Identification and characterization of a ribose 2'-O-methyltransferase encoded by the ronivirus branch of Nidovirales

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1 **Identification and Characterization of a Ribose 2′-O-Methyltransferase Encoded**

2 **by the Ronivirus Branch of *Nidovirales***

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

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22

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

89   as "nsp16" by the analogy with the location and function of coronaviral nsp16), but not

90   torovirus MTase domain (also named as nsp16), possesses the 2′-O-MTase activity. We

91   further demonstrate that ronivirus nsp16 alone can efficiently execute its MTase activity,

92   which is in contrast with coronaviral nsp16 that requires virus-encoded nsp10 as a

93   cofactor for its full function. These results extend the understanding of nidovirus RNA

94   capping and methylation and provide an evolutionary and functional link between

95   roniviruses and coronaviruses.

96

97   **Materials and methods**

98   **Radioactive reagents**. S-adenosyl [methyl-^{3}H] methionine (67.3 Ci/mmol, 0.5 µCi/µl)

99   and [α-^{32}P]-labeled guanosine 5′-triphosphate (3000Ci/mmol, 10mCi/ml) were

100   purchased from PerkinElmer.

101   **Cloning, expression and purification of the related proteins**. The genomic cDNA of

102   shrimp gill-associated nidovirus (GAV), a type species of *Roniviridae*, was kindly

103   provided by Dr. J.A. Cowley and was used as the template to construct the expression

104   constructs. Two pairs of PCR primers were used to amplify the gene fragments of GAV:

105   5′-CGGGATCCCTCAGTTACTCAGGCACCGC-3′   (sense)   and   5′-

106   CCCTCGAGCTCGTGCTTGACTGGGATGG-3′ (antisense) for GAV nsp10, and 5′-

107   CGGGATCCCTCTACCACGTCGCTCCTAC-3′   (sense)   and   5′-

108   CCCTCGAGAAATTTGATGAATCTGGGAG-3′ (antisense) for GAV nsp16, which

109   correspond to the nucleotides 11703-12212 and 19403-20089 of the GAV genome

110   (GI:166851932), respectively. These PCR products were cloned into the *Escherichia*

111   *coli* expression vector pGEX-6p-1, resulting in the expression constructs pGEX-6p-1-

112   GAV nsp10 and pGEX-6p-1-GAV nsp16. The mutant plasmids of GAV nsp16 were

113   generated by one-step PCR with mutagenic primers.

114   *E. coli* BL21 (DE3), transformed with pGEX-6p-1-GAV nsp10 or nsp16, was grown in

115   Luria broth (LB) medium containing ampicillin at 37°C. When the OD_{600nm} value of

116   the culture reached 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a

117   final concentration of 0.4 mM, and induced at 16°C for 16 hours. Bacterial cells were

118   harvested by centrifugation and lysed by sonication. The GST-tagged fusion proteins

119   were purified from the cell lysate by affinity chromatography using Glutathione Resin

120   (GenScript) according to the manufacturer's instructions. GAV nsp10 (pp1a residues

121   3879-4048, 170 aa) fused with GST-tag is about 45 kD and was confirmed by SDS-

122   PAGE. GAV nsp16 (pp1ab residues 6446-6673, 228 aa) fused with GST-tag is about 52

123   kD.

124   The genes of equine torovirus (EToV) were obtained by PCR reactions with the

125   template of EToV genome cDNA (GI:190360102) kindly provided by Eric J. Snijder.

126   We performed the bioinformatic analysis to predict the proteinase cleavage sites and

127   arbitrarily named the most C-terminal cleavage product of pp1a as EToV nsp10 and

128   that of pp1b as EToV nsp16 (the predicted 2'-MTase domain).   The following primers

129   were   used   to   amplify   the   gene   fragments: sense   5′-

130   CGGGATCCGGTGTTAGATTGGGAGGTGT-3′   and   antisense   5′-

131   CCCTCGAGCTGAGGCTTAAAAACTGGA-3′ for EToV nsp10 (nt 13560-14018 of

132   the genome), and 5′-CGGGATCCAAGGATTTTGTAAGGTCGTA-3′ (sense) and 5′-

133 CCCTCGAGATGTTCCATTTGGCACAAC-3′ (antisense) for EToV nsp16 (20597-

134 21391). The PCR fragments were cloned into *E. coli* expression vectors pET30a and

135 pGEX-6p-1 respectively, generating the plasmids of pET30a-EToV nsp10 and pGEX-

136 6p-1-EToV nsp16.

