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Differences in virus receptor for type I and type II feline infectious peritonitis virus

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Summary. Feline infectious peritonitis viruses (FIPVs) are classified into type I and type II serogroups. Here, we report that feline aminopeptidase N (APN), a cell-surface metalloprotease on the intestinal, lung and kidney epithelial cells, is a receptor for type II FIPV but not for type I FIPV. A monoclonal antibody (MAb) R-G-4, which blocks infection of Felis catus whole fetus (fcwf-4) cells by type II FIPV, was obtained by immunizing mice with fcwf-4 cells which are highly susceptible to FIPV. This MAb also blocked infection of fcwf-4 cells by type II feline enteric coronavirus (FECV), canine coronavirus (CCV), and transmissible gastroenteritis virus (TGEV). On the other hand, it did not block infection by type I FIPVs. MAb R-G-4 recognized a polypeptide of relative molecular mass 120–130 kDa in feline intestinal brush-border membrane (BBM) proteins. The polypeptide possessed aminopeptidase activity, and the first 15 N-terminal amino acid sequence was identical to that of the feline APN. Feline intestinal BBM proteins and the polypeptide reacted with MAb R-G-4 (feline APN) inhibited the infectivity of type II FIPV, type II FECV, CCV and TGEV to fcwf-4 cells, but did not inhibit the infectivity of type I FIPVs.

Introduction

Feline infectious peritonitis virus (FIPV), family *Coronaviridae*, genus *Coronavirus*, causes a chronic, progressive, immunologically mediated disease in domestic and exotic cats. The family *Coronaviridae* is divided into 3 distinct antigenic groups on the basis of sequence analysis of their genome and antigenic relationships. One of these includes human respiratory coronavirus 229E (HCV-229E), transmissible gastroenteritis virus (TGEV) of swine, porcine respiratory coronavirus, canine coronavirus (CCV), and feline coronavirus (FCoV) [6, 26]. Feline coronaviruses (FCoVs) are divided into FIPV types I and II, and feline enteric coronavirus (FECV) types I and II, on the basis of disease type; that is, whether

it causes FIP, the ability of the viruses to proliferate in cell cultures, and the antigenic relationship to TGEV and CCV [23]. The type II strains of FIPV and FECV appeared to be more closely related to CCV and TGEV, i.e., immunodominant neutralization epitopes shared by the spike proteins of TGEV, CCV, and the type II FCoV strains seem to be absent in the type I strains [13]. Recently, these findings were confirmed by sequence analysis. The spike genes of type II FCoVs have much closer similarity to those of TGEV and CCV than to the spike genes of type I isolates [19, 20].

Viral infection requires binding of the viruses to cellular receptors. Free virus receptors administered to organisms antagonize the endogenous virus receptors, and suppress proliferation of the virus. Therefore, virus receptors have drawn attention as potential anti-virus drugs, and have been identified for several viruses [16]. The CD13 antigen, which is an important cell-surface marker of myelocytic leukemia, has been widely used for immunophenotyping of leukemia [10]. The CD13 antigen has been identified by gene cloning as the enzyme aminopeptidase N (APN) [18]. CD13/APN is widely distributed in respiratory and digestive epithelial cells and blood cells [14, 25]. The physiological functions of CD13/APN differ depending on cell type. In the intestinal brush border membrane (BBM), for example, CD13/APN cleaves N-terminal amino acids of small peptides in the final steps of digestion, and helps absorption of peptides from the small intestine [25]. In 1992, 2 study groups simultaneously reported that CD13/APN functions as a cellular receptor for TGEV and HCV-229E [8, 31]. In 1993, it was reported that CD13/APN also functions as a receptor of cytomegalovirus [28]. Although TGEV and HCV-229E both use CD13/APN as a receptor, the former cannot use the human APN as a receptor, and the latter cannot use the porcine APN, showing species-specific receptor recognition [7, 17]. It has also been shown that FIPV and CCV, which are antigenically close to TGEV, cannot use the porcine APN as a receptor [9]. Recently, it was reported that mouse and hamster cells, which are intrinsically nonsusceptible to FIPV, are infected with FIPV if transfected with feline APN genes, showing that FIPV also uses CD13/APN as a receptor [29]. However, it has been shown that type II FIPV strain 79–1 146 can use the canine APN as a receptor, while type I FIPV strain TN 406 cannot, suggesting that FIPV receptors are heterogeneous consisting of types [2].

