large-sized nidoviruses excepting toroviruses possess an N7-MTase domain following the exoribonuclease domain (Gorbalenya *et al.*, 2006; Nga *et al.*, 2011; Snijder *et al.*, 2003). We have attempted to address whether the large-sized non-coronaviral nidoviruses also encode methyltransferases involved in viral RNA cap methylation by utilizing the genetic and biochemical methodologies established in our laboratory (Chen *et al.*, 2009; Chen *et al.*, 2011), but we failed to detect any N7-MTase activity of the proteins encoded by roniviruses, mesoniviruses and toroviruses (unpublished results). In this study, we show that the MTase domain of a ronivirus (arbitrarily named

as "nsp16" by the analogy with the location and function of coronaviral nsp16), but not torovirus MTase domain (also named as nsp16), possesses the 2′-O-MTase activity. We further demonstrate that ronivirus nsp16 alone can efficiently execute its MTase activity, which is in contrast with coronaviral nsp16 that requires virus-encoded nsp10 as a cofactor for its full function. These results extend the understanding of nidovirus RNA capping and methylation and provide an evolutionary and functional link between roniviruses and coronaviruses.

Materials and methods

Radioactive reagents. S-adenosyl [methyl-^{3}H] methionine (67.3 Ci/mmol, 0.5 μCi/μl) and [α-^{32}P]-labeled guanosine 5′-triphosphate (3000Ci/mmol, 10mCi/ml) were purchased from PerkinElmer.

Cloning, expression and purification of the related proteins. The genomic cDNA of shrimp gill-associated nidovirus (GAV), a type species of *Roniviridae*, was kindly provided by Dr. J.A. Cowley and was used as the template to construct the expression constructs. Two pairs of PCR primers were used to amplify the gene fragments of GAV: 5′-CGGGATCCCTCAGTTACTCAGGCACCGC-3′ (sense) and 5′-CCCTCGAGCTCGTGCTTGACTGGGATGG-3′ (antisense) for GAV nsp10, and 5′-CGGGATCCCTCTACCACGTCGCTCCTAC-3′ (sense) and 5′-CCCTCGAGAAATTTGATGAATCTGGGAG-3′ (antisense) for GAV nsp16, which correspond to the nucleotides 11703-12212 and 19403-20089 of the GAV genome (GI:166851932), respectively. These PCR products were cloned into the *Escherichia*

coli expression vector pGEX-6p-1, resulting in the expression constructs pGEX-6p-1-GAV nsp10 and pGEX-6p-1-GAV nsp16. The mutant plasmids of GAV nsp16 were generated by one-step PCR with mutagenic primers.

E. coli BL21 (DE3), transformed with pGEX-6p-1-GAV nsp10 or nsp16, was grown in Luria broth (LB) medium containing ampicillin at 37°C. When the OD_{600nm} value of the culture reached 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM, and induced at 16°C for 16 hours. Bacterial cells were harvested by centrifugation and lysed by sonication. The GST-tagged fusion proteins were purified from the cell lysate by affinity chromatography using Glutathione Resin (GenScript) according to the manufacturer's instructions. GAV nsp10 (pp1a residues 3879-4048, 170 aa) fused with GST-tag is about 45 kD and was confirmed by SDS-PAGE. GAV nsp16 (pp1ab residues 6446-6673, 228 aa) fused with GST-tag is about 52 kD.

The genes of equine torovirus (EToV) were obtained by PCR reactions with the template of EToV genome cDNA (GI:190360102) kindly provided by Eric J. Snijder. We performed the bioinformatic analysis to predict the proteinase cleavage sites and arbitrarily named the most C-terminal cleavage product of pp1a as EToV nsp10 and that of pp1b as EToV nsp16 (the predicted 2'-MTase domain). The following primers were used to amplify the gene fragments: sense 5′-CGGGATCCGGTGTTAGATTGGGAGGTGT-3′ and antisense 5′-CCCTCGAGCTGAGGCTTAAAAACTGGA-3′ for EToV nsp10 (nt 13560-14018 of the genome), and 5′-CGGGATCCAAGGATTTTGTAAGGTCGTA-3′ (sense) and 5′-

CCCTCGAGATGTTCCATTTGGCACAAC-3′ (antisense) for EToV nsp16 (20597-21391). The PCR fragments were cloned into *E. coli* expression vectors pET30a and pGEX-6p-1 respectively, generating the plasmids of pET30a-EToV nsp10 and pGEX-6p-1-EToV nsp16.

GST fusion protein of EToV nsp16 (pp1ab residues 6593-6857, 265aa) was expressed and purified similarly to GAV nsp16, and the fusion protein size was 57 kD. The pET30a-EToV nsp10 was transformed into *E. coli* BL21(DE3), and the cells were grown in LB medium containing kanamycin at 37°C. When the OD_{600nm} value of the culture reached 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM, and the culture was grown for additional 16 hours at 16°C. Bacterial cells were harvested by centrifugation and lysed by sonication. The 6-Histidine-tagged protein was purified from the cell lysate by affinity chromatography using Ni-NTA according to the manufacturer's instructions (Qiagen). EToV nsp10 (pp1a residues 4247-4399, 153aa) was 17 kD in size. The predicted sizes of these two proteins were in accordance with their sizes in SDS-PAGE analysis.

SARS-CoV nsp10 and nsp16 were expressed and purified as described in our previous work (Chen *et al.*, 2011).

