Parvovirus capsid structures required for infection: mutations controlling receptor recognition and protease cleavages

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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 ssDNA genome to the nucleus where viral replication occurs. Here, we have examined capsid substitutions that eliminate canine parvovirus (CPV) infectivity, and have identified 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 the transferrin receptor type 1 (TfR), particularly during virus disassociation 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 which 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. (250/250 words)
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 those contribute to viral infectivity. (118/150 words)
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 those 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 fivefold axes, raised surface regions (spikes) around the threefold axes, and surface valley regions located about the twofold axes and fivefold axes of symmetry (the dimple.
The capsid binds the 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 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 threefold 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 threefold 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 N-glycolyl neuraminic acid (Neu5Gc) binds to a site within the twofold 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 (to ~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 may be occurring that result from TfR binding, perhaps in combination with low pH or proteolytic cleavage of a proportion of capsid proteins after assembly, and which are necessary for cell infection by the virus.
While viruses related to feline panleukopenia virus (FPV) have been infecting a wide variety of carnivore hosts for many years (commonly including domestic cats), 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 residues 299 (Gly to Glu or Asp), or 300 (Ala or Gly to Asp) (37-39). The mutation Ala 300Asp 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 that 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 the 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 principle antibody binding sites (the B-site) on the capsid, and may also influence the binding of monoclonal antibodies (MAb) that recognize that 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 VLPs appear structurally highly similar to infectious DNA-containing capsids when different crystal structures are compared, some differences in the structures and dynamics have been revealed (49-51). Residues on the interior of the capsid recognize and interact with the 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, or 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 that are reported for adeno-associated virus (AAV) capsids, and which triggers an autocatalytic cleavage event when those viruses encounter low pHs (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 which make the capsid non-infectious. 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.

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. Plasmids were transfected into NLFK cells using Lipofectamine (Invitrogen) according to the manufacturer's protocol, after which viral infectivity was determined or viruses recovered. Mutations introduced included the VP2 substitutions of H137A, C270S, K271A, K271R, C273S, G299E, G299K, A300D, A300K, and G299K/A300K.

The CPV VP2 sequence was used for production of virus like particles (VLPs) by expression in High Five 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, then transformed into DH10Bac E. coli to generate bacmids. Bacmids were transfected into Sf9 cells with Cellfectin II (Invitrogen) according to the manufacturer's protocol to generate baculovirus. Mutations included the VP2 substitutions of H137A, C270S, K271A, K271R, P272K, C273S, and G299K/A300K.

Cells, viruses and VLP preparations. Norden Laboratory Feline Kidney (NLFK) cells were grown in 1:1 McCoy's/Lebovitz L15 media (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 Media (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 re-suspended in 25 ml of lysis buffer (10mM NaCl; 10mM Tris-HCl (pH 7.5); 15mM
MgCl₂; 5mM Triton X-100; 0.4 × M221 Protease Inhibitor (Amresco)), then freeze-thawed 4 times. Lysates were treated with a Branson sonifier 250 (output 3, duty cycle 50% for 30s) then debris was pelleted at 12,400×g for 30 mins, and the supernatant collected. Capsids were then either purified by 1) polyethylene glycol (PEG) precipitation and sucrose gradient centrifugation as described previously (11), followed by size exclusion chromatography in a BioRad A5M column, or 2) 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 in Jager et. al. (57).

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)/F12 (Lonza) with 10% FCS. DMEM/F12 media was then replaced with production media (Pro293A-CDM (Lonza) with added L-glutamine (2mM) and butyric acid (1mM)), and media was collected at one day intervals (37).

Molecular images and homology modeling. Images of the major capsid protein were generated in UCSF Chimera (version 1.10.1) (RBVI). The homology model of the Gly299Lys/Ala300Lys mutant was generated via the mutagenesis function in PyMOL (version 1.7.4) (Schrödinger, LLC).

