Structural Insight into Molecular Inhibitory Mechanism of InsP$_6$ on African Swine Fever Virus mRNA-Decapping Enzyme g5Rp

Yan Yang, Changhui Zhang, Xuehui Li, Li Li, Yanjuan Chen, Xin Yang, Yao Zhao, Cheng Chen, Wei Wang, Zhihui Zhong, Cheng Yang, Zhen Huang, Dan Su

ABSTRACT Removal of 5' cap on cellular mRNAs by the African swine fever virus (ASFV) decapping enzyme g5R protein (g5Rp) is beneficial to viral gene expression during the early stages of infection. As the only nucleoside diphosphate-linked moiety X (Nudix) decapping enzyme encoded in the ASFV genome, g5Rp works in both the degradation of cellular mRNA and the hydrolyzation of the diphosphoinositol polyphosphates. Here, we report the structures of dimeric g5Rp and its complex with inositol hexakisphosphate (InsP$_6$). The two g5Rp protomers interact head to head to form a dimer, and the dimeric interface is formed by extensive polar and nonpolar interactions. Each protomer is composed of a unique N-terminal helical domain and a C-terminal classic Nudix domain. As g5Rp is an mRNA-decapping enzyme, we identified key residues, including K$_8$, K$_{94}$, K$_{95}$, K$_{98}$, R$_{221}$, and K$_{243}$ located on the substrate RNA binding interfaces of g5Rp which are important to RNA binding and decapping enzyme activity. Furthermore, the g5Rp-mediated mRNA decapping was inhibited by InsP$_6$. The g5Rp-InsP$_6$ complex structure showed that the InsP$_6$ molecules occupy the same regions that primarily mediate g5Rp-RNA interaction, elucidating the roles of InsP$_6$ in the regulation of the viral decapping activity of g5Rp in mRNA degradation. Collectively, these results provide the structural basis of interaction between RNA and g5Rp and highlight the inhibitory mechanism of InsP$_6$ on mRNA decapping by g5Rp.

IMPORTANCE ASF is a highly contagious hemorrhagic viral disease in domestic pigs which causes high mortality. Currently, there are still no effective vaccines or specific drugs available against this particular virus. The protein g5Rp is the only viral mRNA-decapping enzyme, playing an essential role in the machinery assembly of mRNA regulation and translation initiation. In this study, we solved the crystal structures of g5Rp dimer and complex with InsP$_6$. Structure-based mutagenesis studies revealed critical residues involved in a candidate RNA binding region, which also play pivotal roles in complex with InsP$_6$. Notably, InsP$_6$ can inhibit g5Rp activity by competitively blocking the binding of substrate mRNA to the enzyme. Our structure-function studies provide the basis for potential anti-ASFV inhibitor designs targeting the critical enzyme.

KEYWORDS g5Rp, ASFV, mRNA-decapping enzyme, Nudix hydrolases, InsP$_6$
the sole member of the *Asfarviridae*, a family of African swine fever-like viruses that are relatively independent of the host cell transcriptional machinery for viral replication (3, 4). The ASFV infection of domestic swine can result in various disease forms, ranging from highly lethal to subclinical depending on the contributing viral and host factors (5). Since 2018, ASFV has spread into China and led to a high mortality rate in domestic pigs (6, 7). Currently, there are still no effective vaccines or specific drugs available against this particular virus (8, 9).

During an ASFV infection, protein synthesis in the host cell is inhibited as a result of a massive degradation of host cellular mRNAs in the cytoplasm of infected cells (10, 11). As part of its strategy to inhibit host cellular translation and promote viral protein synthesis instead, the virus targets the mRNAs of the host cell using specific enzymes (12). Hydrolysis of the 5′ cap structure (m7GpppN) on eukaryotic mRNAs, a process known as decapping, is considered to be a crucial and highly regulated step in the degradation of mRNA (13). Some viruses including ASFV and vaccinia virus (VACV) can harbor decapping enzymes for control of viral and cellular gene expression (14). Two poxvirus Nudix hydrolases, D9 and D10, have been confirmed with intrinsic mRNA-decapping activity, although the two decapping enzymes appear to have some differences in substrate recognition (15, 16).

Nudix hydrolases (nucleoside diphosphate-linked moiety X) are widely present in bacteria, archaea, and eukarya, where they belong to a superfamily of hydrolytic enzymes that catalyze the cleavage of nucleoside diphosphates and the decapping of the 5′ cap of mRNAs, the latter of which plays a pivotal role in mRNA metabolism (17, 18). Mammalian cells have about 30 different genes with Nudix motifs, including Dcp2, Nudt16, and NUDT3/DIPP1, which cleaves mRNA caps in mRNA degradation by the 5′-3′ decay pathway *in vivo* (19–21). The mRNA-decapping enzyme g5R protein (g5Rp), which is the only Nudix hydrolase in ASFV, shares sequence similarity to the mRNA-decapping enzymes Dcp2 in *Schizosaccharomyces pombe* and D9 or D10 in VACV (22–24). However, g5Rp and its Nudix homologs D9 and D10 exhibit higher hydrolytic activity toward diphosphoinositol polyphosphates and dinucleotide polyphosphates than toward cap analogs (25, 26). Similar to Dcp2, these Nudix hydrolases cleave the mRNA cap attached to an RNA moiety, predicated that RNA binding is crucial for performing its mRNA-decapping activity (16). Recently, structural study has confirmed that the Nudix protein CFIm25 has a sequence-specific RNA binding capability (27). The requirement of RNA binding for the majority of the Nudix decapping enzymes suggest that the members of the Nudix family also belong to RNA binding proteins.

The viral mRNA-decapping enzyme g5Rp is expressed in the endoplasmic reticulum from the early stage of ASFV infection and accumulates throughout the infection process, playing an essential role in the machinery assembly of mRNA regulation and translation initiation (23). Like other members of the Nudix family, g5Rp has a broader range of nucleotide substrate specificity, including that for a variety of guanine and adenine nucleotides and dinucleotide polyphosphates (25). Generally, g5Rp has two distinct enzymatic activities *in vitro* (*viz.* diphosphoinositol polyphosphate hydrolase activity and mRNA-decapping activity), implying that it plays roles in viral membrane morphogenesis and mRNA regulation during viral infections (28). In light of these biochemical observations, the elucidation of the structure of g5Rp is of fundamental importance for our understanding of the molecular mechanisms through which it degrades cellular RNAs and regulates viral gene expression.

Here, we report the crystal structure of g5Rp and its complex structure with InsP₆. Combined with biochemical experiments, the dimeric form of g5Rp and three RNA binding surfaces on each protomer are critical to substrate RNA binding of g5Rp. The g5Rp-InsP₆ complex structure shows that two of the RNA binding surfaces are occupied by InsP₆, indicating that InsP₆ may play a role in its ability to inhibit g5Rp-RNA binding activity. Meanwhile, we evaluate the inhibitor effect of InsP₆ on the mRNA-decapping enzyme activity of g5Rp. Therefore, we proposed that such inhibition could be caused by the competition of InsP₆ with substrate mRNA for binding to g5Rp. Furthermore, we show in detail how InsP₆ inhibits g5Rp activity by occupying the RNA binding interfaces on g5Rp, thereby...
competitively blocking the binding of substrate mRNA to the enzyme. These results suggest InsP₆ or its structural analogs may be involved in the manipulation of the mRNA-decapping process during viral infections and provide an essential structural basis for the development of ASFV chemotherapies in the future.

RESULTS

Characterization of recombinant ASFV g5Rp. Recombinant wild-type (WT) ASFV g5Rp (residues 1 to 250) was expressed in Escherichia coli with an N-terminal His₆ tag. The purified g5Rp was eluted from a Superdex 200 column (GE Healthcare) with a major elution volume of 15.6 mL, indicating an approximate molecular weight of 32.1 kDa (Fig. 1A).

The fractions were further analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and found to have the expected molecular weight (Fig. 1B). The cross-linking of g5Rp by using chemical reagent EGS was also confirmed (Fig. 1C).

The binding abilities of g5Rp to 12-mer and 26-mer ssRNAs were determined by electrophoretic mobility shift assay (EMSA) (Fig. 1D and 1E). The protein concentration is marked on the figure, and the nucleic acid concentration is 0.25 mM. The decapping activity of g5Rp was also measured (Fig. 1F).

The binding abilities of g5Rp to 12-mer and 26-mer ssRNAs were determined by surface plasmon resonance (SPR) (Fig. 1G and 1H). SPR data were analyzed using a 1:1 binding model, and black lines represent curve fits.

