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Parvovirus Infection of Cells by Using Variants of the Feline Transferrin Receptor Altering Clathrin-Mediated Endocytosis, Membrane Domain Localization, and Capsid-Binding Domains

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The feline and canine transferrin receptors (TfRs) bind canine parvovirus to host cells and mediate rapid capsid uptake and infection. The TfR and its ligand transferrin have well-described pathways of endocytosis and recycling. Here we tested several receptor-dependent steps in infection for their role in virus infection of cells. Deletions of cytoplasmic sequences or mutations of the Tyr-Thr-Arg-Phe internalization motif reduced the rate of receptor uptake from the cell surface, while polar residues introduced into the transmembrane sequence resulted in increased degradation of transferrin. However, the mutant receptors still mediated efficient virus infection. In contrast, replacing the cytoplasmic and transmembrane sequences of the feline TfR with those of the influenza virus neuraminidase (NA) resulted in a receptor that bound and endocytosed the capsid but did not mediate viral infection. This chimeric receptor became localized to detergent-insoluble membrane domains. To test the effect of structural virus receptor interaction on infection, two chimeric receptors were prepared which contained antibody-variable domains that bound the capsid in place of the TfR ectodomain. These chimeric receptors bound CPV capsids and mediated uptake but did not result in cell infection. Adding soluble feline TfR ectodomain to the virus during that uptake did not allow infection.

The infection of cells by viruses and their replication and release are the result of a highly evolved series of interactions between the virus and its components and the cellular machinery. Cell entry and infection for many animal viruses initiate with receptor-mediated endocytosis, yet the importance of the specific receptor-capsid interactions or endocytic uptake mechanisms and the subsequent endosomal trafficking pathways appear to vary for different viruses and are often not well understood (56, 59, 71). Enveloped viruses that fuse their envelopes directly to the plasma membrane appear to use multiple receptors acting in concert or in series to allow the viral proteins to induce fusion (11, 74). Most nonenveloped viruses enter cells by endocytosis, which may be clathrin mediated, caveola mediated, or achieved through clathrin- and caveola-independent uptake mechanisms that include macropinocytosis and other less well defined processes (14, 43, 50, 56, 71). The receptor binding to the virus may simply tether it to the cell, allowing uptake, or there may be a more active process where the receptor induces structural changes in the virus (64). Receptor clustering and intracellular signaling may be required for infection, and some nonenveloped viruses engage multiple receptors which control different steps in the infection process (7, 47). For viruses that enter through endosomes, triggers for membrane penetration to allow infection can include struc-

tural changes in the viral proteins induced by receptor binding (64, 80), changes due to the low pH of the endosome (73), or cleavage of viral proteins by the activities of low-pH-dependent endosomal proteases (18).

After endocytosis the virions or their components may traffic through a variety of vesicular compartments before entering the cytoplasm, yet the importance of the endosomal uptake route and the subsequent trafficking of virions or their components are often not well understood. Variables could include the rate of uptake (whether the uptake is clathrin mediated, caveola mediated, or mediated through clathrin- and caveola-independent endosomes [71]), endosomal trafficking to recycling or degradative pathways, and the possible requirement of receptor signaling (24). The association of the receptor or virus with membrane structures of different compositions such as lipid rafts or microdomains may also change the rate and type of endocytosis, the receptor and ligand trafficking, and the membrane fusion or penetration (10, 56, 60). Infecting viruses or their components have been seen to become associated with early endosomes, caveolin-associated endosomes (caveosomes), the endoplasmic reticulum and Golgi compartments, the late endosome or multivesicular endosome, or the lysosome. However, the functional importance of that trafficking for viral infection is generally not well understood.

Canine parvovirus (CPV) and the closely related feline panleukopenia virus (FPV) are small nonenveloped viruses that replicate in the nuclei of cells and depend on cellular S phase for DNA replication. CPV and FPV both use the feline transferrin receptor (TfR) for the binding and infection of feline cells (54), and specific binding of CPV to the canine TfR is associated with the CPV infection of dogs and dog cells, since

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FPV does not bind that receptor (28). The TfR is a type II membrane protein which is expressed on the surface of cells as a homodimer (19). The structure of the human TfR ectodomain shows that each monomer is made up of a protease-like domain, an apical domain, and a helical domain which forms most of the dimer interface (8, 35). The TfR ectodomain is attached to a 32-residue stalk which holds the receptor about 30 Å above the plasma membrane (19), and the human TfR has a 28-residue transmembrane sequence and a 61-residue cytoplasmic domain (32). Tf binding is determined primarily by the TfR helical domain (9, 21, 87), while CPV and FPV binding to the feline or canine TfRs is controlled by interactions between the apical domain of the TfR and a raised region of the capsid surrounding the threefold axis of icosahedral symmetry (the threefold spike) (27, 52).

The TfR-Tf complex is a model for the endocytosis and recycling of receptor-ligand complexes; it is excluded from lipid raft domains in the plasma membrane and is taken up rapidly from the cell surface via clathrin-mediated endocytosis (44). The TfR is constitutively endocytosed although the rate of recycling is increased after Tf binding, and the TfR and Tf recycle efficiently back to the cell surface through vesicular pathways (20, 25, 40, 65, 69, 81, 82, 86). The TfR-Tf complex passes through the early or sorting endosome and then recycles back to the cell surface in a fast process dependent on Rab4 or more slowly through a perinuclear recycling compartment, which is dependent on Rab11 (25, 69). Each TfR may recycle up to 100 times, and the rate of recycling depends on the oligomeric state of the ligand: monomeric Tf recycles in 4 to 10 min, while 10-mer oligomeric Tf is retained in the endosomal system for 60 min or more, and a proportion of the oligomeric Tf-TfR complex is transported to the lysosome and degraded (39, 93). The uptake and recycling of the TfR depend on sequences in the cytoplasmic domain. The tyrosine in the sequence Tyr-Thr-Arg-Phe (YTRF) within the cytoplasmic sequence of the TfR binds adaptor protein 2 (AP-2) (13) via its $\mu 2$ subunit (51), leading to association with the clathrin endocytosis machinery and rapid uptake (29, 34). Mutation or deletion of the YTRF motif in the human TfR reduced the rate of Tf uptake by greater than 80% (13).

Several cell entry and infection steps of CPV capsids have been defined (53, 76–78, 83, 85). CPV capsids bind the TfR on the surface of cells, and in most cases that binding allows entry and infection (28, 54). However, strains of CPV show differences in binding to feline and canine cells, suggesting that some bind additional receptors on some host cells (28). Some combinations of mutant TfR or mutant virus can bind to each other without leading to infection, indicating that the success of the infection process requires specific TfR-capsid interactions (27, 52). The capsids are rapidly endocytosed in a process that can be inhibited by dominant-negative dynamin II mutants or by drugs inhibiting endosomal acidification (53). After cell entry the capsids remain in perinuclear vesicles for up to several hours (53, 78). The capsids also remain functionally associated with the TfR in the endosomes, as it has been shown that infection could be reduced by injecting antibodies binding the cytoplasmic sequences of the TfR 4 h after virus uptake (54). The capsids leave the endosomes by an unknown mechanism, then traffic through the cytoplasm to the nuclear pore, and enter the nucleus for replication (84). Transport from the cytoplasm into the nucleus may occur slowly and require the release of N-terminal sequences of VP1 proteins from within the capsid (77, 84, 85).

