Crystal Structures of Enterovirus 71 (EV71) Recombinant Virus Particles Provide Insights into Vaccine Design*

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Background: No HFMD vaccine is available.

Results: Structures of EV71 recombinant virus particles were determined showing that the inserted foreign peptide is exposed on the particle surface without capsid structural changes and that virus uncoating is not affected.

Conclusion: VP1 BC loop is suitable for foreign peptide insertion for the generation of recombinant EV71 viruses.

Significance: The results provide insights into vaccine development.

Hand-foot-and-mouth disease (HFMD) remains a major health concern in the Asia-Pacific regions, and its major causative agents include human enterovirus 71 (EV71) and coxsackievirus A16. A desirable vaccine against HFMD would be multivalent and able to elicit protective responses against multiple HFMD causative agents. Previously, we have demonstrated that a thermostable recombinant EV71 vaccine candidate can be produced by the insertion of a foreign peptide into the BC loop of VP1 without affecting viral replication. Here we present crystal structures of two different naturally occurring empty particles, one from a clinical C4 strain EV71 and the other from its recombinant virus containing an insertion in the VP1 BC loop. Crystal structure analysis demonstrated that the inserted foreign peptide is well exposed on the particle surface without significant structural changes in the capsid. Importantly, such insertions do not seem to affect the virus uncoating process as illustrated by the conformational similarity between an uncoated virion and an EV71 particle. The results provide insights into vaccine development against HFMD.

Hand-foot-and-mouth disease is an infectious disease found in infants and young children worldwide that may lead to death (1). In recent years, numerous outbreaks of hand-foot-and-mouth disease have occurred in Asia-Pacific regions, causing significant morbidity and mortality. Moreover, no vaccines are available at present. Human enterovirus 71 (EV71) and coxsackievirus A16, both enterovirus species A, have been identified as the major causative agents. Thus, to better control and prevent hand-foot-and-mouth disease, a multivalent vaccine against both EV71 and coxsackievirus A16 is highly desirable.

Several groups, including ours, have reported the construction of recombinant enteroviruses for potential use as novel multivalent vaccines (2–5). When the VP1 BC loop (residues 94–102) used for insertion mutagenesis in a carrier type 1 (P1/Mahoney) poliovirus was exchanged with the amino acid sequences of the same loop (six residues) in a type 2 (P2/Lansing) poliovirus, the resulting virus displayed not only the mosaic of antigenic sites from both viruses but also the mouse adaptation and neurovirulence of the P2/Lansing strain (3). When VP1 residues 90–105 of type 1 poliovirus were replaced by the corresponding region of type 3, the resulting hybrid poliovirus elicited neutralizing antibodies against both types 1 and 3 in rabbits and monkeys (4). When VP1 residues 91–102 of type 1 poliovirus were substituted by 18 HIV-1-specific amino acids, the poliovirus chimera elicited broadly reactive HIV-1-neutralizing antibodies (5). Recently, we demonstrated that the VP1 BC loop in EV71 is also subjected to the insertion of foreign polypeptides of various lengths with the function of the inserted polypeptide and viral replication both preserved (2). Successful rescue of these recombinant viruses demonstrated that the VP1 BC loop exposed on the surface of enteroviruses is able to accommodate a broad range of sequences, leading to the hypothesis that recombinant enteroviruses may be useful as novel vaccines.

Structural studies have been performed to examine the conformational changes upon replacement or insertion in surface loops. The two polioviruses (types 1/2 or types 1/3) contributing to poliovirus chimeras are closely related, and relatively small conformational alterations were found in the exchanged

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§ § The abbreviations used are: EV71, enterovirus 71; HIV-1, human immunodeficiency virus, type 1; r.m.s.d., root mean square deviation.
loop when it was placed within the different context (6). Upon the insertion of 12 amino acids of the HIV-1MN gp120 V3 loop into Ala-59 and Asn-160 of the VP2 puff in human rhinovirus type 14, significant structural differences occurred at the site of the insertion and in a surface loop of VP1 near the canyon, including residues 213–224; such conformational changes in VP1 are similar to the observations when antiviral agents were bound to this virus (7). Cowpea mosaic virus has also been developed into a platform for foreign epitope presentation. When the 14-amino acid-long antigenic epitope (the NIm-IA site) of human rhinovirus type 14 was inserted between amino acids 22 and 23 of the VP1 BC loop of cowpea mosaic virus, some VP1 residues underwent movement, especially residues 24–28 (8). All these structural studies have provided clues for structure-based vaccine design.