FIG 1 Characterization of the African swine fever virus decapping enzyme g5Rp. (A) The elution profile of g5Rp in a Superdex 200 10/300 column and the molecular weight of the standards and g5Rp are shown in this picture. (B) SDS-PAGE result for g5Rp. M is the protein marker. (C) The cross-linking of g5Rp by using chemical reagent EGS. The different concentrations of EGS with g5Rp in the reaction system are indicated above the gel. (D and E) The binding abilities of g5Rp to 12-mer and 26-mer ssRNAs were determined by EMSA; in the reaction, the protein concentration is marked on the figure, and the nucleic acid concentration is 0.25 μM. (F) The decapping activity of g5Rp. (G and H) The binding abilities of g5Rp to 12-mer and 26-mer ssRNAs were determined by surface plasmon resonance. SPR data were analyzed using a 1:1 binding model, and black lines represent curve fits.
TABLE 1 Kinetic analysis of SPR

| Ligand      | Analyte          | $K_D$ (M) | $k_{ob}$ (1/Ms) | $k_{on}$ (1/s) | $R_{max}$ (RU) | Chi-square (RU²) |
|-------------|------------------|-----------|-----------------|----------------|----------------|------------------|
| 26-mer RNA  | g5Rp-WT          | 4.48E-08  | 1.14e+04        | 5.10e-04       | 38.8           | 2.00             |
|             | G5Rp-ΔC          | 3.81E-08  | 1.61e+04        | 6.12e-04       | 33.5           | 3.92             |
|             | G5Rp-I84A/I116A/ | 5.07E-08  | 1.85e+04        | 9.38e-04       | 63.2           | 10.4             |
|             | L200A/I206A/F206A| 4.70E-08  | 1.47e+04        | 6.92e-04       | 46.1           | 4.04             |
| 12-mer RNA  | g5Rp-WT          | 1.64E-07  | 3.20e+03        | 5.25e-04       | 112.9          | 1.23             |
|             | G5Rp-ΔC          | 1.15E-07  | 5.33e+03        | 6.13e-04       | 77.8           | 4.51             |
|             | G5Rp-I84A/I116A/ | 3.90E-08  | 2.12e+04        | 8.28e-04       | 62.3           | 11.6             |
|             | L200A/I206A/F206A| 2.87E-08  | 2.84e+04        | 8.14e-04       | 46.0           | 5.20             |

electrophoresis (SDS-PAGE), showing a g5Rp band of 29.9 kDa (Fig. 1B). A cross-linking assay confirmed that g5Rp exists as a stable homodimer in solution (Fig. 1C).

We first characterized the nucleic acid binding ability of g5Rp with different lengths of single-stranded RNA (12-mer and 26-mer ssRNA). Electrophoretic mobility shift assay (EMSA) results demonstrated that g5Rp binds ssRNA (0.25 M) of single-stranded RNA (12-mer and 26-mer ssRNA). Electrophoretic mobility shift assay (EMSA) results demonstrated that g5Rp binds ssRNA (0.25 μM) at the lowest concentration of 0.5 μM (Fig. 1D and E). Furthermore, we measured the binding affinity of wild-type (WT) g5Rp for ssRNA by using surface plasmon resonance (SPR) (Fig. 1G and H). The enzyme exhibited a stronger binding affinity to ssRNAs with the following equilibrium dissociation constants: 12-mer $K_D = 164.0$ nM and 26-mer $K_D = 44.8$ nM. The kinetic analysis of the binding experiments is shown in Table 1. These results indicate that g5Rp possesses a higher affinity with long ssRNA. Next, we reevaluated the decapping activity of recombinant g5Rp by incubating the protein with a 32P-cap-labeled RNA substrate in a reaction. The products of the reaction were resolved by polyethyleneimine (PEI)-cellulose thin-layer chromatography (TLC) and detected by autoradiography (23). As shown in Fig. 1F, the recombinant g5Rp in the decapping reaction released 7-methylguanosine cap (m7GDP) product efficiently. In contrast, the 32P-cap-labeled RNA substrate as control remained at the origin of the plate. These results suggest that the recombinant g5Rp possesses efficient mRNA-decaping enzyme activity.

**Overview of the ASFV g5Rp structure.** To investigate structural insights into the catalytic mechanism of g5Rp, we determined its dimeric structure by single-wavelength anomalous diffraction (SAD) phases using selenomethionine (SeMet)-labeled protein. As shown in Fig. 2A, the g5Rp dimer is composed of two protomers that each adopt a "boxing glove" shape with a distinct helical domain and Nudix domain (Fig. 2D). The helical domain (residues 36 to 124) forms a globin-fold-like feature composed of six α-helices (α1 to α6) that connects to the Nudix domain by two hinge linkers (linker I, residues 32 to 35; linker II, residues 119 to 139). The Nudix domain (residues 1 to 35 and 125 to 250) consists of a central curved β-sheet (β1, β2, β3, β4) surrounded by five α-helices (α7 to α11) and several loops, thereby forming a classic α·β·α sandwich structure. Linker II splits the top of the β-sheet to connect α6 and α7 (Fig. 2E). The Nudix motif located in the center of the Nudix domain is highly conserved and comprises the loop-helix-loop architecture that contains the Nudix signature sequence extending from residues 133-GKPKEDESLTCAIREFEEETGI154 in g5Rp (Fig. 2F). The sequence of the g5Rp Nudix motif matches the classic pattern of the Nudix motif in the Nudix hydrolase superfamily, that is, GX3EX,REUXEEXGU, where X is any residue and U is Ile, Leu, or Val (29, 30). Using the Dali server (31), we compared the structure of g5Rp with that of other proteins in the Protein Data Bank (PDB), whereupon 46 structures were found to be likely homologous to the enzyme, with Z-scores in the range of 8 to 20 (data not shown). However, all the listed protein structures shared high architectural similarity only with the Nudix domain located in the C terminus of g5Rp. Therefore, a search on the Dali server
was carried out for the helical domain alone, whereupon no homologous structure with a Z-score above 4 was found, suggesting that the helical domain of g5Rp adopts a novel fold. Compared with the structures of Dcp2 in a number of different conformations, g5Rp shows a unique globin-fold-like domain (Fig. 2B and C). A previous study showed that the helical domain of g5Rp is the major mediator of RNA interaction (28). However, the positively charged surface of the g5Rp structure overlaps both the helical domain and the Nudix domain that may exhibit RNA binding activity (Fig. 3A). We proposed that both positively charged regions could contribute to g5Rp-RNA interaction. To test the hypothesis, we measured the binding of the truncation variants g5Rp\(D\)C (helical domain, residues 36 to 124) and g5Rp\(D\)N (Nudix domain, connecting residues 1 to 35 and 125 to 250 directly) to ssRNAs (12-mer and 26-mer), respectively. Our EMSA results showed that both the helical domain (g5Rp\(D\)C) and Nudix domain (g5Rp\(D\)N) of g5Rp are involved in ssRNA interaction (Fig. 3B). The helical domain exhibited \(K_D\) values of 39.0 and 50.7 nM for the surface-immobilized 12- and 26-mer ssRNAs measured by SPR, respectively (Fig. 3C and D). In contrast, the \(K_D\) values of wild-type g5Rp for the ssRNAs (12-mer \(K_D = 164.0\) nM, 26-mer \(K_D = 44.8\) nM) are slightly lower than that of the helical domain with ssRNAs, indicating that both full-length and truncated g5Rp associated with RNA with high affinity.

