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Interspecies Aminopeptidase-*N* Chimeras Reveal Species-Specific Receptor Recognition by Canine Coronavirus, Feline Infectious Peritonitis Virus, and Transmissible Gastroenteritis Virus

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We report that cells refractory to canine coronavirus (CCV) and feline infectious peritonitis virus (FIPV) became susceptible when transfected with a chimeric aminopeptidase-*N* (APN) cDNA containing a canine domain between residues 643 and 841. This finding shows that APN recognition by these viruses is species related and associated with this C-terminal domain. The human/canine APN chimera was also able to confer susceptibility to the porcine transmissible gastroenteritis virus (TGEV), whereas its human/porcine homolog failed to confer susceptibility to CCV and FIPV. A good correlation was observed between the capacity of CCV, FIPV, and TGEV to recognize the different interspecies APN chimeras and their ability to infect cells derived from the relevant species. As an exception, TGEV was found to use a human/bovine APN chimera as a receptor although itself unable to replicate in bovine cells.

Coronaviruses are pathogens that primarily infect the respiratory and the enteric epithelia. Sequence analysis of their genome and antigenic relationships showed that the coronavirus genus could be divided into at least three groups. One of them is comprised of the transmissible gastroenteritis virus (TGEV), the human coronavirus 229E (HCV-229E), the porcine epidemic diarrhea virus, the canine coronavirus (CCV), the feline infectious peritonitis virus (FIPV), and their variants (6, 14). Aminopeptidase-*N*/CD13 (APN) (EC 3.4.11.2) has been shown to act as a receptor for two of the members of this genetic subset, i.e., HCV-229E and TGEV (3, 17). APN/CD13 is highly expressed at the intestinal brush border membrane and plays a role in the final steps of digestion by cleaving small peptides preferentially after N-terminal, neutral amino acids. The enzyme is also expressed in other tissues, in which its physiological role is still ill defined (13). APN is a type II glycoprotein of about 160,000 in molecular weight (160K), and its large extracellular carboxy-terminal domain contains a pentapeptide catalytic sequence (His-Glu-X-X-His), characteristic of members of the zinc-binding metalloprotease family (8).

For coronaviruses TGEV and HCV-229E, the introduction of the species-related APN cDNA into nonpermissive cells was sufficient to render them susceptible to virus infection (3, 17). A series of APN chimeras between porcine APN (pAPN) and human APN (hAPN) was constructed, and the region between residues 717 and 813 was found to be essential for infection by TGEV (5). CCV, FIPV, and TGEV have close genetic and antigenic relationships. However, on the basis of cross-neutralization assays, FIPV can be subdivided into serotypes I and II (12). The former appears to be feline restricted, whereas the second is antigenically highly related to CCV and TGEV (2). FIPV and CCV have never been reported as being able to replicate in porcine cell lines, whereas TGEV can multiply in canine and feline cells (7). Earlier experiments using pAPN-BHK cell clones have shown that FIPV and CCV cannot use pAPN as a receptor in vitro (4).

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Chimeric APN cDNA constructs. In an attempt to see whether coronaviruses other than TGEV and HCV-229E can use APN as a receptor, two different APN chimeras were constructed and tested for their receptor activity in transfected cells. This approach was based on the assumption that the APN domain previously defined as critical for the species-specific interaction with TGEV (5) could play a similar role with other coronaviruses. Two canine and bovine APN cDNA inserts (0.6-kb homolog to the porcine domain of AP18-hph, formerly reported as AP18) (4) (Fig. 1) were obtained by PCR with 2 μl of relevant intestine cDNA library (Clontech) as templates and the oligonucleotides 5′-GGAGGAAGATTC AGACTCAGCTGCAGA (human-derived sequence) (11) and 5′-GGGTGTAACCCAGGTACCTGTTCAGGA (porcine-derived sequence) (5) as primers with internal *Pvu*II and *Kpn*I sites, respectively. The resulting inserts were cloned, sequenced on two independent clones (EMBL accession numbers X98239 and X98240 for canine and bovine APN cDNAs, respectively), and introduced in a hAPN cDNA that had previously been silently mutated at +2740 to destroy a *Pvu*II restriction site (5), by exchanging the homologous sequence (Fig. 1). The chimeric APN constructs were called AP18-hch and AP18-hbh and cloned in pcDNA1 (Invitrogen).