Picornaviruses can produce empty particles, which resemble the mature virion in morphology and antigenicity, sediment at ~80 S, and comprise 60 copies of VP0, VP1, and VP3 (9–11). Although empty particles are not thought to be direct precursors of mature virions, they may function as reservoirs of capsid components (11–13). For poliovirus, empty particles are unstable, readily converting from D-type native antigenicity to a state with altered antigenic properties (C-type) (9, 14, 15). EV71 cultured for vaccine development produces both full virions and empty particles, and the content of empty particles is at least 2-fold more than the full virion in the final production yield (16). To save on the cost for vaccine production, the empty particles of EV71 can be considered as an additional antigen to the full virion. Thus, detailed structure analysis on empty particles of EV71 recombinant viruses can aid structure-based vaccine development.

Recently, we and others have reported the structures of the virion, the naturally occurring empty particle, an uncoating intermediate or the A-particle, and the empty particle after the uncoating of EV71 as determined by x-ray crystallography or cryo-EM techniques (17–21). Despite its elusive role during the viral life cycle, the empty particle shares similar structural features with the virion and the uncoating intermediate. The capsid in all these different particles comprises 60 copies of capsid proteins organized onto a T = 1 (pseudo T = 3) icosahedral lattice. The viral capsid proteins VP1, VP2 (VP0 in the empty particle), and VP3 all fold into a canonical eight-stranded β-barrel structure with the BIDG strands as one edge and the CHEF strands as the other and loops connecting various portions of each capsid protein, including the β-strands. Structural variations are present in the N- and C-terminal regions of capsid proteins and the connecting loops between the virion and the empty particle. Nonetheless, immunological studies have demonstrated that antibodies generated against both particles recognize the same immunodominant linear epitope of VP1 (residues 211–225), suggesting that the structure of the empty particle may not be different enough from that of the virion to influence important antigenic and immunogenic sites (22). Thus, structural analyses of the empty particle of enteroviruses offer an alternative approach to understand the mechanism of capsid assembly and immunogenicity.

Here we report crystal structures of the empty particle from a clinical C4 strain EV71 and one EV71 recombinant virus showing that such insertions into the VP1 BC loop do not affect the overall capsid assembly and structure. Moreover, we present the crystal structure of an uncoating intermediate of another EV71 recombinant virus demonstrating that such insertions do not seem to affect the virus uncoating process and thus virus replication. Our structural studies thus provide insights into the development of more effective epitope presentation and aid in novel vaccine design.

**EXPERIMENTAL PROCEDURES**

*Generation of EV71 Recombinant Viruses—* The coding nucleotides for peptides NE1 (ERKRARLT) and N6 (SVKRGTSVGMKP-SPRP) were each individually cloned into the VP1 BC loop (between residues 100 and 101) of a full-length infectious cDNA clone of a clinical EV71 C4 strain (strain AH08/06) as described previously (2). The engineered viruses carrying these foreign peptides were recovered by transfecting the *in vitro* transcribed RNAs into human rhabdomyosarcoma (RD) cells and were named EV71-NE1 and EV71-N6, respectively. DNA sequencing results confirmed that these peptides were successfully engineered into the VP1 amino acid sequence.

*Virus Purification—* EV71 and the recombinant viruses (EV71-NE1 and EV71-N6) were all grown and purified as described previously (20). The virions and empty particles were separated by sedimentation through a discontinuous 10–50% sucrose gradient by no-brake ultracentrifugation at 26,000 rpm for 3 h. Fractions were collected and resolved by 12% SDS-PAGE. Fractions containing empty particles or virions were separately collected and concentrated by ultracentrifugation at 28,000 rpm for 4 h at 4 °C. The pelleted particles were resuspended in PBS buffer (pH 7.2) to a final concentration of ~3 mg/ml. The quality of the empty particles and virions was examined by negative staining electron microscopy, which showed a high degree of homogeneity.