The dimeric structure of g5Rp. When recombinant g5Rp was subjected to gel filtration chromatography to estimate molecular weight, it migrated as a single population of
FIG 3 Surface charge distribution of g5Rp and the biochemical information of the African swine fever virus decapping enzyme truncated g5Rp. (A) Surface charge distribution of g5Rp. The range of electrostatic surface potential is shown from $-74.712 \text{kT/e}$ in red to $+74.712 \text{kT/e}$ in blue. The three highly positively charged areas are marked by the black dashed lines. (B) The binding abilities of truncated g5Rp (the final concentrations of WT and $\Delta C$ are 2.00 $\mu\text{M}$ while that of $\Delta N$ is 50 $\mu\text{M}$) to 26- and 12-mer ssRNAs (final concentrations, 0.25 $\mu\text{M}$) were determined by EMSA. (C and D) The binding abilities of truncated g5Rp $\Delta C$ to 26- and 12-mer ssRNAs were determined by SPR. SPR data were analyzed using a 1:1 binding model, and black lines represent curve fits.
molecules at a molecular mass consistent with a monomer. However, g5Rp dimerization was consistent with cross-linking experiments (Fig. 1A and C). To obtain more information about the interfaces and likely biological assemblies of g5Rp, we analyzed its structure using the PDB-related interactive tool Proteins, Interfaces, Structures and Assemblies (PDBePISA) (32). The results suggested that g5Rp forms a stable symmetric dimer in crystal packing. The dimer was composed of two protomers (A and B) positioned in an orientation similar to two boxing gloves stuck together back to back (Fig. 2A). The dimer interfaces were stabilized mainly by hydrophobic interactions. Furthermore, a network of hydrogen bonds conferred additional stability on the interface. One interface was composed of four \( \alpha \)-helices (\( \alpha_3 \) and \( \alpha_4 \) from each A and B protomer) from the N terminus of each protomer. Residues Ile64, Asn65, Arg67, Leu68, Leu69, Lys71, Thr72, Arg77, Tyr80, His81, and Ile84 located in helices \( \alpha_3 \) and \( \alpha_4 \) played pivotal roles in stabilizing the dimeric form of the protein. The other dimer interface was located at the linker II portion of the C terminus and one solvent contact surface of the Nudix domain. Residues Ile116, Asn117, Ala119, Lys120, Gly121, Ser122, Gly123, and Thr124 located on linker II and residues Asn195, Met196, Leu198, Ser199, Leu200, Gln201, Ile206, Ser210, Gln215, Glu218, and Ala219 at the Nudix domain formed hydrogen bonds in the dimer interface, with further contributions from a hydrophobic patch composed of Ile206, Ile209, Phe222, and Ile223 (Fig. 4A). To determine the multimeric state of g5Rp in solution and to examine which of its termini is critical for its dimerization, we measured the multimerization of two g5Rp truncation variants (g5Rp\( \Delta N \) and g5Rp\( \Delta C \)) using cross-
linking experiments. The results showed that the wild type, N terminus, and C terminus of g5Rp all formed a dimeric conformation in solution (Fig. 4B and C). The g5Rp mutant I84A/I116A/L200A/I206A/F222A that prepared to dissociate the dimeric form of g5Rp was successful in altering a monomeric state, even the dimeric total buried area of 3,050 Å². Wild-type g5Rp and mutants were subjected to gel filtration chromatography, showing that the mutant I84A/I116A/L200A/I206A/F222A has a larger retention volume, corresponding to a lower molecular weight (Fig. 5A). The protein cross-linking experiment showed that the dimeric conformation was significantly reduced in solution for the mutant (Fig. 5B). The ssRNA binding ability of the monomeric mutant has been measured by SPR and EMSA. The monomeric mutant with analyte concentrations was passed over immobilized ssRNA. The resultant sensorgrams are shown in Fig. 5C and D, and kinetic analysis is shown in Table 1. EMSA data are shown in Fig. 5E. Both measurements produced consistent results indicating that the g5Rp mutant I84A/I116A/L200A/I206A/F222A partially impaired the RNA binding ability. Therefore, we proposed that the dimeric g5Rp is preferred for efficient RNA binding. Meanwhile, mRNA-decapping assays showed that the decapping activity of mutant I84A/I116A/L200A/I206A/F222A dropped greatly (Fig. 5F).

Structure of the g5Rp-InsP₆ complex. g5Rp was originally characterized through its ability to dephosphorylate 5′-P-InsP₆ (InsP₇) to produce InsP₆ (25). We were surprised to find a tight interaction between InsP₆ and g5Rp by microscale thermophoresis (MST) (Fig. 6A). To gain insight into the molecular basis of the interaction, we determined the crystal structure of the g5Rp-InsP₆ complex and found that each asymmetric unit contained one g5Rp-InsP₆ complex in space group P₄₁2₂. PDBePISA analysis revealed that an identical dimeric conformation exists in the g5Rp-InsP₆ complex structure (Fig. 6B). Two InsP₆ molecules were situated on the edge of the β1 strand of each g5Rp protomer through interactions with residues Gln⁶, Lys⁸, and Lys¹³³ (Fig. 6C). Due to the 2-fold symmetry in the crystal, each of the g5Rp protomers shared two InsP₆ molecules (InsP₆ and InsP₆▫) with its neighboring g5Rp protomer in the crystal lattice. Besides the InsP₆ binding on the β₁ strand located on the edge of the Nudix domain, an extra InsP₆ molecule from the neighboring molecule also interacted with g5Rp through residues Lys⁹⁴ and Lys⁹⁸ on the α₅ helix in the helical domain (Fig. 6C). In this way, each InsP₆ molecule is surrounded by four Lys residues in complex structure. The solvent-accessible surface of the InsP₆ binding region of g5Rp was calculated according to the electrostatic potential. It was apparent that both InsP₆ molecules were situated on the highly positively charged area located in the protein cleft between the helical domain and Nudix domain of g5Rp (Fig. 7A). The local conformational changes of g5Rp in the complex structure induced by its interaction with InsP₆ are illustrated in Fig. 7B. In the complex structure, the β₁, β₃, and β₅ strands located in the Nudix domain had moved closer to the helical domain, and α₂ was pushed away from the InsP₆ binding sites. These changes rendered the g5Rp conformation more stable in the complex.

To assess their relative importance in g5Rp-InsP₆ interaction, amino acid residues involved in InsP₆ binding pockets were replaced by single point mutation (Q⁶A, K⁸A, K⁹⁴A, K⁹⁸A, K¹³³A). Each mutant was tested for its binding affinity for InsP₆ by MST. Figure 6D showed that mutants resulted in a notable decrease in g5Rp-InsP₆ interaction, and furthermore, the quintuple mutant Q⁶A/K⁸A/K⁹⁴A/K⁹⁸A/K¹³³A totally lost the binding ability with InsP₆. Taken together, the mutagenesis work indicates that positively charged residues Lys⁸, Lys⁹⁴, Lys⁹⁸, and Lys¹³³ form a cluster to mediate the g5Rp-InsP₆ interaction.

Analysis of residues involved in g5Rp-RNA interfaces. To characterize RNA binding surface on g5Rp, we analyzed the electrostatic potential at the surface of g5Rp, which indicated that three highly positively charged areas (areas I to III) may play roles in g5Rp-RNA interaction (Fig. 3A). Area I is located on the helical domain, containing residues Lys⁹⁴, Lys⁹⁵, Lys⁹₈, Arg¹⁰₀, and Lys¹₀₁ located on helix α₅. Area II is composed of residues Lys⁸, Lys¹₃₁, Lys¹₃₃, Lys¹₃₅, Arg¹₄₆, Lys¹₇₅, Lys¹₇₉, and His¹₸₀ mostly located on the β₁ and β₃ strands, which are close to the Nudix motif; area III is located at the very end of the C terminus of g5Rp, comprising residues Arg²²¹, Lys²²₅,
FIG 5 The biochemical information of the African swine fever virus decapping enzyme monomeric g5Rp. (A) The elution profile of g5Rp-WT and g5Rp-I84A/I116A/L200A/I206A/F222A in a Superdex 200 10/300 column; the molecular weight of the standards and g5Rp is shown in this picture. (B) The cross-linking of g5Rp-I84A/I116A/L200A/I206A/F222A by EGS. The different concentrations of EGS with mutants in the reaction system are indicated above the gel. (C and D) The binding abilities of monomeric g5Rp (I84A/I116A/L200A/I206A/F222A) to 26- and 12-mer ssRNAs were determined by SPR. SPR data were analyzed using a 1:1 binding model, and black lines represent curve fits. (E) The binding abilities of monomeric g5Rp (the final concentrations of WT and I84A/I116A/L200A/I206A/F222A are 2.00 μM) to 26- and 12-mer ssRNAs (final concentration, 0.25 μM) were determined by EMSA. The white star represents the complex precipitation in the gel. (F) The decapping activities of g5Rp-I84A/I116A/L200A/I206A/F222A.
Arg226, Lys243, and Lys247 on helices $\alpha_{10}$ and $\alpha_{11}$ (Fig. 8A). To identify the mRNA binding surfaces on g5Rp further, the residues mentioned above located in three positively charged areas of g5Rp were mutated, respectively. The EMSA pattern showed that some mutants reduce the RNA binding affinity of g5Rp. Specifically, residues Lys8,
Lys94, Lys95, Lys98, Lys131, Lys33, Lys175, Arg221, and Lys243 were critical for single-stranded RNA binding, albeit with different efficiencies (Fig. 8B and C), implying that the g5Rp-RNA interaction interfaces are mainly located at areas I, II, and III. These results also agree with our hypothesis that residues Lys8, Lys94, Lys98, and Lys133 of g5Rp are involved in both RNA and InsP6 interaction.

We further explored whether these key residues were responsible for cap cleavage in a manner dependent on the RNA moiety interaction. Mutant proteins including Q6E/K8E, K94E, K95E, K98E, K175E, R221D, and K243E were expressed and purified. Consistent with our previous data, incubation of the 32P-cap-labeled RNA substrate with wild-type g5Rp resulted in cap cleavage, as observed by m7GDP release. When equivalent amounts of the mutants of g5Rp were included in the decapping reaction, the amount of m7GDP released was reduced variously in each lane. Mutant K95E decreases the decapping activity almost 50% (Fig. 8D and E), indicating that these residues of g5Rp play a pivotal role in mRNA decapping by interacting with substrate mRNA.

