A sensor-adaptor mechanism for enterovirus uncoating from structures of EV71

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Enterovirus 71 (EV71) is a major agent of hand, foot and mouth disease in children that can cause severe central nervous system disease and death. No vaccine or antiviral therapy is available. High-resolution structural analysis of the mature virus and natural empty particles shows that the mature virus is structurally similar to other enteroviruses. In contrast, the empty particles are markedly expanded and resemble elusive enterovirus-uncoating intermediates not previously characterized in atomic detail. Hydrophobic pockets in the EV71 capsid are collapsed in this expanded particle, providing a detailed explanation of the mechanism for receptor-binding triggered virus uncoating. These structures provide a model for enterovirus uncoating in which the VP1 GH loop acts as an adaptor-sensor for cellular receptor attachment, converting heterologous inputs to a generic uncoating mechanism, highlighting new opportunities for therapeutic intervention.

Hand, foot and mouth disease (HFMD) is a serious public health threat across the Asia Pacific region, as evidenced by the >1.7 million cases in China reported by the Chinese Ministry of Health during 2010. The dominant causative agent is EV71, a nonenveloped single-stranded RNA virus (genus Enterovirus, family Picornaviridae) that is closely related to coxsackievirus A16, the other etiological agent of HFMD1. Whereas coxsackievirus A16 infections are not usually serious, acute EV71 infections can cause severe neurological disease2,3 and led to 905 deaths in China in 2010.

The icosahedral capsid of EV71 comprises 60 copies of four protein subunits, VP1–VP4. During assembly, the virally encoded P1 polyprotein is cleaved to yield VP0 (36 kDa), VP1 (32 kDa) and VP3 (27 kDa). VP0 is further processed into VP2 (28 kDa) and VP4 (8 kDa) in a reaction that is autocatalyzed by viral RNA and results in formation of functional subunits. 73S particles are unstable, readily converting from D-type native antigenicity to a state with altered (C-type in the poliovirus nomenclature12,13) antigenic properties.

Structural studies have outlined the processes leading to enteroviral infection of cells14. Cellular receptors attach to the virus, often binding in a canyon-like depression surrounding the five-fold axis15–17. This triggers conformational changes in the virus, resulting in the formation of an expanded intermediate that sediments at ~135S (as compared to about ~160S for the mature virus) and has altered antigenic properties18–20. These conformational changes (which can also be induced by environmental insults such as heating or low pH) lead to the externalization of VP4 and the VP1 N terminus21,22. This is followed by extrusion of the viral genome into the cytoplasm of the target cell, leaving behind an empty ~80S particle23.

Previous low-resolution data indicated that the viral RNA probably exits through a five-fold axis channel14, although recent studies suggest that it exits near a two-fold axis23. Nevertheless, there remains a lack of high-resolution structural detail to illuminate the molecular mechanisms underpinning the dynamic enterovirus cell-entry process. To address this, we have determined structures of both inactivated and infectious EV71 virions. We have also determined structures of expanded natural empty particles that closely resemble the expanded enterovirus uncoating intermediates previously visualized by cryo-EM24,25. Our high-resolution analyses suggest a detailed molecular mechanism for the early stages of enterovirus uncoating.

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RESULTS

Characterization and structure determination

EV71 was isolated from a patient from Anhui, China, and cultivated in Vero cells. When required, the virus was inactivated using formaldehyde before purifying it by centrifugation, ultracentrifugation, PEG precipitation and gel filtration (see Online Methods). Unless otherwise stated, analyses used inactivated virus. We separated out the two particle types of interest by ultracentrifugation and characterized them using SDS-PAGE and analytical ultracentrifugation as either 150S mature virions, containing RNA and a full complement of proteins VP1–VP4, or 82S empty particles containing VP0, VP1 and VP3 (Supplementary Fig. 1).