*Crystallization of Empty Particles and Diffraction Data Collection—* The purified empty particles of EV71 and EV71-NE1 were all subject to crystallization trials. Crystals were obtained by the vapor diffusion method in hanging drops at 16 °C by mixing 2 µl of empty particles (~3 mg/ml) and 2 µl of the reservoir solution. The crystallization condition for the empty particles of both EV71 and EV71-NE1 was 0.1 M imidazole (pH 6.5) containing 1.0 M sodium acetate.

Prior to data collection, cryoprotection of the crystals was achieved by resuspending the crystals in the mother liquor with increasing concentrations of glycerol through six steps: 5, 10, 15, 20, 25, and 30% (v/v). The equilibration time at each concentration was at least 30 s. These crystals were then flash frozen in liquid nitrogen and used for diffraction data collection with an MX-225 (Rayonix) or a Quantum-315 charge-coupled device (Area Detector Systems Corp.) detector at beamline BL17U1 at Shanghai Synchrotron Radiation Facility. For the EV71 empty particle, a data set at 3.15-Å resolution was collected with monochromatic x-rays (λ = 0.97916 Å), a detector to crystal distance of 330 mm using an oscillation angle of 0.3°, and an exposure time of 1 s. For the EV71-NE1 empty particle, a data set at 3.10-Å resolution was collected with monochromatic x-rays (λ = 0.97803 Å), a detector to crystal distance of 300 mm using an oscillation angle of 0.3°, and an exposure time
of 1 s. Indexing, integration, scaling, postrefinement, and reduction of the data were carried out using the HKL2000 software package (23). A total of 100 and 60 diffraction images were used for the data processing of the EV71 and EV71-NE1 empty particles, respectively.

**Crystallization of EV71-N6 Virions and Diffraction Data Collection**—The purified EV71-N6 virions were subjected to crystallization trials. The crystallization condition was 0.1 M MES (pH 6.8) containing 1.6 M sodium acetate. A data set at 3.8-Å resolution was collected with monochromatic x-rays (λ = 1.281579 Å), a detector to crystal distance of 350 mm using an oscillation angle of 0.3°, and an exposure time of 1.0 s. Indexing, integration, scaling, postrefinement, and reduction of the data were carried out using the HKL2000 software package (23). A total of 50 diffraction images were used for data processing.

**Structure Determination and Refinement**—The crystals of the EV71 and EV71-NE1 empty particles all belong to space group P4_3 2_1 with five copies of the protomer as the asymmetric unit. Thus, 5-fold non-crystallographic symmetry was used during the structure determination and refinement. The program GLRF (24) was used to calculate the self-rotation function and was combined with crystal packing analysis to determine the position and orientation of the particle. Each individual structure was then determined by the molecular replacement method using the program package PHENIX (25). To avoid model bias, the crystal structure of the poliovirus type 1 empty particle (Protein Data Bank code 1POV) (9) was taken as the search model, and a single solution was obtained. The crystals of EV71-N6 also belong to space group P4_3 2_1, and the same search model, and a single solution was obtained. The crystals model bias, the crystal structure of the poliovirus type 1 empty particle was combined with crystal packing analysis to determine the position and orientation of the particle. Each individual structure was then determined by the molecular replacement method using the program package PHENIX (25). To avoid model bias, the crystal structure of the poliovirus type 1 empty particle (Protein Data Bank code 1POV) (9) was taken as the search model, and a single solution was obtained. The crystals of EV71-N6 also belong to space group P4_3 2_1, and the same approach was used for the structure determination. After obtaining the initial density map, model building and refinement were carried out iteratively using the Coot (26) and PHENIX (25) programs, respectively. The final refinement statistics are summarized in Table 1. The coordinates and structural factors of the EV71 and EV71-NE1 empty particles and EV71-N6 have been deposited in Protein Data Bank (Protein Data Bank codes 4RQP, 4RR3, and 4RS5). Figures were drawn and rendered using PyMOL (27) and UCSF Chimera (28).

**Characterization of the EV71-N6 Uncoating Intermediate**—To detect the presence of VP4 in the N6 uncoating intermediate, 1 μg of trypsin was added to a 10-μl solution containing 1 μg of the corresponding particles. Samples with or without trypsin were incubated at 16 °C. Digestion was stopped by dilution of the samples with 1/5 volume of 5× SDS loading buffer followed by boiling of the mixture for 10 min. The samples were subjected to 15% SDS-PAGE, and VP4 was visualized by Western blotting using the anti-VP4 antibody (Biorbyt).