In the present study, we further characterized the roles of the feline APN as an FIPV receptor. Our results showed that type II FIPV uses the feline APN as a receptor, but it is not a receptor for type I FIPVs.

Materials and methods

Cell cultures

Felis catus fetus cells (fcwf-4), Crandell feline kidney cells (CrFK), swine testis cells (ST), swine kidney cells (CPK), and African green monkey kidney cells (Vero) were grown in Eagles' minimum essential medium (MEM) containing 50% L-15 medium, 10% fetal calf serum (FCS), 100 unt/ml penicillin and 100 μg/ml streptomycin. Human monocyte cells (U937) were cultured in RPMI 1 640 medium containing 10% FCS and antibiotics as above. The cells were maintained in a humidified 5% CO_2 incubator at 37 °C.

Viruses

FIPV strains 79–1 146, KU-2, Black and UCD-1, FECV strain 79–1 683, TGEV strain To-163, and CCV strain 1–71 were used in this study. FIPV strain 79–1 146 (type II) was supplied by Dr. M. C. Horzinek of State University Utrecht, The Netherlands. FIPV strain KU-2 (type I) was isolated in our laboratory. FIPV strain Black (type I) was supplied by Dr. J. K. Yamamoto of the University of Florida. FIPV strain UCD-1 (type I) was supplied by Dr. N. C. Pedersen of the University of California, Davis. FECV strain 79–1 683 was supplied by Dr. A. J. McKeirnan of Washington State University, Pullman. TGEV strain To-163 was supplied by National Institute of Animal Health of Japan. CCV strain 1–71 was supplied by Dr. E. Takahashi of the University of Tokyo, Japan.

FIPV and FECV, TGEV, and CCV were passaged two or three times in fcwf-4 cells, CPK cells, and CrFK cells, respectively, and used in subsequent experiments.

Production of monoclonal anti-receptor antibody-secreting hybridoma

BALB/c mice, about 5 weeks of age, were inoculated intraperitoneally with a mixture of 1×10^6 fcwf-4 cells collected with a cell scraper and 1×10^9 cells of a pertussis adjuvant. Two and four weeks after inoculation, a booster immunization with fcwf-4 cells alone was performed, and spleen cells were collected 4 days after the last immunization. Fusion was carried out essentially as described by Köhler and Milstein [15]. Polyethyleneglycol-4,000 (Merck, Germany) was used as a fusing agent, and the ratio of mouse spleen cells to mouse myeloma cells (P-3/I-63-Ag8-6, 5, 3) was 10: 1. Screening for anti-receptor antibody-secreting hybridomas was carried out by examining blockade of infection of fcwf-4 cells by type II FIPV strain 79–1 146. Antibody-secreting hybredoma colonies were cloned by limiting dilution method.

Flow cytometric analysis

A total of 1×10^6 cells were incubated with anti-receptor MAb at 4 °C for 1 h. The cells were washed three times in Hanks' balanced salt solution (HBSS) containing 0.1% NaN_3, resuspended and incubated with fluorescein isothiocyanate (FITC)-conjugated Fab of goat anti-mouse IgG antibody at 4 °C for 1 h. The cells were washed three times, and the number of stained cells was determined by counting about 10 000 cells on a fluorescence-activated cell sorter (FACS) 440 (Becton Dickinson).

Preparation of brush-border membrane proteins

Brush-border membrane proteins were purified according to the Booth and Kenny method [3]. The lumen of the small intestine of cats and pigs was washed with mannitol-Tris medium (10 mM mannitol, 2 mM Tris-HCl, pH 7.1), and microvilli were removed with a surgical blade. The mannitol-Tris medium was added to the microvilli to 10% (W/V), and homogenized with a blender. The total volume of the homogenate was 200 ml. Solid $MgCl_2$, $6H_2O$ was added to the homogenate to a final concentration of 10 mM, and occasionally stirred on ice for 15 min. The homogenate was centrifuged at 1 500 × **g** for 12 min, and the supernatant thus obtained was further centrifuged at 15 000 × **g** for 12 min. The pale-pink layer above the pellet was collected, and resuspended in 100 ml mannitol-Tris medium. $MgCl_2$ was added to the suspension to 10 mM, and gently stirred for 15 min. The suspension was centrifuged at 2 200 × **g** for 12 min, and the supernatant was further centrifuged at 15 000 × **g** for 12 min. The pellet was resuspended in 100 ml mannitol-Tris medium.