Here we use mutant and chimeric feline TfRs to examine their effects on CPV endosomal transport and cell infection. We also prepared chimeras between the binding domains of antiviral antibodies and the feline TfR sequences so as to form receptors with different binding sites that could still bind and endocytose the virus.

MATERIALS AND METHODS

Viruses and cells. CPV and FPV were prepared from infectious plasmid clones, grown in feline NLFK cells, and titrated in those cells by using 50% tissue culture infective dose (TCID$_{50}$) assays as previously described (55). Capsids were purified by precipitation with polyethylene glycol 8000 followed by sucrose gradient centrifugation; then they were dialyzed against either phosphate-buffered saline (PBS) or 20 mM Tris-HCl (pH 7.5) and stored at 4°C (1). Capsids and iron-loaded canine Tf (Sigma, St. Louis, Mo.) were labeled with Cy3.5 or Cy5 (Amersham Biosciences, Piscataway, N.J.) as described previously (28). Chinese hamster ovary-derived cells lacking the hamster TfR (TRVb cells) (41) were grown in Ham's F-12 medium containing 5% fetal bovine serum.

TfR clones, mutants, and chimeras. Clones of the cDNA of the feline TfR were prepared in the pcDNA3.1(−) vector (Invitrogen, Carlsbad, Calif.) and expressed under the control of the cytomegalovirus immediate early promoter (28, 54). The cytoplasmic domain sequence of the feline TfR shares features with the cytoplasmic and transmembrane sequences of the human TfR (Fig. 1A). We used the information derived for the human TfR to design mutants of the feline TfR that were likely to alter the receptor endocytosis and infection. The mutant, deleted, and chimeric receptors are diagrammed in Fig. 1B. Mutants or deletions of the feline TfR were prepared directly in the expression plasmid by using the Gene Editor system (Promega, Madison, Wis.). A control mutant feline TfR with residue Leu 221 replaced by Lys (feline TfR L221K) binds CPV but not FPV and does not mediate cell infection (52). Mutants constructed to examine specific receptor endocytosis (TfR-ATAA, TfR-Δ3-32, and TfR-75-81-83) were predicted to affect uptake and trafficking of the Tf and TfR complex (12, 13, 93). The mutant TfR-ATAA had three amino acids in the YTRF internalization motif that were replaced with alanines (Ala-Thr-Ala-Ala), while in the TfR-Δ3-32 mutant residues 3 to 32 were deleted by digesting the feline TfR expression plasmid with XhoI and PshaI and then joining the sites with a DNA adaptor linking codons 2 and 33. Mutant TfR-75-81-83 had three polar residues introduced into the transmembrane sequence in place of Ala 75, Leu 81, and Gly 85, similar to mutants of the human TfR that resulted in increased Tf degradation (93).

The NA-TfR was a chimera between the N-terminal cytoplasmic and transmembrane sequences of the influenza WSN virus neuraminidase (NA) and the feline TfR ectodomain and stalk domain. The chimera was constructed by replacing the first 93 amino acids of the feline TfR with the first 35 amino acids of the NA sequence (31, 33).

Two chimeras fusing the feline TfR cytoplasmic tail, transmembrane, and stalk sequences with antibody single-chain variable fragments (scFv) were prepared by linking feline TfR residues 1 to 122 with the heavy and light chain variable domain sequences derived from antiviral monoclonal antibody (MAb) 8 or MAb 14 linked by a poly-Gly-Ser linker sequence (91). MAb 8 and scFv8 react with both CPV and FPV, while MAb 14 and scFv14 react only with CPV (91).

Baculovirus expression. To prepare the soluble ectodomain of the feline TfR, an N-terminal sequence containing a six-His sequence and a baculovirus gp67 secretion sequence was fused to the feline TfR sequence after residue 120 and expressed in insect cells by using a baculovirus vector as described for the human TfR (36). After the infection of *Trichoplusia ni* (High Five) cells for 4 days, the culture supernatant was dialyzed against 50 mM Tris-HCl (pH 7.5) with 150 mM NaCl; then the TfR ectodomain was isolated on nickel-nitrilotriacetic acid (NTA) resin (QIAGEN, Valencia, Calif.) and eluted with 100 mM imidazole. The receptor-containing fractions were dialyzed against PBS and stored at 4°C.

Receptor expression and membrane localization. Receptors were expressed in TRVb cells by the transfection of plasmids with Lipofectamine reagent (Invitrogen) (54). Cell lines stably expressing receptors were prepared by the selection of cells with 400 μg of G418/ml; then cell clones were picked. The surface expression binding of receptors containing the TfR ectodomain was determined by the incubation of the cells with both CPV capsids (10 μg/ml) and Cy5-labeled iron-loaded Tf in Dulbecco's modified Eagle's medium (DMEM) with 1% bo-
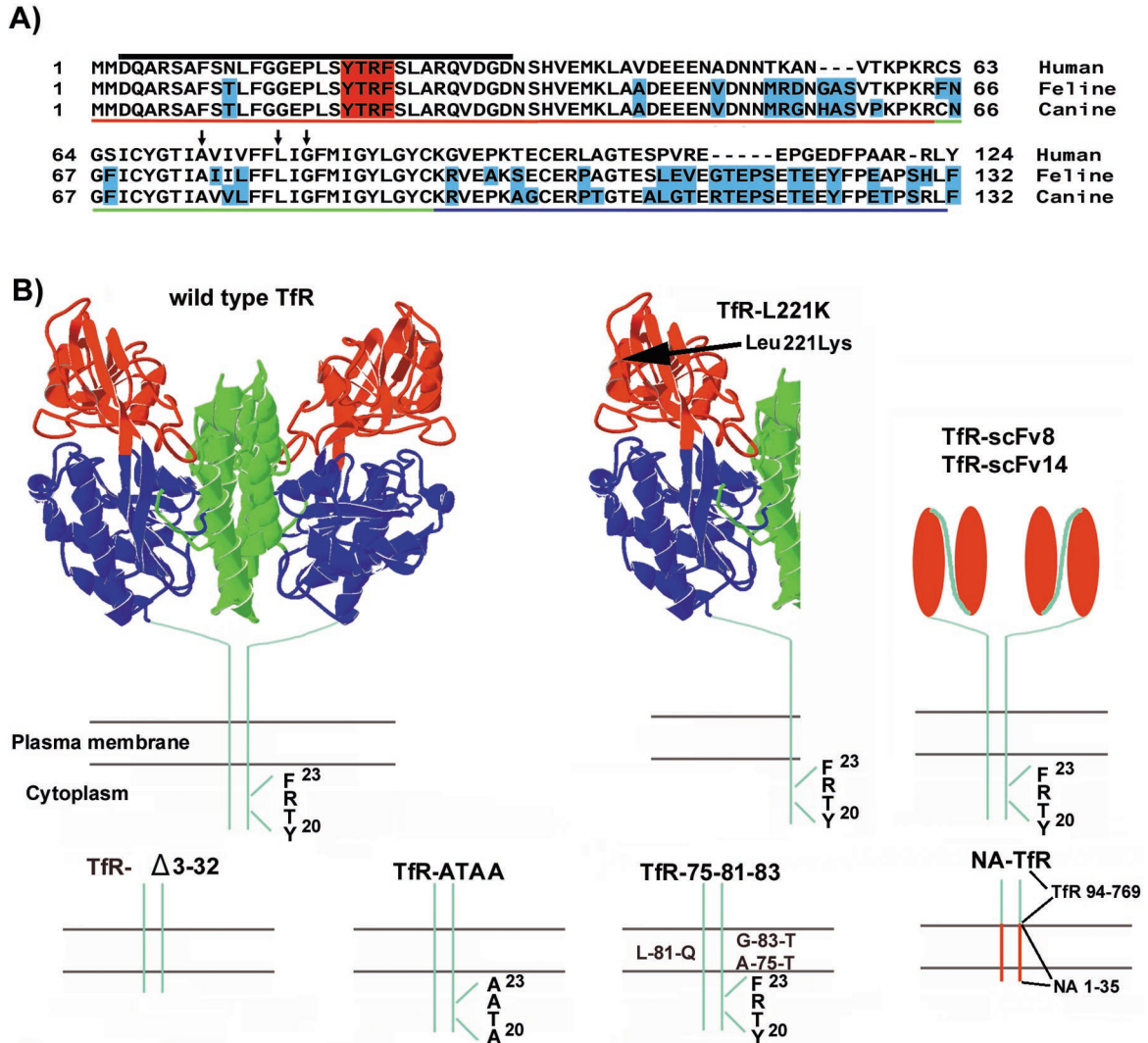
A)