The sedimentation coefficient of the empty particles was considerably greater than that of native-antigenicity enterovirus empty particles (~73S), despite an earlier report that EV71 empty particles were ably greater than that of native-antigenicity enterovirus empty particles (~73S). Therefore, we collected data from small crystals primarily at 21 °C in situ, avoiding the problems of harvesting and cryoprotecting the fragile crystals. We believe this represents the first report of determination of a virus structure using such a method. We solved the structure of the mature inactivated virus in two space groups with data merged from multiple crystals. For the empty particle, we collected a high-resolution data set at 100 K and a lower-resolution data set at 21 °C. This provided reliable atomic models at resolutions of 2.3 Å, 2.6 Å, 2.9 Å and 3.8 Å, respectively, for two independent structure determinations each for full virus and empty particles (see Online Methods; Table 1 and Supplementary Fig. 3). Although particles had been inactivated with formaldehyde, there was no evidence in the electron density for specific covalent cross-linking occurring in a systematic manner, as inactivation did not seem to cross-link the majority of the capsid proteins either to each other or to viral RNA (Supplementary Fig. 1). Subsequent structure determinations of non-inactivated particles (Table 1) showed that inactivation did not perturb the structures and that inactivation presumably operates by the cooperative effect of a few cross-links made in each virus particle. Unless otherwise stated, descriptions below apply to all the structures of that particle type.

Structure of the mature virus

Mature EV71 is well ordered, apart from a few disordered residues in VP4 and VP2 (Fig. 1), and is similar to bovine enterovirus (BEV1), although the inner capsid surface is distinct from that of other enteroviruses (Supplementary Fig. 4). Notably, whereas in structurally characterized enteroviruses residues 1–28 of VP1 proceed toward the five-fold axis, in EV71 they veer across the protomer, presenting a short helix (residues 5–9) underneath the Aα helices of VP2, adjacent to the icosahedral two-fold axes. Additionally, residues 14–31 of VP4 form a loose spiral beneath VP1 rather than lying under the adjacent biological protomer. One RNA molecule base-stacks against VP2 Trp38...
(as in other enteroviruses\textsuperscript{25}), and another may interact with VP1 Gln30 and VP3 Gln48. On the outside of the particle, the VP1 BC, DE and HI loops are flattened away from the five-fold axis (as in BEV1), with residues 96–102 (BC loop) and residues 208–222 (GH loop) being the most exposed (Fig. 1c). Part of the VP2 EF loop (residues 128–148) is unusually extended and surface dominant, whereas the remainder (residues 151–172) is shorter than usual and less accessible. Exposed residues in VP3 include 58–69 and 173–190 (Supplementary Fig. 5).

**An expanded natural empty particle**

The EV71 empty particles are markedly larger than any other picorna-virus particle characterized crystallographically (Fig. 1b), with the r.m.s. capsid radius increasing from 132 Å for the mature virion to 139 Å for the empty particle. Whereas cooling virus particles to 100 K often induces modest isotopic shrinkage (~1%), both the 293-K and 100-K empty particles are ~4% larger than the 293-K mature virus. The 100-K particles are not icosahedral, so refinement imposing icosahedral symmetry at an R factor of 30%, but it improved markedly when this constraint was relaxed. Cryo-cooling pushes C in the crystal’s contact region ~1.3 Å toward the particle center, but the 293-K and 100-K structures are otherwise essentially identical (Supplementary Fig. 5). Such deviations from icosahedral symmetry have never been observed in mature viruses and reflect the extreme flexibility of these expanded particles, explaining why it has been difficult to visualize them at high resolution.

The expansion of the empty particles reflects tectonic movements in the particle, partially separating the protomeric units, disordering >70,000 protein atoms per particle and forming perforations at the icosahedral two-fold axes and at the base of the canyon (Fig. 2a,b). Several external loops, which nestle at the junction of polypeptide chains, become disordered, including five residues at the C terminus of VP2 and residues 211–217 of the GH loop of VP1. The VP3 GH loop also undergoes a major conformational switch, with residues 170–192 converting from loop and helix to almost a β-hairpin upon expansion, becoming less ordered (for residues 174–190, B factors exceed 100 Å\(^2\)) (Supplementary Fig. 5). Inside the particle, the internal festoon comprising VP1’s N-terminal 72 residues and the first 81 residues of VP0 is now disordered (Fig. 1). Overall surface properties are appreciably altered, the interactions that hold the particle together are strongly reduced (Supplementary Table 1) and the capsid in the expanded particle is thinned to 20 Å from 23 Å in the mature virus (Fig. 2c). The core structures of the individual polypeptides are less affected as C\(\alpha\)s superpose with r.m.s. deviations of 1.6 Å, 0.9 Å and 1.3 Å for VP1, VP0(2) and VP3, respectively (Fig. 1c), although some important rearrangements occur.

