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Canine and feline parvoviruses preferentially recognize the non-human cell surface sialic acid *N*-glycolylneuraminic acid

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ABSTRACT

Feline panleukopenia virus (FPV) is a pathogen whose canine-adapted form (canine parvovirus (CPV)) emerged in 1978. These viruses infect by binding host transferrin receptor type-1 (TfR), but also hemagglutinate erythrocytes. We show that hemagglutination involves selective recognition of the non-human sialic acid *N*-glycolylneuraminic acid (Neu5Gc) but not *N*-acetylneuraminic acid (Neu5Ac), which differs by only one oxygen atom from Neu5Gc. Overexpression of α2-6 sialyltransferase did not change binding, indicating that both α2-3 and α2-6 linkages are recognized. However, Neu5Gc expression on target cells did not enhance CPV or FPV infection in vitro. Thus, the conserved Neu5Gc-binding preference of these viruses likely plays a role in the natural history of the virus in vivo. Further studies must clarify relationships between virus infection and host Neu5Gc expression. As a first step, we show that transcripts of *CMAH* (which generates Neu5Gc from Neu5Ac) are at very low levels in Western dog breed cells.

Introduction

Many pathogens mediate infections through receptor-binding proteins that recognize host sialic acids (Sias), resulting in either direct attachment of the microbe leading to uptake, or enhanced binding to the cells, allowing attachment to another ligand (or co-receptor) required for infection (Olofsson and Bergstrom, 2005). For example, influenza A and B viruses infect their hosts, including bird and humans, by recognizing Sias in α2-3 and/or α2-6 linkages (Nicholls et al., 2008; Skehel and Wiley, 2000), and several serotypes of adeno-associated viruses (AAVs) recognize Neu5Ac on human cells in specific linkages (Kaludov et al., 2001; Walters et al., 2001; Wu et al., 2006). Many members of the *Parvoviridae* also use host Sias as direct ligands for cell attachment or infection (Wu et al., 2006). For example, the minute virus of mice (MVM) binds to the common Sia *N*-acetylneuraminic acid (Neu5Ac) in α2-3 or α2-8 linkages, and some MVM strains differ in their infectivity and virulence due to changes in their Sia-binding properties (Nam et al., 2006; Lopez-Bueno et al., 2006). The Sia-binding properties of viruses are often conveniently demonstrated *in vitro* by the hemagglutination (HA) of erythrocytes, which are rich in cell surface Sias.

Feline panleukopenia virus (FPV) and canine parvovirus (CPV) are important pathogens of dogs and cats. While FPV has been known for many years as the cause of diseases in cats, mink and raccoons, CPV is a host range variant of FPV, which emerged as a pathogen of domestic dogs only in the late 1970s, and the host range variation was controlled by a small number of mutations within the capsid protein of the virus (Chang et al., 1992; Hueffer et al., 2003; Palermo et al., 2003). CPV is therefore a rare example of a virus that has extended its host range by mutation and become fully established as an epidemic virus in a previously resistant host (Parrish and Kawaoka, 2005).

The original isolates of CPV (termed CPV type-2; CPV-2) and FPV were shown to use the transferrin receptor type-1 (TfR) for primary binding and infection of host cells (Parker et al., 2001). However, the ability to hemagglutinate (HA) erythrocytes is a highly conserved function of all wild-type CPV and FPV isolates (Simpson et al., 2000) and this appears to involve Sia binding (Barbis et al., 1992; Tresnan et al., 1995). One difference between FPV and CPV is that while FPV HAs erythrocytes only at lower pH values below 6.5, CPV also HAs the same erythrocytes at higher pH values, up to 8.0 (Chang et al., 1992). That pH-dependence of HA is controlled by one of the capsid sequence differences (VP2 residue 323, Asp to Asn), which also controls canine TfR binding and canine host range (Chang et al., 1992). Additionally, the pH dependence of HA is influenced to a lesser degree by VP2 residue 375, which differed between FPV (Asp) and CPV-2 (Asn), but which was an Asp in the more adapted variant of CPV, termed CPV

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type-2a (CPV-2a) which emerged in 1979 and replaced the CPV-2 strain worldwide (Chang et al., 1992; Truyen et al., 1996).