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1  MMDQARSAFSNLFGGEPLSYTRFSLARQVDGDNSHVEMKLAVDEEENADNNTKAN---VTKPKRCS  63   Human
1  MMDQARSAFSTLFGGEPLSYTRFSLARQVDGDNSHVEMKLAADEEENVDNNMRDNGASVTKPKRFN  66   Feline
1  MMDQARSAFSTLFGGEPLSYTRFSLARQVDGDNSHVEMKLAADEEENVDNNMRGNHASVPKPKRCN  66   Canine

64  GSICYGTIAVIVFFLIGFMIGYLGYCKGVEPKTECERLAGTESPVRE-----EPGEDFPAAR-RLY  124  Human
67  GFICYGTIAIILFFLIGFMIGYLGYCKRVEAKSECERPAGTESLEVEGTEPSETEEYFPEAPSHLF  132  Feline
67  GFICYGTIAVVLFFLIGFMIGYLGYCKRVEPKAGCERPTGTEALGTERTEPSETEEYFPETPSRLF  132  Canine
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FIG. 1. Mutant or chimeric receptors analyzed in this study and comparison of the TfR sequences mutated. (A) Aligned sequences of the cytoplasmic, transmembrane, and stalk domains of the human, feline, and canine TfRs. The YTRF internalization motif is shaded in red. Differences from the human TfR are shaded in blue for the feline and canine receptors. Red underlining, cytoplasmic sequence; green underlining, transmembrane sequences; blue underlining, stalk sequences. The arrows indicate residues mutated in TfR-75-81-83, and the black line above the cytoplasmic sequences indicates the deleted residues in TfR-Δ3-32. (B) A model of the human TfR ectodomain structure is shown, along with diagrams of the altered regions of the other receptors. In the TfR-Δ3-32 mutant, residues 3 to 32 of the cytoplasmic domain of the feline TfR were deleted. Three residues of the YTRF internalization motif were changed to Ala in TfR-ATAA. In the TfR-75-81-83 mutant, three nonpolar amino acids in the transmembrane domain were replaced by polar residues. Chimeric receptors prepared included an NA-TfR chimera, which had residues 1 to 35 of the influenza virus NA fused to residues 94 to 769 of the TfR, and TfR-scFv8 and TfR-scFv14, which contained residues 1 to 122 of the feline TfR fused to the variable domains derived from MAb 8 and MAb 14, respectively. In the previously described mutant TfR-L221K, a Leu at position 221 was replaced with a lycine; this mutant receptor binds capsids but does not mediate efficient infection (52). The colors in the TfR model indicate the different domains defined for the human receptor as follows: blue, protease-like domain; red, apical domain; and green, helical domain. For the scFv model, the red ovals represent the light and heavy variable domains of the antibodies, and the line connecting those domains indicates the linker peptide added during the production of the single chains.

vine serum albumin (BSA) for 1 h at 37°C, and then cell-associated capsids were detected by Cy2-labeled MAb 8 after cells were detached with EDTA and fixed with 4% paraformaldehyde (28). Cells expressing the TfR-scFv chimeric receptors were incubated with Cy5-labeled CPV capsids for 1 h at 37°C, and then after detachment and fixation the receptor was detected with MAb H68.4 against the cytoplasmic tail of the TfR (Zymed Laboratories Inc., South San Francisco, Calif.) (54, 88) in PBS containing 0.5% BSA with 0.1% Triton X-100. In all cases the cell-bound virus, Tf, and antibody binding to the TfR were quantified by using a FACScalibur flow cytometer, and the data were analyzed by using Cell Quest software (Becton Dickenson, San Jose, Calif.).

Uptake and intracellular transport of Tf and virus. Canine Tf was radiolabeled with [125]I (Dupont NEN, Boston, Mass.) by using Iodogen-coated beads (Pierce Biotechnology, Rockfort, Ill.), and the unbound iodine was separated from the labeled Tf with a PD10 column (Pharmacia Biotech, Uppsala, Sweden). The monomeric state of the labeled Tf was confirmed by chromatography through a Sephadex 200 column.

To examine the distribution of virus after binding and uptake, stable cell lines expressing the different receptors were incubated with 10 μg of CPV capsids per ml for 1 h at 37°C. After detachment with EDTA, the cells were fixed with 4% paraformaldehyde; then half of the cells were stained with Cy2-labeled MAb 8 in

PBS-BSA with 0.5% Triton X-100 for 20 min, and the remainder were stained in PBS-BSA with no detergent. The percentage of virus internalized was estimated by comparing the total cell-associated virus in the Triton X-100-treated cells with that seen in the nonpermeabilized cells.

To examine the rate of Tf uptake from the cell surface, cells transiently transfected and expressing the different receptors were seeded on coverslips and then incubated with ^{125}I-labeled canine Tf on ice for 30 min. The cells were washed two times with cold DMEM-BSA and then transferred to DMEM-BSA at 37°C. After 0, 3, 5, 10, 20, or 30 min, the coverslips were removed, the cells were washed twice with 0.2 M acetic acid in 0.5 M NaCl for 3 min on ice, and then the proportion of the radioactivity that remained cell associated (intracellular) was determined.

