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Assaying for Structural Variation in the Parvovirus Capsid and Its Role in Infection

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The capsid of canine parvovirus (CPV) was assayed for susceptibility to proteases and for structural variation. The natural cleavage of VP2 to VP3 in CPV full (DNA containing) particles recovered from tissue culture occurred within the sequence Arg-Asn-Glu-Arg ↓ Ala-Thr. Trypsin, chymotrypsin, bromelain, and cathepsin B all cleaved >90% of the VP2 to VP3 in full but not in empty capsids and did not digest the capsid further. Digestion with proteinase K, Pronase, papain, or subtilisin cleaved the VP2 to VP3 and also cleaved at additional internal sites, causing particle disintegration and protein degradation. Several partial digestion products produced by proteinase K or subtilisin were ~31–32.5 kDa, indicating cleavage within loop 3 of the capsid protein as well as other sites. Protease treatment of capsids at pH 5.5 or 7.5 did not significantly alter their susceptibility to digestion. The isoelectric point of CPV empty capsids was pH 5.3, and full capsids were 0.3 pH more acidic, but after proteolysis of VP2 to VP3, the pI of the full capsids became the same as that of the empty capsids. Antibodies against various capsid protein sequences showed the amino termini of most VP2 molecules were on the outside of full but not empty particles, that the VP1-unique sequence was internal, and that the capsid could be disintegrated by heat or urea treatment to expose the internal sequences. Capsids added to cells were localized within the cell cytoplasm in vesicles that appeared to be lysosomes. Microinjected capsids remained primarily in the cytoplasm, although a small proportion was observed to be in the nucleus after 2 h. After CPV capsids labeled with [^{35}S]methionine were bound to cells at 0°C and the cells warmed, little cleavage of VP1 or VP2 was observed even after prolonged incubation. Inoculation of cells with virus in the presence of proteinase inhibitors did not significantly reduce the infection.

INTRODUCTION

Viral capsids protect the viral genome during spread between hosts and cells, and they also deliver the infectious nucleic acid and other necessary components into the cell. During the process of cell entry, specific conformational changes of the viral structural proteins must occur to allow the capsid, nucleocapsid, or nucleic acid to be carried from outside the cell into the cytoplasm and, if necessary, to the nucleus (Marsh and Helenius, 1989). During that process, the capsid must also specifically release the viral nucleic acid or nucleoprotein in the correct form and cellular compartment for replication.

Many viruses undergo specific activation or maturation changes after virus assembly that make the virion infection competent. Those changes may prevent the newly synthesized proteins or virus from undergoing conformational changes during vesicular transport, or they may prevent it from reinfecting the same cell. In many cases, this virion maturation process involves digestion of virion proteins by viral or cellular proteases (Cotten and Weber, 1995; Greber *et al.,* 1996; Lu *et al.,* 1996; Sturzenbecker *et al.*, 1987; Virgin *et al.,* 1994). The viral proteins or capsids may then undergo further structural transitions induced by cell receptor binding or by exposure to low pH during endocytosis (Greber, 1993; Lee *et al.,* 1993; Marsh and Helenius, 1989; Prchla *et al.,* 1994; Varga *et al.,* 1991).

The infection process of most enveloped viruses involves viral proteins fusing the viral envelope with the plasma membrane at the cell surface or within endosomes. For many viruses, the envelope proteins undergo dramatic structural changes in the endosome under the influence of low pH, and those changes make them fusion competent (Doms, 1993a, 1993b). Some well-described examples include influenza virus hemagglutinin, Semliki Forest virus envelope protein, and tick-borne encephalitis virus envelope protein (Bullough *et al.,* 1994; Fuller *et al.,* 1995; Lobigs and Garoff, 1990; Stiasny *et al.,* 1996).

For most nonenveloped viruses, the uptake and infection pathways are less clearly understood. However, there must also be membrane permeabilization or penetration by the particle, resulting in many cases from specific changes in the folding of structural proteins or in some cases from exposure of myristoylated components, as seen for reoviruses, adenoviruses, and picor-

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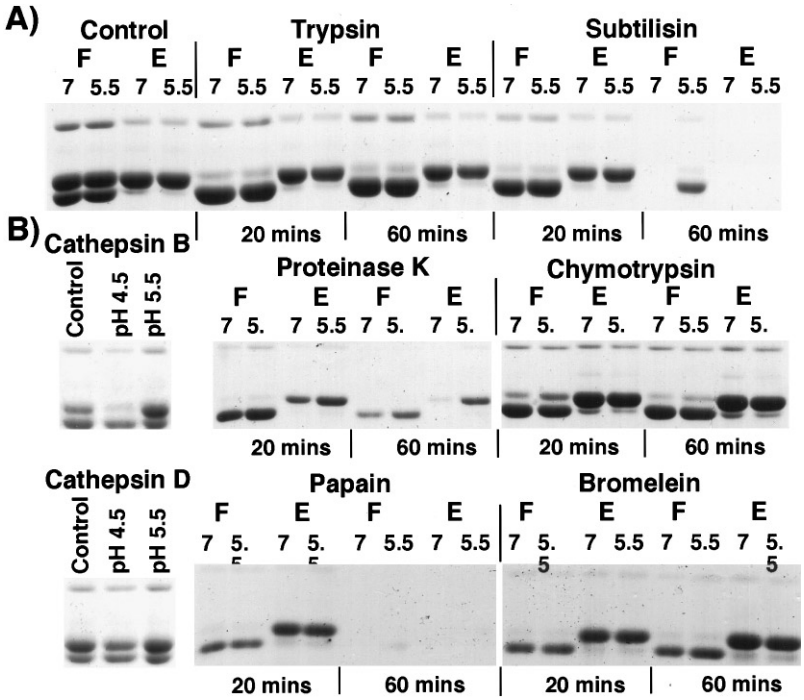

FIG. 1 Proteinase susceptibility of CPV capsids. (A) Sucrose gradient purified CPV full or empty capsids incubated with the proteinases indicated for 20 or 60 min at pH 7 or 5.5 and then eletrophoresed in 7.5% polyacrylamide gels. Gels were stained with Coomassie Blue. (B) Incubation of CPV full capsids with cathepsin B or cathepsin D for 1 h at pH 5.5 or 4.5 and then electrophoresis as in (A). E, Empty capsids; F, full capsids.

naviruses (Dryden *et al.,* 1993; Fricks and Hogle, 1990; Sahli *et al.,* 1993; Sturzenbecker *et al.,* 1987).

