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Citation for published version (APA):
Westerhuis, B. M. (2014). The 3-dimensional play of human parechovirus infection; Cell, virus and antibody.
Structural basis of human parechovirus neutralization by human monoclonal antibodies

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Submitted for publication
Abstract

Human parechoviruses are one of the leading causative agents of neonatal sepsis. Intravenous immunoglobulin treatment is the only treatment available in such life-threatening cases and has given moderate success. Direct inhibition of parechovirus infection using monoclonal antibodies is an alternative treatment. We have developed two therapeutic monoclonal antibodies against human parechovirus 1, namely AM18 and AM28. Here we present the mapping of their epitopes and the mode of their neutralization using peptide scanning, electron cryo-microscopy and fluorescence-based thermal shift assays. We determined by peptide scanning that AM18 recognizes a linear epitope motif including the ‘arginine-glycine-aspartic acid’ on the C-terminus of capsid protein VP1. This epitope is normally used by the virus to attach to host cell-surface integrins during entry. Therefore, AM18 is likely to cause virus neutralization by blocking integrin binding to the capsid. In contrast, we show by electron cryo-microscopy, three-dimensional reconstruction and pseudo-atomic model fitting that AM28 recognizes quaternary epitopes on the capsid composed of VP0 and VP3 loops from neighbouring pentamers. The melting temperature of the virus-AM28 complex increased significantly compared to the virus alone in thermal shift assays indicating stabilization of the capsid by the antibody, thereby preventing virus uncoating. Thus both mAbs show therapeutic promise for HPeV infections.
Introduction

Human parechoviruses (HPeV) are single-stranded, positive-sense RNA viruses in the Parechovirus genus within the Picornaviridae family [1]. HPeV infections mainly cause mild gastrointestinal symptoms, although HPeVs are also associated with more severe central nervous system symptoms, like meningitis and neonatal sepsis [2–5]. The HPeV genome is about 7300 bases in length, enclosed in an icosahedrally-symmetric capsid of 60 copies of each of the three capsid proteins VP0, VP3 and VP1 [1,6]. HPeVs lack the maturation cleavage of the capsid protein VP0 into VP4 and VP2, which is present in most picornaviruses [7]. They have a 30-amino-acid-long extension to the N-terminus of VP3, and a unique non-structural protein 2A, lacking proteolytic activity [8]. HPeV1 contains an arginine-glycine-aspartic acid (RGD) motif close to the C-terminus of VP1 [1] The RGD motif is found in a number of viral capsid proteins which recognize integrin receptors to gain entry into host cells e.g. coxsackievirus A9 (CVA9), echovirus 9 (Echo9) and foot and mouth disease virus [9–11]. The role of the RGD motif for HPeV1 has been shown through blocking experiments with RGD-containing peptides and mutations of the sequence, where deletion of the RGD motif is lethal [12–15]. Studies of the HPeV1 virion in complex with both αVβ3 and αVβ6 integrins confirmed that they have overlapping binding sites on the predicted site of the RGD motif on the capsid surface [6]. The RGD motif has also been shown to be an important antigenic site. Diluted antiserum raised against a peptide containing the RGD motif neutralized 51% HPeV1 infections in a plaque assay compared to a background of 1% in the preimmune serum [16]. When the virion has been used as in antigen in rabbits, the immune sera recognize linear epitopes from VP0 and VP3. 100% neutralization has been shown with rabbit sera raised against virions and VP1 [16,17]. There are several potential neutralizing mechanisms for antibodies that bind specifically to viral capsid surfaces e.g. antibodies may neutralize by obstructing a receptor-binding site, cause viral aggregation as a result of interlinking particles or alternatively by binding bivalently preventing uncoating [18–20].