Preparation of RNA substrates. GAV 68-nt 5′-UTR was transcribed in vitro using the PCR products as a template which was amplified from GAV cDNA (primers as follows, 5′-CAGTAATACGACTCACTATTACGTTACGTTCCACGTAC-3′ and 5′-GGTCGGAAACGCTGGAAAAC-3′). Since GAV genome possesses an adenine at the 5'-proximal first nucleotide position, we used a bacteriophage T7 Φ2.5 promoter to

promote synthesis of RNA which initiated with an adenine. The MEGAscript Kit (Life Technologies) was used to transcribe RNA *in vitro* with 100 ng PCR template in a 20-μl mixture. The reaction mixture was mixed thoroughly and incubated for 16 h at 37°C. After the reaction, 2 units of TURBO DNase was added, followed by further incubation for 15 min at 37°C. For purification of RNA, the transcription reaction was extracted with phenol/chloroform/isoamyl alcohol, precipitated with 75% ethanol which contains sodium acetate, resuspended in RNA-free water (TIANGEN), and quantitated by Nanodrop 2000c (Thermo Scientific). Nonviral RNAs $pppAC_n$, $pppUC_{20}$, $pppGC_{20}$, and $pppCC_{20}$ were transcribed and purified as described previously (Chen *et al.*, 2011). Capped RNAs (GpppRNA and ^{7Me}GpppRNA) were produced using Vaccinia Capping System (New England Biolabs), purified through Sephadex G-25 quick spin columns (Roche), extracted with phenol/chloroform/isoamyl alcohol and precipitated with ethanol.^{32}P-labeled RNA substrates (G*pppRNA and ^{7Me}G*pppRNA) used for thin layer chromatography (TLC) were synthesized as described previously (Chen *et al.*, 2009).

Biochemical assay for methyltransferase activity. The ^{32}P-labeled 2′-O-MTase assay was performed in 8.5-μL reaction system (containing 50 mM Tris-HCl [pH 8.0], 2 mM dithiothreitol [DTT], 2 mM $MgCl_2$, 10 units RNase inhibitor, 2×10^3 cpm of ^{7Me}G*pppA-RNA, 0.2 mM SAM, and 0.5 μg of purified proteins or mutant proteins) at 20°C for 1 h. Control G*pppA-RNA and ^{7Me}G*pppA-RNA were prepared by using the vaccinia virus protein D1-D12, which has the activity of both GTase and N-7-MTase by incubating at 37°C for 1 h and purified as described previously (Chen *et al.*, 2011).

Control ^{7Me}G*pppAm-RNA was produced by incubation of ^{7Me}G*pppA-RNA with the vaccinia virus 2′-O-methyltransferase VP39 (Epicentre) at 37°C. The capped RNAs labeled by radioisotope were digested by nuclease P1 (US Biological) to release cap structures, then spotted onto polyethyleneimine cellulose-F plates (Merck) for TLC analysis, and developed in 0.65 M LiCl. The extent of ^{32}P-labeled cap was determined by scanning the chromatogram with a Phosphorlmager as described previously (Chen *et al.*, 2009; Chen *et al.*, 2011).

The ^{3}H-methyl incorporation MTase activity assays were performed in 20-μL reaction system (containing 50 mM Tris-HCl [pH 8.0], 2 mM DTT, 2 mM $MgCl_2$, 40 units of RNase inhibitor, 0.01 mM SAM, 0.5 μCi of S-adenosyl [methyl-3H] methionine [67.3 Ci/mmol, 0.5 μCi/μl], 1 μg proteins, and 3 μg of ^{7Me}GpppRNA substrates) at 37°C for 1 h. ^{3}H-labeled products were isolated in DEAE-Sephadex A-50 columns and quantitated by liquid scintillation (PerkinElmer) as described previously (Ahola *et al.*, 1997; Chen *et al.*, 2011).

Multiple sequence alignment and homology modeling

The multiple sequence alignment of the nsp16 protein sequence from different ***Nidovirales*** species was performed by using Clustalx, and then the result was imported into ESPript 3.0 together with the three-dimensional structure of SARS nsp16/nsp10 (PDB: 3R24) to assign the secondary structure. The 3D structure of GAV nsp16 was generated on the template structure SARS-CoV nsp16 (PDB 3R24, chain A). The multiple sequence alignment was done by Clustalx, and then uploaded to SWISS-MODEL for structural modeling, which was further analyzed by PyMol.

Results

Ronivirus nsp16 possesses SAM-dependent 2′-O-MTase activity in the absence of another protein co-factor

Bioinformatic analysis revealed that all large-sized nidoviruses encode a putative 2′-O-MTase domain located at the most C-terminal end of the replicase pp1ab polyprotein (Gorbalenya *et al.*, 2006). As coronaviral nsp16 with nsp10 as an essential cofactor has been demonstrated as a functional 2′-O-MTase (Bouvet *et al.*, 2010; Chen *et al.*, 2011; Decroly *et al.*, 2011; Wang *et al.*, 2015), we further characterized the homologous proteins of torovirus and ronivirus for 2′-O-MTase activity. For this purpose, we selected two representative viruses, one being equine torovirus (EtoV) (Smits *et al.*, 2006) that belongs to the subfamily of *Coronaviridae* and is more closely related to coronaviruses, and the other being gill-associated okavirus (GAV) (Cowley *et al.*, 2000; Cowley & Walker, 2002) that belongs to the *Roniviridae* and is distantly related to coronaviruses. As the proteinase cleavage sites are not completely established for the large polyprotein 1ab of these two viruses, their protein counterparts for coronavirus nsp16 and nsp10 are arbitrarily named as EToV or GAV "nsp16" and "nsp10", respectively, in analogy to CoV protein nomenclature. The well characterized 2′-O-MTases SARS-CoV nsp10/nsp16 and vaccinia VP39 were used as controls (Chen *et al.*, 2011; Wang *et al.*, 2015). The locations of the predicted proteinase cleavage sites and nsp10/16 in the polyprotein pp1ab are indicated in Figure 1A.

As shown in Fig. 1B, the recombinant proteins of nsp10 and nsp16 of EToV, GAV and

SARS-CoV were expressed in *E. coli* cells and purified to more than 95% homogeneity by affinity chromatography.

To test the 2′-O-MTase activity possibly possessed by the viral proteins, we first conducted 2′-O-MTase activity assays *in vitro* on a 21-nt RNA substrate (the cap-0 $^{7Me}GpppAC_{20}$) which was capped and methylated at N7 position of the cap. Transfer of ^{3}H-labeled methyl group from SAM to cap-0 RNA substrates resulted in cap-1 RNA ($^{7Me}GpppAmC_{20}$) and could be measured quantitatively by liquid scintillation detection. As the GAV host shrimps (*Penaeus monodon*) keep a low body temperature in nature, all the assays with GAV proteins were performed at 20°C while all other assays were at 37°C. As shown in Figure 1C, GAV nsp16, either in the presence or absence of GAV nsp10, showed remarkable 2′-O-MTase activity to the similar extent as vaccinia VP39 and SARS-CoV nsp10/nsp16, whereas EToV nsp16 or its mixture with nsp10 did not display 2′-O-MTase activity, similarly to the negative control (SARS-CoV nsp10 alone). These results indicate that GAV nsp16 alone possesses the 2′-O-MTase activity, which is different from coronavirus nsp16 that requires nsp10 as a stimulating co-factor.

