Purified Feline and Canine Transferrin Receptors Reveal Complex Interactions with the Capsids of Canine and Feline Parvoviruses That Correspond to Their Host Ranges

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The cell infection processes and host ranges of canine parvovirus (CPV) and feline panleukopenia virus (FPV) are controlled by their capsid interactions with the transferrin receptors (TfR) on their host cells. Here, we expressed the ectodomains of wild-type and mutant TfR and tested those for binding to purified viral capsids and showed that different naturally variant strains of the viruses were associated with variant interactions with the receptors which likely reflect the optimization of the viral infection processes in the different hosts. While all viruses bound the feline TfR, reflecting their tissue culture host ranges, a naturally variant mutant of CPV (represented by the CPV type-2b strain) that became the dominant virus worldwide in 1979 showed significantly lower levels of binding to the feline TfR. The canine TfR ectodomain did not bind to a detectable level in the in vitro assays, but this appears to reflect the naturally low affinity of that interaction, as only low levels of binding were seen when the receptor was expressed on mammalian cells; however, that was sufficient to allow endocytosis and infection. The apical domain of the canine TfR controls the specific interaction with CPV capsids, as a canine TfR mutant altering a glycosylation site in that domain bound FPV, CPV-2, and CPV-2b capsids efficiently. Enzymatic removal of the N-linked glycans did not allow FPV binding to the canine TfR, suggesting that the protein sequence difference is itself important. The purified feline TfR inhibited FPV and CPV-2 binding and infection of feline cells but not CPV-2b, indicating that the receptor binding may be able to prevent the attachment to the same receptor on cells.

Receptor binding is a key step in the life cycles of all animal viruses, and in many cases the virus-receptor interactions determine both the host susceptibility and tissue tropism. Receptors for different viruses include carbohydrates and proteins, and in some cases multiple receptors work in concert to mediate cell binding and infection (33, 37, 66, 71, 73). For non-enveloped viruses the interaction with the receptor initiates a series of steps that leads to capsid endocytosis, sorting of the particle into an endosomal compartment for penetration into the cytoplasm, disassembly to allow release of the infectious material, and transport of the genome and associated proteins within the cell to allow replication (37, 39, 42, 60, 61, 70). Infection of cells by most viruses involves complex interactions and structural changes induced by receptor binding, low pH, or endosomal proteolysis that allow membrane penetration and intracellular delivery (39, 66, 89, 90).

Our studies of the viral-cell interactions and intracellular trafficking pathways of canine and feline paroviruses have shown that the specific interactions between viral capsids and host transferrin receptors (TfR) are critical to infection (24–26, 48, 50). Canine parvovirus (CPV) and the closely related feline panleukopenia virus (FPV) are small nonenveloped viruses that, depending on the strain of virus, infect cats and/or dogs. They provide a model for the process of cell infection and also for the control of viral host range through capsid-receptor interactions (reviewed in reference 27). CPV emerged as a pandemic virus in dogs during the 1970s, and the strain of virus that spread widely in 1978 was designated CPV type-2 (CPV-2) to distinguish it from a previously known but distinct parovirus, minute virus of canines (57, 67). CPV-2 differs from FPV or closely related viruses in only a small number of sequence changes (68, 81), and the ability to infect dogs or dog cells is controlled by changes in a raised region of the capsid surrounding the threefold axis of icosahedral symmetry (the threefold spike) (1). Amino acids affecting host range in natural variants or laboratory-derived mutants included VP2 residues 93 (Lys in FPV; Asn in CPV), 323 (Asp in FPV; Asn in CPV), 299 (Gly in wild-type CPV; Glu in the host range mutant CPV-2-G299E), and 300 (Ala in wild-type CPV; Asp in the mutant CPV-2-A300D) (12, 23, 24, 36, 51).

During 1979 a variant strain of CPV, CPV type-2a (CPV-2a), emerged in dogs and within 1 year had replaced CPV-2 worldwide (56, 58); the variant contained differences of VP2 residues 87, 101, 300, and 305 (53–55). CPV-2a had regained the host range for cats and was also antigenically variant (29, 46, 58, 80). Additional single mutations of CPV-2a that have become widely distributed since 1980 include the substitution of VP2 residue 426 (Asn to Asp) to give the antigenic variant CPV-2b, which appears to be the same as CPV-2a in host range and other properties (74, 81).