Association of the wild-type TfR or NA-TfR chimera with detergent-insoluble membranes. TRVb cells transiently expressing the feline TfR or NA-TfR were incubated on ice with ^{125}I-labeled canine Tf in DMEM-BSA for 30 min. To disrupt lipid rafts, the cells were preincubated with 5 mM methyl-β-cyclodextrin for 1 h at 37°C and then were incubated with ^{125}I-labeled canine Tf in the same medium. Cells were then washed three times with ice-cold DMEM-BSA and extracted for 10 min on ice with PBS containing 1% Triton X-100 and 2× complete protease inhibitor cocktail (Boehringer, Ingelheim, Germany). The cells were then lysed with 1 M NaOH, and the amount of ^{125}I in the extracted and nonextracted fractions was counted.

Degradation of Tf after endocytosis. The degradation of Tf bound to the feline TfR or TfR-75-81-83 mutant was measured in an assay similar to that described by Zaliauskiene et al. (93). TRVb cells transiently expressing those receptors were incubated with ^{125}I-labeled canine Tf in DMEM-BSA for 1 h at 37°C, washed twice with cold DMEM-BSA, and then incubated for 2 h at 37°C in DMEM with 0.1% BSA containing 50 μg of unlabeled Tf per ml. The proteins in the medium were then precipitated in 10% trichloroacetic acid (TCA), and the proportion of the released radioactivity that became TCA soluble was determined as a measure of Tf degradation.

Microscopic analysis of virus localization. TRVb cells were cotransfected with each of the plasmids expressing wild-type or mutant receptors and a plasmid expressing Rab11 fused to green fluorescent protein (Rab11-GFP). The cells were seeded on coverslips the next day; 24 h later they were incubated with Cy3.5-labeled virus for 30, 60, or 120 min at 37°C, washed in cold PBS, fixed on ice with 4% paraformaldehyde in PBS, and examined by confocal microscopy. Confocal sections collected from the center of the cells were analyzed using the Olympus Fluoview software (Melville, N.Y.).

Infection assays. TRVb cells transiently expressing the wild-type feline TfR or mutant TfRs or transfected with the empty pcDNA3.1(-) vector were seeded on coverslips and inoculated for 1 h at 37°C with between 0.001 and 1 TCID$_{50}$ per cell of CPV and then incubated at 37°C for 24 h in growth medium. The cells were then fixed with 4% paraformaldehyde and stained with Cy2-labeled anti-NS1 antibody; the infection rate was determined as the percentage of infected cells expressing each receptor. The transfection rate was determined by the analysis of a parallel culture of the same transfection reaction tested for receptor expression by flow cytometry.

Effect of soluble TfR on infection mediated by nonfunctional receptors. The TfR-scFv8, TfR-scFv14, and TfR-L221K receptors bound virus to cells but did not mediate infection. To determine whether exogenously added feline TfR might compensate for the lack of correct binding, cells expressing these receptors were inoculated with an excess (10 μg/ml) of virus in the presence of 15, 7.5, 3.7, or 1.25 μg of the baculovirus-expressed feline TfR ectodomain per ml, resulting in molar ratios of capsids to receptor of 1:60, 1:30, 1:15, and 1:5, respectively. Viruses were either mixed with the receptor and added to the cells and incubated at 37°C, or virus was incubated with the cells at 4°C for 30 min; then the soluble receptor was added, and cells were incubated for a further 30 min at 4°C. After 24 h at 37°C, the cells were fixed and stained for NS1 protein expression. To confirm that the soluble ectodomain bound the capsids, the TfR domain was bound to enzyme-linked immunosorbent assay plates in twofold dilutions, and each plate was blocked with PBS-BSA containing 0.05% Tween 20 and then incubated with dilutions of CPV capsids. The virus binding the TfR on the plate was detected with rabbit polyclonal anti-CPV antibody and anti-rabbit immunoglobulin G conjugated to horseradish peroxidase, and bound antibody was detected with 2,2'-azinobis(3-ethylbenzthiazoline sulfonic acid) substrate.

RESULTS

Cell infection by CPV initiates with uptake from the cell surface in association with the TfR, and the capsid then traffics within the endosomal systems of the cell before being released
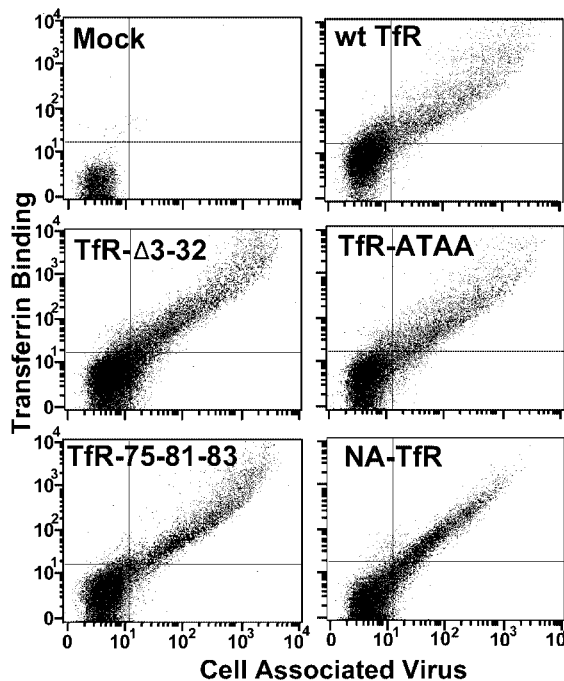

FIG. 2. Flow cytometric analysis of the binding of CPV capsids and canine Tf to TRVb cells transiently expressing the feline TfR or receptors with altered transmembrane or cytoplasmic sequences. Cells were incubated with Cy5-labeled Tf and CPV together for 1 h at 37°C, and then after detachment from the tissue culture dishes and fixation, the cell-associated CPV was detected with Cy2-labeled MAb 8. *y* axis, Tf binding; *x* axis, capsid binding.

into the cytoplasm for transport to the nucleus. However, it is not known which of the receptor binding or endosomal transport steps are required for mediating viral infection. To start to define the important receptor-mediated events in cell infection, we prepared mutants of the feline TfR which would change three components in the infection process: the rate of endocytic uptake, the membrane domain association, and the extracellular capsid binding domain (Fig. 1). The mutant receptors which contained the TfR ectodomain were transported to the cell surface at levels similar to the level of the wild-type TfR, and all of the receptors bound both Tf and CPV capsids (Fig. 2). The level of expression of the NA-TfR chimera was slightly altered compared to the level of other receptors, as fewer cells showed the highest levels of binding to virus and Tf (Fig. 2), perhaps due to differences in the expression or trafficking of the receptor during synthesis or to altered recycling.