Parvoviruses have a stable 25-nm nonenveloped spherical capsid containing a single-stranded DNA (ssDNA) genome of ~5000 nucleotides (Chapman and Rossmann, 1993; Cotmore and Tattersall, 1987; Tsao *et al.,* 1991). Different autonomous parvoviruses infect vertebrate and invertebrate animals, and most have relatively narrow host ranges. The host ranges of CPV, minute virus of mice (MVM), Aleutian mink disease virus (ADV), and porcine parvovirus are controlled, at least in part, by specific sequences within their capsid protein genes (Ball-Goodrich *et al.,* 1991, 1992; Bergeron *et al.,* 1996; Bloom *et al.,* 1993; Chang *et al.,* 1992). Infection of cells by CPV most likely involves an endocytic pathway, and infection can be blocked by cell treatments that raise the endosomal pH or block endosomal trafficking (Basak and Turner, 1992; Vihinen-Ranta *et al.,* 1998). However, it is not known how the stable capsid penetrates the cellular or endosomal membranes, whether the capsid requires specific activation for infection, how it is transported within the cytoplasm, and where in the infectious pathway the DNA is released so it can eventually replicate in the nucleus.

The structures of CPV full (DNA-containing) and empty capsids, FPV empty capsids, MVM full and empty capsids, and empty virus-like particles of human parvovirus B19 have been determined using x-ray crystallography (Agbandje *et al.,* 1994, 1993; Llamas-Saiz *et al.,* 1997; Tsao *et al.,* 1991). The CPV and FPV capsids are T = 1 icosahedrons assembled from 60 copies of a combination of ~10% of the 82-kDa VP1 and 90% of the 67-kDa VP2 (Tsao *et al.,* 1991; Paradiso, 1982). VP1 contains the entire sequence of VP2 and an additional 143 amino-terminal residues. In full capsids, VP2 may be cleaved near the amino terminus by host proteases to form VP3, and in MVM, H1 or CPV capsids that cleavage can be mimicked by trypsin treatment (Clinton and Hayashi, 1975, 1976; Paradiso *et al.,* 1982, 1984; Tattersall, 1977; Tattersall *et al.,* 1976; Tullis *et al.,* 1992). Amino-terminal sequencing showed that trypsin cleaved the VP2 of H1 virus full capsids after VP2 arginine-21, within the sequence ^{16}Asn-Ala-Arg-Val-Glu-Arg ↓ Ser^{22} (Paradiso *et al.,* 1984), although in one study, the natural cleavage site could not be clearly identified by sequencing the VP3 from MVM (Tullis *et al.,* 1992). Mutation or removal of the ^{18}Arg-Val-Glu-Arg^{21} sequence in MVM did not prevent processing of VP2 to VP3-like forms, indicating that proteases can cleave the amino-terminal sequence at various positions in the exposed sequences (Tullis *et al.,* 1992). However, those mutants were somewhat deficient in cell infection and plaquing. The proteins in ADV capsids *in vivo* were found to be largely cleaved into a number of fragments, presumably by host proteases (Aasted *et al.,* 1984).

In the atomic structures of the full and empty CPV and empty FPV capsids, VP1 and VP2 were only resolved from VP2 residue 37, and the structures and locations of the amino termini of VP1, VP2, or VP3 were not seen

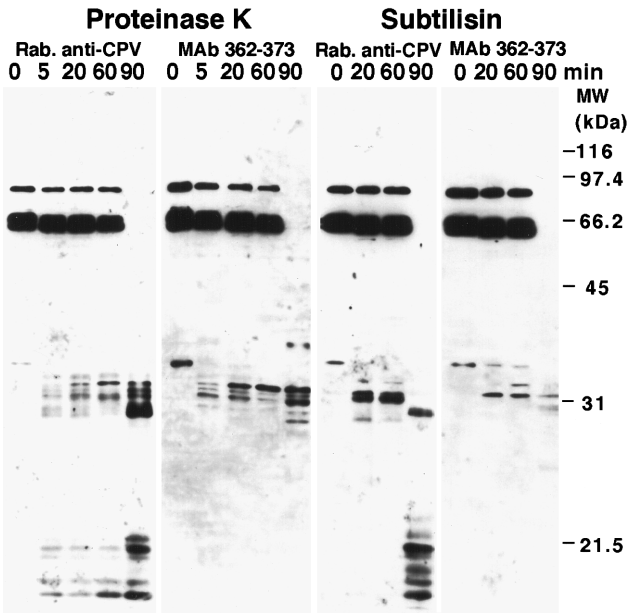

FIG. 2. Limited proteinase digestion of full virus capsids. Purified capsids were incubated with proteinase K or subtilisin at 37°C for 0, 5, 20, 60, or 90 min, and then tubes were placed immediately into boiling water. Proteins were separated by 10% PAGE and transferred to nitrocellulose membranes, and the blots were probed with antibodies against SDS-denatured CPV capsids or with mouse monoclonal antipeptide 362–373. The bound antibodies were detected with anti-species IgG–HRPO and SuperSignal and exposed to x-ray film. Molecular weight standards (MW) are given in kDa.

(Agbandje *et al.,* 1993; Tsao *et al.,* 1991; Wu and Rossmann, 1993). Protease susceptibility indicates that some of the VP2 amino-terminal sequences are outside the full capsid, as does the fact that antibodies against VP2 amino-terminal peptides neutralize the virus (Casal *et al.,* 1995; Langeveld *et al.,* 1994). A pore was seen within the icosahedral five-fold axes of the full and empty capsids, and it contained electron density in full capsids that is presumed to be sequences near the VP2 amino terminus (Tsao *et al.,* 1991). At least one pore per capsid most likely contains DNA in that MVM and AAV full capsids have the 5′ end of the viral DNA outside the particle and attached to the NS1 or Rep protein, respectively (Cotmore and Tattersall, 1989; Prasad and Trempe, 1995). The five-fold pore appears to be of sufficient size to allow only one polypeptide chain to pass to the outside of the capsid, and the conservation of a sequence of glycines and serines between VP2 residues 26 and 40 was suggested to be due to the need for a narrow diameter and unstructured polypeptide sequence within the pore (Tsao *et al.,* 1991; Tullis *et al.,* 1992; Xie and Chapman, 1996). The CPV and FPV capsids are relatively rigid structures, with the main areas of structural flexibility being observed in the cylinder surrounding the five-fold axis (VP2 residues 154–163) and in a partially surface-exposed loop made up of VP2 residues 360–372 (Wu and Rossmann, 1993; Xie and Chapman, 1996).

CPV and FPV are members of the feline subgroup of the autonomous parvoviruses and are >99% identical in DNA sequence, but they differ antigenically and in feline and canine host range (Parrish, 1990, 1994; Truyen *et al.,* 1996, 1995). Sequences controlling canine host range map to at least three regions of the capsid protein gene, and VP2 residues 93 (Lys in FPV, Asn in CPV) and 323 (Asp in FPV, Asn in CPV) and the region around VP2 residue 300 all play roles (Chang *et al.,* 1992; Horiuchi *et al.,* 1994; Parker and Parrish, 1997). In animals, CPV and FPV infect cells of the lymphoid tissues and the intestinal epithelium, and the virions pass through the intestinal lumen and are shed in the feces, remaining infectious in the environment for weeks or months.