FPV and CPV both bind the feline TfR and use that receptor for cell infection; CPV-2, CPV-2a, and CPV-2b can bind the canine TfR and infect dog cells while FPV cannot (26, 50).
The CPV-2 capsids bind to feline and canine cells to much higher levels than do CPV-2a or -2b capsids, suggesting that CPV-2 forms different interactions with the TIR or binds to additional receptors on those cells (26). Cell infection by CPV involves clathrin-mediated endocytosis of the TIR-capsid complex and trafficking within the endosomal system of the cell in a process that requires exposure to low pH (49, 84). Release into the cytoplasm and nuclear transport likely involve the externalization of the N-terminal sequence of the VP1 protein which contains both a phospholipase A2 activity and a nuclear localization sequence (75, 76).

The TIR is a type II membrane protein that is expressed on the surface of cells as a homodimer of ~11 nm in span (17), and each monomer is comprised of protease-like, apical, and helical domains (6, 34). The human TIR ectodomain is attached to a 32-residue stalk that holds the receptor about 30 Å above the plasma membrane (17) and that is connected to transmembrane and cytoplasmic sequences (31). When bound to iron-loaded transferrin (Tf) the TIR-Tf complex enters the cell by clathrin-mediated endocytosis (2, 16, 63), and at the moderately low pH of the recycling endosome the iron is released and the iron-free Tf and TIR remain bound and recycle to the cell surface, where the Tf is released at neutral pH (18, 21, 40, 69, 83, 86). Tf binding occurs primarily through the membrane-facing side of the TIR, with different interactions formed at neutral and acidic pHs (9, 14, 19, 87). The murine TIR is a receptor for the mouse mammary tumor virus, and the human TIR can be used for cell infection by some mutant adenoviruses (65, 91).

Specific binding of CPV to the canine TIR is controlled by changes in three positions on the threefold spike of the capsid that are 20 to 30 Å apart, suggesting that a broad surface of the capsid interacts with the receptor (20, 24, 26). Less is known about the capsid structures involved in feline TIR binding, although binding to both canine and feline TIR was prevented by a mutation of feline TIR residue 221 from Leu to Ser (48). However, the capsid mutations that prevented canine TIR binding did not appear to alter the binding to the feline TIR expressed on cells, indicating that the canine and feline TIR make some different contacts with the viruses (24).

Feline TIR binding to FPV and CPV and canine TIR binding to CPV were affected by changes in three positions in one face of the receptor apical domain (48). Feline and canine TIR differ in an insertion of Asn into the canine TIR apical domain, and deleting that residue allowed the canine TIR to bind FPV capsids, although allowing little cell infection (48). A Lys-to-Asn difference of residue 383 introduces a new N-linked glycosylation site into the apical domain of the canine TIR, and changing that Asn in the canine TIR to Lys (the feline TIR residue) gave a receptor that both bound FPV and mediated infection (48).

The feline and canine TIR binding to CPV and FPV capsids provides specific structural interactions that are required for cell infection, since binding to sialic acid on cells does not result in infection, nor does the binding to some mutant TIRs that can still mediate attachment and endocytosis (48, 79). Artificial receptors with alternative binding sites prepared from antibody variable domains fused to the TIR transmembrane and cytoplasmic sequences also bind and endocytose virus but do not mediate infection (25).

### MATERIALS AND METHODS

Cells, viruses, and antibodies. *Trichoplusia ni* (High Five) cells were grown in shaking flasks in Express5 serum-free medium (Invitrogen, Carlsbad, CA), and *Spodoptera frugiperda* cells (Sf9) cells were grown in Hink's TM-FF medium with 10% fetal bovine serum. Chinese hamster ovary-derived cells lacking the hamster TIR (TRVb cells) (41) were grown in Ham's F12 medium containing 5% fetal bovine serum. Norden Laboratory feline kidney (NFLK) or Crandell-Reese feline kidney (CRFK) cells were grown in a 1:1 mixture of McCoy's 5A and fetal bovine serum. Chinese hamster ovary-derived cells (Sf9) cells were grown in Hink's TNM-FH medium with 10% fetal bovine serum. Norden Laboratory feline kidney (NFLK) or Crandell-Reese feline kidney (CRFK) cells were grown in a 1:1 mixture of McCoy's 5A and Liebowitz L15 medium with 5% fetal bovine serum.