137 GST fusion protein of EToV nsp16 (pp1ab residues 6593-6857, 265aa) was expressed

138 and purified similarly to GAV nsp16, and the fusion protein size was 57 kD. The

139 pET30a-EToV nsp10 was transformed into *E. coli* BL21(DE3), and the cells were

140 grown in LB medium containing kanamycin at 37°C. When the OD_{600nm} value of the

141 culture reached 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final

142 concentration of 0.4 mM, and the culture was grown for additional 16 hours at 16°C.

143 Bacterial cells were harvested by centrifugation and lysed by sonication. The 6-

144 Histidine-tagged protein was purified from the cell lysate by affinity chromatography

145 using Ni-NTA according to the manufacturer's instructions (Qiagen). EToV nsp10 (pp1a

146 residues 4247-4399, 153aa) was 17 kD in size. The predicted sizes of these two proteins

147 were in accordance with their sizes in SDS-PAGE analysis.

148 SARS-CoV nsp10 and nsp16 were expressed and purified as described in our previous

149 work (Chen *et al.*, 2011).

150 **Preparation of RNA substrates.** GAV 68-nt 5′-UTR was transcribed in vitro using the

151 PCR products as a template which was amplified from GAV cDNA (primers as follows,

152 5′-CAGTAATACGACTCACTATTACGTTACGTTCCACGTAC-3′ and 5′-

153 GGTCGGAAACGCTGGAAAAC-3′). Since GAV genome possesses an adenine at the

154 5'-proximal first nucleotide position, we used a bacteriophage T7 Φ2.5 promoter to

155 promote synthesis of RNA which initiated with an adenine. The MEGAscript Kit (Life

156 Technologies) was used to transcribe RNA *in vitro* with 100 ng PCR template in a 20-

157 µl mixture. The reaction mixture was mixed thoroughly and incubated for 16 h at 37°C.

158 After the reaction, 2 units of TURBO DNase was added, followed by further incubation

159 for 15 min at 37°C. For purification of RNA, the transcription reaction was extracted

160 with phenol/chloroform/isoamyl alcohol, precipitated with 75% ethanol which contains

161 sodium acetate, resuspended in RNA-free water (TIANGEN), and quantitated by

162 Nanodrop 2000c (Thermo Scientific). Nonviral RNAs $pppAC_n$, $pppUC_{20}$, $pppGC_{20}$, and

163 $pppCC_{20}$ were transcribed and purified as described previously (Chen *et al.*, 2011).

164 Capped RNAs (GpppRNA and ^{7Me}GpppRNA) were produced using Vaccinia Capping

165 System (New England Biolabs), purified through Sephadex G-25 quick spin columns

166 (Roche), extracted with phenol/chloroform/isoamyl alcohol and precipitated with

167 ethanol.^{32}P-labeled RNA substrates (G*pppRNA and ^{7Me}G*pppRNA) used for thin

168 layer chromatography (TLC) were synthesized as described previously (Chen *et al.*,

169 2009).

170 **Biochemical assay for methyltransferase activity.** The ^{32}P-labeled 2′-O-MTase assay

171 was performed in 8.5-µL reaction system (containing 50 mM Tris-HCl [pH 8.0], 2 mM

172 dithiothreitol [DTT], 2 mM $MgCl_2$, 10 units RNase inhibitor, 2×10^3 cpm of ^{7Me}G

173 *pppA-RNA, 0.2 mM SAM, and 0.5 µg of purified proteins or mutant proteins) at 20°C

174 for 1 h. Control G*pppA-RNA and ^{7Me}G*pppA-RNA were prepared by using the

175 vaccinia virus protein D1-D12, which has the activity of both GTase and N-7-MTase

176 by incubating at 37°C for 1 h and purified as described previously (Chen *et al.*, 2011).

8

177 Control ^{7Me}G*pppAm-RNA was produced by incubation of ^{7Me}G*pppA-RNA with the

178 vaccinia virus 2′-O-methyltransferase VP39 (Epicentre) at 37°C. The capped RNAs

179 labeled by radioisotope were digested by nuclease P1 (US Biological) to release cap

180 structures, then spotted onto polyethyleneimine cellulose-F plates (Merck) for TLC

181 analysis, and developed in 0.65 M LiCl. The extent of ^{32}P-labeled cap was determined

182 by scanning the chromatogram with a PhosphorImager as described previously (Chen

183 *et al.*, 2009; Chen *et al.*, 2011).