Residues Gly132, Lys133, and Glu147 in the Nudix motif impact the decapping activity. The Nudix motif of hydrolases contains crucial residues involved in catalytic activity. However, the residues in the catalytic pocket of g5Rp are still elusive from the viewpoint of structure. To elucidate the function of the key residues in g5Rp, three substrate binding structures from the Nudix superfamily were selected to identify homologous domains with high similarity at the potential catalytic pockets (Fig. 9A to C), as shown in Table 2, viz., Ap4A hydrolase (Aquifer aeolicus, PDB accession no. 3I7V) (33), Nudix hydrolase DR1025 (Deinococcus radiodurans, PDB accession no. 1SZ3), and MTH1 (Mus musculus, PDB accession no. 5MZE) (34, 35) (all belonging to the Nudix superfamily). Superposition of the C terminus of g5Rp with that of MTH1, Ap4A hydrolase, and Nudix hydrolase DR1025 resulted in Cα backbone mean square deviation values of 0.50, 3.08, and 5.6 Å, respectively, despite the low sequence identities among these proteins (Fig. 9D). Therefore, the potential substrate binding site of g5Rp was proposed on the basis of the superpositions of these substrate binding protein structures of the Nudix superfamily. Residues Gly132, Lys133, and Glu147 located on the Nudix motif of g5Rp may be responsible for cap cleavage.

To investigate the potential roles of these key residues located on the Nudix motif in the decapping activity, we replaced g5Rp residues G132, K133, and E147 from the Nudix motif (Fig. 9D and Fig. 10A) with Ala, Glu, and Gln, respectively. As expected, the replacement of the residue K133 with glutamate resulted in a 30% decrease in the decapping activity. And the replacement of the residues G132 and E147 by alanine and
glutamine, respectively, inactivated the decapping function of g5Rp completely (Fig. 10B and C). No m7GDP was observed when the two mutants of g5Rp were included in the decapping reaction, validating that the decapping activity was dependent on these two key residues located in the Nudix hydrolase motif. Interestingly, EMSA results showed that mutant K133E reduces g5Rp’s binding affinity to RNA, which suggests that the loop region of the Nudix motif takes part in substrate mRNA binding (Fig. 10D and E).

InsP6 inhibits the decapping activity by disrupting g5Rp-mRNA interaction.

The finding that residues located on mRNA binding regions of g5Rp are also playing pivotal roles in g5Rp-InsP₆ interaction suggests that InsP₆ may inhibit the g5Rp decapping activity through preventing g5Rp from binding to its mRNA substrate (Fig. 11A). This prediction was confirmed by decapping and EMSAs using recombinant g5Rp, InsP₆, and RNA substrates in vitro. Increasing amounts of InsP₆ were added to the decapping reaction, and the results showed a decrease in m7GDP production, indicating that InsP₆ inhibits the decapping activity by disrupting the g5Rp-mRNA interaction.
reactions to analyze its effect on RNA decapping by g5Rp. As shown in Fig. 11B, the addition of InsP6 significantly affected g5Rp cleavage, suggesting that InsP6 can inhibit the decapping activity of g5Rp in vitro. To investigate if this inhibitory mechanism of InsP6 on g5Rp is due to inositol phosphate competitively inhibiting mRNA binding to the g5Rp, we further measured the competition of InsP6 with nucleic acids for the binding to g5Rp by using EMSA. As expected, the amount of free single-stranded nucleic acids increased with an increasing concentration of InsP6, demonstrating that InsP6 interrupts the g5Rp-mRNA interaction through directly binding to g5Rp (Fig. 11C and D). In addition, all residues involved in InsP6 interaction in g5Rp were mutated into alanine at the same time. The quintuple mutant (Q6A/K8A/K94A/K98A/K133A) of g5Rp lost most of its ability to bind with both InsP6 and RNA (Fig. 6D and see Fig. 13A). These mutations also significantly affected the catalytic ability of g5Rp in vitro (see Fig. 13C and D), suggesting that InsP6 inhibits the mRNA-decapping activity of g5Rp through competing for the substrate mRNA binding surface in g5Rp.

Transient expression of g5Rp decreases levels of mRNA substrates in 293T cells. The above data provide strong in vitro evidence for g5Rp-mRNA interaction being a critical step for the decapping enzyme process. To determine whether changes in g5Rp-mRNA interaction were directly related to the stability of cellular mRNAs in vivo, representative cellular mRNA (eIF4E, eIF4EA, and TP53) levels were tested by

**FIG 9** Superposition and sequence alignment of the African swine fever virus decapping enzyme g5Rp with its homologs. (A to C) Superposition of g5Rp with Ap4A hydrolase in complex with ATP (Aquifex aeolicus V5S, PDB accession no. 317V), Nudix hydrolase DR1025 in complex with phosphoaminophosphonic acid-guanylate ester (Deinococcus radiodurans, PDB accession no. 1SZ3), and MTH1 in complex with 8-oxo-dGTP (Mus musculus, PDB accession no. 5MZE). Ap4A hydrolase is shown in slate, Nudix hydrolase DR1025 is shown in marine, and MTH1 is shown in white. ATP, 8-oxo-dGTP, and GNP (GNP substrate analogue, GMPNP (GNP)) are shown as stick models. (D) Sequence alignment of g5Rp, Ap4A hydrolase, Nudix hydrolase DR1025, and MTH1. The conserved Nudix motif is marked by the black box. The conserved amino acids that bind to the substrates (ATP, 8-oxo-dGTP, and GNP) are marked by red triangles.
quantitative real-time PCR (RT-qPCR) in cells. In 293T cells, the Flag-tagged g5Rp and the g5Rp mutants (K8E, K94E, K95E, K98E, G132A, K133E, E147Q, K175E, R221D, and K243E) were overexpressed, respectively. As shown in Fig. 12A, the g5Rp-WT and mutant proteins were detected by Western blotting. The mRNA levels of target genes (eIF4E, eIF4EA, and TP53) were decreased in 293T cells overexpressing g5Rp-WT. There were no obvious changes in mRNA levels in the catalytic destructive mutants Q132A and E147Q. The overexpression in cells of truncated version g5Rp-DN and mutants K95E and R221D, mutants which significantly lost the RNA binding ability in vitro, had no effect on the mRNA levels of target genes in 293T cells. Mutants K8E and K133E, which had reduced RNA binding in vitro, had various degrees of increase compared

| Parameter or statistic       | g5Rp  | g5Rp-InsP<sub>6</sub> |
|-----------------------------|-------|-----------------------|
| **Data collection**         |       |                       |
| Resolution (Å)              | 2.50  | 2.25                  |
| Space group                 | P2<sub>1</sub>·2<sub>1</sub>·2<sub>1</sub> | P4<sub>2</sub>·2<sub>2</sub> |
| Unit-cell parameters (Å, °) | a = 56.1, b = 105.1, c = 49.5, α = β = γ = 90 | a = 48.4, b = 48.4, c = 220.1, α = β = γ = 90 |
| Resolution (Å)<sup>a</sup>  | 50.00–2.5 (2.54–2.5) | 50.00–2.25 (2.29–2.25) |
| R<sub>merge</sub><sup>b</sup> (%) | 12.7 (30.5) | 6.7 (53.0) |
| R<sub>free</sub><sup>c</sup> (%)   | 4.1 (15.7) | 1.7 (14.7) |
| Avg I/σ(I)                  | 20 (2.1) | 77.5 (12.4) |
| Multiplicity                 | 9.1 (3.4) | 15 (12.2) |
| No. of observed reflections  | 9,721 (248) | 13,490 (668) |
| No. of unique reflections   | 9,678 (506) | 13,350 (1,285) |
| Compleness (%)              | 91.7 (48.80) | 99.37 (98.39) |
| Matthews coefficient (Å<sup>2</sup> Da<sup>−1</sup>) | 2.65 | 2.35 |
| Solvent content (%)         | 53.68 | 47.61 |
| CC<sub>1/2</sub>            | 0.97 (0.92) | 1.00 (0.98) |
| Molecules per asymmetric unit | 1 | 1 |
| **Refinement**              |       |                       |
| Resolution (Å)              | 50.00–2.50 | 50.00–2.25 |
| R<sub>work</sub>/R<sub>free</sub> | 0.2037/0.2744 | 0.1724/0.2307 |
| **MolProbity**              |       |                       |
| Ramachandran favored (%)    | 92.59 | 96.73 |
| Ramachandran allowed (%)    | 7.00  | 3.27 |
| Ramachandran outliers (%)   | 0.41  | 0.00 |
| Rotamer outliers (%)        | 2.20  | 0.00 |
| Clashscore                  | 23.08 | 5.02 |
| Overall score               | 6.1   | 4.5 |
| Avg B factor (Å<sup>2</sup>) | 64.27 | 48.25 |
| Protein                     | 64.32 | 46.61 |
| Water                       | 44.94 | 46.12 |
| Ligand                      | 0     | 148.4 |
| No. of atoms                |       |                       |
| Protein                     | 2,026 | 2,111 |
| Water                       | 28    | 106 |
| Wilson B value              | 43.01 | 37.45 |
| **RMSDs**                   |       |                       |
| Bond lengths (Å)            | 0.010 | 0.008 |
| Bond angles (°)             | 1.186 | 0.925 |

<sup>a</sup>Numbers in the parentheses are for the highest-resolution shell.