**Generation of EV71 Uncoating Particles**—The purified EV71 full virions were concentrated to 0.1 mg/ml, dissolved in the uncoating buffer (10 mM Tris-HCl, 20 mM NaCl, 5 mM MgCl2 (pH 7.5)), and heated at 56 °C for 12 min.

**Proteolytic Digestion of the EV71 Empty Particle**—The proteolytic sensitivity of VP1 in the EV71 empty particle was assessed by adding 5 μg of trypsin to 1 μg of the particles in a 15-μl volume. Trypsin digestion was performed in parallel at 16 and 37 °C for 1 h. Digestion was stopped by dilution of the samples with 1/5 volume of 5× SDS loading buffer followed by boiling of the mixture for 10 min. The samples were then subjected to SDS-PAGE analysis, and VP1 was visualized by Western blotting using an anti-VP1 antibody (Abnova).

To test whether the proteolytic sensitivity of VP1 in the uncoating particle is similar to that in the empty particle, 1 μg of trypsin was added to 1 μg of particles in a 15-μl volume. The samples were incubated at 16 °C for 1 h and then subjected to SDS-PAGE analysis with VP1 visualized by Western blotting using the anti-VP1 antibody. The proteolytic sensitivity of VP0 in the empty particle was assessed by adding 1.6 μg of trypsin to 1.6 μg of particles in a 15-μl volume. The samples were incubated in parallel at 16 and 37 °C for 1 h. Digestion was stopped by dilution of the samples with 1/5 volume of 5× SDS loading buffer followed by boiling of the mixture for 10 min. The samples were then subjected to SDS-PAGE analysis, and VP4 was visualized by Western blotting using the anti-VP4 antibody (Biorbyt).

**N-terminal Sequencing Analysis of VP1 Cleavage Products**—The digested products of VP1 were separated by 12% SDS-PAGE analysis, and VP1 was visualized by Western blotting using the anti-VP1 antibody. To detect the presence of VP4 in the N6 uncoating intermediate, 1 μg of trypsin was added to a 10-μl solution containing 1 μg of the corresponding particles. Samples with or without trypsin were incubated at 16 °C. Digestion was stopped by dilution of the samples with 1/5 volume of 5× SDS loading buffer followed by boiling of the mixture for 10 min. The samples were then subjected to SDS-PAGE analysis, and VP4 was visualized by Western blotting using the anti-VP4 antibody (Biorbyt).

![Image](image-url)

**Table 1**

| Parameter | Value for EV71 | Value for EV71-NE1 | Value for EV71-N6 |
|-----------|----------------|--------------------|-------------------|
| **Space group** | P4_3 2_1 | P4_3 2_1 | P4_3 2_1 |
| **Resolution (Å)** | 49.43-3.15 | 43.49-3.10 | 46.79-3.805 |
| | (3.24-3.15) | (3.19-3.10) | (3.94-3.80) |
| **Unique reflections** | 110105/5658 | 314603/15490 | 66735/6642 |
| **Rmerge (%)** | 18.2 (90.4) | 21.6 (70.1) | 23.2 |
| **Redundancy** | 7.9 (6.0) | 1.5 (1.2) | 3.7 (3.7) |

<sup>a</sup> Values in parentheses refer to the highest resolution shell.
**RESULTS**

**Generation of EV71 Recombinant Viruses**—Previous studies have demonstrated that amino acids 97–105 of the VP1 BC loop in EV71 are well displayed on the particle surface and constitute a neutralizing epitope and is well displayed on the virus surface. EV71 genome organization and design of EV71 insertion mutants. In the case of EV71-NE1, an insertion of 8 amino acids resulted in the loss of residues 101 and 102 (TN) in VP1 in the rescued mutant virus.

blot transfer buffer (10 mM CAPS, 10% methanol) for 20 min and then subjected to electroblotting onto a polyvinylidene fluoride membrane (Bio-Rad). After transfer, the membrane was stained with Coomassie Brilliant Blue R-250 for 1 h and washed with a buffer containing 50% (v/v) ethanol and 10% acetic acid. The appropriate membrane segments were excised and subjected to Edman degradation using an ABI PROCISE® 494 (Shanghai GeneCore BioTechnologies).

