Parvovirus Capsid Structures Required for Infection: Mutations Controlling Receptor Recognition and Protease Cleavages

Heather M. Callaway,^a Kurtis H. Feng,^a Donald W. Lee,^b Andrew B. Allison,^{a,c} Melissa Pinard,^d Robert McKenna,^d Mavis Agbandje-McKenna,^d Susan Hafenstein,^e Colin R. Parrish^a

Baker Institute for Animal Health, Department of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, Ithaca, New York, USA^a; School of Chemical and Biomolecular Engineering, Cornell University, Ithaca, New York, USA^b; Department of Biomedical Sciences and Pathobiology, Virginia-Maryland College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA^c; Department of Biochemistry and Molecular Biology, Center for Structural Biology, McKnight Brain Institute, University of Florida, Gainesville, Florida, USA^d; Department of Medicine, Pennsylvania State University College of Medicine, Hershey, Pennsylvania, USA^e

ABSTRACT Parvovirus capsids are small but complex molecular machines responsible for undertaking many of the steps of cell infection, genome packing, and cell-to-cell as well as host-to-host transfer. The details of parvovirus infection of cells are still not fully understood, but the processes must involve small changes in the capsid structure that allow the endocytosed virus to escape from the endosome, pass through the cell cytoplasm, and deliver the single-stranded DNA (ssDNA) genome to the nucleus, where viral replication occurs. Here, we examine capsid substitutions that eliminate canine parvovirus (CPV) infectivity and identify how those mutations changed the capsid structure or altered interactions with the infectious pathway. Amino acid substitutions on the exterior surface of the capsid (Gly299Lys/Ala300Lys) altered the binding of the capsid to transferrin receptor type 1 (TfR), particularly during virus dissociation from the receptor, but still allowed efficient entry into both feline and canine cells without successful infection. These substitutions likely control specific capsid structural changes resulting from TfR binding required for infection. A second set of changes on the interior surface of the capsid reduced viral infectivity by >100-fold and included two cysteine residues and neighboring residues. One of these substitutions, Cys270Ser, modulates a VP2 cleavage event found in ~10% of the capsid proteins that also was shown to alter capsid stability. A neighboring substitution, Pro272Lys, significantly reduced capsid assembly, while a Cys273Ser change appeared to alter capsid transport from the nucleus. These mutants reveal additional structural details that explain cell infection processes of parvovirus capsids.

IMPORTANCE Parvoviruses are commonly found in both vertebrate and invertebrate animals and cause widespread disease. They are also being developed as oncolytic therapeutics and as gene therapy vectors. Most functions involved in infection or transduction are mediated by the viral capsid, but the structure-function correlates of the capsids and their constituent proteins are still incompletely understood, especially in relation to identifying capsid processes responsible for infection and release from the cell. Here, we characterize the functional effects of capsid protein mutations that result in the loss of virus infectivity, giving a better understanding of the portions of the capsid that mediate essential steps in successful infection pathways and how they contribute to viral infectivity.

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Address correspondence to Colin R. Parrish, crp3@cornell.edu.

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The parvovirus infectious cycle is a complex process that includes cell receptor binding, endosomal and cytoplasmic trafficking, nuclear transport, DNA replication within the nucleus, capsid assembly, nuclear export, and escape or release of the newly formed virions (1, 2). Parvoviral capsid proteins are therefore required to perform a variety of complex functions in order to complete these steps, and the capsids are assembled from only a few forms of a single viral protein. The capsid encloses the viral genome and protects it during transfer between cells and between animal hosts but also performs a variety of functions required to deliver the genome to the nucleus for infection (2–10). Some of these capsid-dependent processes are host specific and result in viral host range variation that determines an animal's susceptibility to different viruses. Many parvovirus capsid structures have been determined to near-atomic resolution (11–14), but the details of how the various components of the capsid and their structures mediate cell infection and the other steps in the replication cycle that involve dynamic changes in the capsids are still poorly understood.

The canine parvovirus (CPV) capsid is a T=1 icosahedron that is assembled from 60 copies of combinations of the viral proteins VP1 and VP2. A VP3 form is generated in full (DNA-containing) capsids through the proteolytic cleavage of ~19 residues from the N terminus of a proportion of the VP2 molecules (15). VP1 is an extended form of VP2 that has 143 additional N-terminal amino acids, which include a nuclear-targeting sequence and a PLA2 enzyme domain (6, 16, 17). VP1 comprises ~10% of the capsid subunits (15, 18). The surface of the CPV capsid is structurally complex and includes features such as pores passing to the capsid exterior at the 5-fold axes, raised surface regions (spikes) around the 3-fold axes, and surface valley regions located about the 2-fold axes and 5-fold axes of symmetry (the dimple and canyon, respectively) (19–21). The capsid binds transferrin receptor type 1 (TfR), and TfR is the essential and apparently only receptor required by CPV and its relatives for binding to and infection of carnivore host cells (22, 23). The capsid may also bind sialic acids, specifically the modified form *N*-glycolylneuraminic acid (Neu5Gc), which is present in some hosts but not others, and while that binding is not an essential receptor interaction, it may reduce the infectivity of cells (24). The capsid stimulates the formation of and is the target for host antibodies that efficiently block infection and aid in recovery from disease (25, 26).

