Feline Lectin Activity Is Critical for the Cellular Entry of Feline Infectious Peritonitis Virus[▽]

Andrew D. Regan, David G. Ousterout,[†] and Gary R. Whittaker*

Department of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, Ithaca, New York, 14853

Received 3 May 2010/Accepted 7 May 2010

Feline infectious peritonitis is a lethal disease of felids caused by systemic infection with a feline coronavirus. Here, we report identification and analysis of the feline homologue to the human lectin DC-SIGN and show that it is a coreceptor for virulent strains of serotype 1 and serotype 2 feline coronaviruses.

Feline infectious peritonitis (FIP) is a disease of felids that has a spectrum of pathological outcomes, all of which are lethal (18). The causative agent of FIP is a feline coronavirus (FCoV) designated feline infectious peritonitis virus (FIPV) which infects immune cells, leading to a systemic infection (25, 28). FCoV is classified as group 1a coronaviruses and circulate as two distinct serotypes (FCoV-1 and FCoV-2) based on the sequences of their S genes (22). FCoV-1 is the more prevalent serotype currently in circulation and therefore represents the dominant cause of clinical FIP (80% to 90%). However, compared to FCoV-2, a disproportionally small amount of data exists on FCoV-1 because of difficulties associated with cultivating these viruses *in vitro*.

Cellular entry is the first step in the infectious cycle of a coronavirus and is mediated by the binding of the S protein to receptors on the surface of target cells (9). Aminopeptidase N ([APN] CD13) was first identified as the primary receptor for

FCoVs (26, 27). However, later studies claimed that APN is utilized only by FCoV-2 strains, while FCoV-1 uses an as yet unidentified receptor (6, 11). It has been proposed that this difference in receptor utilization is responsible for the markedly different behaviors of FCoV-1 and FCoV-2 in cell culture (6). In addition to recognizing a primary receptor, coronaviruses also use of a variety of coreceptors during entry, including C-type lectins (5, 13–15, 23).

The human C-type lectin dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) has been shown to enhance the entry of many viruses including FIPV; however, a feline DC-SIGN (fDC-SIGN) homologue has yet to be identified (21). To identify a potential fDC-SIGN homologue, shotgun sequencing data of the feline genome were searched using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST), with the canine DC-SIGN homologue (EU670674)

FIG. 1. The sequence and phylogenetic analysis of feline DC-SIGN. (A) For each pair of lines, the nucleotide sequence of fDC-SIGN is shown as the top line in regular font, and the amino acid sequence of fDC-SIGN is shown in boldface as the bottom line. The transmembrane domain is boxed, and the glycan binding domain is underlined. (B) A phylogenetic tree of DC-SIGN sequences generated by Clustal W alignment.

* Corresponding author. Mailing address: C4127 Veterinary Medical Center, Department of Microbiology and Immunology, Cornell University, Ithaca, NY 14853. Phone: (607) 253 4019. Fax: (607) 253 3385. E-mail: grw7@cornell.edu.
[†] Present address: Department of Biomedical Engineering, Duke University, Durham, NC 27708.
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FIG. 2. Effect of fDC-SIGN expression on the cellular entry of FIPV-1146 and FIPV-TN406. CRFK or CRFK-fDC-SIGN cells were infected with either FIPV-1146 or FIPV-TN406 at an MOI of 0.1 or 0.5, respectively. Cells were fixed with paraformaldehyde at 6 h and 18 h, respectively; IgG2a anti-group 1 coronavirus monoclonal antibody FIPV3-70 was used to stain infected cells, IgG1 anti-myc monoclonal antibody 9E10 was used to stain myc-tagged fDC-SIGN, and Hoechst stain was used for nuclei. Secondary goat anti-mouse IgG1-Alexa Fluor 568 and IgG2a-Alexa Fluor 488 were used for detection (A). For quantification, cells were infected with FIPV-1146 and FIPV-TN406 at MOIs of 0.1, 0.5, and 1.0 and processed as previously described. At least 1,000 cells were scored from three independent replicates for each experimental condition (B). Error bars represent the standard deviation of the mean.