**Enteroviruses adopt two fundamental configurations**

The ~4% expansion of the EV71 82S empty particle is similar to that seen for the poliovirus 135S and 80S uncoating intermediates\textsuperscript{27}, and the latter has similar hydrodynamic properties. Cryo-EM analyses revealed that the poliovirus 135S and 80S particles are structurally similar to each other\textsuperscript{27} (although the 80S particle has shed the viral genome) and are reconfigured compared to the mature virus\textsuperscript{24,25}. We used VEDA\textsuperscript{28} to compare the EV71 expanded particle with the poliovirus particles. Unexpectedly, the EV71 particle fits the poliovirus 135S and 80S electron density distributions as well as the poliovirus structure previously modeled into this density (Fig. 2d–g); thus, at low resolution the EV71 expanded-assembly byproduct is indistinguishable from poliovirus uncoating intermediates. Furthermore, heat treatment of mature EV71 particles (using a protocol similar to that which produces 135S poliovirus particles) converts them to particles that crystallize isomorphously with 82S immature particles (data not shown). It therefore seems that the plethora of enterovirus particles formed during assembly and uncoating possess only two fundamental configurations, which are both now defined in atomic detail. The mature virus particle, which is rigid and rendered more stable by cleavage of VP0, is generally converted to the second, expanded configuration after cell attachment. This flexible expanded particle can adopt subtly different conformations during the process of uncoating as it progresses from 135S to 80S forms\textsuperscript{25}, but it can also arise from the conversion of unstable VP0 containing natural empty particles, as seen for poliovirus\textsuperscript{10,11} and now EV71.

**Mechanics of particle expansion**

Particle expansion is accompanied by a 5.4° counterclockwise rotation of the protomeric building block (VP1, VP0, VP3), which pivots about the corner of VP3 at the icosahedral three-fold axis (Fig. 3a). This screw-like
In the expanded capsid, the C-terminal stabilizing helix cap (Arg249) becomes poorly ordered, suggesting mechanical strain (the five C-terminal residues beyond it are disordered). Within the protomeric unit, a 7.5° rotation and a 1.4-Å translation of the five-fold proximal end of the VP1 β-barrel, which is jackknifed upward in the mature virus (Fig. 3c), straightens the protomer and moves the GH loop, H strand, CD loop and residues −262–280 beyond strand I of VP1. The trigger for protomer extension seems to be changes centered on a pocket in the VP1 β-barrel.

Mature EV71, as with other enteroviruses, possesses a hydrophobic pocket that penetrates from the surface deep into the interior of the VP1 β-barrel, underlying a canyon-like surface depression and harboring a natural lipid (possibly sphingosine, as seen in poliovirus; Fig. 4 and Supplementary Fig. 3). Notably, the only visible difference between active and inactivated particles is that the occupancy of the ‘pocket factor’ in the active virus is approximately half that of the inactive virus (Supplementary Fig. 6). This suggests that formaldehyde treatment rigidifies the particle, inhibiting the release of the pocket factor and presumably contributing to inactivation, as pocket-factor release seems to be required for the initiation of uncoating (potential antivirals have been discovered that replace natural lipids and inhibit uncoating).

Until now, empty pockets observed in enteroviruses have been largely open (such that a pocket factor might bind without appreciable structural change). In contrast, not only is the EV71 expanded-particle pocket empty, but small conformational changes...
between the mature virus (gray, with pocket factor shown in magenta) and empty particles (blue).

**DISCUSSION**

The mechanism by which the RNA genome is productively released from picornaviruses has long been the subject of speculation and, although the details will vary, the process is likely to be fundamentally similar for all enteroviruses. RNA release in poliovirus is preceded by the egress of the VP1 N terminus and VP4 (ref. 21), which may associate to form a channel through the membrane, allowing the safe transfer of the viral genome to the cell cytoplasm. Candidates for membrane association and channel formation are the myristate group at the N terminus of VP4 (ref. 47) and a region at the N terminus of VP1 that is proposed to form an amphipathic helix. It had been assumed that a five-fold channel would be formed to release these regions, but recent studies suggest instead that VP1 (and possibly VP4) leaves at the base of the canyon or at a two-fold axis. We have defined a channel at the base of the canyon (7 x 9 Å in size) and a larger (8 x 25 Å) opening at the two-fold axis (Fig. 2b), either of which would allow egress of a polypeptide chain while the larger might also allow the exit of RNA.