184 The ^{3}H-methyl incorporation MTase activity assays were performed in 20-µL reaction

185 system (containing 50 mM Tris-HCl [pH 8.0], 2 mM DTT, 2 mM MgCl$_2$, 40 units of

186 RNase inhibitor, 0.01 mM SAM, 0.5 µCi of S-adenosyl [methyl-3H] methionine [67.3

187 Ci/mmol, 0.5 µCi/µl], 1 µg proteins, and 3 µg of ^{7Me}GpppRNA substrates) at 37°C for

188 1 h. ^{3}H-labeled products were isolated in DEAE-Sephadex A-50 columns and

189 quantitated by liquid scintillation (PerkinElmer) as described previously (Ahola *et al.*,

190 1997; Chen *et al.*, 2011).

191 **Multiple sequence alignment and homology modeling**

192 The multiple sequence alignment of the nsp16 protein sequence from different

193 *Nidovirales* species was performed by using Clustalx, and then the result was imported

194 into ESPript 3.0 together with the three-dimensional structure of SARS nsp16/nsp10

195 (PDB: 3R24) to assign the secondary structure. The 3D structure of GAV nsp16 was

196 generated on the template structure SARS-CoV nsp16 (PDB 3R24, chain A). The

197 multiple sequence alignment was done by Clustalx, and then uploaded to SWISS-

198 MODEL for structural modeling, which was further analyzed by PyMol.

199

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

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

222 by affinity chromatography.

223 To test the 2′-O-MTase activity possibly possessed by the viral proteins, we first

224 conducted 2′-O-MTase activity assays *in vitro* on a 21-nt RNA substrate (the cap-0

225 ^{7Me}GpppAC$_{20}$) which was capped and methylated at N7 position of the cap. Transfer of

226 ^{3}H-labeled methyl group from SAM to cap-0 RNA substrates resulted in cap-1 RNA

227 (^{7Me}GpppAmC$_{20}$) and could be measured quantitatively by liquid scintillation detection.

228 As the GAV host shrimps (*Penaeus monodon*) keep a low body temperature in nature,

229 all the assays with GAV proteins were performed at 20°C while all other assays were at

230 37°C. As shown in Figure 1C, GAV nsp16, either in the presence or absence of GAV

231 nsp10, showed remarkable 2′-O-MTase activity to the similar extent as vaccinia VP39

232 and SARS-CoV nsp10/nsp16, whereas EToV nsp16 or its mixture with nsp10 did not

233 display 2′-O-MTase activity, similarly to the negative control (SARS-CoV nsp10 alone).

234 These results indicate that GAV nsp16 alone possesses the 2′-O-MTase activity, which

235 is different from coronavirus nsp16 that requires nsp10 as a stimulating co-factor.

236 We further tested the 2′-O-MTase activity of GAV nsp16 with the capped viral RNA

237 substrates (^{7Me}G*pppA-RNA, where the asterisk-marked phosphate was ^{32}P labeled)

238 containing the 5′-terminal 68nt of GAV genome. GAV nsp16 was incubated with

239 ^{7Me}G*pppA-RNA in the presence of 0.2 mM SAM. After incubation, the RNA was

240 digested with nuclease P1 to release the cap structure and analyzed on TLC plates

241 (Fig.2A, lane 1). The radio-labeled cap analogs G*pppA, ^{7Me}G*pppA and ^{7Me}G*pppAm,

242 generated by vaccinia D1/D12, VP39 and P1 treatment, were used as controls (Fig.2A,

243   lanes 2, 3, 4). When the 2′-O position of substrate′s first nucleotide was methylated, the

244   released cap structure was $^{7Me}G^*pppA_m$, and the 2′-O methylated and unmethylated cap

245   structures could be readily separated on TLC plates. As shown in Figure 2A, the 2′-O

246   methylated cap ($^{7Me}G^*pppA_m$) (lane 4) generated by VP39 migrated a shorter distance

247   than 2′-O unmethylated cap-0 structure ($^{7Me}G^*pppA$) (lane 3), which were used as the

248   position indicators for the reaction products of GAV nsp16. The cap structure released

249   from $^{7Me}G^*pppA$-RNA treated with GAV nsp16 corresponded with $^{7Me}G^*pppAm$,

250   further confirming that GAV nsp16 functions as a methyltransferase that adds a methyl

251   group at the ribose 2′-O position.