Purification of polypeptides recognized by the anti-recepter MAb by affinity chromatography

Polypeptides recognized by the anti-receptor MAb were purified from feline intestinal BBM proteins by affinity chromatography, using HiTrap NHS-Activated Affinity Colums (Pharmacia, Biotech, Sweden) according to the manufacturer's protocol. As the ligand, IgG purified from the culture fluid of anti-receptor MAb R-G-4 with protein A-sepharose CL-4B (Pharmacia Biotech, Sweden) was used. The molecular weights of the purified polypeptides were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining.

Aminopeptidase enzyme activity

The aminopeptidase substrate L-leucyl P-nitroanilide was added at 2 mM to samples, and allowed to react at 37 °C. After 30, 60, 120, and 180 min, the reaction was stopped by adding 0.15 M acetic acid, and the absorbance at 405 nm was measured.

Inhibition of the virus infectivity by BBM proteins or polypeptides recognized by the anti-receptor MAb

The inhibitory effects of receptor proteins on virus infection were tested as follows. The same amounts of a 10-fold dilution of BBM proteins and 200 PFU/0.1 ml of each virus strain were mixed, and allowed to react at 4 °C for 2 h . Aliquots of 0.1 ml of the mixture were inoculated into fcwf-4 cells cultured in 12-well plates, reacted at 37 °C for 1 h, then washed with HBSS, and 2 ml of medium containing 1.5% carboxymethyl cellulose was added.

The cells were incubated in a CO_2 incubator at 37 °C for 3 days, fixed in 10% buffered formalin, and stained with 1% crystal violet. The number of plaques produced by the remaining infectious virus was counted, and the degree of inhibition of each dilution was estimated as compared with the same virus titer when mixed with HBSS.

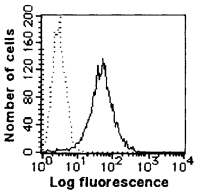
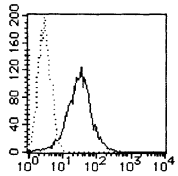
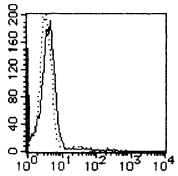
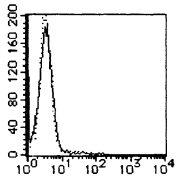
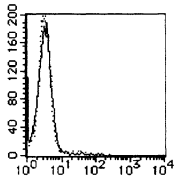
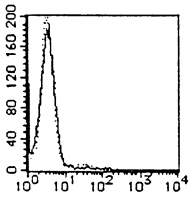
N-terminal amino acid sequencing of the polypeptide recognized by MAb R-G-4

The polypeptide recognized by MAb R-G-4 was separated by SDS-PAGE, and transferred onto a polyvinylidene difluoride (PVDF) membrane. PVDF membranes onto which were transferred the antigenic polypeptides were stained with Coomassie brilliant blue R-250, and the bands cut out. The N-terminal amino acid sequence was determined by APRO SCIENCE Co., Ltd., Japan.

Results

To obtain MAbs against the FIPV receptor, we produced hybridomas against fcwf-4 cells, a feline whole-fetus cell line highly susceptible to FIPV. A MAb which blocked infection of fcwf-4 cells by type II FIPV strain 79–1 146 was obtained and designated R-G-4. Table 1 shows the reactivity of the feline, porcine, human and simian cell lines with the MAb R-G-4 and their susceptibility to FIPV, FECV, CCV, and TGEV. MAb R-G-4 reacted to fcwf-4 and CrFK cells both derived from feline cells, but not to ST, CPK, U937 or Vero cells derived from porcine, human and simian cells. CrFK cells which were susceptible to FIPV strain 79–1 146, FECV strain 79–1 683, CCV strain 1–71, and TGEV strain TO-163 were nonsusceptible to type I FIPV strain KU-2, Black and UCD-1. We examined whether infection of fcwf-4 cells by these 7 coronaviruses was blocked by the

Table 1. Reactivity of feline, porcine, human and simian cell lines with the MAb R-G-4 and their susceptibility to coronaviruses

