1 **Heterologous Expression of Human Norovirus GII.4 VP1 Leads to Assembly of**
2 **T=4 Virus-Like Particles**

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13 SHORT TITLE: Cryo-EM structure of GII.4 norovirus VLPs

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ABSTRACT

Human noroviruses are a leading cause of acute gastroenteritis, yet there are still no vaccines or antivirals available. Expression of the norovirus capsid protein (VP1) in insect cells typically results in the formation of virus-like particles (VLPs) that are morphologically and antigenically comparable to native virions. Previous structural analysis of norovirus VLPs showed that the capsid has a T=3 icosahedral symmetry and is composed of 180 copies of VP1 that are folded into three quasi-equivalent subunits (A, B, and C). In this study, we determined the cryo-EM VLP structures of two GII.4 variants, termed CHDC-1974 and NSW-2012. Surprisingly, we found that greater than 95% of these GII.4 VLPs were larger than virions and 3D reconstruction showed that these VLPs exhibited T=4 icosahedral symmetry. We found that the T=4 VLPs showed several structural differences to the T=3 VLPs. The T=4 particles assemble from 240 copies of VP1 that adopt four quasi-equivalent conformations (A, B, C, and D) that form two distinct dimers, A/B and C/D. The T=4 protruding domains were elevated $\sim 21\text{-}\text{\AA}$ off the capsid shell, which was $\sim 7\text{-}\text{\AA}$ more than the previously studied GII.10 T=3 VLPs. A small cavity and flap-like structure at the icosahedral twofold axis disrupted the contiguous T=4 shell, a consequence of the D-subunit S-domains having smaller contact interfaces with neighboring dimers. Overall, our findings that old and new GII.4 VP1 sequences assemble T=4 VLPs might have implications for the design of potential future vaccines.

39 IMPORTANCE

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41 The discovery that the GII.4 VLPs have a T=4 symmetry is of significance, since this
42 represents the first known T=4 calicivirus structure. Interestingly, the GII.4 2012
43 variant shares 96% amino acid identity with a current GII.4 VLP vaccine candidate
44 sequence, which suggests that this vaccine might also have a T=4 symmetry. Our
45 previous results with these GII.4 VLPs showed functional binding properties to
46 antibodies and Nanobodies that were raised against T=3 (GII.10) VLPs. This suggests
47 that the T=4 VLPs were antigenically comparable to T=3 particles, despite the
48 obvious structural and size differences. On the other hand, these larger T=4 VLPs
49 with novel structural features and possibly new epitopes might elicit antibodies that
50 do not recognize equivalent epitopes on the T=3 VLPs. Further structural and binding
51 studies using a library of GII.4-specific Nanobodies are planned in order to precisely
52 investigate whether new epitopes are formed.

INTRODUCTION

Human noroviruses are members of the *Caliciviridae* family and are a leading cause of outbreaks of acute gastroenteritis. The virus has a positive sense, single stranded RNA genome of ~7.7 kbp. The genome is organized into three open reading frames (ORFs), where ORF1 encodes nonstructural proteins and ORF2 and ORF3 encode a major structural protein (termed VP1) and a minor structural protein (termed VP2), respectively. Noroviruses are genetically diverse and based on VP1 sequences have been classified into seven genogroups (GI-GVII), where GI, GII, and GIV cause infections in humans (1, 2). The GI and GII are further subdivided into numerous genotypes, with GII genotype 4 (GII.4) recognized as the most prevalent and clinically important strain (3, 4).

Recently, two human norovirus cell culture systems were developed (5, 6). However, mechanistic studies of norovirus structure and biology, such as interaction with the host receptor(s), remain challenging owing to difficulties in preparing large-scale virion preparations. Expression of ORF2 alone in insect or mammalian cells can result in the formation of virus-like particles (VLPs) that are antigenically and morphologically similar to native virions (2, 7-10). Expression of norovirus VLPs has permitted studies on host binding factors, interactions with norovirus-specific antibodies, and structural studies (2, 11-16). Indeed, histo-blood group antigens (HBGAs) and bile acids were shown to be important binding co-factors for human norovirus VLPs and/ or virions (17-22).