The TfR binds the capsid through a structure on the 3-fold spikes that appears to center around VP2 residue 300 but involves other residues that are 20 to 30 Å apart, suggesting a broad interaction with the capsid (27, 28). The 3-fold spikes are also the major antibody binding motif, and the antibody binding sites have been divided into two somewhat distinct groupings, which are termed the A and B sites (29, 30). The modified sialic acid Neu5Gc binds to a site within the 2-fold dimple (31, 32).

CPV infects cells by binding to the TfR on the cell membrane, quickly followed by clathrin-mediated endocytosis (8, 23, 33). This results in exposure of the capsid to lower pH as the endosome acidifies, which likely causes the release of some of the capsid-bound calcium ions and movement of capsid loops that also facilitate the release of the VP1 unique region (34). Many changes in the capsid structure resulting from low-pH treatment (approximately pH 5.0 or possibly lower) appear to be reversible (8, 34) and do not appear to alter the structure in a way that changes the overall capsid susceptibility to external protease treatment (15, 35). However, small or asymmetric changes that result from TfR binding, perhaps in combination with low pH or proteolytic cleavage of a proportion of capsid proteins after assembly, and that are necessary for cell infection by the virus may occur.

While viruses related to feline panleukopenia virus (FPV) have been infecting a wide variety of carnivore hosts (commonly including domestic cats) for many years, the variant that infects dogs (CPV) arose during the 1970s, initially through the acquisition of mutations that changed surface residues in two regions of the capsid—VP2 residues

93Lys to Asn (Lys93Asn) and 323Asp to Asn (Asp323Asn) (36). An additional region that controls canine and other host ranges was subsequently defined by examining mutations of residue 299 (Gly to Glu or Asp) or 300 (Ala or Gly to Asp) (37–39). The mutation Ala300Asp has been observed repeatedly after natural transfer of CPV-2 from dogs to raccoons or during passage of CPV strains in raccoon cells (40), and the change also arose (along with a change of residue 301) during CPV-2 passage in feline cells *in vitro* (41, 42). The presence of 300Asp also results in reduced canine *in vivo* and *in vitro* infection and in decreased binding to the feline TfR (43–45). The 300Asp substitution results in more extensive structural changes in the capsid than are seen for 300Ala or Gly, due to the formation of a salt bridge between VP2 Asp300 and Arg81 on adjacent subunits, which stabilizes the extended loop that contains residue 300 in a new position (44). The change of the adjacent VP2 residue 299 from Gly to Glu results in a CPV capsid that no longer binds the canine TfR or infects canine cells but that does not significantly reduce the efficiency of infection of feline cells (39, 41). Residues 299 and 300 fall in the middle of one of the two principal antibody binding sites (the B site) on the capsid and may also influence the binding of monoclonal antibodies (MAbs) that recognize the site (11, 30, 38, 46).

The CPV capsid is very stable and retains infectivity after exposure to temperatures above 60°C (35), yet it likely changes side chain and loop conformations during cell infection. Small differences in the structures and dynamics of various parvovirus and adeno-associated virus (AAV) capsids have been reported (35, 47, 48). Although the crystal structures of empty particles or virus-like particles (VLPs) appear structurally highly similar to those of infectious DNA-containing capsids when different crystal structures are compared, some differences in the structures and dynamics have been revealed (49–51). Residues in the interior of the capsid recognize and interact with the single-stranded DNA (ssDNA) genome by recognizing the splayed out DNA bases (11, 14, 52), and changes in the capsid-DNA interactions may control the packaging of the DNA, as well as its release of the genome during infection. The cleavage of VP2 to VP3 in full capsids may also play a role in facilitating DNA release and has been shown to alter the capsid infectivity of minute virus of mice (MVM) (53–55). Submolar proteolytic cleavages were consistently observed within a small proportion of the capsid proteins (35), although the specific sites cleaved, the origins of the cleavages, and any functions were not defined.

The CPV capsid may also undergo structural changes after assembly, possibly upon binding to the host TfR or to host antibodies, either outside the cell or after uptake into an endosome and exposure to low pH. However, it does not appear to contain a pH-sensitive domain that changes structure in the acidic environment of the endosome like those that are reported for AAV capsids and that trigger an autocatalytic cleavage event when the viruses encounter low pH (48).

Here, we define key positions and functions within the parvovirus capsid involved in the cell infectious process by revealing the functional effects of single or double changes in the CPV capsid protein that make the capsid noninfectious. Some changes altered receptor binding and blocked infection after cell entry, while others were associated with a submolar cleavage of the capsid protein, which altered the interior of the capsid and changed its stability.

RESULTS

To better understand the capsid controls of cell infection, we examined a panel of point mutations in the CPV capsid, among which the VP2 Gly299Lys/Ala300Lys, Pro272Lys, and Cys273Ser mutants did not result in the production of viable viruses and Cys270Ser resulted in 100-fold less viable virus. These mutations were prepared in infectious plasmid clones of the viruses and tested for sustained growth of the viruses after transfection into Norden Laboratory Feline Kidney (NLFK) cells, which are susceptible to all of the natural isolates of CPV. Mutations blocking viral infectivity included those changing residues on the capsid surface (VP2 299 and 300) and others in the interior of the capsid (VP2 270, 272, and 273).