In the mature EV71 virus, the N-terminal region of VP1 lies at the two-fold axis, rather than close to the five-fold axis, perhaps presenting a snapshot of a structural rearrangement that precedes VP1 release in other enteroviruses. Indeed, in the expanded particle, both VP4 and the VP1 N terminus are disordered, and possibly already partly extruded. During exit of the RNA, less structured regions of the single-stranded RNA genome might be transiently unwound through interactions with the inner surface of the capsid. However, the considerable secondary structure at the termini of picornavirus genomes suggests that strain would be required to initiate RNA egress, perhaps accounting for the fact that the RNA does not spontaneously exit 135S particles. This strain could probably be accommodated by the flexibility of the expanded particle, which could allow the two openings to merge by breaking the single weak link between them (Fig. 3e.g). Notably, flexing of this region would render a conserved VP3 sequence (PPGxxxxPxRxR50) reminiscent of a protein-binding motif accessible on the inside of the particle. This motif may be involved in picornavirus assembly or uncoating.

Our results provide two lessons for vaccine production: (i) formaldehyde inactivation leaves the structure, and hence antigenicity, of EV71 essentially unchanged; (ii) when grown in Vero cells, this strain of EV71 produces large amounts of antigenically altered particles, which will, at best, dilute the useful portion of a vaccine. More broadly, our results, with those of others, show that the key structural transitions of enteroviruses are based on just two fundamental states, with different physicochemical and antigenic properties that are matched to their distinct roles in the virus life cycle. Atomic-level descriptions of these states allow us to propose a mechanism by which receptor binding triggers virus uncoating and to highlight points where we might attempt to modulate the switch between the two states, using either small molecules or mutant viruses. One such point is the well-studied VP1 hydrophobic pocket, a second is the pair of VP2 α helices, which are torn apart during expansion; mutations that weaken the link from the adaptor-sensor to this point might be useful in providing stabilized vaccines. Furthermore, key amino acid residues in the proposed uncoating mechanism are conserved beyond the enteroviruses (Supplementary Fig. 4), suggesting that, by targeting elements of the mechanism that are common to other picornaviruses, such as picornavirus genera, it may be possible to develop generic therapies against a number of viruses posing serious threats to health.

**Figure 4** Pocket factor-binding site. (a) The hydrophobic pocket (blue mesh) in VP1 of mature EV71 is occupied by a natural lipid (magenta). The protein cartoon is colored as in Figure 1a. (b) The expanded particle, showing the empty, collapsed pocket. (c) Comparison of the VP1 pockets between the mature virus (gray, with pocket factor shown in magenta) and empty particles (blue).
ONLINE METHODS

Particle production and purification. EV71 (genotype C4), isolated from Fuyang, Anhui Province, China, was used to infect Vero cells at 10^7 50% tissue culture infective dose (TCID50). The virus was collected 5–6 d after infection, inactivated by incubation with 100 μg ml⁻¹ formaldehyde at 37 °C for 3 d, centrifuged to remove cell debris, ultrafiltered and PEG precipitated and subjected to gel filtration. These stages were performed by Sinovac Biotech Ltd and the China National Biotech Group. Crude EV71 concentrate (−0.6 mg in 600 μl PBS (pH 7.4)) was loaded onto a 15–45% (w/v) sucrose density gradient and centrifuged at 103,614 g for 3.5 h in an SW41 rotor at 4 °C. Two sets of fractions were collected and dialyzed against PBS buffer (Supplementary Fig. 1); one comprised empty particles (containing no RNA), the other contained virions. SDS-PAGE analysis used a NuPAGE 4–12% Bis-Tris Gel (Invitrogen) (Supplementary Fig. 1).

Analytical ultracentrifugation. The sedimentation coefficients for both types of particles were determined using a Beckman XL-1 analytical ultracentrifuge at 4 °C (Supplementary Fig. 1).

Antibody D6 antigen-binding (Fab) purification and EV71 immunogenicity. Anti-EV71 monoclonal antibody (mAb) D6 was supplied by Sinovac. Fab fragments were generated (Pierce® Fab preparation kit, Thermo Scientific), dialyzed against 20 mM acetate (pH 5.0) at 4 °C, loaded onto a Mono S column (GE Healthcare) and eluted using a 0–500 mM NaCl gradient. The main peak was collected and dialyzed into PBS buffer. The pure Fab was incubated with the semi-purified EV71 sample containing empty and full particles (at a ratio of ~240 Fab molecules per EV71 virion) at 4 °C for 12 h. The mixture was loaded onto a 15–45% (w/v) sucrose density gradient and centrifuged at 103,614 g for 3.5 h using a SW41 rotor at 4 °C (Supplementary Fig. 2).