Sialic acids are a family of monosaccharides with a shared nine-carbon backbone. The common mammalian Sia *N*-glycolylneuraminic acid (Neu5Gc) is generated from Neu5Ac by the cytoplasmic enzyme cytidine monophosphate-*N*-acetylneuraminic acid hydroxylase (CMAH), which adds a single oxygen atom to the nucleotide donor CMP-Neu5Ac (Chen and Varki, 2010). Humans lack a functional version of the *CMAH* gene and cannot make Neu5Gc (Chou et al., 1998). Although the presence or distribution of Neu5Gc in cat and dog tissues has not been systematically studied, it is known that cat A and B blood groups are determined by the presence or absence of Neu5Gc on certain erythrocyte glycolipids (Andrews et al., 1992), which in turn seems to be determined by mutations in the *CMAH* promoter (Bighignoli et al., 2007). In dogs, Neu5Gc on erythrocytes is found only in certain "ancient" breeds of Asian origin (Hashimoto et al., 1984; Yasue et al., 1978), which diverged from the common Western dog breeds thousands of years ago (Parker et al., 2004). However, to our knowledge no studies examining the expression of CMAH or presence or distribution of Neu5Gc on other tissues or cells of either cats or dogs have been reported. What is clear is that there are differences in the binding of FPV and CPV to the Sias of feline and canine cells, and that the capsid Sia ligands on feline cells can be removed by neuraminidase (sialidase) treatment (Barbis et al., 1992; Basak et al., 1994). Here we ask whether the single oxygen atom difference between Neu5Ac and Neu5Gc is relevant to FPV and CPV binding and/or infection.

Results

Erythrocyte binding of viral capsids is solely dependent on sialic acids

To confirm that Sia is indeed required for binding of FPV and CPV capsids, we initially studied the binding of biotinylated viral capsids to rhesus monkey erythrocytes, with and without prior treatment with *Arthrobacter ureafaciens* sialidase (AUS). This treatment reduced the binding to less than 10% (data not shown), confirming previous results (Barbis et al., 1992; Basak et al., 1994; Tresnan et al., 1995).

Species-specific HA of erythrocytes by FPV and CPV is related to Neu5Gc content

Erythrocytes from a wide range of species were tested in HA assays using viral capsids (Table 1). As often seen with carbohydrate–protein interactions (Cummings and Esko, 2009), HA was as strong as or stronger at 4 °C than 37 °C (Table 1). While both viruses agglutinated erythrocytes at pH 6.2, FPV capsids showed greatly reduced HA at pH 7.2 (data not shown) (Chang et al., 1992). Both viruses also mediated high-titer HA of erythrocytes that were rich in Neu5Gc (chimpanzee, gorilla, cat and Japanese dog) but not with those with little or no Neu5Gc (human, European dog breed and mouse) (Table 1). The requirement for Neu5Gc was further supported by studies showing lack of HA of feline erythrocytes of the rarer feline blood group B which express only Neu5Ac on their surface (Andrews et al., 1992; Bighignoli et al., 2007), but efficient HA of the Neu5Gc expressing feline blood group A erythrocytes (data not shown).

Increased binding of capsids to cells expressing Neu5Gc

We have previously shown that feeding cultured cells with the Neu5Gc monosaccharide results in metabolic incorporation, and greatly increases the amount of Neu5Gc expressed at the cell surface. In contrast Neu5Ac feeding reduced Neu5Gc expression, presumably by metabolic competition (Bardor et al., 2005; Byres et al., 2008). We therefore tested capsid binding to canine A72, canine MDCK and human 293 cells that were fed with 3 mM Neu5Gc, or with Neu5Ac as a control. As expected, the level of Neu5Gc compared to Neu5Ac on these cells increased from $<2\%$ to 50–70% by Neu5Gc feeding (results not shown). Both CPV and FPV capsids bound at significantly higher levels to the cells fed with Neu5Gc, but not to those fed with Neu5Ac (Fig. 1). The increase in binding to cells was dependent on the presence of Sias, as sialidase treatment abolished the binding (Fig. 2). Likewise, Neu5Gc-rich chimpanzee serum blocked binding to a much greater extent than the Neu5Ac containing but Neu5Gc-deficient human serum (Fig. 2).

Capsid binding is not affected by sialic acid linkage type

The above data indicate that both viruses have a conserved preference for recognizing the difference between Neu5Gc and Neu5Ac, and that this does not depend on specific glycoprotein polypeptides found on erythrocytes, nor on any specific species. To further test the role of the linkage of the Sia to the underlying glycan, we studied capsid binding to CHO cells, which primarily express α2-3-linked Neu5Ac, with very small amounts of Neu5Gc. As shown above, Neu5Gc feeding induced a substantial increase in binding. Stable transfection of the CHO cells with a plasmid expressing the sialyltransferase ST6Gal-I results in competition with the ST3Gal sialyltransferases to add α2-6-linked Sia, as shown by a strong increase in binding by α2-6-linked Sia specific lectin SNA (Fig. 3A). However, this had no significant effect on virus binding following Neu5Gc feeding (Fig. 3B), indicating that the α2-3 or α2-6 Sia linkages do not influence binding.

