Crystal structure of 2C helicase from enterovirus 71

Hongxin Guan,1 Juan Tian,1 Bo Qin,1 Justyna Aleksandra Wojdyla,2 Bei Wang,1 Zhendong Zhao,1 Meitian Wang,2 Sheng Cui1*

Enterovirus 71 (EV71) is the major pathogen responsible for outbreaks of hand, foot, and mouth disease. EV71 nonstructural protein 2C participates in many critical events throughout the virus life cycle; however, its precise role is not fully understood. Lack of a high-resolution structure made it difficult to elucidate 2C activity and prevented inhibitor development. We report the 2.5 Å-resolution crystal structure of the soluble part of EV71 2C, containing an adenosine triphosphatase (ATPase) domain, a cysteine-rich zinc finger with an unusual fold, and a carboxyl-terminal helical domain. Unlike other AAA+ ATPases, EV71 2C undergoes a carboxyl terminus–mediated self-oligomerization, which is dependent on a specific interaction between the carboxyl-terminal helix of one monomer and a deep pocket formed between the ATPase and the zinc finger domains of the neighboring monomer. The carboxyl terminus–mediated self-oligomerization is fundamental to 2C ATPase activity and EV71 replication. Our findings suggest a strategy for inhibition of enterovirus replication by disruption of the self-oligomerization interface of 2C.

INTRODUCTION

Enteroviruses are positive-sense single-stranded RNA viruses responsible for many pandemics, posting tremendous public health threats (1). Enterovirus 71 (EV71), coxsackievirus A16, and coxsackievirus A6 are responsible for outbreaks of hand, foot, and mouth disease (HFMD) (2). In 2014, enterovirus D68 outbreak, associated with severe respiratory illness, spread to 49 states in the United States (3). Despite nearly 30 years of extensive research and the introduction of the vaccine against poliovirus (PV), polio still has not been entirely eradicated (4). Recently, a vaccine against EV71 was approved (5); however, there is no evidence of cross protection against other pathogens causing HFMD. It is generally accepted that antiviral agents have an essential role in disease control, which is complementary to vaccination. Unfortunately, there are no anti-enterovirus drugs currently available on the market (6).

Enteroviruses encode a large polyprotein, which is processed into four structural and seven nonstructural proteins. The multifunctional and highly conserved nonstructural protein 2C is arguably the most complex and also the least understood protein in the virus life cycle. Bioinformatic and mutagenesis studies predict that 2C harbors an N-terminal membrane-binding motif, an adenosine triphosphatase (ATPase) domain, a cysteine-rich motif, and RNA binding sites (7). The ATPase domain, which belongs to SF3 helicases of the AAA+ ATPase superfamily, contains Walker motifs and motif C (8). 2C has been shown to participate in many crucial events throughout the virus life cycle, such as uncoating (9), cellular membrane rearrangement (10), RNA replication, immune evasion (11), and encapsidation (12, 13); however, the details of 2C involvement in these processes remain to be understood. Functional dispensability of 2C makes it an attractive target for anti-enterovirus drug development. For decades, these efforts were hindered by lack of structural information about the 2C protein and resulted in identification of several inhibitors, which target 2C (14) without an understanding of the underlying mechanism.

The EV71 2C protein contains all previously identified structural features of picornaviral 2C (Fig. 1A). Because the full-length 2C was insoluble, we conducted systematic construct optimization and successfully overexpressed recombinant EV71 2C (residues 40 to 329) in Rosetta (DE3) competent cells. Next, a trypsin-resistant fragment (residues 116 to 329) was identified by limited proteolysis, which yielded crystals in buffer containing 0.1 M Hepes (pH 7.5), 10% PEG 6000, 5% 2-methyl-2,4-pentanediol (MPD), and 0.5% w/v polyvinylpyrrolidone K15. The crystal belonged to the P212121 space group and diffracted x-rays to 3.3 Å resolution. EV71 2C contains a C-terminal cysteine-rich motif predicted to form a zinc-binding site; therefore, we collected highly redundant multiwavelength anomalous dispersion (MAD) data at the zinc absorption edge for de novo phasing. An initial electron density map was calculated based on the successfully obtained substructure solution, and a preliminary model of 2C was built manually. To improve resolution, we identified a new crystal form, which diffracted the x-rays to 2.5 Å. The structure was solved by molecular replacement using preliminary 2C model and was refined (table S1). The EV71 2C structures determined from two crystal forms were nearly identical; therefore, the high-resolution structure was used for further analysis.

