The 2.3-Angstrom Structure of Porcine Circovirus 2[∇]

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Porcine circovirus 2 (PCV2) is a T=1 nonenveloped icosahedral virus that has had severe impact on the swine industry. Here we report the crystal structure of an N-terminally truncated PCV2 virus-like particle at 2.3-Å resolution, and the cryo-electron microscopy (cryo-EM) image reconstruction of a full-length PCV2 virus-like particle at 9.6-Å resolution. This is the first atomic structure of a circovirus. The crystal structure revealed that the capsid protein fold is a canonical viral jelly roll. The loops connecting the strands of the jelly roll define the limited features of the surface. Sulfate ions interacting with the surface and electrostatic potential calculations strongly suggest a heparan sulfate binding site that allows PCV2 to gain entry into the cell. The crystal structure also allowed previously determined epitopes of the capsid to be visualized. The cryo-EM image reconstruction showed that the location of the N terminus, absent in the crystal structure, is inside the capsid. As the N terminus was previously shown to be antigenic, it may externalize through viral “breathing.”

Circoviruses are small nonenveloped icosahedral viruses, named after their circular single-stranded DNA (ssDNA) genome (~2,000 bases). They infect a variety of animal and plant species. Over the past decade, porcine circovirus 2 (PCV2) has been responsible for significant mortality among swine, severely impacting the swine industry (31). PCV2 is one of the primary etiologies of postweaning multisystemic wasting syndrome (PMWS) (5) and has been associated with porcine dermatitis and nephropathy syndrome (PDNS) (22, 29) as well as porcine reproductive disorders (14, 22, 39). PMWS-affected piglets (between 8 and 16 weeks old) exhibit symptoms including jaundice, weight loss, difficulty in breathing, enlarged lymph nodes, and diarrhea. Morbidity in PCV2-infected piglets varies from 4 to 20%, while mortality is as high as 90% (9). The virus persists in infected herds for extended periods of time and can be controlled by vaccination. Commercial PCV2 vaccines include inactivated PCV2, PCV2 coat proteins expressed using a baculovirus system, or inactivated PCV1/2 chimeric virus (9). PCV1 is an additional serotype of PCV and has not been associated with pathogenicity. It is unknown why the two serotypes cause different clinical symptoms.

Antigenic studies of PCV2 with pathogen-free swine identified six linear epitopes of the coat protein (Table 1) (21). Sequence alignments of PCV2 field isolate capsid proteins have identified a number of variable regions corresponding to the identified epitope sites (7, 12, 34). Indeed, studies have demonstrated that antigenic differences in the capsid protein

exist among the different strains of PCV2 despite the high degree of sequence identity (>90%) shared among their capsid proteins (17). Given the likelihood that antibodies bind to discontinuous rather than continuous epitopes, structural studies of PCV2 are needed to further our understanding of the antigenic difference that exists among the strains of PCV2. Moreover, structural studies of PCV2 are crucial to further expand our knowledge of the immune response to PCV2.

Here we report the 2.3-Å crystal structure of a PCV2 consensus sequence (PCV2^{CS}) virus-like particle (VLP). The choice of a PCV2 consensus sequence was made in a desire to pursue a crystal structure for what would be the most representative of the PCV2 population. Use of the consensus would tend to avoid outlier variants of PCV2, the crystal structure of which could provide nonuniform data particularly with respect to epitopes. The crystal structure provides the first atomic description for the *Circovirus* family. The PCV2^{CS} coat protein was expressed and purified from *Escherichia coli* as monomeric subunits yet assembled to form VLPs during the crystallization trials. We also determined a subnanometer-resolution cryo-electron microscopy (cryo-EM) image reconstruction of VLPs derived from a native PCV2 strain, N12 (PCV2^{N12}), expressed in a baculovirus system to affirm that the PCV2^{CS} subunits assembled into a biologically relevant VLP. PCV2^{N12} VLPs autoassembled *in vivo* and thus are likely to reflect the structure of the infectious virions. The PCV2^{N12} cryo-EM image reconstruction agrees with the PCV2^{CS} crystal structure and displays density corresponding to the packaged nucleic acid and the N terminus of the capsid protein—both absent in the PCV2^{CS} VLP crystal structure.

Combining the structural information gathered from our X-ray crystallographic and cryo-EM studies, we were able to visualize the reported antigenic epitopes of PCV2, identify positively charged clefts that may represent the heparan sulfate binding sites responsible for the attachment of PCV2 to its

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TABLE 1. Epitopes of PCV1 and PCV2

Epitope	Residues ^a	Aligned sequence ^b	MAB binding residue(s) ^c
A	69–83	NV NEL RFNIG Q FLPP V DM RFN IND FLPPG	Asp70, Met71, Asn77, Asp78
B ^d	113–127	T SN QRGVG STV VILD Q GD RGVG SSA VILDD	Gln113, Asp115
C ^d	117–131	RGVG STV VILD AN FV GVG SSA VILDDNFVT	Asp127
D	169–183	DQ TID W FQ PNN KRN Q F TID Y FQ PNN KRN QL	Thr170
E	193–207	NV EHT GLGYAL QN ATT VD HV GLGT AF ENSIY	Glu203, Ile206, Tyr207
F	25–39	RRP YL VHP AF RNR YRWR RRP WL VHP -- RHR YRWR	

^a Residues numbered according to PCV2 numbering.

