Three-dimensional reconstruction of *Heterocapsa circularisquama* RNA virus by electron cryo-microscopy

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*Heterocapsa circularisquama* RNA virus is a non-enveloped icosahedral ssRNA virus infectious to the harmful bloom-forming dinoflagellate, *H. circularisquama*, and which is assumed to be the major natural agent controlling the host population. The viral capsid is constructed from a single gene product. Electron cryo-microscopy revealed that the virus has a diameter of 34 nm and T=3 symmetry. The 180 quasi-equivalent monomers have an unusual arrangement in that each monomer contributes to a ‘bump’ on the surface of the protein. Though the capsid protein probably has the classic ‘jelly roll’ β-sandwich fold, this is a new packing arrangement and is distantly related to the other positive-sense ssRNA virus capsid proteins. The handedness of the structure has been determined by a novel method involving high resolution scanning electron microscopy of the negatively stained viruses and secondary electron detection.

INTRODUCTION

*Heterocapsa circularisquama* is a small dinoflagellate, with a case covering called the theca, that was first observed as the cause of large-scale red-tide blooms occurring at Uranouchi Bay, Kochi Prefecture, Japan in August 1998. It was subsequently observed in other areas of western Japan (Matsuyama, 1999). This algal species specifically kills bivalve molluscs such as pearl oyster (*Pinctada fucata*), manila clam (*Ruditapes philippinarum*), pacific oyster (*Crassostrea gigas*) and blue mussel (*Mytilus galloprovincialis*) (Horiguchi, 1995; Matsuyama, 1999). Hence, *H. circularisquama* is regarded as one of the most noxious bloom-forming microalgae.

*H. circularisquama* is infected by two distinct viruses: *H. circularisquama* virus (HcV; ~200 nm icosahedral DNA virus; Tarutani et al., 2001) and *H. circularisquama* RNA virus (HcRNAV; ~30 nm icosahedral RNA virus; Tomaru et al., 2004). Of the two, HcRNAV, a non-enveloped icosahedral ssRNA virus, is assumed to be the major natural agent controlling *H. circularisquama* population dynamics (Nagasaki et al., 2004; Tomaru et al., 2007).

Infectivity of HcRNAV is strain-specific rather than species-specific (Tomaru et al., 2004). The many strains of HcRNAV can be classified as UA-type or CY-type based on their intra-species strain-specific infectivity. Consequently *H. circularisquama* strains can be classified into three types: those that are lysed by HcRNAV UA-type, those lysed by HcRNAV CY-type, and those that exhibit no lysis for either type of HcRNAV (only two of the 56 strains tested; Tomaru et al., 2004). The two host–virus systems are independent and may coexist in a bloom, thereby affecting both the quality (i.e. the clonal composition) and the quantity (biomass) of the *H. circularisquama* bloom (Tomaru et al., 2007).
The two strains, HcRNAV34 and HcRNAV109, have been characterized as typical type UA and CY virus strains, respectively. HcRNAV34 and HcRNAV109 encapsidate a ~4.4 kb ssRNA genome (4375 and 4391 nt, respectively). Their genomes share 97% nucleotide identity and have two major ORFs (Nagasaki et al., 2005). In both strains, ORF-1 encodes a polyprotein that has domains with significant homology to RNA-dependent RNA polymerase (RdRP), helicase and protease (Nagasaki et al., 2005; E.V. Koonin, personal communication). Recently, the International Committee on Taxonomy of Virus (ICTV) provisionally approved a proposal to place HcRNAV in a new genus ‘Dinornavirus’ within the new class ‘Alvernaviridae’, because phylogenetic analysis of the RdRP showed HcRNAV was deeply branched and apparently distinct from other viruses. A comprehensive phylogenetic analysis of RdRP sequences from picorna-like positive-sense ssRNA viruses places HcRNAV in a clade with the land-based fungal viruses Sclerotaphora macrospora virus A and B (SmVA, SmVB), Mushroom bacilliform virus (MBV), two groups of plant viruses (the Luteoviridae, and the genus Sobemovirus) and the nodaviruses which infect insects, fish and animals (Koonin et al., 2008). ORF-2 encodes the 38.2 and 38.3 kDa single major structural capsid protein of HcRNAV34 and HcRNAV109, respectively (Nagasaki et al., 2005). The most striking differences between the genomes of the two types are four variable regions in capsid protein ORF-2. Phylogenetic analysis of the variable regions from a number of strains coincides with the host-specificity of the viruses, with type UA and CY virus strains divided into distinct groups. This suggests that the specificity is because of the sequence variation in the capsid protein. The functionally important, type-specific amino acids have been modelled to be externally located, based on similarity to black beetle virus (BBV), a member of the nodavirus family. HcRNAV-particle bombardment using a gene gun revealed that the difference in strain-specificity is because of the entry process of the virus into the host cell, presumably because of variations in the sequence of the capsid proteins (Mizumoto et al., 2007).

