Crystal Structure of Enterovirus 71 RNA-Dependent RNA Polymerase Complexed with Its Protein Primer VPg: Implication for a *trans* Mechanism of VPg Uridylylation

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Picornavirus RNA replication is initiated by VPg uridylylation, during which the hydroxyl group of the third tyrosine residue of the virally encoded protein VPg is covalently linked to two UMP molecules by RNA-dependent RNA polymerase (RdRp; also known as 3D^{pol}). We previously identified site 311, located at the base of the palm domain of the enterovirus 71 (EV71) RdRp, to be the site for EV71 VPg binding and uridylylation. Here we report the crystal structure of EV71 3D^{pol} complexed with VPg. VPg was anchored at the bottom of the palm domain of the 3D^{pol} molecule and exhibited an extended V-shape conformation. The corresponding interface on 3D^{pol} was mainly formed by residues within site 311 and other residues in the palm and finger domains. Mutations of the amino acids of 3D^{pol} involved in the VPg interaction (_{3D}L319A, _{3D}D320A, and _{3D}Y335A) significantly disrupted VPg binding to 3D^{pol}, resulting in defective VPg uridylylation. In contrast, these mutations did not affect the RNA elongation activity of 3D^{pol}. In the context of viral genomic RNA, mutations that abolished VPg uridylylation activity were lethal for EV71 replication. Further *in vitro* analysis showed that the uridylylation activity was restored by mixing VPg-binding-defective and catalysis-defective mutants, indicating a *trans* mechanism for EV71 VPg uridylylation. Our results, together with previous results of other studies, demonstrate that different picornaviruses use distinct binding sites for VPg uridylylation.

Picornaviridae members make up one of the largest family of viruses and cause a wide range of infectious diseases in plants, animals, and humans. As the members of *Picornaviridae*, enterovirus 71 (EV71) and coxsackievirus (CV) are the major causative agents of hand, foot, and mouth disease (HFMD) in mainland China (1, 2). These agents caused over 1,000,000 infections and 900 deaths in 2010 (China Ministry of Health, <http://www.moh.gov.cn/publicfiles/business/htmlfiles/mohjbyf/kzj/s3578/201102/50646.htm>). In particular, young children are more susceptible to infection with EV71 (3, 4), which causes severe aseptic meningitis, encephalitis, myocarditis, acute flaccid paralysis, and pulmonary edema, which lead to high fatality rates (2, 5–7).

EV71 is a member of the genus *Enterovirus* within the family *Picornaviridae*. Its genome contains a single-stranded positive-sense polyadenylated RNA (8, 9). Translation of the single open reading frame is initiated by ribosomes that use an internal ribosomal entry site located in the 5' untranslated region of the viral genome, which gives rise to a polyprotein of approximately 250 kDa (10–12). The polyprotein encoded by the viral genome is initially processed into one structural (P1) and two nonstructural (P2 and P3) regions, and then the polyprotein undergoes proteolytic cleavage into various precursors and 11 mature proteins, i.e., VP1 to VP4, 2A, 2B, 2C, 3A, 3B, 3C, and 3D. This process is achieved by the correct function of two proteases, 2A and 3C, together with a protease precursor, 3CD. Among these mature proteins, the 3D^{pol} protein acts as a viral RNA-dependent RNA polymerase (RdRp) and plays a major role in viral negative-strand synthesis and the uridylylation of a few proteins (13–15).

The genome of picornavirus comprises positive-sense single-

stranded RNA (+ssRNA), which could be directly used as mRNA for viral protein translation. However, unlike many other +ssRNA viruses, the genome of picornavirus is not capped at the 5' end and its replication is initiated by the 5' end of the genome covalently linked to a virally encoded nonstructural protein, 3B, which is also known as VPg and comprises 20 to 22 amino acids (16). During replication initiation with the genomic RNA as a template, 3D^{pol} and two copies of the viral 3C protease (possibly in the precursor form of 3CD) (17, 18) uridylylates the VPg protein by processing two UMP molecules to the hydroxyl group of the Y3 residue of VPg, which is a strictly conserved residue within VPg among the members of the *Picornaviridae* (10). Notably, the VPg uridylylation process of picornavirus occurs in a template-dependent manner by use of a small stem-loop structure (*cis*-acting replication element [cre]) first identified in the 2C-coding region of the poliovirus genome (19) but located at various positions in other picornavirus genomes (20, 21) as the natural template. In this process, the structures of cre RNAs are demonstrated to be

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essential for VPg uridylylation and virus replication (19, 22–24). Although 3D^{pol} plays the central role in catalysis of this reaction, there are still a few discrepancies in the mechanism of the VPg uridylylation process. For example, foot-and-mouth disease virus (FMDV) exclusively encodes three VPg peptides in *Picornaviridae*, and its cre is located within the 5′ untranslated region (25). Most interestingly, the distinct locations of VPg-binding sites revealed by the crystal structure of 3D^{pol}-VPg complexes from FMDV and CV type B3 (CVB3) indicate variations in the uridylylation mechanism among the members of the *Picornaviridae* (26, 27), although the picornavirus RdRps (also named 3D^{pol} in *Picornaviridae* members) and VPgs show high primary sequence similarity and structural homology (the root mean square deviation [RMSD] of the overall structures is less than 1.4 Å). In the crystal structure of FMDV 3D^{pol} complexed with its protein primer, VPg1 binds to the F motif of the finger domain and the α13 helix of the thumb domain, spanning residues E166, I167, R168, K172, and R179 and residues T407, A410, and I411, respectively (26). The localization of VPg1 at the catalytic tunnel and the interaction with the UMP molecule suggest that an *in cis* uridylylation mechanism is most likely favored in the FMDV VPg uridylylation process (26). In sharp contrast, Gruez et al. (27) reported that in the crystal structure of CVB3 3D^{pol} complexed with VPg and PP_i, VPg is located at the end palm region of 3D^{pol}, encompassing residues W369, T370, Y378 to E383, V389, V392, P394, and P222. However, the substitutions on such residues affect VPg binding but only slightly influence the uridylylation reaction. This finding indicates a distinct *in trans* VPg uridylylation mechanism in CVB3 and that this VPg-binding position can either act as a substrate in an intermolecular uridylylation complex or stabilize the VPg uridylylation complex (27). Aside from these two complex structures, Lyle et al. reported four surface residues on poliovirus polymerase whose wild-type identity is required for 3AB binding, and two of these that are part of conserved motif E can also affect VPg uridylylation, providing evidence that the binding sites for the membrane tether and the protein primer overlap (28). Together with this work, a few more biochemical and structural studies suggested more sites/residues that could be involved in picornavirus VPg binding or uridylylation (29–32). Moreover, we reported in our previous work that site 311, located on the palm domain of EV71 3D^{pol}, is essential for VPg uridylylation by directly contributing to VPg binding in EV71 3D^{pol} and thus plays an essential role in EV71 replication (33).