The EV71 2C structure consists of three subdomains (Fig. 1A): an ATPase domain, a zinc finger, and a long protruding C-terminal α helix. The ATPase domain exhibits a canonical α/β Rossmann fold (Fig. 1, B and C) with a five-stranded parallel β sheet (β1 to β5) sandwiched by helix α1 on one side and by two α helices (α2 and α3) on the opposite side. Two Walker motifs are located within the ATPase domain; motif A is found between β1 and α1, whereas motif B is found between β3 and α2. The SF3 helicase–specific motif C is located between β4 and α3 (Fig. 1, A to C and fig. S1).

The cysteine-rich motif (residues 270 to 286) of EV71 2C forms a zinc-binding site, which is connected to the ATPase domain via the interaction of antiparallel β sheets between β5 and β6 (Fig. 1, B and C). The zinc coordination has a trigonal bipyramidal geometry, which is unusual in zinc fingers (Fig. 1D) (15, 16). There are five coordinators for zinc. Three S atoms of the invariant cysteines C270, C281, and C286 define an equilateral triangle and lie in the same plane with zinc. Distance from sulfur to zinc ranges from 2.2 to 2.3 Å. The backbone carbonyl oxygen of S282 acts as an axial ligand for zinc (distance, 3.3 Å), stabilizing the metal from the bottom. The second axial ligand opposite to S282 is an ordered water molecule. In addition, E272 and K288 form a salt bridge above the zinc, stabilizing the architecture of the zinc finger (fig. S2).

Genetic and biochemical studies predicted four potential zinc coordination sites (PCS) in the cysteine-rich motif of PV 2C, suggesting a canonical CCCC-type zinc finger (7). In the crystal structure of EV71...
2C, we identified PCS1, PCS3, and PCS4, but not PCS2 (Fig. 1D). Residues N273 and N274 in the predicted PCS2 are neither in contact with zinc directly or through solvent molecules (fig. S2). Although many EVs do not have cysteine or histidine in PCS2, these residues are highly conserved in PCS2 of PV 2C (Fig. 1E) and are important to PV replication (7). In the case of foot-and-mouth disease virus (FMDV) 2C, the cysteine-rich motif is completely missing (17). Collectively, the zinc-binding site presents a key difference among picornaviral 2C homologs.

Analysis of the crystal packing with PISA software (www.ebi.ac.uk/pdbe/psa/) revealed that EV71 2C molecules associate in the crystal via C-terminus–mediated interactions (Fig. 2A). The amphipathic C-terminal helix α6 of EV71 2C is docked inside a hydrophobic pocket on an adjacent 2C molecule in a “knob-into-hole” fashion (Fig. 2B). Side chains of T323, I324, L327, and F328 in the helix α6, which occupy the deep pocket, form the pocket-binding domain (PBD). The pocket is formed between the zinc finger and ATPase helicase domains, comprising mainly hydrophobic residues (Fig. 2C). A salt bridge between E325 from PBD and R144 strengthens the interaction between adjacent 2C molecules. Both the PBD and the pocket are highly conserved in enteroviruses, suggesting that 2C association observed in the crystal structure may be functionally relevant (Fig. 2D and fig. S1).