We further tested the 2′-O-MTase activity of GAV nsp16 with the capped viral RNA substrates (^{7Me}G*pppA-RNA, where the asterisk-marked phosphate was ^{32}P labeled) containing the 5′-terminal 68nt of GAV genome. GAV nsp16 was incubated with ^{7Me}G*pppA-RNA in the presence of 0.2 mM SAM. After incubation, the RNA was digested with nuclease P1 to release the cap structure and analyzed on TLC plates (Fig.2A, lane 1). The radio-labeled cap analogs G*pppA, ^{7Me}G*pppA and ^{7Me}G*pppAm, generated by vaccinia D1/D12, VP39 and P1 treatment, were used as controls (Fig.2A,

lanes 2, 3, 4). When the 2′-O position of substrate′s first nucleotide was methylated, the released cap structure was ^{7Me}G*pppA$_m$, and the 2′-O methylated and unmethylated cap structures could be readily separated on TLC plates. As shown in Figure 2A, the 2′-O methylated cap (^{7Me}G*pppA$_m$) (lane 4) generated by VP39 migrated a shorter distance than 2′-O unmethylated cap-0 structure (^{7Me}G*pppA) (lane 3), which were used as the position indicators for the reaction products of GAV nsp16. The cap structure released from ^{7Me}G*pppA-RNA treated with GAV nsp16 corresponded with^{7Me}G*pppAm, further confirming that GAV nsp16 functions as a methyltransferase that adds a methyl group at the ribose 2′-O position.

GAV nsp16 specifically methylates ATP-initiated N7-methylated cap-0 structure of RNA substrates

Next, we explored whether GAV nsp16 could methylate capped RNA GpppAC$_n$ that was not methylated at N7 position. As shown in Figure 2B, when uncapped RNA oligonucleotide pppAC$_{20}$ and capped RNA oligonucleotide GpppAC$_{20}$ (which is not methylated at the N7 position) were used as substrates, ^{3}H-methyl groups could not be incorporated neither by GAV nsp16 nor by SARS nsp16/nsp10. In contrast, when RNA oligonucleotides containing N7-methylated cap-0 structure ^{7Me}GpppAC$_{20}$ (black bars in Fig. 2B) were used as substrates, there was strong signal for incorporated ^{3}H-methyl groups both by GAV nsp16 and SARS nsp16/nsp10. These data suggest that GAV nsp16 can only methylate cap-0 structure of RNA substrates (^{7Me}GpppA-RNA), similarly to coronavirus 2′-O-MTase.

It had been reported that vaccinia virus VP39 was a 2′-O-MTase which modifies 5′-

capped mRNA in sequence-nonspecific manner (Hodel *et al.*, 1998). However, in our previous study, we found that SARS-CoV nsp16 MTase methylated ^{7Me}GpppA-RNA but not ^{7Me}GpppG-RNA (Chen *et al.*, 2011), indicating that SARS-CoV nsp16 is a sequence-dependent 2′-O methyltransferase for ATP-initiated mRNA. In a similar way, we studied the substrate specificity of GAV nsp16. A variety of capped RNA oligonucleotides (initiated with different nucleotides followed by 20 cytidines, (^{7Me}GpppAC$_{20}$, ^{7Me}GpppCC$_{20}$, ^{7Me}GpppUC$_{20}$, and ^{7Me}GpppGC$_{20}$) and the cap analogue (^{7Me}GpppA) were treated with purified GAV nsp16 in the presence of ^{3}H-labeled SAM, purified and detected by liquid scintillation. As shown in Figure 2C, GAV nsp16 had robust activity on substrate ^{7Me}GpppAC$_{20}$ but no detectable activity on ^{7Me}GpppUC$_{20}$ and^{7Me}GpppGC$_{20}$ (Fig. 2C). Of note, GAV nsp16 showed a marginal activity on the substrate ^{7Me}GpppCC$_{20}$. These results indicate that GAV nsp16 2′-O-MTase has a preference to ^{7Me}GpppA-initiated RNA, similarly to SARS-CoV nsp10/nsp16. Coincidentally, the very 5′-terminal nucleotide of GAV genome is adenylate, consistent with our observation.

There was no activity of GAV nsp16 when the cap analogue ^{7Me}GpppA was used as substrate (Fig. 2C), suggesting that the length of RNA substrate is a determinant for GAV nsp16 2′-O-MTase activity. We next characterized the correlation between MTase activity and RNA substrate length. RNA substrates of different length ^{7Me}GpppAC$_n$ (n=0 to 10, 20) were obtained as described (see Materials and methods), and were treated with the equal amount of purified GAV nsp16. As shown in Figure 2D, there was no detectable 2′-O-MTase activity when the RNA substrates possessed less than 5

nucleotides (n=0 to 4). It showed a marginal activity when the RNA length reached 6 nucleotides (n=5), and the activity increased gradually with the increase of RNA substrate length (n = 5-8). The activity of GAV nsp16 reached a plateau when RNA exceeded 9 nucleotides (n>8). These results imply that GAV nsp16 requires a certain length of RNA substrate for full activity.

Biochemical parameters of GAV nsp16activity

As the natural hosts for roniviruses are invertebrates, we further characterize the biochemical parameters and optimal reaction conditions for GAV nsp16 *in vitro*. First, the optimal temperature of the GAV nsp16 methyltransferase reaction was determined. Since the GAV natural host shrimps live in low temperature or temperature-varying environments, a series of temperatures were (from 10°C to 37°C) tested. As shown in Figure 3A, GAV nsp16 activity adopted typical bell curve of the temperature, with an optimal activity on 20°C, indicating that the GAV enzyme had higher activity at a lower temperature that is similar to its natural ambient temperature. In contrast, the 2′-O-MTase of coronaviruses that infect mammalian hosts adopt a higher temperature for optimal enzymatic activity.

