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A strain-specific epitope of Enterovirus 71 identified by cryoEM of the complex with Fab from neutralizing antibody

Running title: Strain-specific Epitope of EV71

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ABSTRACT:

Enterovirus 71 (EV71) is a picornavirus that causes outbreaks of hand foot and mouth disease (HFMD) primarily in the Asia-Pacific area. Unlike coxsackievirus A16, which also causes HFMD, EV71 induces severe neuropathology leading to high fatalities especially among children under the age of 6 years. Currently, no established vaccines or treatments are available against EV71 infection. The monoclonal antibody (MAb), MA28-7, neutralizes only specific strains of EV71 that have a conserved glycine at amino acid, VP1-145, a surface exposed residue that maps to the five-fold vertex, and has been implicated in receptor binding. The cryo-electron microscopy structure of a complex between EV71 and the Fab fragment of MA28-7 shows that only one Fab occupies each five-fold vertex. A positively-charged patch, which has also been implicated in receptor binding lies within the Fab footprint. We identify the strain-specific epitope of EV71 and discuss the possible neutralization mechanisms of the antibody.

INTRODUCTION:

Enterovirus 71 (EV71) infection causes outbreaks of hand, foot, and mouth disease (HFMD) predominantly in the Asia-Pacific region (1, 2). Whereas most EV71 infections are self-limiting, neurological and systemic complications can develop that range from aseptic meningitis to encephalitis and acute flaccid paralysis. Infection can lead to lethal pulmonary edema and heart failure (2) with mortality especially high in young children under the age of 6 (2, 3). As seasonal outbreaks of HFMD are recurring around the world, development of a vaccine and antiviral therapies for EV71 has become an urgent concern.

A member of the *Picornaviridae* family, EV71 has a non-enveloped, icosahedral capsid comprised of 60 copies of each of four viral structural proteins (VP1-4) (4). Recent studies have solved the structures for three strains of EV71 (MY104 (5), Fuyang (6), and 1095 (7)) demonstrating that EV71 has the general features of picornavirus capsids including the five-fold "mesa" and the depression around the mesa called the "canyon" (5–8). Conserved residues VP1-242K and 244K form positively-charged patches on the five-fold mesa (6), and this symmetry-related clustering of positive charges have been suggested as a common mechanism for haparan sulfate binding in Enteroviruses (9).

Several cellular receptors for EV71 have been identified: Scavenger receptor B2 (SCARB2), P-selectin glycoprotein ligand-1 (PSGL-1), and heparan sulfate (HS) (10–12). SCARB2, which is expressed on a broad variety of cell types, likely binds into the virus canyon and induces the transition of the virion that is required for uncoating (13–15). PSGL-1, which is expressed exclusively on lymphocytes, binds only specific EV71 strains and supports viral replication in lymphocytes in a PSGL-1-dependent manner (11). According to recent studies, PSGL-1 and HS bind the positively-charged patches on the five-fold mesa of EV71 and provide initial attachment on the cell (12, 16, 17). We

recently found that the PSGL-1 binding phenotype of EV71 strains is regulated by a single residue, VP1-145 that maps to the center of five-fold mesa (16).

Linear epitopes of EV71 have been characterized in several studies using synthetic peptides or inactivated whole virus (18–21). The epitopes, which induce cross-neutralization activity, are located at the rim of the canyon on VP1 and VP2, where SCARB-2 binds (18–20). Strain-specific neutralizing antibodies that recognize conformational-dependent epitopes have been generated and classified (22); however, the epitopes have not been mapped. Structural studies can identify and map the conformational epitopes, and elucidate antibody neutralization mechanisms, which support effective vaccine development. Here we have characterized the EV71 MAb MA28-7 as having strain-specific neutralization activity. We used cryo-EM to solve the structure of EV71 complexed with the Fab from MA28-7. The Fab binds across the five-fold symmetry axes such that steric hindrance limits occupation to no more than one Fab per five-fold vertex. The footprint of the Fab includes VP1-145 and residues that map to the positively-charged patches (VP1-98, 242, and 244) around the five-fold axis, overlapping with the binding sites of PSGL-1 and HS. Neutralization and sequence alignment analysis indicate VP1-145 is a determinant for the strain-specific antigenicity of EV71.