FIG 1 Protein structure, surface morphology, and viability of the external capsid surface mutations. (A) CPV VP2 full capsid (PDB ID 4DPV) (exterior surface facing out), with VP2 residues 299 and 300 displayed in yellow (boxed). (B) Magnification of a region of panel A showing surrounding subunits (gray) and residues within 5 Å of Gly299 and Ala300 displayed and labeled. (C) From left to right, WT CPV full capsid (Ala300) (PDB ID 4DPV); Gly300 CPV (PDB ID 4QYK); Asp300 CPV, with salt bridge to Arg81 (PDB ID 1IJS); and a homology model of Gly299Lys/Ala300Lys. (D) Relative infectivity and growth of mutant CPVs compared to the wild type, measured by TCID₅₀ over three passages in NLFK feline cells. The data were collected from 3 independent experiments and analyzed by ANOVA. (E) Reactivity of wild-type or mutant capsids with MAbs 14 (A site), 8 (B site), and E (B site), using an HAI. Data were collected from 3 independent experiments and analyzed by analysis of variance (ANOVA). The error bars display standard errors of the mean; *, $P < 0.05$.

To allow us to produce sufficient particles to study the properties of the capsids containing substitutions that produced nonviable viruses, VLPs were prepared from the VP2 gene expressed in insect cells, and the capsids were purified and compared to both wild-type VLPs and wild-type empty capsids recovered from cell infections.

VP2 residue 299/300 surface mutations allow feline-TfR binding but block infection. VP2 residues 299 and 300 are within a surface-exposed loop (Fig. 1A and B) that previously has been shown to play a crucial role in the infectious interactions with both the canine TfR and feline TfR (fTfR) (22, 44, 56). Some mutations to VP2 residue 300 have been structurally characterized (Gly300 and Asp300) (Fig. 1C) and have been shown to minimally affect the capsid surface structure, with the greatest change being the formation of a salt bridge between Asp300 and Arg81. We predict that other mutations to residues 299 and 300 will similarly affect only the local capsid surface

FIG 2 Binding and uptake of 4- μ g/ml CPV or VLPs into NLFK cells as determined by immunofluorescence assay. Shown are a merge of TfR expression (detecting the cytoplasmic tail) (green), CPV capsids (red), and DAPI/nuclei (blue) for wild-type or mutant CPV or VLPs (top) or CPV capsids only (bottom).

structure, and we have constructed a homology model of what a Gly299Lys/Ala300Lys mutation may look like (Fig. 1C).

To compare the properties of infectious and noninfectious CPV mutants, Gly299Glu and Ala300Asp empty capsids were compared as controls, as both are viable in NLFK cells (Fig. 1D) but show loss of binding to the canine TfR (Gly299Glu) or reduced infectivity in canine cells (Gly299Glu and Ala300Asp) (22, 39, 44). Both of these mutants also showed decreased binding by some MAbs recognizing the B antigenic site (Gly299Glu and Ala300Asp), indicating that they create sufficient alteration of the capsid surface to block antibody binding (30, 39, 44, 46) (Fig. 1E).

The Gly299Lys/Ala300Lys double mutant exhibited a contrasting profile, as the capsid assembled (identified by VLP hemagglutination of feline red blood cells), but it was not viable in NLFK feline cells (Fig. 1D) and VLPs showed a complete loss of binding of the B-site-specific MAb 8 (Fig. 1E). By fluorescence microscopy, the Gly299Lys/Ala300Lys mutant VLPs showed significant binding and uptake into feline cells, and although levels appeared lower than those seen for wild-type CPV or VLPs, they were higher than the levels seen for either Gly299Glu or Ala300Asp, which produced viable and naturally transmissible capsids (Fig. 2). When levels of virus binding and uptake into NLFK cells were determined by flow cytometry (Fig. 3), we observed results similar to those for the immunofluorescence assay, with wild-type CPV capsids and VLPs showing the highest levels of cell association after 60 min of incubation at 37°C, followed by the Gly299Lys/Ala300Lys mutant VLPs, while the Gly299Glu and Ala300Asp mutants showed low levels of binding and uptake that were similar to those of the isotype control (Fig. 3).

In vitro binding analysis. In order to determine whether changing residue 299 or 300 affected the affinity of the virus for the TfR, we used biolayer interferometry to measure the rates of binding and unbinding of CPV and VLPs to TfR-coated probe surfaces. Both CPV and VLPs showed a fast association phase and a biphasic dissociation phase, with an initial fast dissociation followed by a lower dissociation rate (Fig. 4). VLPs with Gly299Lys/Ala300Lys substitutions had association curves comparable to those of wild-type CPV and VLPs, but the dissociation curves for Gly299Lys/Ala300Lys VLPs were much steeper (Fig. 4). Capsids with Gly299Glu and Ala300Asp substitutions

FIG 3 Flow cytometry showing binding and uptake assay of 4 μ g/ml CPV or VLPs in NLFK cells. Representative flow cytometry histogram (A) and quantification of mean fluorescence intensity (MFI) (B) from 3 experiments with 2 replicates, analyzed by ANOVA. The error bars display standard errors of the mean; *, $P < 0.05$.

had diminished association rates and increased dissociation rates. Variations in binding rates could not be explained by variations in capsid concentrations, because they were kept constant for all samples.

