LETTER

Crystal structure of the coxsackievirus A16 RNA-dependent RNA polymerase elongation complex reveals novel features in motif A dynamics

Dear Editor,

Coxsackievirus A16 (CV A16) and enterovirus 71 (EV71) are currently the two primary causative agents of hand-foot-and-mouth disease (HFMD) (Solomon et al., 2010; Mao et al., 2014), threatening health of children worldwide. They both belong to the *Enterovirus* genus of the *Picornaviridae* family, and have single-stranded positive-sense RNA genomes of about 7.5 kilobases (kb) in length. As with other positive-strand RNA viruses, the genome replication process of CV A16 is carried out by a membrane-associated replication complex with the virally encoded RNA-dependent RNA polymerase (RdRP) as the essential catalytic enzyme. Viral RdRPs are a family of nucleic acid polymerases and have unique features in their catalytic mechanisms. They adopt a human right hand global architecture shared by all single-subunit polymerases with palm, fingers, and thumb domains surrounding the active site, but have their finger tips interact with the thumb to make a unique encircled structure. This encirclement restricts the global motion of the RdRP fingers domain to some extent, and they indeed have evolved a palm-domain based conformational change to close the active site during each nucleotide addition cycle (NAC) (Gong and Peersen, 2010), in drastic contrast to A-family polymerases such as *Thermus aquaticus* DNA polymerase and bacteriophage T7 RNA polymerase that close the active site through large-scale rotational movement of their fingers domain (Li et al., 1998; Yin and Steitz, 2004). Although RdRP crystal structures were solved for several enteroviruses, structure of a CV A16 RdRP or its catalytic complex has not been reported. In this work, by solving the first crystal structure of the CV A16 RdRP (also known as 3D^{pol} in picornaviruses) in the form of catalytic elongation complex (EC), we identified a rarely observed conformation in palm domain motif A. The finding enriches the current understandings of this important structural element in controlling viral RdRP catalysis, and further emphasizes the uniqueness of viral RdRPs when comparing to other classes of nucleic acid polymerases.

Using an approach that has been shown to be efficient in picornavirus RdRP EC assembly and crystallization (Gong et al., 2013), we obtained single crystals of CV A16 RdRP EC following protocols described previously (Gong and Peersen, 2010; Shu and Gong, 2016). To prepare protein samples for EC assembly, the CV A16 3D^{pol} gene within the DNA clone of CA16/GD09/24 (GenBank entry: KC117317.1) was cloned into a pET26b-Ub vector (Gohara et al., 1999). Protein expression, purification, and storage were carried out as described previously for EV71 RdRP (Shu and Gong, 2016). The preparation of the RNA construct r2 (Figure 1A), and the EC assembly, purification, and storage were performed following the protocols in the EV71 study (Shu and Gong, 2016). The CV A16 RdRP EC crystals were grown by sitting-drop vapor diffusion at 16 °C using a 7.0 mg/mL EC sample and a drop volume of 0.6–0.8 µL. Crystals grew in 3–7 days with a precipitant solution containing 1.7 mol/L NH₄H₂PO₄, 0.08 mol/L tris (pH 8.5), and 10% (vol./vol.) glycerol. The EC crystals were harvested and transferred to the precipitant solution and were flash-cooled and stored in liquid nitrogen. X-ray diffraction data collection was done at the Shanghai Synchrotron Radiation Facility (SSRF) beamline BL17U1 (temperature=100 Kelvin, wavelength=0.9792 Å). Reflections were integrated, merged, and scaled using D*trek (Pflugrath, 1999) and the structure was solved by molecular replacement using the EC corresponding to chains A–D in the poliovirus (PV) polymerase EC structure (PDB entry: 3OL6) as the search model (Gong and Peersen, 2010). The routine for manual model building, structure refinement, and generation of the 3,500-K composite simulated-annealing (SA) omit electron density map was carried out as previously described in the EV71 work (Shu and Gong, 2016).

The CV A16 RdRP EC structure was determined at 2.5 Å resolution in space group P3₁ (Table 1) and there are two ECs observed in the crystallographic asymmetric unit. The two protein chains adopt highly consistent conformation with a root-mean-square deviation (RMSD) value of 0.2 Å for all superimposable α-carbon atoms. The global protein conformation is also consistent with

that of the recently reported EV71 RdRP EC (PDB entry: 5F8G) (Shu and Gong, 2016), with an RMSD value of 0.8 Å for all superimposable α -carbon atoms (100% coverage for both CV A16 RdRP chains). For each EC, at least 11 base pairs (bp) of RNA duplex were resolved upstream of the active site, while RNA downstream of position + 4 are largely disordered (Figure 1A). According to current understandings of the viral RdRP NAC, both ECs represent the NTP unbound state 1 in the cycle.