77 Structural analysis of GI.1 VLPs revealed that VP1 is separated into two distinct
78 domains: a shell domain (S domain) that encloses the RNA and a protruding domain
79 (P domain) that binds to co-factors, such as HGBAs and bile acids (8, 19, 21). A
80 hinge region, which is typically composed of 10-14 amino acids, connects the S and P
81 domains. The P domain has a β -barrel fold that is structurally conserved in the
82 *Caliciviridae* family. Dimerization of the P domains forms arch shaped protrusions
83 that are visible in electron microscopy images. The P domain is further subdivided
84 into P1 and P2 subdomains, where the P2 subdomain is an insertion in the P1
85 subdomain and is the most variable region on the capsid (8).

86
87 Structural studies have shown that caliciviruses have a common overall organization
88 of T=3 icosahedral symmetry and are comprised of 180 copies of VP1 (7, 8, 23-25).
89 Within the asymmetric unit, VP1 adopts three quasi-equivalent conformations, termed
90 A, B, and C (8). The norovirus A and B subunits assemble into 60 dimers (termed
91 A/B) at the quasi twofold axis, whereas the C subunits assemble into 30 C/C dimers
92 that are located at the strict icosahedral twofold axis. For the GI.1 VLPs, the A/B
93 dimers have a convex S domain conformation, whereas the C/C dimers have a flat S
94 domain conformation (8). The conformational differences within these dimers likely
95 facilitates the curvature of the virus particle to form a closed shell, such features are
96 commonly seen in other T=3 icosahedral viruses (26). Interestingly, smaller norovirus
97 VLPs (~25 nm in diameter) that are assumed to have a T=1 icosahedral symmetry
98 have also been reported (22, 27); however to date, structures of these smaller VLPs
99 have not been published.

100

101 Here, we show the cryo-EM structures of VLPs for two GII.4 variants that were
102 identified in 1974 and 2012, termed CHDC-1974 and NSW-2012, respectively (15,
103 28). We show that these VLPs have a T=4 icosahedral symmetry and are composed of
104 240 copies of VP1. In order to form the T=4 icosahedral structure, VP1 adopts four
105 quasi-equivalent conformations, termed A, B, C, and D, giving rise to two distinct
106 types of dimers, termed A/B and C/D. The VLPs consisted of 60 A/B dimers and 60
107 C/D dimers, with B, C, and D subunits located at the twofold axis, and the A subunit
108 at the fivefold axis. As GII.4 VLPs are currently under consideration as vaccine
109 candidates, our findings might have implications with respect to the likely antigenic
110 properties of such assemblies.

MATERIALS AND METHODS

VLP and virion preparation

The NSW-2012 and CHDC-1974 VLPs (Genebank accession numbers JX459908 and ACT76142, respectively) were expressed in a baculovirus system as previously described (29-31). Briefly, the bacmid containing the recombinant VP1 gene was transfected in Sf9 insect cells. After incubation for five days, the culture medium was centrifuged for 10 min at 3,000 rpm at 4°C. The recovered baculovirus was subsequently used to infect Hi5 insect cells. At five days post infection, the culture medium was centrifuged for 10 min at 3,000 rpm at 4°C and then 1 h at 6,500 rpm at 4°C. The VLPs in the supernatant were concentrated by ultracentrifugation at 35,000 rpm (Beckman Ti45) for 2 h at 4°C and then further purified using CsCl equilibrium gradient ultracentrifugation at 35,000 rpm (Beckman SW56) for 18 h at 4°C. To remove the CsCl, the VLPs were pelleted for 2 h at 40,000 rpm (Beckman TLA55) at 4°C and subsequently resuspended in PBS (pH 7.4). GII.4 virions in stool were also purified using this centrifugation technique, except for the CsCl gradient step.

Negative stain electron microscopy

The integrity of the VLPs was confirmed using negative stain electron microscopy (EM). The VLPs were diluted 1:30 in distilled water and applied to EM grids. The grids were washed with distilled water, stained with 0.75% uranyl acetate, and the excess uranyl acetate was removed with filter paper. Virion samples were applied to EM grids, washed with water, fixed with 4% glutaraldehyde, and then stained as above. EM images were acquired on a Zeiss 910 electron microscope at 50,000× magnification.

