Crystal structure of the mouse hepatitis virus ns2 phosphodiesterase domain that antagonizes RNase L activation

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Prior studies have demonstrated that the mouse hepatitis virus (MHV) A59 strain ns2 protein is a member of the 2H phosphoesterase family and exhibits 2',5'-phosphodiesterase (PDE) activity. During the IFN antiviral response, ns2 cleaves 2',5'-oligoadenylate (2-5A), a key mediator of RNase L activation, thereby subverting the activation of RNase L and evading host innate immunity. However, the mechanism of 2-5A cleavage by ns2 remains unclear. Here, we present the crystal structure of the MHV ns2 PDE domain and demonstrate a PDE fold similar to that of the cellular protein, a kinase anchoring protein 7 central domain (AKAP7 CD) and rotavirus VP3 carboxy-terminal domain. The structure displays a pair of strictly conserved HxT/Sx motifs and forms a deep, positively charged catalytic groove with ß-sheets and an arginine-containing loop. These findings provide insight into the structural basis for 2-5A binding of MHV ns2.

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Innate immunity, which is commonly triggered by dsRNA in eukaryotic cells, plays a crucial role in viral defence (Wang & Carmichael, 2004). The 2',5'-oligoadenylate (2-5A) synthase (OAS)/RNase L system, which is one of the most effective IFN-induced antiviral pathways, utilizes several strategies to inhibit viral infections (Silverman, 2007). Viruses induce the production of type I IFN, resulting in the activation of hundreds of IFN-stimulated genes, including four or more OAS genes (Der et al., 1998; Kristiansen et al., 2011; Schindler & Darnell, 1995; Stark et al., 1998). OASs 1–3 use ATP to synthesize a series of 2-5A molecules consisting of [p3A(2'p5'A)n, n≥2] upon stimulation by viral dsRNA (Hovanessian & Justesen, 2007; Kerr & Brown, 1978). These 2-5A molecules bind to the N-terminal ankyrin repeat and central protein kinase-like domains of RNase L, causing inactive RNase L monomers to form activated dimers with potent RNase activity that preferentially cleave single-stranded viral or cellular RNA after UU and UA repeats (Cole et al., 1997; Dong & Silverman, 1995; Han et al., 2014; Huang et al., 2014).

Both coronavirus and rotavirus families include pathogens that can lead to severe diseases in humans and animals (Desselberger, 2014; Zaki et al., 2012). Prior studies have reported that coronavirus mouse hepatitis virus (MHV) and group A rotavirus (RVA) encode ns2 and VP3 proteins, respectively. Ns2 and VP3 carboxy-terminal domain (CTD) belong to the 2H phosphoesterase superfamily, containing two conserved HxT/Sx motifs (where H represents histidine and x denotes a hydrophobic residue). Both ns2 and VP3 display 2',5'-phosphodiesterase (PDE) activity, which can inhibit the activation of RNase L through degradation of its activator 2-5A, thereby
antagonizing the OAS/RNase L pathway and evading host innate immunity (Zhang et al., 2013; Zhao et al., 2012). AKAP7 is a member of the AKAP family that localizes to nuclei and has a central domain (residues 87-292, AKAP7 CD) that belongs to the 2H phosphoesterase superfamily. AKAP7 exhibits 2',5'-PDE activity that can complement an inactive MHV ns2 gene and degrade 2-5A with kinetics similar to that of MHV ns2 (Gold et al., 2008; Gusho et al., 2014). Although from different origins, these proteins display 2',5'-PDE activity and can prevent the activation of RNase L by cleaving 2-5A.

