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Lineage A Betacoronavirus NS2 proteins and homologous Torovirus Berne pp1a-carboxyterminal domain are phosphodiesterases that antagonize activation of RNase L

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Abstract

Viruses in the family *Coronaviridae,* with the Nidovirus order*,* are etiologic agents of a range of human and animal diseases, including both mild and severe respiratory disease in humans. These viruses encode conserved replicase and structural proteins, and more diverse accessory proteins in the 3' end of their genomes that often act as host cell antagonists. We have previously shown that 2',5' phosphodiesterases (PDE) encoded by the prototypical *Betacoronavirus*, mouse hepatitis virus (MHV), Middle East respiratory syndrome-associated coronavirus antagonize the oligoadenylate – ribonuclease L (OAS-RNase L) pathway. Here we report that additional coronavirus superfamily members including lineage A betacoronaviruses and toroviruses infecting both humans and animals encode 2',5' PDEs capable of antagonizing RNase L. We used a chimeric MHV system, in which exogenous PDEs were expressed from an MHV backbone lacking a functional NS2 protein (MHV^{Mut}), its endogenous RNase L antagonist. In this system, we found that 2',5' PDEs encoded by human coronavirus HCoV-OC43 (OC43), an agent of the common cold, human enteric coronavirus (HECoV), equine coronavirus (ECoV), and equine torovirus-Berne (BEV) are enzymatically active, rescue replication of MHV^{Mut} in bone marrow-derived macrophages and inhibit RNase L-mediated rRNA degradation in these cells. Additionally, PDEs encoded by OC43 and BEV rescue MHV^{Mut} replication and restore pathogenesis in WT B6 mice. This finding expands the range of viruses known to encode antagonists of the potent OAS-RNase L antiviral pathway, highlighting its importance in a range of species, as well as the selective pressures exerted on viruses to antagonize it.

Importance

Viruses in the family *Coronaviridae* include important human and animal pathogens, including the recently emerged SARS-CoV and MERS-CoV. We have shown previously that two viruses within the genus *Betacoronavirus* murine coronavirus (MHV) and MERS-CoV, encode 2',5' phosphodiesterases (PDEs) that antagonize the OAS-RNase L pathway and report here that these proteins are furthermore conserved among additional coronavirus superfamily members including lineage A betacoronaviruses and toroviruses and suggesting they may play critical roles in pathogenesis. As there are no licensed vaccines or effective antivirals against human coronaviruses and few against those infecting animals, identifying viral proteins contributing to virulence can inform therapeutic development. Thus, this work demonstrates that a potent antagonist of host antiviral defenses is encoded by multiple and diverse viruses within *Coronaviridae*, presenting a possible broad-spectrum therapeutic target.

62 **Introduction**
63

64 Coronaviruses (CoV) and closely related toroviruses (ToV) are well known agents of disease in
65 mammals, including humans. Coronaviruses and toroviruses, members of the family
66 *Coronaviridae,* within the Nidovirus order, contain positive sense single stranded (ss)RNA
67 genomes, the longest known RNA genomes ranging from 28-31kb (1). The first two thirds of
68 their genomes encodes the replicase proteins, which include the viral RNA-dependent RNA
69 polymerase and numerous non-structural proteins (NSPs), which are required for replication
70 and in some cases have host immune antagonist activities (2)
71 . The structural proteins are encoded in the 3' third of the genome and consist of spike (S),
72 small membrane protein (E), membrane (M), nucleocapsid (N) and sometimes hemagglutinin-
73 esterase (HE). Interspersed among the structural genes are diverse genes encoding accessory
74 proteins that are not essential for replication but are believed to be required for virulence in vivo
75 (1).
76

77 Mouse hepatitis virus (MHV) is a lineage A *Betacoronavirus* and the prototypical CoV. MHV
78 encodes the accessory protein NS2 which was previously identified as a 2-His (H)
79 phosphoesterase (2H-PE) superfamily member (3) that we have demonstrated has 2',5'-
80 phosphodiesterase (PDE) activity that antagonizes host interferon (IFN) signaling via
81 antagonism of the 2',5'-oligoadenylate synthetase (OAS)-ribonuclease (RNase) L pathway (4).
82 Upon sensing double stranded (ds)RNA, OAS synthesizes 2',5'-oligoadenylates (2-5A) which
83 catalyze the activation of RNase L via homodimerization. RNase L subsequently cleaves host
84 and viral ssRNA leading to termination of protein synthesis and subsequent apoptosis (5). NS2
85 cleaves 2-5A thus preventing the activation of RNase L. NS2 is a critical determinant of MHV
86 strain A59 (A59) liver tropism in C57Bl/6 (B6) mice and is required for the virus to cause
87 hepatitis. A mutant A59 ($NS2^{H126R}$ referred to herein as $NS2^{Mut}$) expressing an inactive

phosphodiesterase is unable to antagonize the OAS-RNase L pathway in the liver of mice. Infection with this virus does not result in hepatitis and $NS2^{Mut}$ replication is reduced at least 10,000 fold compared to wild-type A59. However, in mice genetically deficient for RNase L ($RNase\ L^{-/-}$) $NS2^{Mut}$ replicates to wild-type levels and causes hepatitis (4).