We next tested the influence of pH on GAV nsp16 activity. The pH value of the buffer was adjusted from pH 6.0 to pH 11.0 in the assays, and the activity curve profile of GAV nsp16 at different pH values also fitted into a bell shape with a similar pattern to that of temperature changes (Fig. 3B). The GAV 2′-O-MTase had an optimal activity at pH 8.0, which is different from that of coronaviruses that has an optimal pH value at 7.5 (Decroly *et al.*, 2008).

We also investigated the effect of divalent ions on GAV nsp16 at the optimal temperature and pH, since it has been reported that divalent ions can stimulate the methyltransferase activity of several 2′-O-MTases (Decroly *et al.*, 2008; Jeffery & Roth, 1987). Mn^{2+}, Mg^{2+} and Zn^{2+} were tested in our experiment system. Surprisingly, none of these divalent ions could stimulate the activity of GAV nsp16. On the contrary, high concentration of Mg^{2+} even suppressed the activity of GAV nsp16 remarkably (Fig. 3C). Moreover, we also determined the influence of Na^{+} on GAV nsp16, as some 2'-O-MTases could only tolerate very low concentration of Na^{+} (Selisko *et al.*, 2010). Interestingly, GAV nsp16 was not inhibited by a concentration of Na^{+} as high as 20 mM, which is different from Dengue virus $NS5MTase_{DV}$ 2′-O-MTase (Selisko *et al.*, 2010) and vaccinia virus VP39 (Barbosa & Moss, 1978). This observation is consistent with the fact that the natural hosts for GAV can live in a high salinity environment.

The conserved K-D-K-E catalytic tetrad of 2′-O-MTase is required for the activity of GAV nsp16 2′-O-MTase

The group of 2′-O-methyltransferases contains a conserved K-D-K-E catalytic tetrad, which performs a vital function for RNA 2′-O-MTase (Decroly *et al.*, 2008; Egloff *et al.*, 2002; Feder *et al.*, 2003). To identify the K-D-K-E catalytic tetrad of GAV nsp16, we made a comparative analysis of GAV nsp16 with coronavirus nsp16. The multiple sequence alignment is shown in Figure 4A. In SARS-CoV, the conserved K-D-K-E residues of coronavirus nsp16 had been predicted (Lys-46, Asp-130, Lys-170 and Glu-203) and confirmed by crystal structure analysis (Chen *et al.*, 2011; Decroly *et al.*, 2011). The amino acids marked with black asterisk were K-D-K-E tetrad predicted for GAV

nsp16 (Lys-39, Asp-114, Lys-144 and Glu-178), which are completely conserved in the 2′-O-methyltransferases of the nidoviruses. We next generated a structural model of GAV nsp16 (Fig. 4B) by homology-based modeling with SARS-CoV nsp16 as the template (Fig. 4C). As shown in the schematic diagrams of the structural topology (Fig. 4D), GAV nsp16 and SARS-CoV nsp16 share similar global structure with a parallel arrayed β-strands surrounded by a number of α-helices and loops. The K-D-K-E tetrad residues predicted in GAV nsp16 were located in an α-helix and three parallel arrayed β-strands at similar positions to that of SARS-CoV nsp16. However, the structural model shows that GAV nsp16 possesses a six-stranded β-sheet instead of a seven-stranded β-sheet that exists in many known 2′-O-methyltransferases (Chen *et al.*, 2011; Martin & McMillan, 2002). The missing β-strand of GAV nsp16 corresponds to the β-strand 3 (β3) of SARS-CoV nsp16 (Fig. 4A and 4D). Such structural discrepancy needs to be confirmed by the crystal structure analysis of GAV nsp16 in the future work.

To experimentally confirm the K-D-K-E catalytic tetrad of GAV nsp16, the four residues (K39, D114, K144, and E178) were mutated to alanine or other amino acid residues with similar chemical property. In ^{3}H-methyl incorporation MTase activity assays, we found that the wild type of GAV nsp16 possessed about 50% of the activity of the SARS nsp10/16 which was used as a positive control when tested at their respective optimal conditions (Fig. 5A and 5B). When any of the four residues was mutated to alanine, the 2′-O-MTase activity was completely abolished (Fig. 5A and 5B). When the residues were mutated to the amino acid residues with similar chemical

properties, GAV nsp16 activity was largely disrupted but still retained a trace amount of 2′-O-MTase activity (Fig. 5A and 5B). This indicates that the tetrad residues of GAV nsp16 require both proper charge and steric properties for optimal MTase activity. For these assays, two RNA substrates, 5′-teriminal 68-nt capped untranslated regions (^{7Me}Gppp-5′UTR) of GAV genome (Fig.5A) and the capped oligonucleotides ^{7Me}GpppAC$_{20}$ (Fig. 5B) were tested, and the results were consistent.

The 2′-O-MTase activity of GAV nsp16 mutants were also tested by nuclease P1 treatment and TLC assays. As shown in Fig. 5C, all the GAV nsp16 mutants were crippled in 2′-O-MTase activity, which was in accordance with the results of the ^{3}H-methyl-labeled assays (Fig. 5A & 5B).

Taken together, we concluded that the four amino acid residues (Lys-39, Asp-114, Lys-144 and Glu-178) of GAV nsp16 may function as the K-D-K-E catalytic tetrad essential for 2′-O-MTase activity.

Effects of MTase inhibitors on GAV nsp16 2′-O-MTase activity

As viral 2′-O-MTases have been regarded as potential targets for the antiviral therapy (Kimura *et al.*, 2013; Menachery *et al.*, 2014), we tested whether the activity of GAV nsp16 could be inhibited by the previously characterized MTase inhibitors, such as S-(5′-adenosyl)-L homocysteine (SAH), sinefungin and ribavirin. SAH is the by-product of SAM-dependent methyl transfer reaction. Sinefungin is a SAM analogue and ribavirin is a GTP analogue. All of these three small molecular compounds were often used to inhibit SAM-dependent methyltransferases (Pugh & Borchardt, 1982; Pugh *et al.*, 1978; Selisko *et al.*, 2010). As shown in Figure 6, sinefungin and AdoHcy had

relatively more efficient inhibition, and their half maximal inhibitory concentration (IC_{50}) were determined as 28.7 and 73.9 μM, respectively (Fig. 6A and 6C), whereas ribavirin was less efficient on GAV nsp16 in our system (Fig. 6B). In comparison with the inhibition on other viral 2′-O-MTases, we found that these three compounds have varying effects on different MTases. For examples, the IC_{50} value of AdoHcy is 0.34 μM for the 2′-O-MTase of Dengue virus $NS5MTase_{DV}$ (Selisko *et al.*, 2010) and 144 μM for FCoV nsp16 (Decroly *et al.*, 2008), respectively. Ribavirin did not exert significantly inhibitory effect on 2′-O-MTase of Dengue virus $NS5MTase_{DV}$ (Selisko *et al.*, 2010), similarly to our observation with GAV nsp16.