^b Aligned epitope sequences with PCV1 on the top and PCV2 on the bottom for each epitope (8). Putative residues responsible for discriminating MAb binding to PCV1 or PCV2 are shown in bold type.

^c Residues responsible for discriminating PCV1 or PCV2 epitopes located on the surface of the particle with their side chains exposed to solvent based on a homology model. Residues numbered according to PCV2 numbering.

^d Epitope C is believed to bind to two different MAbs due to its position on the capsid.

target cell, and propose a dynamic N terminus that may be responsible for gaining PCV2 entry into the cell (23).

MATERIALS AND METHODS

Expression, purification, and crystal structure determination of the PCV2^{CS}.

A PCV2 consensus sequence coat protein was produced via sequence alignment of all the PCV2 sequences deposited in GenBank (accession number JF504708). The gene for this sequence was synthesized (DNA 2.0, Menlo Park, CA) and cloned downstream of a hexahistidine and a thrombin cleavage site into a Top10/pET100 vector. The construct was expressed in the *Escherichia coli* BL21(DE3) Star strain (Invitrogen). The cells were grown in Circlegrow (MP Biomedicals) at 37°C till mid log phase. Protein expression was induced by the addition of 0.3 mM isopropyl-β-D-thiogalactoside at 20°C and continued for 16 h. The cells were pelleted and stored at –80°C. The cells were ruptured using a sonicator in lysis buffer (20 mM N-cyclohexyl-3-aminopropanesulfonic acid [CAPS] [pH 10.5], 0.5 M NaCl, 50 mM imidazole [pH 10.5], 0.2 mM phenylmethylsulfonyl fluoride [PMSF], 4 mM β-mercaptoethanol [β-Me], and 20 units of benzonase [EMD4Biosciences]). The lysate was cleared via centrifugation (30 min, 16,000 × g, 4°C) and followed by a 2% streptomycin sulfate cut. The coat protein was purified using nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen) following the manufacturer's protocol, dialyzed overnight in lysis buffer containing 20 mM CAPS (pH 11.1) and supplemented with 200 mM L-Arg (pH 11.1). The samples were concentrated to 50 mg ml^{–1} and further purified by size exclusion chromatography using a Superdex 200 column equilibrated with the dialysis buffer composition (Amersham Biosciences). Fractions pertaining to monomeric capsid protein were pooled, concentrated to 100 mg ml^{–1}, flash-frozen in liquid nitrogen, and stored at –80°C.

Crystals were obtained using the sitting drop method by mixing 15 mg ml^{–1} of the protein in a 1:1 ratio with a mother liquor solution of 6% polyethylene glycol 3350 (PEG 3350), 2.5% isopropanol, and 0.3 M ammonium citrate (pH 5.0). Crystals appeared after several days and were soaked for 30 s in 10% PEG 3350, 2.5% isopropanol, 0.3 M ammonium citrate (pH 5.0) and 12% PEG 400 for cryo-protection. Crystals were flash-frozen in liquid nitrogen for data collection at 100 K. Diffraction data extending to 1.9-Å resolution were collected at the Advanced Photon Source beamline 23-ID-D (λ = 0.88 Å, d = 278 mm) from a single crystal. Data to 2.3-Å resolution were indexed, integrated, merged, scaled, and reduced to a unique reflection set (Table 2) with the HKL suite (26). The crystals have P1 space group symmetry (a = 193.6 Å, b = 202.2 Å, c = 231.0 Å, α = 90.0°, β = 89.3°, γ = 90.1°). There are two particles per asymmetric unit with 120-fold noncrystallographic symmetry (NCS). The virus particle orientations were determined with locked self-rotation functions in the program GLRF (38). Rotation functions computed with data from 5- to 3-Å resolution and a radius of integration of 150 Å clearly resolved the particle alignments, which were sepa-

TABLE 2. Data processing and refinement statistics for the PCV2 crystal structure^a

Statistic or parameter ^b	Value for statistic or parameter	
	Useful data	Outer shell
Data collection statistics		
Resolution range (Å)	40.0–2.35	2.43–2.35
Completeness (%)	68.7	44.8
No. of unique reflections	1,002,451	65,270
R _{merge} (%) ^c	7.1	27.8
Avg I/σ _I	6.2	1.4
Redundancy ^d	1.9	1.3
Refinement statistics		
Resolution range (Å)	40.0–2.35	2.46–2.35
Completeness (%)	68.7	44.9
No. of unique reflections	1,002,451	81,840
R _{cryst} (%) ^e	24.5	39.6
No. of atoms ^f (Ca atoms)	1,740 (194)	
No. of water molecules and ions	131	
Protein geometry and thermal parameters		
RMSD ^b from ideality		
Bond length (Å)	0.007	
Angle (°)	1.4	
Average temperature factor (Å ²)		
Protein	25.8	
Water molecules and ions	38.6	
Ramachandran plot (%)		
Favored	89.2	
Allowed	10.2	
Outliers	0.6	

^a Values given are for data with I/σ_I ≥ 0. The space group is P1 with unit cell dimensions a = 193.6 Å, b = 202.2 Å, c = 231.0 Å, α = 90.0°, β = 89.3°, and γ = 90.1°.