252   **GAV nsp16 specifically methylates ATP-initiated N7-methylated cap-0 structure**

253   **of RNA substrates**

254   Next, we explored whether GAV nsp16 could methylate capped RNA $GpppAC_n$ that

255   was not methylated at N7 position. As shown in Figure 2B, when uncapped RNA

256   oligonucleotide $pppAC_{20}$ and capped RNA oligonucleotide $GpppAC_{20}$ (which is not

257   methylated at the N7 position) were used as substrates, ^3H-methyl groups could not be

258   incorporated neither by GAV nsp16 nor by SARS nsp16/nsp10. In contrast, when RNA

259   oligonucleotides containing N7-methylated cap-0 structure $^{7Me}GpppAC_{20}$ (black bars in

260   Fig. 2B) were used as substrates, there was strong signal for incorporated ^3H-methyl

261   groups both by GAV nsp16 and SARS nsp16/nsp10. These data suggest that GAV nsp16

262   can only methylate cap-0 structure of RNA substrates ($^{7Me}GpppA$-RNA), similarly to

263   coronavirus 2′-O-MTase.

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

265   capped mRNA in sequence-nonspecific manner (Hodel *et al.*, 1998). However, in our

266   previous study, we found that SARS-CoV nsp16 MTase methylated ^{7Me}GpppA-RNA

267   but not ^{7Me}GpppG-RNA (Chen *et al.*, 2011), indicating that SARS-CoV nsp16 is a

268   sequence-dependent 2′-O methyltransferase for ATP-initiated mRNA. In a similar way,

269   we studied the substrate specificity of GAV nsp16. A variety of capped RNA

270   oligonucleotides (initiated with different nucleotides followed by 20 cytidines,

271   (^{7Me}GpppAC$_{20}$, ^{7Me}GpppCC$_{20}$, ^{7Me}GpppUC$_{20}$, and ^{7Me}GpppGC$_{20}$) and the cap analogue

272   (^{7Me}GpppA) were treated with purified GAV nsp16 in the presence of ^{3}H-labeled SAM,

273   purified and detected by liquid scintillation. As shown in Figure 2C, GAV nsp16 had

274   robust activity on substrate ^{7Me}GpppAC$_{20}$ but no detectable activity on ^{7Me}GpppUC$_{20}$

275   and ^{7Me}GpppGC$_{20}$ (Fig. 2C). Of note, GAV nsp16 showed a marginal activity on the

276   substrate ^{7Me}GpppCC$_{20}$. These results indicate that GAV nsp16 2′-O-MTase has a

277   preference to ^{7Me}GpppA-initiated RNA, similarly to SARS-CoV nsp10/nsp16.

278   Coincidentally, the very 5′-terminal nucleotide of GAV genome is adenylate, consistent

279   with our observation.

280   There was no activity of GAV nsp16 when the cap analogue ^{7Me}GpppA was used as

281   substrate (Fig. 2C), suggesting that the length of RNA substrate is a determinant for

282   GAV nsp16 2′-O-MTase activity. We next characterized the correlation between MTase

283   activity and RNA substrate length. RNA substrates of different length ^{7Me}GpppAC$_n$ (n=0

284   to 10, 20) were obtained as described (see Materials and methods), and were treated

285   with the equal amount of purified GAV nsp16. As shown in Figure 2D, there was no

286   detectable 2′-O-MTase activity when the RNA substrates possessed less than 5

287   nucleotides (n=0 to 4). It showed a marginal activity when the RNA length reached 6

288   nucleotides (n=5), and the activity increased gradually with the increase of RNA

289   substrate length (n = 5-8). The activity of GAV nsp16 reached a plateau when RNA

290   exceeded 9 nucleotides (n>8). These results imply that GAV nsp16 requires a certain

291   length of RNA substrate for full activity.