Neu5Gc content does not influence the efficiency of in vitro cell culture infections

The role of the Neu5Gc in live virus infections was studied by examining efficiency of infection in A72 or MDCK cells fed (or for

Table 1
Parvoviruses only Hemagglutinate erythrocytes expressing Neu5Gc.

		CPV		FPV	
Species	**Neu5Gc** (%)	**4** °C	**37** °C	**4** °C	**37** °C
Human	<1	0	0	0	0
Chimpanzee	50	819,200	819,200	20	0
Gorilla	50	102,400	102,400	819,200	409,600
Cat	35–60	$>1,000,000$	$>1,000,000$	$>1,000,000$	6,561
Dog, Japanese breed	95	$>1,000,000$	$>1,000,000$	$>1,000,000$	65,536
Dog, Western breed	<1	0	0	0	0
Mouse (C57Bl/6)	<10	0	0	0	0

Neu5Gc content and HA at pH 6.2. were studied as described in the Methods. Titers are shown as the maximum dilution that gives agglutination of the respective erythrocytes.

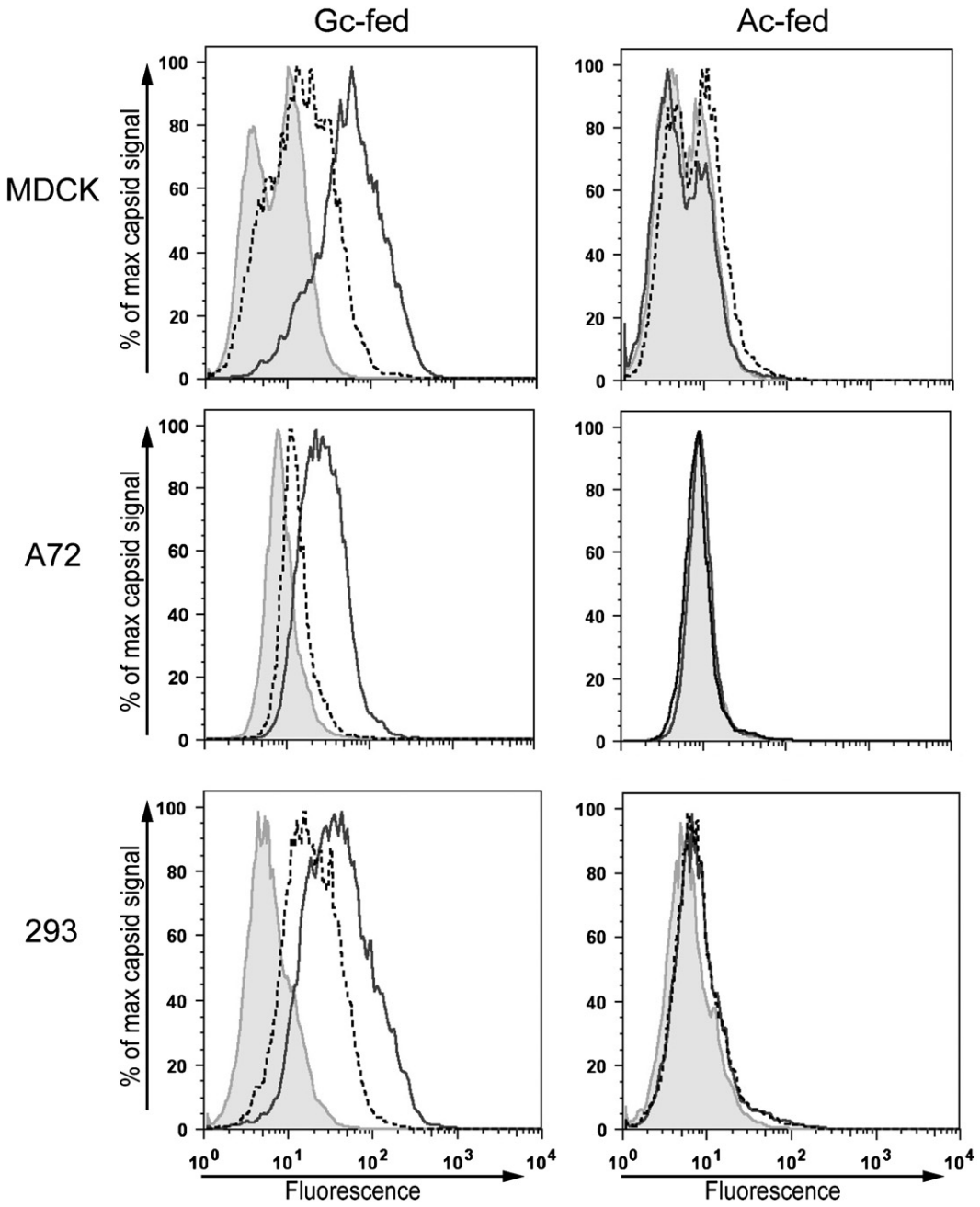

Fig. 1. Feeding of cells with Neu5Gc but not Neu5Ac induces sialic acid-dependent parvovirus capsid binding. The canine MDCK, canine A72 and human 293 cells were incubated with 3 mM Neu5Gc or Neu5Ac for three days to allow metabolic incorporation and then exposed to biotinylated CPV (dashed line), FPV (solid line) or no virus (only fluorescent streptavidin; shaded gray) capsids at pH 6.2 at 4 °C. Binding of capsids was detected by fluorescent Streptavidin binding, using flow cytometry.

