Homologous 2′,5′-phosphodiesterases from disparate RNA viruses antagonize antiviral innate immunity

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Efficient and productive virus infection often requires viral countermeasures that block innate immunity. The IFN-inducible 2′,5′-oligoadenylate (2-5A) synthetases (OASs) and ribonuclease (RNase) L are components of a potent host antiviral pathway. We previously showed that murine coronavirus (MHV) accessory protein ns2, a 2H phosphoesterase superfamily member, is a phosphodies-
terase (PDE) that cleaves 2-5A, thereby preventing activation of RNase L. The PDE activity of ns2 is required for MHV replication in macrophages and for hepatitis. Here, we show that group A rotavirus (RVA), an important cause of acute gastroenteritis in children worldwide, encodes a similar PDE. The RVA PDE forms the carboxy-terminal domain of the minor core protein VP3 (VP3-CTD) and shares sequence and predicted structural homology with ns2, including two catalytic HxT/S motifs. Bacterially expressed VP3-CTD exhibited 2′,5′-PDE activity, which cleaved 2-5A in vitro. In addition, VP3-CTD expressed transiently in mammalian cells depleted 2-5A levels induced by OAS activation with poly(I):poly(C), preventing RNase L activation. In the context of recombinant chimeric MHV expressing inactive ns2, VP3-CTD restored the ability of the virus to replicate efficiently in macrophages or in the livers of infected mice, whereas mutant viruses expressing inactive VP3-CTD (H718A or H798R) were attenuated. In addition, chimeric viruses expressing either active ns2 or VP3-CTD, but not nonfunctional equivalents, were able to protect ribosomal RNA from RNase L-mediated degradation. Thus, VP3-CTD is a 2′,5′-PDE able to functionally substitute for ns2 in MHV infection. Remarkably, therefore, two disparate RNA viruses encode proteins with homologous 2′,5′-PDEs that antagonize activation of innate immunity.

Reoviridae | Nidovirales | RNA capping enzyme | interferon-stimulated gene

The 2′,5′-oligoadenylate (2-5A) synthetase (OAS)–ribonuclease (RNase) L pathway is among the most potent IFN-induced antiviral effectors, blocking viral infections by several mechanisms, including directly cleaving viral single-stranded RNA genomes, depleting viral and host mRNA available for translation, and enhancing type I IFN induction (1). Type I IFNs bind to the cell surface receptor IFNAR (interferon-α/β receptor), initiating JAK-STAT signaling to the OAS genes, which results in elevated levels of OAS proteins. When activated by viral double-stranded (ds)RNA, certain OAS isoforms use ATP to synthesize 5′-triphasphorylated 2-5A. Trimer and longer species of 2-5A bind with high specificity and affinity to the inactive monomeric RNase L, causing it to dimerize and become active (2) (Fig. 1A).

Not surprisingly, viruses have evolved multiple mechanisms to antagonize or prevent activation of RNase L (1). One such virus is mouse hepatitis virus (MHV) (3), a member of the enveloped, positive-stranded (+)RNA coronavirus species. MHV and related 2a-betacoronaviruses (4) encode ns2, a cytoplasmic 30-kDa protein that is dispensable for virus replication in transformed cell lines but serves as an IFN antagonist and is required for the induction of hepatitis (5–7). ns2 is a member of group II (eukaryotic LigT) of the 2H-phosphoesterase superfamily, which contain two HxT/S catalytic motifs (8). ns2 has 2′,5′-phosphodiesterase (PDE) activity that cleaves and inhibits the accumulation of 2-5A, preventing activation of RNase L while enhancing viral growth and pathogenesis (Fig. 1A) (9). Using mice deficient in RNase L expression (RNase L−/−), we demonstrated that ns2 PDE activity allows MHV to replicate to similar titer in the presence or absence of RNase L in vitro in bone marrow–derived macrophages (BMMs) and in the livers of infected mice. However, mutant viruses expressing PDE-inactive ns2 were able to replicate only in RNase L−/−/BMM or in the livers of RNase L−/− mice.