MATERIALS AND METHODS:

Virus production: EV71 was propagated and purified as described previously (8). Briefly, EV71 strain 1095 (23, 24) was propagated in HeLa cells for 24 hours. The media and cells were collected and processed by freezing and thawing three times. Cell debris was pelleted by centrifugation and the supernatant was precipitated with PEG 8K. After ultracentrifugation through a 30% sucrose-buffer cushion, the pellets were re-suspended and applied to a 10-35% tartrate step-gradient. The virus was

collected and dialyzed against 10 mM Tris, 200 mM NaCl, 50 mM $MgCl_2$, pH 7.5, and concentrated to 1.0 mg/ml.

MAb production: Immunization of mice and preparation of hybridomas were performed at the NIPPON BIO-TEST LABORATORIES Inc. (Tokyo, Japan). BALB/c mice were immunized with formalin-inactivated EV71 strain 1095 (50 µg/mouse) three times every two weeks. Splenocytes were isolated, and fused with myeloma cells, and hybridomas were generated. Antibodies from hybridoma cell lines were screened for their ability to bind intact EV71 1095 virions and formalin-inactivated virions in a sandwich enzyme-linked immunosorbent (ELISA) assay. The antibody used for these studies, MA28-7, showed a weak binding activity to native virions of EV71 strains 1095, C7/Osaka and SK-EV006, but did not bind to EV71 strains Nagoya, Bulgaria, and BrCr/tr or CA16 in the ELISA assay (Tano Y, et al. unpublished data).

Fab and virus-Fab complex preparation: MAb of MA28-7 was obtained from ascite fluid after inoculation of the hybridoma to BALB/c mice. The MAb was purified using HiTrap Protein G HP columns (GE healthcare) according to the manufacturer's instructions. The MA28-7 Fab fragment (Fab28-7) was generated by applying the MAb to papain immobilized-beads and purified by protein A column (Pierce Fab Preparation Kit; Thermo Fisher Scientific). Initially purified virus and Fab were incubated for 1 hour with a molar ratio of 1:240 (virus capsid:Fab), providing an excess of 4 Fabs per each of the predicted 60 binding sites. Subsequently, a second incubation of 1:24 (virus capsid:Fab) was done to allow 2 Fabs per each of the 12 possible binding sites of one per five-fold symmetry vertex to reduce the protein background of unbound Fab for the asymmetric reconstruction.

Cryo-EM imaging: The two incubations were processed separately as data set 1 and 2, corresponding to virus:fab ratios of 1:240 and 1:24, respectively (see above). For each, three µl of virus:Fab complex were pipetted onto a Quantifoil grid (Quantifoil Micro Tools GmbH, Jena,Germany),

blotted to remove excess sample, and plunge-frozen into a liquid ethane/propane mixture (25) using a Mk III Vitrobot (FEI, Hillsboro, Oregon). Low dose conditions were used to record images on Kodak SO-163 film (Kodak, Rochester, New York) in an FEI TF-20 electron microscope operating at 200 KV with calibrated magnifications of 30,000x and 50,000x for datasets 1 and 2, respectively. The microscope was equipped with a Gatan 626 cryoholder (Gatan, Inc, Pleasanton, CA). Cryo-EM images were collected with a defocus range of 2.73-4.16µm (dataset 1; Fig. 1A) and 1.9-6.12µm (dataset 2; Fig. 1B). Films were scanned using a Nikon Super Coolscan 9000 (Nikon, Melville, NY) giving a calibrated pixel size at the sample of 2.12 Å/pixel and 1.27 Å/pixel for datasets 1 and 2, respectively. The program suite AUTO3DEM (26) and EMAN2 (27) were used for image processing and 3D reconstructions.