In the present work, we report the crystal structure of the EV71 3D^{pol}-VPg complex. VPg displayed a V-shape conformation and anchored at the bottom of the palm domain of the 3D^{pol} molecule, including residues I311 to E343, as well as Y73, H80, Q84, and Q87. Replacement of L319, D320, and Y335 with alanine significantly disrupted VPg uridylylation by weakening VPg binding to 3D^{pol}. However, the substitutions did not affect the RNA elongation activity of 3D^{pol}. Complementation of VPg uridylylation *in trans* could be achieved by mixing VPg-binding-defective and catalysis-defective mutant 3D^{pol} proteins. These results for EV71, together with the previous results for FMDV and CVB3, demonstrate a variation in the VPg uridylylation process in the *Picornaviridae*.

MATERIALS AND METHODS

Protein production and crystallization. The production of EV71 3D^{pol} was based on a previously reported protocol (15). Briefly, the genomic

region that codes for EV71 3D^{pol} was amplified from the complete genome (GenBank accession number EU131776) of human EV71 strain N3340-TW-02 by PCR, with a tobacco etch virus (TEV) protease cleavage site upstream of the 3D^{pol}-coding region added using the primers 5′-CGGGATCCGAGAATCTTTATTTCAGGGAGAGATCCAGTGGGTTA-3′ and 5′-CGGAATTCCTAAAATAACTCGAGCCAA-3′, and the genomic region was cloned into the pGEX-6p-1 vector (GE Healthcare) using BamHI and EcoRI restriction sites (in boldface), respectively. The recombinant plasmids were transformed into *Escherichia coli* strain BL21(DE3). Ampicillin-resistant colonies were grown in Luria-Bertani medium at 37°C until the optical density at 600 nm reached 0.8. Isopropyl-β-D-1-thiogalactopyranoside was added to a final concentration of 0.1 mM, and the cultures were grown for an additional 20 h at 16°C. Cells were harvested by centrifugation, resuspended, and homogenized in lysis buffer containing 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 1 mM dithiothreitol (DTT), and 0.1 mM EDTA using a low-temperature ultra-high-pressure cell disrupter (JNBIO, Guangzhou, China). The lysate was centrifuged at 20,000 × g for 30 min to remove cell debris. The supernatant was loaded onto a glutathione-Sepharose column (GE Healthcare). After washing with 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 1 mM DTT, the glutathione S-transferase (GST) moiety was cleaved by His-tagged TEV protease at 4°C overnight. The elution was loaded into an Ni²⁺-nitrilotriacetic acid agarose column (Qiagen) to eliminate the His-tagged TEV protease and then eluted into a buffer containing 20 mM Tris-HCl (pH 8.0), 15% glycerol, 100 mM NaCl, and 1 mM DTT by ultrafiltration. The sample was further purified using a ResourceQ column (GE Healthcare) with a linear gradient from 0 mM to 350 mM NaCl with 20 mM Tris-HCl (pH 8.0), 15% glycerol, and 1 mM DTT. The target proteins were concentrated to 10 mg/ml in a buffer with 20 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 14% glycerol for storage.

EV71 VPg from human EV71 strain N3340-TW-02 was synthesized by solid-phase peptide synthesis and then purified to >98% by high-pressure liquid chromatography.

The purified EV71 3D^{pol} and VPg were mixed at a 1:2 molar ratio and pretreated at 4°C for 2 h before crystallization. Crystallization of the EV71 3D^{pol}-VPg complex was performed at 18°C using the hanging-drop vapor-diffusion method. Crystals appeared and reached their final size within 1 week in a well solution containing 1.3 M ammonium sulfate, 0.1 M bis-Tris (pH 6.1), 1 mM DTT, and 10 mM NiCl₂ (to improve the crystal size). Crystals were transferred to a 4.0 M sodium malonate solution for 30 s prior to flash freezing and then stored in liquid nitrogen for data collection.

X-ray diffraction data collection, processing, and structure determination. The diffraction data for the EV71 3D^{pol}-VPg complex were collected to 2.7-Å resolution at 100 K using a MAR165 charge-coupled-device detector on beamline 3W1A of the Beijing Synchrotron Radiation Facility (BSRF) with a wavelength of 1.0000 Å. Data were processed and scaled using the HKL2000 package (34).

The crystals belonged to space group P3₂21 and had one 3D^{pol}-VPg complex molecule per asymmetric unit. The Matthews coefficient was calculated to be 2.75 Å³/Da, which corresponds to a solvent content of 47% (35). The complex structure was determined using PHASER software (36) and the crystal structure of apo EV71 3D^{pol} (Protein Data Bank accession number 3N6L) as the initial search model. The presence of VPg was initially revealed by the difference in the density map. Manual model construction and refinement were performed with the COOT (37) and PHENIX (38) programs following rigid body refinement, energy minimization, and individual B-factor refinement. The quality of the final refined model was verified using the program PROCHECK (39). The final refinement statistics are summarized in Table 1. Structural figures were drawn using the program PyMOL (40).

Site-directed mutagenesis, expression, and purification of mutant EV71 polymerase. Proteins with a single amino acid mutation were constructed by site-directed mutagenesis using an Easy site-directed mutagenesis kit (Transgen, Beijing, China) as described previously (41). The

TABLE 1 Data collection and refinement statistics

Parameter ^a	Value(s) for:				
	EV71 3D ^{pol} -VPg complex ^b	3D ^{pol}	VPg	Ion	Solvent
Data collection statistics					
Cell parameters					
<i>a</i> (Å)	103.9				
<i>b</i> (Å)	103.9				
<i>c</i> (Å)	131.8				
α, β, γ (°)	90.0, 90, 120.0				
Space group	P3 ₂ 21				
Wavelength used (Å)	1.0000				
Resolution (Å)	50.00–2.69 (2.74–2.69)				
No. of all reflections	138,534 (23,580)				
No. of unique reflections	24,730 (1,225)				
Completeness (%)	99.5 (97.6)				
Avg <i>I</i> /σ(<i>I</i>)	12.6 (2.2)				
<i>R</i> _{merge} (%)	11.6 (47.5)				
Refinement statistics					
No. of reflections used (σ <i>F</i> > 0)	22,910				
<i>R</i> _{work} (%)	21.0				
<i>R</i> _{free} (%)	26.7				
RMSD					
Bond distance (Å)	0.009				
Bond angle (°)	1.263				
Average <i>B</i> value (Å ²)		58.7	98.5	80.2	67.4
No. of protein atoms		3,698	144	2	327
Resolution (%) in Ramachandran plot					
Allowed regions		98.5	73.3		
Generously allowed regions		1.0	20.0		
Disallowed regions		0.5	6.7		

^a $R_{\text{merge}} = \sum_h \sum_l |I_{ih} - \langle I_h \rangle| / \sum_h \sum_l \langle I_h \rangle$, where *I* is the intensity of a reflection and $\langle I_h \rangle$ is the mean of the observations *I_{ih}* of reflection *h*. $R_{\text{work}} = \sum (|F_o(\text{obs})| - |F_o(\text{calc})|) / \sum |F_o(\text{obs})|$, where obs and calc are the observed and calculated values, respectively. *R*_{free} is an *R* factor for a preselected subset (5%) of reflections that was not included in the refinement.