As might be expected of antagonists of a potent innate antiviral pathway, 2',5' PDEs are not a host evasion mechanism unique to MHV. We recently showed that the NS4b accessory protein of MERS-CoV and related bat coronaviruses, all lineage C betacoronaviruses, encode the NS4b accessory proteins with 2',5'-PDE activity (6). Additionally, unrelated group A rotaviruses encode a PDE in the C-terminal domain of the VP3 structural protein (7). We show here that lineage A betacoronaviruses closely related to MHV, including the human respiratory HCoV-OC43 (OC43), human enteric CoV-4408 (HECoV), equine ECoV-NC99 (ECoV), and porcine hemagglutinating encephalomyelitis virus (PHEV), as well as the more distantly related equine torovirus (EToV)-Berne (BEV) also encode NS2 homologs with predicted PDE activity. We found that these proteins do possess enzymatic 2',5'-PDE activity that is capable of antagonizing RNase L (with the exception of the PHEV NS2) and thus countering a potent host antiviral response, suggesting that PDE mediated OAS-RNase L antagonism is an important virulence strategy for lineage A betacoronaviruses and toroviruses.

Material and Methods

Cell lines and mice. Murine fibroblast L2 (L2), murine 17 clone 1 (17Cl1) and baby hamster kidney cells expressing MHV receptor (BHK-R) were cultured as previously described (8,9). C57Bl/6 (B6) mice were originally procured from the National Cancer Institutes mouse repository, and RNase $L^{-/-}$ mice on a B6 genetic background were derived by Dr. Robert Silverman (10) and subsequently bred in the University of Pennsylvania animal facility. All

experiments involving mice were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania. Primary bone marrow derived macrophages (BMM) were derived from marrow harvested from the hind limbs (tibia and femur) of four to six week old B6 or RNase $L^{-/-}$ mice as described previously (4,11). Cells were cultured in DMEM (Gibco) supplemented with 10% FBS (Hyclone) and 20% L929 cell-conditioned media for 6 days before infection.

Plasmids. NS2 genes from lineage A betacoronaviruses OC43, HECV-4408, ECoV-NC99 NC99, PHEV and pp1a-carboxyterminal domain (CTD) from the torovirus Berne were synthesized and cloned into pUC57 by BioBasic yielding pUC-OC43NS2, pUC-HECVNS2, pUC-ECoVNS2, pUC-PHEVNS2 and pUC-pp1a. The second catalytic His to Arg substitutions were made by site directed mutagenesis in all plasmids resulting in pUC-$OC43NS2^{H129R}$, pUC-$HECVNS2^{H129R}$, pUC-$NC99NS2^{H129R}$, pUC-$PHEVNS2^{H129R}$ and pUC-$pp1a^{H4516R}$. Select genes were subsequently subcloned into the pMal parallel-2 expression vector resulting in pMAL-OC43NS2, pMAL-$OC43NS2^{H129R}$, pMAL-PHEVNS2, pMAL-$PHEVNS2^{H129R}$, pMAL-pp1a and pMAL-$pp1a^{H4516R}$. MHV NS2 and $NS2^{H129R}$ had been previously cloned into pMAL-c2 (4).

Purification of recombinant PDEs from E. coli and FRET assay. MBP-PDE fusion proteins were expressed from pMAL-plasmids in BL21 T7 expression competent *E. coli* (NEB, Inc., Ipswich, MA) and purified by affinity chromatography followed by ion exchange chromatography on MonoQ GL10/100 using a NaCl gradient from 0 to 1 M in 20 mM NaCl as previously described (4,12). The integrity and the purity of the purified MBP fusion proteins were determined by SDS-PAGE Coomassie Blue R250 staining. The extent of purity was similar for all of the enzymes as accessed by SDS-PAGE analysis. To assess enzymatic activity, purified proteins [10 μM MBP (420 μg/ml) as control or 1 μM OC43 (75 μg/ml); BEV (60 μg/ml) PHEV (65 μg/ml) or MHV (70μg/ml) MBP-PDE fusion proteins] in 150 μl of assay buffer (20 uM

140 HEPES [pH 7.2], 10 mM $MgCl_2$, 1 mM dithiothreitol) were incubated at 30° with (2'-5')p_3A_3 (2-
141 5A). After one hour, reactions were stopped by heat inactivation at 95° for 3 min followed by 30
142 min centrifugation at 20,000 X g (4°) and supernatants carefully removed. A fluorescent
143 resonance energy transfer (FRET) assay was used to assess enzymatic activity by measuring
144 the amount of uncleaved, intact 2-5A left in the reaction, as previously described (13). The
145 abilities of recombinant enzyme to degrade 2-5A were determined by a FRET based RNase L
146 activation assay using an authentic 2-5A (2',5'-p_3A_3) trimer as described earlier (4, 6, 13, 14).
147 Assays were performed three times in triplicate using two separate enzyme preparations.
148
149 **Viruses and chimeric recombinant virus construction.** Wild-type MHV strain A59 and
150 mutant $NS2^{H126R}$ (referred to as MHV and MHV^{Mut} in the data shown herein) were described
151 previously (14). The chimeric viruses were constructed based on the infectious cDNA clone
152 icMHV-A59 (8,15). The wild-type and mutant PDEs genes were PCR amplified from the pUC
153 plasmids constructed above with primers bearing SalI and NotI restricting sites. After purification
154 and digestion with SalI and NotI, the fragments were cloned into icMHV-A59 fragment G, with
155 an $NS2^{H126R}$ mutation, as previously described (14), and confirmed by DNA sequencing. The
156 full-length A59 genome cDNA was assembled, and the recombinant viruses were recovered in
157 BHK-R cells as previously described (8,14,15). When virus cytopathology was observed, virus
158 was plaque purified from the supernatant and amplified on 17CL-1 cells for use. The pairs of
159 chimeric viruses expressing WT and mutant PDEs were named by the source of the PDE,
160 OC43 & $OC43^{Mut}$, HECoV & $HECoV^{Mut}$, PHEV & $PHEV^{Mut}$, $ECoV^{Mut}$ & ECoV and BEV & BEV^{Mut}.
161 The PDE gene and flanking regions were amplified by PCR from the cloned chimeric virus
162 genomes and the sequences verified. The primers used for sequencing were Fns4 (5'-
163 TTGTTGTGATGAGTATGGAG) which maps 136 nucleotides upstream of the ATG start codon
164 for the PDE and Rns4 (5'-GCGTAACCATGCATCACTCAC) which maps 139 nucleotides