To investigate oligomerization of EV71 2C in solution, we performed small-angle x-ray scattering (SAXS) experiments. When the protein concentration was kept constant (~5 mg/ml), Guinier analysis of the SAXS profile of EV71 2C 116–329 resulted in a radius of gyration \( R_g \) of 41 ± 2 Å (Fig. 2E). The SAXS curve and distance distribution function \( p(r) \) suggest that 2C has a rod-like shape and is composed of multiple subunits (Fig. 2F) (18). In contrast, Guinier analysis of the SAXS curve of 2C lacking PBD (EV71 2C 116–319) gives an \( R_g \) of 19 ± 2 Å, indicating that the molecular mass of the truncated 2C is reduced significantly. Moreover, the SAXS profile and the \( p(r) \) function demonstrate that the truncated 2C is a globular single domain (Fig. 2G). SAXS profiles of EV71 2C variants bearing mutations in the PBD are highly similar to that of 2C 116–319 (Fig. 2, E to H, and table S2). In addition, the SAXS profiles of 2C 116–329 measured at different concentrations (3, 5, and 8 mg/ml) show that \( R_g \) values (32 ± 1, 41 ± 2, and 49 ± 1 Å) increase with higher concentrations, a sign of strong intermolecular interaction and concentration-dependent oligomerization, which can be explained by the interaction observed in the crystal. In contrast, SAXS scattering curves of 2C mutants (truncated or mutated PBD) measured at different concentrations were superimposed perfectly, indicating that these mutants are monodispersed in solution and that they lost the ability to self-oligomerize. Consistent with SAXS results, size exclusion chromatography showed that the EV71 2C 116–329 (theoretical molecular mass of 23.6 kDa) elutes as tetramer (110 kDa), whereas 2C 116–319 elutes as monomers (24 kDa) (Fig. 2I). Mutations of residues involved in the 2C-2C interaction impaired or prevented self-oligomerization of 2C. For example, mutations of I324, L327, and F328 resulted in a monomeric protein, as observed with size exclusion chromatography. These results clearly demonstrate that EV71 2C self-oligomerizes in solution. The oligomerization is mediated by a network of specific interactions between the pocket region and the C-terminal PBD.

Fig. 1. Overall structure of EV71 2C 116–329. (A) Ribbon model of EV71 2C 116–329. The ATPase domain is colored in blue, the zinc finger domain is colored in yellow, and the C-terminal long helix is colored in red. Zinc is shown as a gray sphere. (B) Overview of key features of EV71 2C identified in this study. (C) Topology diagram of EV71 2C. Conserved ATPase motifs Walker A and Walker B and motif C are highlighted in black and labeled with A, B, and C in (A) to (C). (D) Zinc-binding site of EV71 2C. Residues involved in zinc coordination are shown with stick model. PCS predicted from PV 2C sequence are indicated. (E) Structure-based protein sequence alignment of the cysteine-rich domain in enterovirus 2C. Residues with red background are invariant; residues with yellow background are conserved.

The helical C terminus of the EV71 2C functions as a structural linkage between molecules. As observed in the crystal structure, the orientation of two linked 2C chains can vary significantly, whereas the interaction between PBD and the pocket remains unchanged (Fig. 3A). This is possible due to the presence of a hinge region formed by residues S318 and A319 in the helix α6. The crystal structure of the EV71 2C with bound adenosine 5′-O-(3-thiotriphosphate (ATP-γ-S) revealed that it occupies the active site formed by two neighboring 2C chains only when they adopt conformation 1 (Fig. 3, A and B). One of the 2C molecules provides Walker A and B motifs to recognize the triphosphate of ATP-γ-S, with motif C located on top of Walker B motif. The second 2C chain delivers an arginine-rich helix (α3) close to the triphosphate moiety, suggesting that either invariant R240 or R241 functions as the "R finger." The adenosine base of ATP-γ-S binds to a hydrophobic cavity at the junction of two 2C chains. In addition, T196 from the second 2C chain forms a hydrogen bond with the base of ATP-γ-S.

To validate our structural findings, we tested ATPase activity of a large selection of 2C variants with mutations in the active site, the zinc finger, the PBD, or the pocket. While EV71 2C 116–329 exhibited potent ATPase activity, mutant K135A (within Walker A) was inactive, serving as a negative control. Two control mutations, R312 and R239, located away from the active site did not affect ATPase activity of 2C. In contrast, any substitution of R240 or R241 aborted ATPase activity, indicating that these arginine residues are essential in hydrolysis (Fig. 3D). Any mutation of the zinc coordinators C270A, C281A, or C286A resulted in insoluble 2C, confirming that the zinc finger is essential to overall correct folding. In contrast, mutations of N273 and N274 in PCS2 or residues stabilizing the zinc finger (P282 and K288) did not affect the folding of 2C (fig. S4). The 2C mutant bearing these mutations remained active in ATPase assay (Fig. 4A). Mutations in the pocket or the PBD that disrupted self-oligomerization of 2C led to an inactive enzyme. The monomeric 2C 116–319 with truncated PBD exhibited similar activity as the negative control K135A. Similarly, the monomeric mutants I141R and S282R (in the pocket) as well as I324K, F328A, F328R, and F328Y (in the PBD) completely lost their ATPase activity. S282A was the only mutation that did not affect self-oligomerization of EV71 2C.