Discussion

Nidoviruses have linear, single-stranded RNA genomes of positive polarity that contain a 5′ cap structure and a 3′ poly(A) tail (Gorbalenya *et al.*, 2006). Several enzymes are involved in the formation of RNA 5′ cap, including RNA triphosphatase, guanylyltransferase, guanine-N7-methyltransferase (N7 MTase) and ribose 2′-O methyltransferase (2′-O MTase) (Bouvet *et al.*, 2012; Decroly *et al.*, 2012; Ghosh & Lima, 2010). In coronaviruses, N-7 MTase and 2′-O MTase have been well studied, and these two methyltransferases play important roles in the modification of the viral cap structure (Chen *et al.*, 2009; Chen *et al.*, 2011; Decroly *et al.*, 2011). However, little is known about the MTases of any other nidoviruses. Although bioinformatic analyses have predicted that roniviruses and toroviruses may encode a methyltransferase at the C-terminal end of ORF1ab in similarity with that of coronaviruses (Gorbalenya *et al.*,

2006), there was no experimental evidence for the predictions. In this study, we expressed and purified the putative methyltransferases of EToV (torovirus) and GAV (ronivirus) according to the bioinformatic prediction for cleavage sites, and we provided experimental evidence that GAV nsp16 possesses the 2′-O-MTase activity. However, we were surprised that the torovirus nsp16, which is more closely related to coronavirus nsp16, did not show any 2′-O-MTase activity in our test settings. The failure to detect 2′-O-MTase activity for torovirus nsp16 may reflect its intrinsic properties or the limitations of our testing systems. Torovirus nsp16 may also adopt an unknown protein cofactor that is different from that of coronavirus.

Our results indicate that GAV nsp16 could discriminate N7 methylated RNA cap from non-methylated ones similarly to coronaviral 2′-O-MTase (Decroly *et al.*, 2008), but in contrast to other viral 2′-O-MTases such as that of dengue virus and Meaban virus which are able to transfer methyl groups from AdoMet to both methylated and unmethylated RNA caps ($GpppAC_n$ and $^{7Me}GpppAC_n$) at the 2′-O position of the first nucleotide (Decroly *et al.*, 2008; Mastrangelo *et al.*, 2007; Peyrane *et al.*, 2007; Selisko *et al.*, 2010). GAV nsp16 can only efficiently methylate ATP-initiated RNA substrates similarly to that of SARS-CoV nsp16, while vaccinia virus VP39 is a 2′-O-MTase that works in a sequence-nonspecific manner (Hodel *et al.*, 1998). It demonstrates that GAV nsp16 is a more specific cap-modifying enzyme in recognition of 5′-capped mRNA.

Interestingly, unlike coronavirus nsp16, GAV nsp16 can function alone in the absence of any other viral protein, suggesting that GAV nsp16 does not require a protein co-factor to stimulate its enzymatic activity. As shown in our previous work, SARS-CoV

nsp10, the co-factor protein to nsp16, acts as a buttress to support the pocket involved in SAM-binding and thus enhances the SAM-binding affinity (Chen *et al.*, 2011).

In modeled 3D structure, GAV nsp16 has remarkable difference to that of SARS-CoV nsp16. First, the SAM-binding pocket GAV nsp16 appears to have a more rigid structure, and may be stable enough to bind with the methyl donor SAM. In contrast, SARS-CoV nsp16 SAM-binding pocket possesses a flexible loop100-108 (Fig. 4). Second, the β3-strand of SARS-CoV nsp16 is missing in GAV nsp16. Third, the surface electrostatic potential analysis shows that the RNA-binding groove of GAV nsp16 is longer than that of SARS nsp16 (Fig. 4E), the former being about 32Å in length and fitting well with the 6-nt RNA substrate ($^{7Me}GpppAC_5$), which is consistent with our observation that the shortest RNA substrate with which GAV nsp16 showed 2'-O-MTase activity is 6-nt long (Fig.2D). This observation may in part explain why GAV does not need a viral protein co-factor for its enzymatic activity whereas SARS-CoV nsp16 requires nsp10 to extend its RNA-binding groove for accommodating substrate RNA efficiently as demonstrated in our previous study (Chen et al., 2011). These structural differences may contribute to the distinct properties of coronavirus and ronivirus 2'-O-MTases.

Through sequence alignment and mutational analysis, we identified the conserved K-D-K-E catalytic tetrad of GAV nsp16, which is similar to that of the typical RNA cap-1 structure capping enzymes. The catalytic tetrad is K_{39}-D_{114}-K_{144}-E_{178} (Fig.4A). Homology modeling results show that GAV nsp16 has six-stranded β-sheet parallel arrayed in the middle of the protein surrounded by α-helices and loops, while the β-sheet is seven-stranded in SARS-CoV nsp16 and many other known 2′-O-