**Structures of EV71 Recombinant Virus Particles**

**Purification of EV71 and Recombinant Viruses**—The EV71 and mutant viruses were each propagated and purified as described under “Experimental Procedures.” Two different forms of particles were obtained by discontinuous sucrose gradient ultracentrifugation: one is the naturally occurring empty particle, and the other is the mature virion (Fig. 2, A–C). Biochemical analyses showed that the empty particle contains VP0, VP1 (or mutant VP1), and VP3, whereas the virion contains VP1–4 (20). EM analyses showed that the empty particle exhibits only an empty shell, whereas the virion displays a dark center within the shell due to the RNA genome inside (Fig. 2, D and E).

**Crystal Structure of the EV71 Empty Particle**—The crystal structure of EV71 empty particle was determined at 3.15-Å resolution (Fig. 3A). After iterative model building and structure refinement, the electron density is visible in Fig. 4, B, C, and D, for residues 73–296 in VP1, 82–319 in VP0 (13–250 in VP2), and 1–175 and 189–238 in VP3. This structure has a higher resolution (3.15 Å) compared with the reported empty particle structure (4.0 Å) (Protein Data Bank code 3VBU) (17). Although these two viruses used for the structural study are both C4 strains, an amino acid sequence comparison showed a total of 5 amino acid changes in the capsid proteins (residues 98 and 225 in VP1, residue 144 in VP2, and residues 93 and 227 in VP3). The overall structures of the empty particles from both strains are similar, including the lack of ordered structures in the VP4 portion of VP0, the RNA genome, and the N terminus of VP1; the loss of pocket factors in the VP1 pocket region; and the opening of channels at the 2-fold symmetry axis. Despite these similarities, some conformational variations are observed. Notable differences are mapped to the GH loops of VP1 and VP3. The GH loop of VP1 has been identified as a major neutralization epitope (31) and modeled as a disordered region in the reported structure (Protein Data Bank code 3VBU); in our structure, the main chain backbone of this loop can still be traced, although part of the side chain atoms are invisible. Conversely, the GH loop of VP3 modeled as a loop region in the reported structure appears to be disordered in our density map. Superimpositions of the individual VP1, VP0, and VP3 proteins, onto these two particles resulted in calculated r.m.s.d. values of 1.28 (217 Ca), 1.03 (237 Ca), and 1.09 Å (225 Ca), respectively, for equivalent Ca atoms.

The empty particle structure was also compared with that of the virion (Protein Data Bank code 4N53) (20). The calculated r.m.s.d. is 2.77 (224 Ca), 1.80 (238 Ca), and 2.11 Å (225 Ca) for equivalent Ca atoms in VP1, VP2 (portion of VP0), and VP3, respectively. Interestingly, the same hydrogen bonding network at the 5-fold axis channel is also maintained in the empty particle, similar to those of the virion and the uncoating intermediate (Fig. 3B).

The empty particle structure is rather similar to that of the uncoating intermediate. One notable difference lies in the N-terminal portion of VP2 (amino acids 47–52) near the bottom of the junction, which was modeled as a loop region in the empty particle but is disordered in the uncoating intermediate, suggesting more drastic conformational changes during uncoating (20).
Crystal Structure of the EV71-NE1 Empty Particle—The crystal structure of the EV71-NE1 empty particle was determined to assess the effects of the insertion on the capsid structure. Despite insertion of the NE1 peptide, the structure of the EV71-NE1 empty particle is rather similar to that of the EV71 empty particle, including the lack of ordered structure in the RNA or VP4 portion of VP0 and the N-terminal extensions of VP1 and the loss of pocket factors.

The structure of the EV71-NE1 empty particle (8-amino acid insertion into the VP1 BC loop) was determined at 3.1-Å resolution (Fig. 4A). After iterative model building and structure refinement, the electron density is visible for residues 72–303 for VP1, 80–321 for VP0 (11–252 of VP2), and 1–178 and 189–240 for VP3 (Fig. 4, B, C, and D). The inserted NE1 epitope is well exposed on the particle surface (Fig. 4A). Despite a relatively weak electron density, most of the NE1 main chain atoms can still be traced. Superimposition of the individual VP1, VP0, and VP3 proteins of the empty particle of EV71-NE1 with those of EV71 resulted in calculated r.m.s.d. values of 1.39 (224 Ca), 2.04 (238 Ca), and 0.61 Å (225 Ca), respectively, for equivalent Ca atoms. The most significant structural differences are as follows: 1) at and near the site of the BC loop insertion between residues 97 and 106 of VP1, 2) residues 209–221 of VP1 (located in the GH loop) that were previously identified as the neutralizing epitope (31), 3) residues 93–100 of VP0 (residues 34–41 of VP2) (eight Ca atoms; r.m.s.d., 2.75 Å), 4) residues 112–122 of VP0 (residues 43–53 of VP2), and 5) residues 204–212 of VP0 (135–143 of VP2) that are near the identified linear cross-genotype neutralizing epitope (141–150 of VP2) (22) (Fig. 4, B, C, and D).