**Fig. 2.** Self-oligomerization of EV71 2C. (A) Top: 2C in the crystal polymerizes through a specific interaction indicated with arrows. Bottom: Magnified view of the intermolecular interaction. (B) Magnified view of the interaction between two 2C molecules. The C terminus of EV71 2C (orange ribbon model) forms the pocket-binding motif, which binds to the hydrophobic pocket (shown as molecular surface) formed between zinc finger and ATPase domains. Residues 318 to 329 are shown with stick model. Residues lining the pocket (F328, L327, I324, and T323) are highlighted in blue. The molecular surface of EV71 2C harboring the pocket is colored according to the hydrophobic scale of residues, from white (hydrophobic) to green (hydrophilic). (C) Semitransparent surface of the hydrophobic pocket [color scheme as in (B)]. Residues forming the pocket are shown with stick model. (D) Structure-based sequence alignment of C-terminal amphipathic helix of enteroviral 2C. (E) SAXS profiles of 2C 116–329, 2C mutants without PBD, and mutant F328A. (F to H) Distance distribution function $ρ(r)$ of the SAXS measurements shown in (E). (I) Molecular weights of EV71 2C 116–329 and various mutants as measured by size exclusion chromatography.
2C (Fig. 2I), whereas the ATPase activity of this mutant was largely retained (Fig. 4A).

Because of the insolubility of full-length 2C, biochemical characterization was performed using a fragment of 2C without the N-terminal membrane-binding domain. To validate whether our data reflect the function of the intact 2C protein, we introduced a selection of mutations (similar to biochemistry study) to an infectious clone of EV71 and evaluated the EV71 production. The results of the EV71 production experiment are consistent with the biochemical data (Fig. 4, B and C). Mutations at Walker motifs (K135A and D176N) halted virus production, demonstrating that ATPase activity of 2C is essential for EV71 replication. Mutations that disrupted self-oligomerization of 2C also abolished EV71 production, showing that self-oligomerization of 2C is fundamental to virus replication. In contrast, EV71 bearing S282A that did not affect self-oligomerization of 2C exhibited similar activity of EV71 replication as wild-type (WT) virus. The E325A mutation was designed to disrupt a salt bridge formed between two 2C molecules. E325A is dimeric instead of tetrameric or monomeric (Fig. 2I), suggesting some level of impairment of the self-oligomerization. Although 2C E325A has residual ATPase activity in vitro, the mutation was lethal to EV71 production. These results demonstrate that self-oligomerization of 2C is essential to EV71 replication, and virus replication is highly sensitive to defects in self-oligomerization of 2C.

The first step in autoproteolytic processing of enteroviral polyprotein by 3C protease (3Cpro) is cleavage between 2C and 3A proteins (Fig. 4D) (19). Deletion of the PBD, which contains a cleavage site between 2C and 3A, prevents correct processing of polyprotein and was shown to be lethal to EV71 in our in vivo experiments. Nevertheless, single

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**Fig. 3. Structural basis for ATP recognition and hydrolysis.** (A) Adjacent EV71 2C linked by C terminus $\omega_6$ adopt different orientations in crystal. A hinge region allows significant rotations. EV71 2C molecules on the right side are superimposed. We denote the 2C-2C interactions containing orange, blue, and red molecules as conformation 1, conformation 2, and conformation 3, respectively. (B) ATP-$\gamma$-S (cyan) occupies the active site only when adjacent EV71 2C adopt proper orientation [conformation 1 in (A)]. One EV71 2C molecule (green) provides Walker A (pink) and Walker B (blue) motifs to recognize ATP-$\gamma$-S. Motif C (red) is located above the Walker B motif. Second EV71 2C molecule (orange) provides R finger (magenta) required for hydrolysis. Adenosine base docks into a hydrophobic cavity at the junction of the two molecules. Residues involved in ATP recognition are shown with stick model. (C) Diagram illustrating recognition of ATP-$\gamma$-S by EV71 2C. Hydrogen bonds are shown with dashed lines. (D) ATPase activity of EV71 2C and single mutants (including arginine finger).
mutations E325A, L327A, F328A, or F328Y in the PBD region, which do not affect substrate specificity of 3C\textsuperscript{pro} (20, 21), severely affected virus production. Our results clearly indicate that disruption of 2C self-oligomerization, and consequent loss of ATPase activity, has a detrimental effect on EV71 life cycle. Using the structure of 3C in complex with peptide [Protein Data Bank (PDB) ID: 3SJ9], we modeled an extended structure of 2C terminus bound to the substrate-binding site of 3C\textsuperscript{pro} (fig. S6). On the basis of comparison of PBD bound to 3C\textsuperscript{pro} and in EV71 116–329 structure, we propose the following scenario of polyprotein processing (Fig. 4D). Upon cleavage, the C terminus of 2C is released from 3C\textsuperscript{pro} and quickly switches from the extended conformation (fig. S6) to the compact \( \alpha \) helix with functional PBD. This in turn facilitates C terminus–mediated self-oligomerization and, after release of mature N terminus (cleavage between 2B and 2C), allows formation of 2C hexamers in the presence of RNA and nucleotide.