^b Numbers in parentheses are the corresponding values for the highest-resolution shell.

target constructs, proved by sequencing, were used in the transformation, expression, and purification processes.

GST pulldown assay. Glutathione-Sepharose beads (wet volume, 15 μl) coupled with approximately 5 μg of the purified GST-VPg were incubated for 30 min at 4°C in phosphate-buffered saline (PBS) buffer containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 2 mM KH₂PO₄. The GST-VPg beads were incubated for 30 min at 4°C with 2 μg of wild-type (WT) EV71 3D^{pol} or relevant mutants. After the beads were washed four times with 1 ml of PBS buffer, the proteins bound to the beads were analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue.

In vitro VPg uridylylation assay. Although *cre* is known to be the natural template of VPg uridylylation in the picornavirus family, the *cre* sequence of EV71 is predicted only according to the primary sequence homology with other picornaviruses (20) and is not experimentally defined. Therefore, we used poly(rA) as the template in this study, as that was used in the structural study of CVB3 3D^{pol}-VPg (27) and FMDV 3D^{pol}-VPg1 (26). The assays were performed following the reported procedure (33). Briefly, the uridylylation assay mixture, containing 50 mM HEPES (pH 7.5), 0.5 mM magnesium chloride, 7% glycerol, 1 μg of poly(rA), 50 μM VPg, 1 μg of WT EV71 3D^{pol} or mutants, and 1 μCi [α -³²P]UTP, was incubated at 30°C for 1 h. The reaction products were separated by Tris-Tricine SDS-PAGE. A PhosphorImager (Molecular Dynamics, Storm 860) and ImageQuant TL software (GE Healthcare) were used to produce and analyze the image file.

RNA elongation assay. The detailed protocols for the RNA elongation of EV71 3D^{pol} have been previously reported (15). The reactions with mixtures containing 20 nM WT EV71 3D^{pol} and relevant mutants, as well

as 0.01 μg of poly(rA) template (300 nucleotides in average length), 0.005 μg of oligo(dT) (6), 50 mM HEPES (pH 7.5), 25 μM UTP, 4 mM DTT, 1.5 mM MgCl₂, 60 μM ZnCl₂, 0.01% NP-40, and 0.01 μCi of [α -³²P]UTP were performed in a final volume of 50 μl. The reactions were then quenched at 10-min intervals by adding the samples directly onto DEAE filter mat papers (Wallac-PE Life Sciences). The amount of radiolabeled RNA bound to the membrane was quantified using a PhosphorImager and ImageQuant TL software.

Cells, viruses, and antibodies. African green monkey kidney cells (Vero) were cultured in Dulbecco's modified Eagle's medium (DMEM) (42) with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in 5% CO₂ at 37°C. WT and mutant viruses were produced by the electroporation of Vero cells with *in vitro*-transcribed RNA from an infectious cDNA clone, pACYC-EV71 (43). EV71 rabbit anti-VP1 polyclonal antibody was provided by the group of Hua-Lin Wang (Wuhan Institute of Virology, Chinese Academy of Sciences) (43). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG was purchased from ProteinTech Group Inc.

Plasmid for mutant EV71 infectious clone construction. EV71 genome-length cDNA clones with different mutations were constructed using pACYC-EV71. Shuttle vector pUC19-3D^{pol} was used to perform genetic engineering on the mutations. This vector was constructed by engineering the SpeI-HindIII fragment of EV71 [representing nucleotide position 5878 at the 3' end with a poly(A) tail] into the pUC19 plasmid. An Easy site-directed mutagenesis kit (Transgen, Beijing, China) was used to engineer all mutations into the shuttle vector pUC19-3D^{pol}. The mutant DNA fragments were then pasted back into pACYC-EV71 at the SpeI and HindIII sites.

FIG 1 EV71 VPg binds with 3D^{pol} *in vitro*. (A) GST pull-down assay for interactions of EV71 3D^{pol} with VPg *in vitro*. Glutathione-Sepharose beads coupled with approximately 5 μg of GST-VPg were incubated with wild-type 3D^{pol}. After the beads were washed, proteins that bound to the beads were analyzed by 12% SDS-PAGE, followed by staining with Coomassie blue. The positions of 3D^{pol} and GST-VPg are indicated by arrows on the left. The results shown are representative of at least three independent experiments. Lane M, a standard protein marker. (B) Binding affinity of VPg to wild-type 3D^{pol} measured by ITC. The binding of VPg to wild-type 3D^{pol} (at a concentration of 0.1 mM) was determined by ITC by stepwise injection of VPg at a concentration of 0.5 mM. K_d was calculated to be 57.5 μM.

***In vitro* RNA transcription, transfection, and plaque assay.** All methods for *in vitro* RNA transcription, transfection, and plaque assay were performed as described previously (33). Briefly, genome-length WT and mutant viral RNAs were transcribed from the HindIII-linearized cDNA plasmids using a MEGAscript T7 kit (Ambion). Vero cells were transfected with 5 μg of RNA as described previously (33), and the transfected Vero cells were incubated at 37°C with 5% CO₂. The viruses in culture fluids were collected at 48 h posttransfection (p.t.). Aliquots of the viruses were stored at −80°C. Plaque assays were performed by crystal violet staining, and plaque morphology and numbers were recorded after the plates were washed with tap water.

IFA. Vero cells transfected with EV71 genome-length RNA were seeded on a chamber slide (Nalge Nunc). At about 48 h p.t., the cells were fixed using cold (−20°C) 5% acetic acid in methanol for 20 min at room temperature. The fixed cells were used for immunofluorescence assay (IFA) with EV71 VP1 polyclonal antibody as described previously (33). Cell images were taken at a ×200 magnification.

Protein structure accession number. The coordinates and structure factors for the protein were deposited in the Protein Data Bank under accession number 4IKA.

RESULTS

EV71 VPg interaction with 3D^{pol} *in vitro*. We measured the binding affinity of EV71 VPg to WT EV71 3D^{pol}. Consistent with our previous study, EV71 VPg bound with 3D^{pol} in the GST pull-down assay (Fig. 1A). However, the molar ratio of GST-VPg to 3D^{pol} was not 1:1, and only a small amount of 3D^{pol} protein bound to the GST-VPg-bound resin. This finding suggested that the interaction between EV71 VPg and 3D^{pol} is not highly stable and can be weakened by the extensive washing step during the pull-down assay. Isothermal titration calorimetry (ITC) analysis revealed that the dissociation constant (K_d) between EV71 VPg and 3D^{pol} was 57.5 μM (Fig. 1B). The low binding affinity was not surprising because the VPg polypeptide should be released from 3D^{pol} after uridylylation. Interestingly, the ITC result indicated a stoichiometry of 1:1 between VPg and 3D^{pol} (Fig. 1B).