Parvoviruses were derived from infectious plasmid clones, and included three strains: FPV (FPV-b), CPV-2 (CPV-d), and CPV-2b (CPV-39) (26, 52). Plasmids were transfected into NLFK cells, and the viruses isolated were titrated in those cells using 50 tissue culture infective dose assays (51). In addition to the wild-type viruses, viral mutants tested included FPV-K93N, FPV-K93N/D323N, CPV-2-N93K, and CPV-2-N93K/N323D (24) (where the numbers indicate the VP2 residues altered, the first letter indicates the wild-type residue in single-letter code, and the second letter indicates the residue in the mutant). Virus capsids were concentrated by polyethylene glycol precipitation followed by sucrose gradient centrifugation and then dialyzed against phosphate-buffered saline (PBS; pH 7.5) and stored at 4°C (1). For some experiments capsids were passed through a 0.6- by 60-cm column of Sepharose CL-2B, and the monomeric capsid peak was collected.

Antibodies against the TIR included a monoclonal antibody (MAb) recognizing the soluble feline and canine TIR (MAB T4F3), a rabbit polyclonal antibody against a peptide comprising residues 558 to 571 of the feline TIR (rabbit anti-TIR), or MAb 6 (see reference 48). The Fab fragments were produced from MAb 6 recognizing intact CPV and FPV capsids, a rabbit polyclonal antibody (rabbit anticalicivirus) recognizing CPV and FPV capsids, and MAB CE10 against the NS1 protein (92).

**TIR clones, mutants, and ectodomain expression.** The TIR clones tested (listed in Table 1) contained cDNAs of the feline and canine TIR or were mutants of those clones (26, 48, 50). The ectodomains of the TIR were cloned into baculoviruses as previously described for the human TIR (35). The clones contained an N-terminal baculovirus g68 signal sequence that was cleaved off and a His6 sequence fused to residue 121 of the feline and canine TIR. After infection of High Five cells for 3 days, the culture medium was dialyzed against 50 mM Tris-CI (pH 7.5) and 150 mM NaCl; then the TIR ectodomain was

### TABLE 1. Wild-type and mutant forms of the feline and canine TIR analyzed in this study, along with the previously described effects of the mutations

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Description of the receptor structure</th>
<th>Virus binding on cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feline TIR</td>
<td>Wild type</td>
<td>+</td>
</tr>
<tr>
<td>Canine TIR</td>
<td>Wild type</td>
<td>+</td>
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<tr>
<td>Feline TIR-L221S</td>
<td>Substitution in the apical domain</td>
<td>−</td>
</tr>
<tr>
<td>Feline TIR-L221K</td>
<td>Substitution in the apical domain</td>
<td>−</td>
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<tr>
<td>Canine TIR-del220N</td>
<td>Deletion of insertions N230 in the canine TIR</td>
<td>+ +</td>
</tr>
<tr>
<td>Canine TIR-N383K/T385S</td>
<td>Changes to the feline TIR residues, removing a potential glycosylation site in the apical domain</td>
<td>+ +</td>
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*(See reference 48.)*
isolated by binding to Ni\(^{2+}\)-nitrilotriacetic acid resin (QIAGEN, Valencia, CA) in either 0 or 10 mM imidazole, 10% (vol/vol) glycerol, and 300 mM NaCl. The TfR was eluted with between 25 and 100 mM imidazole, and then the nonaggregated TfR dimer was prepared by chromatography in a Sephacryl S300 column in 50 mM Pipes-Cl (pH 7.5) with 0.15 M NaCl. The TfR protein was concentrated at 22°C by filtration in Ultra 15 10,000-Da cutoff filters (Millipore, Billerica, MA) and stored at 4°C.