Hemagglutination Inhibition (HAI) assays. HAI assays were used to determine the relative activity of monoclonal antibodies (MAbs) for VLPs. Four HA units of VLP were added to MAbs 14, E and 8 (25, 26) that were diluted two-fold in Bis-Tris Buffered Saline (BTBS) (25 μl total volume) in a 96-well V-bottom plate and incubated for 1 h at room temperature, then 50 μl of 0.5% feline red blood cells were then added to each well and the plates were incubated at 4°C for 2 h.
**TCID\textsubscript{50} assays.** 2.5μg of mutant virus infectious plasmid was transfected into NLFK cells seeded at $2 \times 10^4$ cells/cm\textsuperscript{2} in 9cm\textsuperscript{2} plates, as described above. 4 days after transfection, supernatant was collected and serially passaged twice at 1:4, with supernatant collected at 4 days after each passage. For virus titrations, NLFK cells seeded at $2 \times 10^4$ cells/cm\textsuperscript{2} in 96-well plates were incubated with 50 μl of 10-fold diluted viruses for 1 h at 37°C, then 200 μl of tissue culture media was added and cells were incubated at 37°C. After 2 days, cells were fixed, incubated for 1h with rabbit polyclonal anti-FPV antibody, washed, incubated with goat anti-rabbit horse radish peroxidase (HRP) antibody, and stained with amino ethyl carbazol (AEC) substrate (20mg AEC in 0.035M sodium acetate pH 5.0, with 0.015% H\textsubscript{2}O\textsubscript{2}). Infected wells were determined and used to calculate TCID\textsubscript{50} 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 (58). Three separate transfections and serial passages were performed for each virus.

**Bio-layer 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 bio-layer interferometer, using 240 μg/ml of capsid (as CPV empty capsids or insect cell expressed VLPs). The assay procedure is as follows: 1) hydration of Nickel Nitrilotriacetic Acid (NiNTA) tips for 10 min in kinetics buffer (PBS with 0.02% ovalbumin and 0.02% Tween-20), 2) 30s baseline wash in kinetics buffer, 3) 300s TfR loading, 4) 60s baseline wash in kinetics buffer, 5) 300s capsid association, and 6) 300s analyte disassociation 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, and that was loaded onto tips to give 0.8nm of binding. 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 cover slips at $2 \times 10^4$ cells/cm$^2$ were incubated in DMEM with 0.1% bovine serum albumin (BSA) for 30 min at 37°C. Cells were incubated with 40 μl of 4 μg/ml CPV capsids or VLPs diluted in DMEM with 0.1% BSA for 1 h at 37°C, then fixed in 4% paraformaldehyde (PFA) for 10 min at room temperature. 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. Cells were then washed with PBS, incubated with goat anti-rabbit Alexa594 and goat anti-mouse Alexa 488 for 1 h at room temperature and mounted on slides using Prolong Gold Antifade with 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies). Cells were imaged using a Nikon TE300 fluorescence microscope.

Flow cytometry. NLFK cells seeded at $2 \times 10^4$ cells/cm$^2$ in 9 cm$^2$ plates were incubated in DMEM with 0.1% BSA for 30 min at 37°C. Cells were then incubated with 500μl of 4μg/ml of CPV or VLP diluted in DMEM with 0.1% BSA for 1 h at 37°C. Cells were trypsinized and centrifuged at 500 × 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% NaN$_3$) and incubated with 1.5 μg/ml MAb 14 for 30 min at room temperature. Cells were washed with permeabilization buffer, incubated with goat anti-mouse Alexa647 for 30 min at room temperature, 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 $2 \times 10^4$ cells/cm$^2$ on cover slips in 9 cm$^2$ plates were transfected with 2.5μg 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, 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. Cells were
washed with PBS, incubated with secondary antibody (goat anti-rabbit Alexa488 or goat anti-
mouse Alexa 488 as appropriate) for 1 h at room temperature, then mounted on slides using
Prolong Gold Antifade with DAPI (Life Technologies). Cells were imaged with a Nikon TE300
fluorescence microscope.

**Quantitative western blotting.** Samples of 40 μg CPV or VLP were run on 10% SDS-
PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked in Odyssey
blocking buffer (LI-COR) for 1 h at room temperature, then incubated with primary antibody:
polyclonal rabbit anti-FPV antibody, polyclonal mouse antibody prepared against VP2 peptide
258-270 (15), or monoclonal mouse antibody P1F6 against the CPV-2 C-terminus (15).
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. 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 a 10% SDS-PAGE gel
and stained with Coomassie blue. Gels were stored in distilled water and sent to the Cornell
Biotechnology Resource Center Proteomics Core, where the N-terminal ~30kDa 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/cm^2 in 9 cm^2 plates were transfected with 2.5 μg of wild-type or Cys273Ser infectious plasmid with Lipofectamine (Invitrogen) according to the manufacturer's instructions. Cells were fixed with 4% paraformaldehyde 24 h later, then stained with MAb8 (recognizing assembled capsids) and goat anti-mouse Alexa488 secondary antibody in permeabilization buffer (see above), and mounted with ProLong Anti-Fade Gold containing DAPI (Life Technologies). Cells imaged using a Nikon TE300 fluorescence microscope were scored according to whether assembled capsids localized only to the nucleus or were also visible in the cytoplasm. Data were from 6 replicates.