The restrictions in viral host ranges occur at a stage in the infection pathway between virus binding to the cell surface and DNA replication (Horiuchi *et al.,* 1992; Parker and Parrish, 1997). However, the stages in infection that might influence the viral host ranges are poorly understood, and little is known about the role of the capsid structures or its variation in this process. Empty and full capsids differ in the presence of symmetrically arranged ssDNA in full particles and some associated differences in the protein structures near the DNA (Chapman and Rossmann, 1995; Tsao *et al.,* 1991; Xie and Chapman, 1996). The amino terminus of VP2 can be removed by proteolysis of full capsids with little effect on virus infectivity, and any function of that peptide exposure or cleavage is not understood (Tsao *et al.,* 1991; Tullis *et al.,* 1992). Two forms of the MVM and H1 full capsids have been defined in which newly synthesized capsids have densities of 1.47 g/cm^3 in CsCl gradients, and after prolonged incubation in culture, those band at ~1.42 g/cm^3. The cause of that density conversion is unknown, but it is not directly related to the VP2-to-VP3 cleavage (Clinton and Hayashi, 1975, 1976; Paradiso, 1981).

To further examine the capsid structure and to further define capsid functions that may play roles in cell infection, we examined the CPV capsid structure using protease treatments and specific antibody probes. Variation in the capsid charge was also determined, as was the exposure of the VP2 amino terminus and other sequences. The processing and transport of the capsids and their proteins within cells were examined by natural infection or microinjection. The capsids proved to be very stable and structurally invariant in most of the assays used, and any functions involved in cell infection must be very subtle or only observed in association with cellular molecules.

RESULTS

Protease treatments

Incubation of purified parvovirus capsids with proteases that represent a variety of cleavage specificities at pH 7.0 or 5.5 resulted in two types of cleavage: con-

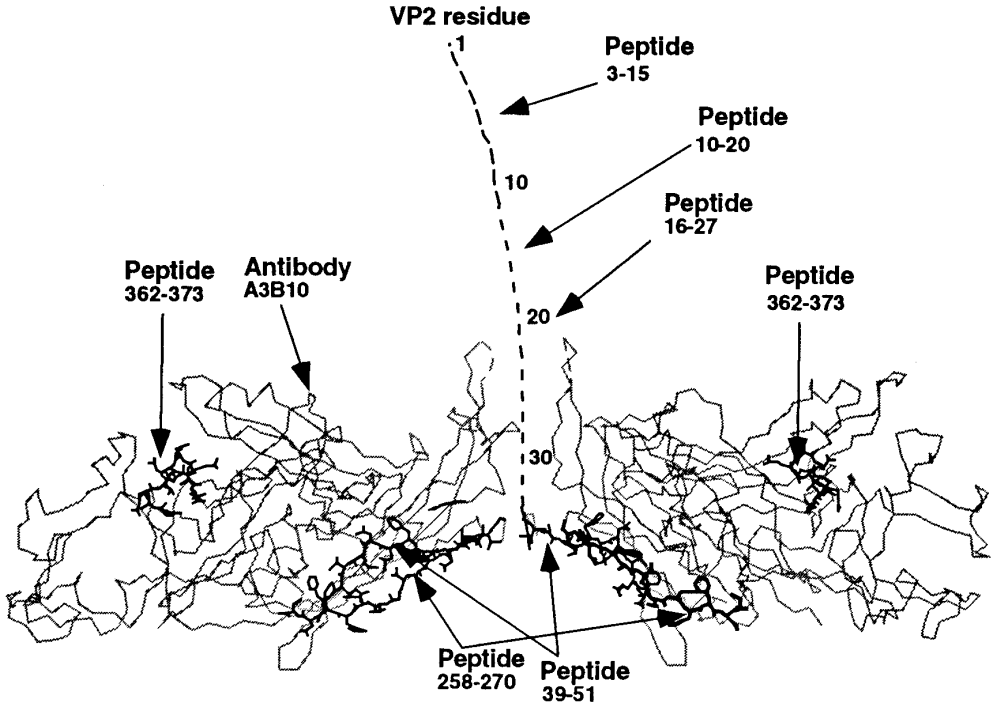

FIG. 3. Diagrammatic representation of part of the structure of the CPV capsid showing two adjacent VP2 molecules surrounding the pore at the five-fold axis of symmetry. A linear sequence approximating the extended length of the amino-terminal 36 amino acids of VP2 is shown; after proteolysis of VP2, the amino terminus of VP3 is at residue 20. Positions of the peptides used to prepare structure specific antibodies or the epitope recognized by MAb A3B10 are indicated.

version of VP2 to VP3 and particle degradation. The full virus preparations used contained approximately equal amounts of VP2 and VP3, and most proteases tested rapidly converted >90% of the remaining VP2 to VP3 (Fig. 1). No reduction in VP1 was consistently observed until the VP3 was degraded, when amounts of VP1 were reduced in parallel. Essentially no cleavage of VP2 to VP3 was observed in empty virus capsids (Fig. 1A). Cathepsins B and D were tested in similar assays at pH 5.5 and 4.5, and although those enzymes rapidly digested control proteins or virus heated to 100°C, neither digested VP2 to VP3 in capsids at pH 5.5. At pH 4.5, only cathepsin B digested the VP2 to VP3 (Fig. 1B).

Virus heated to 100°C for 2 min before the proteinase treatment was rapidly degraded by all proteinases tested (results not shown). After incubation of full capsids with trypsin, chymotrypsin, or bromelain, only the VP2-to-VP3 cleavage was detected. Treatment with proteinase K, subtilisin, or papain rapidly converted VP2 in full but not empty capsids to VP3, and then both capsids forms were degraded (Fig. 1A). Pronase rapidly degraded both full and empty capsids (results not shown).

To determine the cleavages that were occurring and the cleavage products that were released, full capsids were subjected to limited digestion with proteinase K or subtilisin for varying times. Both proteases generated fragments of 31–35 kDa that were detected by Western blotting using antibodies against SDS-denatured capsids (Fig. 2), and a subset of those fragments were also detected by anti-peptide 362–373 (Fig. 2) or anti-peptide 39–50 sera (not shown). Several fragments of <20 kDa were also generated that were recognized only by the anti-SDS-denatured capsid antibodies. Those products appeared to be primarily protease-resistant subfragments of the capsid protein. The fact that the cleavages occurred during the incubation of the capsid with the protease, and not during the process of denaturation of the capsid before electrophoresis, is shown by the 0 time result. Although the protease was included in that reaction, none of the specific degradation products were detected by Western blotting. A product of 31 kDa reacting with the anti-39–50 antibody indicated that at least one major cleavage occurred between VP2 residues 290–310, in a region associated with control of canine host range (Llamas-Saiz *et al.,* 1996; Parker and Parrish, 1997).

Amino-terminal sequencing of VP3 prepared from full virus without additional protease digestion showed the sequence Ala-Thr-Gly-Ser-Gly, indicating that cleavage occurred after VP2 residue 19, within the sequence ^{16}Arg-Asn-Glu-Arg ↓ Ala-Thr-Gly-Ser^{23}. No other amino acids were detected at >20% the level of the strongest amino acid peak during the first five sequencing cycles.