Cell	Susceptibility[a]							Reactivity to[b]
	FIPV				FECV	TGEV	CCV	
	79–1146	KU-2	Black	UCD-1	79–1683	TO-163	1–71	MAb R-G-4
fcwf-4	+[c]	+	+	+	+	+	+	
CrFK	+	−[d]	−	−	+	+	+	
ST	−	−	−	−	−	+	−	
CPK	−	−	−	−	−	+	−	
U937	−	−	−	−	−	−	−	
Vero	−	−	−	−	−	−	−	

[a]Cells were passaged if CPE was not detected after inoculation of viruses, and after 3 days, the virus-specific antigen was detected by indirect fluorescent antibody assay

[b]Detection by flow cytometry

[c]CPE and virus antigen-positive

[d]CPE and virus antigen-negative

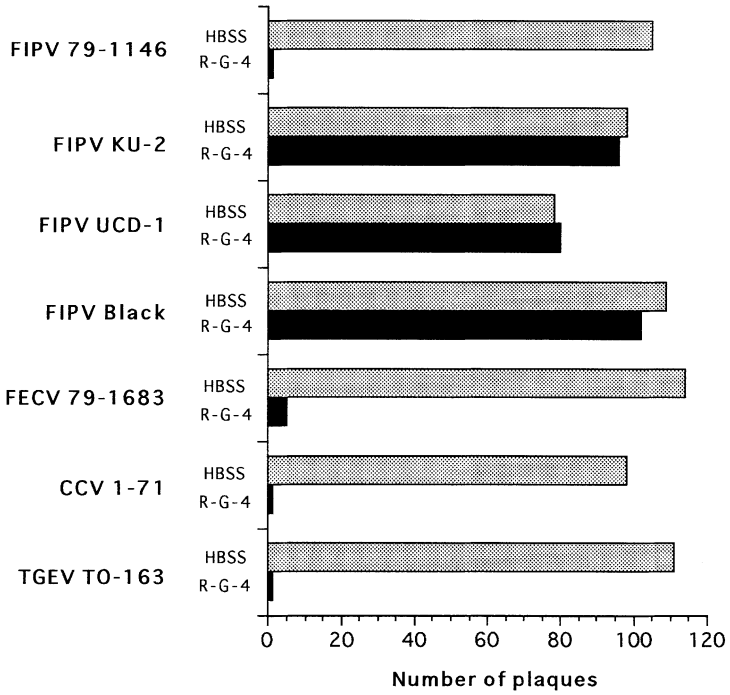


Fig. 1. Blocking of virus infection by the MAb R-G-4. The culture fluid of MAb R-G-4 secreting hybridoma or HBSS was added to fcwf-4 cells cultured in 12-well plates, and incubated at 37 °C for 1 h. The cells were washed with HBSS, inoculated with about 100 PFU/0.1 ml of each virus, and incubated at 37 °C for 1 h. The cells were washed with HBSS, then medium containing 1.5% corboxymethyl cellulose was added, and incubated at 37 °C for 3 days. The cells were fixed with 10% buffered formalin, and stained with 1% crystal violet, then the number of plaques was counted

MAb R-G-4. As shown in Fig. 1, MAb R-G-4 blocked infection by FIPV strain 79–1 146, FECV, CCV, and TGEV, but did not block that by KU-2, Black or UCD-1 which are classified into type I FIPV.

By flow cytometric analysis, we examined whether binding of the MAb R-G-4 to fcwf-4 cells was blocked by feline or porcine intestinal BBM proteins. As shown in Fig. 2, the reactivity of MAb R-G-4 which had been preincubated with porcine intestinal BBM proteins was not blocked as in the MAb R-G-4 which had been reacted with HBSS. On the other hand, the reactivity of MAb R-G-4 which had been preincubated with feline intestinal BBM proteins was blocked, and the peak was shifted closer to that of control cells. Therefore, we attempted to purify polypeptides recognized by the MAb R-G-4 from feline intestinal BBM proteins. Purification was performed using the MAb R-G-4 as the ligand by affinity chromatography. As shown in lane 3 of Fig. 3, the MAb recognized a polypeptide with a relative molecular mass of 120–130 kDa. The polypeptide was examined for aminopeptidase activity using L-Leucyl P-nitroanilide as a substrate. As shown in Fig. 4, the absorbance at 405 nm increased with time, and free P-nitroanilide