We have isolated two different human HPeV1 monoclonal antibodies (mAb) (Westerhuis et al., submitted), of which AM18 was shown to be a broadly cross-neutralizing mAb against HPeV1, 2, 4, 5 and 6, and AM28 neutralized HPeV1 and HPeV2. These results indicated two different neutralizing epitopes for AM18 and AM28. Here, we present the epitopes identified for AM18 and AM28 and also their neutralization mechanism. The location of the virus neutralization epitopes on the capsid surface was revealed by peptide scanning for AM18. For AM28, we generated homology models of the HPeV1 capsid and the AM28 antibody as there are no atomic models available, and used these to interpret data from electron cryo-microscopy (cryoEM) and three-dimensional (3D) image reconstruction of AM28 complexed with HPeV1.
Results

Peptide scanning

To determine the specific binding region of the mAbs AM18 and AM28, overlapping 12mer peptides designed to cover the P1 (VP0, VP3 and VP1) sequence of HPeV1 were used in a peptide ELISA (biotin was bound to the N-terminus of the peptide without a linker) (Figure 1). The AM18 antibody showed strong binding to one peptide containing the RGD motif (peptide number 85; VTSSRALRGDMA), binding to a lesser extent to the second peptide containing the RGD motif (peptide number 86; ALRGDMANLTNQ), no binding to the preceding peptide (number 84: FFFPLPAPKVTS), and low binding to a peptide in the VP0 region (YGQSRYFAAVRC). This difference in binding between the two ‘RGD’ containing peptides indicates that the residues VTSSRN-terminal to the RGD increase the binding specificity, most probably by increasing the accessibility of the RGD motif due to the position of the RGD in the peptide (as no flexible linker was used between the biotin and the peptide of interest), not due to sequence specificity as peptide 84 showed no binding. The AM28 antibody showed no binding to the linear overlapping peptides in the ELISA (Figure 1), strongly suggesting that the epitope recognized by it is a non-linear, conformational-dependent epitope, hence we progressed with three-dimensional epitope mapping on the intact virions.

Figure 1. Peptide-scanning ELISA of AM18 and AM28. Plot of peptide number versus absorbance. The x-axis shows the peptides (P1 region) used to test binding with AM18 (A) or with AM28 (B) and the y-axis shows the absorbance at 540 nm after background subtraction from absorbance at 620 nm. Peptide number 85 (VTSSRALRGDMA) showed significant binding to AM18 compared to peptide number 86 (ALRGDMANLTNQ) even though both the peptides contain the ‘RGD’ motif.
Virus-antibody complex

In order to understand the neutralization mechanisms and the exact epitopes of the mAbs AM18 and AM28, we imaged HPeV1 virions complexed with either mAb in a transmission electron microscope under cryogenic conditions. The micrographs did not show any noticeable disruption of HPeV1 virions in the presence of the mAbs antibodies AM18 or AM28, but they did induce strong aggregation of the HPeV1 virions (Figure 2).

Since no linear epitopes were determined for AM28 using peptide scanning, we used cryoEM, 3D reconstruction, modelling and fitting to determine the binding site of AM28 on HPeV1. Due to aggregation of the virus in the presence of the mAb, we prepared AM28 Fab-labelled virus. The reconstruction statistics of HPeV1-AM28 Fab are summarized in Table 1 and the reconstruction is shown in Figure 3. In comparison with the virus reconstruction alone, the HPeV1-AM28 Fab reconstruction showed clear additional density attributable to the Fab density either side of the two-folds, bridging neighbouring pentamers (Figure 3B-C). This Fab footprint is distinct from the integrin footprint encompassing the VP1 RGD epitope that we have shown previously [6] (Figure 3D).

**Figure 2.** AM18 and AM28 antibodies recognise virus capsids leading to aggregation. Raw micrographs showing the aggregation (red arrow) of HPeV1 when mixed with AM18 Ab (A) or AM28 (B) at a molar ratio of 1(capsid):5 (antibody). (A & B) Scale bar in (A) is 100 nm.