**Capsid binding assays.** These assays were performed in two ways. In most cases the purified receptor ectodomains were bound to polystyrene plates, blocked with 0.5% ovalbumin, and then incubated with the virus in PBS (pH 7.0). In some cases the bound TfR was preincubated with pH 5.5 buffer for 30 min before being returned to pH 7.0 buffer. After incubation at 37°C for 1 h, the wells were washed and incubated with Fab from MAb 6, and the bound immunoglobulin G (IgG) was detected with goat anti-mouse Fab conjugated to horseradish peroxidase (HRP). ABTS ([2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)]) (Sigma, St. Louis, MO) was then added, and absorbance values were determined at 405 nm. To test the effect of carbohydrate composition on virus binding, the purified ectodomains of feline and canine TfR bound to polystyrene plates were incubated for 1 h with 5 U/ml of peptide N-glycosidase F (PNGase-F) (New England Biolabs, Beverly, MA) or 5 U/ml of sialidase from *Clostridium perfringens* (Sigma). After being washed with PBS, samples were incubated with viral capsids, and binding was examined as described above. To examine for the presence of N-linked carbohydrates, samples of the soluble feline and canine TfR and the mutant canine TfR-N838K/T385S were boiled for 10 min in sodium dodecyl sulfate (SDS) sample buffer, digested with 50 U of PNGase-F for 1 h at 37°C in the presence of 1% Nonidet P-40, and then electrophoresed in a 7.5% polyacrylamide gel electrophoresis (PAGE) gel. The proteins were detected by Western blotting with rabbit anti-TfR and then with anti-rabbit IgG as described above.

In a second solid-phase binding assay using a different orientation, the capsids (20 μg/ml) were bound to the plates, and then after blocking as above, dilutions of the feline or canine TfR were incubated with the wells. The bound TfR was detected with MAb T4F3 and then with anti-mouse IgG as described above.

**Capsid and TfR complex formation.** To estimate the sizes of the TfR-capsid complexes, FPV, CPV-2, or CPV-2b capsids were incubated with soluble feline TfR at a receptor-to-virus ratio of 30:1 for 30 min at 22°C and then passed through a 0.6- by 60-cm Sepharose CL-2B column in Pipes-Cl (pH 7.5) and 0.15 M NaCl. Protein was detected by \(A_{280}\), and the results for the mixtures were compared to those obtained for the virus or TfR alone. The amounts of the TfR and capsids in each fraction were determined by dot blotting samples onto nitrocellulose filters, along with samples of known amounts of capsids or feline TfR. The filters were incubated with rabbit anti-TfR, rabbit anti-CPV, anti-rabbit IgG HRP, and then the Supersignal substrate (Pierce Biotechnology, Rockford, IL) and the filters were exposed to X-ray film. The amounts of protein in each

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**FIG. 1.** Purification of the feline and canine TfR ectodomains from baculovirus cultures and locations of mutations analyzed. (A) Chromatographic analysis of the feline and canine TfR in a Sepharose S300 column, compared to protein standards of known sizes. Some aggregated protein was detected, particularly for the canine TfR, but the purified 150-kDa dimer was separated by this method. (B) Purified feline, canine, or mutant TfR proteins analyzed by SDS-PAGE and detected by Coomassie blue staining or by Western blotting with rabbit anti-TfR. (C) Sizes of the feline and canine TfR ectodomains and of the canine TfR-N838K/T385S and the effects of PNGase-F treatment of the proteins. The proteins were electrophoresed in a 7.5% SDS-PAGE gel and transferred to a membrane, and the TfR were detected with rabbit anti-TfR. (D) Structure of the human TfR ectodomain showing the locations of the changes in the feline or canine TfR which were tested for their effects on virus binding (Table 1). AU, absorbance units.
fraction were determined by scanning the films and comparing the protein concentrations with the standards using the quantitative functions of the program ImageJ.

**Virus and Tf labeling.** Canine Tf (Sigma) was iron loaded as previously described (4, 5). Purified CPV capsids, FPV capsids, or canine Tfs were labeled with Texas red (Molecular Probes, Eugene, OR), Cy2, or Cy5 (Amersham Biosciences, Piscataway, NJ) as previously described (26).