^b I/σ_I, measured intensity divided by measured error; RMSD, root mean square deviation.

^c R_{merge} = (Σ_hΣ_i (I_{hi} – <I_h>)/Σ_hΣ_i I_{hi}) × 100 where <I_h> is the mean of the I_{hi} observations of reflection h.

^d Redundancy is the number of observations/number of unique reflections.

^e R_{cryst} = (Σ_h|F_o – F_c|/Σ_hF_o) where F_o and F_c are the observed and calculated structure factors, respectively.

^f Number for all nonhydrogen atoms, including water molecules and ions.

rated by approximately 3° . PCV2 has poor sequence similarity with capsid proteins in known T=1 virus structures; therefore, several models were tested to generate initial phases. The particle positions were initially determined with a modified version of TF (37) using the T=1 particle coordinates from an amino-terminal deletion of Sesbania mosaic virus (SeMV) coat protein (PDB accession number 1X36). The first particle position is arbitrary in a P1 cell and was placed at the origin. The second particle was located at the center of the cell (0.5, 0.5, 0.5) with an R-factor of 49.1% for data between 8- to 4-Å resolution. Initial phases were computed from 40- to 15-Å resolution using the coordinates of SeMV oriented and positioned in the PCV2 unit cell. The phases were refined and extended with a combination of CCP4 (6) and RAVE (13) programs to 3.0-Å resolution using the 120-fold noncrystallographic symmetry for real-space averaging ($R_{\text{ave}} = 31.3\%$, $CC_{\text{ave}} = 0.75$). Electron density quality in the initial averaged electron density map was sufficient to recognize the canonical viral jelly roll and large side chains. Iterative model building, mask adjustments, and NCS averaging resulted in a contiguous trace from residues 40 to 231. This initial PCV2 model was refined as a rigid body using CNS v1.2 to an R-factor of 44.8% for data between 50- and 3.5-Å resolution (2). The resulting particles were slightly skewed and had close subunit contacts, which indicated the particle positions needed a small adjustment. Using the initial PCV2 model in TF resulted in a refined second particle position of (0.497, 0.5, 0.497) with an R-factor of 32.5% between 8- and 4-Å resolution. The electron density was averaged at 3.0-Å resolution after adjustments to the model and matrices, producing an improved map with excellent details ($R_{\text{ave}} = 18.0\%$, $CC_{\text{ave}} = 0.91$). After a cycle of rebuilding and coordinate refinement, the remaining data up to 2.3 Å were included in all subsequent steps. The final round of refinement with 1,741 protein atoms, 128 water molecules, and 4 sulfate ions gave an R_{cryst} of 24.5% (Table 2), and the final averaging cycle gave an R_{ave} of 25.8% and CC_{ave} of 0.88.

Procheck was used to examine and validate polypeptide geometry (16). Subunit interactions were identified using ViperDB analysis and the hydrogen bond tool in Chimera for OS X (4, 28).

Assembly protocol of PCV2^{CS} coat protein. VLPs were assembled by mixing 100 mg ml⁻¹ PCV2 capsid protein with 6% PEG 3350, 2.5% isopropanol, and 0.3 M ammonium citrate (pH 5.0) in a 1:19 ratio overnight at 4°C. The reaction mixture was cleared using centrifugation (12,000 × g, 10 min, 4°C), laid under a 35% (wt/wt) CsCl solution, and centrifuged in a SW41 rotor (Beckman) at 36,000 rpm (160,030 × g) for at least 18 h at 4°C. PCV2 VLPs formed a band approximately halfway down the CsCl gradients. Particles were harvested by puncturing the tube with a needle and drawing the band into a syringe. CsCl was removed by dialysis against 20 mM Tris-HCl (pH 8.0) and 150 mM NaCl and visualized using negative stain (2% uranyl acetate) electron microscopy.

Construction of the PCV2^{N12} VLPs. A DNA fragment containing the coat protein coding sequence of PCV2 N12 strain (GenBank accession number AAN06827) was amplified by PCR from plasmid pJ201:12772, cloned into vector pBacPAK9 (Clontech), and sequenced. Recombinant baculovirus encoding PCV2 coat protein was generated with the BacPAK expression system kit (Clontech) using protocols provided by the manufacturer.

Spodoptera frugiperda cells (line IPLB-Sf21) were maintained in TC100 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and penicillin and streptomycin antibiotics. Sf21 cells were used for generating baculovirus stocks and titration by plaque assay. *Trichoplusia ni* cells ("High 5"; Invitrogen) were propagated in serum-free ESF921 medium (Expression Systems) supplemented with penicillin and streptomycin. *T. ni* cells were used for synthesis and purification of PCV2 virus-like particles.