**Table 1.** Statistics of the reconstruction

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HPeV1-AM28 Fab reconstruction</th>
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<tbody>
<tr>
<td>No. of micrographs</td>
<td>65</td>
</tr>
<tr>
<td>No. of particles used in the reconstruction</td>
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</tr>
<tr>
<td>Underfocus range</td>
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<tr>
<td>Resolution (Å)</td>
<td>19.76</td>
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Figure 3. HPeV1-AM28 Fab reconstruction. A) Raw micrograph of HPeV1 in complex with AM28 Fab. Bar, 50 nm. B) Central cross-section of HPeV1-AM28 Fab complex with two-fold (2f), five-fold (5f) and three-fold (3f) symmetry axes marked. Scalebar 15 nm. C) Three-dimensional radially depth-cued reconstruction of the HPEV1 capsid with 60 Fab molecules bound. The reconstruction is colored according to the distance from the center of the particle. The color key is shown below the reconstruction. D) Overlay of the integrin-bound form of HPEV1 (EMD-1689; greenish blue) with the HPEV1-AM28 (grey) complex showing different binding sites for integrin and antibody. A red arrow indicates one of the Fab molecules on the capsid surface and a black arrow indicates one of the positions where the integrin is bound.
Figure 4. Epitopes on HPeV1 for AM28. A) Homology models of VP1 (red), VP0 (yellow) and VP3 (green) built using I-TASSER. B) Final fits of VP1, VP0 and VP3 homology models into an asymmetric unit of HPeV1 (EMD-1690). C) Superimposing asymmetric units of echovirus 1 (PDB ID: 1EV1; magenta), poliovirus 1 (PDB ID: 1POV; grey), enterovirus 71 (PDB ID: 3VBF; orange) and foot mouth disease virus (PDB ID: 1QQP; cyan) on final fits of HPeV1 VP1 (red), VP0 (yellow) and VP3 (green). D) Mapping epitopes for AM28 on HPeV1 surface by fitting AM28 Fab variable region homology model into the Fab density seen in HPeV1-AM28 Fab reconstruction and superimposing VP1 (red), VP0 (yellow) and VP3 (green) fits for HPeV1 (EMD-1690) into the HPeV1-AM28-Fab reconstruction (mesh). AM28 variable heavy chain is shown in magenta and variable light chain is shown in blue. E) Roadmap showing the density of AM28 Fab (red line contour, radius 155-156Å) and the epitopes HEWTPSWA (VP0; yellow), HQDKP (VP0; cyan), PLISIPTGSANQVD (VP0; magenta), MADSTTPSENHG (VP3; blue), ATTAPQSIVH (VP3; green) and FFPNATTDST (VP3; red). An asymmetric unit is marked by black lines. F) Distance between the symmetry-related Fab shown as wire.
In order to approximate the AM28 binding site, a homology model of the HPeV1 capsid was generated (Figure 4A) and subsequently superposed on the 20Å resolution HPeV1-AM28 Fab reconstruction. The highest confidence model was obtained for VP3 with an I-TASSER based confidence score (C-score) of -0.38 followed by VP0 with a C-score of -1.60. In contrast, the VP1 had a C-score of only -3.77. The typically C-score ranges from -5 to 2 with high score means better confidence in the quality of modelling [21]. In general, a C-score of -1.5 means more than 90% of the quality predictions are correct, thus, the VP1 model was only used to constrain the fitting of VP0 and VP3 in the asymmetric unit (Figure 4B-C). All the models had the characteristic eight-stranded β-barrels found in all picornaviruses capsid proteins (Figure 4C). Since the termini in picornaviruses are least conserved in the 3D conformation within the capsids and prediction was unreliable, we truncated the termini of the homology models. The placement of the individual capsid proteins within the capsid shell was improved using flexible fitting, resulting in improved fitting of the β-barrels and long helices of the models [22–24]. In addition a model of the AM28 Fab variable region was also generated and fitted into the Fab density in the HPeV1-AM28 Fab reconstruction. This showed that the antibody recognizes a conformational epitope which has contributions from both VP0 and VP3 (Figure 4D).