The exposure of the amino terminus of VP2 was also confirmed with various specific anti-peptide antibodies with the specificities shown in Fig. 3. As seen in Fig. 4, antibodies against VP2 peptides 3–12, 10–20, and 16–27 all recognized intact full CPV capsids, and that reactivity did not

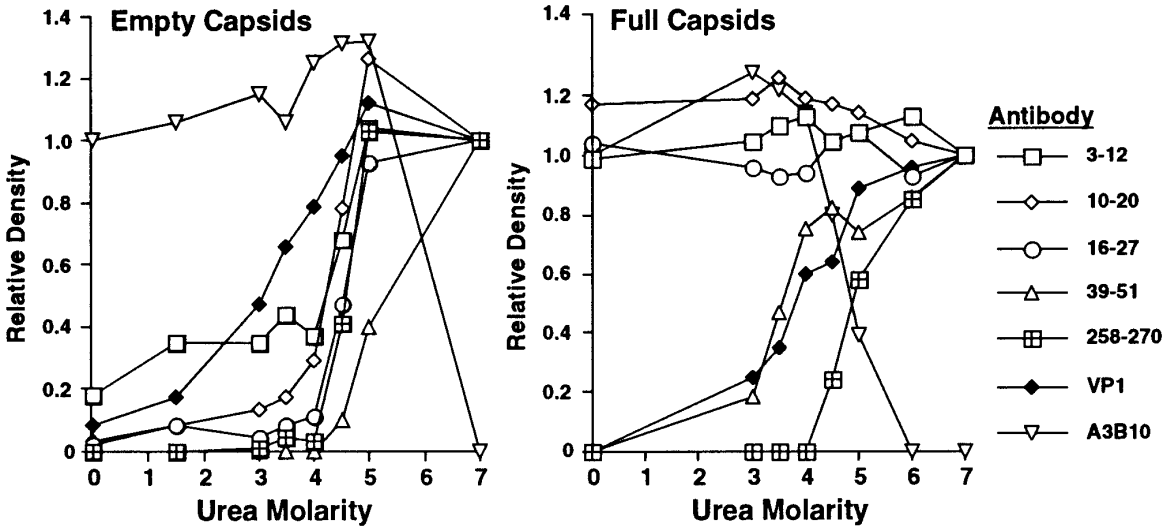

FIG. 4. Exposure of various virus sequences in empty or full virus capsids after either no treatment or incubation with 1.5–7 M urea. The capsids were then spotted onto a membrane and detected with the antibodies against the VP1 unique region (VP1), against various VP2 peptides, or MAb A3B10, which recognizes only assembled capsids. The bound antibodies were detected with anti-species IgG-HRPO and SuperSignal and exposed to x-ray film, and the films were scanned to determine the relative binding.

increase after urea denaturation of the capsid. In contrast, the antibodies against peptides 39–51 and 258–270 reacted to low or undetectable levels with the intact virus capsids, but those epitopes reacted strongly after capsids were treated with urea of $>\sim$3 M. Monoclonal antibody (mAb) A3B10 reacted with native capsids but not with virus that had been heated to 100°C or treated with >6 M urea (Figs. 4 and 5). Two mAbs prepared against peptide 362–373 reacted to low levels with native CPV or FPV but reacted to 8- to 10-fold higher levels against viruses denatured with heat or urea (Fig. 5A). Because the structure of the loop between VP2 residues 360 and 375 may be affected by pH and that may cause the difference in pH dependence of hemagglutination seen between CPV and FPV (Agbandje *et al.,* 1993; Chang *et al.,* 1992), those antibodies were also incubated with capsids at pH 4.5–7.5. A significant effect on mAb binding was seen only at pH 4.5, where the reactivity with capsids was reduced to a greater degree than the binding to peptide 362–373 conjugated to bovine serum albumen (Fig. 5B).

Capsid pI

Isoelectric focusing of CPV empty capsids showed them to have a pI of $\sim$5.3, indicating that the particle is negatively charged at physiological pH. The pI of the full

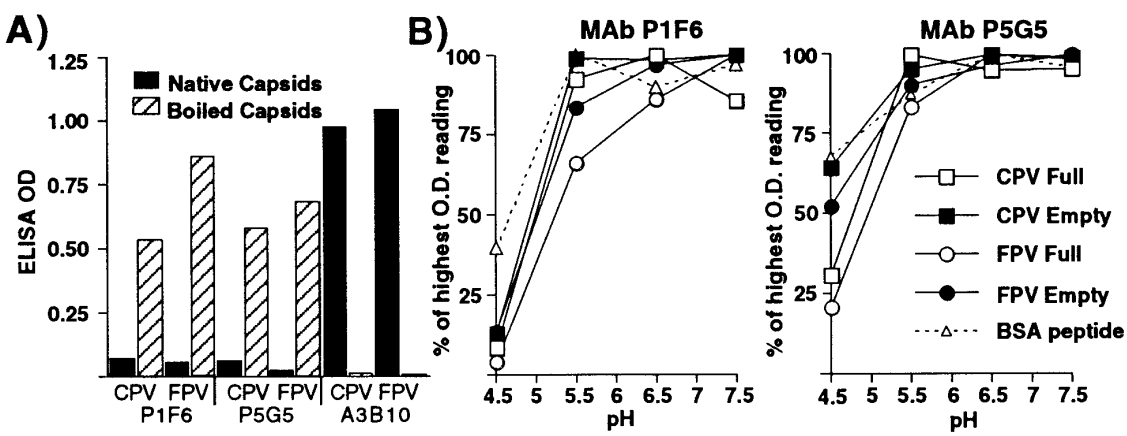

FIG. 5. Reactivity in ELISA of mAb P1F6 or P5G5, which recognize peptide 362–373, a variable loop between CPV and FPV. (A) Reaction of the mAb with either native or boiled CPV capsids from either CPV or FPV or reaction of mAb A3B10, which recognizes only intact capsids. (B) Effect of pH on the binding of mAb P1F6 and P5G5 to CPV or FPV full and empty capsids or with peptide 362–373 conjugated to BSA. Antibodies were incubated for 1 h with the various antigens at pH 4.5, 5.5, 6.5, and 7.5 and then washed and reacted with anti-mouse IgG HRPO and substrate in the normal incubation and wash solutions. The optical density results were normalized to the highest optical density reading obtained for each virus–antibody combination.

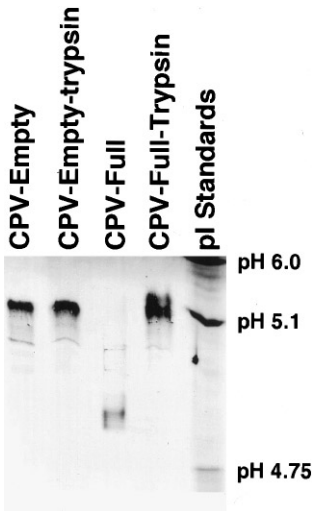

FIG. 6. Isoelectric focusing of purified full or empty CPV capsids in agarose gels. Samples of virus were either untreated or pretreated with trypsin. pI standard markers are indicated.

capsid was ~0.3 pH less than that of the empty particle, but trypsin treatment converted that full virus to a form with the pI of the empty particle (Fig. 6).