292   **Biochemical parameters of GAV nsp16activity**

293   As the natural hosts for roniviruses are invertebrates, we further characterize the

294   biochemical parameters and optimal reaction conditions for GAV nsp16 *in vitro*. First,

295   the optimal temperature of the GAV nsp16 methyltransferase reaction was determined.

296   Since the GAV natural host shrimps live in low temperature or temperature-varying

297   environments, a series of temperatures were (from 10°C to 37°C) tested. As shown in

298   Figure 3A, GAV nsp16 activity adopted typical bell curve of the temperature, with an

299   optimal activity on 20°C, indicating that the GAV enzyme had higher activity at a lower

300   temperature that is similar to its natural ambient temperature. In contrast, the 2′-O-

301   MTase of coronaviruses that infect mammalian hosts adopt a higher temperature for

302   optimal enzymatic activity.

303   We next tested the influence of pH on GAV nsp16 activity. The pH value of the buffer

304   was adjusted from pH 6.0 to pH 11.0 in the assays, and the activity curve profile of

305   GAV nsp16 at different pH values also fitted into a bell shape with a similar pattern to

306   that of temperature changes (Fig. 3B). The GAV 2′-O-MTase had an optimal activity at

307   pH 8.0, which is different from that of coronaviruses that has an optimal pH value at

308   7.5 (Decroly *et al.*, 2008).

14

309   We also investigated the effect of divalent ions on GAV nsp16 at the optimal

310   temperature and pH, since it has been reported that divalent ions can stimulate the

311   methyltransferase activity of several 2′-O-MTases (Decroly *et al.*, 2008; Jeffery & Roth,

312   1987). Mn^{2+}, Mg^{2+} and Zn^{2+} were tested in our experiment system. Surprisingly, none

313   of these divalent ions could stimulate the activity of GAV nsp16. On the contrary, high

314   concentration of Mg^{2+} even suppressed the activity of GAV nsp16 remarkably (Fig. 3C).

315   Moreover, we also determined the influence of Na^+ on GAV nsp16, as some 2'-O-

316   MTases could only tolerate very low concentration of Na^+ (Selisko *et al.*, 2010).

317   Interestingly, GAV nsp16 was not inhibited by a concentration of Na^+ as high as 20 mM,

318   which is different from Dengue virus NS5MTase$_{DV}$ 2′-O-MTase (Selisko *et al.*, 2010)

319   and vaccinia virus VP39 (Barbosa & Moss, 1978). This observation is consistent with

320   the fact that the natural hosts for GAV can live in a high salinity environment.

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

322   **of GAV nsp16 2′-O-MTase**

323   The group of 2′-O-methyltransferases contains a conserved K-D-K-E catalytic tetrad,

324   which performs a vital function for RNA 2′-O-MTase (Decroly *et al.*, 2008; Egloff *et*

325   *al.*, 2002; Feder *et al.*, 2003). To identify the K-D-K-E catalytic tetrad of GAV nsp16,

326   we made a comparative analysis of GAV nsp16 with coronavirus nsp16. The multiple

327   sequence alignment is shown in Figure 4A. In SARS-CoV, the conserved K-D-K-E

328   residues of coronavirus nsp16 had been predicted (Lys-46, Asp-130, Lys-170 and Glu-

329   203) and confirmed by crystal structure analysis (Chen *et al.*, 2011; Decroly *et al.*, 2011).

330   The amino acids marked with black asterisk were K-D-K-E tetrad predicted for GAV

331 nsp16 (Lys-39, Asp-114, Lys-144 and Glu-178), which are completely conserved in the

332 2′-O-methyltransferases of the nidoviruses. We next generated a structural model of

333 GAV nsp16 (Fig. 4B) by homology-based modeling with SARS-CoV nsp16 as the

334 template (Fig. 4C).   As shown in the schematic diagrams of the structural topology

335 (Fig. 4D), GAV nsp16 and SARS-CoV nsp16 share similar global structure with a

336 parallel arrayed β-strands surrounded by a number of α-helices and loops. The K-D-K-

337 E tetrad residues predicted in GAV nsp16 were located in an α-helix and three parallel

338 arrayed β-strands at similar positions to that of SARS-CoV nsp16. However, the

339 structural model shows that GAV nsp16 possesses a six-stranded β-sheet instead of a

340 seven-stranded β-sheet that exists in many known 2′-O-methyltransferases (Chen *et al.*,

341 2011; Martin & McMillan, 2002).   The missing β-strand of GAV nsp16 corresponds

342 to the β-strand 3 (β3) of SARS-CoV nsp16 (Fig. 4A and 4D). Such structural

343 discrepancy needs to be confirmed by the crystal structure analysis of GAV nsp16 in

344 the future work.