**Virus binding to feline or canine TfR expressed on mammalian cells.** The intact feline or canine TfR were expressed from plasmids in TRVb cells by transfection as previously described (26, 48). Two days later the cells were incubated with either 10 μg/ml of purified virus capsids or Cy5-labeled canine Tf or with both ligands together. Some of the cells were fixed for 10 min with 4% paraformaldehyde prior to incubation with virus to prevent cellular endocytosis, and then after incubation virus was detected by staining the cells with Cy2-labeled MAb 8. Another set of cells was fixed as described above and permeabilized, and the total TfR expression was detected with anti-TfR-cyt antibody. The amounts of cell-associated virus, Tf, and antibody binding to the TfR were determined using a FACScalibur flow cytometer (Becton-Dickinson, San Jose, CA).

In some studies the cells were incubated for 1 h at 37°C with 5 U/ml PNGase-F or 5 U/ml sialidase. After the cells were washed, Cy2-labeled virus or Texas red-labeled Tf was added in the presence of the enzymes and incubated for a further 30 min. The cells were then fixed, and the cell-associated virus was examined by fluorescence microscopy.

**Soluble feline TfR effects on cell binding and infection.** To determine the effects of soluble feline TfR on virus binding to the TfR, we incubated 10 μg/ml of FPV, CPV-2, or CPV-2b capsids with purified feline TfR at a ratio of 1:30 for 1 h at 22°C and then added those mixtures to TRVb cells expressing the feline TfR from expression plasmids for 60 min at 37°C. The cells were then washed with PBS and fixed with 4% paraformaldehyde, and virus was detected as described above.

To measure the effects of soluble feline TfR on virus infection, FPV, CPV-2, or CPV-2b in culture medium (which contained 5 μg/ml of capsids) was incubated with feline TfR at a receptor-to-capsid ratio of 30:1 and then added to CRFK cells. After a 24-h incubation, cells were fixed with 4% paraformaldehyde in PBS for 10 min and then permeabilized with 0.1% Triton X-100 in PBS with 1% bovine serum albumin. Infected cells were detected by immunostaining with a Cy2-conjugated MAb against the minute virus of mice nonstructural protein-1 (92).

**RESULTS**

**TfR expression and binding.** The wild-type or mutant versions of the feline and canine TfR ectodomains were expressed in insect cells, and the dimeric protein was purified free of aggregates using nickel binding of the His-tagged proteins,
followed by gel filtration (Fig. 1A). The samples were largely free of contaminating proteins; the TIR was a dimer of ~150 kDa by gel filtration, and the monomeric protein showed the expected molecular size of ~75 kDa on denaturing SDS-PAGE (Fig. 1B). The proteins produced were glycosylated, with the canine TIR-N383K/T385S being slightly smaller than the wild-type canine TIR, and digestion with PNGase-F significantly reduced the sizes of all of the proteins (Fig. 1C). The locations of the mutations tested in the TIR are shown on the structure of the human TIR (Fig. 1D).

Capsid binding to purified feline and canine TIR ectodomains or their mutants was tested in solid-phase assays detecting the bound virus (Fig. 2A to C). Both FPV and CPV-2 bound at similar levels to the feline TIR in this assay, while CPV-2b capsids bound to much lower levels (Fig. 2A). None of the viruses bound significantly above background levels to the purified canine TIR in this assay (Fig. 2A). Of the mutant receptor ectodomains tested, feline TIR-L221S and TIR-L221K showed no binding to any of the viruses (Fig. 2B), while the canine TIR-N383K/T385S bound CPV-2 at a similar level, FPV to a lower level, and CPV-2b to a slightly higher level than the feline TIR (Fig. 2C). The canine TIR-del205N mutant showed little binding to any of the capsids (Fig. 2C). The low binding to the canine TIR was not due to a mutation in the baculovirus clones, as we sequenced the insert from the baculovirus after PCR amplification, and there were no differences from the canine TIR plasmid clone that allows virus binding and entry after expression in mammalian cells (26). These results were confirmed in a reversed assay, where the capsids were bound on the plate and incubated with the soluble TIR; this showed essentially the same levels of binding as the alternative assay, with similar levels for CPV-2 and FPV and significantly lower binding of CPV-2b (Fig. 2D).