Table 2. Mapping conformational epitopes for AM28 and linear epitopes from peptide scanning of sera [13] on the capsid protein amino acid sequence.

<table>
<thead>
<tr>
<th>Capsid protein</th>
<th>Amino acid sequence*</th>
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<tbody>
<tr>
<td>VP0 (Gerbank id: L02971, amino acids 1-289)</td>
<td>METIKSIADMATGVSSVSDSTINAVNEKVESVGNEIGGNLLTKVADDASNILPGN CFATTAEPEKNKVQATTTVNTLTQHPASPTMPFPSDFSBNVDNFSHAYMDIT TGDKNSPKLVRLETHEWTPSWARGYQITHVELPKVFVHDQDKPAAGQSRYFA AVRCQFHFOQVFVQNVNGQTG3ALVYVEPKPYTVYDSKLEFGAFTNLPHTVLMLNL AETTQADLCIPYVADTNVYKTDSSDLGQLKVYYWTPLSIPTGSANSVDTILGSL LQLDQNPVRFAQDVNYIYDN</td>
</tr>
<tr>
<td>VP3 (Gerbank id: L02971, amino acids 290-542)</td>
<td>APNGKNNWKKIMTMSTKYKWTRTKIDIAEGPSMNMANLCLTGACSGVALVG ERAFYDPRTAGSKSRFDLLKIAQLFSSVMADSTTPSENHVDAKGYFKWSATT APGSIVHRNIVYLRFLPNLNFVNFNSYFRGSLVRSLSVYASTFRNRGRLRMGFFP NATTDSTSLDNAIYTDIGSDNSFEITIPYFSSTWMRKTNQPIGLFQIEVLRNL TYNSSSPSEVYCVGKGMGDARFFCPTGVSSTFQ</td>
</tr>
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</table>

*Green: Antigenic sites identified by peptide scanning in [13]. Red: Antigenic sites for AM28 identified by fitting pseudo-atomic model fitting in EM density of HPeV1-Fab AM2. The loops in consecutive order are βB-βC (VP0), αA-βD (VP0), βI-βH (VP0), αZ-βB (VP3), βB-βC (VP3) and βE-αB (VP3).
Modelling and fitting of the HPeV1 VP0, VP1 and VP3 proteins indicated that amino acids in the following loops in HPeV1 were involved in the footprint: βB-βC (VP0), αA-βD (VP0), βI-βH (VP0), αZ-βB (VP3), βB-βC (VP3) and βE-αB (VP3) (Figure 4D and E and summarized in Table 2 in red). These identified antigenic regions are distinct from linear epitopes of VP0 and VP3 that have been described previously by peptide scanning (Table 2, amino acids indicated in green) [16]. The fitting of the Fab variable region was unambiguous, with a cross correlation value of 0.88 compared to the 0.84 if the molecule was rotated by 180°. The distance between the two Fab molecules across the two-fold symmetry axis was on average about ~53Å (Figure 4F).

**Conservation of the conformational epitope recognised by AM28**

We compared amino acid sequence alignments of the newly identified VP0 and VP3 antigenic regions from different HPeV1 isolates with those of HPeV2-6. They were well conserved in HPeV1, moderately conserved in HPeV2 and poorly conserved in HPeV3-6 (Figure 5) which explains why mAb AM28 cross-binds and cross-neutralizes HPeV2, but no reactivity was detected against HPeV3-6 (Westerhuis et al., submitted).