A search of the protein database using pGenTHREADER (Lobley et al., 2009) did not find any candidates for homology modelling of the capsid protein. As there are no homologues to the HcRNAV capsid protein with known structures, the structure prediction based on BBV may not be accurate and therefore structural studies of HcRNAV are necessary to assess the validity of these claims.

Here, we performed an electron cryo-microscopy (cryoEM) 3D reconstruction of one of the two HcRNAV strains – HcRNAV109 – to a resolution of 18 Å and provide the first structural information on this marine virus family. The correct handedness was identified by reconstructing surface-images in three dimensions from a negatively stained sample (Woodward et al., 2009). This is the first time that this technique has been applied to an icosahedral sample. The handedness ambiguity usually associated with projection images was overcome by measuring the secondary electron (SE) signal, in this case using a scanning electron microscope (SEM) equipped with an in-lens SE detector.

**RESULTS AND DISCUSSION**

**CryoEM and 3D reconstruction**

One hundred and eight micrographs, of approximately 5900 x 9200 pixels, with a sampling resolution of 1.808 Å pixel⁻¹ were recorded using cryoEM. After interpolation, this resulted in images with sampling resolution of 3.616 Å pixel⁻¹. In total, 2593 homogeneous HcRNAV109 particles, with diameters between 318 and 340 Å (88–94 pixels), which were situated in the vitreous ice and were clearly distinct from the neighbouring particles, were picked from 102 micrographs. HcRNAV particles, which exhibit the characteristic hexagonal and circular outlines that are consistent with icosahedral particles, are shown in the micrographs in Fig. 1.

The two independent starting models, generated by the image processing program, EMAN (Ludtke et al., 1999), or the common lines method employed by the MRC–LMB software (Crowther, 1971; Crowther et al., 1996) produce similar refined models. The two independent reconstructions have a Fourier shell correlation (FSC) of 0.5 at a resolution of 18 Å and 0.3 at a resolution of 15.2 Å (Fig. 2). A reconstruction from negative-stain data from 3511 particles (Supplementary Fig. S1, available in JGV Online) converges to a very similar model. Two reconstructions from independent subsets of the negative-stain data had a Fourier shell correlation of 0.5 at a resolution of 26 Å and 0.3 at a resolution of 20 Å.

The handedness of the model is indeterminate (Supplementary Fig. S2, available in JGV Online) because the images represent projections of the sample. In particular, the top and bottom of the particles are indistinguishable from one another. This means that two possible reconstructions, being mirror images of one another, are consistent with the data. In the past, tilting or metal-shadowing experiments were required to eliminate this ambiguity. We have used the SE signal from an SEM to image the ‘top’ surface of the negatively stained sample and thus identify the correct handedness (Woodward et al., 2009). Reconstruction of the negatively stained sample shows that the uranyl acetate ‘shell’ is essentially the same shape as the outer surface of the cryo-reconstruction and is thus a valid representation of the virus (Fig. 3b). The SEM works by sweeping a focused electron beam across the sample in a grid. In our case, an in-lens SEM detector measured the SE flux at each grid position.

SE are formed all along the primary electron beam trajectory, which extends through the entire sample. However, only those SE that arise sufficiently near the surface are able to escape and be detected. This information is used to reconstitute an image of the sample. This means that structural information is acquired from one side of the sample only, thus eliminating the ambiguity of a projection image. In some cases, this image has sufficient signal to...
determine the correct handedness from single images. In this case, several images needed to be aligned and their average calculated. The correct handedness was applied to the negative-stain and cryoEM reconstructions (Fig. 3).