Group II 2H phosphoesterases contain cellular proteins, including A-kinase-anchoring protein-7 (AKAP7), and other viral proteins, including VP3 (C-terminal domain) of group A rotavirus (RVA) and polypeptide 1a of torovirus (C-terminal protein), each of which contains two HxT/S catalytic motifs separated by similar distances (8). Based on this homology, and on evidence that RVA VP3 is a virulence factor (10, 11), we hypothesized that RVA VP3 contains an antagonist of RNase L activation, similar to MHV ns2.

Rotaviruses, nonenveloped viruses with dsRNA genomes, are a common cause of severe diarrhea in infants and young children. Despite using a strategy of gene expression and replication very different from that of coronavirus, we show that RVA uses a similar mechanism as MHV to antagonize the activation of RNase L. We found that the C-terminal domain (CTD; ~143 amino acids) of RVA VP3, a minor protein component of the virion core chiefly involved in RNA capping (12, 13), has a predicted structure homologous to ns2 (Fig. 1B and C). Accordingly, VP3-CTD has 2′,5′-PDE activity that cleaves 2-5A in vitro and in intact cells, thereby antagonizing RNase L activation. Furthermore, in chimeric recombinant MHV strains, VP3-CTD is able to effectively substitute for a mutant ns2 gene, permitting viral replication in BMMs and in the livers of infected mice, even in the presence of RNase L. Our results reveal a previously unknown function for RVA VP3, one that has potential importance in countering antiviral innate immunity.


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PyMol (http://pymol.org) was used for molecular graphics. Ribbon representation of rat AKAP7 amino acids 89–291 compared with the predicted models of the C-terminal region of SA11 VP3. Alignments with AKAP7 were used to predict a structural homology and predicted structural similarity suggested that VP3-CTD, like MHV ns2, was capable of degrading 2-5A. To determine whether VP3-CTD is a 2-5’-PDE, the protein was expressed in Escherichia coli, purified, and incubated with the 2-5A trimer, (2′-5′)pApA. Following incubation, the 2-5A substrate was separated from the reaction products by HPLC (Fig. 24). VP3-CTD produced a profile of reaction products with the expected degradation of (2′-5′)pApA to (2′-5′)pApA, ATP, and AMP as a function of incubation time (Fig. 24). However, mutation of the two predicted catalytic His residues of VP3-CTD (H718A and H797R) produced protein that was deficient in 2-5A degradation (Fig. 2B). MHV ns2 generated a profile of reaction products like that of WT VP3-CTD (Fig. 2A and C) (9). The similar activities of MHV ns2 and rotavirus VP3-CTD were apparent from the kinetics of 2-5A cleavage (Fig. 2C).

**VP3-CTD Degrades 2-5A in Intact Cells.** To determine whether VP3-CTD was capable of cleaving 2-5A in intact cells, vectors encoding WT VP3-CTD, mutant VP3-CTD (H718A and H797R), and ns2 were individually transfected into human Hey1b ovarian carcinoma cells. Afterward, cells were treated with poly(rI):poly(rC) to activate OAS. The presence of functional forms of 2-5A in the intact cells was determined by adding nucleotide pools isolated from the treated cells to a specific FRET-based RNase-L activation assay (17) (SI Materials and Methods). Protein expression was monitored by Western blot (Fig. 3A). A standard RNase-L activation profile with (2′-5′)pApA produced a 50% effective concentration (EC50) of about 0.3 nM (2′-5′)pApA (Fig. 3B). Although no 2-5A was detected in untreated cells transfected with empty vector, cells transfected with empty vector and poly(rI):poly(rC) accumulated 2-5A as determined by RNase-L activation (Fig. 3C). Remarkably, there was no accumulation of 2-5A in cells transfected with VP3-CTD vector and poly(rI):poly(rC). In contrast, 2-5A accumulated in cells transfected with the mutant VP3-CTD (H718A and H797R) vector and poly(rI):poly(rC), resulting in RNase-L activation. MHV ns2 prevented 2-5A accumulation in poly(rI):poly(rC)-treated cells to a similar extent as VP3-CTD. These data indicate that VP3-CTD can degrade 2-5A in intact cells.