Expression and purification of PCV2^{N12}. A 1-liter suspension culture of *T. ni* cells (2×10^6 cells/ml) was infected at a multiplicity of infection (MOI) of 0.1 to 1 with recombinant baculovirus encoding the PCV2 coat protein. Infected cells were incubated at 27°C on an orbital shaker set at 100 rpm. Cell death was monitored by trypan blue staining, and the culture was harvested when at least 50% of the cells had died, typically 7 to 10 days postinfection. The cells were lysed by the addition of 50 ml 10% (vol/vol) NP-40 and incubation for 10 min at 4°C. Nuclei and cell debris were pelleted at 4°C for 15 min at 15,300 × g. Pooled supernatants were underlaid with 3 ml of 30% (wt/wt) sucrose in 50 mM HEPES (pH 7.5) and centrifuged at 45,000 rpm (184,048 × g) for 2.5 h at 11°C in a 50.2 Ti rotor (Beckman). The supernatants were removed, and each pellet was resuspended in 0.75 ml of 50 mM HEPES (pH 7.5). Resuspended pellets were clarified by centrifugation at 16,100 × g for 10 min at 4°C. The supernatants were pooled, and VLPs were banded using CsCl gradients as described above. CsCl was removed by dialysis against 50 mM HEPES (pH 7.5), and purified particles were stored at -20°C.

Cryo-EM data collection and image reconstruction of PCV2^{N12}. Frozen hydrated samples of PCV2 (N12 strain) VLPs were prepared on C-flat CF-22-4C grids (Portochips) covered by a thin layer of carbon. Briefly, a thin layer of

FIG. 1. PCV2^{CS} VLP assembly. (A) Micrograph of a crushed PCV2^{CS} crystal stained with 2% (wt/vol) uranyl acetate. (B) The condition was optimized to produce VLPs without crystal growth.

carbon was deposited on freshly cleaved mica using an Edwards Auto 308 carbon evaporator, and floated onto C-flat grids. A 4-μl sample of the PCV2^{N12} VLP (at 10 mg ml⁻¹) was applied to the grid, blotted for a short period of time, and dipped into a liquid ethane bath using an FEI Vitrobot instrument. The grids were stored and handled in liquid nitrogen for data collection. Data were collected at cryogenic temperatures on an FEI Tecnai F20 electron microscope operating at 120 kV using the LEGION suite and less than 16 e⁻ Å⁻² (36). Images were captured on a Gatan 4K by 4K charge-coupled device (CCD) at a nominal magnification of ×80,000 with defocus values from -1.5 to -2.5 μm. The CCD pixel size was calibrated using the diffraction pattern of a two-dimensional (2D) catalase crystal with known cell parameters. Contrast transfer function (CTF) estimation and correction were done using ACE2 through the Appion package (15). A template-based particle picker was used to automatically select and extract 11,085 particles from the images to a box size of 240 by 240 pixels at 1.370 Å pixel⁻¹ (30). The particles were binned by 2-Å spherical shell with the size and thickness of the particles (measured from an average of centered particles) used as the starting model for image reconstruction. Image reconstruction was carried out using the EMAN package (20). The resolution of the final image reconstruction, as determined by a Fourier shell correlation (FSC) of 0.5, is 9.5 Å.

A difference map between the cryo-EM image reconstruction and a calculated map from the PCV2 consensus crystal structure was produced using a previously described procedure (11).

Accession numbers. We have deposited the structure factors and coordinates for the PCV2 icosahedral asymmetric unit in the Protein Data Bank (PDB) with accession number 3R0R. The structure is also available from the VIPER database (viperdbs.scripps.edu). We have deposited the nucleotide sequence for the consensus construct in GenBank (accession number JF504708).

RESULTS

Expression and assembly of PCV2^{CS} capsid subunit. A construct possessing the consensus sequence of deposited PCV2 capsid protein sequences was generated. This construct, containing an engineered N-terminal hexahistidine tag and thrombin cleavage site lacked the N-terminal 40 residues of PCV2. It was expressed and purified as monomeric subunits from *E. coli*. Over 20 mg of purified protein was obtained from a liter of *E. coli* culture. This construct has 88% sequence identity to the N12 strain (see below). Crystal screens of the monomeric subunit produced crystals formed by assembled VLPs as revealed by negative stain electron microscopy (Fig. 1). An assembly procedure was optimized to produce soluble VLPs. These remained assembled for 2 weeks at 4°C as determined by electron microscopy.

Crystal structure of the PCV2^{CS} VLP. The interpretable density for PCV2^{CS} begins at residue 40 (located in the interior of the capsid) and is contiguous to residue 231 (located on the exterior of the capsid). The PCV2 subunit fold is a canonical viral jelly roll first described for tomato bushy stunt virus and

FIG. 2. Crystal structure of PCV2^{CS} VLP. (A) Ribbon diagram of the PCV2 capsid protein with the secondary structures labeled according to the convention first described for tomato bushy stunt virus (TBSV). The N and C termini are labeled using blue and red spheres, respectively. (B) Stereoscopic representation of a ribbon diagram of the PCV2^{CS} VLP. The loops connecting the different strands have been color coded according to the bar diagram at the top right. N-term, N termini.

subsequently found in many icosahedral viruses (Fig. 2) (10, 35). The fold is a β -sandwich composed of two sheets. Four antiparallel β -strands form each sheet (labeled B to I). The sheet formed from strands BIDG is approximately two times longer than the sheet formed by strands CHEF. Each viral jelly roll is oriented roughly radial with respect to the spherical particle, and the majority of subunit-subunit interactions occur between residues forming the loops of neighboring subunits. Of the approximately 4,600-Å² buried surface area per subunit in the assembled VLP, the loops contribute 3,480 Å² while the strands contribute 1,120 Å². The loops connecting the eight β -strands alternate between short and long. Loops connecting β -strands BC/DE/FG/HI are four to nine residues long, while loops connecting strands CD/EF/GH are 21 to 36 residues long. The long loops contribute to the capsid surface features and contact neighboring loops to stabilize the capsid.