136

137 **Cryo-EM data sample preparation and data collection**

138 UNSW-2012 and CHDC-1974 VLPs (3 μ l) were applied to freshly glow discharged
139 Quantifoil holey carbon support films (R1.2/1.3) and blotted for 18 seconds at 100%
140 humidity and 10°C before being plunged in liquid ethane using an FEI Mark IV
141 Vitrobot (Thermo Fischer Scientific). Vitrified specimens were imaged on a Titan
142 Krios microscope (Thermo Fisher scientific) operated at 300 keV. NSW-2012
143 micrographs were acquired with a K2 direct electron detector with Latitude S
144 software (Gatan) at 64,000 $\times$ magnification corresponding to a pixel size of 2.27 Å/px,
145 while CHDC-1974 micrographs were collected using a K3 direct electron detector at
146 64,000 $\times$ magnification, corresponding to a pixel size of 1.375 Å/px.

147

148 **Cryo-EM data processing**

149 Initially, the movies containing 16 frames for NSW-2012 and 40 frames for CHDC-
150 1974 were motion corrected using motioncor2 software (32) and defocus estimation
151 was performed using ctffind 4.1 software (33). All further image-processing steps
152 were performed using Relion 2.1 software for NSW-2012 and cryoSPARC software
153 for CHDC-1974 (34, 35). An initial set of 1,000 particles was manually picked for 2D
154 classification to produce averages suitable as references for automated particle
155 picking. The autopicked particles were sorted in a 2D classification step and the best
156 particles were used for calculation of an initial starting model, followed by 3D
157 classification. A subset of particles that generated the highest resolution was selected
158 for further refinement. The 3D refinement and post-processing of NSW-2012 from
159 10,548 T=4 particles produced a final map at 7.3-Å resolution with icosahedral (I2)
160 symmetry imposed (0.143 FSC cutoff). Smaller NSW-2012 T=3 particles were

manually picked and sorted in 2D classification. In total, 391 particles were selected for further 3D structure determination. Refinement of these smaller NSW-2012 T=3 particles yielded a map of 15-Å resolution. For CHDC-1974, a subset of 42,485 particles for refinement led to the calculation of a map at 6.1-Å resolution using the 0.143 FSC cutoff. Cryo-EM T=4 VLP structures for CHDC-1974 (accession number: EMD-4549) and NSW-2012 (EMD-4550) were deposited at EMDB. The cryo-EM VLP structure for NSW-2012 with T=3 icosahedral symmetry is available on request.

Fitting of the X-ray structures into the density maps

Crystal structures of NSW-2012 P domain (4OOS) and CHDC-1974 P domain (5IYN) were fitted into the respective densities using the “fit in map” command in the UCSF Chimera software (36). Since a high-resolution GII.4 shell domain structure was unavailable, the GI.1 Norwalk virus S domain was extracted from the X-ray crystal structure (1IHM) and fitted into the GII.4 cryo-EM densities using UCSF Chimera software.

RESULTS AND DISCUSSION

The purpose of this study was to analyze GII.4 VLP architecture and compare these assemblies to the previously solved GI.1 and GII.10 VLP structures. The NSW-2012 VP1 sequence had a single amino acid insertion at position ~394 (NSW-2012 numbering) compared to CHDC-1974 (Fig. 1). Overall, NSW-2012 and CHDC-1974 shared 89% amino acid identity, with most (45 of 54) amino acid substitutions occurring in the P domain. Negative stain EM images revealed that the VLPs exhibited characteristic norovirus morphology (Fig. 2). However, the diameter of these VLPs was measured to be ~52 nm, which suggested that the GII.4 VLPs were larger than GII.10 and GI.1 VLPs that had diameters of ~43 nm and ~38 nm, respectively.