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*Figure 5. Conservation of epitopes. Amino acid sequences of HPeV1-5 used for neutralization in Westerhuis et al., (submitted) were aligned against complete genome sequences for HPeV1-6. The sequence annotation on the left hand side is ‘virus genotype/GenBank ID’. The epitopes are marked in black on the HPeV1-Harris strain (GenBank ID: L02971) that was used as the basis for the HPeV1 homology modelling. The alignment is coloured according to percent sequence identity, from a scale of white (no identity) to dark blue (full identity). The conservation panel in yellow below the alignment gives the numerical values for the conservation based on the BLOSUM 62 score of the alignment and physicochemical conservation where * is 100% identity. The blue arrowheads indicate irrelevant regions of the sequence that have been hidden in the final representation for simplicity (1-120, 160-246, 270-372, 413-453). The figure was made with Jalview.*
**Mode of neutralization**

In order to understand the mechanism of neutralization, we performed a capsid thermal stability assay using an RNA binding dye, which has previously been used to explain the mode of action of EV71 neutralizing antibodies [19]. The Tm of the HPeV1, HPeV1-AM18 and HPeV1-AM28 were 53°C, 54°C and 56°C respectively (Figure 6). The 3°C shift in Tm for HPeV1-AM28 compared to HPeV1 alone indicates that the bivalent binding of AM28 across neighbouring pentamers stabilizes the capsid, inhibiting RNA release from the capsid. In contrast, a shift of 1°C in Tm for HPeV1-AM18 suggests that increasing the capsid stability is not the primary reason for neutralization by AM18.

**Figure 6.** Thermofluor assay. Plot of temperature (x-axis) versus first derivative of fluorescence (y-axis) showing the change in Tm when HPeV1 is bound to antibodies AM18 or AM28 compared to HPeV1 alone. Arrows indicate the Tm for each sample. AM18 and AM28 were used as the negative control for the RNA binding dye.
Discussion

HPeV infections can be severe and even life threatening, especially for neonates. Severe infections in neonates can be due to a lack of protective maternal Abs. No treatment is currently available against these severe HPeV infections, making it an unmet medical need [25]. For HPeVs, Ab-based therapies are a feasible option as the HPeV genus is a relatively small group of highly similar viruses for which cross-reactive, neutralizing polyclonal Abs have already been described [16,17]. For a successful antiviral approach, knowledge of specific and overlapping viral antigenic sites is important and mAbs are preferred. We developed two different mAbs AM18 and AM28 which were able to neutralize HPeV1 efficiently at IC50s of 2.6 to 5.5 ng/ml (Westerhuis et al., submitted). These mAb were cross-reactive with HPeV2 and AM18 also showed cross-reactivity against HPeV4. Peptide-scanning ELISA analysis confirmed that the site of the AM18 binding was within the sequence ‘ALRGDMA’ as this was the common sequence between two positive overlapping peptides from the VP1 C-terminus. Of this, “RGDMA” is also common to HPeV4 VP1 and “RGD” is common to HPeV2 VP1; it is missing from HPeV3. Hence, the minimally-recognised epitope for neutralization is “RGD” and AM18 most likely neutralises by directly competing with the cellular receptor (integrins) for this binding site, diminishing the ability to infect cells through this route. AM18 recognises and neutralizes other RGD containing viruses like coxsackievirus A9 (Westerhuis et al. submitted), but it did not neutralize Echoviruses presumably because they can use alternative receptors to enter the host cells. For HPeV1 it has been shown that RGD-less mutants with reduced binding to integrin are less viable [12], it may therefore be difficult for the virus to generate escape mutants resistant to AM18 neutralization. Hence AM18 shows potential as a useful therapeutic molecule.