Tryptophan fluorescence spectroscopy. CPV empty capsids or VLPs were diluted to 0.5 μg/ml in 500 μl 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 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 Fluorometry (DSF). CPV empty capsids or VLPs were diluted to 0.1-0.2 mg/ml with PBS (pH 7.4), 100 mM citric acid (pH 5.5), or 10mM EGTA (pH 8.0) to a total volume of 22.5 μl and incubated for one h at room temperature. 2.5 μl of 1% SYPRO Orange dye (Life Technologies) was added to samples in a 96-well qPCR plate, and samples were then loaded into a StepOne Plus qPCR machine (Applied Biosystems). Measurements were taken every 0.2°C as samples were heated from 25°C to 85°C (1°C/min). 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.).

RESULTS

To better understand the capsid controls of cell infection, we examined a panel of point mutations in the CPV capsid, of which VP2 mutants Gly299Lys/Ala300Lys, Pro272Lys, and Cys273Ser 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 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 on the interior of the capsid (VP2 270, 272, and 273).

To allow us to produce sufficient particles to study the properties of the capsids containing substitutions that produced non-viable 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 (Figs. 1A and 1B) that previously has been shown to play a crucial role in the infectious interactions with both the canine and feline TfRs (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 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 non-infectious 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 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 similar results to the immunofluorescence assay, with wild-type CPV capsids and VLPs showing the highest levels of cell association after 60 mins 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 the isotype control (Fig. 3).

**In vitro binding analysis.** In order to determine whether changing residues 299 or 300...
affected affinity of virus for the feline transferrin receptor (fTfR), we used bio-layer interferometry to measure the binding and unbinding rates of CPV and VLPs to fTfR-coated probe surfaces. Both CPV and VLPs showed a fast association phase and a biphasic disassociation phase, with an initial fast disassociation followed by a slower disassociation rate (Fig. 4). VLPs with Gly299Lys/Ala300Lys substitutions had association curves comparable to wild-type CPV and VLPs, but the dissociation curves for Gly299Lys/Ala300Lys VLPs were much steeper (Fig. 4). Capsids with Gly299Glu and Ala300Asp substitutions had diminished association rates and increased dissociation rates. Variations in binding rates could not be explained by variations in capsid concentrations, because these 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, 59). This model assumes that the binding force can change with contact time, perhaps due binding-triggered conformational changes of the capsid (43). However, the initial adhesion-strengthening model only applies 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 (Eq. 1) as explained elsewhere (59).

\[
N_{\text{diss}}(t) = \sum_{t'=0}^{t=t_{\text{diss}}-\Delta t} B \left[ \ln \frac{t-t'}{\Delta t} \right]^{-A}
\]  
Eq. 1
In Eq. 1, parameter $A$ reflects how fast a virus-receptor contact can transition into a stronger contact, with lower $A$ values representing faster 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. Results of the fits are provided in Table 1. We are mainly interested in comparing $A$ values amongst 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 both have the lowest $A$ 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 high delay time for forming strong contacts with the TfR. Capsids with Gly299Glu and Ala300Asp mutations have the highest $A$ value, suggesting that these capsids have the most difficulty undergoing changes that stabilizes contact with TfR.

Overall, these results agree with those derived from the virus binding and uptake studies shown above.

**Mutations on the interior capsid surface that affect infection.** Other mutations that affected viral infectivity were found on the interior of the capsid (Fig 5A-C), and did not affect receptor binding by the VLPs. Mutations examined were clustered in a region that contains the only four Cys residues in the capsid (VP2 residues 270, 273, 490, 494). Cys490 and Cys494 form an intra-chain disulfide bond, while Cys270 and Cys273 are free and point away from each other in crystal structures of the capsid (Figs. 5A-C) (14). The 270-273 amino acid 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 490-494, 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 the minute virus of mice (MVM) or porcine parvovirus (PPV) (Fig. 5D), where residues that align with 270 are Thr and 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 viral titer after 3 passages compared to wild-type CPV, while the Cys273Ser mutation completely eliminated infection in NLFK cells (Fig. 6B). Pro272Lys mutation also eliminated viral infectivity in NLFK cells (Fig. 6B), but the low infectivity appears to be due to that 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 the wild-type (Figs. 6A and 6B).

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 in the VLPs were examined by Western blotting (Fig. 6C). This cleavage product occurs in ~10% of the wild-type VP2 proteins in either capsids or VLPs, and mass spectrometry peptide mapping after trypsin and Glu-C protein digestion of wild-type capsids showed that the cleavage generating that peptide occurred between Asp269 and Cys270 (Fig. 6D and 6E).