To further elucidate the mechanism by which MHV ns2 binds and cleaves 2-5A, we resolved the crystal structure of the PDE domain of MHV-A59 ns2. The full-length ns2 protein containing residues 1–261 readily aggregates, possibly due to its cysteine-rich (six cysteines in a 62 aa segment, approximately 10 %) carboxy-terminal region. Thus, to select an ns2 fragment suitable for crystallization, we designed a series of C-terminally truncated constructs of ns2 based on a secondary structure prediction for this protein. An ns2 fragment containing residues 1–199 (ns21–199), including the PDE activity domain (residues 6–186) exhibited improved expression and solubility compared with the full-length protein (Fig. 1a, b and data not shown). To purify the ns21–199 protein, the codon-optimized sequence of truncated MHV-A59 ns21–199 was cloned into pET-42b vector (Novagen) with a 6 x His tag at the C terminus. Expression of ns21–199 protein was induced in Escherichia coli Rosetta BL21(DE3) (at an OD600 of 0.7) with 0.5 mM IPTG. After incubation for 18 h at 18 °C, the cells were collected and homogenized in PBS. After centrifugation at 10 000 g for 10 min, the supernatant was applied to Ni2+ affinity resin (Ni-NTA; GE) and further purified by gel filtration chromatography on Superdex 75 (GE Healthcare). The final protein preparation (at 10 mg ml-1) containing 20 mM Tris/HCl (pH 7.4) and 200 mM NaCl was crystallized using the hanging-drop vapour-diffusion method at 4 °C with a reservoir solution containing 16 % (w/v) polyethylene glycol 3350, 0.1 M succinic acid (pH 7.0) and 20 mM hexamethinebalt(III) chloride. Selenomethionine (SeMet)-labelled protein was overexpressed in Escherichia coli and cleaved by 2-5A. After incubation for 18 h at 18 °C, the cells were collected and homogenized in PBS. After centrifugation at 10 000 g for 10 min, the supernatant was applied to Ni2+ affinity resin (Ni-NTA; GE) and further purified by gel filtration chromatography on Superdex 75 (GE Healthcare). The final protein preparation (at 10 mg ml-1) containing 20 mM Tris/HCl (pH 7.4) and 200 mM NaCl was crystallized using the hanging-drop vapour-diffusion method at 4 °C with a reservoir solution containing 16 % (w/v) polyethylene glycol 3350, 0.1 M succinic acid (pH 7.0) and 20 mM hexamethinebalt(III) chloride. Selenomethionine (SeMet)-labelled protein was overexpressed in M9 minimal salts, and purified in the same way as the unmodified protein. The first crystal appeared 2 days post-seeding and the crystals were harvested for X-ray diffraction analysis 2 weeks later. However, the resolution was not high enough for data collection (3.6 Å), so dehydration was applied according to the protocols described by Heras & Martin (2005). The crystals were soaked in dehydration buffer containing 0.5 M succinic acid (pH 7.0) and 25 % polyethylene glycol 3350 for 1 month. Diffraction data were collected at the Shanghai Synchrotron Radiation Facility (SSRF) and then integrated and scaled using the HKL2000 package (Otwinowski & Minor, 1997). The structure of ns21–199 was determined by single-wavelength anomalous diffraction (SAD) phasing using SeMet-labelled ns21–199 and four SeMet sites in ns21–199 were identified. Model building and refinement were iteratively conducted using PHENIX and COOT (Adams et al., 2002; Emsley & Cowtan, 2004). Data collection and refinement statistics for the ns21–199 crystal structure are provided in Table S1 (available in the online Supplementary Material). The ns21–199 structure was determined to within 3.05 Å. Since the residues of 1–6 and 168–199 were disordered and could not be resolved, we chose the ordered region (residues 7–167) as the final model, defined as ns27–167 (Fig. 1c–e).