345 To experimentally confirm the K-D-K-E catalytic tetrad of GAV nsp16, the four

346 residues (K39, D114, K144, and E178) were mutated to alanine or other amino acid

347 residues with similar chemical property. In ^{3}H-methyl incorporation MTase activity

348 assays, we found that the wild type of GAV nsp16 possessed about 50% of the activity

349 of the SARS nsp10/16 which was used as a positive control when tested at their

350 respective optimal conditions (Fig. 5A and 5B). When any of the four residues was

351 mutated to alanine, the 2′-O-MTase activity was completely abolished (Fig. 5A and 5B).

352 When the residues were mutated to the amino acid residues with similar chemical

353   properties, GAV nsp16 activity was largely disrupted but still retained a trace amount

354   of 2′-O-MTase activity (Fig. 5A and 5B). This indicates that the tetrad residues of GAV

355   nsp16 require both proper charge and steric properties for optimal MTase activity. For

356   these assays, two RNA substrates, 5′-teriminal 68-nt capped untranslated regions

357   (^{7Me}Gppp-5′UTR) of GAV genome (Fig.5A) and the capped oligonucleotides

358   ^{7Me}GpppAC$_{20}$ (Fig. 5B) were tested, and the results were consistent.

359   The 2′-O-MTase activity of GAV nsp16 mutants were also tested by nuclease P1

360   treatment and TLC assays. As shown in Fig. 5C, all the GAV nsp16 mutants were

361   crippled in 2′-O-MTase activity, which was in accordance with the results of the ^{3}H-

362   methyl-labeled assays (Fig. 5A & 5B).

363   Taken together, we concluded that the four amino acid residues (Lys-39, Asp-114, Lys-

364   144 and Glu-178) of GAV nsp16 may function as the K-D-K-E catalytic tetrad essential

365   for 2′-O-MTase activity.

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

367   As viral 2′-O-MTases have been regarded as potential targets for the antiviral therapy

368   (Kimura *et al.*, 2013; Menachery *et al.*, 2014), we tested whether the activity of GAV

369   nsp16 could be inhibited by the previously characterized MTase inhibitors, such as S-

370   (5′-adenosyl)-L homocysteine (SAH), sinefungin and ribavirin. SAH is the by-product

371   of SAM-dependent methyl transfer reaction. Sinefungin is a SAM analogue and

372   ribavirin is a GTP analogue. All of these three small molecular compounds were often

373   used to inhibit SAM-dependent methyltransferases (Pugh & Borchardt, 1982; Pugh *et*

374   *al.*, 1978; Selisko *et al.*, 2010). As shown in Figure 6, sinefungin and AdoHcy had

375 relatively more efficient inhibition, and their half maximal inhibitory concentration

376 (IC_{50}) were determined as 28.7 and 73.9 μM, respectively (Fig. 6A and 6C), whereas

377 ribavirin was less efficient on GAV nsp16 in our system (Fig. 6B). In comparison with

378 the inhibition on other viral 2′-O-MTases, we found that these three compounds have

379 varying effects on different MTases. For examples, the IC_{50} value of AdoHcy is 0.34

380 μM for the 2′-O-MTase of Dengue virus NS5MTase$_{DV}$ (Selisko *et al.*, 2010) and 144

381 μM for FCoV nsp16 (Decroly *et al.*, 2008), respectively. Ribavirin did not exert

382 significantly inhibitory effect on 2′-O-MTase of Dengue virus NS5MTase$_{DV}$ (Selisko *et*

383 *al.*, 2010), similarly to our observation with GAV nsp16.