The capsid surface can be described as a mountainous terrain with its base at the icosahedral 3-fold axes and two protrusions emanating from the icosahedral 5- and 2-fold axes. Loops DE from neighboring subunits pack against one another on the outer surface of the particle to form the 5-fold axes. Each loop contributes 460 Å² of buried surface area. Loops BC and HI define the knob-like protrusions extending furthest from the capsid surface and decorating the 5-fold axes. Loops CD from the subunits related by the icosahedral 2-fold axis form the second highest elevation on this terrain. Each loop contributes 340 Å² of buried surface area. Loops GH are related by the icosahedral 3-fold axis and make extensive contact with one another. Each loop contributes 1,010 Å² of buried surface area for a total of 3,030 Å² (Fig. 2).

A number of sulfate anions interact with arginine residues on the interior and exterior surface of the capsid. Sulfate interaction sites on the interior surface may represent the phosphate binding sites of the packaged ssDNA genome, while sites on the exterior may identify the heparan sulfate binding sites of the PCV2 (see Discussion).

Identifying the MAb binding sites on PCV2. PEPSCAN analysis of antisera from PCV2-infected swine by Mahe et al. (21) identified 15-residue peptides of the PCV2 capsid that

were targeted by the immune system. However, these epitopes are linear and do not necessarily identify what capsid residues are involved in immune recognition. Therefore, a combination of sequence alignment, homology modeling, and structural visualization were employed to identify the specific residues in each epitope responsible for monoclonal antibody (MAb) binding. To identify these residues, a sequence alignment of the PCV1 and PCV2 capsid protein sequences used by Mahe et al. (21) (GenBank accession numbers AF012107 and AF201311) was generated with the T-Coffee server (24). A homology model of the PCV2 capsid used by Mahe et al. (21) was then generated using the PCV2^{CS} crystal structure as a template with the I-TASSER server (40). The residues that are different in the two PCV strains were then mapped onto the PCV2 homology model using the AL2CO server (27). Finally, the locations of the epitopes on the surface of the PCV2 homology model were visualized, and the residues in each epitope responsible for MAb binding were identified in accordance with the following logic: residues that are different in PCV1 and PCV2 are responsible for discriminating between MAbs that bound only to the PCV2 epitopes, and residues that are conserved between PCV1 and PCV2 are responsible for binding MAbs that bound to both PCV1 and PCV2 epitopes.

Four of the epitopes map to the exterior surface of the capsid (residues 69 to 83, 113 to 127, 117 to 131, and 193 to 207), while two others map to the subunit interface at the icosahedral 3-fold axes (residues 169 to 183) and to the interior of the capsid shell (residues 25 to 39). These epitopes are labeled A to F (Table 1). Residues 70 and 71 and residues 77 to 78 may be responsible for discriminating the binding of MAbs to PCV2 versus PCV1 in epitope A. These residues are present on the surface of the particle, exposed to solvent, and located in close proximity to one another. Similarly, residues 113 and 115 may be responsible for binding MAbs to epitope B, residue 127 responsible for binding MAbs to epitope C, residues 170 to 172 and residues 174 and 175 responsible for binding MAbs to epitope D, and residue 203 and residues 206 and 207 responsible for binding MAbs to epitope E (Table 1 and Fig. 3 and 4).

FIG. 3. Surface mapping of the PCV2^{CS} VLP. Sulfate ions are shown as sphere models. (A) Sequence conservation among the deposited PCV2 capsid protein sequences. The sequences are shown colored from a gradient of red (no conservation) to blue (absolute conservation). The PCV2 residues responsible for discriminating the reported MAb (20) are color coded as follows: epitope A is blue, epitope B is red, epitope C is magenta, epitope D is yellow, and epitope E is cyan. Epitope F is not shown. (B) Electrostatic potential map of the PCV2 crystal structure. Sulfates surrounding the icosahedral 3-fold axes sit within a canyon of positively charged residues that may bind heparin sulfate. This figure was made using University of California, San Francisco Chimera (28).

Epitopes D and F (residues 169 to 183 and residues 25 to 43, respectively) exhibit cross-reactivity to PCV1 but map to regions of the structure that appear to be protected from the immune system. Residues in epitope D (loop GH) map to the subunit interface at the icosahedral 3-fold axes and interact with equivalent residues in the neighboring symmetry-related subunits. With the exception of residues 169 and 170, the side chains of the remaining residues are completely buried and contribute ~1,000 Å² of buried surface area. Such an extensive interaction suggests that the buried residues are unlikely to interact with MAbs, unless they transiently externalize and become exposed to solvent or the virions disassemble and expose these regions to the immune system. Residue 170 is conserved in the PCV1 and PCV2 epitopes and is located at the crest of the GH loop where it is fully exposed to solvent and able to interact with MAbs. Residues in epitope F are located in the interior of the PCV2 capsid shell where they may be interacting with the viral genome in an infectious particle.