<sup>b</sup>R<sub>merge</sub> is approximated by multiplying the R<sub>merge</sub> value by the factor [1/(N – 1)]<sup>1/2</sup>, where N is the overall redundancy of the data set.

<sup>c</sup>R<sub>free</sub> is approximated by multiplying the R<sub>merge</sub> value by the factor [1/(N – 1)]<sup>1/2</sup>, where N is the overall redundancy of the data set.

<sup>d</sup>RMSDs, root mean square deviations.
with the mRNA levels of the g5Rp-WT group. However, the changes in mRNA levels of target genes observed in mutants K94E, K98E, K175E, and K243E did not have statistical differences from those in g5Rp-WT (Fig. 12B to D). Taken together, these results suggest that key residues K8, K95, K133, and R221, playing pivotal roles in g5Rp-RNA interaction, are also important to the g5Rp-related cellular RNA degradation in vivo.
DISCUSSION

Given that an ASFV outbreak in China would potentially devastate the world’s largest pork producer, significant efforts have been made to determine the structures and functions of essential viral proteins that may be used as targets for new anti-ASFV drugs. Several structures of ASFV-encoded enzymes and associated proteins that are involved in viral transcription and replication have been reported, including AP endonuclease (36), the histone-like protein pA104R (37), pS273R protease (38), DNA ligase (39), and dUTPase (40, 41). However, the structures and functions of some critical ASFV proteins remain elusive, including those of g5Rp, a decapping enzyme that is crucial for viral infection (23). Our structures of g5Rp alone and in complex with InsP₆ provide the molecular basis for g5Rp substrate recognition and reveal that inositol phosphate was involved in the regulation of cellular mRNA degradation through direct interaction with the ASFV decapping enzyme g5Rp. Three potential RNA binding regions are identified, including a novel folding domain located on the helical domain of g5Rp and the Nudix motif on its C terminus. More importantly, identification of the major nucleic acid binding surfaces as well as the binding pocket of InsP₆ on g5Rp provides important structural information and a novel strategy for future anti-ASFV drug design.

To explore the nucleic acid binding properties of g5Rp, we conducted a series of nucleic acid binding experiments. Results indicated that an intact dimeric interface is efficient for g5Rp-RNA interaction. Meanwhile, the helical domain and Nudix domain of g5Rp are both involved in ssRNA interaction. Our EMSA and SPR measurements show that the helical domain of g5Rp can bind with ssRNA with equally high affinity as the full-length protein. Six α-helices form a globin-fold-like helical domain, which is different from the traditional RNA binding domain that prefers to adopt the alpha/beta topologies (42–45). According to the g5Rp structure, the surface electrostatic potential characteristics of the N terminus present a highly positively charged area on helix α5.

FIG 11 The influence of InsP₆ on mRNA binding and decapping activity of g5Rp. (A) The surface charge of g5Rp in complex with InsP₆. (B) The influence of InsP₆ on decapping activity of g5Rp. (C and D) The influence of InsP₆ on binding abilities of g5Rp with 12-mer and 26-mer ssRNAs was determined by EMSA, respectively. The different concentrations of InsP₆ with g5Rp in the reaction system are indicated above the gel; the concentrations of protein and nucleic acid were 2.00 μM and 0.25 μM, respectively.
FIG 12 The influence of g5Rp and mutants on mRNA substrate level in 293T cells. (A) The expression of various g5Rp mutants in 293T cells was analyzed by Western blotting. (B) The mRNA levels of eIF4E in 293T cells when a series of g5Rp mutants was overexpressed. (C) The mRNA level of eIF4A in 293T cells when a series of g5Rp mutants was overexpressed. (D) The mRNA level of TP53 in 293T cells when a series of g5Rp mutants was overexpressed (mean ± standard deviation, n ≥ 3; *, P < 0.05; **, P < 0.01, unpaired t test).
The single point mutations of positively charged residues in the N terminus significantly reduced the nucleic acid binding activity of g5Rp with ssRNA (Fig. 8B and C). Furthermore, there are two positive areas located on the C terminus of g5Rp, including the Nudix motif, participating in the substrate RNA interaction. We mutated the two positively charged regions (K8A/K131A/K133A and R221A/K225A/R226A/K243A/R247A) located in the Nudix domain; the EMSA data showed that the nucleic acid binding ability of these two mutants was significantly reduced (Fig. 13B), and the Fig. 13E and F data showed that the substantial decline in capacity of K8A/K131A/K133A removed the m7Gppp RNA cap. These results predicted that the Nudix motif of g5Rp possesses substrate selectivity at the step of mRNA binding.

Previously, studies revealed that the Nudix motif (residues 132 to 154) is an essential component of the α-β-α sandwich in the catalytic center of g5Rp. Several of the conserved catalytic amino acids and glutamate residues (E147, E149, E150, and E151) located on the α-helix of the Nudix motif of g5Rp have been found to be important for the activity of Nudix hydrolases (23, 28). However, the function of the loop region within the Nudix motif is exclusive, leading us to predicate that the loop region may...

FIG 13 The decapping activity and RNA binding abilities of g5Rp mutants. (A) The comparison in binding abilities of g5Rp and Q6K/K8A/K94A/K98A/K133A (final concentration, 2.00 μM) with 26-mer and 12-mer ssRNAs (final concentration, 0.25 μM). (B) The binding abilities of K8A/K131A/K133A and R221A/K225A/R226A/K243A/R247A (final concentration, 2.00 μM) with 12-mer and 26-mer ssRNAs (final concentration, 0.25 μM). (C and E) The decapping activities of g5Rp mutants (Q6K/K8A/K94A/K98A/K133A, K8A/K131A/K133A, and R221A/K225A/R226A/K243A/R247A). (D and F) The semiquantitative of m7GDP by GraphPad Prism 8 (mean ± standard deviation, n ≥ 3; *, P < 0.05; **, P < 0.01, unpaired t test).
contribute to binding with mRNA. Therefore, we mutated several residues in this loop region, including the mutations K133E and G132A, and examined the effects on the protein’s interaction with single-stranded nucleic acids. It is interesting to find that substitutes for the conserved residues K133 and G132 are highly sensitive to g5Rp-RNA interaction. Compared with glutamate residues located on the α-helix of the Nudix motif of g5Rp involved in mRNA cap structure interaction, residues K133 and G132 are important for binding with the RNA moiety on the substrate. In this way, we provided a demonstration that the short loop in the Nudix motif is required for g5Rp-RNA interaction. Including the Nudix motif, three positively charged patches on the g5Rp surface were mapped as mRNA binding regions. Furthermore, we also investigated the importance of the residues involved in mRNA interaction in g5Rp-mediated decapping. The g5Rp mutants K8E, K94E, K95E, K98E, K175E, and R221D showed a strong reduction in decapping activity, demonstrating the importance of the mRNA binding residues for catalysis. The dimeric form of g5Rp is also important to the decapping activity. We constructed mutant g5Rp-I84A/I116A/L200A/I206A/F222A in which the dimerization surface was destroyed. mRNA-decapping assays showed that the decapping activity of mutant g5Rp-I84A/I116A/L200A/I206A/F222A decreased drastically (Fig. 5F). It will be of profound interest to elucidate the structural basis of the enzymatic activity of g5Rp by solving the structure of g5Rp in complex with mRNA in the future.

The other important finding in this study was that InsP₆ is able to inhibit the decapping activity of g5Rp. As we know, InsP₆ is widespread in cells with diverse biological functions (46–49). Here, we found that InsP₆ competes with mRNA substrates for binding to g5Rp and inhibits its decapping activity. A previous study reported that g5Rp is a diphosphoinositol polyphosphate phosphohydrolase (DIPP), which preferentially removes the 5-β-phosphate from InsP₇ to produce InsP₆ with unclear functional significance (25). Later, Parrish and colleagues identified that g5Rp can hydrolyze the mRNA cap when tethered to an RNA moiety in vitro (23). Our results show that InsP₆ as the product of g5Rp playing the role of DIPP can directly inhibit the mRNA-decapping activity of g5Rp. To illustrate the structural basis of the inhibitory mechanism of InsP₆ for the decapping activity of g5Rp, we solved the structure of the complex of g5Rp with InsP₆ and also the enzyme-product complex in the Nudix superfamily. To our surprise, InsP₆ is located on the mRNA binding region instead of in the catalytic center of the g5Rp. Furthermore, we superposed the catalytic domain of g5Rp-InsP₆ complex with the structures of human DIPP1 in complex with the substrate InsP₇ (50, 51). The visualized result showed that the substrate InsP₇ is located in the catalytic center of DIPP1, unlike InsP₆, which sits on the edge of the catalytic domain of g5Rp (Fig. 14A). Therefore, the structure of the g5Rp-InsP₆ complex may represent an intermediate in the release of the product of the enzymatic reaction (52). We also noticed that InsP₆ decreased the temperature value (B factor) around the binding sites compared with B factor in the same regions of the g5Rp wild-type structure, suggesting that the flexible loop closed to the catalytic center is locked in place by InsP₆ (Fig. 14B). InsP₆ itself was refined with a correspondingly high B factor that exceeded the average B factor of the protein in complex. Considering that the g5Rp-InsP₆ interaction has a dissociation constant (Kd) in the 22.5 μM range, the ligand achieves only a reasonable occupancy of 70% (53). To avoid an instance of overenthusiastic interpretation of ligand density, we tested the InsP₆ binding site by using single point mutations. Residues involved in the InsP₆ binding surface of g5Rp replaced by alanine (Q6A/K8A/K94A/K98A/K133A) reduced its InsP₆ binding capacity and RNA interaction, indicating the destructive InsP₆ binding site has the capability to abolish the substrate RNA binding ability of g5Rp (Fig. 13A, C, and D).