Cryo-EM structure of NSW-2012 T=4 VLPs

The structure of the NSW-2012 VLPs was determined using cryo-EM and 3D icosahedral image reconstruction. The VLPs were mono-disperse in vitreous ice and appeared mostly homogenous in size (Fig. 3A). From the 364 images, 10,548 particles were used for image reconstruction and refined to 7.3-Å resolution (Fig. 3C). Unexpectedly, NSW-2012 VLPs were discovered to have T=4 icosahedral symmetry (Fig. 4). Our data revealed that these VLPs were composed of 240 copies of VP1, rather than 180 VP1 copies as in GI.1 and GII.10 VLPs. The inner diameter of NSW-2012 shell was measured as 32 nm, while the outer capsid diameter was 50 nm. The larger diameter of the T=4 VLPs corresponded to a capsid volume of ~12,724 nm³, which was ~2.1 times the volume of the GII.10 VLPs that had an inner diameter of 23 nm, corresponding to a volume of ~5,985 nm³ (7).

201
202 Another interesting structural feature of these NSW-2012 VLPs was a small cavity
203 and flap-like structure on the contiguous shell (Fig. 5). This feature was associated
204 with the S domain and found on opposing sides at the twofold axis. This feature may
205 arise as a consequence of the C/D dimer having a stronger curvature, preventing the
206 S-domain from forming the expected contacts with neighboring B and C-type
207 subunits. Alternatively, there might be insufficient space to allow the D subunit S-
208 domain to pack, such that it lies in the plane of the shell without causing steric
209 collision with neighboring subunits.

210
211 Structural analysis of the T=4 VLPs indicated that VP1 adopted four quasi-equivalent
212 conformations, termed A, B, C, and D. These four subunit conformations formed two
213 distinct dimer classes: A/B and C/D (Figs. 4 and 6). The B, C, and D subunits
214 alternated about the two-fold symmetry axis, while the A subunit was positioned at
215 the fivefold axis (Fig. 4). The T=4 VLPs were composed of 60 A/B dimers and 60
216 C/D dimers, which was distinct from the T=3 VLPs that have been shown to be
217 assembled from 60 A/B and 30 C/C dimers. We also observed that both A/B and C/D
218 dimers had a convex S domain conformation, which was in contrast to the GI.1 VLPs
219 that consisted of both convex (A/B) and flat (C/C) dimers (8).

220
221 In order to better comprehend how VP1 assembled into the T=4 VLPs, the X-ray
222 crystal structure of NSW-2012 P domain (4OOS) and GI.1 S domain (1IHM) were
223 fitted into the VLP density map. We found that the NSW-2012 P domain dimer could
224 be unambiguously positioned into the VLP density, with a cross correlation
225 coefficient of 0.96 (Fig. 7A). This result indicated that the P domain dimers on the

T=4 VLPs had not undergone any major structural modifications. In the case of the S domain, the GI.1 S domain needed to be manually positioned into the density map. The GI.1 S domain fitted well into to A/B dimer and the C subunit, while the D subunit needed to be further repositioned (Fig. 7B). This additional fitting in the D subunit was necessary in order to occupy the elevated density of the flap-like regions.

Unfortunately, it was problematic to fit the hinge region, since the hinge region on the X-ray crystal structure of NSW-2012 P domain was excluded from the expression construct and the hinge region of the GI.1 VLPs was flattened (7, 8). Interestingly, an apparent point of contact was also observed between the S domain and the C-terminus of the P domain on the D subunit (Fig. 8). This may stabilize the convex conformation of the C/D dimer and possibly the T=4 VLPs. Indeed, the C-termini of VP1 on the GI.1 VLPs and the GII.4 P domain were found to be flexible (8, 37). Moreover, the C-terminus of VP1 was previously shown to be important for the size and stability of VLPs (38).

Another interesting feature that we observed with the T=4 VLPs was the raised P domains (Figs. 4 and 8). We found that the T=4 P domain was elevated ~ 21 -Å off the shell by an extended hinge region, which was higher than the P domains on the GII.10 VLPs, which were raised ~ 14 -Å (7). The hinge region in NSW-2012 and GII.10 (7) were both ~ 10 amino acids and mainly conserved (39). This result suggested that the raised P domains might be a structural feature of GII noroviruses, since the P domains on the GI.1 VLPs were essentially resting on the shell (8).