165 downstream of the PDE ORF The regions sequenced region includes the SalI and NotI
166 restriction sites as well as the transcription regulatory sequence (TRS) for ORF4 and ORF5a.
167
168 **Chimeric MHV infections of bone marrow derived macrophages (BMM).** BMM were mock
169 infected or infected at a multiplicity of infection (MOI) of 1 PFU/cell (in triplicate) and allowed to
170 adsorb for 1 hour at 37 °C. Cultures were washed with PBS (3 times) and fed with medium. At
171 the times indicated, cells were lysed and analyzed for degradation of RNA (described below) or
172 supernatants were harvested for quantification of viral titers by plaque assay on L2 cells (14).
173
174 **Immunoblotting.** L2 cells were infected with MHV or chimeric viruses (MOI=1PFU/cell). At 10
175 hours post infection, cells were lysed in nonidet P-40 (NP-40) buffer (1% NP-40, 2 mM EDTA,
176 10% glycerol, 150 mM NaCl and 50 mM Tris pH 8.0) containing protease inhibitors (Roche).
177 Protein concentrations were measured using a DC protein assay kit (Bio-Rad). Supernatants
178 were mixed 3:1 with 4X SDS-PAGE sample buffer. Samples were boiled, separated by 4-15%
179 SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Blots were blocked
180 with 5% nonfat milk and probed with the following antibodies: anti-Flag M2 mice monoclonal
181 antibody (Agilent, 1:1000); anti MHV nucleocapsid mouse monoclonal antibody (a gift from Dr.
182 Julian Leibowitz; 1:400) and anti GAPDH mouse monoclonal antibody (Thermo Scientific,
183 1:1000). Anti-mouse HRP (Santa Cruz; 1:5000) secondary antibodies were used to detect the
184 primary antibodies. The blots were visualized using Super Signal West Dura Extended Duration
185 Substrate (Thermo Scientific). Blots were probed sequentially with antibodies with blots being
186 stripped between antibody treatments.
187
188 **Analysis of RNase L mediated rRNA degradation.** RNA was harvested from B6 WT BMM
189 infected with MHV and chimeric viruses encoding WT and catalytically inactive PDEs at the
190 indicated time points using a Qiagen RNeasy kit. RNase was denatured at 70° for 2 min and

191 analyzed with an Agilent BioAnalyzer 2100 on a eukaryotic total RNA nanochip. The
192 BioAnalyzer converts the electropherogram generated for each sample into the pseudogel as
193 depicted in Fig 6 (4).
194
195 **Replication in mice.** Four week old B6 or RNase $L^{-/-}$ mice (5-7) were anesthetized with
196 isoflurane (Abbott Laboratories; Chicago, IL) and inoculated intrahepatically with 2000 PFU in
197 50 µL of DPBS (Gibco) containing 0.75% bovine serum albumin (Sigma). Mice were euthanized
198 with CO_2, perfused with DPBS (Gibco) and livers harvested at day five post inoculation. Part of
199 the liver was fixed for histology below and the rest was homogenized and viral titers were
200 determined by plaque assay of liver homogenates on L2 cells (16). A piece of each liver was
201 fixed overnight in 4% paraformaldehyde, embedded in paraffin and sectioned. Sections were
202 stained with hematoxylin and eosin (H&E) or alternatively blocked with 10% normal donkey
203 serum and immunostained with a 1:20 dilution of a monoclonal antibody against MHV
204 nucleocapsid (N) protein (1:1000 dilution). Staining was developed using avidin-biotin-
205 immunoperoxidase (Vector Laboratories).
206
207
208 **Results**
209 **Alignment and modeling of coronavirus and torovirus NS2 proteins.** To determine whether
210 the MHV-related betacoronaviruses encode proteins with 2',5'-PDE activity we first analyzed the
211 primary amino acid sequence of the NS2 proteins from OC43, HECoV, ECoV-NC99, PHEV and
212 the pp1a-CTD of BEV. While the NS2 homologs are encoded within ORF2a, the PDE of BEV is
213 encoded at the 3' end of the ORF1a and processed from the pp1a polyprotein (2). All of these
214 proteins contain two conserved HxS/Tx motifs spaced by ~80 residues, where x is any
215 hydrophobic residue, characteristic of 2H-phosphoesterase superfamily proteins (3,4,14) (Fig 1).
216 Interestingly the carboxytermini of the PHEV and BEV PDEs are truncated relative to the other