Thermofluor assay. Thermofluor experiments were performed with an MX3005p RT-PCR instrument (Agilent). SYTO9 and SYPROred (both Invitrogen) were used as fluorescent probes to detect the presence of RNA and the exposed hydrophobic regions of proteins, respectively. We set up 50-μl reactions in a thin-walled PCR plate (Agilent), containing 5.0–10 μl of either the virus or empty particles, 5 μM SYTO9 and 3X SYPROred in PBS (pH 7.4) and ramped the temperature from 25 °C to 99 °C, with fluorescence recorded in triplicate at 1 °C intervals. The melting temperature, Tm, was taken as the minimum of the negative first derivative of the denaturation curve (Supplementary Fig. 2).

Crystallization. Crystallization used nanoliter vapor diffusion in Greiner CrystalQuick X plates. Purified particles were concentrated to 2 mg ml⁻¹ in PBS (pH 7.4). Crystal Screen1 (Hampton Research) condition 13 (30% (v/v) PEG400, 0.2 M tri-sodium citrate, 0.1 M Tris-HCl (pH 8.5)) gave small irregular crystal morphologies: rhomboid plates, and cubic and triangular prisms. Crystals of empty particles grew in two conditions: (i) 200 mM ammonium phosphate monobasic, 24% (v/v) isopropanol and 100 mM sodium cacodylate (pH 6.5); (ii) 1.4 M sodium acetate and 0.1 M sodium cacodylate (pH 6.5). The crystallization conditions were similar for inactivated and non-inactivated virus.

Structure determination. Diffraction data were taken at room temperature (293 K) from crystals in crystallization plates (in situ), a method developed at beamline 124, Diamond. Diffraction images of 0.05° or 0.1° oscillation were recorded on a Pilatus 6M detector using a 0.02 × 0.02 mm² or 0.05 × 0.05 mm² beam according to crystal size. The X-ray beam was focused downstream from the crystal. Using a 0.05-s exposure time and 100% beam transmission, typically six to ten useful images could be collected from one position on a crystal (up to six positions for larger crystals). Data for the EV71 inactivated full particles were collected from the rhomboid plates and cubic crystals (space groups R32 and I23, respectively; Table 1). Room temperature data were also collected for the empty EV71 inactivated particles to 3.8Å resolution. The data were weak (by the usual current standards of macromolecular crystallography), and this is reflected in the poor merging R factors. Subsequently, data were collected, to lower resolution, from non-inactivated full and empty particles (Table 1).

Cryo-cooled (100 K) data for crystals of empty EV71 particles were measured using 0.1°–1° exposure oscillations. Crystals were soaked for 20 min in a cryo-protectant solution (80% (v/v) reservoir solution and 20% (v/v) glycerol) before being plunged into liquid nitrogen.

Data were analyzed using HKL2000 (ref. 54). The structures of both empty and full inactivated particles were determined by molecular replacement. For the empty particle (P4,32, a = b = c = 353.1 Å (100 K data)) there is an icosahedral pentamer in the crystallographic asymmetric unit. For the full virus (R32 a = b = 330.0 Å and c = 748.4 Å, and I23 a = b = c = 600.1 Å) there are two and four pentamers, respectively, in the asymmetric unit. Molecular replacement used BEV (PDB 1BEV) as the search molecule (most orientation and positional parameters were defined by the alignment of icosahedral and crystallographic symmetry axes). For the 23 crystals, the center of the virus is at (0.25, 0.25, 0.25). In each case, the crystallographic asymmetric unit was rigid-body refined. Cyclic positional, simulated annealing and B-factor refinement used strict NCS constraints with CNS41. Averaging was done using GAP (D.L.S., J. Grimes and I. Diprose, unpublished data) and models were rebuilt with COOT56. Refinement of the empty-particle cryo-structure stuck at an R factor of 0.30. Rigid-body refinement of individual protomers (breaking the icosahedral symmetry) reduced the R factor to 0.25 (all data to 2.88 Å). Inspection revealed that, at the crystallographic contacts, the structure was squashed 1.3 Å inward, presumably as a result of crystal freezing. BUSTER refinement using NCS restraints gave an R factor of 0.187. Recalculated NCS matrices were used as constraints with CNS, resulting in the final structure (Table 1). The non-inactivated structures were isomorphous with the inactivated structures and were refined directly from those structures, using strict NCS constraints in CNS. Models were verified with PROCHECK58 (Table 1). Structural comparisons used SHP51. Unless otherwise noted, structural figures were prepared with PyMOL (DeLano Scientific).

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