Effects of altered rate of uptake. All mutations that changed or removed the tyrosine-based motif in the cytoplasmic domain (TfR-ATAA, TfR-Δ3-32, and NA-TfR) would prevent the association of the receptor with AP-2, and any uptake would be by nonclathrin-mediated uptake. Compared to clathrin-mediated uptake levels, these receptors all had their initial rate of uptake from the cell surface reduced by about 85% (Fig. 3A) and also showed lower levels of steady-state intracellular Tf after 30 min of incubation (Fig. 3B), as was seen for the human TfR with similar changes (13). The reduced internalization confirms the expected phenotypes of the receptors with a reduced endocytosis rate due to a lack of interaction between the
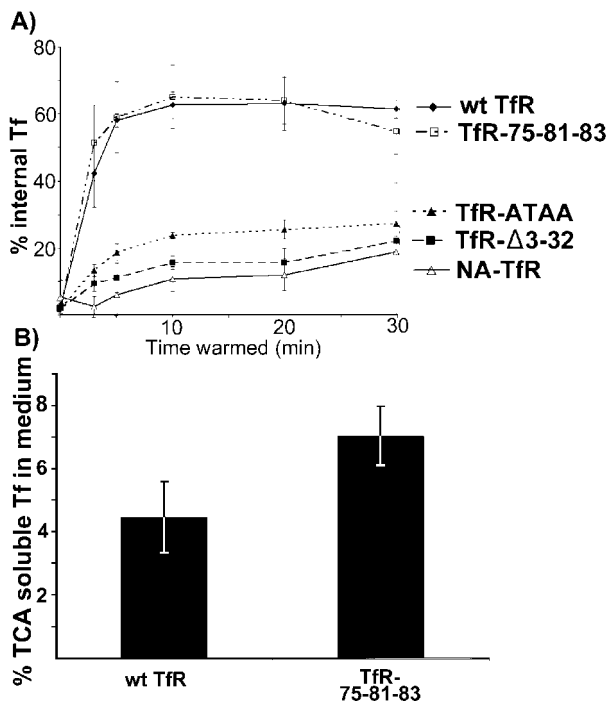
FIG. 3. Distribution and uptake of Tf bound to TRVb cells expressing the wild-type or mutant feline TfRs. (A) Rate of ^{125}I-labeled canine Tf uptake into TRVb cells expressing various mutant receptors. Tf was incubated with the cells on ice, and then after warming to 37°C for various times, the extracellular Tf was removed by acid wash. The data are shown for three independent experiments ±1 standard deviation. (B) Degradation of Tf in TRVb cells expressing TfR-75-81-83 or wild-type TfR. Cells were incubated with ^{125}I-labeled Tf at 37°C for 1 h, washed on ice, and then incubated for a further 2 h at 37°C. The protein released into the supernatant was precipitated by using 10% TCA, and the amounts of TCA-soluble and insoluble radioactivity were counted. Data are for three independent experiments ±1 standard deviation. wt, wild type.
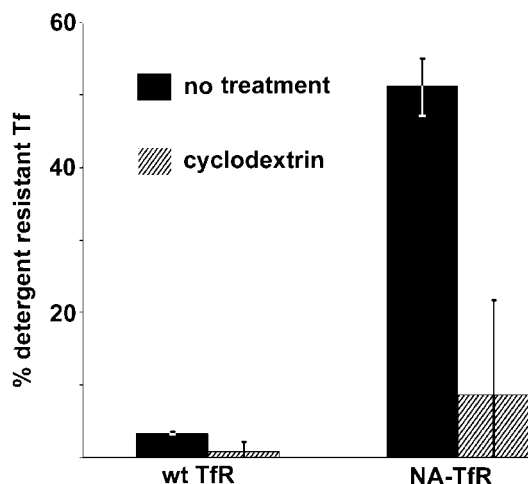

FIG. 4. Association of wild-type (wt) TfR and NA-TfR with detergent-insoluble lipid rafts. TRVb cells expressing either feline TfR or NA-TfR were incubated with ^{125}I-labeled Tf for 30 min on ice and then extracted with PBS containing 1% Triton X-100. The radioactivity in the extracted supernatant and in the cells was measured. To disrupt lipid rafts the cells were pretreated with 5 mM methyl-β-cyclodextrin prior to incubation with the labeled Tf. The data are shown for three independent experiments. Error bar, ±1 standard deviation.

receptor and AP-2. However, in the absence of clathrin-mediated uptake, receptors are endocytosed by other mechanisms (14), and these vesicles generally intersect with the same endocytic pathways (15, 30).

The human TfR with mutations equivalent to those in TfR-75-81-83 showed less efficient recycling (93). When the degradation of ^{125}I-labeled canine Tf endocytosed by that receptor was examined by testing for release of TCA-soluble Tf fragments after 2 h, the cells expressing the TfR-75-81-83 mutant showed a modest increase in TCA-soluble released radioactivity to 7%, compared to 4.5% for the wild-type TfR, although that difference was not statistically significant (Fig. 3B).

The human TfR is a classical non-raft domain-associated receptor, and feline TfR also proved to be non-raft-associated, as >95% of the ^{125}I-labeled canine Tf bound to the feline TfR could be extracted by 1% Triton X-100 at 0°C, while only ~50% of the Tf bound to the NA-TfR was extracted under the same conditions (Fig. 4) (4, 33). The resistance to extraction by cold Triton X-100 was largely eliminated by treatment of the cells with the cholesterol-depleting drug methyl-β-cyclodextrin, which reduced the Triton X-100-resistant Tf to around 10%, close to the levels of the wild-type TfR (Fig. 4).

Examining the uptake and endocytosis of virus in TRVb-

derived cell lines expressing mutants TfR-Δ3-32, TfR-ATAA, and TfR-75-81-83 showed that the internalization of the virus in 1 h was 70% for the wild-type TfR and 60% for the TfR-75-81-83 mutant, but that level was reduced to ~35% for the mutants with changes which decreased the rate of Tf uptake (Fig. 5). Multiple attempts failed to generate stable cell lines expressing levels of the TfR-scFv chimeras or the NA-TfR fusion proteins high enough for detection; therefore, this assay could not be performed for these receptors. The reduction in the intracellular virus taken up with the TfR-Δ3-32 and TfR-ATAA mutants was not as great as that seen for the canine Tf
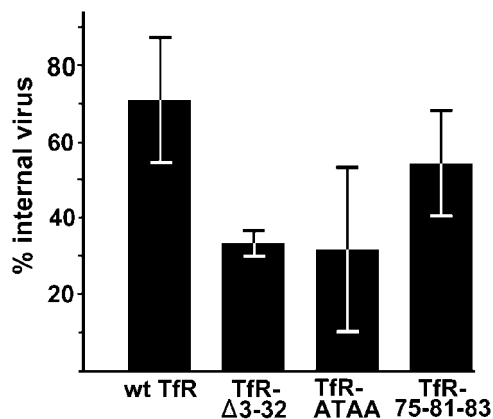

FIG. 5. Cell surface and intracellular distribution of capsids in TRVb cell lines stably expressing the wild-type (wt) or mutant feline TfR. The cells were incubated for 1 h at 37°C with CPV capsids and then fixed. Cell-associated virus was detected with Cy2-labeled MAb 8 in Triton X-100-permeabilized or nonpermeabilized cells. The ratio of total virus and surface-accessible virus was determined by flow cytometry. Data are for three independent experiments ±1 standard deviation.