Virus uptake and microinjection

[^{35}S]Methionine-labeled virus was bound to the cells at 0°C for 1 h, and then the cells were washed and incubated at 37°C. About 80% of the cell-associated virus was released into the supernatant after warming (Fig. 7; results not shown). The remaining bound capsid proteins were recovered in similar amounts over the next 24 h (Fig. 7). Conversion of VP2 to VP3 in the full capsids was detected only after 6- to 24-h incubation with the cells (Fig. 7A), and trypsin treatment of the capsids used in those studies showed that 50% of the VP2 could be converted to VP3 (Fig. 7B).

Microscopic examination of virus uptake was followed by incubation of cells with purified full capsids for various times. After 2 h, native and biotinylated capsids were detected within vesicles in the perinuclear region of the cell (Fig. 8). Those vesicles also costained for lysosomal glycoprotein, suggesting that the virus primarily became localized in lysosomes (Fig. 8C), and 24 h after uptake, the capsid antigens were still readily detected in such vesicles (results not shown).

After microinjection of purified full CPV capsids into the cytoplasm of A72 cells, most of the antigen remained in the cytoplasm (Fig. 9). By 2 h, a small proportion of the injected antigen was seen within the nucleus, as demonstrated by confocal microscopy, where it was excluded from the nucleoli (Fig. 9).

Proteinase inhibitors

Infection of cells with freshly prepared full CPV capsids containing no VP3 was examined in the presence of the proteinase inhibitors *trans*-epoxysuccinyl-L-leucylamide(4-guanidino)-butane (E64) (100 or 400 μM), leupeptin (100 μM), aprotinin (1 mg/ml), or pepstatin A (0.75 mM). There was little decrease in infection titer when the inhibitor was added 1 or 4 h before virus addition compared with cultures with no inhibitor or compared with cultures in which the inhibitor was added to the cultures 1 h after virus addition (Table 1).

DISCUSSION

A variety of approaches were taken to investigate the structural variation and stability of the CPV capsid and its role in cell infection. Variation in the susceptibility of viral proteins to protease treatment and structure-specific antibodies have been widely used to investigate the variation of other viruses and their proteins (Baer *et al.,* 1997; Bothner *et al.,* 1998; Fricks and Hogle, 1990; Ketterlius and Wiegers, 1994; Stiasny *et al.,* 1996; Virgin *et al.,* 1994).

The cleavage of the VP2 to VP3 in CPV and related parvoviruses has been widely reported in previous studies, but the mechanisms involved and any associated functions are not completely understood. Here we show that the natural cleavage of VP2 to VP3 in CPV occurs after VP2 residue 19, in the equivalent position to that determined for trypsin-treated H1 virus (Paradiso *et al.,*

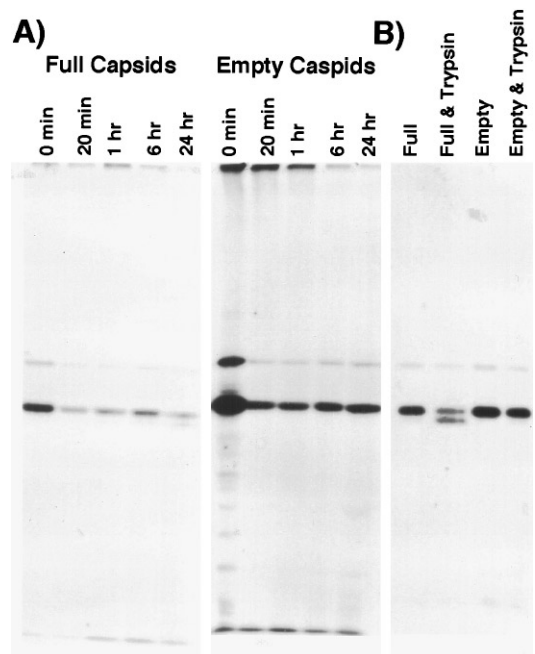

FIG. 7. (A) Uptake of [^{35}S]methionine-labeled CPV full and empty capsids into cells. The virus was bound on ice for 1 h, and then the cells washed, and one sample was collected (0 min). The remaining cultures were warmed to 37°C, and the cells were lysed at the times indicated. Materials collected at each time point were electrophoresed in 10% acrylamide gels and subjected to fluorography. (B) Control analysis of capsids used in the uptake studies. Samples of the full or empty capsids were left untreated or treated with trypsin and then analyzed as described above.

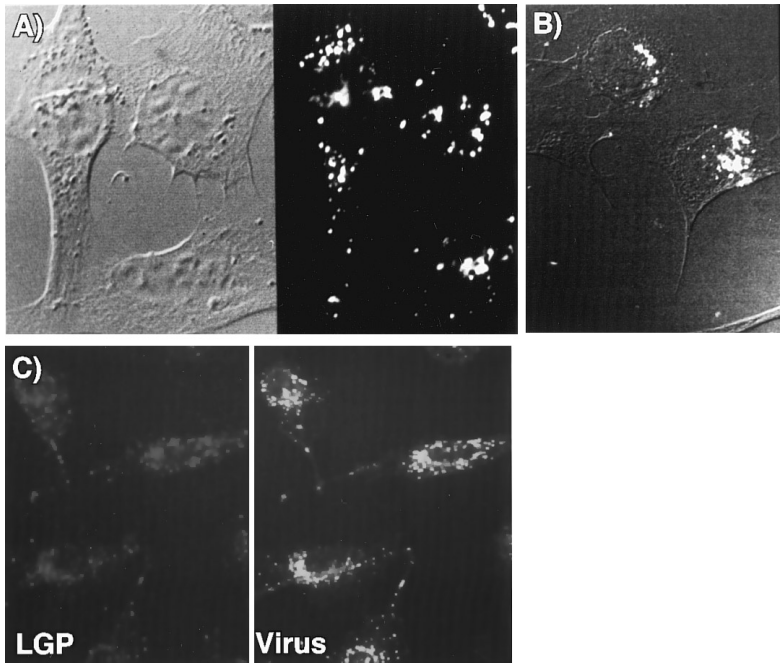

FIG. 8. Localization of biotinylated full CPV capsids in cells at 4 (A) and 16 (B) h after inoculation of A72 cells. In each case, the fluorescent label shows the virus capsids, and this is shown along with a Nomarski DIC view of the calls, either separately (A) or overlaid (B). (C) Colocalization of native CPV capsids and lysosomal glycoprotein in A72 cells after 2-h incubation of the virus with the cells.