Surprisingly, the palm domain motif A in the CV A16 RdRP EC adopts an apparent different backbone conformation when compared to the EV71 and CV B3 RdRP state 1 “open” catalytic complexes (Figure 1B, EV71_S1

and CV B3_S1) (Gong et al., 2013; Shu and Gong, 2016). The CV A16 RdRP motif A backbone conformation around residue D233 is more consistent with that of the state 4 “closed” catalytic complex of the EV71 RdRP (Figure 1B, EV71_S4). Note that this unusual motif A backbone conformation is probably not due to motif A sequence difference between RdRPs of CV A16 and related viruses, as the motif A residues 231–242 of the CV A16, CV B3, and EV71 RdRP EC structures are identical. Previously this unusual motif A backbone conformation was only observed under two situations. The first situation is when a viral RdRP is in the form of a catalytic complex and in the presence of a correct NTP substrate. The correct substrate induces the conformational switch of the motif A backbone to close the active site for catalysis, and such “closed” catalytic complexes have been captured either as the pre-catalysis state 3 complex (3BSO) (Zamyatkin et al., 2008) or as the post-catalysis state 4 complex (PDB entries: 3OL7, 3OL8, 5F8J, and 5F8M) (Gong and Peersen, 2010; Shu and Gong, 2016). During NTP binding and catalysis, this conformational switch is likely initiated by the interactions between the NTP ribose hydroxyls, a conserved motif A aspartic acid (D238 in CV A16), and a conserved motif B serine (S289 in CV A16), establishing a defined hydrogen bonding network involving side chain rotamer changes of these conserved residues (Figure 1B). Following this rearrangement around the ribose hydroxyls, the other conserved aspartic acid (D233 in CV A16) moves toward the NTP substrate and a conserved motif C aspartic acid (D329 in CV A16) (Shu and Gong, 2016). These two aspartic acid residues (D233 and D329 in CV A16) are universally conserved for nucleic acid polymerases and both coordinate the catalytic divalent metal ions (naturally being magnesium ions, a and b in Figure 1B) during the phosphoryl transfer reaction. As documented in the PV study describing the RdRP active site closure (Gong and Peersen, 2010), the “open” to “closed” conformational switch allows the formation of two additional β -type hydrogen bonds between motif C and motif A accompanied by a side chain rotamer change of residue F232, thus forming a complete 3-strand β -sheet (Figure 1C).

When the nucleic acid and NTP substrate are absent, the “apo” form of viral RdRP usually adopts the “open” conformation (Figure 1, D and E) with the placement of motif A backbone consistent with previously reported state 1 catalytic complexes. However, a couple of exceptions occurred when an ion or a ligand was bound to motif A. This is the second known situation in which the “closed” motif A backbone conformation is observed, in parallel to the situation of the “closed” catalytic complex. In the rabbit hemorrhagic disease virus (RHDV) RdRP crystal structures, a lutetium ion (Lu^{3+}) or two manganese ions (Mn^{2+}) bound to the motif A aspartic

Table 1. X-ray diffraction data collection and structure refinement statistics

PDB	5Y6Z
Data collection^a	
Space group	P3 ₁
Cell dimensions	
a, b, c(Å)	93.7, 93.7, 167.6
α , β , γ (°)	90, 90, 120
Resolution (Å) ^b	46.0–2.50 (2.59–2.50)
R_{merge}	0.132 (0.372)
$\ \sigma \ $	4.7 (2.1)
Completeness (%)	99.5 (99.9)
Redundancy	2.9 (3.0)
Structure refinement	
Resolution (Å)	46.0–2.50
No. unique reflections	56, 671
$R_{\text{work}}/R_{\text{free}}$ (%)	21.8/25.9
No. atoms	
Protein/RNA	7247/1204
Ligand/Ion/Water	36/21/377
B-factors (Å ²)	
Protein	44.5/55.9
Ligand/Ion/Water	48.6/56.2/44.5
R.m.s. deviations	
Bond lengths (Å)	0.008
Bond angles (°)	0.985
Ramachandran stat. ^d	93.6/6.0/0.2/0.2

Note: ^a One crystal was used for data collection. ^b Values in parentheses are for the highest-resolution shell. ^c 5% of data are taken for the R_{free} set. ^d Values are in percentage and are for most favored, additionally allowed, generously allowed, and disallowed regions in Ramachandran plots, respectively.