To provide a more quantitative comparison of dissociation behaviors, we sought kinetic models that could fit the dissociation curves. Characterizing the dissociation curves can also be useful for determining if adhesion-strengthening mechanisms are present, as demonstrated by related work on tracking contact times between individual parvoviruses and TfRs (43). Because the dissociation curves here did not fit the basic 1:1 binding model, we used the adhesion-strengthening model (43, 57). This model assumes that the binding force can change with contact time, perhaps due to binding-triggered conformational changes of the capsid (43). However, the initial adhesion-strengthening model applies only to studies where contact times for each capsid particle can be measured, whereas here, we measured the collective loss of many capsids from the surface over time. We therefore used the ensemble-averaged form of the model (equation 1), as explained previously (57):

FIG 4 Binding of CPV or VLPs to soluble feline TfR using biolayer interferometry. The sensorgram data show the association of CPV or VLPs with feline TfR for 5 min of incubation, followed by dissociation for 5 min. The curves and error bars represent the averaged data from 6 replicates. Fitted dissociation curves using the adhesion-strengthening model are shown as black lines. The error bars display standard errors of the mean.

TABLE 1 Fitting constants for CPV and VLP external capsid mutants derived from fitting biolayer interferometry sensorgrams to equation 1

CPV or VLP	Fit of averaged curve ^a		R ²
	A	B	
WT CPV	2.12 ± 0.21	0.026 ± 0.004	0.996
G299E CPV	4.68 ± 0.86	1.100 ± 1.054	0.952
A300D CPV	4.51 ± 0.46	1.000 ± 0.215	0.962
WT VLP	1.02 ± 0.33	0.003 ± 0.002	0.936
G299K/A300K VLP	3.56 ± 0.50	0.333 ± 0.224	0.985

^aValues ± SD.

$$N_{\text{diss}}(t) = \sum_{t'=0}^{t'=t_{\text{diss}}-\Delta t} B \left[\ln \frac{t-t'}{\Delta t} \right]^{-A} \quad (1)$$

In equation 1, parameter *A* reflects how fast a virus-receptor contact can transition into a stronger contact, with lower *A* values representing shorter transition times and stronger contacts. Parameter *B* reflects how many binding attempts are made by virus particles, which include diffusion-limited transport of the virus to the surface and also rebinding attempts made by weakly bound viruses. The results of the fits are provided in Table 1. We are mainly interested in comparing *A* values among the mutated capsids to determine if certain residues affect the ability of parvovirus to form stronger bonds with the TfR. Wild-type VLPs and CPV have minimum *A* values of ~1 and 2, respectively, which suggests that these capsids quickly form strong contacts with the TfR. Capsids with the Gly299Lys/Ala300Lys double mutation have a moderately high *A* value, suggesting a long delay time for forming strong contacts with the TfR. Capsids with Gly299Glu and Ala300Asp mutations have the highest *A* values, suggesting that these capsids have the most difficulty undergoing changes that stabilize contact with TfR. Overall, these results agree with those derived from the virus binding and uptake studies shown in Fig. 2 and 3.

Mutations on the interior capsid surface that affect infection. Other mutations that affected viral infectivity were found in the interior of the capsid (Fig. 5A to C) and did not affect receptor binding by the VLPs. The mutations examined were clustered in a region that contains the only four Cys residues in the capsid (VP2 residues 270, 273, 490, and 494). Cys490 and Cys494 form an intrachain disulfide bond, while Cys270 and Cys273 are free and point away from each other in crystal structures of the capsid (Fig. 5A to C) (14). The amino acid 270-to-273 region of CPV VP2 is exposed on the internal surface of the capsid, and while it is not known to be in direct contact with the DNA or to differ greatly in position between full and empty capsids, the position of residue 269 is influenced by the loop containing VP2 residues 490 to 494, which directly interacts with viral DNA (Fig. 5C) (11, 19, 34, 52). Cys490 is conserved in other parvoviruses, but the sequences surrounding this region of the capsid are not conserved in other parvoviruses, such as MVM or porcine parvovirus (PPV) (Fig. 5D), where residues that align with 270 are Thr and those that align with 273 are either Val or Leu.

The Cys270Ser and Cys273Ser substitutions in CPV allowed capsid expression and assembly (Fig. 6A) but significantly impaired viral infectivity. The Cys270Ser change resulted in a 100-fold decrease in the viral titer after 3 passages compared to wild-type CPV, while the Cys273Ser mutation completely eliminated infection in NLFK cells (Fig. 6B). The Pro272Lys mutation also eliminated viral infectivity in NLFK cells (Fig. 6B), but the low infectivity appears to be due to the substitution preventing capsid assembly, leading to a degradation of capsid subunits in the cytoplasm after transfection of the infectious plasmid (Fig. 6A) and greatly decreased VLP production after baculovirus expression of the mutant VP2 (data not shown). The Lys271Ala and Lys271Arg substitutions were also located between Cys270 and Cys273, but they showed capsid assembly and viral infectivity similar to those of the wild type (Fig. 6A and B).