441 methyltransferases (Chen *et al.*, 2011; Martin & McMillan, 2002). Nevertheless, the
442 six-stranded β-sheet is also reported for some MTases, e.g. the RNA methyltransferase
443 TlyA (Arenas *et al.*, 2011), whose missing β-strand is the third one located at the edge
444 of the seven-stranded β-sheet structure. The four amino acid residues in the catalytic
445 tetrad K_{39}-D_{114}-K_{144}-E_{178} of GAV nsp16 are located at similar positions compared with
446 the tetrad K_{46}-D_{130}-K_{170}-E_{203} of SARS-CoV nsp16.
447 It is well-known that the cap structure is very important to mRNA stability and protein
448 translation. It has been demonstrated that the 2′-O methylation of viral RNA cap is also
449 important for RNA viruses to evade host innate immune recognition (Daffis *et al.*, 2010;
450 Garcia-Sastre, 2011; Zust *et al.*, 2011). Therefore, viral capping enzymes may be used
451 as potential targets for the therapeutics against viruses. Recently, we demonstrated that
452 coronavirus nsp10-derived short peptide can potently inhibit nsp16 2′-O-MTase activity
453 in vitro and viral replication in animals (Wang *et al.*, 2015). In the future, small
454 molecule inhibitors can be developed to target the 2′-O-MTases of other nidoviruses. In
455 this study, we showed that the well-known MTase inhibitor sinefungin possessed
456 inhibitory activity on GAV nsp16.
457 In summary, we have identified and characterized the 2′-O-methyltransferase of GAV,
458 extending the understanding of RNA cap formation and methylation beyond
459 coronaviruses in the order of *Nidovirales*. The ronivirus 2′-O-MTase shows similar and
460 unique features in comparison with coronavirus 2′-O-MTase. The K-D-K-E catalytic
461 tetrad of GAV nsp16 is well conserved but ronivirus 2′-O-MTase does not require a
462 protein co-factor for stimulating its activity and possibly possesses a six-stranded β-

463 sheet instead of a canonical seven-stranded β-sheet in the core of the MTase structure.

464

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471 ## References

472 **Ahola, T., Laakkonen, P., Vihinen, H. & Kaariainen, L. (1997).** Critical residues of Semliki Forest virus
473 RNA capping enzyme involved in methyltransferase and guanylyltransferase-like activities.
474 *Journal of virology* **71**, 392-397.
475 **Arenas, N. E., Salazar, L. M., Soto, C. Y., Vizcaino, C., Patarroyo, M. E., Patarroyo, M. A. & Gomez, A.
476 (2011).** Molecular modeling and in silico characterization of Mycobacterium tuberculosis TlyA:
477 possible misannotation of this tubercle bacilli-hemolysin. *BMC structural biology* **11**, 16.
478 **Barbosa, E. & Moss, B. (1978).** mRNA(nucleoside-2'-)-methyltransferase from vaccinia virus.
479 Characteristics and substrate specificity. *The Journal of biological chemistry* **253**, 7698-7702.
480 **Bouvet, M., Debarnot, C., Imbert, I., Selisko, B., Snijder, E. J., Canard, B. & Decroly, E. (2010).** In vitro
481 reconstitution of SARS-coronavirus mRNA cap methylation. *PLoS pathogens* **6**, e1000863.
482 **Bouvet, M., Ferron, F., Imbert, I., Gluais, L., Selisko, B., Coutard, B., Canard, B. & Decroly, E. (2012).**
483 [Capping strategies in RNA viruses]. *Medecine sciences : M/S* **28**, 423-429.
484 **Chen, Y., Cai, H., Pan, J., Xiang, N., Tien, P., Ahola, T. & Guo, D. (2009).** Functional screen reveals SARS
485 coronavirus nonstructural protein nsp14 as a novel cap N7 methyltransferase. *Proceedings of
486 the National Academy of Sciences of the United States of America* **106**, 3484-3489.
487 **Chen, Y. & Guo, D. (2016).** Molecular mechanisms of coronavirus RNA capping and methylation.
488 *Virologica Sinica*.
489 **Chen, Y., Su, C., Ke, M., Jin, X., Xu, L., Zhang, Z., Wu, A., Sun, Y., Yang, Z., Tien, P., Ahola, T., Liang, Y.,
490 Liu, X. & Guo, D. (2011).** Biochemical and structural insights into the mechanisms of SARS
491 coronavirus RNA ribose 2'-O-methylation by nsp16/nsp10 protein complex. *PLoS pathogens* **7**,
492 e1002294.
493 **Chen, Y., Tao, J., Sun, Y., Wu, A., Su, C., Gao, G., Cai, H., Qiu, S., Wu, Y., Ahola, T. & Guo, D. (2013).**
494 Structure-function analysis of severe acute respiratory syndrome coronavirus RNA cap
495 guanine-N7-methyltransferase. *Journal of virology* **87**, 6296-6305.
496 **Cowley, J. A., Dimmock, C. M., Spann, K. M. & Walker, P. J. (2000).** Gill-associated virus of Penaeus
497 monodon prawns: an invertebrate virus with ORF1a and ORF1b genes related to arteri- and

coronaviruses. *The Journal of general virology* **81**, 1473-1484.

Cowley, J. A. & Walker, P. J. (2002). The complete genome sequence of gill-associated virus of Penaeus monodon prawns indicates a gene organisation unique among nidoviruses. *Archives of virology* **147**, 1977-1987.

Daffis, S., Szretter, K. J., Schriewer, J., Li, J., Youn, S., Errett, J., Lin, T. Y., Schneller, S., Zust, R., Dong, H., Thiel, V., Sen, G. C., Fensterl, V., Klimstra, W. B., Pierson, T. C., Buller, R. M., Gale, M., Jr., Shi, P. Y. & Diamond, M. S. (2010). 2'-O methylation of the viral mRNA cap evades host restriction by IFIT family members. *Nature* **468**, 452-456.

Decroly, E., Debarnot, C., Ferron, F., Bouvet, M., Coutard, B., Imbert, I., Gluais, L., Papageorgiou, N., Sharff, A., Bricogne, G., Ortiz-Lombardia, M., Lescar, J. & Canard, B. (2011). Crystal structure and functional analysis of the SARS-coronavirus RNA cap 2'-O-methyltransferase nsp10/nsp16 complex. *PLoS pathogens* **7**, e1002059.

Decroly, E., Ferron, F., Lescar, J. & Canard, B. (2012). Conventional and unconventional mechanisms for capping viral mRNA. *Nature reviews Microbiology* **10**, 51-65.

Decroly, E., Imbert, I., Coutard, B., Bouvet, M., Selisko, B., Alvarez, K., Gorbalenya, A. E., Snijder, E. J. & Canard, B. (2008). Coronavirus nonstructural protein 16 is a cap-0 binding enzyme possessing (nucleoside-2'O)-methyltransferase activity. *Journal of virology* **82**, 8071-8084.