Our study raises the possibility that g5Rp hydrolyzes InsP₇ to upregulate the level of InsP₆, which is a key regulator of g5Rp-mediated mRNA decapping during ASFV infection in vivo (54). Very recently, Sahu and colleagues reported that InsP₇ regulates the NUDT3-mediated mRNA decapping and also observed the phenomenon that InsP₆ inhibits mRNA decapping by NUDT3 (54). There are emerging signs that the functions
of InsP₆ are associated with mRNA transportation and degradation in ASFV-infected cells. Further studies on the function of InsP₆ and the regulation mechanism in the inositol-based cell signaling family during viral infection are required.

MATERIALS AND METHODS

Cell culture. The human 293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (HyClone) supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C under a humidified 5% CO₂ atmosphere (Thermo).

Plasmid construction, protein expression, and purification. The gene encoding ASFV g5Rp (D250R) was synthesized and subcloned into pSMART-1 and pcDNA3.1, respectively. The amino acid sequence of g5Rp comes from UniProt (https://www.uniprot.org/), with accession no. P32092. The point mutants and truncation variants of g5Rp (viz., Q6E/K8E, K94E, K95E, K98E, R100D, K101E, K131E/K133E, R146G/E147Q, K175E/K179E/H180D, R221D, K225E, K243E/R247D, Q6A/K8A/K94A/K98A/K133A, K8A/K131A/K133A/K135A, R221A/K225A/R226A/K243A/R247A, I84A/I116A/L200A/I206A/F222A, g5Rp-ΔC [helical domain, residues 36 to 124], and g5Rp-ΔN [Nudix domain, connecting residues 1 to 35 and 125 to 250 directly]) were generated using the Fast Mutagenesis V2 kit (Vazyme Biotech, China). The primers used in this study are listed in Table 3. The recombinant plasmids were confirmed by sequencing (Sangon Biotech, China) before being introduced into E. coli BL21(DE3) (Invitrogen, USA) or human 293T cells. The bacterial cells were cultured in Luria broth medium at 35°C until the optical density at 600 nm reached 0.6 to 0.8. Protein expression was then induced by the addition of isopropyl-β-D-1-thiogalactopyranoside for 16 h at 16°C. The g5Rp molecules were purified by Ni-nitrilotriacetic acid (NTA) (Qiagen, Germany) affinity chromatography, followed by heparin affinity chromatography (GE Healthcare, USA). The peak fractions containing the target proteins were pooled, concentrated to 1 mL, and finally loaded onto a Superdex 75 column (GE Healthcare, USA) for further purification and characterization. Selenomethionine-labeled g5Rp (SeMet-g5Rp) was then prepared using a previously described protocol (55). The purity of all proteins was above 95% on the SDS-PAGE gel.

Protein crystallization and optimization. The prepared SeMet-g5Rp was concentrated to 12 mg/mL for the crystallization trials. The crystals were grown using the hanging-drop vapor diffusion method at 16°C in a reservoir solution containing 0.1 M sodium citrate tribasic dihydrate (pH 5.8), 0.54 M magnesium formate dihydrate, and 10% (vol/vol) 1,2-butanediol as an additive reagent. The g5Rp-InsP₆ complexes were prepared by mixing g5Rp with InsP₆ at a stoichiometric ratio of 1:3. Then, using the hanging-drop vapor diffusion method, crystals of the complexes were grown from 1 M imidazole (pH 7.0) at 16°C. All crystals were transferred into solutions containing 20% (vol/vol) glycerol prior to being frozen and stored in liquid nitrogen.

Data collection, processing, and structure determination. The single-wavelength anomalous dispersion (SAD) data were collected using synchrotron radiation of an 0.98-Å wavelength under cryogenic conditions (100 K) at the BL18U1 beamline, Shanghai Synchrotron Radiation Facility. All diffraction data sets including g5Rp-WT and the complex with InsP₆ were indexed, integrated, and scaled by using the HKL-2000 package (56). The selenium atoms in the asymmetric unit of SeMet-g5Rp were located and
refined, and the SAD data phases were calculated and substantially improved through solvent flattening with the PHENIX program (57). A model was built manually into the modified experimental electron density using the model-building tool Coot (58) and then further refined in PHENIX. The model geometry was verified using the program MolProbity (59). Molecular replacement was used to solve the structure of the g5Rp-InsP6 complex, using Phaser in the CCP4 program suite with an initial search model of SeMet-g5Rp (60). Structural figures were drawn using PyMOL (DeLano Scientific). The data collection and refinement statistics are shown in Table 1.

### Table 3: Primer sequences for generation of the g5Rp mutants

| Primer name | Sense sequence (5’ → 3’) | Orientation |
|-------------|--------------------------|-------------|
| R146G/E147Q-F | ATCTGACCTGCAATTTCCGAAATTGGA | Forward |
| R146G/E147Q-R | GCGCAAATTGTGCAATTTCCGAAATTGGA | Reverse |
| Q6E/K8E-F | GAGCCTGAAACCCAGAATTCGCTGATATCCCAGGCTCCG | Forward |
| Q6E/K8E-R | CGCTGGACCTGCAATTTCCGAAATTGGA | Reverse |
| K131E/K133E-F | GAATTTCCGGAAGTGAACCGAAAGAAAGATGAAA | Forward |
| K131E/K133E-R | CACCTTCCGAAATTTCCCGCAACAGGCGGTTCCACT | Reverse |
| K175E/K180E-F | GTCGCTCCGAAATTTCCCGCAACAGGCGGTTCCACT | Forward |
| K175E/K180E-R | TTCTCGCTCAGATTTCCCGCAACAGGCGGTTCCACT | Reverse |
| K94E-F | CTTTGAACCTGTATCATGAAGAAAGATGAAAAGAAGGGGTTCCACT | Forward |
| K94E-R | CTTTGAACCTGTATCATGAAGAAAGATGAAAAGAAGGGGTTCCACT | Reverse |
| R100D-F | ATATGAACTGTATCATGAAGAAAGATGAAAAGAAGGGGTTCCACT | Forward |
| R100D-R | CTTTGAACCTGTATCATGAAGAAAGATGAAAAGAAGGGGTTCCACT | Reverse |

Structural and Functional Insights into g5Rp Journal of Virology May 2022 Volume 96 Issue 10 10.1128/jvi.01905-21
Surface pluron resonance analysis. The SPR analyses were carried out using the Biacore 8K system with a streptavidin-coated (SA) chip (catalog no. BR-1005-30; GE Healthcare) at 16°C. To reduce effects attributed to mass transport, low levels of biotin-labeled ssRNA (5'-GCUUUGAUUGUGCAUCAUGAGG-3' and 5'-GCUUUGAUUUUG-3') ligands (given in relative units [RU]) were immobilized on the SA chip. Given the apparent variation in RU of immobilized ligands used in different binding studies, this RU was determined by adding glycine at a concentration of 0.15 M. The reaction products were separated by surface pluron resonance spectroscopy. In brief, g5Rp was first labeled using the Monolith NT protein labeling kit RED-NHS (fluorescent dye NT-647-NHS) (NanoTemper Technologies), and the labeled protein was then diluted to 20 μM with buffer containing 50 mM HEPES, 300 mM NaCl, and 0.05% (vol/vol) Tween 20 (pH 7.0). Then, a series of concentrations of InSP6, diluted in a buffer composed of 50 mM HEPES, 300 mM NaCl, and 0.05% (vol/vol) Tween 20 (pH 7.0) was added. The mixtures were loaded into capillaries and measured at ambient temperature using 20% LED (light-emitting diode) and medium MST power in a Monolith NT.115 system (NanoTemper Technologies). The data were analyzed using NanoTemper analysis software (v.1.2.101).