controls, not fed) with Neu5Gc. Despite the clear difference in binding to cells induced by the presence of Neu5Gc, we did not observe any change in the rate of infection by FPV, CPV-2 or CPV-2b, over a range of infective doses (Table 2).

Canine CMAH transcripts and protein were expressed at very low level

Quantitative Real-Time RT-PCR analysis was performed on Western-dog derived canine cells using actin as an internal control and feline NLFK cell line as a positive reference. Expression levels of canine *CMAH* transcripts were 0.003 for Cf2TH, less than 0.0001 for A72, and 0.036 for MDCK cell lines each compared with the levels seen in feline NLFK cells which were set at 1.0 (Table 3). While the CMAH protein was detected in lysates of feline cells by Western blot analysis, no protein was detected in the canine cells (Fig. 4).

Discussion

Here we show that CPV and FPV bind specifically to Neu5Gc but not to Neu5Ac, and that binding occurs in either α2-3 or α2-6 linkages. This specificity was strongly suggested by the patterns of HA seen for erythrocytes from different animals that reflect Neu5Gc content, although the HA titers did not totally correlate with NeuGc content on the surface of the RBCs (e.g. chimpanzee and gorilla). However, this might be explained by a different distribution of

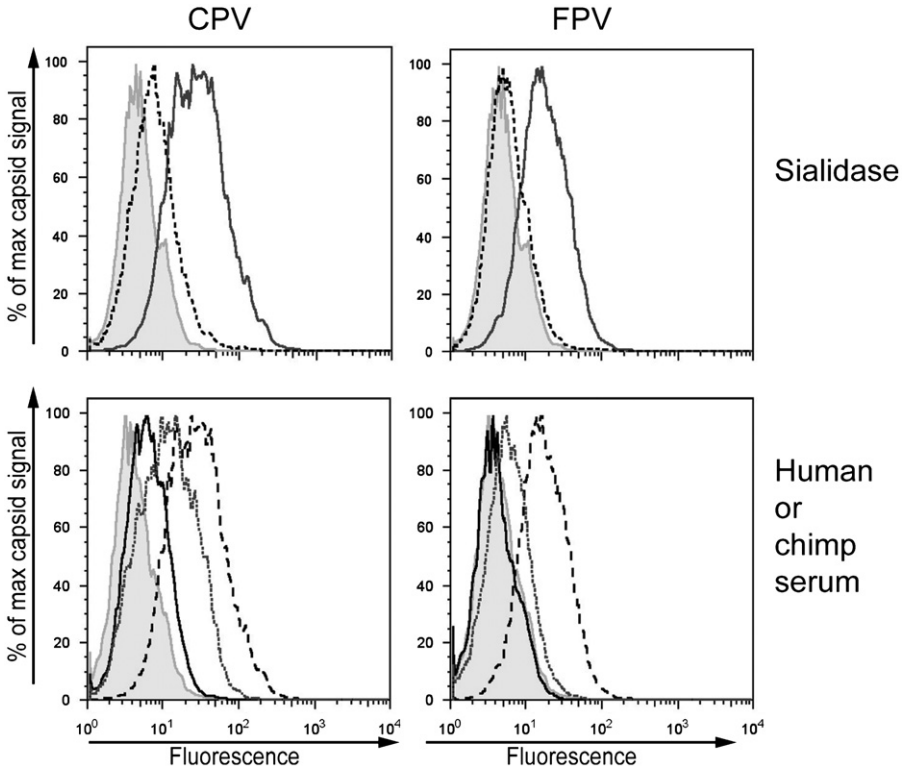

Fig. 2. Confirmation of the Neu5Gc-specificity of capsid binding. Human 293 cells were incubated with 3 mM Neu5Gc for three days and analysis of biotinylated capsid binding studied at pH 6.2, as described in Fig. 1. The binding specificity was tested by blocking using chimpanzee (solid black line) or human (dotted dark gray line) sera, which primarily differ in Neu5Gc content, or by addition of PBS (dashed black line); or after sialidase (dashed line) or mock (solid line) treatment for 30 min. Cells exposed to only fluorescent streptavidin is shown in shaded gray.