Icosahedral reconstruction of the complex: Virus-Fab complexes from 16 micrographs of dataset 1 were used for calculating a three-dimensional reconstruction (Table 1). Semi-automatic particle selection was performed using EMAN2's e2boxer.py to obtain the particle coordinates, followed by particle extraction, linearization, normalization, and apodization of the images using Robem (26). Defocus and astigmatism values to perform contrast transfer function (CTF) correction were assessed using Robem for the extracted particles (Table 1). The icosahedrally-averaged reconstruction was initiated using a random model generated from the raw data (26) and reached 10.5 Å resolution estimated where the Fourier Shell Correlation, FSC, dropped below 0.5. The absolute pixel size (2.08 Å/pixel) and handedness were confirmed using UCSF Chimera (28) to compare the density map with one calculated from the crystal structure of EV71 Fuyang strain (PDB ID : 3VBS).

Asymmetric reconstruction: Images of the virus-Fab complex of dataset 2 were selected with the EMAN2's e2boxer.py. An icosahedrally-averaged reconstruction and assessment of absolute pixel size (1.23 Å/pixel) and handedness were performed as described above. The resulting 3D map was used as an initial model for generating an asymmetric reconstruction with the following modifications: the Fab

density (radii >165 Å) of the 3D map was removed using AUTO3DEM's PCUT program and a small sphere of density, 15 pixels in radius, was built on the capsid shell adjacent to one of the five-fold symmetry axes using Robem (26). The modified map was rotated to the EMAN2 standard orientation and the EMAN2 e2ctf.py module was used to manually determine the CTF parameters. The phases were rectified for 6,539 images selected from 45 micrographs and the images were low-pass filtered at 10 Å by e2proc2d.py. The 3D orientation of each particle was determined using standard refinement procedures in EMAN2 with the 'breaksym=1' flag on the orientation generator to generate projections for C1 symmetry. Each particle contributed to the five best scored classes by a 'sep=5' flag to generate a smoother map and roughly 20% of the particles were discarded during the refinement process (27). The resolution of the map was determined by e2eotest.py (Fig. 1C).

Fitting of a Fab structure and roadmap of the Fab footprint: Fitting of the atomic structure of murine antibody Fab 3GK8 (29) was done first by eye and then refined in UCSF Chimera (28). The refined fit of the Fab (correlation coefficient value of 0.743) was used to identify all atoms within 4 Å of the fitted virus structures to generate the Fab-binding footprint. Stereographic projections were done using the program RIVEM (30) and Coulombic representations of the surface were generated using chimera (28).

Neutralization assay: The neutralizing titer of the EV71 antibodies was measured by following a standard micro-neutralization assay. Briefly, in quadruplicate, the original ascitic fluid of BALB/c mice was serially diluted in a 96-well tissue culture plate and each well mixed with an equal volume of EV71 (100 $TCID_{50}$ /50 µl). The plate was incubated at 36ºC for 2 hours. After incubation, a cell suspension of Vero cells (2×10^4 cells/100µl) was added to each well and the mixtures were incubated at 36ºC. CPE was read under an inverted microscope for 10 days. The neutralizing titer was recorded as the highest

dilution of ascitic fluid, which protects 50% of the wells showing CPE, and expressed as the reciprocal (Table 2). The end point of each neutralizing titer was calculated by the Kärber formula.

RESULTS:

Cryo-EM reconstruction of Fab28-7 bound to EV71. Fab fragments of MA28-7 were used to make virus-fab complex for visualization by cryoEM. Initial experiments were aimed at saturating 60 potential binding sites on the capsid with bound Fab to produce a homogeneous population of virus-Fab complexes. In the resulting cryoEM images, electron density protruded visibly from the capsid edges, but only a few Fab appeared to be bound per capsid (Fig. 1A). In addition, the cryoEM images had a strong background likely to be unbound Fab protein, raising the possibility that 60 binding sites on the capsid were not occupied.

The 10.5 Å (Fig. 1C) icosahedrally-averaged 3D reconstruction revealed irregularly shaped and weak density located above the exterior surface of the capsid at each five-fold vertex (Fig. 2A), in accordance with a Fab occupancy of fewer than 60 per capsid. In cases where the Fab footprint overlaps a five-fold icosahedral symmetry axis, steric hindrance combined with the imposition of icosahedral symmetry results in the Fab density appearing as a bi-lobed turret (31, 32). In our reconstruction, however, the density on each five-fold vertex was smaller than expected and disconnected from the capsid (Fig. 2A and B). This lack of five-fold averaged Fab density is likely due to averaging occupied epitopes with those that are empty. However, the disconnection between the capsid and Fab may be an effect of the Fresnel fringe or incomplete correction of the CTF. Further tests indicated that the “loss” of Fab density is resulted from the phase-flipping application for CTF correction (data not shown). An additional icosahedrally-averaged reconstruction was done with full CTF deconvolution applied by Wiener filter, which showed Fab connected to virus, but the distal Fab densities were greatly reduced in