Cryo-EM structure of NSW-2012 T=3 VLPs

To determine the structure of a recent GII.4 isolate (NSW-2012), 10,548 particles were imaged by cryo-EM, and a 3D image reconstruction was calculated. Unexpectedly, this analysis revealed a previously unknown T=4 assembly. In this preparation however, a very small number of particles (391 particles) appeared smaller (~46 nm) than the ~50 nm VLPs (Fig. 4A). Reconstruction of these particles at ~15-Å resolution revealed the expected T=3 icosahedral symmetry (Fig. 9). At this resolution, the hinge region was not visible. Also, the cavity and flap-like structures that characterized the T=4 VLP were not present. Based on the T=3 geometry, these VLPs were likely composed of 180 copies of VP1, however the VP1 dimers or subunits were not clearly resolved. Overall, these results indicated that NSW-2012 VP1 formed VLPs of which ~5% of the population (391 of 10,548) were T=3 particles. This proportion was confirmed over several VLP preparations. Unfortunately, owing to the low numbers it remains challenging to improve on the achieved resolution, since only few T=3 VLPs were expressed and were not able to be separated from the T=4 VLPs using CsCl or sucrose gradient ultracentrifugation.

Cryo-EM structure of GII.4 CHDC-1974 T=4 VLPs

To test whether assembly of VLPs with T=4 icosahedral symmetry is a property of VP1 from all GII.4 strains, we proceeded to determine the cryo-EM structure of VLPs produced by VP1 of CHDC-1974. The VLPs were mostly mono-disperse and homogenous in size (Fig. 10A). From 591 images, 42,485 particles were processed to calculate a reconstruction with a final resolution of 6.1-Å (Fig. 10B). The CHDC-1974 VLPs also had T=4 symmetry (Fig. 11) and their structure closely resembled that of NSW-2012 VLPs. Unlike NSW-2012 however, CHDC-1974 VP1 only assembled into T=4 VLPs and no T=3 VLPs were identified.

276

277 We found that CHDC-1974 T=4 VLPs were also composed of 240 copies of VP1 that
278 formed the quasi-equivalent subunits A, B, C, and D and A/B and C/D dimeric
279 capsomeres. The inner diameter of the shell was 32 nm, whereas the outer diameter of
280 the capsid was 50 nm. As in NSW-2012 VLPs, cavity and flap-like structures were
281 also present on the CHDC-1974 VLPs (Fig. 12). The CHDC-1974 A/B and C/D
282 dimers showed a similar convex conformation as NSW-2012 dimers, although
283 slightly less pronounced (Fig. 13). The X-ray crystal structure of CHDC-1974 P
284 domain (5IYN) was easily fitted into the CHDC-1974 VLP density map (Fig. 14A).
285 The GI.1 S domain also fitted into the A, B, and C subunits, whereas the GI.1 S
286 domain was repositioned to occupy the D subunit density (Fig. 14B). Similar to
287 NSW-2012 VLPs, a possible contact interface was observed between the S and P
288 domains on the D subunit (Fig. 15). Lastly, we found that CHDC-1974 P domain was
289 also lifted off the shell ~21-Å by an extended hinge region (Figs. 11 and 15).

290

291 Overall, these results showed that GII.4 VP1 sequences isolated over three decades
292 apart remained structurally conserved. This could imply that other GII.4 VP1
293 sequences would also form T=4 VLPs when expressed in insect cells, especially since
294 these two sequences had only 89% amino acid identity.

295

296 **Previous binding studies using GII.4 capsids**

297 GII.4 VLPs and their corresponding P domains have been extensively examined for
298 binding to co-factors (HBGAs and bile acid), antibodies, human milk
299 oligosaccharides (HMOs), and Nanobodies (21, 31, 37, 39-41). NSW-2012 P domains
300 were capable of binding numerous HBGA types and the VLPs cross-reacted with

Nanobodies and antibodies that were raised against other genotypes or GII.4 variants. However, the NSW-2012 VLPs did not bind bile acid and poorly bound HMOs (unpublished). Overall, these results suggested that T=4 VLPs were indeed antigenically relevant and capable of functioning similarly to GII.4 virions with respect to antigenicity and HBGA interactions.