Egloff, M. P., Benarroch, D., Selisko, B., Romette, J. L. & Canard, B. (2002). An RNA cap (nucleoside-2'-O-)-methyltransferase in the flavivirus RNA polymerase NS5: crystal structure and functional characterization. *The EMBO journal* **21**, 2757-2768.

Feder, M., Pas, J., Wyrwicz, L. S. & Bujnicki, J. M. (2003). Molecular phylogenetics of the RrmJ/fibrillarin superfamily of ribose 2'-O-methyltransferases. *Gene* **302**, 129-138.

Furuichi, Y. & Shatkin, A. J. (2000). Viral and cellular mRNA capping: past and prospects. *Advances in virus research* **55**, 135-184.

Garcia-Sastre, A. (2011). 2 methylate or not 2 methylate: viral evasion of the type I interferon response. *Nature immunology* **12**, 114-115.

Ghosh, A. & Lima, C. D. (2010). Enzymology of RNA cap synthesis. *Wiley interdisciplinary reviews RNA* **1**, 152-172.

Gorbalenya, A. E., Enjuanes, L., Ziebuhr, J. & Snijder, E. J. (2006). Nidovirales: evolving the largest RNA virus genome. *Virus research* **117**, 17-37.

Hodel, A. E., Gershon, P. D. & Quiocho, F. A. (1998). Structural basis for sequence-nonspecific recognition of 5'-capped mRNA by a cap-modifying enzyme. *Molecular cell* **1**, 443-447.

Snawar Hussain, Ji'an Pan, Yu Chen, Yalin Yang, Jing Xu, Yu Peng, Ying Wu, Zhaoyang Li, Ying Zhu, Po Tien, Deyin Guo (2005) Identification of novel subgenomic RNAs and noncanonical transcription initiation signals of severe acute respiratory syndrome coronavirus. Journal of Virology 79, 5288-95.

Jeffery, D. R. & Roth, J. A. (1987). Kinetic reaction mechanism for magnesium binding to membrane-bound and soluble catechol O-methyltransferase. *Biochemistry* **26**, 2955-2958.

Kimura, T., Katoh, H., Kayama, H., Saiga, H., Okuyama, M., Okamoto, T., Umemoto, E., Matsuura, Y., Yamamoto, M. & Takeda, K. (2013). Ifit1 inhibits Japanese encephalitis virus replication through binding to 5' capped 2'-O unmethylated RNA. *Journal of virology* **87**, 9997-10003.

Lauber, C., Ziebuhr, J., Junglen, S., Drosten, C., Zirkel, F., Nga, P. T., Morita, K., Snijder, E. J. & Gorbalenya, A. E. (2012). Mesoniviridae: a proposed new family in the order Nidovirales formed by a single species of mosquito-borne viruses. *Archives of virology* **157**, 1623-1628.

Martin, J. L. & McMillan, F. M. (2002). SAM (dependent) I AM: the S-adenosylmethionine-dependent methyltransferase fold. *Current opinion in structural biology* **12**, 783-793.

Mastrangelo, E., Bollati, M., Milani, M., Selisko, B., Peyrane, F., Canard, B., Grard, G., de Lamballerie, X. & Bolognesi, M. (2007). Structural bases for substrate recognition and activity in Meaban virus nucleoside-2'-O-methyltransferase. *Protein science : a publication of the Protein Society* **16**, 1133-1145.

Menachery, V. D., Yount, B. L., Jr., Josset, L., Gralinski, L. E., Scobey, T., Agnihothram, S., Katze, M. G. & Baric, R. S. (2014). Attenuation and restoration of severe acute respiratory syndrome coronavirus mutant lacking 2'-o-methyltransferase activity. *Journal of virology* **88**, 4251-4264.

Nga, P. T., Parquet Mdel, C., Lauber, C., Parida, M., Nabeshima, T., Yu, F., Thuy, N. T., Inoue, S., Ito, T., Okamoto, K., Ichinose, A., Snijder, E. J., Morita, K. & Gorbalenya, A. E. (2011). Discovery of the first insect nidovirus, a missing evolutionary link in the emergence of the largest RNA virus genomes. *PLoS pathogens* **7**, e1002215.

Peyrane, F., Selisko, B., Decroly, E., Vasseur, J. J., Benarroch, D., Canard, B. & Alvarez, K. (2007). High-yield production of short GpppA- and 7MeGpppA-capped RNAs and HPLC-monitoring of methyltransfer reactions at the guanine-N7 and adenosine-2'O positions. *Nucleic acids research* **35**, e26.

Pugh, C. S. & Borchardt, R. T. (1982). Effects of S-adenosylhomocysteine analogues on vaccinia viral messenger ribonucleic acid synthesis and methylation. *Biochemistry* **21**, 1535-1541.

Pugh, C. S., Borchardt, R. T. & Stone, H. O. (1978). Sinefungin, a potent inhibitor of virion mRNA(guanine-7-)-methyltransferase, mRNA(nucleoside-2'-)-methyltransferase, and viral multiplication. *The Journal of biological chemistry* **253**, 4075-4077.

Selisko, B., Peyrane, F. F., Canard, B., Alvarez, K. & Decroly, E. (2010). Biochemical characterization of the (nucleoside-2'O)-methyltransferase activity of dengue virus protein NS5 using purified capped RNA oligonucleotides (7Me)GpppAC(n) and GpppAC(n). *The Journal of general virology* **91**, 112-121.

Smits, S. L., Snijder, E. J. & de Groot, R. J. (2006). Characterization of a torovirus main proteinase. *Journal of virology* **80**, 4157-4167.

Snijder, E. J., Bredenbeek, P. J., Dobbe, J. C., Thiel, V., Ziebuhr, J., Poon, L. L., Guan, Y., Rozanov, M., Spaan, W. J. & Gorbalenya, A. E. (2003). Unique and conserved features of genome and proteome of SARS-coronavirus, an early split-off from the coronavirus group 2 lineage. *Journal of molecular biology* **331**, 991-1004.

Wang, Y., Sun, Y., Wu, A., Xu, S., Pan, R., Zeng, C., Jin, X., Ge, X., Shi, Z., Ahola, T., Chen, Y. & Guo, D. (2015). Coronavirus nsp10/nsp16 Methyltransferase Can Be Targeted by nsp10-Derived Peptide In Vitro and In Vivo To Reduce Replication and Pathogenesis. *Journal of virology* **89**, 8416-8427.