184 strength compared to the strong capsid and RNA core densities (data not shown). This lack of Fab
185 density relative to that of the capsid suggests partial occupancy of less than one Fab per five-fold vertex.
186 **Asymmetric reconstruction.** For each of the icosahedrally-averaged reconstructions**,** the resulting
187 Fab electron density (Fig. 2) was not large enough to accommodate a model of a Fab molecule due to
188 the partial occupancy and 60-fold symmetry averaging. Consequently we calculated an asymmetric
189 reconstruction where Fab-labeled vertices were aligned with each other to maximize the Fab signal (27)
190 (EM database accession number EMD-27331). The starting model was an icosahedral capsid in which a
191 small sphere of density was positioned on one of the five-fold vertices, and each particle was oriented so
192 that a Fab-labeled vertex aligned to the sphere-occupied vertex. The resulting 23.4 Å (Fig. 1C)
193 asymmetric reconstruction had noticeable icosahedral features and a strong asymmetric density
194 corresponding to one Fab protruding from a single five-fold vertex (Fig. 3A). The volume of the Fab
195 density was about 3 times larger than the volume of the small sphere used in the starting model. There is
196 also weak density corresponding to Fab at the opposite vertex.
197 **Pseudo-atomic model and footprint of the Fab.** The X-ray crystal structures of EV71 MY104
198 strain (5) and Fuyang strain (6) were fitted separately into the asymmetric virus-Fab cryoEM
199 reconstruction by superimposing symmetry elements. The crystal structure of a murine antibody Fab
200 (3GK8)(29) was fitted into the Fab density to identify contacts on the virus surface (Fig. 3B) (PDB
201 accession code 3J3Z). All contacts within the Fab footprint mapped to VP1, with most residues located
202 on the BC- and HI-loops of two adjacent VP1 proteins. Six of the predicted contacts occur twice within
203 the Fab footprint (Fig. 4A and B), so that the effects of a single point mutation would be multiplied and
204 the effect on the footprint enhanced by the symmetrical five-fold environment. Residues of the five-fold
205 positively-charged patch (VP1-98K, 242K, 244K) (6) contribute significantly to the Fab footprint. The
206 two X-ray crystal structures have oppositely charged residues for VP1-98 (MY104:Glu and 3VBS:Lys),

207 which alters the electrostatic potential of the antibody binding site between the two strains (16) (Fig. 4C
208 and D). Within the antibody-binding footprint, the surface potential of the 1095 strain would more
209 closely resemble that of the MY104 strain.

210 **Strain-specific neutralization activity.** The neutralization activity of MA28-7 was tested against
211 six EV71 strains, including genotypes A, B1, B3, B4, and C2 (Table 2). EV71 strain C7/Osaka (B4),
212 SK-EV006 (B3), and 1095/Shiga (C2), were sensitive to neutralization by MA28-7, whereas strains
213 Nagoya (B1), Bulgaria (B1) and BrCr/tr (A) were resistant. The VP1 protein sequences of the six strains
214 were aligned to find any relationship between the antibody neutralization specificities (data not shown).
215 93.6% (278 out of 297) of amino acid residues were conserved among the six strains. Within the
216 antibody-binding footprint, only three residues varied in identity (Table 2). A glycine at VP1-145 is
217 conserved between neutralization-sensitive strains, whereas the strains that were resistant to antibody
218 neutralization have VP1-145E/A. In addition, neutralization-sensitive strains have VP1-98E and 242K,
219 whereas neutralization-resistant strains have VP1-98E/K and VP1-242K/E, suggesting that Glu98 and
220 Lys242 may be necessary, but not sufficient for antibody neutralization (16).
221
222 **DISCUSSION:**