Microscale thermophoresis. The binding between g5Rp and InSP6 was measured by microscale thermophoresis. In brief, g5Rp was first labeled using the Monolith NT protein labeling kit RED-NHS (fluorescent dye NT-647-NHS) (NanoTemper Technologies), and the labeled protein was then diluted to 20 μM with buffer containing 50 mM HEPES, 300 mM NaCl, and 0.05% (vol/vol) Tween 20 (pH 7.0). Then, a series of concentrations of InSP6, diluted in a buffer composed of 50 mM HEPES, 300 mM NaCl, and 0.05% (vol/vol) Tween 20 (pH 7.0) was added. The mixtures were loaded into capillaries and measured at ambient temperature using 20% LED (light-emitting diode) and medium MST power in a Monolith NT.115 system (NanoTemper Technologies). The data were analyzed using NanoTemper analysis software (v.1.2.101).

Electrophoretic mobility shift assay. EMSAs were performed to determine the nucleic acid affinity of the wild-type and truncated variants of g5Rp. The single-stranded nucleic acids used were 6-carboxyfluorescein-labeled ssRNA (5'-GCUUUGAUUUGCAUCAUGAGG-3' and 6-carboxyfluorescein-labeled ssRNA (5'-GCUUUGAUUUUG-3') (Sheng Gong, Shanghai, China). Initially, 0.25 μM ssRNA was incubated on ice for 30 min with different concentrations of wild type in a buffer composed of 20 mM HEPES, 60 mM KCl, 0.5 mM EDTA, 0.1% Triton X-100, 4 mM dithiothreitol (DTT), 2 mM MgCl2, and 5% (vol/vol) glycerol (pH 7.9); the experimental concentration of mutant g5Rp was 2 μM. To determine the effect of InSP6 on nucleic acid binding ability of g5Rp, we also used the EMSA to test the nucleic acid binding ability of the enzyme mixed with different concentrations of InSP6 (0.03 mM, 0.06 mM, 0.12 mM, 0.25 mM, 0.5 mM, 1 mM, 1.50 mM, and 2.00 mM). All samples were incubated on ice for 30 min and then electrophoresed on 4.0% native PAGE gels for 45 min at a voltage of 100 V. The results were determined with a Bio-Rad ChemiDoc MP imaging system (Bio-Rad, USA).

Capping of the mRNA body. For decapping assays with g5Rp, uncapped RNA was transcribed using the T7 RNA transcription kit (Promega) with the linear DNA (5'-CATATATGCGCTGAAAATAATGATTACTACAACAACGAGATTGTGTGTTAAAAATACCA-3') as a template. The RNAs were capped in a 50-μL reaction system typically containing 0.4 nM RNA, 12 μM 3,000 Ci/μmol [α-32P]GTP, 60 mM MgCl2, 6 mM KCl, 1 mM DTT, and 75 U vaccinia capping enzyme (NEB) at 37°C for 2 h. Cap-labeled RNAs were then separated from free [α-32P]GTP nucleotide by chromatography through a G-50 column (Sigma).

mRNA-decapping assays. All decapping experiments were carried out in a buffer containing 50 mM Tris-HCl (pH 7.0), 1 mM DTT, and 2 mM MnCl2. Generally, 100 ng of wild type or g5Rp mutants was used in each 5-μL reaction mixture. To determine the correlation between InSP6 and the decapping ability of g5Rp, we also tested the decapping ability of the enzyme mixed with different concentrations of InSP6 (0.03 mM, 0.06 mM, 0.12 mM, 0.25 mM, 0.5 mM, 1 mM, 2 mM, and 4 mM). The decapping reaction was carried out at 37°C for 60 min and then stopped with 25 mM EDTA. The products of the reaction were separated by PEI-cellulose thin-layer chromatography developed in 0.45 M (NH4)2SO4 and detected by autoradiography.

Cross-linking assay. A cross-linking assay was carried out by incubating 1 mg/mL of wild-type g5Rp or each g5Rp truncation variant and mutant 184A/I116A/L200A/I206A/F222A in a buffer containing 20 mM HEPES, 200 mM NaCl, 1 mM dithiothreitol, and 10% (vol/vol) glycerol (pH 7.5) and different concentrations of ethylene glycol bis(succinimidyl succinate) (EGS) for 15 min at 4°C; EGS was dissolved in 100% dimethyl sulfoxide (DMSO), and its reservoir concentration was 25 mM. The reaction was terminated by adding glacial acetic acid to a final concentration of 0.15 M. The reaction products were separated by 12% SDS-PAGE and detected by Coomassie brilliant blue staining.

Western blotting and analysis. Two hundred thousand cells per well were seeded in a 6-well plate (Nest) and cultured for 24 h. Then, plasmids (2 μg/well) were transfected into cells using Lipofectamine 3000 (Invitrogen). After 48 h, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Biohit) containing protease inhibitor cocktail (MedChemExpress) and quantified by the bicinchoninic acid (BCA) kit (Sangon). About 20 μg protein was loaded in 12% SDS-PAGE gels and transferred to a polyvinylidene difluoride (PVDF) (0.22 μm) membrane (Millipore). Membranes were incubated with primary antibodies including anti-β-actin (1:10,000 dilution; HuaBio) and anti-Flag (1:1,000 dilution; CST) at 4°C overnight. After the membranes were washed three times for 10 min with Tris-buffered saline with Tween 20 (TBST) and incubated with secondary antibodies for 2 h at room temperature, they were washed three times again in TBST. Then, the blots were detected with enhanced chemiluminescence reagents (Millipore) using MiniChemi (Sagecreation, China).

RNA extraction and quantitative real-time PCR. Briefly, 100,000 cells were seeded in a 12-well plate (Nest) and cultured for 24 h before transfection with plasmid (1 μg/well). Total RNA was extracted using RNAiso reagent (TaKaRa), and 500 ng of total RNA was reverse transcribed using the PrimeScript
RT reagent kit (TaKaRa). Subsequently, real-time PCR amplification was performed with SYBR Premix ExTaq (TaKaRa) on a QuantStudio 3 system (Applied Biosystem). The touchdown 3 settings were as follows: 95°C for 30 s and then 40 cycles of 95°C for 5 s and 60°C for 34 s. Relative mRNA levels were determined using the threshold cycle (2^–ΔΔCt) (ΔΔCt = ΔCt [test] – ΔCt [calibrator]) method.

Data availability. The structure factors and atomic coordinates of apo-g5R and g5R-InsP6 have been deposited in the Protein Data Bank under the PDB ID codes 7DNT and 7DNU, respectively.

ACKNOWLEDGMENTS

We thank the staff at the State Key Laboratory of Biotherapy, Sichuan University, who assisted with our research work during the period of the COVID-19 epidemic. The X-ray diffraction experiments were carried out at the Shanghai Synchrotron Radiation Facility (SSRF) at BL18U1. We also thank the beamline staff for their technical help during the data collection. We appreciate helpful advice from and discussion with Xiaoyu Xue from Texas State University.

This work was supported by the National Key Research and Development Program of China (2017YFA0505903), the National Natural Science Foundation of China (31370735 and 31670737 to D.S.), the Science and Technology Department of the Tianjin Foundation (19FYCSN00470), the Special Research Fund on COVID-19 of Sichuan Province (2020YSF0010), and the urgent project on COVID-19 of West China Hospital, Sichuan University (HX-2019-nCoV-044).

We declare that we have no conflicts of interest surrounding the contents of this article.