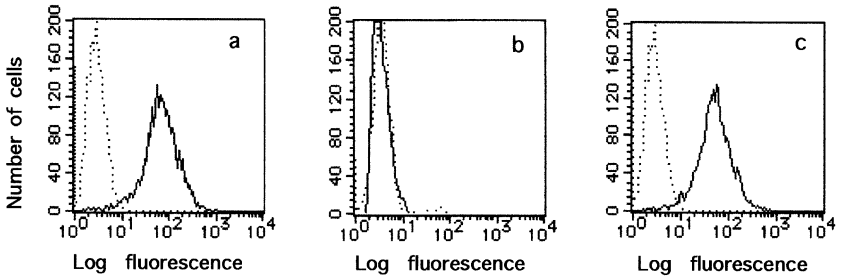


Fig. 2. Flow cytometric analysis of the MAb R-G-4 binding to feline and porcine intestinal brush-border membrane proteins. The MAb R-G-4 was mixed with HBSS (**a**), feline (**b**) or porcine (**c**) intestinal brush-border membrane proteins, and allowed to react at 37 °C for 1 h. The mixture was added to 1×10^6 fcwf-4 cells, and allowed to react at 4 °C for 1 h. After rinsing, FITC-conjugated Fab of goat anti-mouse IgG antibody was added, and the number of stained cells was determined with FACS 440. — fcwf-4 cells treated with the mixture; - - - control fcwf-4 cells (reacted with HBSS instead of the mixture)

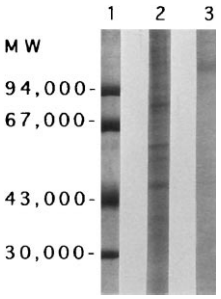


Fig. 3. Polypeptide recognized by the anti-receptor MAb R-G-4. Polypeptides recognized by the MAb R-G-4 were purified from feline intestinal brush-border membrane proteins by affinity chromatography using MAb R-G-4 as the ligand. *1* Marker proteins; *2* feline intestinal brush-border membrane proteins; *3* polypeptide recognized by MAb R-G-4

was detected. This enzyme activity was completely blocked by addition of the inhibitor, 1, 10-phenanthroline or Bestatine (Fig. 4). The aminopeptidase activity was not detected in the eluted fraction (data not shown). The first 15 amino acids of the polypeptide were determined by N-terminal sequencing: NH2-Ala-Lys-Gly-Phe-Tyr-Ile-Ser-Lys-Pro-Val-Ala-Ile-Leu-Ala-Ile-COOH. This sequence agreed with the N-terminal sequence of feline aminopeptidase N at 93.3%.

The virus-binding capacities of feline and porcine intestinal BBM proteins or the polypeptide recognized by the MAb R-G-4 were examined by neutralization of the virus infectivity. Ten fold-diluted samples were reacted with 200 p.f.u. FIPV strain 79–1 146, KU-2, Black, UCD-1, FECV strain 79–1 683, TGEV strain TO-163 or CCV strain 1–71 at 4 °C for 2 h, and the amounts of infectious viruses in the mixtures were determined by plaque assay. Feline intestinal BBM protein and the polypeptide recognized by the MAb R-G-4 inhibited the infectivity of FIPV strain 79–1 146, FECV strain 79–1 683, TGEV strain TO-163 and CCV strain 1–71. On the other hand, they did not inhibit the infectivity of the type I FIPV strains KU-2, Black, and UCD-1 (Fig. 5a, c). The infectivity of all viruses tested,

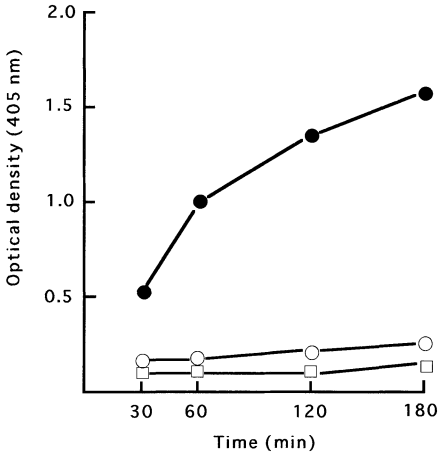


Fig. 4. Aminopeptidase activity of the polypeptide recognized by MAb R-G-4. 0.03 mg/ml polypeptide recognized by the MAb R-G-4 was mixed with the same amount of PBS (●), 3.0 M 1, 10-phenanthroline (○) or 0.1 mg/ml Bestatine (□), and allowed to react at 37 °C for 1 h. An aminopeptidase substrate, L-leucy1 P-nitroanilide, was added to each misture, and after 30, 60, 120, and 180 min, absorbance at 405 nm was measured