GII.4 VLPs in vaccine trials

VLP vaccines against norovirus that are currently in clinical trials use a combination of GI.1 VLPs and modified GII.4 VLPs (termed GII.4c) (42, 43) Negative stain EM images of these GII.4c VLPs showed the typical norovirus morphology. However, the size determination and the structure are not available. Interestingly, GII.4c and NSW-2012 shared 94% amino acid identity with most substitutions (28 of 31) located in the P domain (Fig. 1). Therefore, it is tempting to speculate that GII.4c VP1, which closely matched NSW-2012 VP1 sequence, might also form T=4 VLPs particles. In both VLPs, VP2 is not present.

To test whether GII.4 virions are T=3 or T=4 assemblies, we used negative stain EM of authentic GII.4 virions. These virion images revealed a smaller diameter of ~44 nm compared to the T=4 VLPs (~50 nm) (Fig. 16). This size corresponds to the diameter determined for the NSW-2012 T=3 VLPs and indicates that GII.4 virions likely exhibit T=3 icosahedral symmetry.

In general, studies have shown that norovirus-specific antibody titers were raised after vaccination with VLPs, but the levels of protection were not strongly improved compared to placebo groups (44). This might indicate that vaccination leads to

production of neutralizing antibodies against epitopes on T=4 VLPs that are not accessible on T=3 virions (i.e., when challenged) and that therefore vaccine efficacy could be lowered by a sub-optimal antigen. Clearly, further studies are needed in order to determine whether the GII.4c VLPs form T=4 particles and if the T=4 VLPs are antigenically identical to T=3 VLPs.

Summary

We have shown that upon heterologous expression of human norovirus GII.4 VP1 in insect cells, VLPs are formed that adopt T=4 icosahedral symmetry. This is at odds with the likely T=3 symmetry virions encoded by this virus. There are two important consequences of this outcome. Firstly, the assembly of GII.4 T=4 VLPs may impact on results from previous studies in which it was assumed that GII.4 norovirus VLPs were morphologically similar to virions. Secondly, the cavity and flap-like structures on the T=4 could elicit antibodies that are not capable of recognizing T=3 virions. Further structural and binding studies using a library of GII.4-specific Nanobodies are planned in order to investigate these novel epitopes.

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343

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FIGURE LEGENDS

Figure 1. Amino acid sequence alignment of GII.4 VP1. NSW-2012 (JX459908), CHDC-1974 (ACT76142), and GII.4c (42) VP1 amino acid sequences were aligned using ClustalX. The S domain (orange), hinge region (green), P1 subdomain (light blue), and P2 subdomain (navy) were labeled accordingly. Compared to CHDC-1974, NSW-2012 and GII.4c VP1 had a single amino acid insertion (arrow) at position 394 (NSW-2012 numbering). The S domain and hinge region were mainly conserved, whereas most amino acid substitutions were located in the P2 subdomain.

Figure 2. EM images of GI and GII VLPs. Negative stain EM images of norovirus VLPs show the characteristic norovirus virion morphology (50,000 $\times$ magnification). The diameter of the CHDC-1976 and NSW-2012 VLPs was $\sim$ 52 nm, whereas the diameter of GI.1 West Chester (AY502016.1) and GII.10 Vietnam026 (AF504671) VLPs were $\sim$ 38 nm and $\sim$ 43 nm, respectively (45, 46). The bar represents 100 nm.

Figure 3. NSW-2012 cryo-EM data processing. (A) A representative cryo-EM micrograph of NSW-2012 VLPs at 64,000 $\times$ magnification. The blue and green arrows show examples of VLPs measuring $\sim$ 46 nm and $\sim$ 50 nm, respectively. The scale bar represents 100 nm. (B) 2D classification of GII.4 NSW-2012 T=4 VLPs. (C) Gold standard FSC plot of the icosahedral reconstruction of NSW-2012 indicates a resolution of 7.3-Å.

Figure 4. Cryo-EM reconstruction structure of NSW-2012 T=4 VLPs. The left side shows NSW-2012 VLPs have a T=4 icosahedral symmetry (symmetry axis

labeled 2, 3, and 5). These VLPs were composed of 240 copies of VP1 and VP1 adapted four quasiequivalent conformations (A, B, C, and D) that gave rise to two distinct dimers (A/B and C/D). At the icosahedral twofold axis, the B, C, and D subunits were alternating, while the A subunits are positioned at the fivefold axis. The right side shows a cutaway section of these VLPs and indicates that the inner and outer diameters are 32 nm and 50 nm, respectively. The P domains are elevated ~21 Å off the S domain.