as a template. This identified the fDC-SIGN gene with 98% exon coverage derived from four contiguous sequences (NCBI accession numbers ACBE01052350.1, AANG01184685.1, ACBE01052349.1, and AANG01554893.1). Reverse transcription-PCR (RT-PCR) primers were designed from these sequences (fDC-Fwd, ATGTGTGACCCCAAGGAGCCGGAT; fDC-Rev, TCAGAGGCCCGGGCAGGGGGACGA). Since human DC-SIGN has been shown to be highly expressed in dendritic cells (DCs) (10), fDC-Fwd and fDC-Rev were used to perform RT-PCR on RNA purified from feline monocyte-derived DCs prepared as previously described (8). In short, primary feline blood-derived monocytes were purified and pooled from two specific-pathogen-free (SPF) cats (Cornell East Campus Research Facility) by standard Ficoll-Paque gradient centrifugation. Cells were allowed to attach to glass coverslips and were incubated with recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF; 50 ng/ml), recombinant interleukin-4 (IL-4; 10 ng/ml), and 3% feline serum at 37°C in an atmosphere of 5% CO₂. Typical DC morphology was observed after 7 days, and differentiation was

confirmed with anti-major histocompatibility complex class II (MHC-II) and anti-CD14 antibodies. RNA was purified from feline DCs using a Qiagen RNeasy Kit, and RT-PCR was performed using a Qiagen One-Step RT-PCR Kit, both used as specified by the manufacturer. This yielded a 0.8-kb band which was submitted for direct nucleotide sequencing, resulting in a sequence with a single open reading frame (ORF) coding for a protein with 85.7% sequence homology to canine DC-SIGN. After sequence verification, the fDC-SIGN ORF was subcloned into a mammalian expression vector with a C-terminal c-myc tag to facilitate detection in downstream applications.

The 756-nucleotide (nt) fDC-SIGN ORF codes for a 251-amino-acid (aa) protein (Fig. 1A) that is predicted to be a type II transmembrane protein according to the dense alignment surface method (3). Similar to human DC-SIGN, the cytoplasmic tail of fDC-SIGN contains a dileucine motif and a tyrosine-based endocytosis motif (1). However, the triacidic amino acid cluster present in human DC-SIGN has been disrupted by a proline residue (Fig. 1A). The extracellular domain

consists of a characteristic neck region 56 aa in length, followed by a highly conserved 132-aa carbohydrate recognition domain (CRD) (7). The CRD of human DC-SIGN has been shown to bind two Ca^{2+} ions using two distinct motifs (7), both of which are fully conserved in the fDC-SIGN homologue. In addition, eight cysteines which are highly conserved in all reported DC-SIGN homologues are also present in the fDC-SIGN homologue. fDC-SIGN possesses a high level of amino acid sequence homology with canine DC-SIGN (85.7%) but shares only 41.8% homology with human DC-SIGN. An analysis of protein phylogeny places fDC-SIGN in a distinct lineage separate from primates (Fig. 1B). fDC-SIGN most closely groups with homologues from other nonprimate mammals (canine DC-SIGN, bovine DC-SIGN, porcine DC-SIGN, and equine DC-SIGN).

To investigate whether FCoV can utilize fDC-SIGN during cellular entry, CRFK cells stably expressing fDC-SIGN were created (CRFK-fDC-SIGN) using standard selection techniques with the antibiotic G418. We first tested the effect of fDC-SIGN expression on the cellular entry on the FCoV-2 isolate FIPV-1146. Infection of CRFK-fDC-SIGN cells by FIPV-1146 at a multiplicity of infection (MOI) of 0.1 resulted in an infection rate of 36%, an enhancement of 4.5-fold compared to CRFK cells (8%) (Fig. 2). At a higher MOI of 0.5, the enhancement effect was less but still discernible (57% infection of CRFK-fDC-SIGN cells versus 36% infection CRFK cells). At an MOI of 1 there was virtually no difference in infection rates (Fig. 2B).

Although the FCoV-1 strain FIPV-TN406 was first isolated in cell culture by coculturing uninfected CRFK cells with peritoneal macrophages from a cat infected with FIPV (2), CRFK cells alone do not efficiently support FCoV-1 propagation (6, 19). In support of this observation, infection of CRFK cells by FIPV-TN406 at MOIs of 0.1, 0.5, and 1 resulted in minimal levels of infection (<1%) (Fig. 2). However, expression of fDC-SIGN substantially rescued infection by FIPV-TN406 at an MOI of 0.1 (12% infection), 0.5 (23% infection), and 1 (43% infection) (Fig. 2B). We theorized that the few cell lines which have been reported to support growth of FCoV-1 *in vitro* may therefore express high levels of mannose-binding lectins. It has recently been reported that AKD (feline lung) cells support the efficient growth of FIPV-TN406 *in vitro* (17), and this observation has been verified in our laboratory. AKD cells were therefore pretreated with mannan and infected with FIPV-TN406 at a range of MOIs. As shown in Fig. 3, pretreatment with 100 $\mu\text{g}/\text{ml}$ of mannan reduced infection of AKD cells by FIPV-TN406 inoculated at an MOI of 0.1 (9% reduction), 0.5 (23% reduction), and 1 (54% reduction) compared to untreated cells.