To further examine the levels of binding of the TIR, we examined the same receptors expressed on the hamster-derived TRVb cells. The feline and canine TIR expression...
levels on the cells were similar when cells were tested by antibody staining for the TIR or for the ability to bind and endocytose canine Tf (Fig. 3A). Both the feline and canine TIR bound CPV-2 capsids at 37°C when expressed on live cells, although the cells expressing the feline TIR showed higher levels of binding and uptake compared to those expressing the canine TIR (Fig. 3A). The binding of CPV-2 capsids to the fixed cells expressing the canine TIR was also at a much lower level than to cells expressing the feline TIR (Fig. 3A and 4). When CPV-2 capsids were incubated with cells expressing the canine TIR, the virus bound and was taken into cells expressing high levels of receptor (Fig. 3B). We tested the cell binding and uptake of the three viruses, and as expected all bound and entered the cells expressing the feline TIR, while only CPV-2 and CPV-2b capsids bound and entered the cells expressing the canine TIR (26, 48). However, when capsids were incubated with fixed cells, binding was detected only for CPV-2, while FPV or CPV-2b capsids showed little binding above background (Fig. 4). To test the effects of differences in the canine TIR glycosylation site on virus binding, we treated feline or canine cells or TRVb cells expressing the feline or canine TIR with PNGase-F or sialidase and then incubated the cells with capsids, and the bound virus was detected by fluorescence microscopy. These treatments did not affect virus binding to the canine TIR, and we obtained the same results when treating the soluble canine TIR with the enzymes and testing for virus binding in the solid-phase assay (data not shown).

Effects of viral host range mutations on TIR binding. VP2 residues 93 and 323 are primary determinants of the canine host range of CPV (24). CPV-2 and FPV capsids containing reciprocal replacements of these mutations bound the purified feline TIR to the same levels seen for the wild-type capsids (Fig. 5). CPV-2 capsids containing both changes showed significantly reduced binding to the canine TIR-N383K/T385S. However, mutations of residues 93 and 323 did not alter FPV binding to that receptor (Fig. 5A), and none of the viruses tested showed detectable binding to the canine TIR (Fig. 5A).

Preexposure of the feline TIR to pH 5.5 prevented the binding of any of the capsids, but capsids already bound to the TIR were not eluted when the complexes were exposed to pH 5.5 (Fig. 6). To further define the nature of the feline TIR complexes with capsids, we analyzed mixtures at 30:1 ratios of TIR to capsids in a Sepharose CL-2B column, which can separate complexes up to 4 × 10^5 Da. For CPV-2 or FPV mixtures, a portion of both the capsids and TIR was found in the void volume of the column, which would therefore contain ~8 or more capsids. CPV-2b capsids did not appear to complex with the TIR in this assay. In addition to the peak at the void volume, smaller complexes were seen for both FPV and CPV-2, as well as peaks around the same size as the purified capsids without TIR (Fig. 7). More CPV-2 capsids were found in the large complexes, which may reflect the higher affinity of that virus. Testing for the relative amounts of virus and TIR by antibody dot blot analysis showed ratios of TIR to capsids of 5:1 to 16:1 in all virus-containing fractions eluted.

Receptor competition. Incubating soluble feline TIR with FPV or CPV-2 capsids before it was added to cells expressing the canine TIR reduced virus binding and uptake by ~80%, while the same amount of TIR had no effect on CPV-2b capsids (Fig. 8A). The soluble feline TIR incubated with the viruses had various effects on the viral infection of feline cells, as adding 30 TIR per capsid completely inhibited infection with FPV, reduced infection with CPV-2 by ~80%, but had little effect on infection with the CPV-2b virus (Fig. 8B).