Overall structure of the EV71 3D^{pol}-VPg complex. Crystals of the EV71 3D^{pol}-VPg complex were obtained by the cocrystallization of EV71 3D^{pol} and VPg with a molar ratio of 1:2. The crystal structure of the EV71 3D^{pol}-VPg complex was determined by the molecular replacement method and refined to 2.7-Å resolution with a final R_{work} value of 21.0% (R_{free} = 26.7%; Fig. 2A and Table 1). The crystal belonged to space group P3₂21, and one complexed molecule in an asymmetric unit with a Matthews coefficient of 3.75 Å³/Da (corresponding to a 67% solvent content) was found (35). The first 20 residues of the VPg molecule bound to 3D^{pol} were constructed according to the unambiguous electron density, except for S4 of VPg (VPgS4)-VPgK8, which were not covered by the visible density (Fig. 2B), indicating the structural flexibility of this region because of the lack of interaction with 3D^{pol}.

In the complex structure of EV71 3D^{pol}-VPg, the 3D^{pol} molecule presents a structure highly similar to the apo structure (RMSD, 0.6 Å of the C-α atoms from a total of 462 residues). No obvious conformational shift was observed in the complex structure, indicating that the interaction with VPg did not change the overall structure of 3D^{pol}, which was similar to the structures in FMDV and CVB3 3D^{pol} complexed with their VPgs (26, 27). VPg was anchored at the bottom of the palm domain of the 3D^{pol} molecule and displayed an extended V-shape conformation (Fig. 2). Consistent with our previous results, the bound VPg (particularly the Y3 residue) was located a long distance from the catalytic center of 3D^{pol}, indicating that a *trans* uridylylation mechanism may be favored in EV71.

Interaction between EV71 3D^{pol} and VPg. The VPg molecule was located at the base of the palm domain of 3D^{pol} and presented a V-shaped extended conformation from the front side of the catalytic center to the back side (Fig. 2 and 3). The residues from G1 to T20 were described on the basis of their electron density, whereas the last two residues of VPg were invisible, indicating their intrinsic flexibility because of the lack of interaction with

FIG 2 Overall structure of the EV71 3D^{pol}-VPg complex. (A) The crystal structure of the EV71 3D^{pol}-VPg complex is shown as a colored ribbon covered with a transparent molecular surface in two perpendicular views. The finger, palm, and thumb domains are colored light blue, cyan, and green, respectively, and the bound VPg molecule is colored orange. The active center of 3D^{pol} is indicated by a red arrow. The catalytic base, D238, and the key tyrosine residue, Y3, are shown as colored spheres. (B) Omit map of the bound VPg molecule. The bound VPg molecule is shown as colored sticks and is covered by the omit map at 1.0 σ . The figure is shown in stereo mode.

3D^{pol}. To ensure consistency with the low binding affinity, the VPg molecule contributed an interacting surface area of 767 Å²; its total molecular surface area is 2,700 Å².

The corresponding interface on 3D^{pol} was mainly contributed by residues I311 to E343, as well as Y73, H80, Q84, and Q87. Eight hydrogen bonds, one salt bridge, and a number of van de Waals interactions helped stabilize the formation of the EV71 3D^{pol}-VPg complex (Table 2). On the basis of the positions anchoring VPg in 3D^{pol}, two major regions (named contacts I and II) were identified (Fig. 3). Contact I mainly consisted of residues H80, Q84, Q87, Y204-I205, R307, and D320 to N323 in 3D^{pol}. Contact I formed a negatively charged deep cavity to attract the first four residues (i.e., G1 to S4) of VPg (Fig. 3A and B). The carbonyl group of the VPg G1 hydrogen bond with the side chain N^ε2 atom of Q84 of 3D^{pol} (3D^{pol}Q84) (2.7 Å) and exhibited a hydrophilic interaction with the imidazole group of 3D^{pol}H80. In contrast, the amino group of VPg G1 formed a salt bridge with the 3D^{pol}D320 side chain O^δ1 atom. The amide nitrogen of VPg A2 interacted with the carbonyl group of 3D^{pol}L322 and the side chain of 3D^{pol}N323. VPg Y3, the key residue to be uridylylated, was stabilized by hydrogen bonds between its amide nitrogen and the side chain of 3D^{pol}N323.

Contact II was formed by a set of residues located in the palm domain of 3D^{pol}, including 3D^{pol}Y73, 3D^{pol}I311 to 3D^{pol}L319, and 3D^{pol}Y335 to 3D^{pol}D340 (Fig. 3C and D). This relatively hydrophobic bridge spanned the bottom of the palm domain of 3D^{pol} and clamped the last half of VPg. The amide nitrogen atom and carbonyl group of VPg A15 made contact with the carbonyl group and amide nitrogen of 3D^{pol}K315, respectively. 3D^{pol}K315 was also stabilized by interacting with VPg R17, which participated in the formation of a complicated network of hydrogen bonds for the 3D^{pol}-VPg interaction

FIG 3 Structural details of the interface between EV71 3D^{pol} and VPg. The structural details of contacts I (A and B) and II (C and D) are shown in the plane (A and C) and ball-and-stick (B and D) modes. Dashed lines indicate hydrogen-bonding and salt bridge interactions. The palm and finger domains of the EV71 3D^{pol} molecule are colored following the same color scheme described in the legend to Fig. 2, whereas contacts I and II areas are green and red, respectively. The VPg molecules are represented as yellow sticks. The superscripts indicate the origins of the labeled residues.

TABLE 2 Hydrogen bonds and salt bridge mediating the interaction between EV71 3D^{pol} and VPg

Site	VPg		3D ^{pol}		Distance (Å)
	Residue	Atom	Residue	Atom	
Hydrogen bonds	Ala2	N	Leu322	O	2.82
	Tyr3	N	Asn323	O ^{δ1}	3.10
	Ala15	N	Lys315	O	2.70
	Arg17	N	Thr313	O	2.93
	Arg17	N ^e	Tyr73	OH	3.15
	Arg17	N ^{o1}	Phe314	O	2.84
	Gly1	O	Gln84	N ^{e2}	2.70
	Ala15	O	Lys315	N	2.38
Salt bridge	Gly1	N	Asp320	O ^{δ1}	3.93

through the hydroxyl group of _{3D}Y73 as well as the carbonyl groups of _{3D}I311, _{3D}K312, and _{3D}F314 (Table 2). The carbonyl group of _{3D}I311 was also bound with the N^{ε2} atom of _{VPg}R17. This N^{ε2} atom was further stabilized by binding with the carbonyl group of _{3D}F314. _{VPg}L11 to _{VPg}L16 and the side chain of _{VPg}R17 were hydrophobically covered by residues _{3D}F314 to _{3D}I317 and _{3D}Y335 to _{3D}I339. _{3D}Y335, _{3D}P337, and _{3D}I339 were located at one side of VPg, and their large aromatic side chains were stretched outward, stacking against the main chain of all four residues from _{VPg}L11 to _{VPg}L16. In contrast, residues _{3D}F314 to _{3D}I317 were located on the other side of VPg and helped form a large, semisurrounded hydrophobic concave to interact with the VPg segment from _{VPg}L11 to _{VPg}L16.