Capsid structure

The capsid structure reveals a particle of ~34 nm diameter with an exterior surface covered by a symmetrical arrangement of 180 bumps. Within this outer capsid shell is another less well-defined shell of density, with an inner diameter of 12 nm and an outer diameter of 22 nm, which is presumed to be the encapsidated ssRNA genome. The outer capsid shell structure is consistent with 180 protomers arranged with $T=3$ icosahedral symmetry. In Fig. 4(a) the superposition of a $T=3$ icosahedral cage is shown. The different protomers in pentameric and hexameric arrangements are clearly visible when the model is coloured radially (Fig. 4b, c). A volume calculation indicates that there is one protein subunit per protomer. Using a threshold value of double the SD from the mean, the volume of the capsid portion of the virus (with the central RNA portion masked out for the calculation) was measured as being $1.037 \times 10^7 \text{ Å}^3$, 22% larger than the predicted volume for 180 subunits of the 38 kDa protein of $8.055 \times 10^6 \text{ Å}^3$ (using a protein density of 1.41 g.cm$^{-3}$). Factors which contribute to the larger-than-predicted protein volume are a combination of incomplete masking out of the RNA and using a threshold value for the volume map that is too low. There is also some variation in protein densities, thus the protein may occupy more than the calculated volume.

The $T=3$ symmetry implies that there must be three distinct copies of the protein subunit in the asymmetrical unit. A trimer of the subunits forms a 'biological asymmetric unit', which does not overlap with the icosahedral asymmetrical unit. The trimer is shown in Fig. 4(d), where it can be seen that there are different interactions between the quasi-equivalent subunits. The two distinct I and III subunits alternate in the formation of the hexamer around the threefold axis.

The density linking the subunits at the fivefold and threefold axes is stronger than the association of the bumps to form the trimer. This is evident when the map is rendered with a higher threshold (as is shown in Supplementary Fig. S3, available in JGV Online). Therefore, the icosahedral shell is probably formed from pentameric and hexameric capsomeres, rather than a trimer. A network of arm-like links joins the capsomeres at the shell level.

Examination of the HcRNAV map indicates that the protein subunit may have several domains. The largest domain forms the main bump that defines each subunit. It is also evident that there is a separate, smaller, oligomerization domain responsible for the interactions at the fivefold and threefold (pseudo-sixfold) axes. The interactions between the subunits are tightest in these regions, making it likely that the shells form from pentameric and hexameric capsomeres. In addition, there are one or two small segments or domains that form links between the pentameric and hexameric substructures. A more detailed interpretation is limited by the resolution of the map. The arrangement of the density, predominantly in subunit-sized lumps in rings around the fivefold and sixfold symmetry axes, indicates a monomeric arrangement of the subunit, which is unusual.

The capsid proteins of all known positive-sense ssRNA plant viruses have the ‘jelly roll’ β-sandwich fold (Chapman & Liljas, 2003), as do nodaviruses. Structures

Fig. 1. Comparison of cryoEM, negatively-stained and SEM images. (a) Representative cryoEM images of HcRNAV109 particles embedded in vitreous ice. (b) Negative-stain images. (c) Contrast inverted in-lens secondary electron SEM images. The signal/noise ratio of these images is excellent, allowing direct visualization of some features without averaging. In this case however, direct handedness determination was not possible without reconstructing the particle in three dimensions. Bar, 20 nm.
of the fungal capsid proteins are not known; however, a BLAST (Sayers et al., 2010) search of the predicted MBV capsid sequence gives high identity with plant virus capsid proteins (~30%). Similarly, sequence identities of 17–22% were observed between the core protein of SmVA and the tombusvirus family (Yokoi et al., 2003), which are positivesense ssRNA plant viruses. Therefore it is likely that HcRNAV capsid structure shares the ‘jelly roll’ β-sandwich fold with these other related virus families.