The Ser270 mutation directly altered that cleavage recognition site, but the source of the proteolytic activity was not clear. We asked whether Cys270 might itself be a component of a cysteine protease, and therefore examined the surrounding region for His or Asp/Glu residues.
which could complete a catalytic triad of residues typically observed in cysteine proteases (60, 61). There were no Asp or Glu residues within 5Å of Cys270, but His137 was close enough to Cys270 to interact with it, and in some cases, Cys/Ser-His dyads have formed functional proteases (60). However, changing His137 to Ala did not change the formation of the VP2 peptide compared to wild-type VLPs (Fig. 6C) or result in a loss of infectivity (data not shown).

The Cys270Ser substitution significantly increased the melting temperature of the viral capsid compared to wild-type VLPs, as measured by both tryptophan fluorescence spectroscopy and differential scanning fluorometry (Fig.7). This increase was also present when capsids were incubated at pH 5.5, which has previously been shown to induce a conformational change in the capsid (34), and when capsids were incubated in 10mM EGTA, which removes two calcium ions per subunit (34).

The Cys273Ser substitution allowed capsids to assemble efficiently as VLPs (data not shown), but resulted in complete loss of viability. At 24 h after transfecting NLFK cells with infectious plasmid, unassembled capsid subunits were localized throughout the cell cytoplasm, but assembled capsids were located primarily in the nucleus, unlike wild-type virus, where assembled capsids were found in both the nucleus and cytoplasm (Fig. 8A and 8B).

DISCUSSION.

Here we studied two capsid structures, one surface exposed and one internally located, to reveal new details about how the parvovirus capsid controls the successful 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 non-infectious particle, despite the mutant being able to bind the TfR and to enter cells at apparently normal rates. Those changes therefore appear to interfere with a
capsid structural change that is required for infection after the initial receptor binding. The internal capsid substitution of Cys270 to 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, but 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 Gly299Lys/Ala300Lys mutant bound to TfR and entered feline cells to 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 conformation 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 scFv (single-chain variable fragment) allows CPV to enter cells, but prevents infection (62). 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 (63). The TfR-capsid interaction is likely highly asymmetric, with only a small number of TfRs binding per capsid – indeed, capsids were shown to only engage one or two
TfRs during initial cell binding and entry (33), and biochemical and imaging analysis also show fewer than 5 TfR 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 include residue 221 of the feline TfR, and the three-fold spike surface, including residue 300.

These results add to our previous understanding of how the specific structures of the CPV capsid controls infection and host cell tropism, where variation in the capsid controls the host range (23, 36, 37, 40, 44). Residue 300, which we showed 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 the gaining of the canine host range by CPV in the 1970s (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, 64). 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 are 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 on the interior of
the capsid. We confirmed that about 10% 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 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 (65), rhinovirus (66), flockhouse virus (67), and adenovirus (68). It is unclear what introduces the submolar proteolytic cleavage of the CPV VP2 at Cys270, but this must be either an autocatalytic cleavage or due to a cellular protease. The Cys270 cleavage occurred in capsids from virus-infected cells as well as in VLPs to similar levels, so any cellular protease must be present in feline (NLFK) cells and insect (High Five) 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, and 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 are nearby. Although there are reports of cysteine/serine/threonine residues using the N-terminal amine group as a base during proteolysis (60, 61), it is much more common to find the conserved catalytic triad (serine/cysteine, histidine and glutamate/aspartate) (60). 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 auto-proteolytic 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 non-viable 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 nucleus. Similar results were seen in the minute virus of mice (MVM) when serine residues on the N-terminus of VP2 were mutated to prevent phosphorylation, and the capsids could no longer be recognized by nuclear export machinery (69). Residue 273 is on 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 – 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.

**ACKNOWLEDGEMENTS.**

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REFERENCES


### Table 1

Fitting constants for CPV and VLP external capsid mutants, derived from fitting bio-layer interferometry sensorgrams to Eq.1.