NS2 proteins, similar to rotavirus VP3-CTD PDEs (14,17). We further entered the primary amino acid sequence of these proteins into Phrye[2] to predict their tertiary structures (Fig 2). All of these proteins scored highly for homology with the published structure of the A-kinase anchoring protein 7 (AKAP7) central domain (CD) (18), a previously identified host-encoded 2H-PE with 2',5'-PDE (7). We have previously shown that the MHV NS2 and group A rotavirus (RVA) VP3 proteins, also structural homologs of AKAP7 CD, exhibit 2',5' PDE activity and can antagonize RNase L (4,7,14).

Coronavirus and torovirus putative 2',5' PDEs are enzymatically active and cleave 2-5A. To determine whether the predicted Nidovirus PDEs (OC43, BEV, PHEV) are enzymatically active, the genes encoding them as well as their corresponding mutants with an Arg substitution of the second predicted catalytic His residue were expressed in *Escherichia coli* as maltose binding protein (MBP) fusion proteins and purified by affinity chromatography followed by ion exchange chromatography and size exclusion chromatography as described in Materials and Methods (4). Purified wild type or catalytic mutant proteins were incubated with 2-5A substrate and an indirect fluorescent resonance energy transfer (FRET) assay was used to assess activation of RNase L, in which higher RLUs represent active RNase L as described in Materials and Methods and in detail previously (13). MHV NS2 was utilized as a positive control for inhibition of RNase L (Fig 3). OC43 and BEV proteins reduced RNase L activation to a similar degree as MHV NS2, while PHEV NS2 was significantly less active. The mutant proteins containing a His → Arg mutation in the second catalytic motif did not inhibit RNase L, as expected and consistent with previously results describing MHV NS2 (4).

Coronavirus and Torovirus PDEs inhibit RNase L when are expressed from a chimeric MHV NS2 mutant backbone To investigate whether the NS2 proteins of OC43, HECoV, ECoV, PHEV, and BEV pp1a-CTD can antagonize RNase L during infection, we constructed chimeric

243 viruses expressing each exogenous PDE from ORF4 (encoding NS4a, 4b) of an MHV backbone
244 (Fig 4). The MHV-A59 backbone we utilized encodes H126R substitution in NS2 (MHV^{Mut}
245 referred to in literature as $NS2^{H126R}$) that abrogates its enzymatic activity and its ability to
246 antagonize RNase L. MHV^{Mut} exhibits minimal replication in primary bone marrow-derived
247 macrophages (BMM) or in vivo (4). The chimeric viruses we constructed express either the
248 exogenous PDE protein or its catalytic mutant from the ORF4 locus of MHV, which is
249 dispensable for MHV replication *in vitro* and *in vivo* (19). Each exogenous protein was
250 constructed with a C-terminal Flag-tag to allow verification of expression from the chimeric
251 viruses.
252
253 To assess expression of PDEs by western blot, we infected L2 cells with the chimeric viruses
254 and harvested protein lysates at 10 hours post-infection (hpi). We probed for the exogenous
255 PDEs using a primary antibody directed against the Flag-tag, and utilized GAPDH as a loading
256 control (Fig 5). The OC43, HECV and ECov PDEs were detectable by western blot at a high
257 level of abundance, while detection of BEV pp1-CTD expression was less robust. PHEV NS2
258 expression from multiple viral clones as well as the swarm of uncloned recombinant virus could
259 not be detected by western blot.
260
261
262 **Exogenous coronavirus and torovirus PDEs rescue replication of MHV^{Mut} in primary B6**
263 **BMMs through inhibition of RNase L activation.** To determine if the exogenous PDEs can
264 antagonize RNase L in the context of infection, we infected BMMs from WT B6 and RNase $L^{-/-}$
265 mice with MHV, MHV^{Mut} and the chimeric viruses and measured replication by plaque assay at
266 6, 9, 12, and 24 hpi. As expected, MHV^{Mut} is significantly attenuated in WT BMMs but replicates
267 to equivalent titers as MHV in RNase $L^{-/-}$ BMMs (Fig 6A). All of the chimeric viruses encoding
268 WT exogenous PDEs from OC43, HECoV, ECoV and BeV, replicated to a similar extent as WT