The Cys270Ser mutant assembled normal levels of capsids in NLFK cells and VLPs in insect cells but lacked a submolar proteolytic cleavage fragment when capsid proteins

FIG 6 Capsid assembly, viability, and proteolytic cleavage of the mutations changing residues on the internal capsid surface. (A) Immunofluorescence assay of NLFK cells 4 days after transfection with WT or mutant CPV infectious plasmid. The cells were stained with MAb 8 (which recognizes only assembled capsids) or a polyclonal anti-VP1/2 antibody (recognizing both monomeric VPs and capsids). (B) Relative infectivity of mutant CPVs compared to the wild type, measured by TCID₅₀, over three passages in NLFK feline cells. The data were collected from 3 independent experiments and analyzed by ANOVA. (C) Western blot of wild-type CPV empty capsids or wild-type or mutant VLPs. Peptides were stained with polyclonal anti-VP1/2 antibody (top), monoclonal antibody recognizing a peptide containing residues 362 to 373 (middle, boxed in red), or a polyclonal antibody recognizing a peptide containing residues 258 to 270 (bottom, boxed in green). (D) Diagram showing results of peptide-mapping experiments, with the identified proteolytic cleavage site located between residues Asp269 and Cys270. (E) Diagram showing the proteolytic cleavage site in the context of the surrounding protein structure. The error bars display standard errors of the mean.

infection of cells. In both cases, the effects appear to be due to changes in the dynamic properties of the capsid that are required for successful cell infection. The Gly299Lys/Ala300Lys mutations on the outer surface of the capsid resulted in a noninfectious particle, despite the mutant being able to bind the TfR and to enter cells at apparently normal rates. The changes, therefore, appear to interfere with a capsid structural change that is required for infection after the initial receptor binding. The internal capsid replacement of Cys270 with Ser also inhibited cell infection, but it was associated with loss of a proteolytic cleavage product and increased capsid stability, again indicating a structure that functions after receptor binding and cell entry. The Pro272Lys change likely decreased infectivity because it interfered with efficient capsid assembly, while the specific role of the Cys273Ser mutation is not clear, although it appears to prevent egress of assembled capsids from the nucleus.

Key structural interactions between the TfR and the capsid are required for infection. The external surface changes show that there are essential interactions between the TfR and the CPV capsid, in addition to simple binding, that are required for cell infection. Most of our studies were of the Gly299Lys/Ala300Lys double mutant, but the Ala300Lys mutation alone also eliminated infectivity. Since the nonviable

FIG 7 Stability of wild-type or mutant VLPs determined using tryptophan fluorescence spectroscopy or differential scanning fluorimetry. (A) Tryptophan fluorescence spectroscopy of wild-type (WT), C270S, and C273S VLPs heated from 25°C to 85°C at 0.5°C/min at pH 7.4. (B) Melting temperatures of different capsids determined from the results of tryptophan fluorescence spectroscopy experiments shown in panel A. (C) DSF of WT, C270S, and C273S VLPs heated from 25°C to 85°C at 0.5°C/min at pH 7.4. (D) Melting temperatures of different capsids determined from DSF experiments at pH 7.4 (as in panel C) and pH 5.5 and with 10 mM EGTA. The data were taken from 3 independent experiments and analyzed by ANOVA. The error bars display standard errors of the mean; *, $P < 0.05$; ****, $P < 0.001$. a.u., arbitrary units.

Gly299Lys/Ala300Lys mutant bound to TfR and entered feline cells at higher levels than either of the viable Gly299Glu or Ala300Asp mutants, and residues 299 and 300 are located directly on the receptor binding site, there must be an additional interaction between CPV and the TfR that is required for infection. We suspect that the 300 residue governs a conformational change that is triggered by specific receptor binding events that precede viral genome delivery. In support of this hypothesis, previous work has suggested that parvoviruses require structural cues from the receptor to infect cells, as replacement of the TfR ectodomain with a single-chain variable fragment (scFv) allows CPV to enter cells but prevents infection (60). A substitution in the apical domain of the feline TfR (Leu221Lys) also results in reduced infection despite allowing efficient cell binding and uptake of CPV capsids (61). The TfR-capsid interaction is likely highly asymmetric, with only a small number of TfRs binding per capsid; indeed, capsids were shown to engage only one or two TfRs during initial cell binding and entry (33), and biochemical and imaging analyses also showed fewer than 5 TfRs bound per capsid *in vitro* (27). In summary, one or a few TfRs bind the capsid to allow uptake and infection, but the structural interaction with the capsid is highly specific and involves contacts between the TfR apical domain, which likely includes residue 221 of the feline TfR, and the 3-fold spike surface, including residue 300.

These results add to our previous understanding of how the specific structures of the CPV capsid control infection and host cell tropism, where variation in the capsid

FIG 8 Intracellular localizations of the Cys273Ser mutant compared to wild-type capsids. (A) IFA showing nuclear localization of capsid subunits detected with a polyclonal anti-VP1/2 antibody (left) and assembled capsids detected with MAb 8 (right) 24 h posttransfection. (B) Percentages of cells that had assembled capsids only in the nucleus out of all transfected cells, from a total of 6 transfections in 3 independent experiments, analyzed by ANOVA. The error bars display standard errors of the mean; ****, $P < 0.001$.

controls the host range (23, 36, 37, 40, 44). Residue 300, which we show here controls the virus after cell entry, has also been shown in other cases to control receptor binding, tropism, and host range. One of those processes was when CPV gained the host range for dogs and spread worldwide (45). The structural challenge encountered appears to have been particularly difficult, as the canine TfR has an additional glycan within the virus-binding region of its apical domain, and changes in three different areas of the capsid have been shown to influence binding to that TfR and infection of canine cells (36, 39, 62). The Lys93Asn and Asp323Asn substitutions in the original CPV allowed canine TfR binding and canine cell infection, while the single change of Gly299 to Glu blocked those activities (39). Variation of VP2 residue 300 and nearby residues is associated with the specific adaptation to other hosts or their cultured cells, most likely due to small accommodations to allow efficient binding and infection using the variant TfRs of the different host animals (37). Altogether, it is clear that infection requires specific interactions between the capsid and the TfR that involve not just binding, but also the ability to induce small asymmetric allosteric changes in the capsid that are essential for successful infection.