ligands (Cohen et al., Blood 2009), or the influence of differential expression of another ligand, such as the transferrin receptor (Parker et al., 2001). The specificity was also suggested by the binding seen to feline A but not B blood group erythrocytes, a difference known to be determined by the absence of Neu5Gc on the B blood type cells (Andrews et al., 1992). Notably, these A and B blood groups are also found in various wild felids (Griot-Wenk and Giger, 1999), suggesting an ongoing selection pressure to maintain this polymorphism in erythrocyte Neu5Gc expression. For dogs, certain Asian breeds showed Neu5Gc on their red cell glycolipids, which contrasts with most European breeds, which showed Neu5Ac on their red cell glycoplipids (Hashimoto et al., 1984).

We specifically confirmed the role Sia plays in binding by showing that increased expression of Neu5Gc on cells resulted in enhanced binding of FPV and CPV capsids. However, this did not enhance the process of infection *in vitro*. This result fits with prior studies that examined the infection of cells treated with sialidases or studied viruses that contained single mutations that prevented Sia binding (Basak et al., 1994; Tresnan et al., 1995; Truyen et al., 1996). Indeed, it has been previously shown that passage of CPV type-2 in feline cells selects for non-Sia binding mutants (Parrish et al., 1988a), suggesting that the binding of that virus to the Sia on cells at the neutral pH of the culture reduces the efficiency of virus infection, while FPV would not be subject to such selection as it binds only below pH 6.5 (Parrish et al., 1988b).

Sias are expressed at high levels on the glycoproteins and glycolipids on the plasma membranes of many vertebrate cells, and are also components of the mucus and glycocalyx present at mucosal and endothelial surfaces. They are often used as ligands for cell binding by many different pathogens, including viruses, bacteria and parasites (Esko and Sharon, 2009; Varki, 2007). For many viruses, including some parvoviruses and related adeno-associated viruses, Sia binding is an essential part of the cell infection process, either by leading directly to productive cell entry, or by acting as part of a series of binding events that includes other receptors (Esko and Sharon, 2009; Harbison et al., 2008). Many viruses show highly regulated specificities for different forms of Sia, including Neu5Ac, Neu5Gc, 4- or 9-O-acetylated Sia, or their various α2-3 or α2-6 linked versions (Langereis et al., 2009; Schwegmann-Wessels and Herrler, 2006; Zeng et al., 2008). These differences are often host specific; for example while the primate-derived Simian virus 40 binds to Neu5Gc better than Neu5Ac and the stronger binding also leads to increased infection (Campanero-Rhodes et al., 2007), the human derived JC virus may bind preferentially to Neu5Ac (Gee et al., 2004). The parvovirus MVM also shows differences in Sia binding specificity and affinity, which are associated with changes in tissue tropism and pathogenicity (Lopez-Bueno et al., 2006).

The regulation of CMAH expression in feline and canine species is a complex issue that requires further study. However, in three different canine cells tested the *CMAH* gene was expressed at 300–10,000 times lower levels compared with the levels present in feline cells. The canine CMAH protein was also not detected by Western blotting in lysates of either in the A72 or MDCK canine cell lines. Possible reasons of low expression levels of canine *CMAH* include changes in the 5′UTR region or promoter region of canine *CMAH* that allow only low mRNA expression, and/or lack of a correct starting codon as suggested by Bighignoli et al. (2007), likely together with a weaker promoter.

Given the conserved Neu5Gc binding specificity of CPV and FPV capsids, the lack of involvement in *in vitro* infections was surprising. On the other hand the strong preference for Neu5Gc over Neu5Ac (differing by one oxygen atom) has remained conserved in these viruses. Thus, the role of this binding is most likely related to *in vivo* events during active infection. The pathogenic processes of CPV and FPV infections in dogs and cats are similar, with the viruses infecting by an oro-nasal route, spreading systemically via a plasma viremia