269 A59 in B6 BMMs, indicating that these proteins effectively compensate for an inactive NS2^{H126R}
270 in MHV (Fig 4B-E). In contrast, and similarly to MHV^{Mut}, the chimeras expressing catalytically
271 inactive exogenous PDEs fail to replicate robustly in B6 BMMs but do replicate efficiently in
272 RNase L$^{-/-}$ BMMs (Fig 6A-E). The chimeric virus encoding PHEV NS2 were not assessed for
273 replication in BMMs due to our inability to confirm its expression (Figure 5).
274
275 To directly link antagonism of RNase L to the ability of the exogenous PDEs to rescue MHV^{Mut}
276 replication, we assessed rRNA degradation in infected cells by Bioanalyzer. We have previously
277 used this assay to demonstrate that MHV NS2, but not NS2^{H126R}, inhibits RNase L-mediated
278 RNA degradation, and that a deficiency in RNase L obviates the requirement for NS2 in MHV
279 replication (4). We infected B6 WT and RNase L$^{-/-}$ BMMs with MHV and the chimeric viruses and
280 harvested total RNA 9 hpi. We ran the total RNA on a Bioanalyzer to visualize the integrity of
281 rRNA during infection with MHV and the chimeric viruses. MHV and the chimeric viruses
282 encoding exogenous PDEs encoded by MHV, OC43, HECoV, ECoV and BEV prevented rRNA
283 degradation in B6 WT BMMs, while the corresponding catalytically inactive PDEs failed to do so
284 (Fig 6F). This directly links the ability of the exogenous PDEs to rescue MHV^{Mut} replication to
285 their antagonism of RNase L activation.
286
287 **OC43 NS2 and BEV pp1a-CTD restore MHV^{Mut} replication and pathogenesis *in vivo*.** MHV
288 causes profound hepatitis and associated liver pathology in B6 mice, with its liver replication
289 and pathogenesis dependent on NS2-mediated antagonism of RNase L (Fig 7) (4). To
290 determine whether exogenous viral PDEs can rescue replication and restore pathogenesis to
291 MHV^{Mut}, we infected B6 and RNase L$^{-/-}$ mice with MHV, MHV^{Mut} and the chimeric viruses
292 expressing either WT or catalytic mutant PDEs from OC43 (NS2) and BEV (pp1a-CTD). Five
293 days post-infection, at the time of peak titer, the mice were sacrificed and livers harvested for
294 virus titration by plaque assay. In WT B6 mice chimeric viruses expressing either WT OC43

NS2 or BEV pp1-CTD replicated robustly in the liver, similarly to MHV. In contrast, and like MHV^{Mut}, the chimeric viruses expressing mutant OC43 NS2 (Fig 7B) or BEV pp1a-CTD (Fig 7C) are dramatically restricted, replicating only to titers below or just above the limit of detection, whereas all of the chimeric viruses replicated robustly in the livers of RNase L$^{-/-}$ mice (Fig 7A-C).

To assess hepatitis in these infected mice, livers sections were assessed for viral antigen and pathological changes. Like A59, chimeric viruses expressing WT OC43 NS2 or BEV pp1a-CTD caused hepatitis in B6 mice, indicated by pathologic foci in H&E stained livers, with viral antigen staining widely observed (Fig 7D,E). Chimeric viruses expressing catalytically inactive OC43 NS2 or BEV pp1a-CTD did not cause liver pathology in B6 mice and viral antigen was absent, consistent with the lack of replication (Fig 7D,E). In RNase L$^{-/-}$ mice, all of the chimeric viruses replicated robustly and caused pathology similar to MHV A59 (Fig 7D,E), further demonstrating that the restriction of the viruses expressing mutant PDEs in B6 mice is RNase L-mediated and that the exogenous PDEs function equivalently to MHV NS2.

Discussion

We have previously demonstrated 2-5A cleavage and RNase L antagonism by 2',5' PDEs encoded by a lineage A and a lineage C betacoronavirus (MHV and MERS-CoV respectively) and group A rotaviruses as well as by cellular AKAP7 CD (4,6,7,14). Here, we extend these findings to show that additional lineage A betacoronaviruses as well as a related torovirus family member encode 2',5' PDEs capable of antagonizing RNase L by cleaving 2-5A. The presence of genes encoding these proteins in multiple lineage A betacoronaviruses suggests that this gene was acquired by an ancient common ancestor of this lineage. Whether this virus was also ancestral to toroviruses and lineage C betacoronaviruses, or whether 2',5' PDEs were acquired by viruses in multiple independent events is unclear. The maintenance of this highly conserved protein throughout lineage A betacoronaviruses supports the idea that this protein mediates an

essential function in the diverse natural hosts of these viruses, spanning multiple mammalian families. Our finding of a homologous PDE in some groups of rotaviruses (14), a virus family, unrelated to coronaviruses is intriguing. A coronavirus recently isolated from bats was found to encode a protein likely to have originated from a bat orthoreovirus, which like rotaviruses has a dsRNA genome, suggesting the possibility of recombination between coronavirus and a dsRNA virus (20). Further support for this idea comes from a recent report of isolation of a MERS like coronavirus and a rotavirus in the feces of Korean bats (21). Additionally, the viruses encoding the PDEs we have described here infect different tissues within their hosts (1,22,23), indicating that RNase L antagonism may be required for robust replication in diverse cell types. For example, although MHV is hepatotropic, OC43 infects the upper airway, while other PDEs described here are encoded by enterotropic viruses (1,22,23).