Shang et al. used MAbs raised against PCV1 and PCV2 subunits to identify epitopes (32). In addition to the epitopes reported by Mahe et al. (21), they identified a number of additional epitopes. The additional epitopes map to the interior surface of the capsid. These MAbs were raised against unassembled subunits and are probably not immunologically relevant; hence, they are not discussed.

The cryo-EM image reconstruction of the PCV2 N12 strain VLP. The cryo-EM image reconstruction of the PCV2^{N12} determined to 9.6-Å resolution was comparable to a calculated map of the PCV2^{CS} crystal structure at the same resolution and established that the consensus sequence VLP assembles similarly to the N12 strain VLP. A difference map between the cryo-EM image reconstruction and the fitted atomic structure did not identify any posttranslational modifications to the capsid. This was in agreement with our peptide mass fingerprinting experiments of the purified N12 VLP samples. However, density for what we postulate to be packaged nucleic acid and the

FIG. 4. PCV2 coat protein sequence conservation and epitope mapping. The aligned coat protein sequences of the PCV2^{N12}, PCV2^{CS}, and the PCV1 and PCV2 strains used by Mahe et al. (21). The secondary structure for the PCV2^{CS} crystal structure is shown at the top (β-strands are shown as cyan arrows, α-helices as yellow ovals, and loops as gray bars). A BLAST search of the PCV2^{N12} coat protein sequence identified 199 coat protein sequences belonging to various strains of PCV2. Sequence alignment using the T-Coffee server produced the conservation bars shown above. The blue, red, magenta, yellow, and cyan boxes highlight the epitopes identified by Mahe et al. (21), and the correspondingly colored circles identify the surface residues responsible for discriminating MAb binding to PCV1 and PCV2. This figure was produced using Jalview (38).

N terminus (deleted in the construct used for the crystal structure) could be seen in the difference map. The difference density was strongest near the icosahedral 3- and 5-fold axes, while it was weakest near the 2-fold axes. In fact, the sulfates observed in the PCV2^{CS} crystal structure closely followed the difference density from the 3-fold axes to the 5-fold axes. These sulfates make intimate hydrogen bond interactions with basic residues and may chemically mimic the backbone phosphates of the PCV2 ssDNA genome. The absence of sulfates near the strong density at the icosahedral 5-fold axes suggested that a portion of the N-terminal 40 residues missing in the crystal structure may indeed be located proximal to the icosahedral 5-fold axes. The N terminus is highly basic, possesses a nuclear localization signal, and is predicted to be helical with the I-TASSER server (33, 40).

DISCUSSION

The epitope PEPSCAN studies conducted by Mahe et al. (21) offer a general guideline to where MAbs may bind to the PCV2 surface. Therefore, the six immunogenic epitopes were mapped onto the crystal structure of PCV2 (Table 1) (21). The residues within epitopes A, B, C, and E that may be responsible for MAbs binding only to PCV2 and the residues within epitope D that may be responsible for MAbs capable of binding to both PCV2 and PCV1 were identified (Fig. 3B). This provides a more detailed map of MAb binding sites on PCV2.

The sequence conservation among the deposited PCV2 capsid proteins was mapped onto the surface of the PCV2 particle to correlate the MAb binding sites with the sequence variability that the PCV2 particle can tolerate yet maintain infectivity (Fig. 3A). Figure 3A clearly shows that with the exception of residues 169-ST-170 and 228-DP-229, the loops decorating the capsid surface can tolerate mutations. The conserved regions of the capsid lie in the plateau surrounding the icosahedral 3- and 5-fold axes and appear not to be readily accessible to MAb binding.

Residues in epitope F are located on the interior surface of the capsid. The immune system can generate Abs against these residues under the following two conditions. (i) The disassembled capsid elicits an immune response against the exposed N terminus. (ii) The N terminus of an intact capsid is transiently externalized. While the former cannot be ruled out, the latter possibility is supported by “breathing” observed in a number of animal viruses. The exposure of internal polypeptides was shown to be crucial for the infectivity of these viruses (1, 18, 19). The N terminus of PCV2 may also be transiently exposed and may be involved in the infectivity of the virion. The PCV2 N terminus is highly basic, and structure predictions indicate a helix with one positively charged surface. Internal amphipathic helices in other viruses have membrane-disrupting activity essential for infectivity (8, 25). Future studies will test PCV2 VLPs for membrane lysis activity and establish the role that the N terminus may play in it.

PCV2 binds heparan sulfate during its entry into cells (23); therefore, the PCV2 crystal structure was examined for candidate binding sites. The predicted PCV2 heparan sulfate binding site was 98-IRKVKV-103 and follows the XBBXBH heparan sulfate (HS) binding motif (where X and B correspond to hydrophobic/neutral and basic residues, respectively) (3, 23).