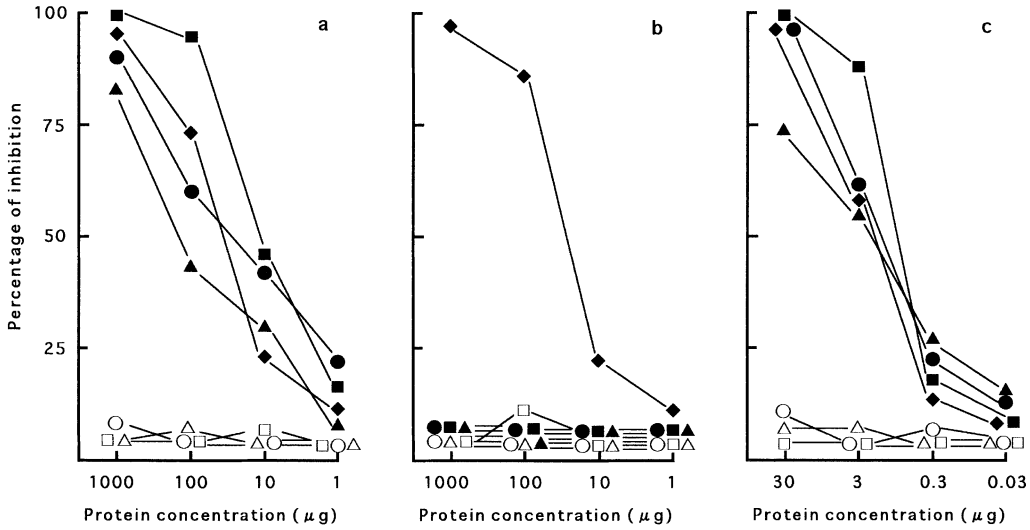


Fig. 5. Neutralization kinetics of coronaviruses by feline and porcine intestinal brush-border membrane protein, or polypeptide recognized by MAb R-G-4. Feline intestinal brush-border membrane protein (**a**), porcine intestinal brush-border membrane protein (**b**), or polypeptide recognized by MAb R-G-4 (**c**) were mixed with FIPV strain 79–1 146 (●), KU-2 (○), Black (□), UCD-1 (△), FECV strain 79–1 683 (▲), TGEV strain TO-163 (◆), or CCV strain 1–71 (■) and incubated at 4 °C for 2 h. The virus titers in the mixtures were plaque assayed on fcwf-4 cells

FIPV strain 79–1 146, FECV strain 79–1 683, TGEV strain TO-163 and CCV strain 1–71, was not inhibited by the eluted fraction which was not absorbed by the affinity column with the MAb R-G-4 as the ligand (data not shown). In the porcine intestinal BBM proteins, the infectivity of TGEV strain TO-163 alone was inhibited (Fig. 5b).

Discussion

We immunized mice with fcwf-4 cells which are highly susceptible to FIPV, and obtained MAb R-G-4 against the FIPV receptor. This MAb completely blocked the infectivity of type II FIPV strain 79–1 146, type II FECV strain 79–1 683, CCV strain 1–71, and TGEV strain TO-163 to fcwf-4 cells. Type II FIPV is serologically similar to type II FECV, CCV and TGEV, and the deduced amino acid sequence of S protein shows 95.4% homology with type II FECV, 90.9% homology CCV, and 80.9% homology TGEV [13, 19, 20]. It was reported that in cats experimentally infected with TGEV or CCV, the virus proliferated in the small intestine and caused asymptomatic infection [1, 24, 27]. The cellular receptor of these viruses including type II FIPV has been shown to be CD13/APN [2, 8, 29, 31]. The polypeptide recognized by the MAb R-G-4 which was purified from feline intestinal BBM proteins had aminopeptidase activity, and the first 15 N-terminal amino acid sequence was similar to that of feline APN. This shows that the polypeptide recognized by the MAb R-G-4 was the feline APN. However, MAb R-G-4 could not block infection of fcwf-4 cells by type I FIPVs.