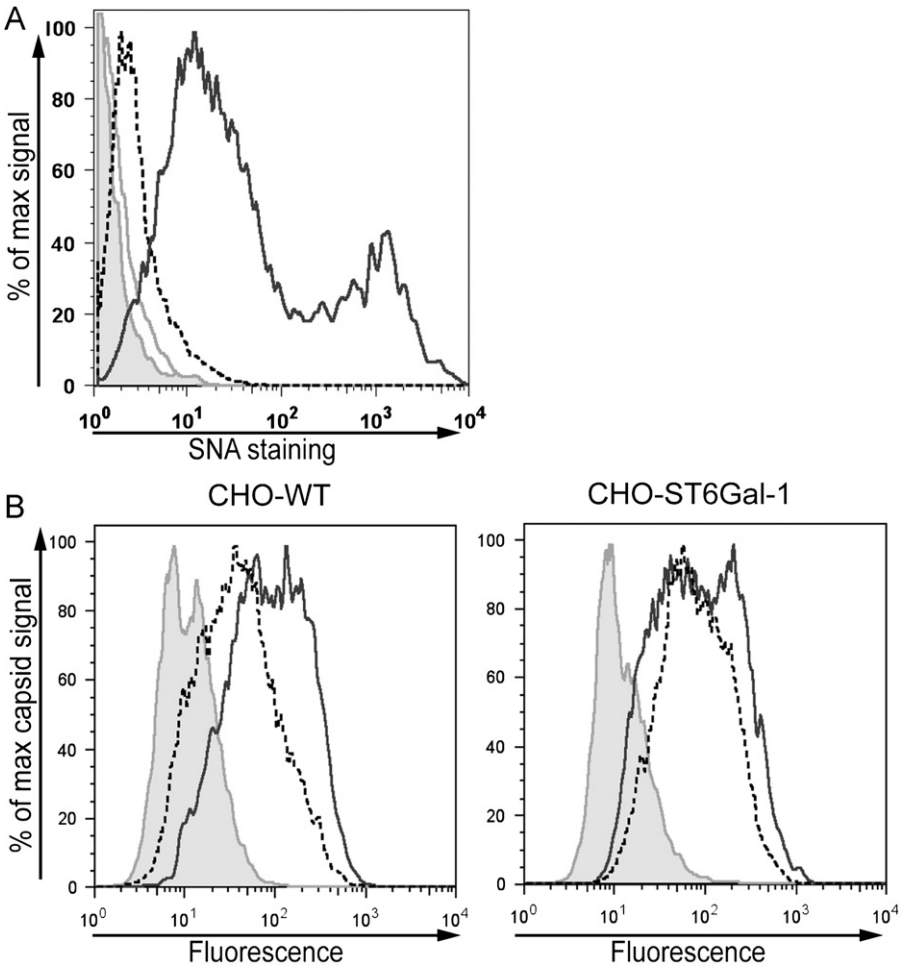

Fig. 3. Neu5Gc-dependent parvovirus binding shows little or no preference for sialic acid linkage. (A) The Sia linkage pattern of wild-type CHO-K1 cells (which express primarily α2-3-linked Sias; solid light gray for control cells and dashed line for SNA-stained cells) was altered by stable transfection with ST6Gal-I, giving increased α2-6-linked Sias, as shown by increased binding of SNA lectin (shaded gray for control cells and solid dark gray for SNA-stained cells). (B) Both cell types were fed with 3 mM Neu5Gc and binding preference of the CPV and FPV capsids were tested and analyzed as in Fig. 1.

Table 2
Primary infectivity of FPV and CPV is not affected by the Neu5Gc content of target cells.

Cell line	Sia Fed	CPV-2	CPV-2b	FPV
A72	None	5.59	4.27	2.22
A72	Neu5Gc	6.22	6.21	2.22
A72	Neu5Ac	6.22	5.48	2.72
NLFK	None	4.72	4.95	5.95
NLFK	Neu5Gc	5.72	5.54	5.95
NLFK	Neu5Ac	5.22	5.72	5.72
MDCK	None	3.22	2.36	<2
MDCK	Neu5Gc	1.57	3.13	<2
MDCK	Neu5Ac	1.57	1.57	<2

The $TCID_{50}$ titers of CPV-2, FPV, and CPV-2b viruses in cells grown without added Sia, or with Neu5Gc or Neu5Ac for 4 days before inoculation. The Sia was left in the culture medium during after inoculations of the cells (for 36 h).

Table 3
The relative expression levels (RQ) of CMAH in canine and feline cell lines treated with different concentrations of $MgSO_4$. In all cases, the relative mRNA levels of CMAH transcript in canine cell lines are mRNA levels related to those in feline NLFK cell line as reference, and using actin mRNA as an endogenous control. Data are presented as means ± standard deviation from two biological replicates.

Cell lines		Relative mRNA levels (+/−1 SD)
Feline	NLFK	1
	CRFK	1.17 (+/−1.37)
Canine	Cf2TH	0.003 (+/−0.003)
	A72	<0.0001 (+/− <0.0001)
	MDCK	0.036 (+/−0.01)

and likely entering many host tissues through a systemic spread, with replication initially being seen mostly in the primary or secondary lymphoid tissues that contain large numbers of dividing cells that can support DNA replication (Parrish, 1995). Two to three days after infection the virus infects the rapidly dividing intestinal epithelial progenitor cells in the crypts of the intestinal epithelium, and virions are then shed in the feces for transmission to other host animals. In this cycle the virus would be exposed to the Sias on many tissues, fluids and in the feces, as well as those expressed on cell surfaces.