The substitutions on the interface eliminated the EV71 3D^{pol}-VPg interaction. A complicated interaction network is crucial to the formation of the EV71 3D^{pol}-VPg complex. Accordingly, we performed a mutagenesis study on the residues located at the interface to verify the interaction between 3D^{pol} and VPg through the GST pulldown assay, in addition to our previous result (Table 3).

The residues in the contact I region on EV71 3D^{pol}, including _{3D}H80, _{3D}Q84, _{3D}Q87, _{3D}Y204-_{3D}I205, _{3D}R307, and _{3D}D320 to _{3D}N323, contributed to the stabilization of _{VPg}G1-_{VPg}S4 of VPg. We first performed mutational analysis of these residues (Fig. 4A and B). _{3D}Y204A, _{3D}I205A, and _{3D}R307A cannot produce a soluble protein (data not shown), indicating that these mutants may affect the overall structure of EV71 3D^{pol}. _{3D}E321A and _{3D}L322A had no evident effect on the interaction between 3D^{pol} and VPg, whereas _{3D}H80A, _{3D}Q84A, and _{3D}Q87A slightly affected this interaction. In contrast, _{3D}D320A and _{3D}N323A completely disrupted the interaction with 3D^{pol}.

The contact II region was formed by _{3D}Y73, _{3D}I311 to _{3D}L319, and _{3D}Y335 to _{3D}E343 to stabilize the major parts of the VPg molecule. In our previous study, we demonstrated that I311, T313, F314, and I317 play a major role in the 3D^{pol}-VPg interaction (33). Further analysis revealed that _{3D}Y73 exhibited a distinct but insignificant effect on this interaction (Fig. 4A), whereas the _{3D}L319A mutant also completely eliminated the 3D^{pol}-VPg interaction, similar to _{3D}I317A (Fig. 4B). When _{3D}Y335 and _{3D}F337 were replaced by alanine residues, they significantly disrupted the 3D^{pol}-VPg interaction, whereas _{3D}D340 and _{3D}E343 exhibited only a slight effect. In contrast, mutant _{3D}I339A retained the same amount of binding as WT 3D^{pol}, suggesting that this position was not critical for VPg binding.

The VPg residues on the interface also showed an obvious impact on the 3D^{pol}-VPg interaction (Fig. 4D). Among them, _{VPg}A2F, _{VPg}P14F, _{VPg}P14L, _{VPg}L16A, and _{VPg}R17A obviously demolished the 3D^{pol}-VPg interaction, which is consistent with the structural observation. In contrast, _{VPg}Y3F and the substitutions on _{VPg}S4 to _{VPg}K8 did not affect the binding of VPg. Since the side chain of _{VPg}Y3 is exposed to solvent and has no interaction with 3D^{pol} residues, this observation is not surprising. _{VPg}K12A also retained VPg binding like the _{VPg}Y3F mutant, which is consistent with the structural information that the side chain of _{VPg}K12 is exposed to solvent and has no contribution to the VPg-3D^{pol} interaction. Moreover, the GST pulldown results for _{VPg}S4 to _{VPg}K8 are consistent with their structural flexibility.

All these results indicate that the substitutions on the 3D^{pol}-VPg interface can significantly eliminate the physical interaction between 3D^{pol} and VPg.

TABLE 3 Summary of mutagenesis analysis^a

Mutation	IFA result	Plaque formation	% uridylation	% RNA elongation	GST pulldown assay result
WT	++++	Yes	100	100	++++
D238H	—	No	0	0	++++
Y73A	++++		85	>95	+/-
H80A	++++		70	>95	+/-
Q84A	++++		150	>95	++
Q87A			110	>95	++
I311A	—	No	40	98	+/-
K312A	++++	Yes	>100	>100	++++
T313A	+/-	No	<5	>100	+/-
F314A	—	No	<5	>100	+/-
K315A	+++	Yes	>100	>100	++++
G316A	++++	Yes	>100	90	++++
I317A	—	No	<5	>95	+/-
L319A	—	No	20	>95	—
D320A	—	No	5	>95	—
E321A	++++	Yes	200	>95	++++
L322A	—	No	75	>95	++++
N323A	+++	Yes	120	>95	+/-
Y335A	—	No	15	>95	+/-
F337A	—	No	40	>95	+/-
I339A			90	>95	++++
D340A			130	>95	++
E343A			120	>95	++

^a Summary of mutagenesis analysis of 3D^{pol}-VPg interface on EV71 replication. The replication levels of WT and mutant RNAs are categorized into nonreplicative (—), weak (+/-), medium (++), and strong (++++). The binding affinities between 3D^{pol} mutants with VPg are summarized as not observed (—), weak (+/-), medium (++), and strong (++++).

FIG 4 GST pulldown assay for EV71 3D^{pol} and relevant mutants with VPg interactions *in vitro*. Glutathione-Sepharose beads coupled with approximately 5 μ g of GST-VPg (or the relevant mutants) were incubated with either wild-type 3D^{pol} or the relevant mutants. After the beads were washed, proteins bound to the beads were analyzed by 12% SDS-PAGE, followed by staining with Coomassie blue. (A to E) Interaction between VPg and 3D^{pol} mutants. The positions of the 3D^{pol} protein and GST-VPg are indicated by the arrows on the left. The results shown are representative of at least three independent experiments.

The residues on the interface directly affected VPg uridylylation. To determine whether the residues located on the 3D^{pol}-VPg interface affected the EV71 VPg uridylylation process, we next analyzed the mutational effects on VPg uridylylation activity. The corresponding mutants with single amino acid mutations were further constructed and subjected to an *in vitro* enzymatic assay using a single-stranded poly(rA) that comprised approximately 200 to 300 nucleotides, similar to a previously described template (33). Given the instability of the D238A protein during protein purification and enzymatic assay, we replaced the catalytic base D238 with histidine to obtain a stable protein and use it as a negative control in the enzymatic assay.

In our previous study, we demonstrated that 3D^{T313A}, 3D^{F314A}, and 3D^{I317A} completely disrupted *in vitro* VPg uridylylation, whereas the 3D^{I311A} mutant retained 40% of the WT VPg uridylylation activity (33). In the current uridylylation assay, the 3D^{L319A}, 3D^{D320A}, and 3D^{Y335A} mutants showed the most significant effect on VPg uridylylation activity, whereas the 3D^{F337A} mutant exhibited 50% reduced uridylylation activity compared with that of WT 3D^{pol} (Fig. 5). The mutants with replacements at

3D^{H80} and 3D^{L322} to alanine residues retained 70% uridylylation activity, whereas the mutants with mutations at 3D^{Y73}, 3D^{Q87}, 3D^{N323}, 3D^{I339}, 3D^{D340}, and 3D^{E343} showed uridylylation activity comparable to that of WT 3D^{pol}. Unexpectedly, the 3D^{Q84A} and 3D^{E321A} mutants displayed an evident increase in uridylylation activity; in particular, 3D^{E321A} presented over 200% uridylylation activity.