To our knowledge, the crystal structure of the PDE domain of ns2 is the first coronavirus 2',5'-PDE structure to be reported. Based on an overview of the structure of ns27–167, we found that this protein features a globular α/β-type architecture consisting of three α-helices and nine β-sheets with approximate dimensions of 48 Å × 46 Å × 16 Å (Fig. 1c, d). The structure is bilobal, with β1/2/3/5/6/7/9 and an arginine (R)-containing loop (R-loop, the loop between x3 and β7) that forms a concave surface where the electrostatic potential is positive and presumed to be the domain in which the negatively charged 2-5A binds and is cleaved (Fig. 1e) (Gold et al., 2008). In accordance with the definition of the 2H phosphoesterase family, the β3 strand harbours an HxSx (where x is a hydrophobic residue) motif, and the β7 strand harbours an HxT motif (Fig. 2a, b). This pair of HxT/Sx motifs play a key role in 2',5'-PDE activity and are conserved in the ns2 homologues of other group 2a betacoronaviruses, including human virus OC43 and toroviruses (Silverman & Weiss, 2014). Prior research has demonstrated that mutations at the catalytic residues H46 and H126 in conserved HxT/Sx motifs of ns2 abolish PDE activity in vitro and severely impact virus replication in vivo (Zhao et al., 2011, 2012).

MHV ns2, VP3-CTD and AKAP7 CD are representative 2H phosphoesterases (Fig. 1a) (Gusho et al., 2014; Zhang et al., 2013; Zhao et al., 2012). Based on the sequence alignment, we identified a number of conserved residues in these 2H phosphoesterases (Fig. 2a). The structures of AKAP7 CD (PDB ID 2VFY) and VP3-CTD (PDB ID 5AF2), which exhibit substantial structural homology, have previously been determined (Brandmann & Jinek, 2015; Ogden et al., 2015). The structural alignment of ns27–167, AKAP7 CD and VP3-CTD indicated that all of these structures form similar positive electrostatic concave regions in the 2',5'-PDE activity domain, which is putatively involved in the binding and cleavage of 2-5A (Fig. 2b, c). In addition, the two conserved HxT/Sx motifs at the H46/S48/H126/T128 residues of ns2 were located in positions similar to those of the H132/T134/H224/T226 residues of AKAP7 CD and the H718/T720/H797/T799 residues of VP3-CTD (Fig. 2d). The arginine that is important for stabilizing 2-5A has previously been reported to be present in the R-loop region and is also strictly conserved in those three proteins (Fig. 2a, d) (Ogden et al., 2015). All of the aforementioned characteristics demonstrate the structural conservation of the PDE catalytic domain of these three 2H phosphoesterases, despite lacking significant sequence homology (Fig. 2a).

Both MHV ns2 and RVA VP3 are viral proteins that display 2',5'-PDE activity, although there are subtle differences
between the ns2\(^{7-167}\) and VP3-CTD structures. The ns2\(^{7-167}\) structure displays \(\alpha\)-helices in the region from S19 to M35 (\(\alpha1\)), which is similar to AKAP7\(^{CD}\), whereas VP3-CTD shows a loop in this region that is much shorter compared with ns2\(^{7-167}\) (Fig. 2b). The regions from residues D79 to D83 (\(\beta4\)) and Y144 to P148 (\(\beta8\)) display two \(\beta\)-sheets that are similar to those of AKAP7\(^{CD}\), whereas the similar regions of VP3-CTD show two loops. Although some differences are seen between ns2\(^{7-167}\) and VP3-CTD, these two proteins show greater structural similarities in their PDE catalytic domains between themselves than compared with AKAP7\(^{CD}\) [AKAP7\(^{CD}\), with a root mean square deviation (RMSD) of 2.41 Å between the 149 Cz atoms. VP3-CTD, with the RMSD of 1.67 Å between the 148 Cz atoms. Analysed by PDBeFold.], having nearly identical \(\beta\) sheet coordinates (\(\beta1/3/6/7\)) (Fig. 2e). Prior studies have demonstrated that the L758 residue plays a key role in VP3-CTD activity (Ogden et al., 2015); ns2\(^{7-167}\) has an L88 in this position, although AKAP7\(^{CD}\) contains a Q residue in this position (Fig. 2a). Two \(\alpha\)-helices at the position of \(\alpha2/\alpha3\) in ns2\(^{7-167}\) are also conserved in these three proteins, although ns2 and VP3 have shorter \(\alpha\)-helices in \(\alpha3\) than does AKAP7\(^{CD}\). The sequence alignment of ns2\(^{1-199}\), VP3-CTD and AKAP7\(^{CD}\) illustrates that ns2\(^{1-199}\) and VP3-CTD share greater sequence homology compared with AKAP7\(^{CD}\) (Fig. 2a). According to these features, we can conclude that ns2\(^{7-167}\) not only displays structural features of AKAP7\(^{CD}\) but also of VP3-CTD, and ns2\(^{7-167}\) shares greater sequence or structural homology with VP3-CTD compared with AKAP7\(^{CD}\).