Zust, R., Cervantes-Barragan, L., Habjan, M., Maier, R., Neuman, B. W., Ziebuhr, J., Szretter, K. J., Baker, S. C., Barchet, W., Diamond, M. S., Siddell, S. G., Ludewig, B. & Thiel, V. (2011). Ribose 2'-O-methylation provides a molecular signature for the distinction of self and non-self mRNA dependent on the RNA sensor Mda5. *Nature immunology* **12**, 137-143.

584 **Figure legends:**
585
586 **Figure 1.** Identification of nidovirus 2′-O-MTases. (A) Domain organization of the
587 replicase pp1ab polyprotein for selected nidoviruses: SARS-CoV (coronavirus), EToV
588 (torovirus) and GAV (ronivirus). The predicted domains are indicated, and the cleavage
589 sites are marked with triangles. The domains include: ADRP, ADP-ribose-1′-
590 phosphatase; PLpro, papain-like proteinases; 3CLpro, chymotrypsin-like proteinase;
591 RdRp, RNA-dependentRNA polymerase ; Hel, helicase; ExoN, exonuclease; N7MT,
592 N7-methyltransferase; Ne, uridylate-specific endoribonuclease (also abbreviated
593 NendoU); 2OMT, 2′-O-methyltransferase. The coronavirus nsp10 and its similarly
594 located counterparts in EToV and GAV are depicted in purple color, and RFS stands for
595 the ribosomal frameshift site. (B) Expression and purification of recombinant viral
596 proteins. The gels of SDS-PAGE were stained with Coomassie brilliant blue. Lanes 2-
597 7 are EToV nsp10, GAV nsp10, SARS nsp10, EToV nsp16, GAV nsp16 and SARS
598 nsp16, respectively. GAV nsp10, GAV nsp16 and EToV nsp16 are GST fusion proteins,
599 and the others are 6-histidine-tagged. (C) The activity assays of potential 2′-O
600 methyltransferases in ^{3}H-methyl incorporation assay. Vaccinia virus VP39 and SARS-
601 CoV nsp10/16 were used as positive control, while SARS-CoV nsp10 acted as a
602 negative control for 2'-O-MTase activity. The CPM amounts reflect the 2′-O-MTase
603 activity which was detected by liquid scintillation.
604
605 **Figure 2.** RNA substrate specificity of GAV nsp16 2'-O-MTase. (A) The first 68
606 nucleotides of the GAV genome was capped to form ^{7Me}G*pppA-RNA, incubated with

GAV nsp16, digested to release cap structures, and analyzed by TLC. (B) SAM-dependent methyltransferase activity of GAV nsp16 and SARS nsp16. Equal amounts of proteins were incubated with AC_{20} (white), $GpppAC_{20}$ (gray) and $^{7Me}GpppAC_{20}$ (black) in the presence of ^{3}H-labeled SAM for 1 h, and radioactive incorporation was detected by liquid scintillation. (C) MTase activity of GAV nsp16 for capped RNAs with different initiating nucleotide. $^{7Me}GpppAC_{20}$, $^{7Me}GpppCC_{20}$, $^{7Me}GpppUC_{20}$, $^{7Me}GpppGC_{20}$ and $^{7Me}GpppA$ were used as substrates to test GAV nsp16 activity. (D) GAV nsp16 2′-O-MTase activity on capped RNA substrates of different length. The activity was formulated as percentage (100% corresponds to that of $^{7Me}GpppAC_{20}$).

Figure 3. The optimal reaction conditions of GAV nsp16. The GAV nsp16 activity was measured in the presence of ^{3}H-labeled SAM by counting transferred ^{3}H-methyl. (A) Effect of temperature on the enzymatic activity. Reactions were performed in Tris buffer (pH8.0) and incubated at various temperatures. RNAs were purified by Sephadex A-50 and detected by liquid scintillation. (B) Enzymatic activity of GAV nsp16 at different pH values, including citric acid-NaOH (pH6.0), Tris-HCl buffer (pH7.0-9.0), and Na_2CO_3-NaOH buffer (pH10-11). 100% activity corresponds to that at pH8.0. (C) The influence of positive valence metal ions on enzymatic activity. The reactions were conducted at pH8.0 on 20°C.

Figure 4. Sequence alignment and homology modeling of GAV nsp16. (A) The multiple sequence alignment of selected 2′-O-methyltransferases of nidoviruses. (B)

Ribbon representation of GAV nsp16 model structure generated modeled on the template structure of SARS nsp10/nsp16 (PDB: 3R24) (C). SAM was added in GAV nsp16 model by superimposition of the model and the structure of SARS nsp10/nsp16 (3R24). β-sheets were marked in accordance with alignment result. (D) Schematic diagrams of the topology of GAV nsp16 (modeled structure) and SARS nsp16. (E) The surface electrostatic potentials of GAV nsp16 (model) and SARS nsp16. The surface electrostatic potential diagram was generated by PyMol, and blue areas represent positive charge areas while red represents negative charge areas.

Figure 5. The activity of GAV wild type nsp16 and its K-D-K-E mutants. (A) MTase activity of GAV nsp16 (WT and mutants) detected by using $^{7Me}GpppAC_{20}$ as substrates in ^{3}H-methyl incorporation assay. (B) MTase activity of GAV nsp16 (WT and mutants) detected by using ^{7Me}Gppp-RNA (GAV 5′UTR) as substrates in ^{3}H-methyl incorporation assay. (C) MTase activity of GAV nsp16 (WT and mutants) analyzed by RNA digestion and TLC assays. Lane 4 represents the WT of GAV nsp16, lane 2 as a negative control and lane 3 as positive control treated with vaccinia virus VP39, and the lanes 5-12 are the mutants of GAV nsp16.

Figure 6. Inhibition of GAV nsp16 2′-O-MTase activity by methyltransferase inhibitors. Increasing concentration of sinefungin (A), ribavirin (B) and AdoHcy (by-product of the reaction) (C) were added in the reaction mixtures, and the activity was measured by using ^{3}H-methyl incorporation MTase activity assays.