**VP3-CTD Rescues Replication of ns2 Mutant Viruses in B6 BMMs.** After observing that rotavirus VP3-CTD cleaves 2-5A in vitro and in intact cells, we hypothesized that VP3-CTD could compensate for the loss of ns2 function in mutant MHV-infected B6 BMMs. To test this hypothesis, we constructed a series of recombinant viruses using an infectious cDNA clone of MHV strain A59 (18) (SI Materials and Methods; Fig. 4A). The recombinant viruses included WT A59 and A59 expressing ns2 in which the catalytic His residues were individually mutated (ns2H126R, ns2H220R, and ns2H2146R). In addition, we constructed isogenic chimeric A59-VP3 viruses expressing ns2H2146R and either WT VP3-CTD or VP3-CTD in which the catalytic His residues were individually mutated (ns2H126R, VP3, ns2H2146R-VP3H718A, and ns2H2146R-VP3H797R) in place of the nonessential ns4 protein (19) (Fig. 4A).

The replication kinetics of these viruses were evaluated in mouse L2 fibroblasts, a cell type in which MHV does not induce IFN-β expression (20). All mutant and chimeric viruses replicated with similar kinetics and to similar final titers as A59, indicating that neither the mutations in ns2 nor the insertion of VP3-CTD in place of ns4 had any detectable effect on virus replication (Fig. S3A). Western blot analysis demonstrated that WT and mutant ns2 and VP3-CTD proteins were expressed in the virus-infected L2 cells (Fig. S3B). Immunofluorescent staining residues were located in homologous locations in each structure, suggesting a similar PDE function (Fig. 1C; Fig. S2).
of virus-infected murine 17CL-1 cells further demonstrated that both ns2 and VP3-CTD localized to the cytoplasm (Fig. S3C).

We next assessed viral replication in BMMs from B6 mice, a cell type in which MHV induces IFN expression, and ns2 PDE activity is needed to ensure efficient replication (21). Replication of ns2 mutant viruses (ns2H46A and ns2H126R) was severely impaired in B6 BMMs in comparison with that of A59 (Fig. 4B). Intriguingly, the chimeric virus ns2H126R-VP3, expressing VP3-CTD and a mutant ns2, replicated to nearly the same level as A59, suggesting that VP3-CTD was able to replace the function of ns2 within the A59 genome. In contrast, chimeric viruses expressing an inactive VP3 PDE in addition to mutant ns2, ns2H126R-VP3H718A, and ns2H126R-VP3H797R replicated only to the same extent as ns2 mutant viruses, with a final titer almost 100-fold less than ns2H126R-VP3. Because 2-5A is responsible for triggering RNase L activation, accumulation of 2-5A should have no effect on virus replication in RNase L−/− cells. As expected, all tested mutant and chimeric viruses replicated efficiently, with similar kinetics and final titer, in RNase L−/− BMMs (Fig. 4B) (9). These results demonstrate that the expression of VP3-CTD can functionally replace A59 ns2 during infection of B6 BMMs.