DISCUSSION

The interactions between the capsids of FPV and CPV and the host TIR play diverse and complex roles in the biology of the viruses through effects on the virus and TIR structures and by mediating cell surface binding leading to endosomal uptake, trafficking, and infections; these interactions also control the canine and feline host ranges of the viruses. The acquisition of new natural host ranges by viruses leading to a new pandemic virus is a very rare event, and so the acquisition in this case of canine host range by the CPV ancestor is an important example of a virus successfully crossing a host range barrier to create a self-sustaining epidemic in a new host. It is now clear that this host jump involved the natural selection of an initial group of mutations that allowed the CPV-2 ancestor to bind the canine TIR, and then further selection in dogs resulted in the replacement of the original CPV-2 strain by the better adapted CPV-2a strain and its descendants (68).

In previous studies we have examined the interactions of the capsids with the naturally expressed receptors on feline and canine cells and also examined the binding to receptors expressed on the hamster TRVb cells from plasmids (24–26, 48). While the TRVb cell expression system recreates many of the features of the interactions seen for host cells, this approach does not allow the biochemical and biophysical features of the different interactions to be examined in detail. Here, we show that the purified components can indeed be used to analyze the interactions between the feline and canine TIR and the FPV, CPV-2, and CPV-2b capsids, as well as several mutant viruses.
and TfR that vary in these interactions. The results obtained give further important details of the interactions seen between the different capsids and receptors and lay the foundations for further studies of the biophysical bases of cell infection processes and variant host ranges.

The results confirmed that the feline TfR binds to all the viral capsids tested, and the continued binding at low pH is consistent with the finding that CPV-2 capsids and the feline TfR remain together in the endosomal system of the cells for long periods after uptake (24, 26, 48, 49, 76). However, these

![Graphs showing binding data](image1)

**FIG. 5.** (A) Binding of FPV, CPV-2, CPV-2b, or the viral mutants CPV-2-N93K/N323D or FPV-K93N/D323N to the purified ectodomains of the feline TfR, canine TfR-N383K/T385S, or the canine TfR bound to polystyrene plates. Capsids were detected with Fab from MAb 6, followed by an anti-Fab-HRP and substrate. Error bars indicate ±1 standard deviation in the data from five experiments. (B) The locations in the structure of the capsid of the mutations or natural variations tested here. The surface of the capsid is shown as a projection, and the position of the asymmetric unit of the capsid is shown as a triangle. Residues 93 and 323 differ between CPV and FPV and control canine host range and influence canine TfR binding, while residues 87, 300, and 305 differ between CPV-2 and CPV-2a/b viruses (along with residue 101, which is not surface exposed). OD, optical density.

![Graphs showing binding data](image2)

**FIG. 6.** Binding of FPV, CPV-2, or CPV-2b capsids to the purified feline TfR kept at neutral pH or pretreated for 60 min at pH 5.5 prior to incubating with virus in neutral pH buffer. Alternatively, capsids were bound to the TfR at neutral pH and then treated at pH 5.5 for 60 min before capsids detection. The bound capsids were detected with Fab from MAb 6 and anti-Fab conjugated with HRP. Error bars indicate ±1 standard deviation in the data from five experiments. OD, optical density.
studies showed distinct features and structural responses that indicated differences in the details of the binding of each TfR and virus. The first unexpected result was that there was only very low level binding of CPV-2 and CPV-2b capsids to the canine TfR ectodomain and that was similar to the level of binding to the same receptor expressed on cells, although sufficient to allow uptake into the cell and infection. Although the canine and feline TfR differ in ~11% of their amino acid sequences, the low level of binding of the canine TfR for CPV-2 and CPV-2b capsids and its inability to bind FPV are in large part determined by only a few differences in their apical domain sequences; indeed, the change of only residues 383 and 385 in the canine TfR (the feline TfR counterparts) allowed that receptor to bind FPV to levels similar to those seen for feline TfR binding and also increased binding of FPV and CPV-2b capsids. These residues make a potential glycosylation site on the canine TfR. However, although this site appears to be glycosylated, the increased binding appears to be due to the differences in amino acid sequence, since PNGase-F deglycosylation did not give any increased binding of CPV-2 or CPV-2b capsids.