1984). The amino-terminal sequence is cleaved by enzymes with many different cleavage specificities (Fig. 1), as has also been suggested by studies involving mutagenesis of the RVER sequence in MVM (Tullis *et al.,* 1992). Analysis of CPV capsids by x-ray crystallography shows the VP1 and VP2 structures from VP2 residue 37 to the carboxyl-terminal residue 584 (Tsao *et al.,* 1991; Wu and Rossmann, 1993), with the most obvious structural difference between the full and empty particles being several DNA bases within each asymmetric unit on the inside of the capsid (Chapman and Rossmann, 1995; Tsao *et al.,* 1991). The capsid structures show that the only pore where the amino termini could be exposed to the outside was at the five-fold axis of symmetry, although the pore seen appeared to be too small (minimum diameter, 8 Å) to allow more than one chain to pass through. About 50% of the protein in the full capsids tested was VP2, and most proteases tested rapidly digested >90% of that VP2 to VP3 (Fig. 1). The antibodies recognizing the VP2 amino terminus also did not show increased reactivity when the capsid was broken with urea (Fig. 4), indicating that most of the remaining VP2 amino termini were exposed to the outside of the capsid simultaneously. When newly synthesized capsids containing ~100% VP2 were digested with trypsin, only 50% of the VP2 converted to VP3, suggesting that only 50% of the VP2 amino termini were exposed simultaneously (Fig. 7B).

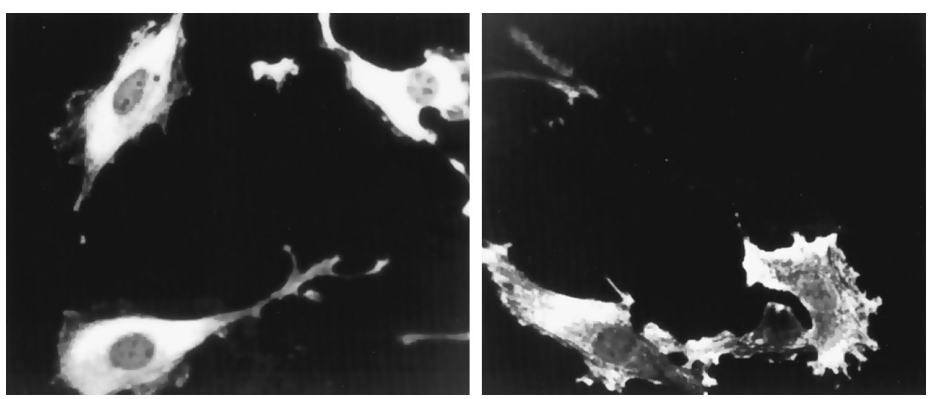

FIG. 9. Two fields of A72 cells showing the localization of full capsids after microinjection into the cytoplasm and incubation for 2 h at 37°C and then fixation and staining with rabbit anti-CPV capsid antibodies.

TABLE 1

Effects of Proteinase Inhibitors on CPV Infection of NLFK Cells

Inhibitor	$TCID_{50}$	
	Inhibitor added before	Inhibitor added after
E64 100 μM	4.7	4.8
E64 400 μM	4.5	4.49
Leupeptin 100 μM	5.27	4.45
Aprotinin 1 mg/ml	4.9	4.25
Pepstatin (0.75 mM) and E64 (400 μM)	3.76	3.96

Note. Data are given as $\log_{10}$ $TCID_{50}$ titers. The CPV full virus ($TCID_{50}$ $10^{4.8}$) purified from a short-term culture was added to cells either in the presence of the inhibitor (added 1 or 4 h before inoculation) or when the inhibitor was added 1 h after the virus. The inhibitors were then left in the culture medium for the remaining incubation time.

It has been proposed that a glycine-rich sequence between VP2 residues 22 and 37 would pass through the pore in each five-fold cylinder, and electron density was seen within the pore of the full capsids (Tsao *et al.,* 1991). The upper portions of the loops surrounding the five-fold cylinder do not have any interloop bonds, so they can most likely move apart. The capsid structure may therefore involve exposure of two VP2 amino termini through each of 11 five-fold axes of the capsid (the 12th axis presumably is occupied by DNA). Alternatively, one VP2 may be exposed, and after cleavage, the VP3 amino terminus may withdraw into the particle and be replaced by a new VP2 amino terminus that is subsequently cleaved. It is possible that aspects of both models apply, with one terminus being exposed at any one time, and that after cleavage to VP3, there is a slower exchange with some of the remaining VP2 termini, during which process a number of large charged side chains would need to transit the pore. That most of the full particles had similar numbers of VP2 termini exposed was shown by the fact that before trypsin treatment, their pI value was fairly uniformly reduced by ~0.3 pH (Fig. 6).

The structure of the capsid was very stable at pH of 5.5–7.5, and no significant differences were seen in the cleavages of the capsids when they were incubated with the proteases at those pH values. The decreased cleavage by subtilisin and proteinase K at pH 5.5 probably reflects lower protease activity under that condition. In other studies, we have observed that the capsid stays intact between ~pH 3.0 and 11.0 (W. Yuan and C. R. Parrish, unpublished results).

The CPV capsids were also completely degraded by proteases with several different specificities (Fig. 1), and digestion products of 31–32.5 kDa were detected after limited digestion with proteinase K and subtilisin (Fig. 2). Probing with peptide-specific antibodies showed that one fragment released early contained the sequence VP2 39–50 and another contained the sequence 362–373. A 32,500-kDa fragment containing residues 39–55 and derived from VP3 would be cleaved between VP2 residues 305 and 310, within an extended surface loop that overlaps the three-fold related VP2 molecule (Tsao *et al.,* 1991), and residues 300–302 and 307–309 are all highly accessible on the capsid surface (Xie and Chapman, 1996). Sequences on the shoulder of the three-fold spike of the capsid are associated with the host range for canine cells (Llamas-Saiz *et al.,* 1996; Parker and Parrish, 1997), although any relationships between the protease cleavage and host range are not known.

CPV infection of cells may be blocked by treatments that raise the endosomal pH (Basak and Turner, 1992). Because we can demonstrate that there were no major changes in the structure of the capsid at pH 5.5, a possible mechanism might be that acid-dependent endosomal proteases are required for infection. We therefore incubated capsids at pH 4.5 and 5.5 with the endosomal proteases cathepsin B and cathepsin D (Authier *et al.,* 1996; Blum *et al.,* 1989). The full capsids were resistant to cathepsin D, whereas cathepsin B cleaved the VP2 to VP3 only at pH 4.5 and did not further cleave the protein (Fig. 1B). We were also unable to show a role for proteolysis in the process of cell infection when we examined the fate of [^{35}S]methionine-labeled virus taken up into cells (Fig. 7) or inoculated cells with virus in the presence of protease inhibitors (Table 1). Pepstatin A and E64 are potent inhibitors of cathepsin D and cathepsin B, respectively, and inhibit those enzymes in the endocytic pathway under the conditions used (Baer and Dermody, 1997; Fiani *et al.,* 1993). In both assays, full viruses used for infection were purified after short periods and contained no detectable VP3 (Fig. 7; unpublished results). When observed microscopically, CPV entered cells through endocytosis and accumulated in vesicles that resemble lysosomes (Fig. 8), as has been shown by others (Basak and Turner, 1992; Vihinen-Ranta *et al.,* 1998). The infecting virus or its DNA must escape from the endosome into the cytoplasm during infection. The mechanisms by which that occurs were not revealed in these studies, but the capsid must undergo structural changes during that process because intact capsids microinjected into the cytoplasm of the cell are only inefficiently transported into the nucleus (Fig. 9) (Vihinen-Ranta *et al.,* 1998). In previous studies examining the uptake of H1 parvovirus purified from longer-term cultures and labeled with ^{125}I using chloramine T, degradation products of VP3 were detected only after several hours of incubation with the cells, and no functional role for that cleavage in cell infection was demonstrated (Paradiso, 1981).