Self-oligomerization is a common feature of picornaviral 2C and is essential in the formation of functional replication complex. The oligomeric structure of 2C is affected by the N terminus (22) and ligands of 2C (17). Although the region around the pocket-binding motif is conserved among entroviruses, sequence alignment of 2C from different picornaviruses shows that there are clear differences in the C-terminal region of FMDV 2C, which include deletions and a position shift of the conserved phenylalanine in the pocket-binding motif of 2C (fig. S1). To investigate whether C terminus–mediated oligomerization is a common feature of picornaviral 2C proteins, we used Phyre2 server (www.sbg.bio.ic.ac.uk/phyre2) to predict the secondary structures of FMDV 2C. The resulting model contains a long C-terminal helix formed by residues 293 to 316, which is homologous to the C-terminal helix of EV71 2C (fig. S1). It has been previously reported that FMDV 2C lacking the N-terminal membrane-binding motif self-assembles into oligomers of different sizes.
Fig. 5. The hexameric ring model of EV71 2C 116–329. (A) Top view of the modeled EV71 2C 116–329 hexamer, colored by chains. ATP-γ-S molecules are shown with stick model in red. C-terminal helices (α6), zinc atoms, and predicted C-terminal RNA binding motif are labeled. Inset: Magnified view of the interaction between PBD and the pocket. Slight rotation around the hinge allows binding of the C terminus of EV71 2C to the pocket on the adjacent subunit. Blue ribbon indicates the structure before rotation, and gray ribbon indicates the structure after rotation. (B) Side view of the modeled EV71 2C 116–329 hexamer with the dimension of the hexameric ring indicated. (C) Superposition of EV71 2C 116–329 hexameric model (red ribbon model) onto cryo-EM structure of human p97 (PDB ID: 5FTK), which is colored by domains (green, N-domain; blue, D1-domain; gray, D2-domain). (D) Electrostatic surface of the cytoplasmic side of EV71 2C 113–329 hexamer model (left) and membrane proximal side (right; negative charge, red; positive charge, blue). The central channel has a funnel-like shape. The diameters of the narrowest and widest openings are indicated. (E) Structure comparison 2C-2C conformation 1 observed in the crystal structure with the 2C-2C interaction in hexameric model of EV71 2C 116–329. The 2C monomers providing Walker motifs to the active site are superimposed. The 2C-2C interaction in the hexameric model is shown with gray and red molecules; the 2C-2C interaction in conformation 1 is shown with gray and green molecules. ATP-γ-S and the R finger residues are shown with stick model.
(17), which is reminiscent of the oligomerization of EV71 2C 116–329 presented in the current study. Together, this evidence suggests that FMDV 2C also undergoes C-terminal–mediated oligomerization; however, the mode of interaction between 2C–2C might be different because the zinc-binding cysteines are not present in FMDV 2C. The crystal structure of EV71 2C provides structural insights into C-terminal–mediated oligomerization, which has not been previously shown in the AAA+ ATPase superfamily. Moreover, it is likely that this type of oligomerization is conserved in picornaviral 2C.