Internal capsid structures controlling infection. A second structural region that appeared to undergo key changes that were required for infection was identified in the interior of the capsid. We confirmed that about 10% of the VP2 (and presumably VP1) molecules in assembled capsids were cleaved between Asp269 and Cys270. The substitution of Ser for Cys at residue 270 resulted in loss of infectivity, and there was little or no proteolysis of that site and a small but significant increase in the capsid melting temperature, indicating increased capsid stability. This suggests that proteolytic cleavage of a small number of capsid subunits may give the capsid a specific structure or conformational flexibility required for infection. Many different viruses use proteolytic cleavage of one or more of the viral capsid proteins for maturation into an infectious form, including poliovirus (63), rhinovirus (64), flock house virus (65), and adenovirus (66). It is unclear what causes the submolar proteolytic cleavage of the CPV VP2 at Cys270, but it must either be autocatalytic cleavage or due to a cellular protease.

The Cys270 cleavage occurred in capsids from virus-infected cells, as well as in VLPs at similar levels, so any cellular protease must be present in feline (NLFK) cells and insect (High5) cells. The identity of a catalytic structure in the capsid that would cleave at Asp269/Cys270 is not obvious. The His137Ala substitution had no effect on formation of the submolar cleavage product, the presence of a Cys/Lys dyad protease was not supported by the finding that the Lys271Ala mutation had no effect on the formation of the submolar cleavage product, and there are no other lysine residues nearby. Although there are reports of cysteine/serine/threonine residues using the N-terminal

amine group as a base during proteolysis (58, 59), it is much more common to find the conserved catalytic triad (serine/cysteine, histidine, and glutamate/aspartate) (58). The cleavage in CPV has parallels in the capsids of various AAV serotypes, which have a variety of low-pH-activated protease cleavages that may also activate the capsid for infection, where the sources of the apparent autoproteolytic activities are still unclear (48).

Of the other mutations examined, the Pro272Lys mutation prevented assembly of capsids both in the virus and in VLPs, likely due to disruption of the local structure of the capsid. The Cys273Ser mutation resulted in a nonviable virus that could not be passaged successfully, and those capsids appeared to be primarily nuclear localized, in contrast to the wild-type CPV or other mutations, where capsids were distributed in both the cytoplasm and the nucleus. Similar results were seen in MVM when serine residues on the N terminus of VP2 were mutated to prevent phosphorylation and the capsids could no longer be recognized by the nuclear export machinery (67). Residue 273 is in the interior of the capsid and distant from the 5-fold pores, so the Cys273Ser change seems unlikely to act through such a mechanism, and additional experiments will be needed to determine its role in the CPV infection and replication process.

Summary. These studies confirm that the parvovirus capsid is a finely tuned molecular machine, and its highly specialized functioning can be disrupted by as little as a single amino acid change in critical positions. Here, we show that small and likely submolar asymmetric changes within the capsid control key steps of infection, with some being triggered by receptor binding while others appear to involve proteolytic cleavages. Because all the steps in infection, replication, and release are mediated by a single protein structure (shared by VP1 and VP2), understanding those functions will require analysis of fine-scale and often submolar processes and defining their different roles in the replication cycle and in controlling cell-cell and animal-animal transmission.

MATERIALS AND METHODS

Mutant viruses and VLPs. Mutations were introduced into the full-length infectious plasmid clone of the CPV-2 genome (56) using the Phusion mutagenesis method (New England BioLabs) according to the manufacturer's instructions and then sequenced to confirm the mutations. The plasmids were transfected into NLFK cells using Lipofectamine (Invitrogen) according to the manufacturer's protocol, after which viral infectivity was determined or viruses were recovered. The mutations introduced included the VP2 substitutions H137A, C270S, K271A, K271R, C273S, G299E, G299K, A300D, A300K, and G299K/A300K.

The CPV VP2 sequence was used for production of VLPs by expression in High5 insect cells with the FastBac plasmid system (Invitrogen), and mutations were also introduced using the Phusion mutagenesis method. Plasmids were sequenced to verify the presence of the mutations and then transformed into *Escherichia coli* DH10Bac to generate bacmids. The bacmids were transfected into Sf9 cells with Cellfectin II (Invitrogen) according to the manufacturer's protocol to generate baculovirus. Mutations included the VP2 substitutions H137A, C270S, K271A, K271R, P272K, C273S, and G299K/A300K.

Cells, viruses, and VLP preparations. NLFK cells were grown in 1:1 McCoy-Lebovitz L15 medium (Corning) with 5% fetal calf serum (FCS). Sf9 and High5 insect cells were grown in Grace's insect medium (Invitrogen) with 10% FCS and Express Five serum-free medium (Invitrogen), respectively.