The ‘jelly roll’ β-sandwich virus fold is modified in the capsids of different virus families, and sometimes within a family. The sobemoviruses, (e.g. southern bean mosaic virus) have a fairly minimal, uninterrupted ‘jelly roll’ β-sandwich fold. In the tombusvirus family (e.g. tomato bushy stunt virus; TBSV) an additional domain is appended to the carboxy terminus. These dimerize and form projections from the shell domain (Olson et al., 1983; Rossmann, 1984). Insect nodaviruses have an inserted sequence known as the P loop between the strands of the β-sandwich. These trimerise and project from the shell structure. In a virus related to the nodaviruses, Nudaurelia capensis omega virus (NoV), a tetravirus (Munshi et al., 1996), the P loop is replaced by an inserted immunoglobin domain that faces outwards from the capsid. The structures of the insect nodaviruses and NoV are otherwise very similar: they have the same shell domain fold, both have another inner alpha-helical domain formed from alpha helices at the amino- and carboxy-terminal side of the shell domain and, finally, post-assembly autocatalytic cleavage of a carboxy terminus alpha-helical region from the main ‘jelly roll’ β-sandwich domain is required for infectivity.

Fig. 2. (a) Twofold, (b) threefold and (c) fivefold surface views of the cryo reconstruction of HcRNAV with the density mapped onto an icosahedral surface below them. The density increases from black, red, yellow, green and aquamarine to blue as shown in the key. The reconstruction was performed with SPIDER (Frank et al., 1996) and the images created with CHIMERA (Pettersen et al., 2004). (d) FSC (solid line) and differential phase residual (DPR) (dashed line) of the final cryoEM reconstruction indicating resolution values of 18.1 Å using the FSC=0.5 criterion, 15.2 Å using the FSC=0.3 criterion and 22.6 Å using a 45° threshold for DPR.
In a search of the available virus structure databases (VIPERdb, Carrillo-Tripp et al., 2009; EMDB, Lawson et al., 2011; PDB, Berman et al., 2000) for other \( T=3 \) capsid structures showing similar morphology to HcRNAV, no similar matches were found. At this resolution of \(~20\) Å, the appearance of the capsids is governed mainly by their quaternary structure, as the densities of closely associated subunits or domains will blend together. Hence, the single bump feature in this \( T=3 \) map gives a strikingly different capsid appearance compared with the spikes formed from dimers or trimers, as in the tombusvirus or nodavirus families, though the underlying capsid fold is probably very similar. Those spikes form at the interface of the dimers or trimers, whereas the HcRNAV bump sits centrally on a single subunit. The smaller sobemovirus structures do not have a feature that matches the bump of HcRNAV either.

A similar morphology could be seen, however, in the procapsid form of NoV (Canady et al., 2000) even though this is a \( T=4 \) capsid. NoV is remarkable in that during the transition from the procapsid to the mature capsid form there is a switch in the subunit interactions within the shell domain, from being a dominantly dimeric to a predominantly trimeric subunit interaction (Canady et al., 2000, 2001). In the procapsid form of NoV there are relatively weak dimer associations in the protomer, which allows the individual subunits to be distinguished in the 28 Å resolution cryoEM map. When individually resolved, the subunit packing resembles that of HcRNAV. The shapes of the regions forming the interactions around the fivefold and threefold symmetry axes also look similar.

Nagasaki et al. (2005) modelled the HcRNAV capsid protein based on \(~10\%\) sequence homology to the BBV capsid protein (Wery et al., 1994) to determine where the species-specific amino-acid substitutions occur. The capsid plate was modelled as a trimer of ORF-2 products. Seven and eight of the 14 significantly different residues were predicted to be on the outside of the UA and CY capsid proteins, respectively, with no significant amino acid substitutions on the interior of the capsid protein (Nagasaki et al., 2005). The comparison of high resolution structures of the members of the nodavirus family (of which BBV is a member) and the NoV capsid folds solved by X-ray crystallography (Munshi et al., 1996) shows a common fold is conserved in these related virus families though they are distant in evolutionary terms, and significant adaptation has occurred. In HcRNAV the subunit is monomeric, and not trimeric or dimeric as in BBV and NoV. Therefore, it is likely that HcRNAV contains a related and similar fold, though it may be modified. This provides justification for the model of the HcRNAV capsid protein based on the shell domain in the BBV structure, even though the sequence identity is weak. Supplementary Fig. S4 (available in JGV Online) shows an alignment of the sequences of BBV and HcRNAV together with the actual and predicted secondary structures, respectively. The quaternary structure and capsomere unit of HcRNAV is different from BBV, but the morphological relationship of the capsid to the related NoV makes it very likely that they have a related fold. It is also probable that the orientation of the fold is similar, with the carboxy and amino termini facing the inside of the shell. Therefore, the structure is consistent with the homology modelling, placing the species-specific mutations on the exterior of the capsids.