AM28 did not recognize linear epitopes from denatured proteins or overlapping peptides in Western blot or ELISA, indicating that the epitopes are conformational. We used homology models of the VP0, VP1, VP3 and AM28 fitted into a HPeV1-AM28 Fab reconstruction to identify a conformational epitope which has contributions from VP0 and VP3 of neighbouring pentamers. Although we have little experimental data to show the validity of the homology models, as there are no atomic models for parechoviruses that we are aware of, the position of the RGD epitope in VP1 agrees to within 5 Å with the position of the integrin footprint found earlier [6] and the fit of the obvious elements of secondary structure, such as the β-barrels and the VP0 helices forming the interface at the two-fold are consistent with what is expected from the literature. Comparison of known picornavirus structures with that of our capsid model indicates that this conformational epitope is a region commonly occupied by the equivalent loops from VP2 and VP3, defined by the conserved position of the β-barrels. Hence, even though we could not trace the chain of
the capsid proteins, or identify amino acid side chains, we are confident in the prediction of the loops contributing to the footprint. The sequence conservation between HPeV1 and HPeV2 supports this prediction, as both are neutralized by AM28. From the reconstruction AM28 appears to staple neighbouring pentamers together thereby stabilizing the whole capsid. This hypothesis was tested by measuring the fluorescence of an RNA binding dye when the virus capsid was heated in a step-wise manner from 25°C to 95°C. The idea was that upon heating, the capsid would destabilize at a certain temperature, thus allowing the dye increased access to the RNA. AM28 had a stabilising effect on the capsid. Thus the Tm was increased by 3°C compared to the virus alone. Significantly, breathing of picornavirus capsids for RNA release has been shown to be dependent on domain movements that open up the interfaces at the two-folds [26–32]. Hence, the monovalent binding of one Fab arm of AM28 across the two-folds, would prevent this movement, stabilize the capsid, prevent uncoating of RNA from the capsid on cell entry, and thus neutralize the virus. The avidity of AM28 would be higher because both the Fab arms can bind the neighbouring epitopes as the distance between the two Fab arms is about 53Å when bound to the capsid [20,33]. Interestingly, this conformational epitope did not overlap with any of the linear immunogenic epitopes identified previously by peptide scanning [16] indicating that such single dimensional epitope mapping techniques may miss some of the crucial epitopes on the capsid surface which are only presented in the tertiary form. The importance of this area of the capsid in capsid assembly and RNA delivery is shown in the conservation of these loops in multiple HPeV1 and HPeV2 isolates, but not in the other serotypes. Hence this mAb also shows therapeutic promise for HPeV1 and HPeV2 infections offering a glimmer of hope to the many patients that are afflicted. The atomic model of HPEV1 will be of use in understanding mutations in the capsid that affect the tropism of the virus – an area of great interest in understanding the transmission from the respiratory and gastrointestinal tract to the central nervous system.

### Material and Methods

#### Virus culture and purification

HPeV1-Harris was provided by the Dutch National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands and grown on a human colon carcinoma cell line (HT29). The HT29 cells were maintained in Eagle’s Minimum Essential Medium (EMEM) with L-glutamic acid (0.2X), non-essential amino acid (1X), streptomycin (0.1 μg/ml) and ampicillin (0.1 μg/ml), supplemented with 8% heat-inactivated fetal calf serum (FCS). The virus concentration was determined by the median tissue culture infective dose (TCID50) and calculated by the Reed and Muench method [34]. For the large scale
virus purification 90% confluent cell layers in T-175 flasks were infected with HPeV1 at a multiplicity of infection 0.1. After 75-100% infection of the cell monolayer evident by the cytopathic effect, the cells and spent media were freeze-thawed twice at -80°C/+37°C, centrifuged at 4000 rpm for 15 minutes in an Eppendorf A-4-62 swing bucket rotor at 4°C and the supernatant was filtered using a 0.22 µm filter. The virus was pelleted by ultracentrifugation at 32000 rpm for 2 h at 4 °C in a Beckman SW32Ti rotor. The pellet was dissolved in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl2 (1X TNM buffer) and loaded on to a cesium chloride step gradient with a 5ml 40% (w/v) bottom layer and a 5ml 15% (w/v) top layer and centrifuged at 32000 rpm, for 16 h at 4 °C in a Beckman SW41Ti rotor. The fraction containing the virus was concentrated with a 100kDa cutoff filter (Millipore) in 1X TNM.

**Antibody preparation and virus labeling**

The human AM18 and AM28 antibodies were prepared as described previously (Westerhuis et al., submitted). In brief, human memory IgG+ B cells were cultured using the AIMSelect method [35,36] and antibody-containing culture supernatants were used to directly screen for HPeV1 neutralization. Fab fragments from AM28 were produced using a Pierce Fab micro preparation kit according to the manufacturer’s protocol. The resulting Fab was mixed with HPEV1 capsids at a molar ratio of 5:1 in 1x TNM buffer for 30 minutes at room temperature.