The electron density for the inserted residues is somewhat weaker or even missing when compared with the density for residues not close to the modification. This suggested that conformational flexibility at the insertion may be static (slightly different, but rigid, conformations in the 60 subunits) or dynamic (a flexibility corresponding to the continuous mobility...
of that portion of the polypeptide in the crystal) (Fig. 4E). Nonetheless, the inserted residues form an extended loop on the particle surface (Fig. 4A).

In summary, insertions in the VP1 BC loop did not affect EV71 assembly, and the overall structure of the EV71 empty particle was maintained. The disposition of the VP1–3 subunits is largely maintained in the recombinant EV71 empty particle compared with that in the EV71 empty particle.

**Modeling of the Recombinant Virus EV71-NE1 Structure**—Structural modeling using the Phyre2 (Protein Homology/Analogy Recognition Engine 2) server (32) showed that the inserted peptide NE1 protrudes from the natural VP1 subunit to be uniformly displayed on the surface of EV71 without affecting the original structure of the EV71 virion (Fig. 5).

**Crystal Structure of EV71-N6 (an Uncoating Intermediate) —**
The structure of EV71-N6 was determined at a resolution of 3.8 Å (Fig. 6A). The refined model includes residues 73–98 and 119–313 of VP1, 11–47 and 54–252 of VP2, and 1–174 and 190–238 of VP3 (Fig. 6, B–D). Residues 99–118 of VP1 (including the N6 peptide), residues 48–53 of VP2, and residues 175–189 of VP3 are disordered. The N-terminal region (residues 1–72) of VP1 is disordered, and the pocket region is empty. We previously demonstrated that an EV71 uncoating intermediate can be obtained through crystallization experiments (20). This determined EV71-N6 structure shares some structural features with the EV71 uncoating intermediate, such as the opening of 2-fold channels and the lack of ordered densities in the N-terminal regions of VP1 and VP4. Furthermore, a Western blot analysis showed that VP4 is still present in the EV71-N6 crystal (Fig. 6E), suggesting that this particular crystallization condition converted the EV71-N6 virion into an uncoating intermediate.

Structural comparison with that of the EV71 uncoating intermediate was performed. Superimpositions of the individual VP1, VP2, and VP3 proteins of these two particles resulted in r.m.s.d. values of 1.39 (220 Cα), 1.59 (229 Cα), and 1.14 Å (221 Cα), respectively, for equivalent Cα atoms. The most significant structural differences are as follows: 1) at the site of the BC loop insertion between residues 98 and 103 of VP1 (ordered in the EV71 structure; disordered in EV71-N6) and residues 209–221 of VP1 (13 Cα, 2.26 Å), 2) residues 26–32 (at the inner side of the capsid) and 43–47 of VP2 (near the pseudo-3-fold junction), 3) residues 135–145 of VP2 (surface loop) that are near the identified linear cross-genotype neutralizing epitope (141–150 of VP2) (22), and 4) residues 159–165 of VP3 (Fig. 6, B–D).