acid residue equivalent to CV A16 RdRP D233 induced a similar backbone conformational switch of motif A to reach the “closed” state (Figure 1F, left) (Ng et al., 2002). In the encephalomyocarditis virus (EMCV) RdRP crystal structure, a glutamine was bound to the C-terminal half of motif A (corresponding to the region around D238 in CV A16) and caused the N-terminal half of motif A (corresponding to the region around D233 in CV A16) to adopt the “closed” conformation (Figure 1F, right) (Vives-Adrian et al., 2014). Moreover, partially “closed” motif A backbone conformations were observed in apo CV B3 RdRP bearing mutation in motif D known to undergo coordinated movement with motif A during active site closure (McDonald et al., 2016). In the CV A16 EC structure, the “closed” conformation of motif A seems not to be caused by similar factors that are capable of inducing conformational changes. The only ion or ligand found near motif A is a magnesium ion (Mg^{2+}) bound to motif C residue D330 (the second D in the picornavirus RdRP YGDD signature sequence) in

one of RdRP chains, and it does not have direct interaction with motif A.

All structural evidences to date, including the current work, emphasize the dynamic features of viral RdRP motif A. Note that motif A is structurally conserved in all single subunit polymerases, but its backbone conformational diversity related to the open and closed states has been found only in viral RdRPs. Other classes of polymerases have the “motif A-motif C” 3-strand β -sheet completely formed even in the absence of nucleic acids and NTP substrate, and their catalysis-related dynamic features are instead found within the fingers domain (Li et al., 1998; Yin and Steitz, 2004; Gong and Peersen, 2010). Therefore, the dynamic nature of the viral RdRP motif A is a key to understanding how RdRP catalysis is unique compared to other nucleic acid polymerases. We have previously defined the formation of two interaction clusters during RdRP active site closure: the ribose cluster involves the C-terminal half of motif A (corresponding to the region around D238 in CV A16);

Figure 1. An unusual motif A conformation observed in the CV A16 RdRP EC crystal structure. (A) The schematic of the RNA construct in the EC and the global views of EC. Top: Cyan and green letters represent template and product nucleotides, respectively, that are resolved in both ECs in the asymmetric unit. The template nucleotide at position + 1 is in orange. The boxed “GAGA” tetra-nucleotide was added to the product chain in the EC assembly reaction. Bottom: Cartoon representations of EC structure viewing into the front channel (i.e. the template-product RNA duplex exiting channel) (left) or viewing into the NTP entry channel (right). Part of the fingers domain is rendered semi-transparent in the NTP view for clarity of the active site. Coloring scheme: palm in grey, fingers in pink, thumb in light blue, the picornavirus RdRP signature sequence YGDD within motif C in magenta, template RNA in cyan with + 1 nucleotide in orange, and product RNA in green. (B) Stereo-view comparison of the CV A16 RdRP EC (black) with the EV71 RdRP state 1 and state 4 complexes (top) and the CV B3 state 1 complex (bottom). (Protein is colored grey. Nucleic acids coloring scheme is the same as in panel A). Key interactions established in the “closed” state 4 complex are indicated by dashed lines. Structures are shown as stereo-pair images. The two magnesium ions (a and b) are shown as magenta spheres. (C) Stereo-pair images of CV A16 and CV B3 EC structures show the completion of the 3-strand β -sheet upon the conformational switch from the “open” state (grey, CV B3) to the “closed” state (black, CV A16). Dashed lines indicated hydrogen bonding interactions. (D) A comparison of CV A16 EC structure (protein in grey, nucleic acids coloring scheme same as in panel A) with RdRPs from other representative enteroviruses in their apo form (green). RV: rhinovirus. Composite SA omit electron density map (contoured at 1.4 σ) of the CV A16 RdRP motif A is overlaid onto the structural model in the magnified view on the right. (E) A comparison of the RdRP in CV A16 EC (grey) and RdRPs from several representative positive-strand RNA viruses (yellow). FMDV: foot-and-mouth disease virus; NV: norovirus; HCV: hepatitis C virus. (F) A comparison of the RdRP in CV A16 EC (grey), the RHDV RdRP with a Lu^{3+} (in sphere) bound (in wheat, left panel), the RHDV RdRP with two Mn^{2+} (in spheres) bound (in brown, left panel), and the EMCV RdRP with a glutamine (Gln, in ball-and-stick) bound (in olive, right panel). For panels B–F, RdRP motifs are indicated by white capital letters with grey oval background and all amino acid residue labels are according to CV A16 RdRP. All PDB entries and related references in this figure are listed in [Supplemental Table 1](#). Structure superimpositions were carried out using the maximum likelihood superpositioning program THESEUS (Theobald and Wuttke, 2006) with the entire RdRP molecule (panels B, C, and D) or only the RdRP motif C (panels E and F) included in the alignment.