It has previously been reported that AKAP7\(^{CD}\) can bind 5’AMP (Gold et al., 2008). Ns2 and AKAP7 present similar
Fig. 2. Sequence and structural comparisons of ns2<sup>7–167</sup>, AKAP<sub>7</sub><sup>CD</sup> and VP3-CTD. (a) Structure-based sequence alignment of the PDE domains of ns2<sup>1–199</sup>, AKAP<sub>7</sub><sup>CD</sup> and VP3-CTD. Sequence comparison of rat AKAP7 (residues 76–292), MHV-A59 ns2 (residues 1–199) and rotavirus A VP3 (residues 697–835). The sequence homology of ns2<sup>1–199</sup> compared with AKAP<sub>7</sub><sup>CD</sup> and VP3-CTD was 13% and 22%, respectively (calculated by PDBeFold). Two conserved HxT/Sx motifs...
protein AKAP7 exhibit similar 2'-5'-PDE enzymic activity after the key histidine was mutated. Truncated ns2 (residues 1–199) retained 2',5'-PDE activity, although reduced compared with full-length WT ns2. It is possible that some residues in the carboxy-terminal domain of ns2 may enhance 2',5'-PDE activity.

The structural analysis showed that the key residues in HxT/Sx motifs of ns2 protein were strictly conserved and located at similar coordinates compared with those of AKAP7CD and VP3-CTD. This structure also revealed that the conserved arginine in the R-loop region might play a key role in stabilizing the adenine moiety. These structural features suggest that these proteins bind and degrade 2-5A in a similar way. Based on the structural alignment of ns27–167, AKAP7CD and VP3-CTD, we demonstrate that ns27–167 exhibits features of both AKAP7CD and VP3-CTD, whereas ns22–167 and VP3-CTD show greater sequence and structural homology (Fig. 2a, e). Previously, it has been speculated that MHV and some other viruses acquired ancestral precursors of viral 2',5'-PDE genes from their hosts. Homologous recombination might have occurred between the MHV genome and host RNA, thus endowing viruses with the ability to antagonize innate immunity by degrading 2-5A (Gusho et al., 2014; Luytjes et al., 1988). Here, we provide further evidence for this concept at the structural level.

The family Coronaviridae, a large family of viruses, have the largest genomes among RNA viruses. The precise mechanisms by which these viruses evade innate immunity are complicated and remain to be fully elucidated. Several emerging infectious disease outbreaks in recent years were caused by coronaviruses, including severe acute respiratory syndrome (SARS), porcine epidemic diarrhea virus and Middle East respiratory syndrome (MERS). MHV, MERS and SARS all belong to the betacoronavirus family. To our knowledge, the crystal structure of the truncated PDE domain of MHV ns2 is the first ns2 PDE domain structure of any coronavirus to be solved. By resolving and analysing the structure of ns27–167, we demonstrated that ns27–167 cleaved 2-5A and that the mutant ns21–199 H126R protein lacked 2',5'-PDE activity. Levels of 2',5'-PDE activity were compared among ns21–199, full-length ns2, and AKAP7CD (Fig. 3f). The results demonstrated that full-length ns2 and the AKAP7CD displayed similar 2',5'-PDE activity, whereas they lost catalytic activity after the key histidine was mutated. Truncated ns2 (residues 1–199) retained 2',5'-PDE activity, although reduced compared with full-length WT ns2. It is possible that some residues in the carboxy-terminal domain of ns2 may enhance 2',5'-PDE activity.
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