The CPV-2 strain of virus was used to prepare full (DNA-containing) and empty particles by growth of the virus in NLFK cells, and capsids were purified as described previously (11). VLPs were prepared by expressing CPV-2 VP2 in High5 cells using baculoviruses as described above, followed by purification. Briefly, after 4 days of incubation with baculovirus, High5 cells were resuspended in 25 ml of lysis buffer (10 mM NaCl, 10 mM Tris-HCl [pH 7.5], 15 mM MgCl₂, 5 mM Triton X-100, 0.4× M221 protease inhibitor [Amresco]) and then freeze-thawed 4 times. The lysates were treated with a Branson sonifier 250 (output, 3; duty cycle, 50% for 30 s), and then the debris was pelleted at 12,400 × *g* for 30 min and the supernatant was collected. Capsids were then purified by (i) polyethylene glycol (PEG) precipitation and sucrose gradient centrifugation, as described previously (11), followed by size exclusion chromatography in a Bio-Rad A5M column, or (ii) by centrifuging on a CsCl step gradient (3.5 ml of 1.25 g/cm³, 3 ml of 1.35 g/cm³, and 0.5 ml of 1.5 g/cm³ CsCl), followed by banding in an isopycnic gradient formed from 1.35 g/cm³ CsCl, as described by Jager et al. (68).

Production of soluble feline Tfr ectodomain. BHK cells expressing the soluble 6-His-tagged feline Tfr ectodomain were grown in roller bottles with Dulbecco's modified Eagle medium (DMEM)/F-12 (Lonza) with 10% FCS. DMEM/F-12 medium was then replaced with production medium (Pro293A-CDM [Lonza] with added L-glutamine [2 mM] and butyric acid [1 mM]), and medium was collected at 1-day intervals (37).

Molecular images and homology modeling. Images of the major capsid protein were generated in UCSF Chimera (version 1.10.1) (Resource for Biocomputing, Visualization, and Informatics [RBVI]). The

homology model of the Gly299Lys/Ala300Lys mutant was generated via the mutagenesis function in PyMOL (version 1.7.4) (Schrödinger, LLC).

HAI assays. Hemagglutination inhibition (HAI) assays were used to determine the relative activities of MABs for VLPs. Four HA units of VLP were added to MABs 14, E, and 8 (25, 26), which were diluted 2-fold in Bis-Tris-buffered saline (BTBS) (25 μ l total volume) in a 96-well V-bottom plate and incubated for 1 h at room temperature; then, 50 μ l of 0.5% feline red blood cells was added to each well, and the plates were incubated at 4°C for 2 h.

TCID₅₀ assays. Mutant virus infectious plasmid (2.5 μ g) was transfected into NLFK cells seeded at 2×10^4 cells/cm² in 9-cm² plates, as described above. Four days after transfection, the supernatant was collected and serially passaged twice at 1:4, with supernatant collected 4 days after each passage. For virus titrations, NLFK cells seeded at 2×10^4 cells/cm² in 96-well plates were incubated with 50 μ l of 10-fold-diluted viruses for 1 h at 37°C, and then, 200 μ l of tissue culture medium was added, and the cells were incubated at 37°C. After 2 days, the cells were fixed, incubated for 1 h with rabbit polyclonal anti-FPV antibody, washed, incubated with goat anti-rabbit horseradish peroxidase (HRP) antibody, and stained with aminoethyl-carbazole (AEC) substrate (20 mg AEC in 0.035 M sodium acetate, pH 5.0, with 0.015% H₂O₂). Infected wells were determined and used to calculate the 50% tissue culture infective dose (TCID₅₀) by the automatic drop through method using the NIH ID50 server (available at http://www.ncbi.nlm.nih.gov/CBBresearch/Spouge/html_ncbi/html/id50/id50.html) (69). Three separate transfections and serial passages were performed for each virus.

Biolayer interferometry of capsid-TfR binding kinetics. Binding kinetics between the CPV capsids or VLPs and the purified feline TfR ectodomain were determined in a ForteBio BLITz biolayer interferometer, using 240 μ g/ml of capsid (as CPV empty capsids or insect cell-expressed VLPs). The assay procedure was as follows: (i) hydration of nickel nitrilotriacetic acid (NiNTA) tips for 10 min in kinetics buffer (phosphate-buffered saline [PBS] with 0.02% ovalbumin and 0.02% Tween 20), (ii) 30 s baseline wash in kinetics buffer, (iii) 300 s TfR loading, (iv) 60 s baseline wash in kinetics buffer, (v) 300 s capsid association, and (vi) 300 s analyte dissociation in PBS with 0.02% Tween 20. The feline TfR was added as a culture supernatant prepared from BHK cell expression, which contained the expressed dimeric ectodomain with a 6-His tag. The total amount of feline TfR was consistent between runs, with TfR attachment to NiNTA tips reaching 0.8 nm of binding during step (iii). For each capsid or VLP sample, experiments were repeated 6 times to obtain an averaged kinetic curve with standard error of the mean.

Binding and uptake analysis using immunofluorescence assays (IFA). NLFK cells seeded on coverslips at 2×10^4 cells/cm² were incubated in DMEM with 0.1% bovine serum albumin (BSA) for 30 min at 37°C. The cells were incubated with 40 μ l of 4- μ g/ml CPV capsids or VLPs diluted in DMEM with 0.1% BSA for 1 h at 37°C and then fixed in 4% paraformaldehyde (PFA) for 10 min at room temperature. The cells were washed with PBS and incubated with polyclonal rabbit anti-FPV and mouse anti-TfR cytoplasmic tail (Affymetrix) antibodies for 1 h at room temperature. The cells were then washed with PBS, incubated with goat anti-rabbit Alexa 594 and goat anti-mouse Alexa 488 for 1 h at room temperature, and mounted on slides using Prolong Gold Antifade with DAPI (4',6-diamidino-2-phenylindole) (Life Technologies). The cells were imaged using a Nikon TE300 fluorescence microscope.