The PDEs encoded by OC43, HECoV, ECoV and BEV antagonized RNase L and rescued replication of MHV^{Mut} in primary WT B6 BMMs, indicating that not only are they enzymatically active 2',5' PDEs, but that they functionally compensate for an inactive MHV NS2 (Fig 3,6,7). Interestingly the BEV encoded PDE was able to antagonize RNase L and rescue virus MHV^{Mut} replication both *in vitro* and *in vivo* despite the apparently low level of expression (Fig 5,6,7). This is not surprising as MERS NS4b PDE can rescue MHV^{Mut} despite its very low expression level in the cytoplasm (6). PHEV NS2, is less enzymatically active than the other PDEs (Fig 3), suggesting it may be less able to antagonize RNase L. However since we could not detect expression by western blot of the PHEV PDE from a chimeric virus (Fig 5), further work will be needed to determine if it has RNase L antagonist activity in the context of an infection. Interestingly both the BEV and PHEV PDEs are truncated at the carboxytermini similar to the rotavirus PDE [Fig 3; (14)]; clearly the carboxyterminal sequences are not required for cleavage of 2-5A or RNase L antagonism as the rotavirus VP3-CTD and BEV PDEs have similar activity to MHV NS2 [Fig3,(14)]. Nevertheless, the diminished enzymatic activity of PHEV NS2 relative

347 to the other PDEs, suggests that while PDE may have been essential in the PHEV ancestor, it
348 may not be required in the cells targeted by PHEV in its porcine host. However, RNase L is
349 likely actively antiviral in other porcine tissues or stages of development, as suggested by the
350 presence of an RNase L antagonist in protein 7 of transmissible gastroenteritis virus (24).
351
352 Although the chimeric MHVs encoding OC43-NS2 and BEV pp1a-CTD do not replicate quite as
353 well as MHV in vivo (Fig. 6), this is unlikely due to disruption of the ORF4 gene by insertion of
354 the exogenous PDEs as ablation of ORF4 expression within the genome of MHV strain JHM,
355 had no effect on replication *in vitro* and *in vivo* pathogenesis and the MHV strain A59 ORF 4 is
356 disrupted by a termination codon (25). Nevertheless, these chimeric viruses replicated robustly
357 *in vivo* causing hepatitis and their respective mutants replicated to wild-type titers in the livers of
358 RNase $L^{-/-}$ mice, indicating that restriction of the mutants in WT B6 mice is due to RNase L
359 activity.
360
361 Overall, we have demonstrated that active 2',5' PDEs are a conserved feature of lineage A
362 *Betacoronavirus* genomes, and that a homologous domain is encoded in the first open reading
363 frame of a related nidovirus, BEV. This suggests that RNase L is a potent antiviral effector in
364 diverse species and tissues, due to the wide host range represented by the viruses encoding
365 these now-characterized PDEs. This thus far includes the lineage A and lineage C
366 betacoronaviruses as well as the related toroviruses and the unrelated group A Rotaviruses
367 (4,14). Finally since 2',5'-PDEs are potent antagonist of host antiviral defenses encoded by
368 multiple and diverse viruses within *Coronaviridae*, this class of protein may have the potential to
369 be a broad-spectrum therapeutic target for human viruses including the OC43, a ubiquitous
370 agent of the common cold and MERS.
371

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375

376 **References**

377 1. **Weiss SR, Leibowitz JL**. 2011. Coronavirus pathogenesis. Adv Virus Res **81**: 85-164.
378 2. **Snijder EJ, Bredenbeek PJ, Dobbe JC, Thiel V, Ziebuhr J, Poon LL, Guan Y, Rozanov**
379 **M, Spaan WJ, Gorbalenya AE**. 2003. Unique and Conserved Features of Genome and
380 Proteome of SARS-coronavirus, an Early Split-off From the Coronavirus Group 2
381 Lineage. J Mol Biol **331**: 991-1004.
382 3. **Mazumder R, Iyer LM, Vasudevan S, Aravind L**. 2002. Detection of novel members,
383 structure-function analysis and evolutionary classification of the 2H phosphoesterase
384 superfamily. Nucleic Acids Res **30**: 5229-5243.
385 4. **Zhao L, Jha BK, Wu A, Elliott R, Ziebuhr J, Gorbalenya AE, Silverman RH, Weiss SR**.
386 2012. Antagonism of the interferon-induced OAS-RNase L pathway by murine
387 coronavirus ns2 protein is required for virus replication and liver pathology. Cell Host
388 Microbe **11**: 607-616.
389 5. **Silverman RH**. 2007. Viral encounters with 2',5'-oligoadenylate synthetase and RNase L
390 during the interferon antiviral response. Journal of virology **81**: 12720-12729.
391 6. **Thornbrough JM, Jha BK, Yount B, Goldstein SA, Li Y, Elliott R, Sims AC, Baric RS,**
392 **Silverman RH, Weiss SR**. 2016. Middle East Respiratory Syndrome Coronavirus NS4b
393 Protein Inhibits Host RNase L Activation. MBio **7**.
394 7. **Gusho E, Zhang R, Jha BK, Thornbrough JM, Dong B, Gaughan C, Elliott R, Weiss SR,**
395 **Silverman RH**. 2014. Murine AKAP7 Has a 2',5'-Phosphodiesterase Domain That Can
396 Complement an Inactive Murine Coronavirus ns2 Gene. MBio **5**: e01312-01314.
397 8. **Yount B, Denison MR, Weiss SR, Baric RS**. 2002. Systematic assembly of a full-length
398 infectious cDNA of mouse hepatitis virus strain A59. Journal of virology **76**: 11065-
399 11078.
400 9. **Roth-Cross JK, Stokes H, Chang G, Chua MM, Thiel V, Weiss SR, Gorbalenya AE,**
401 **Siddell SG**. 2009. Organ-specific attenuation of murine hepatitis virus strain A59 by
402 replacement of catalytic residues in the putative viral cyclic phosphodiesterase ns2.
403 Journal of virology **83**: 3743-3753.
404 10. **Zhou A, Paranjape J, Brown TL, Nie H, Naik S, Dong B, Chang A, Trapp B, Fairchild R,**
405 **Colmenares C, Silverman RH**. 1997. Interferon action and apoptosis are defective in
406 mice devoid of 2',5'-oligoadenylate-dependent RNase L. The EMBO journal **16**: 6355-
407 6363.
408 11. **Caamano J, Alexander J, Craig L, Bravo R, Hunter CA**. 1999. The NF-kappa B family
409 member RelB is required for innate and adaptive immunity to Toxoplasma gondii.
410 Journal of immunology **163**: 4453-4461.
411 12. **Sheffield P, Garrard S, Derewenda Z**. 1999. Overcoming expression and purification
412 problems of RhoGDI using a family of "parallel" expression vectors. Protein Expr Purif
413 **15**: 34-39.
414 13. **Thakur CS, Xu Z, Wang Z, Novince Z, Silverman RH**. 2005. A convenient and sensitive
415 fluorescence resonance energy transfer assay for RNase L and 2',5' oligoadenylates.
416 Methods Mol Med **116**: 103-113.
417 14. **Zhang R, Jha BK, Ogden KM, Dong B, Zhao L, Elliott R, Patton JT, Silverman RH,**
418 **Weiss SR**. 2013. Homologous 2',5'-phosphodiesterases from disparate RNA viruses
419 antagonize antiviral innate immunity. Proc Natl Acad Sci U S A **110**: 13114-13119.
420 15. **Sperry SM, Kazi L, Graham RL, Baric RS, Weiss SR, Denison MR**. 2005. Single-amino-
421 acid substitutions in open reading frame (ORF) 1b-nsp14 and ORF 2a proteins of the
422 coronavirus mouse hepatitis virus are attenuating in mice. Journal of virology **79**: 3391-
423 3400.