A plot of radial density distribution comparing the structure of the insect nodavirus Pariacoto virus (PaV, Tang et al., 2001) and a fish nodavirus (as shown by Tang

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**Fig. 3.** Handedness determination. (a) Surface-display representation of the cryoEM reconstruction orientated along the icosahedral fivefold axis. The individual protein subunits making up a face are indicated by red asterisks. (b) Negative-stain map in the same orientation as (a), the individual subunits (red asterisks) show evidence of flattening, but are clearly in the same positions as the cryoEM map. (c) 3D SEM reconstruction. While the individual protein subunits are less distinct than in (a) or (b), their relative orientations can be clearly seen (red asterisks). The 3D SEM reconstruction preserves the handedness of the original sample and has therefore been used to assign the correct handedness to the negative-stain and cryoEM reconstructions.
et al., 2002) provides further evidence of similarities in capsid structure between HcRNAV and members of the family Nodaviridae. The radius where the shell domain meets the packaged RNA (11.2 nm) is similar for these nodaviruses, and compares closely with HcRNAV (11 nm, Fig. 4f). The outer shell measurements vary slightly owing to the different projection domains. Harrison (1980) reports the same inner radius for the capsid of TBSV, 11 nm. Therefore, the inner $T=3$ shell created by the ‘jelly roll’ $\beta$-sandwich fold is remarkably consistent across diverse families, though it is modified in different ways.

The fish nodavirus shows a stronger homology with TBSV than with insect nodaviruses and has a carboxy-terminal P domain rather than the P loop. PaV has an icosahedrally ordered shell of RNA, whereas the other members of the nodavirus family do not. These comparisons indicate a range of diversity that occurs within virus families. Another example of diversity in virus components is shown in Drosophila A virus (DAV; Ambrose et al., 2009). Though first described as a picorna-like virus, it has components evolutionarily related to the families Nodaviridae, Tetraviridae and Tombusviridae. The DAV capsid protein shows strongest sequence similarity with the nodavirus family, but the capsid domain folds are more similar to members of the tombusvirus family. The evolution of the coat protein gene and other genes is not always in synchrony; they evolve at different rates owing to host
selection pressure, function and evolution through recombination. Like fish nodavirus, the structure of DAV particles shows an inner $T=3$ icosahedral core surrounded by a less-well-defined outer protein domain. The radius of the RNA–capsid boundary is similar, ~11 nm. In HcRNAV the bump is firmly in the middle of the protein subunit, not loosely on a tail like DAV or fish nodavirus, therefore the fold of HcRNAV is likely to be more similar to the nodaviruses/tetraviruses than the plant tombusviruses, e.g. TBSV.

**RNA packing**

A cross-sectional view of the HcRNAV map reveals an inner double shell of RNA (Fig. 4e), which is connected to the outer protein shell by thin connections. The two inner RNA layers form a spherical shell of 22–12 nm in diameter, with their interface at a radius of 8.5 nm. The peak-to-peak spacing between the two RNA shells is ~22 Å. Fig. 4(f) shows a plot of the radial density distribution of the structure, on which each of these layers is indicated. RNA usually has a higher density than protein, however since the RNA is not icosahedrally symmetrical, and although icosahedrally averaged during the reconstruction, the RNA shell appears to have a lower density on the map.

In Fig. 5, cross-sectional views of the model are coloured according to the density (i.e. the value of the volumetric pixel). Areas of higher density between the RNA and the capsid indicate areas of RNA that are more ordered and that are likely to be where the RNA binds to the protein. By slicing the density plot at a higher plane along the $z$-axis (Fig. 5), it is revealed that ordered-RNA regions occur near the threefold axes. There are also ordered connections between the protein shell and the inner RNA shell near the fivefold symmetry axes, which can be seen in Fig. 4(e). The amino-terminal region of the capsid protein contains an arginine-rich region (12 of the 18 aa from residues 22–39; Supplementary Fig. S4), which is most likely to be the RNA binding region. This suggests that the amino terminus is positioned towards the interior of the virus. Similar links joining an inner RNA shell with an outer protein shell were seen in a cryoEM map of hepatitis B virus core shells containing encapsidated RNA (Roseman et al., 2005).