The mutations on the interface did not affect the RNA elongation of EV71 3D^{pol}. We have demonstrated that the mutations on site 311 do not affect the RNA elongation activity of EV71 3D^{pol} (33). To determine whether other VPg-binding sites can function in RdRp activity, we subsequently analyzed the effects of VPg-binding-defective mutants on EV71 3D^{pol} polymerase activity (RNA elongation activity) (Fig. 6).

Consistent with the long distance between the VPg-binding site and the catalytic center of EV71 3D^{pol}, all mutants retained more than 90% of the WT RNA elongation activity. The comparable elongation activities between the WT protein and mutants allowed us to conclude that the mutations on VPg-binding sites did not change the overall conformation or catalytic activity of

FIG 5 Effect of 3D^{pol}-VPg interface mutants on VPg uridylylation. (A) The VPg uridylylation assay measured the radioactivity generated by [α -³²P]UTP-VPg. (B) The relative activities were calculated by comparing the activities of the EV71 3D^{pol} mutants with the activity of the WT protein (which was set equal to 100%). Results were obtained from three independent experiments performed in duplicate, and standard deviations ($n = 3$) are presented.

EV71 3D^{pol}. One exception was mutant L322A, whose RNA elongation activity was less than 50%. Given that L322 is located far from the catalytic center residue, we proposed that the substitution on this residue may affect the overall conformation of EV71 3D^{pol} and, thus, its enzymatic activity. Interestingly, the mutants with substitutions on H80, Q84, and Q87, as well as I317, Y335, F337, and D340, showed higher elongation activity (more than 150%) than those on the WT protein. All of these residues were localized far from the catalytic center and were unlikely to affect RNA binding. Accordingly, we speculate that they affected the overall structure of 3D^{pol} and increased the elongation activity. Nonetheless, these results revealed that the residues in the structurally identified VPg-binding site did not reduce the enzymatic

activity of EV71 3D^{pol}. Thus, the substitutions on these residues were confirmed to weaken VPg binding directly.

The interaction between VPg and 3D^{pol} played an essential role in EV71 replication. The residues located on the 3D^{pol}-VPg interface evidently affected VPg *in vitro* uridylylation; consequently, we analyzed whether these residues affected EV71 replication. We selected a subset of amino acids for mutagenesis analysis, i.e., 3D^{pol}Y73, 3D^{pol}H80, 3D^{pol}Q84, 3D^{pol}L319, 3D^{pol}D320, 3D^{pol}L322, 3D^{pol}E321, 3D^{pol}N323, 3D^{pol}Y335, and 3D^{pol}F337 from 3D^{pol} and VPgA2, VPgY3, VPgS4, VPgA6, VPgP7, VPgK8, VPgK12, VPgP14, VPgL16, and VPgR17 from VPg, according to their representative effects on VPg binding or uridylylation. Together with these residues, 3D^{pol}D238H was used as a negative control to demonstrate the total effect on EV71 3D^{pol} activity.

First, each of the selected residues on 3D^{pol} was mutated to alanine in the infectious clone of EV71. Equal amounts of WT and mutant genome-length RNAs were electroporated into Vero cells. Viral protein synthesis, plaque morphology, and viral reproduction were compared.

For viral protein synthesis, IFAs (detecting viral VP1 protein) showed similar percentages of IFA-positive cells for WT and mutant 3D^{pol}E321A, 3D^{pol}N323A, 3D^{pol}Y73A, 3D^{pol}H80A, and 3D^{pol}Q84A RNAs at 48 h p.t. In contrast, 3D^{pol}L319A, 3D^{pol}D320A, 3D^{pol}L322A, 3D^{pol}Y335A, and F337A mutants remained free of IFA-positive cells at 48 h p.t. Culture fluids were also examined at 48 h p.t. for the presence of infectious viruses by the plaque assay. No infectious virus was recovered from mutant 3D^{pol}L319A, 3D^{pol}D320A, 3D^{pol}L322A, 3D^{pol}Y335A, and 3D^{pol}F337A RNA-transfected cells (Fig. 7A).

Similar to the crucial role of the interacting residues on 3D^{pol}, mutants with substitutions on VPgA2, VPgP14, VPgL16, and VPgR17 showed obvious defects in virus replication (Fig. 7B). Although mutant VPgY3F showed the same binding to 3D^{pol} as WT VPg, the virus with the VPgY3F mutation could not replicate. Since the side chain of VPgY3 is exposed to solvent but the hydroxyl group is the exact position for uridylylation, this is an expected result. Moreover, the mutations on VPgS4 to VPgK8, as well as the VPgK12A mutation, which had no interaction with 3D^{pol} and showed no

FIG 6 Effect of VPg-binding-defective mutants on EV71 3D^{pol} RNA elongation activity. The RNA elongation assay measured the radioactivity generated by [α -³²P]UTP, and the relative activities were calculated by comparing the activities of the EV71 3D^{pol} mutant proteins with the activity of the WT protein (which was set equal to 100%). D238A and D238H mutants were used as negative controls. Results were obtained from three independent experiments performed in duplicate, and standard deviations ($n = 3$) are presented. The activity of the WT protein, which was set equal to 100%, is indicated by the dashed line.

FIG 7 Mutagenesis analysis of the VPg-3D^{pol} interface on EV71 replication. Results for the mutants of the 3D^{pol} protein (A) and VPg (B) are shown. (Top) Vero cells were transfected with WT and mutant genome-length RNAs (5 μ g) and analyzed for viral VP1 protein expression by IFA 48 h p.t.; (bottom) the plaque morphologies of WT and mutant viruses are also shown. N.D., not detectable.

obvious effect in the GST pulldown assay, did not impact virus replication.

These results suggested that the residues inside the VPg-binding site, which are essential to VPg uridylylation, play critical roles in EV71 replication.

Complementation *in trans* enhanced the uridylylation activity of VPg-binding-defective 3D^{pol} mutants. We further performed a *trans* complementation assay to determine whether EV71 replication and VPg uridylylation could be recovered. The assay involved testing the *in vitro* VPg uridylylation activity and *in vivo* viral replication with equal amounts of mutant D238H, which had a loss of activity, and VPg-binding-defective mutants (Fig. 8).

Consistent with our previous result (33), most of the VPg-binding-defective mutants retained *in vitro* uridylylation activity when combined with the same amount of mutant D238H, which

had a loss of activity (Fig. 8). Specifically, the mutant pairs D238H-Y335A and D238H-F337A recovered VPg uridylylation to more than 70% compared with that of WT 3D^{pol}.