A consequence of activation of the OAS–RNase L pathway is RNase L–mediated RNA degradation, usually assessed by analysis of rRNA integrity (22). To further verify that restoration of ns2 mutant virus replication results from blocking the OAS-RNase L pathway, we compared the state of rRNA in B6 BMMs infected with each recombinant virus by RNA chip analysis using an Agilent analyzer. By 12 h postinfection, degradation of rRNA by RNase L was apparent in cells infected with viruses expressing ns2H46A, ns2H126R, ns2H126R-VP3H797R, or ns2H126R-VP3H797R (Fig. 4C). In contrast, rRNA remained intact in cells infected with A59 viruses expressing a functional PDE, either ns2 or VP3-CTD.
Expression of VP3 Enhances Replication of ns2 Mutant Virus in Liver.
Because VP3 was able to functionally replace ns2 function in BMMs, we hypothesized that replication of ns2 mutant virus in the liver would be restored by expression of VP3-CTD from the viral genome. To test this idea, B6 and RNase L−/− mice were infected intrahepatically with A59 and mutant viruses, and the virus titers were determined in the livers of mice killed at day 5 after infection, which is the peak of A59 replication in this organ (23, 24). As expected, ns2 H126R replicated minimally in B6 mice but robustly and to the same level as A59 in RNase L−/− mice (Fig. 4D). We compared replication of chimeric viruses expressing either WT VP3-CTD (ns2 H126R, VP3) or mutant VP3-CTD (ns2 H126R, VP3H797R). Although the titers of ns2 H126R, VP3 virus reached 10^4 plaque forming units (PFU)/g tissue in B6 mice, ns2 H126R·VP3H797R, expressing inactive PDEs, was undetectable. All of the chimeric viruses replicated equally well in RNase L−/− mice, regardless of the presence or absence of a functional PDE (Fig. 4D). These results show that expression of the active VP3 PDE confers increased replication of ns2 mutant virus in livers of WT B6 animals. Therefore, our results identify a previously unknown PDE activity of rotavirus VP3-CTD that functions in countering the OAS–RNase L pathway in vivo and in BMMs in vitro.

Discussion
2-5A degradation by virus-encoded 2′,5′-PDEs to prevent RNase L activation is a newly recognized viral strategy for evading innate immunity, discovered during studies on the coronavirus MHV (9). Here, we have shown that two highly similar 2′,5′-PDEs capable of cleaving 2-5A and antagonizing RNase L are produced by two very different RNA viruses; one virus (MHV) is pathogenic for mice, whereas the other virus (RVA) is an important gastrointestinal pathogen in young children. As there are relatively few similarities between the enveloped, positive-stranded RNA coronavirus MHV and the nonenveloped, segmented, dsRNA RVA, it is remarkable that both viruses encode homologous 2H PDE domains.

ns2 is a nonstructural protein translated from a monocistronic mRNA, whereas the rotavirus 2′,5′-PDE is found as the C-terminal domain of a larger structural protein that also contains rotavirus capping activities. ns2 resides in the cytoplasm (7), the site of replication for most RNA viruses. This localization agrees with ns2 function in 2-5A cleavage and RNase L antagonism. In contrast, VP3 is a structural protein that is encapsidated in RVA virions and caps nascent transcripts synthesized within transcriptionally active subviral particles. Following translation, VP3 accumulates in viroplasms, sites of new particle assembly, RNA packaging, dsRNA synthesis, and secondary rounds of transcription (25). Although it is not known whether VP3 is also localized diffusely within the cytoplasm, we hypothesize that a non–particle-associated form of VP3 mediates its RNase L antagonistic activities. Because different isoforms of OAS function at different subcellular locations and may respond to different dsRNA triggers to synthesize different forms of 2-5A (1), future studies to determine the localization of these viral PDEs might enhance an understanding of their function.

Due to the essential role of VP3 in RNA capping (12, 13) and lack of a robust reverse genetics system for rotaviruses, it is not currently possible to directly assess the role of VP3 PDE activity in the context of rotavirus infection. Nonetheless, several lines of
evidence suggest that, like ns2, RVA VP3-CTD functions to prevent RNase L activation via 2-5A degradation. First, in vitro, both VP3-CTD and ns2 degrade 2-5A from the 2′,3′-termini of 2-5A molecules, releasing 5′-AMP residues one at a time until the 5′-terminal ATP moiety is released (9) (Fig. 2). The minimum active species of 2-5A is triadenylate; therefore, once a single 5′-AMP residue is cleaved from trimer 2-5A to yield the dimeric species, (2′,5′)-pApA, the ability to activate RNase L is lost (26). VP3-CTD and ns2 also have a remarkable ability to prevent activation of RNase L in cells transfected with the potent OAS activator, poly(rI):poly(rC) (Fig. 3C). To be effective, 2′,5′-PDE activity needs to deplete 2-5A to levels lower than those necessary to maintain RNase L activation. Our findings emphasize the ability of VP3-CTD and ns2 to rapidly and efficiently degrade 2-5A at a rate that exceeds the ability of OAS to synthesize 2-5A. A chimeric MHV recombinant expressing both VP3-CTD and ns2 at a rate that exceeds the ability of OAS to synthesize 2-5A.