Proteins of the AAA+ superfamily often assemble into hexameric ring structures. Electron microscopy (EM) studies have shown that picornaviral 2C helicases also form ring-like structures (17, 22, 23). EV71 2C shares multiple structural features with SF3 helicase, suggesting that its functional assembly is likely a hexameric ring. We compared the crystal structure of EV71 2C 116–329 with all structures in the PDB using the Dali server (http://ekhidna2.biocenter.helsinki.fi/dali_server/). The best hit was large T antigen (LTAg) of the human polyomavirus John Cunningham virus (JCV), a hexameric DNA helicase sharing a 75% sequence identity with SV40 LTAg (24). The root mean square deviation value between EV71 2C 116–329 and JCV LTAg (PDB code: 5J4Y) is 2.7 Å for 117 superimposed Ca atoms. Next, we built a hexameric model of EV71 2C 116–329 based on the crystal structure of JCV LTAg. The model shows six adenosine 5′-triphosphate (ATP) domains of EV71 2C assembling into a flat ring with the C-terminal helices (o6) and the zinc-binding sites lining up the rim (Fig. 5, A and B). The PBD at the C terminus of o6 is located very close to the pocket on the neighboring subunit, and only a slight rotation around a flexible hinge of o6 (indicated in Fig. 3A) was necessary for it to bind inside the pocket (Fig. 5A). The hexameric model of EV71 2C 116–329 shows that all 2C monomers are connected via the interaction between the C-terminal PBD and the pocket. Six C-terminal helices form a circle around the hexamer, stabilizing its conformation. Comparison of the hexameric model of EV71 2C 116–329 with the cryo-EM structure of a well-characterized member of AAA+ protein p97 shows that the EV71 2C 116–329 hexamer can be superimposed onto the D2 hexamer of p97 (PDB ID: 5FTK, Fig. 5C).

The hexameric model of EV71 2C 116–329 has an external diameter of 110 Å, which is similar to the dimensions of the hexamers of echovirus 30 2C (120 Å) and FMDV 2C (140 Å). The ring-like oligomers of PV 2C are larger (150 to 200 Å) than that of the EV71 2C 116–329 hexamer, most likely due to the presence of the maltose-binding protein tag in PV 2C. The N terminus of EV71 2C 116–329 is located on the upper side of the hexameric ring model (Fig. 5B), suggesting that the missing N-terminal membrane-binding portion (amino acids 1 to 115) of EV71 2C makes contact with this side. Therefore, the upper side of the hexamer is the membrane-proximal side, whereas the lower side is the cytoplasm side. The central channel of the hexameric ring has a funnel-like shape with a narrow opening (13.2 Å) on the membrane side and a wide opening (49.1 Å) on the cytoplasm side (Fig. 5D). In contrast to the positively charged central channel of the hexamers of SF3 DNA helicases (25, 26), the central channel of EV71 2C 116–329 hexamer is negatively charged. It is therefore unlikely that this channel can bind RNA. Rodríguez et al. (27) found that N-terminal (amino acids 21 to 45) and C-terminal (amino acids 312 to 319) motifs of PV 2C are involved in RNA binding. Deletion of either the N-terminal or C-terminal motif abolishes RNA binding, indicating that these two motifs act in concert to bind RNA. The N-terminal RNA binding motif is missing in the EV71 2C 116–329 structure, whereas the C-terminal RNA binding motif is situated in the middle of the C-terminal helix (Fig. 5A). The hexameric model of EV71 2C 116–329 suggests that the C-terminal RNA binding motif may be located on the rim of the ring structure. It is possible that one RNA strand simultaneously interacts with multiple RNA binding sites on the hexamer and plays an essential role in stabilizing the 2C hexamer. This hypothesis is supported by a biochemical and structural characterization of FMDV 2C, which demonstrated that FMDV 2C hexamers were only isolated in the presence of ATP and RNA (17).