Flow cytometry. NLFK cells seeded at 2×10^4 cells/cm² in 9-cm² plates were incubated in DMEM with 0.1% BSA for 30 min at 37°C. The cells were then incubated with 500 μ l of 4- μ g/ml CPV or VLPs diluted in DMEM with 0.1% BSA for 1 h at 37°C. The cells were trypsinized and centrifuged at $500 \times g$ for 5 min, washed with PBS and fixed in 4% PFA, washed with permeabilization buffer (PBS with 0.1% BSA, 0.1% Triton X-100, and 0.1% Na₂S₂O₃), and incubated with 1.5 μ g/ml MAB 14 for 30 min at room temperature. The cells were washed with permeabilization buffer, incubated with goat anti-mouse Alexa 647 for 30 min at room temperature, and then washed with permeabilization buffer and resuspended in PBS. Measurements were taken using an A94291 Gallios flow cytometer (Beckman Coulter) running Kaluza analysis software (Beckman Coulter), and graphs were prepared using FlowJo V10 (FlowJo, LLC).

Immunofluorescence assays to monitor capsid assembly. NLFK cells seeded at 2×10^4 cells/cm² on coverslips in 9-cm² plates were transfected with 2.5 μ g of infectious plasmid with Lipofectamine (Invitrogen) according to the manufacturer's instructions. Four days later, the cells were fixed in 4% PFA for 10 min at room temperature, washed with PBS, and then incubated with polyclonal rabbit anti-FPV antibody (recognizing VP2 subunits and assembled capsids) or mouse MAB 8 (recognizing only the assembled capsid) for 1 h at room temperature. The cells were washed with PBS, incubated with secondary antibody (goat anti-rabbit Alexa 488 or goat anti-mouse Alexa 488 as appropriate) for 1 h at room temperature and then mounted on slides using Prolong Gold Antifade with DAPI (Life Technologies). The cells were imaged with a Nikon TE300 fluorescence microscope.

Quantitative Western blotting. Samples of 40 μ g CPV or VLPs were run on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. The membranes were blocked in Odyssey blocking buffer (LI-COR) for 1 h at room temperature and then incubated with primary antibody: polyclonal rabbit anti-FPV antibody, polyclonal mouse antibody prepared against VP2 peptide residues 258 to 270 (15), or monoclonal mouse antibody P1F6 against the CPV-2 C terminus (15). The membranes were incubated either for 1 h at room temperature or overnight at 4°C, washed in PBS, and incubated with secondary antibody (goat anti-rabbit IRDye 800CW or goat anti-mouse IRDye 680RD [LI-COR]) for 1 h at room temperature. The membranes were washed again in PBS and imaged on an Odyssey infrared imager (LI-COR).

Peptide mapping. Wild-type CPV capsid proteins were run on an SDS-10% PAGE gel and stained with Coomassie blue. The gels were stored in distilled water and sent to the Cornell Biotechnology Resource Center Proteomics Core, where the N-terminal ~30-kDa proteolytic cleavage fragment was

excised from the gel, digested in separate preparations with trypsin and Glu-C enzymes, and peptide mapped using an Orbitrap fusion or Orbitrap elite mass spectrometer.

Quantification of capsid nuclear or cytoplasmic localization. NLFK cells seeded at a density of 4×10^4 cells/cm² in 9-cm² plates were transfected with 2.5 μ g of wild-type or Cys273Ser infectious plasmid with Lipofectamine (Invitrogen) according to the manufacturer's instructions. The cells were fixed with 4% paraformaldehyde 24 h later and then stained with MAb 8 (recognizing assembled capsids) and goat anti-mouse Alexa 488 secondary antibody in permeabilization buffer (see above) and mounted with ProLong Anti-Fade Gold containing DAPI (Life Technologies). The cells imaged using a Nikon TE300 fluorescence microscope were scored according to whether the assembled capsids localized only to the nucleus or were also visible in the cytoplasm. The data were from 6 replicates.

Tryptophan fluorescence spectroscopy. CPV empty capsids or VLPs were diluted to 0.5 μ g/ml in 500 μ l PBS (pH 7.4). Tryptophan fluorescence was measured using 1-cm-path-length cuvettes in a Cary Eclipse spectrophotometer (Agilent Technologies) with 5-nm excitation and emission bandwidths. Samples were excited at 295 nm and read at 331 nm, as described previously (35), and measurements were taken every 0.5°C as the samples were heated from 25°C to 85°C at 0.5°C/min. The melting temperature (T_m) for each sample was determined by finding the minimum of the first derivative of the resulting curve using GraphPad Prism 6 (GraphPad Software, Inc.).

Differential scanning fluorimetry (DSF). CPV empty capsids or VLPs were diluted to 0.1 to 0.2 mg/ml with PBS (pH 7.4), 100 mM citric acid (pH 5.5), or 10 mM EGTA (pH 8.0) to a total volume of 22.5 μ l and incubated for 1 h at room temperature; 2.5 μ l of 1% SYPRO orange dye (Life Technologies) was added to samples in a 96-well quantitative-PCR (qPCR) plate, and the samples were then loaded into a StepOne Plus qPCR machine (Applied Biosystems). Measurements were taken every 0.2°C as the samples were heated from 25°C to 85°C (1°C/min). The T_m for each sample was determined by finding the maximum of the first derivative of the resulting curve using GraphPad Prism 6 (GraphPad Software, Inc.).

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