16. **Gombold JL, Hingley ST, Weiss SR**. 1993. Fusion-defective mutants of mouse hepatitis virus A59 contain a mutation in the spike protein cleavage signal. Journal of virology **67**: 4504-4512.
17. **Ogden KM, Hu L, Jha BK, Sankaran B, Weiss SR, Silverman RH, Patton JT, Prasad BV**. 2015. Structural basis for 2' -5' -oligoadenylate binding and enzyme activity of a viral RNase L antagonist. Journal of virology.
18. **Gold MG, Smith FD, Scott JD, Barford D**. 2008. AKAP18 contains a phosphoesterase domain that binds AMP. J Mol Biol **375**: 1329-1343.
19. **Ontiveros E, Kuo L, Masters PS, Perlman S**. 2001. Inactivation of expression of gene 4 of mouse hepatitis virus strain JHM does not affect virulence in the murine CNS. Virology **289**: 230-238.
20. **Huang C, Liu WJ, Xu W, Jin T, Zhao Y, Song J, Shi Y, Ji W, Jia H, Zhou Y, Wen H, Zhao H, Liu H, Li H, Wang Q, Wu Y, Wang L, Liu D, Liu G, Yu H, Holmes EC, Lu L, Gao GF**. 2016. A Bat-Derived Putative Cross-Family Recombinant Coronavirus with a Reovirus Gene. PLoS pathogens **12**: e1005883.
21. **Kim HK, Yoon SW, Kim DJ, Koo BS, Noh JY, Kim JH, Choi YG, Na W, Chang KT, Song D, Jeong DG**. 2016. Detection of Severe Acute Respiratory Syndrome-Like, Middle East Respiratory Syndrome-Like Bat Coronaviruses and Group H Rotavirus in Faeces of Korean Bats. Transbound Emerg Dis **63**: 365-372.
22. **Navas-Martin S, Weiss SR**. 2003. SARS: lessons learned from other coronaviruses. Viral Immunol **16**: 461-474.
23. **Navas-Martin SR, Weiss S**. 2004. Coronavirus replication and pathogenesis: Implications for the recent outbreak of severe acute respiratory syndrome (SARS), and the challenge for vaccine development. J Neurovirol **10**: 75-85.
24. **Cruz JL, Sola I, Becares M, Alberca B, Plana J, Enjuanes L, Zuniga S**. 2011. Coronavirus gene 7 counteracts host defenses and modulates virus virulence. PLoS pathogens **7**: e1002090.
25. **Weiss SR, Zoltick PW, Leibowitz JL**. 1993. The ns 4 gene of mouse hepatitis virus (MHV), strain A 59 contains two ORFs and thus differs from ns 4 of the JHM and S strains. Arch Virol **129**: 301-309.
26. **Luytjes W, Bredenbeek PJ, Noten AF, Horzinek MC, Spaan WJ**. 1988. Sequence of mouse hepatitis virus A59 mRNA 2: indications for RNA recombination between coronaviruses and influenza C virus. Virology **166**: 415-422.
27. **St-Jean JR, Jacomy H, Desforges M, Vabret A, Freymuth F, Talbot PJ**. 2004. Human respiratory coronavirus OC43: genetic stability and neuroinvasion. Journal of virology **78**: 8824-8834.
28. **Zhang J, Guy JS, Snijder EJ, Denniston DA, Timoney PJ, Balasuriya UB**. 2007. Genomic characterization of equine coronavirus. Virology **369**: 92-104.
29. **Vijgen L, Keyaerts E, Lemey P, Maes P, Van Reeth K, Nauwynck H, Pensaert M, Van Ranst M**. 2006. Evolutionary history of the closely related group 2 coronaviruses: porcine hemagglutinating encephalomyelitis virus, bovine coronavirus, and human coronavirus OC43. Journal of virology **80**: 7270-7274.
30. **Snijder EJ, den Boon JA, Bredenbeek PJ, Horzinek MC, Rijnbrand R, Spaan WJ**. 1990. The carboxyl-terminal part of the putative Berne virus polymerase is expressed by ribosomal frameshifting and contains sequence motifs which indicate that toro- and coronaviruses are evolutionarily related. Nucleic Acids Res **18**: 4535-4542.