The volume for this ~4.4 kb RNA genome, if it were tightly packed, is theoretically predicted to be $1.33 \times 10^6 \text{Å}^3$ (Voss & Gerstein, 2005). The volume bounded by the radius limits of the spherical RNA shells is large enough to accommodate twice the volume required for the genome and 180 copies of the ~40 aa amino-terminal protein tails. Therefore it appears that the internal RNA and protein are arranged in a loose, less ordered configuration.

PaV is a nodavirus with an ordered dodecahedral inner RNA shell. X-ray and cryoEM studies show details of interactions of a single layer of RNA with the subunits positioned around the icosahedral fivefold axis (A type). Usually such detail is not apparent in X-ray or cryoEM maps, as the RNA is generally not icosahedrally ordered. CryoEM studies on another ssRNA plant virus, turnip yellow mosaic virus (Böttcher & Crowther, 1996) also indicated a single layer of packaged ssRNA. We observe all three quasi-symmetrical subunits linking to an RNA shell, though in a less ordered and defined way. The structure of the amino-terminal region that interacts with the RNA will be ordered where it connects with the capsid domain, but

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**Fig. 5.** View of the cryoEM model of HcRNAV109 down the twofold axis coloured by density and (a) sliced half way up, (b) sliced higher up and (c) surface view in order to show the separate density of the protein and RNA. The density increases from red, yellow, green and aquamarine to blue as shown in the key. Visualizations and images were created with CHIMERA (Pettersen et al., 2004).
since it can be flexible it can become less ordered further away.

There are multiple shells of nucleic acid in many other dsRNA- and dsDNA-containing viruses, but we are not aware of another structure showing a double layer of RNA in a ssRNA virus. The spacing of the shells of dsRNA range from 25 Å [partitivirus-F (PsV-F); Pan et al., 2009] to 35 Å (PsV-S; Tang et al., 2010), and in some cases three or four layers are present. The closer spacing of 22 Å we have observed for the ssRNA HcRNAV could reflect the different properties of ssRNA compared with dsRNA. An example of a dsDNA virus with multiple shells of nucleic acid is cauliflower mosaic virus (CaMV; Cheng et al., 1992).

Another feature that can be compared is the distance of the first shell of RNA from the protein shell, which can be very small (as in Penicillium chrysogenum virus, Luque et al., 2010), indicating many direct interactions of RNA with the shell, or consist of a gap (of 25 Å as in PsV-S and PsV-F). Interactions of basic residues on the carboxy or amino termini with the nucleic acid can reduce or neutralize the charge density of the phosphate backbone, and have a role in organizing it. Linker regions could control the spacing. In PsV-F and PsV-S, defined linker regions connect the capsid shell with the observed RNA layer, while in PcV there is almost no gap. In HcRNAV, there is a distinct gap between the capsid and the RNA shell, indicating that the RNA interacts mainly with the protein-linker region and not directly with the capsid shell. The resolution of the map and lack of an atomic model does not allow a more detailed analysis of the protein–RNA interactions in HcRNAV to be made.

CONCLUSION

Three 3D reconstructions of HcRNAV109 were produced (Fig. 2 and Supplementary Fig. S1). Both the negative-stain and cryoEM 3D reconstruction resulted in similar models (Fig. 3). A third method applied SEM and SE detection to the imaging of negatively stained icosahedral viruses for the first time and enabled the handedness of the reconstruction to be unambiguously assigned. This reconstruction revealed that HcRNAV109 forms a T=3 virus with each of the 180 subunits in the structure representing a separate morphological subunit. This number of subunits is consistent with the volume calculated from the capsid component of the reconstruction.

While the surface features are present in the negative-stain reconstruction, the method does not allow for internal structure to be seen because the stain only coats the exterior of the virus. In contrast, in the cryoEM reconstruction of HcRNAV109 the capsid protein and RNA are clearly separated by a trough of low density. Areas of more ordered RNA, where the RNA and protein interact more closely, are visible around the threefold and fivefold symmetry axes. This arrangement of connections is consistent with T=3 symmetry and strongly suggests that the RNA interacts with the capsid protein by binding to the arginine-rich sequence near the amino terminus of the protein.