the metal cluster includes the N-terminal half of motif A (corresponding to the region around D233 in CV A16) ([Figure 1B](#)) (Shu and Gong, 2016). It is conceivable that the formation of the ribose cluster interactions precedes that of the metal cluster interactions upon NTP binding and active site closure, as the ribose cluster formation provides an ideal pre-chemistry fidelity checkpoint. In the time-resolved NTP soaking experiments in the EV71 RdRP EC structural study, the formation of the ribose cluster was indeed observed prior to the formation of the metal cluster (Shu and Gong, 2016). The current work provides the evidence of the “closure” of viral RdRP motif A N-terminal half, independent of conformational changes in its C-terminal half and conformational-change-inducible NTP substrates, ions, and ligands. Collectively, the dynamics of N- and C-terminal regions of motif A may be relatively independent, and whether their conformational changes coordinate with each other or follow a particular sequence is likely dependent on but not limited to the presence of RNA and the aforementioned conformational-change-inducible factors in the vicinity of motif A.

FOOTNOTES

This work was supported by the National Key Basic Research Program of China (2013CB911100), the National Key Research

and Development Program of China (2016YFC1200400), the Open Research Fund Program of Wuhan National Bio-Safety Level 4 Laboratory of Chinese Academy of Sciences, China (NBL2017009), and the “One-Three-Five” Strategic Programs, Wuhan Institute of Virology, Chinese Academy of Sciences (Y605191SA1). We thank Dr. Bo Zhang for providing the CV A16 infectious clone as the cloning template, Dr. Craig Cameron for providing the *Escherichia coli* BL21 (DE3) pCG1 cells for CV A16 RdRP expression, Dr. Jeffrey Kieft and Dr. Robert Batey for sharing the pRAV23 plasmid for RNA preparation, Dr. Guoliang Lu, Xuping Jing, and Liu Deng for laboratory assistance, and The Core Facility and Technical Support, Wuhan Institute of Virology, for access to instruments. This article does not contain any studies with human or animal subjects performed by any of the authors. The authors declare that there are no conflicts of interest.

Supplementary materials are available on the websites of *Virologica Sinica*: www.virosin.org; link.springer.com/journal/12250.

Peng Bi^{1,2}, Bo Shu^{1,2}, Peng Gong^{1✉}

1. Key Laboratory of Special Pathogens and Biosafety, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, China

2. University of Chinese Academy of Sciences, Beijing 100049, China

✉Correspondence:

Phone: +86-27-87197578

Email: gongpeng@wh.iov.cn

ORCID: 0000-0002-8264-7523

Published online: 20 November 2017

REFERENCES

- Gohara DW, Ha CS, Kumar S, et al. 1999. *Protein Expr Purif*, 17: 128–138.
- Gong P, Kortus MG, Nix JC, et al. 2013. *PLoS One*, 8: e60272.
- Gong P, Peersen OB. 2010. *Proc Natl Acad Sci U S A*, 107: 22505–22510.
- Li Y, Korolev S, Waksman G. 1998. *EMBO J*, 17: 7514–7525.
- Mao Q, Wang Y, Yao X, et al. 2014. *Hum Vaccin Immunother*, 10: 360–367.
- McDonald S, Block A, Beaucourt S, et al. 2016. *J Biol Chem*, 291: 13999–14011.
- Ng KK, Cherney MM, Vazquez AL, et al. 2002. *J Biol Chem*, 277: 1381–1387.
- Pflugrath JW. 1999. *Acta Crystallogr D Biol Crystallogr*, 55: 1718–1725.
- Shu B, Gong P. 2016. *Proc Natl Acad Sci U S A*, 113: E4005–4014.
- Solomon T, Lewthwaite P, Perera D, et al. 2010. *Lancet Infect Dis*, 10: 778–790.
- Theobald DL, Wuttke DS. 2006. *Bioinformatics*, 22: 2171–2172.
- Vives-Adrian L, Lujan C, Oliva B, et al. 2014. *J Virol*, 88: 5595–5607.
- Yin YW, Steitz TA. 2004. *Cell*, 116: 393–404.
- Zamyatkin DF, Parra F, Alonso JM, et al. 2008. *J Biol Chem*, 283: 7705–7712.