The unique amino-terminal domain of VP1 was clearly inside both empty and full capsids in that it was not cleaved by any protease before the degradation of VP2 and VP3 (Fig. 1) and it was recognized by antibodies only

after capsids had been treated with urea (Fig. 4). This is in contrast to results reported for the human parvovirus B19 assembled in insect cells after baculovirus expression, where the VP1 unique region was on the outside of particles containing VP1 and VP2. Those particles were recognized by anti-VP1 antibodies, the antibodies neutralized the virus infectivity (Anderson *et al.,* 1995; Kawase *et al.,* 1995), and fusion of lysozyme with the amino-terminal sequence of VP1 resulted in that enzyme being displayed outside the capsid after baculovirus expression (Miyamura *et al.,* 1994).

These results confirm that the parvovirus capsid is very stable, and they also show that there is little structural variation over the physiological range of pH and temperature. The changes that are observed, such as the cleavage of the VP2 amino terminus or internal sequences or the shift in buoyant density of full virus after prolonged incubation, have not yet been shown to play critical roles in infection, although it is possible that those functions are expressed only after the virus has bound to a cell receptor or entered the cell. We are now using further antibody and crystallographic analyses to look for more subtle changes in the capsids that may be important for cell infection and host range, as well as further examining the virus–host cell interactions.

MATERIALS AND METHODS

Viruses and cells

Stocks of CPV-d strain of CPV type 2 and FPV-b strain of FPV were prepared by transfection of infectious plasmid clones and propagated in Norden laboratories feline kidney (NLFK) cells (Parrish, 1991). Canine A72 cells were used for most of the studies of virus uptake and infection (Binn *et al.,* 1980). Cells were grown in a 50% mixture of McCoy's 5A and Liebovitz L15 with 5% fetal bovine serum (FBS). Virus was purified from lysed cells by precipitation with 3.5% polyethylene glycol (PEG) 8000 and 0.5 M NaCl at 4°C, pelleted through a 30% sucrose cushion at 100,000 *g* for 6 h, and then banded twice in 10–40% sucrose gradients in 10 mM Tris–Cl (pH 7.5). The virus was dialyzed against 10 mM Tris–Cl (pH 7.5) and then stored at 4°C. For the antibody-binding studies, virus was not PEG precipitated but instead was concentrated through filtration of the culture supernatant through a 10^6-Da cutoff filter, followed by banding twice in 10–30% glycerol gradients in 20 mM Tris–Cl (pH 7.5) and 0.15 M NaCl (Tris–saline) for 5 h at 100,000 *g* at 20°C followed by dialysis against Tris–saline.

Antibodies

Polyclonal rabbit sera were prepared by immunization with either purified CPV capsids or CPV capsids that had been boiled in SDS. The capsid regions recognized by site-specific antibodies are shown in Fig. 3. mAb 8 (A3B10) recognizes a conformational epitope present only on intact CPV and FPV capsids (Strassheim *et al.,* 1994; Wikoff *et al.,* 1994). Mouse sera were prepared against peptides containing VP2 sequences along with an amino-terminal cysteine, including peptide 16–27 (VP2 residues 16–27), peptide 39–51, peptide 258–270, and peptide 362–373. Peptides were conjugated to sulfosuccinimidyl-4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate-activated keyhole limpet hemocyanin (KLH) (Pierce Chemicals, Rockford, IL). Mice were immunized once with KLH-conjugated peptides in Freund's complete adjuvant and then repeatedly with the conjugate in Freund's incomplete adjuvant. To prepare mAbs against peptide 362–373, immunized mice were injected intravenously with KLH-conjugated peptide, and spleen cells recovered 4 days later were used to prepare hybridomas. Hybridomas were screened in ELISA against boiled CPV-d capsids and BSA-conjugated peptide 362–373. Two positive hybridomas (P1F6, P5G5) were cloned, and the mAbs were prepared as tissue culture supernatants.

Rabbit sera against CPV amino-terminal sequences (peptides 3–12 and 10–20) were provided by J. I. Casal (Casal *et al.,* 1995; Langeveld *et al.,* 1994). A rabbit antiserum against the entire VP1-specific region of MVM was provided by P. Tattersall and S. Cotmore (Cotmore *et al.,* 1997). Antibodies diluted in 5% nonfat dried milk, 50 mM Tris–Cl (pH 7.5), and 200 mM NaCl were incubated with virus bound on nitrocellulose filters and detected with goat anti-mouse or anti-rabbit IgG horseradish peroxidase (HRPO) conjugate and SuperSignal substrate (Pierce) and then exposed to x-ray film.

Protease treatments

Full or empty CPV capsids were diluted in 50 mM Tris–Cl (pH 7.0) or 50 mM Na acetate (pH 5.5 or 4.5), both with 100 mM NaCl. Most proteases were obtained from Sigma Chemical (St. Louis, MO). Subtilisin Carlsberg (EC 3.4.21.62), bromelain (EC 3.4.22.4), papain (EC 3.4.22.2), proteinase K (EC 3.4.21.64), trypsin (EC 3.4.21.4), α-chymotrypsin (EC 3.4.21.1), and *Streptomyces griseus* protease (Pronase) (EC 3.4.24.31) were used at amounts equal to the capsid concentration. Cathepsin B (EC 3.4.22.1) and cathepsin D (EC 3.4.23.5) were obtained from Calbiochem (San Diego, CA). Bromelain and papain were activated with 14 μM 2-mercaptoethanol, and cathepsin B was activated with 2 mM cysteine. SDS sample buffer was added after 20 or 60 min at 37°C, the samples were heated to 100°C for 5 min and separated by 7.5% SDS–PAGE, and then proteins were stained with Coomassie Blue R250. To show that the enzymes were all active against the viral proteins, control digestions were performed on virus that had previously been heated to 100°C for 2 min.

Partial proteolytic cleavage products were identified by treating 12 μg of full capsids at 37°C with proteinase K or subtilisin at virus-to-proteinase ratios of 1:1 or 1:2, respectively. After various times up to 90 min, samples were removed, mixed with SDS sample buffer, heated to 100°C for 5 min, and then separated by 10% SDS–PAGE and electrophoretically transferred to nitrocellulose membranes. Viral proteins were detected with rabbit antiserum against SDS-denatured CPV or with mouse anti-peptide 362–373, 258–270, or 39–51 antibodies. Bound antibodies were detected as described above.