(Fig. 3A). This result is likely to be due to the differences in receptor-ligand interaction and recycling in each case, since the Tf binds as a monomer and most of it recycles, while the capsid contains multiple TfR binding sites (possibly up to 60), resulting in receptor clustering that would both alter the endocytosis of the receptor and reduce any recycling to the cell surface (39).

In addition, after incubation for 30 min at 37°C, there were differences in the cellular localization of CPV capsids. In cells expressing the feline TfR and TfR-75-81-83 along with Rab11-GFP, virus and Rab11 were colocalized in perinuclear vesicles (Fig. 6). In contrast, the TfR-Δ3-32, TfR-ATAA, and NA-TfR mutants showed little Rab11 colocalization at that time point, and much of the virus was on the cell surface or in the periphery (Fig. 6). At later times (60 and 120 min) after uptake, some virus endocytosed by TfR-Δ3-32, TfR-ATAA, and NA-TfR was seen to colocalize with Rab11, although most of the capsids were still not in the Rab11 compartment (data not shown).

After transient expression in TRVb cells and inoculation with various titers of CPV, the TfR-Δ3-32, TfR-ATAA, and TfR-75-81-83 mutants all allowed CPV infection at levels to similar to the level for the wild-type TfR (Fig. 7). In contrast, cells expressing NA-TfR were only infected to very low levels, with only 4% as many cells infected compared to cells expressing the wild-type TfR (Fig. 7).

Antibody binding domain-TfR chimera characterization. To examine the role of the ligand binding domain of the receptor, we prepared chimeras of the cytoplasmic, transmembrane, and stalk sequences of the feline TfR with the binding domains of two different MAbs, TfR-scFv8 and TfR-scFv14 (Fig. 1B). These MAbs were compared to the wild-type feline TfR and to a feline TfR with a mutation in its apical domain (TfR-L221K) which showed reduced binding to CPV and very low levels of cell infection (52). The TfR-scFv chimeric receptors did not bind Tf, and their expression was therefore determined by staining with an antibody against the TfR cytoplasmic sequence (Fig. 8A). These receptors were all expressed in cells to similar levels, but TfR-scFv8 and TfR-scFv14 both showed lower levels of virus binding compared to the level of the wild-type TfR, indicating that they did not bind with the same affinities. The apical domain mutant of the feline TfR (TfR-L221K) showed CPV binding only when the receptor was expressed at levels 10-fold higher than either the wild type or the TfR-scFvs (Fig. 8A). The antibody detection of the TfR cytoplasmic sequences was not sensitive to the lowest level of receptor, as some cells showed virus binding but did not show receptor expression. These cells must, however, express the receptors, as mock-transfected cells never showed any virus binding (Fig. 8A). MAb 8, which was used to prepare scFv8, bound to both CPV and FPV, while MAb 14, used to prepare scFv14, bound to only CPV and not FPV (91). We therefore tested for the specificity of virus binding, and the TfR-scFv8 bound both CPV and FPV, while TfR-scFv14 bound CPV but not FPV (Fig. 8B). No infections were detected in cells expressing TfR-scFv8 or TfR-scFv14, while only low levels of infection were seen in cells expressing TfR-L221K (Fig. 9); adding soluble feline TfR ectodomain to the inoculations did not change the result (data not shown).

DISCUSSION

The process of cell infection is a subject of investigation for many nonenveloped viruses. For the parvoviruses and adeno-associated viruses, many studies have shown that uptake and infection require receptor-mediated endocytosis, but the details of the later steps in the infection pathway and the roles played by the endosomal trafficking and processing of the capsids are less clearly defined. For CPV the infection pathway that uses TfR initiates with rapid uptake from the cell surface, and at later times the CPV capsids are seen in both recycling endosomes and late endosomes, where they colocalize with TfR and Tf or with late endosomal markers such as Lamp2 (53, 78, 83). The endosomal processing of CPV capsids leading to infection can be slow, and the virus is retained in the endosomal system for several hours prior to entering the cytoplasm; thus, antibodies binding the cytoplasmic sequence of the TfR could block many infections by CPV in feline cells even when they are injected 4 h after uptake of the virus (54).

Here we sought to define the cellular controls of cell infection by CPV by taking advantage of the properties of the TfR, anti-CPV antibodies, and CPV capsids to manipulate the receptor binding and endosomal entry steps of the infection pathway. The TfR and mutant receptors were expressed in the Chinese hamster ovary-derived TRVb cells, which normally do not bind any virus and completely resist infection. After transfection with plasmids expressing the TfR, over 20% of the transfected cells were infected by CPV, similar to the levels seen in nonsynchronized feline cells (28).

Here we showed that the rapid clathrin-mediated uptake of the TfR was not required for infection, since the TfR mutants with changes that would affect AP-2 binding still mediated infection to levels similar to the level of the wild-type TfR, despite the slow uptake of the receptors. The results parallel results reported for the uptake of other viruses where alternative modes of endocytosis can allow infection: influenza virus infection can occur via several different entry pathways (72), adenoviruses infect cells by using both clathrin-mediated uptake and macropinocytosis (42, 43), and Moloney murine leukemia virus infection is not affected when clathrin-mediated endocytosis is blocked (37).

The requirements for particular routes of endosomal trafficking of the viruses was not specifically examined in these studies. However, capsids entering through the wild-type TfR became colocalized with the Rab11-positive endosome within 30 min, while capsids entering with the TfR-ATAA and TfR-Δ3-32 mutants did not colocalize with Rab11 at 30 min, although some did colocalize by 60 or 120 min. However, entering the Rab11-positive endosome by itself was not sufficient to ensure cell infection, as both TfR-scFv8 and TfR-scFv14 transported capsids to that compartment but neither mediated infection (Fig. 6 and 9).