Morphological similarities of the capsid shell and protomer arrangement with the procapsid form of Nodavirus, and its relationship to one of the more closely related families of positive-sense ssRNA viruses (the family Nodaviridae) indicates that the fold is distantly related to the capsid folds of these families, and is distinct since the HcRNAV subunits pack as monomers rather than as dimers or trimers, as observed in the related virus families.

METHODS

Isolation and purification: virion purification The virus was collected from infected H. circularisquama culture using the method described by Tomaru et al. (2004). Briefly, 450 ml of exponentially growing H. circularisquama strain, HCLG-1, was inoculated with 3 ml of HcRNAV109 producing approximately 1 × 107 infectious unit ml⁻¹. The cells were lysed and filtered sequentially through 8.0, 0.8 and 0.2 μm filters to remove cell debris. Polyethylene glycol was added to the filtrates to obtain a 10 % (w/v) final concentration and stored in the dark overnight at 4 °C. The suspension was centrifuged at 57 000 g for 90 min. The viral pellet was then washed with phosphate buffer (10 mM Na₂HPO₄ and 10 mM KH₂PO₄ in distilled water) and centrifuged again at 217 000 g for 4 h. The virus particles thus collected were then resuspended in 500 μl of 10 mM phosphate buffer and concentrated using a 100 000 kDa cut-off mini spin filter (PALL Life science) by centrifugation at 4000 g for 2–3 min.

Negative-stain electron microscopy. The sample was prepared by applying 3 μl of virus sample to a glow-discharged carbon-coated copper grid and allowing it to be absorbed for 30 s before blotting. The grid was then successively floated on two drops of water and three droplets of 2 % uranyl acetate placed on Parafilm, and blotting with filter paper between washings. The air-dried grid was then viewed with a Leo 912 transmission electron microscope operating at 120 kV. Data were recorded using low dose methods on Kodak electron image film SO-163 at a nominal magnification of × 50 000. The film was developed in Kodak Professional D19, fixed with Ilford Rapid Fixer and digitised using an Ilford Leafscan 45 by scanning at 10 μm pixel⁻¹ for micrographs with a sampling rate of 2 Å pixel⁻¹.

Handedness determination. The viral particles were absorbed onto glow-discharged, carbon-coated (8 nm) copper grids and allowing it to be absorbed for 30 s before blotting. The grid was then successively floated on two drops of water and three droplets of 2 % uranyl acetate placed on Parafilm, and blotting with filter paper between washings. The air-dried grid was then viewed with a Leo 912 transmission electron microscope operating at 120 kV. Data were recorded using low dose methods on Kodak electron image film SO-163 at a nominal magnification of × 50 000. The film was developed in Kodak Professional D19, fixed with Ilford Rapid Fixer and digitised using an Ilford Leafscan 45 by scanning at 10 μm pixel⁻¹ for micrographs with a sampling rate of 2 Å pixel⁻¹.

Six virus particles were manually selected (128 pixels²) using BOXER (Ludke et al., 1999), interpolated to 3.4 Å pixel⁻¹ and contrast inverted. A selected particle view with distinct threefold symmetry was identified by angular auto-correlation and back-projected into a volume on which icosahedral symmetry was imposed. This initial structure was iteratively refined by projection matching using SPIDER (Frank et al., 1996). To maintain the handedness of the reconstructed volume, only the ‘facing side’ was back-projected and aligned to the input data (Woodward et al., 2009).
CryoEM. CryoEM sample preparation was undertaken in a humidity-controlled fume hood as described previously by Baker et al. (1999). Briefly, 3 μL of sample was applied to glow-discharged 300 mesh copper R2/2 Quantifoil grids (Quantifoil Micro Tools), blotted with filter paper for 1–3 s and immediately submerged (by plunging) into liquid ethane slush cooled by liquid nitrogen. This resulted in the virus particles being embedded in a thin layer of vitrified ice over the holes of the carbon supporting grid. Liquid nitrogen temperatures (−176 °C) were maintained throughout the subsequent data collection steps by transferring the sample grid to a side-entry Gatan 626 cold stage (Gatan) to be loaded into the electron microscope.