Figure 5. NSW-2012 T=4 VLPs shows several new structural features. The cavity and flap-like structures are observed at the twofold axis and are found on opposing sides. The cavity and flap-like structures are associated with the S domain on the D subunit.

Figure 6. NSW-2012 T=4 VLPs are formed with 60 A/B and 60 C/D VP1 dimers. The A/B and C/D dimers show an equivalent convex conformation of the S domain. An additional connection was also observed between the D subunit of the S and P domain.

Figure 7. The X-ray crystal structures of NSW-2012 P domain and GI.1 S domain were fitted into the T=4 VLP density map. (A) The X-ray crystal structure of NSW-2012 P domain (4OOS, cartoon) could be fitted into the A/B and C/D P domain dimers, indicating little conformational change. (B) The X-ray crystal structure of the GI.1 S domain (1IHM, cartoon) fitted into the A/B and C/D S domain dimers. However, the cavity and flap-like structures on the D subunit suggests a large conformational change compared to typical T=3 particles.

Figure 8. A close-up view of NSW-2012 C/D dimer. The fitted X-ray crystal structures of the GI.1 S domain (cartoon) and the GII.4 P domain (cartoon) into the cryo-EM map shows the how the hinge region connects the S and P domains. Also, the new connection between the S domain and the C-terminus of the P domain is shown. The asterisk represents the missing hinge region on the X-ray crystal structures that connects of the S and P domains for the C subunit (blue) and D subunit (red).

Figure 9. Cryo-EM reconstruction structure of NSW-2012 T=3 VLPs. (A) 2D classification of 1780 manually picked particles. The first class (red circle) shows particles of a smaller diameter, compared to averages of other classes. These smaller particles were used for further refinement. (B) FSC plot of the icosahedral reconstruction of T=3 VLPs indicates a resolution of 15-Å. (C) The left side shows NSW-2012 exhibiting T=3 icosahedral symmetry (symmetry axis labeled 2, 3, and 5). These VLPs were composed of 180 copies of VP1 that forms A/B and C/C dimers. The cutaway section (right) shows the inner and outer diameter of the particle, which measured 24 and 46 nm, respectively. At this resolution the hinge region could not be resolved, but the large gap between S and P domain indicates that the P domains are raised up from the shell.

Figure 10. CHDC-1974 cryo-EM data processing. (A) A representative cryo-EM micrograph of CHDC-1974 VLPs at 64,000× magnification. The scale bar represents 100 nm. (B) 2D classification of CHDC-1974 particles. (C) FSC plot of the

icosahedral reconstruction of CHDC-1974 indicates a resolution of 6.1-Å at 0.143 cutoff.

Figure 11. Cryo-EM structure and analysis of CHDC-1974 VLPs. The image on the left side shows that CHDC-1974 VLPs has a T=4 icosahedral symmetry (symmetry axis labeled 2, 3, and 5) and was composed of 240 copies of VP1. The VP1 exhibited four quasiequivalent conformations (A, B, C, and D) that gave rise to two distinct dimers (A/B and C/D). At the icosahedral twofold axis, the B, C, and D subunits were alternating, while the A subunit was located around the fivefold axis. The right side shows a cutaway section of these VLPs and indicates that the inner and outer diameters are 32 nm and 50 nm, respectively. The P domains are elevated ~21 Å off the S domain.

Figure 12. CHDC-1974 T=4 VLPs shows several new structural features. The cavity and flap-like structures are observed at the twofold axis and are found on opposing sides. The cavity and flap-like structures are associated with the S domain on the D subunit.

Figure 13. CHDC-1974 T=4 VLPs are formed with 60 A/B and 60 C/D VP1 dimers. The A/B and C/D dimers show an equivalent convex confirmation on the S domain. Also, the additional connection between the D subunit of the S and P domain was found on the CHDC-1974 T=4 VLPs.