<table>
<thead>
<tr>
<th></th>
<th>A ± SD</th>
<th>B ± SD</th>
<th>$R^2$</th>
</tr>
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<tbody>
<tr>
<td><strong>WT CPV</strong></td>
<td>2.12 ± 0.21</td>
<td>0.026 ± 0.004</td>
<td>0.996</td>
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<tr>
<td><strong>G299E CPV</strong></td>
<td>4.68 ± 0.86</td>
<td>1.100 ± 1.054</td>
<td>0.952</td>
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<tr>
<td><strong>A300D CPV</strong></td>
<td>4.51 ± 0.46</td>
<td>1.000 ± 0.215</td>
<td>0.962</td>
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<tr>
<td><strong>WT VLP</strong></td>
<td>1.02 ± 0.33</td>
<td>0.003 ± 0.002</td>
<td>0.936</td>
</tr>
<tr>
<td><strong>G299K/A300K VLP</strong></td>
<td>3.56 ± 0.50</td>
<td>0.333 ± 0.224</td>
<td>0.985</td>
</tr>
</tbody>
</table>

*a Values are ± SD.
FIGURE LEGENDS

Figure 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. (B) Magnification of region of (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) Reactivity of wild-type or mutant capsids with mAbs 14 (A-site), 8 (B-site), and E (B-site), using a hemagglutination inhibition assay (HAI). Data were collected from 3 independent experiments and analyzed via ANOVA. (E) Relative infectivity and growth of mutant CPVs compared to wild-type, measured by TCID50, over three passages in NLFK feline cells. Data was collected from 3 independent experiments and analyzed via ANOVA. Error bars display standard error of the mean, and asterisks denote p<0.05.

Figure 2.
Binding and uptake of 4μg/mL CPV or VLP into NLFK cells determined by immunofluorescence assay. (Top) Images show a merge of TfR expression (detecting the cytoplasmic tail) (green), CPV capsids (red) and DAPI/nuclei (blue) for wild-type or mutant CPV or VLP or (bottom) CPV capsids only.

Figure 3.
Flow cytometry showing binding and uptake assay of 4μg/mL CPV or VLP into NLFK cells. (A)
Representative flow cytometry histogram and (B) quantification of mean fluorescence intensity (MFI) from 3 experiments with 2 replicates, analyzed via ANOVA. Error bars display standard error of the mean, and asterisks denote p<0.05.

Figure 4.
Binding of CPV or VLP to soluble feline TfR using bio-layer interferometry. Sensorgram data shows association of CPV or VLPs to feline TfR for 5 min of incubation, followed by disassociation for 5 min. Curves and error bars represent the averaged data from 6 replicates. Fitted dissociation curves using the adhesion-strengthening model are shown as black lines. Error bars display standard error of the mean.

Figure 5.
Structure and sequence conservation of the capsid region on the interior surface of CPV that was mutated. (A) CPV VP2 empty capsid (PDB ID: 2CAS) (interior surface facing out), with VP2 residues 270-273 and 137 colored orange, residues within 5 Å of 270-273 and 137 colored green, and associated DNA colored red. (B) Magnification of (A), with residues 270-273 and 137 labeled. (C) Diagram of (B), showing the overall structural arrangement, the relative positions of residues 270-273, 137 and residues within 5 Å of those. (D) Amino acid sequence alignment generated using Clustal Omega for CPV (UniProtKB Q11213) and the paroviruses porcine parovirus (PPV) (UniProtKB P18546) and minute virus of mice (MVM) (UniProtKB P07320). Residues labeled in (C) are underlined.

Figure 6.
Capsid assembly, viability, and proteolytic cleavage of the mutations changing residues on the internal capsid surface. (A) Immunofluorescence assay of NLFK cells four days after transfection with WT or mutant CPV infectious plasmid. 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 wild-type, measured by TCID$_{50}$, over three passages in NLFK feline cells. Data was collected from 3 independent experiments and analyzed via 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 362-373 (middle, red outline), or a polyclonal antibody recognizing a peptide containing residues 258-270 (bottom, green outline). (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. Error bars display standard error of the mean.

Figure 7.

Stability of wild-type or mutant VLPs determined using tryptophan fluorescence spectroscopy or differential scanning fluorometry. (A) Tryptophan fluorescence spectroscopy of 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 the different capsids determined from tryptophan fluorescence spectroscopy experiments in (A). (C) Differential scanning fluorometry (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 the different capsids determined from DSF experiments at pH 7.4 (as in C), pH 5.5, and with 10mM EGTA. Data was taken from 3
independent experiments and analyzed via ANOVA. Error bars display standard error if the mean, and asterisks denote p<0.5.

Figure 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 column) and assembled capsids detected with MAb 8 (right column) 24 h post-transfection. (B) Percentage 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 via ANOVA. Error bars display standard error of the mean, and asterisks denote p<0.5.
%Cells with Cytoplasmic Capsid at 24h post transfection

WT CPV C273S CPV

0 20 40 60

Antigen-VP1/2 Polyclonal
Mab 8 (Capsid)

A

B

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