Neu5Gc is likely found on most cat cells and tissues, but as FPV binds that Sia only at pH values below 6.5, and the blood and tissue pH of cats are consistently >7.0 (Hochberg et al., 1978), it would not interact with the Neu5Gc of the feline tissues. In contrast, while CPV-2 binds to Neu5Gc at pH values of 7.0 and above, that Sia appears not to be present in most breeds of dogs. Although CPV-2 strains infect and replicate in feline cells, that virus does not replicate in cats, while the more recently emerged strains of CPV (such as CPV-2a) both infect and replicate in cats (Truyen et al., 1994; Truyen and Parrish, 1992).

The neutral pH-dependence of Sia binding by CPV appears to be an evolutionary trade-off, as that was largely controlled by a mutation in the capsid protein (VP2 residue Asp323 to Asn) that

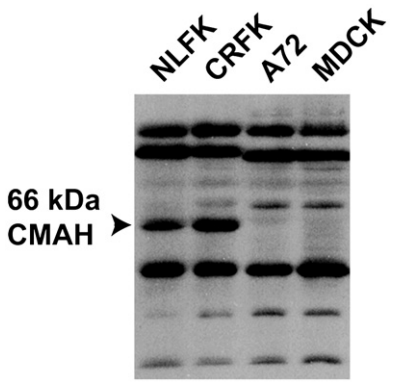

Fig. 4. Analysis of putative canine *CMAH-like* protein expression. Lysates from feline (CRFK and NLFK) or canine (A72 and MDCK) cells were electrophoresed in SDS-PAGE, and the CMAH enzyme detected using a specific antibody. The specific CMAH protein band is indicated by the arrowhead.

was required for CPV binding to the canine TfR, an essential step in the acquisition of the canine host range (Hueffer et al., 2003; Palermo et al., 2006; Palermo et al., 2003). The pH dependence of Sia binding of CPV-2 was also affected by VP2 residue 375 (Asp to Asn), and during the evolution of CPV that residue reverted to an Asp in the CPV-2a variant, giving a virus that binds to Sia at lower pH values (Chang et al., 1992).

Despite all these subtle evolutionary changes in the binding properties of the virus, the strong preference of the virus for binding Neu5Gc over Neu5Ac has remained conserved through numerous passages within and between species. Thus, while it does not appear to play a role in primary infectivity in vitro, this binding property has presumably remained critical for the life cycle of the virus in the hosts where Neu5Gc is present, which are likely to be most of the carnivores that are naturally susceptible to the virus. In this regard, we have previously suggested a role for circulating erythrocytes as sinks or carriers for viruses that enter the bloodstream (Gagneux and Varki, 1999), and others have recently shown a specific example, involving a different molecule (Seiradake et al., 2009).

In addition to erythrocytes in the bloodstream, other potentially relevant sites of virus binding to Sia would be in the upper airways, in the lumen of the small and large intestine, or in the feces. The luminal contents of the intestines of the cat or dog are between pH 5.5 and 6.6 (Brosey et al., 2000), which would allow binding of virus to Sias on intestinal mucus, tissue fragments or dietary materials. Such binding could modulate the shedding and persistence of the viruses in the intestine and the feces, perhaps enhancing their persistence in the environment and transmission. This may be analogous to the situation seen for the Transmissible Gastroenteritis Virus (TGEV) from pigs, which binds to Neu5Gc *in vitro*, but without changing the infection rate (Schwegmann-Wessels and Herrler, 2006; Schwegmann-Wessels et al., 2003). In that case the Sia binding is suggested to protect the virus *in vivo*, a property that may also act for FPV and CPV through association with materials in the gastro-intestinal tract or the feces. A further complexity arise from the fact, that in carnivores including dogs and cats, the dietary materials would contain significant amounts of Neu5Gc.

Materials and methods

Cells and viruses

Feline Norden Laboratory Feline kidney (NLFK) and Crandell Rees feline kidney (CRFK) cells, canine Cf2TH, A72, and Madin Darby canine kidney (MDCK) cells, human 293 or hamster CHO-K1 cells originated from ATCC and were all cultured as recommended. CPV and FPV viruses were recovered from infectious plasmid clones by transfection of NLFK cells, and were grown in those cells (Parrish, 1991). The capsids from infected cells were purified by sucrose gradient centrifugation using standard methods (Agbandje et al., 1993). Capsids were biotinylated (Tresnan et al., 1995) and infectious assays were conducted using tissue culture infectious dose 50 ($TCID_{50}$) assays in 96 cells trays, as described previously (Parker and Parrish, 1997). Briefly, cells were incubated with various dilutions of virus for 60 min at 37 °C, then incubated with growth medium for 48 h prior to fixing and staining for infected cells by detecting viral capsid proteins.