FIG. 7. Chromatographic analysis of the sizes of mixtures of the feline TfR ectodomain and different virus capsids in a Sepharose CL-2B gel. Purified feline TfR was incubated for 30 min at 22°C with FPV or CPV-2 capsids at a 30:1 ratio and then passed through the column, and the A_{280} absorbance of the eluate was determined. The profiles of the TfR run without capsids and of the CPV-2 capsids without TfR are also shown; capsids of the other viruses ran at the same position. AU, absorbance units.

FIG. 8. Competition for capsid binding between the purified feline TfR ectodomain and the same TfR expressed on TRVb cells and the effects of soluble TfR on virus infection of cells. (A) Capsids of FPV, CPV-2, or CPV-2b were incubated with the cells with or without preincubation with 30 molecules per capsid of purified feline TfR for 60 min at 22°C. The virus or virus-TfR mixtures were incubated at 37°C for 1 h with TRVb cells expressing the feline TfR along with Cy5-labeled canine Tf. The amounts of cell-associated virus and Tf were determined by flow cytometry. The virus-specific mean fluorescence intensity (MFI) and the percentage of Tf- and capsid-bound cells in the upper right quadrants are indicated. (B) Effect of the soluble feline TfR on infection of CRFK cells by FPV, CPV-2, or CPV-2b. Virus inocula each contained equivalent amounts of viral capsids, and each inoculum was first incubated with a 60-fold excess of the purified feline TfR ectodomain for 1 h at 22°C and then with cells at 37°C. After 24 h the cells were fixed, and the percentages of cells infected were determined by immune staining for the NS1 protein and compared to equivalent control inoculations without TfR. Error bars indicate ±1 standard deviation in the data from three experiments.
CPV-2a, CPV-2b, and their variants infect cats while the CPV-2 does not replicate detectably (28, 29, 80, 82). A possible connection with feline TfR binding is suggested by our finding that the CPV-2b capsids bind to only very low levels in these studies and are not neutralized. Soluble TfR ectodomains shed from cells are found in the circulation of animals at levels that vary depending on their iron status (15, 30), and the feline TfR could therefore potentially inactivate CPV-2 in circulation. However, there is obviously not a simple relationship between feline TfR inactivation and cat infection, as the soluble feline TfR also inhibited FPV binding and infection of cells. Explanations would be that FPV circulates in a cell-associated form in cats, while CPV-2 circulates as a free virion (as it does in dogs) and therefore is readily inactivated (11, 43, 62). However, the comparative pathogenesis of the different viruses is not sufficiently well understood to allow us to determine the mechanisms involved.

These in vitro studies also revealed new details about the capsid interactions with the canine TfR which control canine host range. VP2 residues 93 and 32 are central canine host range differences between FPV and CPV-2 (12, 24). The finding that CPV-N93K/N323D capsids (with both the FPV changes in a CPV background) showed greatly reduced binding to the canine TfR-N383K/T385S indicates that mutant receptors retain properties which can discriminate among the host range determining residues of the capsids. The apical domain of the TfR is clearly involved in the interactions with all of the viruses, as changing feline TfR residue Leu221 to Ser or Lys prevented that receptor from binding any of the capsids. Although the feline TfR-L221K bound and endocytosed all of the viruses, as changing feline TfR residue Leu221 to Ser, the receptor binding acts as more than a simple tether and induces specific structures in the TVA receptor (72). The receptor binding acts as more specific ligand for multiple ligand recognition by the human transferrin receptor. PLOS Biol. 6858–6867.

Cell infections by many other viruses involve complex interactions between viral structural proteins and their receptors, including the major group of rhinoviruses and intercellular adhesion molecule 1 (7, 66), the minor group of rhinoviruses and very-low-density lipoprotein receptor (47), poliovirus and poliovirus receptor (8, 22), human immunodeficiency virus gp120 and CD4 (13, 32), and avian sarcoma and leucosis virus and the TVA receptor (72). The receptor binding acts as more than a simple tether and induces specific structures in the proteins that are required for cell infection by the viruses. The results for the FPV and CPV capsids suggest that receptor affinity, specific TfR-capsid interactions, and exposure to low pH are all involved in allowing cell infection and controlling viral host range, and the details of these interactions will be accessible using the purified components described here.

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