223 **Low occupancy of Fab and asymmetric reconstruction.** The Fab binds across the five-fold
224 symmetry axis such that only one Fab can bind each vertex. Previously two cryo-EM studies solved
225 structures of virus-Fab complexes in which Fab binds the five-fold axes of cucumber mosaic virus
226 (CMV) and human rhinovirus serotype 2 (HRV2) (31, 32). In both studies, steric hindrance limited the
227 number of Fabs occupying each five-fold vertex to one or two, depending on how closely the Fab bound
228 to the symmetry axis. The density of Fab relative to capsid was 20% for CMV and 40% for HRV2,
229 which correlates to the one and two Fab molecules binding per each five-fold vertex, respectively. In our

study, we find a situation similar to the CMV-Fab study, however the averaged Fab28-7 density was <10% that of the EV71 capsid, indicating that not all EV71 five-fold vertexes were occupied. This low occupancy was confirmed by comparison to a simulated model in which we reduced the Fab density to 10% (data not shown). Possibly, Fab 28-7 binds with low affinity. Consequently, an asymmetric reconstruction was necessary to improve the accuracy of the Fab-fitting. Similar approaches with different reconstruction packages have been used previously to visualize successfully unique features of icosahedral capsids (33–35). Using this approach, a single Fab was reconstructed bound to one of the five-fold vertices, providing a better quality map for fitting a Fab structure and identifying the antigenic epitope.

Neutralization mechanisms. The orientation of bound Fab relative to the capsid surface allows only monovalent-attachment of the antibody, which could result in cross-linking of virions (Fig. 5). Additionally, about half the residues within the footprint map to the positively-charged patches (Fig. 4), which are necessary for PSGL-1 binding (16) and predicted to confer HS binding (12). This overlap of binding sites suggests that the antibody might compete with receptors during initial attachment.

VP1-145 as an EV71 antigenicity-controlling switch. Our data shows that the conserved glycine at VP1-145 located within the Fab buried surface (Fig. 4B), correlates with MA28-7 neutralization-sensitivity (Table 2). The identity of amino acid 145 also confers the PSGL-1 binding phenotype by regulating the configuration of electrostatic potential of the positively-charged patch (16). This same mechanism may influence the MA28-7 binding; however, the specific identity at VP1-145 dictates Mab neutralization (Table 2). Additionally, Chen et al. classified antibodies that neutralize VP1-145E strains (PSGL-1-nonbinding) but not VP1-145G/Q strains (PSGL-1-binding phenotype) (Fig. 6) (16, 36). VP1-145 has been found to be under positive selection (37, 38) and has been implicated as one of the possible determinants of virulence in humans (39, 40) and adaptation in the mouse (41–43). It is likely that the

EV71 antigenicity, as well as receptor-binding phenotype, is altered with a single mutation at VP1-145, which may confer an advantage to EV71 for effective host infection and transmission.

Here we have identified the strain-specific conformational epitope of an EV71 neutralizing antibody. The antibody footprint maps to the five-fold vertex and encompasses the positively-charged patch and residue VP1-145. Our data indicate that the identity of VP1-145 is a determinant of the MA28-7 neutralization-sensitivity. MA28-7 can cross-link virions and might inhibit initial attachment of EV71 virions to the cell surface. Our study gives insights into how EV71 can regulate strain-specific antigenicity and provides meaningful information for the development of EV71 vaccines.

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FIGURE LEGENDS

Figure 1. CryoEM micrographs of data and the resolution of reconstructions. (A and B) CryoEM datasets of Fab28-7 incubated in excess with EV71 in a ratio of 240:1 and 24:1 (Fab:capsid) for the Dataset 1(A) and 2(B), respectively. Arrows indicate where fab can be seen bound to capsid. The scale bar represents 50 nm. (C) Plot showing Fourier shell correlation (FSC) vs spatial frequency of the icosahedrally-averaged reconstruction (Dataset 1; black line) and asymmetric reconstruction (Dataset 2; red line). Resolution of the reconstructions is assessed where the FSC curve crosses below a correlation value of 0.5.

Figure 2 The 3D image reconstruction and central section of the map. (A and B) Surface rendered map and central section from the 10.5 Å icosahedrally-averaged reconstruction of EV71-Fab complex. The averaged Fab density indicates that steric hindrance allows no more than one Fab to bind per five-fold vertex. The surface rendered map is radially colored according to the key and displayed at a contour level of 0.4 σ. Icosahedral symmetry axes are indicated in the central section.