REFERENCES

1. Wardle RC, de M Andrade C, Black DN, de Castro Portugal FL, Enjuanes L, Wilkinson PJ, Moura Nunes JF, Thomson G. 1983. African swine fever virus. Brief review. Arch Virol 76:73–90. https://doi.org/10.1007/BF01311692.
2. Wang N, Zhao D, Wang J, Zhang Y, Wang M, Gao Y, Li F, Wang J, Bu Z, Rao Z, Wang X. 2019. Architecture of African swine fever virus and implications for viral assembly. Science 366:640–644. https://doi.org/10.1126/science.aaz1439.
3. Cackett G, Matelska D, Sykora M, Portugal R, Malecki M, Bähler J, Dixon L, Werner F. 2020. The African swine fever virus transcriptome. J Virol 94: e00119-20. https://doi.org/10.1128/JVI.00119-20.
4. Rodriguez JM, Salas ML. 2013. African swine fever virus transcription. Virus Res 173:15–28. https://doi.org/10.1016/j.virusres.2012.09.014.
5. Netherton CL, Connell S, Beniston S, Sefton-BT, Dixon LK. 2019. The genetics of life and death: virus-host interactions underpinning resistance to African swine fever, a viral hemorrhagic disease. Front Genet 10:402. https://doi.org/10.3389/fgene.2019.00402.
6. Zhao D, Liu R, Zhang X, Li F, Wang J, Zhang J, Liu X, Wang L, Zhang J, Wu X, Guan Y, Chen W, Wang X, He X, Bu Z. 2019. Replication and virulence in pigs of the first African swine fever virus isolated in China. Emerg Microbes Infect 8:438–447. https://doi.org/10.1002/emm2.15900128.
7. Dixon LK, Stahl K, Jori F, Vial L, Pfeiffer DJ. 2020. African swine fever epidemiology and control. Annu Rev Anim Biosci 8:221–246. https://doi.org/10.1146/annurev-animal-021419-083741.
8. Teklu T, Sun Y, Abid M, Luo Y, Qiu HJ. 2020. Current status and evolving approaches to African swine fever vaccine development. Transbound Emerg Dis 67:529–542. https://doi.org/10.1111/tbed.13364.
9. Arabyan E, Kotsynyan A, Hakobyan A, Kiledjian M. 2017. New insights into decapping enzyme of vaccinia virus contributes to decay of cellular and viral mRNAs activity, providing a mechanism for control of host and viral gene expression. Proc Natl Acad Sci U S A 104:2139–2144. https://doi.org/10.1073/pnas.0611685104.
10. Gao A, Vasilyev N, Kaushik A, Duan W, Serganov A. 2020. Principles of RNA and nucleotide discrimination by the RNA processing enzyme RppH. Nucleic Acids Res 48:3776–3788. https://doi.org/10.1093/nar/gkaa024.
11. Song MG, Bail S, Kiledjian M. 2013. Multiple Nudix family proteins possess mRNA decapping activity. RNA 19:390–399. https://doi.org/10.1261/rna.037309.112.
12. Wang Z, Jiao X, Curr-Schmid A, Kiledjian M. 2002. HNdcp2 protein is a mammalian mRNA decapping enzyme. Proc Natl Acad Sci U S A 99:12663–12668. https://doi.org/10.1073/pnas.192445599.
13. Grudzien-Nogalska E, Kiledjian M. 2016. New insights into decapping enzymes and selective mRNA decay. Wiley Interdiscip Rev RNA 8:e1379.
14. Gao A, Vasilyev N, Kaushik A, Duan W, Serganov A. 2020. Principles of RNA and nucleotide discrimination by the RNA processing enzyme RppH. Nucleic Acids Res 48:3776–3788. https://doi.org/10.1093/nar/gkaa024.
15. Song MG, Bail S, Kiledjian M. 2013. Multiple Nudix family proteins possess mRNA decapping activity. RNA 19:390–399. https://doi.org/10.1261/rna.037309.112.
16. Gao A, Vasilyev N, Kaushik A, Duan W, Serganov A. 2020. Principles of RNA and nucleotide discrimination by the RNA processing enzyme RppH. Nucleic Acids Res 48:3776–3788. https://doi.org/10.1093/nar/gkaa024.
17. Song MG, Bail S, Kiledjian M. 2013. Multiple Nudix family proteins possess mRNA decapping activity. RNA 19:390–399. https://doi.org/10.1261/rna.037309.112.
18. Gao A, Vasilyev N, Kaushik A, Duan W, Serganov A. 2020. Principles of RNA and nucleotide discrimination by the RNA processing enzyme RppH. Nucleic Acids Res 48:3776–3788. https://doi.org/10.1093/nar/gkaa024.
19. Song MG, Bail S, Kiledjian M. 2013. Multiple Nudix family proteins possess mRNA decapping activity. RNA 19:390–399. https://doi.org/10.1261/rna.037309.112.
20. Gao A, Vasilyev N, Kaushik A, Duan W, Serganov A. 2020. Principles of RNA and nucleotide discrimination by the RNA processing enzyme RppH. Nucleic Acids Res 48:3776–3788. https://doi.org/10.1093/nar/gkaa024.
21. Gao A, Vasilyev N, Kaushik A, Duan W, Serganov A. 2020. Principles of RNA and nucleotide discrimination by the RNA processing enzyme RppH. Nucleic Acids Res 48:3776–3788. https://doi.org/10.1093/nar/gkaa024.
22. Gao A, Vasilyev N, Kaushik A, Duan W, Serganov A. 2020. Principles of RNA and nucleotide discrimination by the RNA processing enzyme RppH. Nucleic Acids Res 48:3776–3788. https://doi.org/10.1093/nar/gkaa024.
decapping enzyme. Nucleic Acids Res 38:7599–7610. https://doi.org/10.1093/nar/gkq638.

27. Yang Q, GilmarTM GM, Double S. 2010. Structural basis of UGUA recognition by the Nudix protein CFlm(25) and implications for a regulatory role in mRNA 3’ processing. Proc Natl Acad Sci U S A 107:10062–10067. https://doi.org/10.1073/pnas.1000848107.

28. Quintas A, Pérez-Núñez D, Sánchez EG, Nogal ML, Hentze MW, Castelló A, Revilla Y. 2017. Characterization of the African swine fever virus decapping/Zn2+/cytochrome d3 infection. J Virol 91:e00990-17. https://doi.org/10.1128/JVI.00990-17.

29. McLennan AG. 2006. The Nudix hydrolase superfamily. Cell Mol Life Sci 63:123–143. https://doi.org/10.1007/s00018-005-5386-7.

30. Bessman MJ. 2019. A cystic activity in the Nudix hydrolase superfamily. Protein Sci 28:1494–1500. https://doi.org/10.1002/pro.3666.

31. Holm L. 2020. DALI and the persistence of protein shape. Protein Sci 29:127–143. https://doi.org/10.1002/pro.3749.

32. Krissinel E, Henrick K. 2007. Inference of macromolecular assemblies from crystalline state. J Mol Biol 372:774–797. https://doi.org/10.1016/j.jmb.2007.05.022.

33. Jeyakanthan J, Kanaujia SP, Nishida Y, Nakagawa N, Praveen S, Shinkai A, Revilla Y, Joris I. 2020. Structure and RNA binding of the third KH domain of poly(C)-binding protein L7Ae has a 3D structure very similar to that of its eukaryal counterpart while having a broader RNA-binding specificity. J Mol Biol 342:757–773. https://doi.org/10.1016/j.jmb.2004.07.046.

34. Menotti FS, Oliver KG, Putney JW, Jr, Shears SB. 1993. Inositol phosphates and cell signaling: new views of InsP3 and InsP6. Trends Biochem Sci 18:53–56. https://doi.org/10.1016/0968-0004(93)90053-P.

35. Morrison RS, Shi E, Kan M, Yamaguchi F, McKewen W, Rudnicka-Nawrot M, Palczewski K. 1994. Inositolhexakisphosphate (InsP6): an antagonist of fibroblast growth factor receptor binding and activity. In Vitro Cell Dev Biol Anim 30A:783–789.

36. Van der Kaay J, Wesseling J, Van Haaster PJ. 1995. Nucleus-associated phosphorylation of Ins1,4,5P3 to Ins6P in Dicytostelium. Biochem J 312:911–917. https://doi.org/10.1042/bj3120911.

37. Verbitsky J, Majerus PW. 2005. Increased levels of inositol hexakisphosphate (InsP6) protect HEK293 cells from tumor necrosis factor alpha- and Fas-induced apoptosis. J Biol Chem 280:29263–29268. https://doi.org/10.1074/jbc.M503366200.

38. Döllins DE, Bai W, Fridy PC, Otto JC, Neubauer JL, Gattis SG, Mehta K, York JD. 2017. Vif is a kinase and pyrophosphatase switch that regulates inositol diphosphate signaling. Proc Natl Acad Sci U S A 114:9336–9346. https://doi.org/10.1073/pnas.1712927117.

39. Thourell A-G, Persson C, Graslund S, Hammarström M, Busam RD, Hallberg BM. 2009. Crystal structure of human diphosphoinositol phosphate 1. Proteins 77:242–246. https://doi.org/10.1002/prot.22489.

40. De Simone A, Aprile FA, Dhulesia A, Dobson CM, Vendruscolo M. 2015. Structure of a low-population intermediate state in the release of an enzyme product. Elife 4:e02777. https://doi.org/10.7554/eLife.02777.

41. Yang Q, Gilmartin GM, Doublie S. 2010. Structural basis of UGUA recognition by the Nudix protein CFlm(25) and implications for a regulatory role in mRNA 3’ processing. Proc Natl Acad Sci U S A 107:10062–10067. https://doi.org/10.1073/pnas.1000848107.

42. Suryadi J, Tran EJ, Maxwell ES, Brown BA, II. 2005. The crystal structure of the Methanocaldococcus jannaschii multifunctional L7Ae RNA-binding protein reveals an induced-fit interaction with the box C/D RNAs. Biochemistry 44:9657–9672. https://doi.org/10.1021/bi050568q.

43. Sidiqui M, Wilce JA, Vivian JP, Porter CJ, Barker A, Leadman PJ, Wilce MJ. 2005. Structure and RNA binding of the third KH domain of poly(C)-binding protein 1. Nucleic Acids Res 33:1213–1221. https://doi.org/10.1093/nar/gkz265.

44. Kumarevel T, Fujimoto Z, Karthe P, Oda M, Mizuno H, Kumar PKR. 2004. Crystal structure of activated HuP; an RNA binding protein that regulates transcription of the hou operon in Bacillus subtilis. Structure 12:1269–1280. https://doi.org/10.1016/j.str.2004.05.005.