We also performed concentration-dependent *trans* complementation by mixing 0.5 μ M Y335A mutant (a VPg-binding-defective mutant) with 0, 0.5, 1, 2, and 4 μ M D238H (Fig. 9). The uridylylation activities of 0.5 and 5 μ M Y335A, 0.5 and 5 μ M D238H, as well as 0.5 and 1 μ M WT 3D^{pol} were set as controls. As negative controls, no uridylylated VPg was detected for 0.5 and 5 μ M Y335A or 0.5 and 5 μ M D238H. In contrast, mixing Y335A and D238H remarkably increased VPg uridylylation in a D238H-concentration-dependent manner. The addition of 0.5 μ M D238H to 0.5 μ M Y335A caused a 15-fold increase in uridylylation activity, reaching 64% of the uridylylation activity of 0.5 μ M WT 3D^{pol} VPg. The addition of 1, 2, and 4 μ M D238H generated 160%, 280%, and 210% of the activity of 0.5 μ M WT 3D^{pol}. Con-

FIG 8 Complementation assay for EV71 3D^{pol} and relevant mutants in *trans*. (A) Radioactive agent-based VPg uridylylation assay; (B) summary of the results, with the activity of WT EV71 3D^{pol} being set equal to 100%. Results were obtained from three independent experiments performed in duplicate, and standard deviations (*n* = 3) are presented.

sidering that D238H cannot catalyze the polymerase reaction, this result indicated that a *trans* mechanism is favored for EV71 uridylylation.

On the other hand, the *in vivo* replication assay showed that EV71 replication cannot be recovered by *trans* complementation (data not shown). Specifically, when two genome-length RNAs (5 μg of each) containing distinct point mutations (e.g., D238H plus D320A and D238H plus Y335A) were cotransfected into Vero

cells, no IFA-positive cells were recovered at various days post-transfection. We also blindly passaged the transfected cells for three rounds (to maximize the possibility to detect cells that were cotransfected with both mutant RNAs); unfortunately, no IFA-positive cells were detected. The failure of *trans* complementation in cell culture agrees with our recently published results (33). One possible reason for the failure of virus recovery in cell culture was the low efficiency of the cotransfection with two mutant RNAs. However, Tiley et al. reported that a defect in the cre structure in FMDV, which is critical for viral replication, can be complemented in *trans* through coinfection with other mutants (24). According to the same work from Tiley et al. (24), we speculate that the mutations may disrupt the genomic RNA structure, which is crucial for viral replication. However, the sequences of the mutants used in our *in vivo* complementation assay did not include the possible cre portion of the EV71 genome. Therefore, we hypothesize that the discrepancy between *in vitro* and *in vivo trans* complementation in our work could be due to compartmentalization of the replication complex inside cells, preventing the exchange of distinct mutant 3D^{pol} molecules between two replication complexes.

DISCUSSION

Variation in protein primer binding sites among *Picornaviridae* members. The replication of picornavirus is characterized by the VPg uridylylation process to generate a protein primer by linking two UMPs to the third tyrosine residue of the virally encoded protein VPg by RdRp. The crystal structures of the FMDV and CVB3 3D^{pol} molecules complexed with their VPgs revealed a significantly variable VPg-binding model in picornavirus, although

FIG 9 Complementation in *trans* shows that uridylylation occurs in a D238H-concentration-dependent manner. The results of the radioactive agent-based *trans* complementation assay are summarized, with the activity of 0.5 μM WT EV71 3D^{pol} being set equal to 100%. Results were obtained from three independent experiments performed in duplicate, and standard deviations (*n* = 3) are presented.

four residues overlap the CVB3 VPg-binding site but can affect the VPg uridylylation reaction, which is discrepant with the effect on CVB3. Although the key residues of RdRps, particularly the residues involved in the catalytic reaction, were highly conserved in the members of the *Picornaviridae* family, the residues that participated in the reported VPg-binding sites showed less sequence conservation, suggesting the intrinsic variation in VPg binding.

Combined with our previous result, we identified a novel VPg-binding site on picornavirus 3D^{pol} by structurally and biochemically investigating the VPg uridylylation process of EV71. The VPg molecule was located at the base of the palm domain of EV71 3D^{pol} and presented an extended conformation from the front side of the catalytic center to the back side. The interface between EV71 3D^{pol} and VPg was mainly contributed by residues _{3D}I311 to _{3D}E343, as well as _{3D}Y73, _{3D}H80, _{3D}Q84, and _{3D}Q87. This interface comprised two major regions, i.e., contacts I and II. Contact I was a negatively charged deep cavity that stabilized the first four residues of VPg, whereas contact II was formed by _{3D}Y73, _{3D}I311 to _{3D}L319, and _{3D}Y335 to _{3D}D340 to clamp the last half of VPg. Biochemical and *in vivo* viral replication analysis revealed that the substitutions on the key residues on the interface, which accounted for the interaction between 3D^{pol} and VPg, evidently affected VPg uridylylation and EV71 replication, indicating that this binding site is a functional site for the VPg uridylylation process of EV71. None of these mutants abrogated the RNA elongation activity, which suggested that this site is involved in VPg binding but not in the catalytic reaction.

As mentioned in our previous work, we propose that site 311 likely binds specifically with _{VPg}V10–_{VPg}L11 of EV71 VPg because the major cavity in site 311 is mainly formed by hydrophobic residues (33). In the crystal structure of EV71 3D^{pol}-VPg, the residues in site 311 directly interacted with the _{VPg}L11, _{VPg}P14, _{VPg}A15, and _{VPg}L16 residues of the VPg molecule (Fig. 2C and 2B). Although the VPg residues anchored at site 311 were inconsistent with our previous speculation, the electric properties of _{VPg}P14–_{VPg}L15 were consistent with the hydrophobic interaction between site 311 and VPg residues. For example, _{3D}F314 had extensive intermolecular interactions with _{VPg}R17 of the VPg molecule. _{3D}F314 also made contact with the carbonyl group of _{3D}I311 (3.89 Å) and _{3D}K312 (3.4 Å) to stabilize the conformation of these two residues and enhance the interaction between EV71 3D^{pol} and VPg. The large aromatic side chain of _{3D}F314 contributed to establishment of the hydrophobic concave and interaction with the middle segment within VPg. This structural information refined our biochemical findings.

Another notable observation was that residues _{VPg}S4 to _{VPg}K8 of the VPg molecule had no density in the crystal structure of EV71 3D^{pol}-VPg, indicating the intrinsic structural flexibility because of the lack of interaction with 3D^{pol}. This finding was consistent with that of the *in vivo* mutagenesis analysis, which revealed that the replication of mutants _{VPg}S4A, _{VPg}A6L, _{VPg}P7A, and _{VPg}K8A was comparable to that of WT virus (Fig. 7B). Thus, these residues may not be included in the formation of the EV71 replication complex and are thus unnecessary for EV71 replication.