The TfR-75-81-83 mutant with additional polar residues in the transmembrane sequence was predicted to be diverted into the late endosomal and lysosomal compartment at a greater rate than the wild type, since for the human TfR with equivalent mutations 8% of the bound Tf became degraded in 1 h, compared to 2% for the wild type (93). We saw only small differences in the processing of TfR-75-81-83, as 7% of the Tf was degraded after uptake compared to 4.5% for the wild type
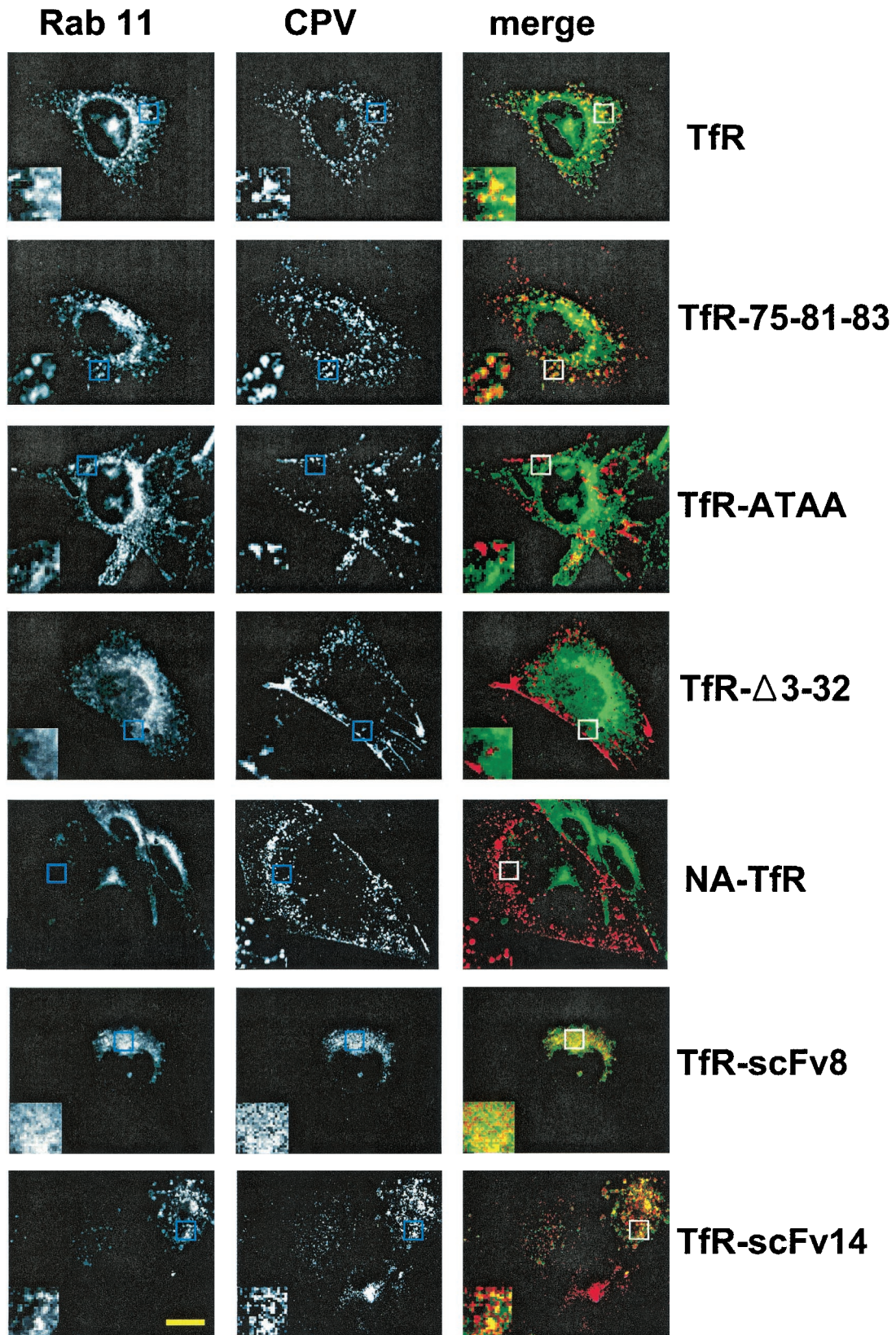
FIG. 6. Localization of CPV capsids in TRVb cells expressing the feline TfR or altered receptors and Rab11-GFP (green). Cells were incubated with Cy3.5-labeled capsids (red) for 30 min at 37°C and then fixed and examined by confocal microscopy. One section through the center of the cell body is shown. Scale bar, 10 μm. The inset boxes show threefold enlargements of a portion of each image.
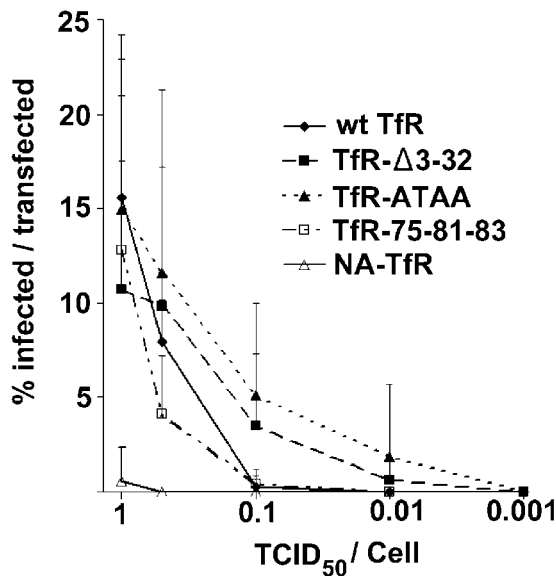
FIG. 7. Infection of TRVb cells expressing altered receptors containing the feline TfR ectodomain. Cells were inoculated with between 0.001 and 1 $TCID_{50}$ of CPV per cell for 1 h at 37°C and then incubated in growth medium for 24 h at 37°C. The proportion of cells expressing each TfR was determined by flow cytometry in parallel cultures. The data show the percentages of cells expressing the mutant TfR that became infected. Error bars represent data for at least five independent experiments ± standard deviations. wt, wild type.

(Fig. 3B). The level of uptake did not affect infection; the mutant receptor still allowed endocytosis of canine Tf and virus, the virus became colocalized with Rab11, and the cells were infected to levels similar to the level of the wild-type receptor.

In contrast to these findings, CPV infection mediated by the NA-TfR mutant was reduced to less than 5% of the level seen for the wild-type TfR (Fig. 9). While the wild-type TfR is a prototype non-raft-associated receptor, the NA sequences caused the TfR to associate with detergent-insoluble lipid rafts (Fig. 4), as predicted from the properties of the NA and from previous studies of the human TfR (4, 32). Lipid rafts are membrane domains enriched in cholesterol and sphingolipids (4, 26, 58). Various viruses can infect cells by using receptors associated with different membrane environments including those associated with lipids rafts, such as caveolae (38, 57). However, the effects of the alterations in membrane association have been examined in only a few studies. The avian sarcoma and leukosis virus can infect cells through either the transmembrane form of the Tva receptor or through a form that becomes glycosylphosphatidylinositol anchored. One study found that the glycosylphosphatidylinositol-anchored receptor became localized to raft domains in the membrane and that the rate of infection was slower but that the same number of infections occurred (46). Endocytic pathways initiating from lipid rafts may deliver their contents to the endoplasmic reticulum or Golgi complexes (45, 48, 49) or to a tubular-vesicular compartment that has been termed the caveosome (57), and some of these pathways intersect with those starting from clathrin-mediated endocytosis (68). The reasons for the lack of infection with the NA-TfR mutant could, therefore, include
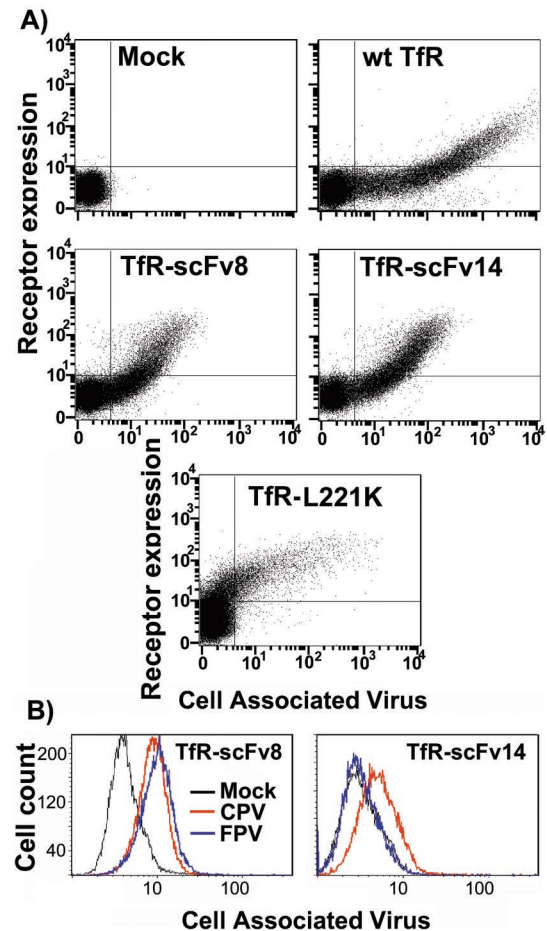