**The N-terminal Regions of VP1 Are Externalized**—A structural analysis of the EV71 empty particle revealed conformational flexibility at the pseudo-3-fold junction region among VP1, VP0, and VP3 that is reminiscent of that observed in the structure of the EV71 uncoating intermediate (20) (Fig. 7A). We
thus speculate that this junction site in the empty particle is an intrinsically dynamic region and the site for capsid protein externalization. To test this hypothesis, the purified empty particles were digested by trypsin at 16 and 37 °C. A series of truncated products (VP1-a, VP1-b, VP1-c, and VP1-d) were obtained when probed with anti-VP1 antibodies (Fig. 7B). A VP0-truncated product (VP0-a) was also detected with anti-VP4 antibodies (Fig. 7C). In contrast, VP3 in the empty particle is resistant to trypsin digestion as N-terminal sequencing results showed that the N-terminal amino acid residues of the digested VP3 are GFP(Q/T)E(K/L)P (two possibilities at positions 4 and 6), which corresponds to residues 1–7 of VP3. In addition, the digestion profile of the empty particles was similar to that of the uncoating particle obtained by heating (Fig. 7D). Similar trypsin-sensitive sites are present in the N-terminal regions of both the VP1 empty particles and the uncoating particles. The presence of these truncated products suggests that the VP0 and N terminus of VP1 may externalize from the junction channel.

To determine how many residues in the N terminus of VP1 are externalized, N-terminal sequencing was carried out. The sequenced N terminus of VP1-a is ALTHA, corresponding to residues 19–23 of VP1 (Fig. 7B), suggesting that the cleavage occurs between amino acids Arg-18 and Ala-19. Thus, at least 18 residues from the N-terminal region of VP1 are externalized, possibly through the junction channel.

FIGURE 4. Structures of empty particles from EV71 insertion mutants. A, surface representation of the EV71-NE1 empty particle viewed along the 2-fold axis. EV71 capsid proteins VP1, VP0, and VP3 are colored in magenta, yellow, and cyan, respectively. The inserted amino acids are colored in blue. B, superposition of VP1 from EV71 and EV71-NE1 empty particles. Residues 73–296 are modeled in the EV71 empty particle and colored in red. C, superimposition of VP0 (VP2) from EV71 and EV71-NE1 empty particles. The color scheme is the same as in B. Residues 82–319 (VP2, 13–250) are modeled in the EV71 empty particle. Residues 80–321 (VP2, 11–252) are modeled in the EV71-NE1 empty particle. D, superimposition of VP3 from EV71 and EV71-NE1 empty particles. The proteins are colored the same as in B. Residues 1–175 and 189–238 are modeled in the EV71 empty particle. Residues 1–178 and 189–240 are modeled in the EV71-NE1 empty particle. E, the electron densities corresponding to the inserted NE1 peptide. AA, amino acids.
Here we present the crystal structure of the EV71 empty particle from a clinical C4 strain. Compared with the structure reported previously (Protein Data Bank code 3VBU) that corresponds to a laboratory-adapted C4 strain, this structure exhibits differences in the GH loops of VP1 and VP3, both of which are situated close to the pseudo-3-fold junction. Such conformational variations between the two strains may arise from changes in the amino acid sequence of the capsid proteins and/or differences in the crystallization conditions. Nonetheless, these observations suggest that the junction situated at the pseudo-3-fold axis of an enterovirus capsid may be intrinsically dynamic.

Studies on both poliovirus and EV71 have provided evidence that the BC loop is not a primary determinant of infectivity in primate cells. Characterization of poliovirus chimeras demonstrated that there is minimal loss of infectivity when the BC loop of VP1 is cleaved with trypsin (33) or replaced by extraneous (non-poliovirus) sequences (3–5, 34–37). The structural characterization of poliovirus chimeras indicated that replacements in the VP1 BC loop caused only minor conformational changes, whereas insertions appeared to lead to significant conformational alterations. Similar to what was observed for the poliovirus chimera, insertions in the VP1 BC loop of EV71 caused minor changes in viral replication with significant structural variations in some local regions. It appears that such insertions in the VP1 BC loop caused more significant structural changes in VP0 than in VP1 as observed for EV71-NE1 empty particle and EV71-N6 uncoating intermediate in comparison with their respective parental EV71 particles. Nonetheless, our structural analysis indicated that amino acids 100 and 101 in the VP1 BC loop could serve as an ideal insertion site for the display of foreign epitopes on the particle surface without much perturbation in the capsid structure. In addition, these inserted residues mostly adopt rather flexible conformations, which may partially account for the preservation of the functionality associated with the inserted polypeptide. Thus, recombinant EV71 viruses can be developed into a presentation form for polypeptides with a broad spectrum of uses ranging from antigens for epitope-based vaccines to inhibitors of specific protein interactions.