**Peptide-scanning enzyme linked immunosorbent assay**

Streptavidin-coated ELISA plates (Greiner bio-one) were blocked with 2% BSA in PBS for 2 h at room temperature. N-terminally biotin-labelled HPeV1 overlapping peptides (12aa in length with 6aa overlap and no flexible linker) (Antonie van Leeuwenhoek, Netherlands cancer institute, Peptide Synthesis) were diluted (1:500) in 1% BSA in PBS and bound to the plate for 1 h at room temperature. The plate was incubated with 2 µg/ml of AM18 or AM28 for 1 h at room temperature and washed 3 times with PBS/0.1% Tween. Anti-human IgG HRP-labeled (0.3 µg/ml) was used as the secondary Ab, incubated for 1 h at room temperature and washed three times with PBS/0.1% Tween. The substrate solution containing 3,3',5,5'-tetramethylbenzidine was added and incubated for 10 min at room temperature in the dark. The reaction was stopped by the addition of 0.8 M H2SO4. The absorbance at 450 nm and 620 nm was measured with a microplate reader.
Electron cryo-microscopy

Aliquots of the Fab-virus mixture were vitrified on Quantifoil R2/2 holey carbon nickel grids in a home-built guillotine by plunging into liquid ethane maintained in a liquid nitrogen bath. After vitrification, the grids were stored in liquid nitrogen until use. The grids were examined in a FEI F20 transmission electron microscope at 200 keV using a Gatan 626 cryostage. The images were recorded on a Gatan Ultrascan 4000 under low dose conditions at a magnification of 69000X with a sampling size of 2.17 Å per pixel.

The contrast transfer function of each micrograph was estimated using CTFFIND3 and images containing drift or astigmatism were discarded [27]. Particles were picked using the program ETHAN [37] with a box size of 401 pixels and inspected by eye in the program suite EMAN [38]. A previous reconstruction of HPeV1 from Seitsonen et al., 2010 (EMD-1690) was used as a starting model to initiate full orientation and origin determinations of the Fab labelled set of images using AUTO3DEM ver4.03.1 [39]. The final reconstructions calculated to the Nyquist frequency were used to estimate the B-factors with EM-Bfactor, and then the reconstructions were truncated to the resolution indicated by the Fourier shell correlation analysis with a threshold criterion of 0.5 [40,41]. The HPeV1-AM28 Fab density map was deposited in the Protein Databank in Europe (accession number EMD-XXXX).

Homology modelling and fitting of models into cryoEM maps

The structures of the three HPeV1 capsid proteins were predicted by multiple-template comparative modeling using the I-TASSER server [30]. The template structures for VP0 were foot and mouth disease virus (PDB ID: 1QQP, 1FMD and 1BBT) [42,43], poliovirus 1 (PDB ID: 1POV) [44], bovine enterovirus (PDB ID: 1BEV) [45] and Seneca Valley virus-001 (PDB ID: 3CJI) [46]. For VP1, they were triatoma virus (PDB ID: 3NAP) [47], human rhinovirus 14 (PDB ID: 1D3I) [48], cricket paralysis virus (PDB ID: 1B35) [49], rabbit hemorrhagic disease virus (PDB ID: 4EJR) [50], echovirus 7 (PDB ID: 1M11) [51] and bovine enterovirus (PDB ID: 1BEV) [45]. For VP3, they were human enterovirus 71 (PDB ID: 3VBF) [28], Seneca Valley virus-001 (PDB ID: 3CJI) [46], human rhinovirus 16 (PDB ID: 1AYM) [52], poliovirus Mahoney strain (PDB ID: 1HXS) [53].