473 **Figure Legends**

474 Fig 1. Alignment of lineage A betacoronavirus and Berne torovirus PDEs. PDEs with Genbank
475 assession numbers are MHV NS2 (P19738.1) (26), OC43 NS2 (AAT84352.1) (27), HECoV NS2
476 (ACJ35484.1), ECoV NS2 (ABP87988.1) (28), PHEV NS2 (AAY68295.1) (29) and BEV
477 (CAA36600.1) (30). Conserved catalytic HxS/Tx motifs are indicated by boxes.
478
479 Fig 2. Known and predicted structures of nidovirus PDEs. (A) Crystal structure of MHV NS2 (PDB:
480 4Z5V) and predicted structures of OC43 NS2 (B) and BEV pp1a-CTD (C). Predicted structures were
481 generated using Phyre^2 then visualized and annotated using UCSF Chimera 1.8. Catalytic His and
482 conserved Ser/Thr residues are indicated.
483
484 Fig 3. Assay of PDE activity of coronavirus and torovirus PDEs. Recombinant WT and mutant PDEs
485 were incubated with 2-5A for 60 minutes and the remaining substrate was quantified using an
486 indirect FRET based assay as described in Materials and Methods. RFU= relative fluorescence
487 units, is proportional to 2-5A remaining. Data shown are from one representative of three
488 independent experiments, each carried out in triplicate with separate enzyme preparations and are
489 expressed as means ± SEM; *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.
490
491 Fig 4. NS2 organization and construction of chimeric viruses. (A) Depiction of the NS2 protein of
492 HCoV-OC43. Shown are the catalytic His residues at positions 49 and 129, with the His->Arg
493 mutation shown below. (B) Genome organization of MHV with NS2 and NS4 loci indicated. Also
494 shown are replicase open reading frames 1a and 1b, genes encoding structural proteins HE, S, E,
495 M, N and I as well as nonstructural protein 5a. In chimeric viruses MHV NS2 residue 126 is mutated
496 from H->R, rendering NS2 catalytically inactive (NS2^{Mut}). The gene encoding the exogenous PDE
497 or its catalytically inactive mutant is inserted in place of MHV NS4.
498

499 Fig 5. Expression of exogenous PDEs from chimeric viruses. L2 cells were infected with MHV or
500 chimeric viruses and protein harvested 10 hpi and analyzed by western immunoblotting. Blots were
501 probed with antibody against Flag to detect PDEs, anti-nucleocapsid antibody to assess chimeric
502 viral infection and GAPDH as a protein loading control. MHV NS2 (lanes 1-2) is not Flag-tagged.
503 Flag-tagged WT and mutant PDEs of OC43, HECV, PHEV, ECoV and BEV are detected as
504 indicated. This blot was performed two times using proteins from independent infections with similar
505 results.
506
507 Fig 6. Replication and activation of RNase L of chimeric viruses in bone marrow derived
508 macrophages (BMM). (A-E) BMMs derived from WT B6 or RNase $L^{-/-}$ mice were infected with (A)
509 MHV or chimeric MHV viruses expressing WT or mutant (B) OC43 NS2, (C) HECoV NS2, (D)
510 ECoV NS2 and (E) BEV pp1a-CTD. Virus at each time point was titrated by plaque assay. Each
511 time point is represented by three biological replicates, titrated in duplicate and variance expressed
512 as SEM. Statistical significance was calculated by 2-way ANOVA in GraphPad Prism. **, P <0.01;
513 ***, P <0.001. (F) Total RNA was isolated from WT B6 BMMs 9 hpi and rRNA integrity assayed using
514 an Agilent BioAnalyzer. These data are from one of at least two independent experiments with
515 similar results
516
517 Fig 7. Replication and pathogenesis of chimeric viruses *in vivo*. (A-C) WT B6 or RNase $L^{-/-}$ mice (n=
518 5-7) were infected intrahepatically with (A) MHV and MHV^{Mut} or chimeric viruses encoding WT or
519 mutant (B) OC43 NS2 or (C) BEV pp1a-CTD. Five days post-infection livers were harvested and
520 virus titrated by plaque assay. Each data point represents a single mouse liver, titrated in duplicate
521 with variance expressed as SEM. Statistical significance determined by 1-way ANOVA in GraphPad
522 Prism. ***, P <0.001. Liver sections from infected mice were stained with (D) H&E to identify hepatic
523 pathology or (E) antibody to detect MHV nucleocapsid protein.
524