FIG. 8. Flow cytometric analysis of the binding of virus to cells expressing receptors with altered ectodomains. (A) CPV binding to TRVb cells expressing TfR-scFv8 or TfR-scFv14 or to the control feline TfR-L221K which has a mutation in the apical domain that affects capsid binding and infection. Cells were incubated with Cy5-labeled CPV for 1 h at 37°C and then detached; TfR expression was detected with a MAb against the cytoplasmic tail of the TfR. y axis, receptor expression; x axis, CPV binding; wt, wild type. (B) Specificity of capsid binding to TfR-scFv chimeras. Cloned cell lines expressing TfR-scFv8 or TfR-scFv14 constructs were incubated with 10 μg of CPV or FPV capsids per ml for 1 h at 37°C; after detachment the cells were fixed and permeabilized, and the cell-associated virus was detected with Cy2-labeled MAb 8. Mock, mock-transfected cells.

the possibility that the lipid environment of the receptor is unfavorable for virus penetration from the endosome, that the receptor-capsid complexes are transported to alternative endosomal compartments which do not provide the correct environment for infection, or that other unidentified factors required for CPV infection do not colocalize with the TfR-virus complex in the raft domains. In future studies we will examine these different possible effects to determine which one(s) changes the virus infection of the cell.

Other influences on the endosomal trafficking of CPV by the receptors with altered tails could include increased TfR transport after receptor clustering due to capsid binding, as is seen after oligomeric Tf binding (65), while the phospholipase A_2 activity of the VP1-unique region may alter the structure and function of endosomal compartments (16, 92). For other par-
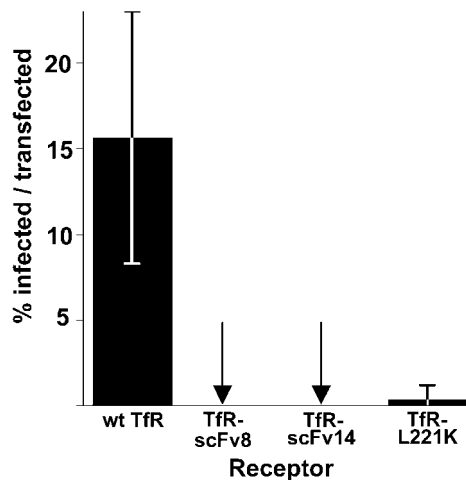
FIG. 9. Infection of TRVb cells expressing TfR-scFv8, TfR-scFv14, or TfR-L221K after inoculation with 1 $TCID_{50}$ per cell of CPV, as described in the legend of Fig. 8. The data are shown for three independent experiments ±1 standard deviation.

voviruses and adeno-associated viruses, the capsids appear to spend various periods of time in late endosomes, recycling endosomes, the Golgi compartment, and perhaps other compartments, but little is known about either the specific endosomal requirements for infection or the ability of the viruses to exploit alternative pathways (2, 6, 17, 63, 66, 67).

Antibody-derived receptor ligands do not allow virus infection. The TfR-scFv receptors were expressed on cells at levels similar to the level of the wild type TfR, and they bound CPV capsids and transported them into cells where they became colocalized with Rab11-positive compartments, but no cell infection resulted. Both of the antibodies used to prepare the chimeric receptors were neutralizing, and each associated with a binding site on the surface of the capsid in regions that are involved in binding to the canine TfR and, most likely, also to the feline TfR (23, 27, 75). From cryoelectron microscopic studies, MAb 8 is known to bind to the shoulder of the three-fold spike, while escape mutant analysis shows that MAb 14 binds near the top of that structure (27). The scFv of each antibody also neutralized CPV, although with an efficiency of 10% of that of the intact immunoglobulin G of the same antibody, but incubating the scFv with the capsids did not prevent CPV binding and uptake into feline cells (91). Changes in the interactions between the receptor and capsid that allowed binding to occur but that did not lead to infection have been seen previously for mutants of both the TfR and the virus (27, 52). For example, CPV and FPV can bind sialic acids on some host cells, but that binding is not required for infection. Indeed, passaging CPV type 2 in feline cells which express sialic acids that bind the capsid selects for viruses that no longer bind the sugar, suggesting that the sialic acid binding inhibits infection (3, 79). Differences in the TfR-scFv interaction with the capsid and that of the feline TfR ectodomain leading to the lack of infection could include binding at too high or low affinity to allow release at the appropriate point in the endocytic pathway or an intrinsic neutralization effect of the scFv, or the scFv may not induce the structural changes in the capsid needed for infection. Less virus was bound to cells

expressing similar levels of TfR-scFv compared to cells expressing the wild-type TfR (Fig. 8), suggesting a lower binding affinity to the antibody domains compared to the TfR. Adding exogenous soluble feline TfR to the virus during binding to the cells did not rescue infection of the virus by the TfR-scFv chimeras or the control TfR-L221K, indicating that if any structural effects of the receptor on the capsids were needed for infection, they could not be readily supplied in *trans*.

Compared to some other viruses, CPV appears fastidious in its receptor requirements for infection. Adeno-associated virus type 2 can use a variety of carbohydrate or glycoprotein receptors or capsid-receptor cross-linking systems to mediate cell transduction (5, 22, 61, 70, 89, 90). Foot and mouth disease virus can infect through an antiviral scFv fused to intracellular adhesion molecule 1 (62). In contrast, poliovirus requires the poliovirus receptor for infection, and binding to the N-terminal domain of the receptor leads to the changes in the capsid structure required for infection, including the loss of the VP4 protein and exposure of hydrophobic portions of VP1 (80).

These studies provide new insights into the importance of receptor binding, endocytic uptake mechanisms, and the intracellular trafficking of CPV and into their roles in mediating infection. In future studies we will examine in detail the steps of viral infection revealed by these altered receptors, focusing on the structural role of the receptor on the capsid and its role in infection, the alternative trafficking of the capsids within the cells after capsid entry, and the effects of the NA-TfR chimera on infection through the localization of capsids in alternative compartments such as the caveosome or the endoplasmic reticulum.

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