The electron density of the inserted residues was somewhat weaker or even absent when compared with the density of residues not close to the modification. It appears that the length of the inserted peptide has an impact on the extent of its own conformational flexibility. In the case of NE1 (8 amino acids), most of the main chain atoms could still be traced, whereas in the case of N6 (16 amino acids), a portion of the inserted pep-
tide exhibited significant mobility in the crystal, leading to the loss of an ordered structure. Such a relationship between the polypeptide length and the conformational flexibility was substantiated by our crystal structural analysis on the empty particle from another recombinant EV71: all 19 residues inserted in the VP1 BC loop of this EV71 recombinant are disordered (data not shown). Nonetheless, such insertions at the VP1 BC loop had no significant impact on the overall structure of the capsid.

Another issue we addressed in this study is that an insertion in the VP1 BC loop has little or no effect on the structural changes associated with uncoating as shown from the structural characterization of the EV71-N6 uncoating intermediate. Studies from our group and others have demonstrated that drastic conformational changes occur during the uncoating process, particularly at the icosahedral 2-fold and pseudo-3-fold axes (20). The BC loop of VP1 is situated near the icosahedral 5-fold axis with only moderate shifting occurring along the 5-fold axis direction during the uncoating process. Our current study showed that the uncoating process of the recombinant virus EV71-N6 carrying an epitope in the VP1 BC loop did not appear to be affected as demonstrated from similar conformations shared between the uncoating intermediates of EV71 and EV71-N6.

This is the first structure report of EV71 recombinant viruses, including both empty particles and uncoating intermediate. Our structural study provided an explanation for why the VP1 BC loop is an ideal insertion site for foreign peptides or epitopes for generation of recombinant EV71 viruses. Rescue of viable EV71 recombinant viruses has not been reported for insertion into other VP1 surface loops. The reasons for insertion into the VP1 BC loop may be that 1) such insertions do not affect the overall capsid assembly and structure and 2) such
sections do not seem to affect virus uncoating process and thus virus replication.

At least four different forms of EV71 particles generated during the life cycle have been structurally characterized to date: the virion, the naturally occurring empty particle, the uncoating intermediate, and the empty capsid after uncoating; of these, the role of the naturally occurring empty particle is the least understood. The hypothesis that the empty particle is an intermediate for the mature virion does not gain support from previous studies on poliovirus that demonstrated that only the uncoating intermediate or that of EV71-N6. We thus speculate that the empty particle junction is surrounded by VP1 and VP3 (magenta). VP0 (yellow), and VP3 (cyan). VP1 from a neighboring protomer is colored in red. The visible N terminus of VP1 is located at the base of the junction (from the top view). B, a series of truncated products (VP1-a, VP1-b, VP1-c, and VP1-d) were detected with anti-VP1 antibodies. Lane 1, empty particles (1 μg) incubated at 16 °C for 1 h. Lane 2, empty particles (1 μg) digested by trypsin at 16 °C for 1 h. Lane 3, empty particles (1 μg) incubated at 37 °C for 1 h. Lane 4, empty particles (1 μg) digested by trypsin at 37 °C for 1 h. VP0-a, VP1-b, VP1-c, and VP1-d were detected. The N-terminal amino acid sequence of VP1-a is “ALTHA” (residues 19–23 of VP1).

A band smaller than VP0 (VP0-a) was detected with anti-VP4 antibodies. g) incubated at 16 °C for 1 h. Lane 2, empty particles (1.6 μg) incubated at 16 °C for 1 h. Lane 3, empty particles (1.6 μg) digested by trypsin at 16 °C for 1 h. g) digested by trypsin at 16 °C for 1 h. Lane 4, empty particles (1.6 μg) incubated at 37 °C for 1 h. Lane 4, empty particles (1.6 μg) digested by trypsin at 37 °C for 1 h. VP1-a and VP1-b were detected. D, the digestion profile of the empty particles is similar to that of the uncoating particles obtained by in vitro heating. Lane 1, uncoating particles (1 μg) incubated at 16 °C for 1 h. Lane 2, uncoating particles (1 μg) digested by trypsin at 16 °C for 1 h. VP1-a and VP1-b were detected. Lane 3, empty particles (1 μg) incubated at 16 °C for 1 h. Lane 4, empty particles (1 μg) digested by trypsin at 16 °C for 1 h. VP1-a and VP1-b were detected.

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