Considering that DCs are known to highly express DC-SIGN, we also sought to investigate if feline DCs could be infected by FIPV *in vitro* and whether infection would be dependent on cellular mannose-binding activity. As shown in Fig. 4 feline DCs were highly susceptible to infection by both FIPV-1146 and FIPV-TN406. Infection was marked by a high proportion of syncytia not previously observed in undifferentiated blood-derived mononuclear cells (20). To determine the effect of mannose-binding lectins on DC entry, cells were pretreated with mannan and infected with FIPV-1146 or FIPV-TN406. Pretreatment with 100 $\mu\text{g}/\text{ml}$ of mannan reduced in-

FIG. 3. Effect of mannan pretreatment on the entry of FIPV-1146 and FIPV-TN406 in CRFK-fDC-SIGN and AKD cells. CRFK-fDC-SIGN or AKD cells were pretreated with mannan (100 $\mu\text{g}/\text{ml}$) or medium only (control) and then infected with either FIPV-1146 or FIPV-TN406 at MOIs of 0.1, 0.5, and 1.0. Cells were fixed and stained for viral infection as previously described. At least 1,000 cells were scored from three independent replicates for each experimental condition. Error bars represent the standard deviation of the mean.

fection of feline DCs by both FIPV-1146 (64% reduction) and FIPV-TN406 (84% reduction) (Fig. 4).

In this study, we show that expression of fDC-SIGN enhances the cellular entry of the FCoV-2 isolate FIPV-1146

FIG. 4. Effect of mannan pretreatment on the entry of FIPV-1146 and FIPV-TN406 in primary feline-derived dendritic cells. Primary feline monocytes were purified by standard Ficoll-Paque gradient centrifugation and differentiated into DCs. DCs were pretreated with mannan (100 μ g/ml) or medium only (control) and infected with either FIPV-1146 or FIPV-TN406 at an MOI of 10. Cells were fixed with paraformaldehyde at 24 h and stained with the anti-group 1 coronavirus monoclonal antibody FIPV3-70, and Hoechst stain was used for nuclei. Secondary goat anti-mouse Alexa Fluor 488 was used for detection of infected cells (A). For quantification, at least 450 cells were scored from three independent replicates for each experimental condition (B). Error bars represent the standard deviation of the mean.

(Fig. 2). These data are in agreement with previous work from our laboratory showing that FIPV-1146 and an additional FCoV-2 isolate, FIPV-DF2, can utilize human DC-SIGN as a coreceptor in conjunction with their primary cellular receptor, APN. In the current study we also show that the FCoV-1 isolate FIPV-TN406 can utilize fDC-SIGN as a coreceptor for cellular entry. Specifically, expression of fDC-SIGN renders the feline cell line CRFK permissive to infection by FIPV-TN406. This is notable, considering that previous studies have concluded that CRFK cells lack the primary receptor for FCoV-1 (6). Data presented here suggest that CRFK cells do, in fact, possess the primary receptor necessary for the entry of FCoV-1 but that viruses of this serotype may depend heavily on the mannose-binding activity of lectins (or other coreceptors) to efficiently utilize their primary receptor. The use of feline cell lines engineered to overexpress specific coreceptors (or combinations of coreceptors) may enhance our ability to cultivate and study these underrepresented, yet more clinically relevant, strains.

This study also suggests that feline mannose-binding lectins may play an important role in the development of FIP *in vivo*. Mannose-binding lectins are expressed in intestinal epithelial tissue, the initial site of feline coronavirus infection (4, 16). Within this region DC-SIGN has been shown to be expressed within the lymphoid tissue of the Peyer's patch (12). Peyer's patch tissue contains a high concentration of DCs and macrophages, cells which have previously been shown to disseminate viruses systemically throughout their host (24, 29). Taken together with data presented in this study, the Peyer's patch

represents an area where enteric feline coronaviruses may attempt to extend their tropism, a process which could depend, in part, on the enhancing effect of fDC-SIGN. Further studies on feline mannose-binding lectins such as fDC-SIGN and their role in feline coronavirus pathogenesis could help to clarify events which occur early during the development of FIP.

Nucleotide sequence accession number. The GenBank accession number for the FDC-SIGN sequence is HM237358.

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