Figure 14. The X-ray crystal structures of CHDC-1974 P domain and GI.1 S domain were fitted into the VLP density map. (A) The X-ray crystal structure of

NSW-2012 P domain (5IYN, cartoon) easily fitted into the A/B and C/D P domain dimer densities. (B) The X-ray crystal structure of the GI.1 S domain (1IHM, cartoon) fitted into the A/B and C/D S domain dimers. However, the cavity and flap-like structures on the D subunit suggests a large conformational change from typical T=3 particles.

Figure 15. A close-up view of CHDC-1974 C/D dimer. The fitted X-ray crystal structures of the GI.1 S domain (cartoon) and the GII.4 P domain (cartoon) into the cryo-EM map shows the how the hinge region connects the S and P domains. Also, the new connection between the S domain and the C-terminus of the P domain is shown. The asterisk represents the missing hinge region on the X-ray crystal structures that connects of the S and P domains for the C subunit (blue) and D subunit (red).

Figure 16. EM images of GII.4 virions. Negative stain EM images of GII.4 virions show that the virions exhibit a smaller diameter (~44 nm) than GII.4 VLPs expressed in insect cells that were ~52 nm (see Figure 2).

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Figure 1

NSW-2012	1	MKMASSDANPSDGSANLVPEVNNEVMALEPVVGAATAAPVAGQQNVIDPWIRNNFVQAP	60
CHDC-1974	1	T.....I.....	60
GII.4c	1	T.....	60

NSW-2012	61	GGEFTVSPRNAPGEILWSAPLGPLNPLYSHLARMYNGYAGGFVQVILAGNAFTAGKVI	120
CHDC-1974	61	S.....S.....I.....	120
GII.4c	61	I.....	120

NSW-2012	121	FAAVPPNFPTGLSPSQVTMFPHIVVDVRQLEPVLIPDVRNNFYHYNQSNPTIKLIA	180
CHDC-1974	121	...I.....I.....AH.S.L...	180
GII.4c	121	I.....	180
		*** ***** *	
NSW-2012	181	MLYTPLRANNAGDDVFTVSCRVLTRSPDFDFIFLVPPTVESRTKPFVSPVLTVEEMTNS	240
CHDC-1974	181	T..I.....S..	240
GII.4c	181	T..I.....	240

NSW-2012	241	RFPIPLEKLFTGPSSAFVVQPQNGRCTTDGVLGGTQLSPVNICTFRGDVTHITGSRNYT	300
CHDC-1974	241	Y.....A...N.....RVGI..HD..	300
GII.4c	241	G.....A.TQE..	300

NSW-2012	301	MNLASQNWNDYDPTTEEIPAPLGTPDFVGKIQGVLQTTRTDGSTRGHKATVYTGSADFAP	360
CHDC-1974	301	...V....N.....L.....A....A....S...VH.T.	360
GII.4c	301	N.....G.....S...VH.T.	360
		*** ***** *	
NSW-2012	361	KLGRVQFETDTRDFEANQNTKFTPVGVIQDGGTTHRNEPQQWVLPSYSGRNTHNVLAP	420
CHDC-1974	361	...S...T...NN..QTG.....DH-.Q.....N...TSG.....	419
GII.4c	361	...S...S...SN...TG.....V...S...Q.....D...DS.....	420
		*** ** * * * ***** *	
NSW-2012	421	AVAPTFPGEQLLFFRSTMPGCSGYPNMDLCLLPQEWVQYFYQEAAPASDVALLRFVNP	480
CHDC-1974	420	N.....SH.....	479
GII.4c	421	N.....H.....	480

NSW-2012	481	DTGRVLFECKLHKSGYVTVAHGTGQHDLVIPPNGYFRFDSWVNQFYTLAPMNGTGRRRAV	540
CHDC-1974	480	I.....PY.....S...K.VL	539
GII.4c	481	L	540

GI.4 NSW-2012

GI.4 CHDC-1974

GI.1 West Chester

GI.10 026-Vietnam

Figure 3

A

B

C

Figure 4

Figure 5

Figure 6

A

A/B dimer

C/D dimer

B

A/B dimer

C/D dimer

Figure 8

Figure 9

A

B

C

Figure 10

A

B

C

Figure 11

Figure 12

Figure 13

A

A/B dimer

C/D dimer

B

A/B dimer

C/D dimer

Figure 15

Figure 16