To compare the geometry of the ATPase active site between 2C–2C in the crystal structure of EV71 2C 116–329 (Fig. 3A) and that between 2C–2C in the hexameric model, we superimposed different 2C–2C conformations from the crystal structure (indicated in Fig. 3A) with the hexameric model of EV71 2C 116–329. The 2C monomers providing the Walker A and B motifs of the ATPase active site were superimposed. The 2C–2C conformation 1 (the only 2C–2C interaction allowing full occupation of ATP–γ-S) is very similar to the 2C–2C interaction in the hexameric model (Fig. 5E). In both models, the catalytically important R240 and R241 are oriented close to the ATP–γ-S. In the hexameric model, R241 is located closer to ATP–γ-S than R240, suggesting that R241 may act as the R finger in hydrolysis. The closest distance from the R finger (R241) to ATP–γ-S (γ-thiophosphate) is shorter in the hexameric model (4.9 Å) than in conformation 1 (11.9 Å), suggesting that the hexameric model of EV71 2C 116–329 has a better geometry for ATP hydrolysis. On the contrary, distances from the R finger to the ATP–γ-S in conformation 2 (23.7 Å) and conformation 3 (27.4 Å) are too long to allow formation of the functional ATPase active sites (fig. S7, A and B), suggesting that these conformations are artifacts of crystal packing. The hexameric model of EV71 2C 116–329 suggests that ATP binding to the phosphate-binding loop in one 2C subunit and the R finger of the neighboring 2C subunit may strengthen the 2C–2C interaction and stabilizes the hexamer.

Proteolytic processing of the 2C-3A site occurs in the early phase of the virus replication cycle. It is possible that the C terminus of 2C mediates oligomerization of the precursor 2BC, as well as 2C. Both 2C and 2BC have ATPase activity and are indispensable components of the large RNA replication complex attached to the membrane (7). Thus, the C terminus–mediated oligomerization may be important in the correct assembly of the high-order membrane-bound RNA replication complex. Our results suggest a strategy for inhibition of enterovirus replication by disrupting the 2C oligomerization interface. The conserved 2C pocket, which PBD binds, represents a possible target for the structure-based design of inhibitor.

**MATERIALS AND METHODS**

**Cells and reagents**

Vero and rhabdomyosarcoma (RD) cells were purchased from American Type Culture Collection and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. EV71 infectious clone was a gift from S. Cen (Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College). The mouse anti-EV71 monoclonal antibody was purchased from Millipore. Donkey anti-mouse immunoglobulin G secondary antibody was purchased from LI-COR Biosciences.

**Protein preparation**

The gene of EV71 2C was amplified by polymerase chain reaction using an infectious clone as template. The fragment encoding residues 116 to 329 of EV71 2C was inserted into plasmid pET-28a-SUMO. The plasmid
was transformed to Rosetta (DE3) competent cells (Novagen). The bacteria were grown at 37°C to an OD_{600} (optical density at 600 nm) = 0.8 and cooled to 18°C before induction with isopropyl-β-D-thiogalactopyranoside (0.5 mM). The induction continued by shaking at 18°C overnight. Bacteria were harvested by centrifugation and resuspended in lysis buffer containing 50 mM tris-HCl (pH 8.0) and 100 mM NaCl. Bacteria cells were disrupted by ultrasonication and clarified by centrifugation. The supernatant was loaded to Ni-NTA (nitrilotriacetic acid) resin (Invitrogen), and the resin was treated with Ulp1 peptidase overnight to remove the SUMO tag. The nontagged protein was washed off from the column by lysis buffer and finally purified using Superdex 200 HR10/30 column (GE Healthcare) pre-equilibrated with buffer containing 20 mM tris-HCl Hepes (pH 7.5) and 100 mM NaCl.

**Crystallization and structure determination**

EV71 2C 116–329 was concentrated to ~10 mg/ml before crystallization. The 2C was crystallized by mixing 1 μl of sample with 1 μl of buffer containing 0.1 M Hepes (pH 7.5), 10% PEG 6000, 5% MPD, and 0.5% w/v polyvinylpyrrolidone K15 in a hanging drop vapor diffusion system at 22°C. Crystals were flash-frozen in liquid nitrogen before measurement. Because EV71 2C harbors a putative zinc-binding site, highly redundant MAD data near zinc absorption edge (peak, 1.28283 Å; in-flection point, 1.28283 Å; high energy remote, 1.26980 Å) were collected at X06DA beamline Swiss Light Source (SLS). The data were processed with XDS package (28). The crystal has the space group of P2_1_2_1, and contains six molecules in asymmetric unit. Zinc was bound by each molecule. The initial phase and electron density map was calculated using SHELEX/D/E (29), which allowed manual model building using Coot (30). To improve resolution, we discovered a new crystal form (in the same mother liquor) that diffracted the x-ray to 2.5 Å using higher x-ray energy (λ = 0.978 Å). The crystal has the space group of P2_1. Crystals of EV71 2C 116–329 bound with ATP-γ-S were prepared by back soaking in the same mother liquor containing 5 mM ATP-γ-S overnight on ice. Native data sets for these crystals were collected at the Shanghai Synchrotron Radiation Facility (SSRF). The crystal structures were solved by molecular replacement using Phaser-MR (31). All above structures were finally refined with PHENIX (32). Data collection, refinement parameters, and model validation are summarized in table S1.