A Tecnai F30 FEG microscope (FEI) operating at 300 kV and a defocus range of 1.6–5 μm was used to collect images of HcRNAV109 under low-dose (about 10 electrons Å²⁻¹) conditions at × 55 299.5 magnification (× 59 000 nominal magnification). The images were recorded on Kodak SO163 film. The micrographs were visually examined to discard those containing few or no particles and too much ice contamination. Of the micrographs recorded, 102 of 108 were scanned at 10 μm pixel⁻¹ using an Ilford Leafscan 45 to give sampling resolution of 1.808 Å pixel⁻¹, although the micrographs were interpolated to yield a final sampling resolution of 3.616 Å pixel⁻¹ for further processing.

Three-dimensional reconstruction. The power spectra of the micrographs, computed by SPIDER (Frank et al., 1996), were used to discard those which were astigmatic or showed drift. BOXER, an EMAN routine (Ludtke et al., 1999), was used to automatically pick and manually refine virus particles from each micrograph into 200 × 200 pixel boxes for a total of 2593 particles.

The defocus of a montage of the particles of each micrograph was determined using CTFFIND3 (Mindell & Grigorieff, 2003) and confirmed by generating power spectra using SPIDER scripts (Frank et al., 1996) with the TF ED command and using CTFmatch (Frank et al., 1996; Penczek et al., 1997) to visually confirm the defocus. The micrographs were grouped into 19 defocus groups with a range of <1000 Å and average defocus values for each group were determined. The particles from the micrographs in each defocus group were then combined into separate stacks of images and pre-processed with SPIDER (Frank et al., 1996) and EMAN (Ludtke et al., 1999).

One starting model was generated with the STARTICOS program of EMAN (Ludtke et al., 1999). A second starting model was computed using the common lines method implemented in the MRC program, REFIN (Crowther et al., 1996), to determine the orientations of 30 class averages created using rotational alignment of all the particles with a k-means clustering algorithm from SPIDER (Frank et al., 1996). All subsequent steps of the reconstruction were performed using the SPIDER image processing system (Frank et al., 1996). These orientations were used to produce models by the iterative algebraic reconstruction technique to back-project the class averages individually with icosahedral symmetry. The most defined model was subjected to one iteration of projection matching and back-projection using the merged set of cryoEM-data from all the defocus groups to improve the model. The data were randomly split into two groups for independent reconstructions from the two starting models.

Refinement of the models was carried out in separate defocus groups. After each iteration a contrast transfer function (CTF)-corrected model was produced by combining the models (weighted by the number of particles in the defocus group) of all the defocus groups (Penczek et al., 1997; Zhu et al., 1997). The projections of this model, with the appropriate calculated CTF applied, were then used as references for multi-reference alignment of the images to determine the orientations of the particles in each defocus group, which were back-projected with icosahedral symmetry to produce an improved model. The number of projections used for the alignment were increased and the images were gradually discarded based on their cross correlation with the projections. The resolution was calculated by comparing the two independent reconstructions and determining the FSC using a threshold of 0.5 or 0.3 for resolution determination and DPR using a 45° threshold.

The final negative-stain reconstruction was obtained using 2457 of the 3511 images originally picked, to yield a final resolution of 25 Å. In total, 1275 of the original 2593 particles from cryoEM micrographs were used to produce a model with a resolution of 18.5 Å. Two adjustments to the initial method were found to greatly improve the resolution of the reconstruction: (i) aligning the particles to projections of the CTF-corrected model (with the appropriate defocus applied) rather than the individual models produced in the defocus groups and (ii) increasing the number of projections to which the images were aligned by decreasing the angular step size. For the negative-stain reconstruction, an increase in the number of projections did not increase the resolution of the reconstruction. While the resolution of the cryoEM reconstruction improved when the angular step was decreased from 5 to 2°, a further decrease of the angular step to 1° only improved the resolution slightly. The resolution of the cryoEM reconstruction was limited by the number of images of HcRNAV109 particles that were collected, owing to limited sample with a low concentration of virus particles.

Three-dimensional models were visualized and images generated using UCSF CHERMA (Pettersen et al., 2004).

The three-dimensional EM map has been deposited at the Electron Microscopy Data Bank, accession number EMD-1870.

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