384

385 **Discussion**

386 Nidoviruses have linear, single-stranded RNA genomes of positive polarity that contain

387 a 5′ cap structure and a 3′ poly(A) tail (Gorbalenya *et al.*, 2006). Several enzymes are

388 involved in the formation of RNA 5′ cap, including RNA triphosphatase,

389 guanylyltransferase, guanine-N7-methyltransferase (N7 MTase) and ribose 2′-O

390 methyltransferase (2′-O MTase) (Bouvet *et al.*, 2012; Decroly *et al.*, 2012; Ghosh &

391 Lima, 2010). In coronaviruses, N-7 MTase and 2′-O MTase have been well studied,

392 and these two methyltransferases play important roles in the modification of the viral

393 cap structure (Chen *et al.*, 2009; Chen *et al.*, 2011; Decroly *et al.*, 2011). However, little

394 is known about the MTases of any other nidoviruses. Although bioinformatic analyses

395 have predicted that roniviruses and toroviruses may encode a methyltransferase at the

396 C-terminal end of ORF1ab in similarity with that of coronaviruses (Gorbalenya *et al.*,

397 2006), there was no experimental evidence for the predictions. In this study, we

398 expressed and purified the putative methyltransferases of EToV (torovirus) and GAV

399 (ronivirus) according to the bioinformatic prediction for cleavage sites, and we

400 provided experimental evidence that GAV nsp16 possesses the 2′-O-MTase activity.

401 However, we were surprised that the torovirus nsp16, which is more closely related to

402 coronavirus nsp16, did not show any 2′-O-MTase activity in our test settings. The

403 failure to detect 2′-O-MTase activity for torovirus nsp16 may reflect its intrinsic

404 properties or the limitations of our testing systems. Torovirus nsp16 may also adopt an

405 unknown protein cofactor that is different from that of coronavirus.

406 Our results indicate that GAV nsp16 could discriminate N7 methylated RNA cap from

407 non-methylated ones similarly to coronaviral 2′-O-MTase (Decroly *et al.*, 2008), but in

408 contrast to other viral 2′-O-MTases such as that of dengue virus and Meaban virus

409 which are able to transfer methyl groups from AdoMet to both methylated and

410 unmethylated RNA caps (GpppAC$_n$ and ^{7Me}GpppAC$_n$) at the 2′-O position of the first

411 nucleotide (Decroly *et al.*, 2008; Mastrangelo *et al.*, 2007; Peyrane *et al.*, 2007; Selisko

412 *et al.*, 2010). GAV nsp16 can only efficiently methylate ATP-initiated RNA substrates

413 similarly to that of SARS-CoV nsp16, while vaccinia virus VP39 is a 2′-O-MTase that

414 works in a sequence-nonspecific manner (Hodel *et al.*, 1998). It demonstrates that GAV

415 nsp16 is a more specific cap-modifying enzyme in recognition of 5′-capped mRNA.

416 Interestingly, unlike coronavirus nsp16, GAV nsp16 can function alone in the absence

417 of any other viral protein, suggesting that GAV nsp16 does not require a protein co-

418 factor to stimulate its enzymatic activity. As shown in our previous work, SARS-CoV

419   nsp10, the co-factor protein to nsp16, acts as a buttress to support the pocket involved

420   in SAM-binding and thus enhances the SAM-binding affinity (Chen *et al.*, 2011).

421   In modeled 3D structure, GAV nsp16 has remarkable difference to that of SARS-CoV

422   nsp16. First, the SAM-binding pocket GAV nsp16 appears to have a more rigid structure,

423   and may be stable enough to bind with the methyl donor SAM. In contrast, SARS-CoV

424   nsp16 SAM-binding pocket possesses a flexible loop100-108 (Fig. 4). Second, the β3-

425   strand of SARS-CoV nsp16 is missing in GAV nsp16. Third, the surface electrostatic

426   potential analysis shows that the RNA-binding groove of GAV nsp16 is longer than that

427   of SARS nsp16 (Fig. 4E), the former being about 32Å in length and fitting well with

428   the 6-nt RNA substrate (^{7Me}GpppAC$_5$), which is consistent with our observation that the

429   shortest RNA substrate with which GAV nsp16 showed 2'-O-MTase activity is 6-nt long

430   (Fig.2D). This observation may in part explain why GAV does not need a viral protein

431   co-factor for its enzymatic activity whereas SARS-CoV nsp16 requires nsp10 to extend

432   its RNA-binding groove for accommodating substrate RNA efficiently as demonstrated

433   in our previous study (Chen et al., 2011). These structural differences may contribute

434   to the distinct properties of coronavirus and ronivirus 2'-O-MTases.