Figure 3. The asymmetric reconstruction and fitted Fab structure. (A) An asymmetric approach aimed at reconstructing one Fab bound to the capsid surface resulted in strong Fab density ~30% the magnitude of the capsid density. Shown here displayed at 1.0 σ, the icosahedral features of the capsid are easily discerned and the surface rendered map is radially colored using same key as Fig. 2. (B) The refined fit of the Fab structure with heavy chain (grey) and light chain (yellow) shown in wire demonstrates the fit of a single Fab onto one five-fold mesa, which identifies the interactions of the Fab with two subunits of the virus capsid. The fitted virus crystal structure is shown colored in the convention for picornavirus: VP1-blue, VP2-green, VP3-red, and VP4-yellow.

Figure 4. Roadmaps of virus surface at the five-fold vertex. (A-D) A five-fold view of a pentamer of the EV71 (4AED) viral surface is shown as a stereographic projection where the polar angles θ and φ

represent latitude and longitude, respectively (30). The virus surface is represented as a quilt of amino acids (44). The asymmetric unit is shown in black lines. (A) Residues are colored according to radius. A section of radial density, 158-160 Å, corresponding to Fab bound adjacent to the viral capsid, is projected onto the five-fold road map with a yellow contour of density greater than 0.8. (B) One Fab footprint is shown with dark blue contacts in one protomer (light blue) and red contacts in the adjoining protomer (pink). (C and D) The five-fold projections of MY104 strains (5) (4AED) and Fuyang (6) (3VBU) are shown with Coulombic surface coloring and an outline of the Fab binding footprint to illustrate the differences.

Figure 5. Monovalent-interaction of the Fab. Using the refined fitted Fab structure, all 60 symmetry-related capsid-bound Fabs were generated. The distances between the heavy chain C-terminus of adjacent Fabs and between the centers of each Fab footprint, show that adjacent Fabs are too far apart to allow bivalent binding. Heavy chain and light chain are shown in grey and yellow wire, respectively, with virus protomers colored according to VP, with the picornavirus convention VP1, 2, and 3 as blue, green, and red, respectively. The two-fold symmetry axis is indicated by the arrow.

Figure 6. Sequence alignments of the seventeen EV71 virus strains used in Chen et al (22)**.** Strains in the red box (antigenic type 1, 2.1 and 2.2) are neutralized by class IIb 12 MAbs, whereas strains in blue box (antigenic type 2.3 and 2.4) are not neutralized by the same class. VP1-145 is the only residue that is conserved among strains in the red box exclusively. Clustal W (45) was used to align the VP1 sequence from 96-155 for 17 EV71 strains that are listed by genbank accession ID code. Each dot (.) refers to conserved sequence and the single letter identifier is used for residue identity.

Table 1. Image data for cryoEM reconstruction

Parameter	Dataset 1	Dataset 2
Virus : Fab ratio	1:240	1:24
No. of micrographs	16	45
Defocus level range (μm)	2.73-4.16	1.90-6.12
No. of particles selected from micrographs	2,864	6,539
No. of particles used for reconstruction	2,807	6,277 (27,026)
Final resolution (Å)	10.5	9.72 (23.4)

*Values in ( ) are for asymmetric reconstruction.

Table 2. Characterization of EV71 strains

Strains	Genogroup	Neutralization titer	Amino acid residue				PSGL-1 binding
			98	145	241	242	
SK-EV006	B3	1350	Glu	Gly	Leu	Lys	PB
C7/Osaka	B4	1350	Glu	Gly	Leu	Lys	PB
1095/Shiga	C2	1130	Glu	Gly	Ser	Lys	PB
Bulgaria	B1	<200	Glu	Ala	Ser	Lys	*PB
BrCr/tr	A	<200	Lys	Glu	Ser	Gln	Non-PB
Nagoya	B1	<200	Glu	Glu	Ser	Lys	Non-PB

PB = PSGL-1-binding
Non-PB = PSGL-1-nonbinding
* a predicted phenotype based on (16).