Amino-terminal sequence of VP3 protein

CPV full capsids purified from infected NLFK cells after 5 days of culture were heated in SDS sample buffer, electrophoresed in 7.5% SDS–PAGE, and then electrophoretically transferred to PVDF membranes. After Coomassie Blue staining, the VP3 band was excised from the membrane and sequenced in an Applied Biosystems Procise protein sequencer for six cycles of Edman degradation.

Isoelectric focusing of capsids

Capsids (5 μg) that were either untreated or pretreated with 2 μg of trypsin for 60 min were isoelectric focused in gels containing 1% w/v low-molecular-weight agarose, 5% w/v sorbitol, 10% v/v glycerol, and 2% w/v of pH 4–6 ampholytes (BioRad, Hercules, CA). Gels were focused for 15 min each at 100 and 200 V and then for 60 min at 450 V; fixed with a mixture of 5% w/v trichloracetic acid, 30% v/v methanol, and 3.5% w/v sulfosalicylic acid; washed with 100% ethanol; and then stained with Coomassie Blue.

Antibody recognition of capsid structures

Purified CPV full or empty capsids were left untreated or treated with 1–8 M urea for 5 min and then bound to nitrocellulose membranes in a slot blot apparatus. The capsids were detected with anti-peptide sera or mAbs, and the bound antibodies were detected with HRPO-conjugated anti-species antibodies as described above. Films were scanned, and the antibody bindings after different treatments were compared.

The loop between VP2 residues 360–378 varies in structure between CPV and FPV capsids (Agbandje *et al.,* 1993). To examine for conditions that cause variation of that loop, mAbs P1F6 and P5G5 against peptide 362–373 were incubated with virus bound to ELISA plates. Some virus was also pretreated at 100°C, and then the antibodies were incubated with the virus in PBS at pH 7.2. The antibodies were also incubated for 60 min with capsids at pH 4.5–7.5, and the plates were washed and incubated in PBS at pH 7.2 with anti-mouse HRPO antibodies and then with substrate 2,2′-azino-bis-(3-ethylbenzthiazidine-6-sulfonic acid) diammonium.

[^{35}S]Methionine labeling and cell uptake of virus capsids

NLFK cells were inoculated with 5 $TCID_{50}$/cell of CPV-d and incubated for 48 h. The cultures were then incubated for 1 h with methionine-free medium, for 6 h with 0.2 mCi/ml of [^{35}S]methionine, and then in complete growth medium for 2 h. Cells were lysed with 1% v/v Nonidet P-40 in 0.1 M Tris–Cl (pH 8.0) containing 1 μM pepstatin, 25 μM phenylmethylsulfonyl fluoride, and 100 μM leupeptin; frozen and thawed; and centrifuged for 10 min at 10,000 *g*, and the supernatant virus was pelleted at 100,000 *g* for 3 h. The virus was resuspended in 1 0mM Tris–Cl (pH 8.0) and centrifuged in a 10–40% sucrose gradient at 100,000 *g* for 6.5 h. Empty and full capsids were pelleted at 100,000 *g* for 2 h and then resuspended in Dulbecco's modified Eagle's medium. Labeled virus was incubated with A72 cells on ice for 1 h; the cells washed three times with the same medium and then incubated for 0–24 h at 37°C in growth medium. The cells were washed with PBS, lysed in SDS sample buffer, scraped from the dishes, and boiled, and half of the sample was electrophoresed in 7.5% SDS–PAGE. Gels soaked in Amplify (Amersham, Arlington Heights, IL) were dried and exposed to preflashed x-ray film at −70°C. In control studies, the purified virus samples were incubated with 2 μg/ml trypsin for 30 min at 37°C before electrophoresis as described above.

To examine the uptake of the capsids microscopically, 10 μg/ml of full capsids conjugated with NHS–biotin were incubated with A72 cells in chamber slides for 1 h at 37°C. The cells were washed with medium at 37°C, incubated for various periods, fixed with acetone on ice for 10 min, and stained with FITC–streptavidin. In other studies, cells were incubated with 4 μg/ml concentration of native virus on ice for 1 h and then warmed to 37°C for 10 min to 6 h. After fixation for 10 min with 2% paraformaldehyde in PBS, they were rinsed in PBS with 1 mg/ml glycine and permeabilized with 0.05% Saponin. Virus antigen was stained with rabbit anti-CPV capsids conjugated with either FITC or RITC, and in some cases the cells were also stained with a mouse monoclonal anti-lysosomal glycoprotein (Nabi *et al.,* 1991), followed by goat anti-mouse FITC. Cells were examined under visible light by differential interference contrast and for fluorescence with a BioRad MRC 600 confocal imaging system, and 1-μm optical sections were recorded from the midsection of the cells.

Purified full CPV capsids at a concentration of 1 mg/ml were injected into A72 cells using essentially the protocol described for microinjection of SV40 capsids by Clever *et al.* (1991). Briefly, the capsids were dialyzed against a buffer of 48 mM K_2HPO_4, 14 mM NaH_2PO_4, and 4.5 mM KH_2PO_4 (pH 7.2) made up to 1 mg/ml, and $\sim 2 \times 10^{-11}$ ml ($\sim$1000 particles) was injected into the cytoplasm of each cell. After 2 or 6 h at 37°C, the cells were

fixed with 1:1 acetone and methanol at 0°C and then incubated with rabbit anti-CPV capsid antibody and goat anti-rabbit FITC.

Proteinase inhibitors and infection

Virus stocks containing no detectable VP3 were prepared from short-term culture. NLFK cells were synchronized in two stages: first by growth in isoleucine-free medium for 30 h, and then by incubation with 12 μg/ml aphidicolin in complete medium for 16 h. The cells were inoculated with CPV and incubated with complete medium containing 1 μM pepstatin, 100 μM leupeptin, and 10 μM E64 for 12 h. After freezing and thawing of the cells, the virus was pelleted at 100,000 *g* for 3 h and then banded onto a 10–40% sucrose gradient at 100,000 *g* for 6 h. Fractions containing full capsids were pooled and used as an inoculum. NLFK cells seeded at $1 \times 10^4/cm^2$ in a 96-well plate were treated with the proteinase inhibitors E62 (100 or 400 μM), with leupeptin (100 μM), with aprotinin (1 mg/ml) 1 h before or 1 h after inoculation, or with pepstatin (0.75 mM) 4 h before or 1 h after virus inoculation. Inhibitors were left in the cultures, and the cells were fixed after 46 h with a mixture of acetone and methanol, incubated with rabbit-anti CPV and then with goat anti-rabbit HRPO, and developed with amino ethyl carbazole. $TCID_{50}$ titers of the virus in the presence or absence of the inhibitors were calculated.

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