The feline and porcine intestinal BBM proteins purified according to the Booth and Kenny method [3] had aminopeptidase activity which was inhibited by 1, 10-phenanthrolin or Bestatine (data not shown). CD13/APN is expressed at high levels in intestinal brush border membranes, and plays a role in the final steps of digestion by cleaving small peptides preferentially after N-terminal neutral amino acids. Feline intestinal BBM proteins and polypeptides recognized by the MAb R-G-4 which had been purified from the feline intestinal BBM proteins inhibited the infectivity of type II FIPV, type II FECV, CCV, and TGEV, but not of type I FIPVs. It was reported that TGEV can use the feline and canine APN as a receptor, but FIPV and CCV cannot use the porcine APN as a receptor [9]. This agrees with the observation that the infectivity of FIPVs, FECV, and CCV was not inhibited by porcine intestinal BBM proteins. MAb R-G-4 blocked the infectivity of TGEV toward fcwf-4 cells, but not that of TGEV toward porcine ST and CPK cells (data not shown). MAb R-G-4 reacted with feline fcwf-4 and CrFK cells, but did not react with feline ST or CPK cells. It was suggested that the MAb R-G-4 recognized the antigenic domain specific to the feline APN.

Tresnane et al. reported that both type I FIPV and type II FIPV infected feline APN-transfected hamster cells [29]. However, in the present study: 1) The MAb R-G-4 blocked infection of fcwf-4 cells by type II FIPV strain 79–1 146, but did not block infection by type I FIPVs; 2) MAb R-G-4 recognized the feline APN; 3) CrFK cells which reacted with MAb R-G-4 were infected by type II FIPV, but not by type I FIPVs; and 4) Feline intestinal BBM proteins with the aminopeptidase activity and polypeptides recognized by the MAb R-G-4 inhibited the infectivity of type II FIPV, but not of type I FIPVs. These results showed that type II FIPV uses the feline APN as a receptor, but it is not a receptor for type I FIPVs. In the experiment by Tresnan et al., the infection rate of type I FIPV to feline

APN-transfected hamster cells was much lower than that of type II FIPV (2% and 30%, respectively) [29]. Benbacer et al. showed that type II FIPV can use the canine APN as a receptor but type I FIPV cannot, supporting our results [2]. The amino acid sequences of S proteins of type I and type II FIPVs showed obly about 46% homology [20], which may have been responsible for the differences in the receptor between these FIPVs. The homology in the N-terminal 700 amino acids of S proteins is very low, and there are few domains where the sequence of 5–6 consecutive amono acids is maintained. Neither type I nor type II FIPVs infected porcine ST or CPK cells. However, Woods et al. showed that type I FIPV strain UCD-1 infected newborn pigs [30]. This result suggested that strain UCD-1 may use receptor proteins other than the APN because FIPV cannot use the porcine APN as receptors. It has not been verified whether type II FIPV infects newborn pigs, or whether proteins other the APN can be used as receptors. However, the study by Woods et al. [30] supports our observation that type I FIPV does not use the APN as a receptor unlike type II FIPV.

We previously reported that in the phylogenetic tree of the M and N protein-deduced amino acid sequences, type I and type II FIPVs belong to the same group as type II FECV, and these viruses are evolutionarily distant from CCV and TGEV. In the phylogenetic tree of the S protein-deduced amino acid sequences, however, type I FIPV strains form a group, which is very distant from the type II FIPV, type II FECV, CCV, and TGEV groups [20]. In the present study, we demonstrated that type II FIPV, type II FECV, CCV, and TGEV form a group because these viruses use CD13/APN as a receptor, while type I FIPV alone uses a different receptor, indicating that it is distant from those groups. Although the receptor binding site of FIPV is unknown, there are important antigenic domains in S proteins such as the neutralization and enhancement sites [4, 5, 11–13, 21, 22]. Generally, the distribution of cells expressing virus receptors is an important factor determining in viro proliferation of viruses (pathogenicity). Although type I and type II FIPVs use different receptors, these FIPVs cause the same diseases because FIP is likely immune-mediated and not directly related to virus replication. It is hoped that the receptor (CD13/APN) binding site of type II FIPV and the cellular receptor of type I FIPV will be identified in the near future.

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