FIG. 5. Subnanometer resolution cryo-EM image reconstruction of the PCV2^{N12} VLPs. (A) Surface cutaway view showing the capsid (transparent gray) with the fitted subunits (ribbon diagram) and packaged cellular nucleic acid (red). (B) The crystal structure of the PCV2^{CS} is overlaid onto the difference map (see text). The N and C termini are identified by blue and red spheres, respectively. Sulfate ions in the vicinity of the difference map are shown as sphere models and believed to represent the phosphate backbones of the packaged cellular nucleic acid in the PCV2^{N12} VLPs used for the cryo-EM image reconstruction. The N-terminal segment, not present in the crystal structure, is believed to be located proximal to the icosahedral 5-fold axes.

However, these residues are located on the interior surface of the capsid shell and are unlikely to be the binding sites unless they externalize. Docking the PCV2^{CS} crystal structure into the PCV2^{N12} cryo-EM image reconstruction revealed that these residues are in close proximity to the difference peaks attributed to the nucleic acid in the PCV2^{N12} particles (Fig. 5). To identify the putative sites that may be responsible for binding heparan sulfate, we analyzed the sequence conservation and charge distribution of the clefts that bind sulfate ions on the exterior surface of the PCV2^{CS} crystal structure. These clefts are located proximal to the icosahedral 2- and 5-fold axes. An electrostatic potential map of the capsid shows that the clefts surrounding the icosahedral 2-fold axis are positively charged at neutral pH (Fig. 3B). Residues composing this cleft are highly conserved among the PCV2 sequences. Hence, heparan sulfate may bind to this region of the PCV2 capsid. Future cryo-EM and image reconstruction studies of PCV2 in complex with heparan sulfate will test this hypothesis.

The simplicity of the PCV2 capsid protein (233 residues in length), the T=1 capsid symmetry, and the three identified open reading frames of its ~2,000-base genome make PCV2 one of the most economical “autonomously replicating” animal viruses encountered thus far. Here, the subnanometer resolution cryo-EM image reconstruction of a PCV2 VLP (N12 strain) produced in insect cells was described along with the 2.3-Å crystal structure of a consensus sequence VLP purified from an *E. coli* expression system. The two structures allowed the mapping of previously determined immunogenic epitopes onto a three-dimensional platform for understanding how the host immune system neutralizes this pathogen. The location of the heparan sulfate binding sites to PCV2 was determined as well as a proposed mechanism by which the internalized N terminus may facilitate PCV2 entry into the cell.