An atomic model of echovirus 1 capsid (PDB ID: 1EV1) [54] was placed into an 8.5 Å resolution HPeV1 map (EMD-1690) [6]. The fitting was done using a modification of a protocol described elsewhere [24]. The homology models were aligned with the echovirus 1 capsid, before being rigidly fitted into the HPeV1 map (EMD-1690) [6] using the ‘fit in map’ feature in UCSF-Chimera [55]. The N-terminus of VP0, VP3 and VP1 were truncated to avoid inter-subunit clashes. Using the ‘zoning’ feature in UCSF-Chimera [55], the HPeV1
capsid map was zoned to an asymmetric unit with a radius of 6 Å using the truncated VP0-VP3-VP1 rigidly-fitted model. RIBFIND based rigid bodies were identified for the truncated VP0-VP3-VP1 model [24] and the model was flexibly fitted into the asymmetric unit using one iteration in FlexEM [22] followed by iMODfit based flexible fitting using the default settings [23]. The resulting homology model of the complete HPeV1 capsid was then placed directly into the Fab-labelled reconstruction to identify the probable binding sites. The variable regions of AM28 Fab were modelled using the WAM webserver [56] and manually fitted into the corresponding Fab density in the HPeV1-AM28 Fab reconstruction and the fit was optimized by ‘fit in map’ feature in UCSF-Chimera. All the visualization was carried out in UCSF-Chimera [55]. The fitted models are deposited in the Protein Databank in Europe with the PDB ID: XXXX.

Sequence alignment

The P1 amino acid sequences of HPeV1 (GenBank ID: L02971, GQ183023, GQ183022, GQ183021, GQ183020, GQ183019, GQ183018, GQ183025, GQ183024), HPeV2 (GenBank ID: NC_001897), HPeV3 (GenBank ID: GQ183026), HPeV4 (GenBank ID: DQ315670) and HPeV5 (GenBank ID: AM235749) used for AM18 and AM28 neutralization assays (Westerhuis et al., submitted) were aligned using Clustal Omega [57] with additional HPeV strains for which the complete genome sequences were available in GenBank (GenBank IDs for HPeV1 are JX441355, JX575746, S45208, EF051629, FJ840477, GQ183035, GQ183034, HQ696574, HQ696572, HQ696570, HQ696573, HQ696571, FM178558; for HPeV3 are GQ183027, GQ183028, GQ183029; for HPeV4 are AB433629, AM235750; for HPeV5 is AM235749; for HPeV6 are EU077518, AB252583). The alignment was visualized with Jalview [58].

Thermofluor assay

In order to test the capsid stability in the presence of antibodies, AM18 and AM28 mAb were mixed with HPeV1 virions (so capsids containing RNA) at a molar ratio of 66:1 and incubated at room temperature for 30 min. Dye-accessibility to the RNA increasing with heat was detected with a fluorescent dye. The reaction volumes were set up per well in a 96-well PCR plate and each reaction contained 2.5 µl of 200X Sybr Safe DNA gel stain (Invitrogen, also binds RNA) and the protein sample which was one of the following HPeV1 (10 µl of 1mg/ml stock), AM18 (10 µl of 2 mg/ml stock), AM28 (10 µl of 2 mg/ml stock), HPeV1-AM18 complex (20 µl) or HPeV1-AM28 complex (20 µl). The total volume was made up to 25 µl for each reaction volume using 1X TNM buffer. The assay was run from 25°C to 95°C with readout every 0.33 s in an Mx3005P qPCR instrument (Agilent Technologies). The Sybr Safe DNA gel stain dye was excited at 492 nm and emission was read at 516 nm.
Acknowledgements

We thank Pasi Laurinmäki, Konstantin Kogan and Eevakaisa Vesanen for excellent technical assistance and Susan Hafenstein for helpful discussions. The Biocenter Finland National Cryo Electron Microscopy Unit, the Crystallization Facility in the Institute of Biotechnology, Helsinki University and the CSC-IT Center for Science Ltd. are thanked for providing facilities.
References