To prevent cellular and tissue damage from excessive activation of RNase L, certain host proteins are able to degrade 2-5A molecules. Unlike virally encoded 2′,5′-PDEs, cellular enzymes that mediate 2-5A decay, most notably the exonuclease-endonuclease-phosphatase PDE12 (2′-PDE) (36–38), are often insufficient to prevent RNase L activation in response to potent stimulation of OAS proteins by viral dsRNA. Host 2-5A degrading enzymes described to date have amino acid sequences that are unrelated to ns2 and VP3. Thus, it is possible that there may be other viral non–2H-PDEs with the same function as ns2 and VP3-CTD that would be missed by the searches for proteins with 2H-PDE homology (8).

Eight species of rotavirus (RVA-RVH) have been proposed, with RVA causing the majority of human disease (29, 30). Based on alignment of VP3 sequences (Fig. 5A), all RVA strains, including those of both human and animal isolates, encode a similar PDE domain. The presence of similar 2H-PDE motifs and numerous other conserved residues suggests that a PDE domain is also present at the VP3 C terminus of RBV and RVG strains. The presence of a conserved PDE-like domains for RV, RCV, and RVH strains accounts for the comparable decreased size of their VP3 proteins. Because VP3 PDE cleaves 2-5A and antagonizes RNase L and it is absent from some rotavirus species, this feature may enhance viral infection, spread, and pathogenesis in specific cell types. RVA viremia is more common than previously thought, with virus detected in the blood and extra-intestinal tissues of many infected children (34). Additionally, RVA is capable of infecting a subpopulation of human plasma-cytoid dendritic cells (pDCs) (35); pDCs contribute to the success of the RVA.

Materials and Methods

Cell Lines and Mice. Murine L2 fibroblast (L2), murine 17 clone-1 fibroblast (17C1-1), and baby hamster kidney cells expressing MHV receptor (BHK-MHV) were cultured as described previously (18, 31). Plaque assays were performed on L2 cells (39). B6 mice were purchased from Jackson Laboratory. RNase L−/− mice (B6, 10 generations of back-crossing) (40) were bred in the University of Pennsylvania animal facility. Hey1b human ovarian carcinoma cell lines were cultured as described previously (a gift of A. Marks, University of Toronto, Toronto, Canada) (41) and standardized for studies on the OAS–RNase L pathway (42).

Plasmids and Recombinant Viruses. The 3′ terminus of VP3 encoding 143 amino acids was amplified from the plasmid p8Smold (g3(VP3-SA11)) derived from SA11-4F virus strain (GenBank accession no. DQ838641) and cloned into mammalian expression vector pCAAGS, resulting in the pC-VP3. The His residues at positions 46 and 126 were both mutated resulting in pC-VP3H46A, H126R. The ORFs for VP3 and mutant VP3H46A, H126R were cloned into E. coli expression vectors pMAL-c2, pMAL-c5, pMAL-c5e, and pMAL-c5x and expressed in E. coli. The purified His6- or His10-tagged proteins were used for the experiments described in this study.

2′,5′-PDE Activity Assays. Expression and purification of VP3 are described in SI Materials and Methods. The 2′,5′-PDE activities of ns2 and VP3 were determined with purified (2′,5′)-pApA as substrate followed by HPLC to separate the substrate from the products (43) (for details, see SI Materials and Methods).

Quantification of 2-5A by FRET Assays. 2-5A present in cell extracts was measured by activation of recombinant, human RNase L in FRET assays (44).
Briefly, RNase L was incubated with (2'-5')-oligoadenylate synthetase and RNase L during the interferon antiviral response. J Virol 81(12):12720-12729.