Cell binding assays

Cells were grown for three days in 75 cm^2 flasks in the presence of 3 mM of either Neu5Ac or Neu5Gc, to modify cell surface Sia patterns, as described (Bardor et al., 2005; Byres et al., 2008). For binding assays the cells were then detached with trypsin, resuspended in culture medium, then spun down at $200 \times g$ for 3 min and washed twice with Bis–Tris buffered saline of pH 6.2 or 7.2, respectively, and finally resuspended at a concentration of 5×10^6 cell/ml. 10 μl of 10 ng/μl biotinylated parvovirus capsids were added to 100 μl of cell suspension and incubated at 4 °C for 30 min, then the cells were washed once with Bis–Tris buffered saline (pH 6.2). This was followed by staining with 5 μg/ml of phycoerythrin labeled-streptavidin for 30 min at 4 °C in the dark. The cells were washed once again and analyzed in a Becton-Dickinson FACScalibur, counting 10,000 events. In all experiments shown, biotinylated capsids were used, but we saw the same results when using non-labeled capsids and detecting the cell associated capsids with a parvovirus-specific antibody (not shown).

Hemagglutination of erythrocytes

This was done in Bis–Tris buffered saline adjusted to different pHs as previously reported (Simpson et al., 2000).

Sialidase treatment of cells

The sialidase from *Arthrobacter urefaciens* was used at a concentration of 100 mU/ml in cell culture medium for 30 min at 37 °C and then the cells were washed in buffer and used for binding assays.

Sialic acid pattern of erythrocytes

This was determined by release with mild acid hydrolysis, derivatization by DMB, and separation on reverse-phase HPLC with fluorescent detection, as described (Hara et al., 1986).

Testing the role of Sia linkage for virus binding to cells

To test the role of the linkage of the Sia within the glycan, we examined the effect of capsid binding to wild-type CHO cells that had been fed Neu5Gc (which express predominantly α2-3 linked Sia) and also to CHO-ST6Gal-I cells that had been stably transfected with a plasmid expressing the enzyme ST6 beta-galactoside α2-6sialyltransferase 1 (ST6Gal-I), and thus express predominantly α2-6 linked Sia (Shi et al., 1996).

Real-time PCR

Total RNA samples from the cell culture of NLFK, CRFK, Cf2TH, A72, MDCK was reversely transcribed into cDNA by SuperScript III

RNase H-Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. The CMAH oligod(T) derived first strand cDNAs were used as templates in real-time PCR assays using a SYBR green reagent (Applied Biosystems, Foster City, CA). The CMAH gene specific PCR primers used were canine and feline CMAH-F (5′-CAA GGA GGC CTG TTC ATA AAA GAC AT-3′) and canine and feline CMAH-R (5′-CAT CCT CTT GTC TCC CAG CTT GAG GTC CAT-3′). Actin gene specific primers used were ACTIN-F (5′-GGC TGG GGT GTT GAA GGT CTC-3′) and ACTIN-R (5′-GAT ATC GCC GCG CTC GTC GTC-3′). The reactions were performed in an Applied Biosystems StepOnePlus Real-Time PCR System (Applied Biosystems). The RT-PCR products were sequenced. The PCR program consisted of a first step of denaturation and Taq activation (95 °C, 10 min), followed by 40 cycles of denaturation (95 °C, 15 s) and annealing/extension (55 °C, 15 s; 60 °C, 1 min). The relative quantification of the *CMAH* transcript levels was calculated using the $\Delta\Delta C_T$. In brief, ΔC_T value of each sample was first calculated using the formula $\Delta C_T = C_T$ (CMAH) $- C_T$ (ACTIN), followed by the determination of $\Delta\Delta C_T$ by comparing with the NLFK as reference and actin as endogenous control. The relative expression level was obtained using the equation $RQ = 2^{-\Delta\Delta C_T}$. The reported values are averages of three independent trials with triplicate repeats.

Antibody analysis of CMAH expression

A rabbit anti-CMAH polyclonal antibody (M-240) against amino acids 3-242 mapping near the N-terminus of CMAH of mouse CMAH were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). For Western blot analysis of CMAH protein expression, feline cells and canine cells were lysed in sodium dodecyl sulfate (SDS) sample buffer (2% SDS, 60mM Tris-HCl [pH6.8], 5% 2-mercaptoethanol). Proteins separated on 10% SDS-PAGE gels were transferred to nitrocellulose membrane and incubated with CMAH antibody (M-240). The membranes were incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase and then, visualized with Supersignal substrate (Pierce Chemicals, Rockford, Ill, USA)

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