435   Through sequence alignment and mutational analysis, we identified the conserved K-

436   D-K-E catalytic tetrad of GAV nsp16, which is similar to that of the typical RNA cap-

437   1 structure capping enzymes. The catalytic tetrad is K_{39}-D_{114}-K_{144}-E_{178} (Fig.4A).

438   Homology modeling results show that GAV nsp16 has six-stranded β-sheet parallel

439   arrayed in the middle of the protein surrounded by α-helices and loops, while the β-

440   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-

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

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582

583

Figure legends:

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

607 GAV nsp16, digested to release cap structures, and analyzed by TLC. (B) SAM-

608 dependent methyltransferase activity of GAV nsp16 and SARS nsp16. Equal amounts

609 of proteins were incubated with AC_{20} (white), $GpppAC_{20}$ (gray) and $^{7Me}GpppAC_{20}$

610 (black) in the presence of ^3H-labeled SAM for 1 h, and radioactive incorporation was

611 detected by liquid scintillation. (C) MTase activity of GAV nsp16 for capped RNAs

612 with different initiating nucleotide. $^{7Me}GpppAC_{20}$, $^{7Me}GpppCC_{20}$, $^{7Me}GpppUC_{20}$,

613 $^{7Me}GpppGC_{20}$ and $^{7Me}GpppA$ were used as substrates to test GAV nsp16 activity. (D)

614 GAV nsp16 2′-O-MTase activity on capped RNA substrates of different length. The

615 activity was formulated as percentage (100% corresponds to that of $^{7Me}GpppAC_{20}$).

616

617 **Figure 3.** The optimal reaction conditions of GAV nsp16. The GAV nsp16 activity was

618 measured in the presence of ^3H-labeled SAM by counting transferred ^3H-methyl. (A)

619 Effect of temperature on the enzymatic activity. Reactions were performed in Tris buffer

620 (pH8.0) and incubated at various temperatures. RNAs were purified by Sephadex A-50

621 and detected by liquid scintillation. (B) Enzymatic activity of GAV nsp16 at different

622 pH values, including citric acid-NaOH (pH6.0), Tris-HCl buffer (pH7.0-9.0), and

623 Na_2CO_3-NaOH buffer (pH10-11). 100% activity corresponds to that at pH8.0. (C) The

624 influence of positive valence metal ions on enzymatic activity. The reactions were

625 conducted at pH8.0 on 20°C.

626

627 **Figure 4.** Sequence alignment and homology modeling of GAV nsp16. (A) The

628 multiple sequence alignment of selected 2′-O-methyltransferases of nidoviruses.　(B)

629 Ribbon representation of GAV nsp16 model structure generated modeled on the

630 template structure of SARS nsp10/nsp16 (PDB: 3R24) (C). SAM was added in GAV

631 nsp16 model by superimposition of the model and the structure of SARS nsp10/nsp16

632 (3R24). β-sheets were marked in accordance with alignment result. (D) Schematic

633 diagrams of the topology of GAV nsp16 (modeled structure) and SARS nsp16. (E) The

634 surface electrostatic potentials of GAV nsp16 (model) and SARS nsp16. The surface

635 electrostatic potential diagram was generated by PyMol, and blue areas represent

636 positive charge areas while red represents negative charge areas.

637

638 **Figure 5.** The activity of GAV wild type nsp16 and its K-D-K-E mutants. (A) MTase

639 activity of GAV nsp16 (WT and mutants) detected by using ^{7Me}GpppAC$_{20}$ as substrates

640 in ^{3}H-methyl incorporation assay. (B) MTase activity of GAV nsp16 (WT and mutants)

641 detected by using ^{7Me}Gppp-RNA (GAV 5′UTR) as substrates in ^{3}H-methyl

642 incorporation assay. (C) MTase activity of GAV nsp16 (WT and mutants) analyzed by

643 RNA digestion and TLC assays. Lane 4 represents the WT of GAV nsp16, lane 2 as a

644 negative control and lane 3 as positive control treated with vaccinia virus VP39, and

645 the lanes 5-12 are the mutants of GAV nsp16.

646

647 **Figure 6.** Inhibition of GAV nsp16 2′-O-MTase activity by methyltransferase inhibitors.

648 Increasing concentration of sinefungin (A), ribavirin (B) and AdoHcy (by-product of

649 the reaction) (C) were added in the reaction mixtures, and the activity was measured by

650 using ^{3}H-methyl incorporation MTase activity assays.