**ATPase assay**

The reaction mixture (50 μl) contained 100 mM Hepes (pH 7.5), 4 mM MgCl_2, 20 μM ATP, and a trace amount of [γ-32P]-labeled ATP. The reaction was initiated with addition of 15 μM enzyme. The mixture was incubated at 37°C for 10 mins, and the reaction was stopped with addition of 2 μl of quenching buffer (0.2 M EDTA). The resulting mixtures were resolved by thin-layer chromatography using polyethyleneimine-cellulose plate (Sigma-Aldrich) and running buffer containing 0.8 M acetic acid and 0.8 M LiCl. The plate was dried and exposed to phosphor screen. The results were visualized and quantified using Typhoon Trio Variable Mode Imager (GE Healthcare). The ATP hydrolysis rate was calculated by the amount of released Pi (in nmol) per minute per milligram of enzyme.

**Small-angle x-ray scattering**

SAXS experiments were conducted using the laboratory source BioSAXS-1000 (Rigaku) equipped with a photon-counting PILATUS 100K detector (Dectris). The wavelength of the x-ray is 1.54 Å. The sample-to-detector distance is 500 mm and yields the range of momentum transfer (s = 4π sinθ/λ, where 2θ is the angle between the incident and scattered waves)

from 0.008 to 0.65 Å⁻¹. Bovine serum albumin solution (5 mg/ml) was prepared with 50 mM Hepes (pH 7.5) and used for calibration. Scattering data were collected for at least two different sample concentrations (2.5 and 5 mg/ml) in a buffer containing 20 mM Hepes (pH 7.5) and 100 mM NaCl. SAXS data reduction and analysis were performed using ATSAS data analysis (ATSAS package).

**Virus production efficiency from EV71 infectious clones**

Vero and RD cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. The EV71 infectious clone (pEV71) contained full-length cDNA of WT virus. Mutations were introduced to the infectious clone by site-directed mutagenesis. The sequence of the resulting plasmids was verified by DNA sequencing. Plasmids were linearized at a unique Hind III restriction site and used as templates for in vitro RNA transcription using the MEGAscript T7 Kit (Ambion). The RNAs were purified and quantified by ultraviolet spectrophotometer (NanoDrop Technologies). The RNAs (2 μg) were transferred into Vero cells. At 72 hours after transfection, supernatants of cell culture were collected to infect RD cells seeded in a 24-well plate. At 24 hours after infection, the RD cells were fixed. Immunofluorescence analysis was performed to detect EV71 virus production using anti-EV71 antibody and anti-mouse secondary antibody. At least eight different visual fields from each well were randomly photographed under the fluorescence microscope. The positive dots were counted.

**Size exclusion chromatography**

The Superdex 200 10/300 GL column (GE Healthcare) was equilibrated with buffer containing 20 mM Hepes (pH 7.5) and 100 mM NaCl. The column was precalibrated with molecular mass standards containing γ-globulin (158 kDa), ovalbumin (45 kDa), myoglobin (17 kDa), and vitamin B12 (1.35 kDa). The purified EV71 2C or mutants (5 mg/ml) was loaded and eluted with a flow rate of 0.5 ml/min.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/3/4/e1602573/DC1

fig. S1. Structure-based multiple sequence alignment of 2C proteins from different enteroviruses.

fig. S2. Structure of three-ligated zinc finger of EV71 2C.

fig. S3. SAXS experiments of various EV71 2C with mutations or deletions at pocket-binding motifs.

fig. S4. Circular dichroism spectroscopy of EV71 2C mutants and truncations.

fig. S5. Structure of ATP-binding site of EV71 2C.

fig. S6. Model of the C terminus of EV71 2C bound to 3C proteinase.

table S1. Data collection and refinement statistics.

table S2. Parameters in SAXS experiments.

**REFERENCES AND NOTES**


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