The residues located on the 3D^{pol}-VPg interface showed different effects on VPg uridylylation. The VPg-interacting residues in EV71 3D^{pol} evidently affected VPg uridylylation and viral replication. Among these VPg-interacting residues, _{3D}L319, _{3D}D320, and _{3D}Y335 showed the most significant disruptive effect on VPg uridylylation, consistent with a similar disruptive effect on VPg

binding and viral replication. These observations revealed that these three residues affected EV71 replication by directly binding with VPg, which played the same role as the key residues in site 311. Notably, _{3D}D320 formed only a very weak bond (3.93 Å) with _{VPg}G1 of VPg through the O⁰¹ atom of _{3D}D320 in the crystal structure, suggesting that this residue is not likely to directly contribute to the 3D^{pol}-VPg interaction through stabilization of the VPg residue. However, _{3D}D320 formed a few more bonds with the N^{e2} atom of _{3D}H80 via its O⁰¹ atom (3.34 Å) and the amide nitrogen of _{3D}L322 via its carbonyl group (3.82 Å). These phenomena helped stabilize the proper conformation of _{3D}L322 and _{3D}H80, thereby facilitating the correct interaction of these two residues with VPg. It is noticeable that the _{VPg}G1 residue is partially buried in a charged cavity in the EV71 3D^{pol}-VPg complex structure, but the fusion protein GST-VPg, in which several flexible linker residues are connected to the N terminus of VPg, is still working in the measurement of the VPg-3D^{pol} interaction in the GST pulldown assays. This discrepancy could be due to the weak contribution of _{VPg}G1 to the 3D^{pol}-VPg interaction.

Similar to _{3D}I311 in site 311, the replacement of _{3D}F337 by alanine resulted in 40% retained VPg uridylylation activity, although VPg binding was significantly weakened. However, all of these mutants showed complete disruption on EV71 replication. The other residues on the EV71 3D^{pol}-VPg interface showed no evident effect on VPg binding, uridylylation, or viral replication. This observation revealed that these residues are not critical to VPg uridylylation, which was attributed to the weak interaction contributed by these residues to the VPg molecule.

A few of the most interesting findings were those on the interacting residues. _{3D}L322, which was located near site 311, did not affect VPg binding and retained more than 70% uridylylation activity once it was mutated to an alanine residue. However, the _{3D}L322A mutant showed a significant effect on viral replication. No IFA signal or plaque could be observed at 48 h p.t. In contrast, although the _{3D}L322A mutant did not weaken VPg binding (Fig. 4B), the VPg uridylylation activity of this mutant was comparable to (or even greater than) that of WT 3D^{pol} (Fig. 5).

Moreover, the replacements on _{VPg}A2, _{VPg}P14, _{VPg}L16, and _{VPg}R17 obviously caused defects in virus replication. Since the side chain of _{VPg}Y3 is exposed to solvent but the hydroxyl group is the exact position for uridylylation, this is an expected result, in that the mutant _{VPg}Y3F showed the same binding to 3D^{pol} as WT VPg but the virus with the _{VPg}Y3F mutation could not replicate. Moreover, the mutations on _{VPg}S4 to _{VPg}K8, which had no interaction with 3D^{pol} and showed no effect in the GST pulldown assay, did not affect virus replication.

A *trans* mechanism was favorable for EV71 uridylylation. The localization of the key tyrosine residue of VPg is one of the major indicators of whether a *cis* or a *trans* uridylylation mechanism is favored by *Picornaviridae* members. In the crystal structure of FMDV 3D^{pol}-VPg1, VPg1 fits the RNA binding cleft of the polymerase and projects the key residue Y3 into the active site of 3D^{pol} (26). In contrast, although the first six residues of VPg (including the key tyrosine residue) are missing in the crystal structure of the CVB3 3D^{pol}-VPg complex, the position of the CVB3 VPg P7 residue relative to the front entry point of RdRp is too far to allow direct VPg uridylylation by 3D^{pol} in an *in cis* mode (27). This observation indicates that two 3D^{pol} molecules may be necessary for VPg uridylylation and the first six VPg residues should

not strongly interact with the carrier 3D^{pol} but, rather, should be stabilized by uridylylating 3D^{pol} in CVB3.

Our previous biochemical data suggested that an *in trans* VPg uridylylation mechanism was favored in EV71. Consistent with this biochemical finding, the key tyrosine residues were located at the back entry point of EV71 3D^{pol}, which was far from the catalytic center at D238. The bound VPg cannot access the catalytic center and be uridylylated by the carrier 3D^{pol}. This structural observation, which was similar to that for CVB3 (27), was further supported by observations that poliovirus 3D^{pol} forms extensive oligomeric arrays *in vitro* and *in vivo* and that dimerization or oligomerization is necessary for primer-dependent RdRp activity *in vitro* (44, 45). The uridylylation activity of VPg-binding-defective 3D^{pol} mutants can be enhanced by *trans* complementation with the D238H mutant, and this *trans* complementation depends on the D238H concentration. Given that D238H cannot catalyze the polymerase reaction, we concluded that an *in trans* uridylylation mechanism was favored by EV71. VPg residues _{VPg}S4 to _{VPg}K8 were covered with a weak electron density in the crystal structure of the EV71 3D^{pol}-VPg complex, suggesting the lack of interaction with the carrier 3D^{pol}. These residues were also dispensable for EV71 replication.

According to these observations, we hypothesized that VPg may play two roles in the EV71 replication complex. First, VPg may play a scaffolding role in the formation of replication oligomeric arrays through interacting with 3D^{pol} via the well-ordered residues in the crystal structure. However, the structurally invisible fragment from _{VPg}S4 to _{VPg}K8 was not involved in the interaction with 3D^{pol} and may also not bind with the other plausible factors in the EV71 replication complex. Second, VPg may function as a substrate of another uridylylating 3D^{pol} in the arrays and may be released from the carrier 3D^{pol} and able to access the catalytic center of the adjacent uridylylating 3D^{pol}. In this hypothesis, the structurally missing and functionally dispensable fragment from _{VPg}S4 to _{VPg}K8 may interact with the 3D^{pol} residues and help stabilize VPg for further uridylylation, similar to that in the FMDV 3D^{pol}-VPg1 complex (26). Larger EV71 replication complexes are necessary to finalize our insight into the entire process of EV71 replication.

In summary, we reported the structure of EV71 3D^{pol} complexed with its protein primer VPg and then confirmed the presence of a novel VPg-binding site combined with our previous biochemical results. VPg was anchored at the bottom of the palm domain of the 3D^{pol} molecule and showed a V-shaped extended conformation. The corresponding interface on 3D^{pol} was mainly contributed by residues I311 to E343, as well as Y73, H80, Q84, and Q87. A subsequent *in vitro* uridylylation assay revealed that L319A, D320A, and Y335A significantly disrupted VPg uridylylation; however, they did not evidently affect the 3D^{pol} RNA elongation activity. Further analysis revealed that *trans* complementation can enhance the uridylylation activity and that an *in trans* uridylylation mechanism was favored in EV71. HFMD is one of the major infectious diseases in Asia, particularly in China, and no effective therapeutic regimen is currently available for HFMD, caused by EV71. Although a number of studies on vaccines and inhibitors targeting EV71 3C^{pro} have been conducted (46–49), no report on EV71 3D^{pol} has been presented. The blockage of this VPg-binding site by either chemical compounds or peptides derived from EV71 VPg could potentially lead to the inhibition of the EV71 VPg-3D^{pol} interaction and suppress viral replication.

These results indicate a novel site for potential inhibitors of EV71 replication, apart from traditional nucleotide analog inhibitors.

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