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REFERENCES

- Bothner, B., X. F. Dong, L. Bibbs, J. E. Johnson, and G. Siuzdak. 1998. Evidence of viral capsid dynamics using limited proteolysis and mass spectrometry. *J. Biol. Chem.* **273**:673–676.
- Brunger, A. T., et al. 1998. Crystallography & NMR system: a new software suite for macromolecular structure determination. *Acta Crystallogr. D* **54**: 905–921.
- Cardin, A. D., and H. J. Weintraub. 1989. Molecular modeling of protein-glycosaminoglycan interactions. *Arteriosclerosis* **9**:21–32.
- Carrillo-Tripp, M., et al. 2009. VIPERdb2: an enhanced and web API enabled relational database for structural virology. *Nucleic Acids Res.* **37**: D436–D442.
- Clark, E. G. 1997. Post-weaning multisystemic wasting syndrome, p. 499–501. *In* Proceedings of the 28th Annual Meeting of the American Association of Swine Practitioners, Quebec City, Quebec, Canada. American Association of Swine Practitioners, Perry, IA.
- Collaborative Computational Project. 1994. The CCP4 suite: programs for protein crystallography. *Acta Crystallogr. D* **50**:760–763.
- de Boisseson, C., et al. 2004. Molecular characterization of Porcine circovirus type 2 isolates from post-weaning multisystemic wasting syndrome-affected and non-affected pigs. *J. Gen. Virol.* **85**:293–304.
- Futaki, S. 2005. Membrane-permeable arginine-rich peptides and the translocation mechanisms. *Adv. Drug Deliv. Rev.* **57**:547–558.
- Gamage, L. N., J. Ellis, and S. Hayes. 2009. Immunogenicity of bacteriophage lambda particles displaying porcine circovirus 2 (PCV2) capsid protein epitopes. *Vaccine* **27**:6595–6604.
- Harrison, S. C., A. J. Olson, C. E. Schutt, F. K. Winkler, and G. Bricogne. 1978. Tomato bushy stunt virus at 2.9 Å resolution. *Nature* **276**:368–373.
- Khayat, R., C. Y. Fu, A. C. Ortmann, M. J. Young, and J. E. Johnson. 2010. The architecture and chemical stability of the archaeal *Sulfolobus* turreted icosahedral virus. *J. Virol.* **84**:9575–9583.
- Kim, J. H., and Y. S. Lyoo. 2002. Genetic characterization of porcine circovirus-2 field isolates from PMWS pigs. *J. Vet. Sci.* **3**:31–39.
- Kleywegt, G. J., and T. A. Jones. 1996. xdlMAPMAN and xdlDATAMAN - programs for reformatting, analysis and manipulation of biomacromolecular electron-density maps and reflection data sets. *Acta Crystallogr. D* **52**:826–828.
- Ladেকjaer-Mikkelsen, A. S., et al. 2001. Transplacental infection with PCV-2 associated with reproductive failure in a gilt. *Vet. Rec.* **148**:759–760.
- Lander, G. C., et al. 2009. Appion: an integrated, database-driven pipeline to facilitate EM image processing. *J. Struct. Biol.* **166**:95–102.
- Laskowski, R. A., M. W. MacArthur, D. S. Moss, and J. M. Thornton. 1993. PROCHECK: a program to check the stereochemical quality of protein structures. *J. Appl. Cryst.* **26**:283–291.
- Lefebvre, D. J., et al. 2008. Antigenic differences among porcine circovirus type 2 strains, as demonstrated by the use of monoclonal antibodies. *J. Gen. Virol.* **89**:177–187.
- Lewis, J. K., B. Bothner, T. J. Smith, and G. Siuzdak. 1998. Antiviral agent blocks breathing of the common cold virus. *Proc. Natl. Acad. Sci. U. S. A.* **95**:6774–6778.
- Li, Q., A. G. Yafal, Y. M. Lee, J. Hogle, and M. Chow. 1994. Poliovirus neutralization by antibodies to internal epitopes of VP4 and VP1 results from reversible exposure of these sequences at physiological temperature. *J. Virol.* **68**:3965–3970.
- Ludtke, S. J., P. R. Baldwin, and W. Chiu. 1999. EMAN: semiautomated software for high-resolution single-particle reconstructions. *J. Struct. Biol.* **128**:82–97.
- Mahe, D., et al. 2000. Differential recognition of ORF2 protein from type 1 and type 2 porcine circoviruses and identification of immunorelevant epitopes. *J. Gen. Virol.* **81**:1815–1824.
- Meehan, B. M., et al. 2001. Isolation and characterization of porcine circovirus 2 from cases of sow abortion and porcine dermatitis and nephropathy syndrome. *Arch. Virol.* **146**:835–842.
- Misinzio, G., P. L. Delputte, P. Meerts, D. J. Lefebvre, and H. J. Nauwynck. 2006. Porcine circovirus 2 uses heparan sulfate and chondroitin sulfate B glycosaminoglycans as receptors for its attachment to host cells. *J. Virol.* **80**:3487–3494.
- Notredame, C., D. G. Higgins, and J. Heringa. 2000. T-Coffee: a novel method for fast and accurate multiple sequence alignment. *J. Mol. Biol.* **302**:205–217.
- Oren, Z., and Y. Shai. 1998. Mode of action of linear amphipathic alpha-helical antimicrobial peptides. *Biopolymers* **47**:451–463.
- Otwinski, Z., and W. Minor. 1997. Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol.* **276**:307–326.
- Pei, J., and N. V. Grishin. 2001. AL2CO: calculation of positional conservation in a protein sequence alignment. *Bioinformatics* **17**:700–712.
- Petersen, E. F., et al. 2004. UCSF chimera - a visualization system for exploratory research and analysis. *J. Comput. Chem.* **25**:1605–1612.
- Rosell, C., et al. 2000. Identification of porcine circovirus in tissues of pigs with porcine dermatitis and nephropathy syndrome. *Vet. Rec.* **146**:40–43.
- Roseman, A. M. 2004. FindEM—a fast, efficient program for automatic selection of particles from electron micrographs. *J. Struct. Biol.* **145**:91–99.
- Segales, J., G. Allan, and M. Domingo. 2005. Porcine circovirus diseases. *Anim. Health Res. Rev.* **6**:119–142.
- Shang, S. B., et al. 2009. Fine mapping of antigenic epitopes on capsid proteins of porcine circovirus, and antigenic phenotype of porcine circovirus type 2. *Mol. Immunol.* **46**:327–334.
- Shuai, J., et al. 2008. Mapping of the nuclear localization signals in open reading frame 2 protein from porcine circovirus type 1. *Acta Biochim. Biophys. Sin. (Shanghai)* **40**:71–77.
- Shuai, J., et al. 2007. Genetic characterization of porcine circovirus type 2 (PCV2) from pigs in high-seroprevalence areas in southeastern China. *Virus Genes* **35**:619–627.
- Speir, J. A., and J. E. Johnson. 2008. Virus particle structure: nonenveloped viruses, p. 380–393. *In* B. W. J. Mahy and M. H. V. Van Regenmortel (ed.), *Encyclopedia of virology*, vol. 5. Elsevier, Oxford, United Kingdom.
- Suloway, C., et al. 2005. Automated molecular microscopy: the new Legimon system. *J. Struct. Biol.* **151**:41–60.
- Tong, L. 1993. REPLACE, a suite of computer programs for molecular-replacement calculations. *J. Appl. Cryst.* **26**:748–751.
- Tong, L., and M. G. Rossmann. 1997. Rotation function calculations with GLRF program. *Methods Enzymol.* **276**:594–611.
- West, K. H., et al. 1999. Myocarditis and abortion associated with intrauterine infection of sows with porcine circovirus 2. *J. Vet. Diagn. Invest.* **11**:530–532.
- Zhang, Y. 2008. I-TASSER server for protein 3D structure prediction. *BMC Bioinformatics* **9**:40.