CCV can use APN as a receptor. BHK-21 cells, which are naturally resistant to CCV, FIPV, TGEV, and bovine coronavirus (BCV) infections, were cotransfected by lipofection with the plasmids encoding the AP18-chimeric cDNAs and pSV2neo. For each chimera, three independent clones resistant to the neomycin analog G418 were then selected through flow cytometric analysis by using the anti-hAPN antibody SJ-1D1 (Immunotech, Marseille, France). The results of infections by CCV strain IRC5 (provided by G. Chappuis, Rhône-Mérieux, Lyon, France) or BCV strain G110 (provided by J.-F. Vautherot, INRA, Jouy-en-Josas, France) of the APN-expressing clones are summarized in Table 1 and illustrated in Fig. 2. Of hAPN, pAPN, and AP18-hch, only the lattcr allowed CCV replication in BHK cells. Hence, this result established that CCV can recognize the APN molecule as a receptor in a species-specific manner and that species specificity is essen-

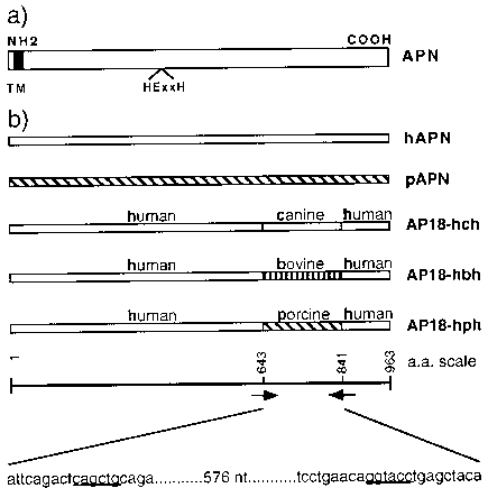

FIG. 1. Interspecies APN chimeras. (a) Schematic linear representation of the primary structure of APN. The positions of the transmembrane domain (TM) and the enzymatic site (HExxH) are indicated. (b) Structures of chimeric APNs. hAPN sequences are represented by open boxes, and canine, bovine, or porcine sequences are represented by different motifs. The numbers of the amino acids are given according to the pAPN sequence (Z29522). Positions of primers are indicated by arrows.

tially associated with residues of the 643- to 841-amino-acid stretch. A low proportion of BHK cells became infected by BCV regardless of whether they expressed hAPN, AP18-hbh, or no recombinant protein (Table 1). It was therefore concluded that AP18-hbh does not facilitate BCV infection.

Receptor activity of APN chimeras to CCV, FIPV, and TGEV. In order to establish to what extent interaction with the region 643 to 841 modulates the species spectrum of the above viruses, we examined their infectivity towards cells stably expressing any one of the three AP18 chimeras (-hch, -hbh, and -hph). As reported in Table 1 and in Fig. 2, BHK cells transfected with AP18-hph or with AP18-hbh (three independent clones per chimera) showed no infection 18 h postinfection with CCV in contrast to the AP18-hch-expressing clones, as revealed by an indirect immunofluorescence assay. TGEV Purdue 115 strain replication was demonstrated in the AP18-hph-expressing cells as previously described (5), and also in the AP18-hch- and AP18-hbh-chimera-expressing cells. The same observation was made when using transfected MDCK cell clones instead of BHK clones (Table 1). In addition, the expression of AP18-hch was able to confer susceptibility to the FIPV strain 79-1146 (serotype II; provided by M. Horzinek, University of Utrecht), but we failed to detect any infection at 40 h postinfection with the TN406 FIPV strain (serotype I; provided by G. Chappuis, Rhône-Mérieux) revealed by using a polyclonal anti-FIPV ascites provided by M. Eloit (ENV, Maisons-Alfort, France). The bovine and porcine AP18 homologs did not confer susceptibility to the FIPV strain 79-1146. From these data, the following conclusions were drawn: (i) As with CCV, the FIPV strain 79-1146 can use an APN molecule as a receptor, and this interaction is essentially mediated by the 643- to 841-amino-acid domain. (ii) TGEV is able to accommodate the heterologous human/canine chimera and, less expectedly, the human/bovine chimera.

Virus replication in species-heterologous cell lines. The above findings raise the question of whether CCV, TGEV, and FIPV have the capacity to replicate in species-heterologous cell lines and to gain entry into the cell by binding on the endogenous APN. As presented in Table 2, the canine A72 cell line (provided by W. S. K. Chalmers, Intervet) was able to replicate not just CCV but also TGEV and the FIPV strain 79-1146. These observations extended data previously obtained on the susceptibility of canine kidney cells and the canine A72 cell line to replicate CCV and TGEV (1, 16). Observations carried out by flow cytometric analysis using the six antibodies recognizing pAPN showed that only G3 was able to recognize the canine APN. However, assays aiming at blocking A72 cell infection by TGEV, FIPV, and CCV with this antibody failed, probably due to its low intrinsic neutralization activity (not shown).

Considering that AP18-hbh expression conferred TGEV susceptibility, it was of interest to examine whether TGEV was able to replicate in a bovine cell line. Two bovine cell lines, MDBK (Madin Darby bovine kidney) and EBTr (embryonic trachea from *Bos taurus*), were infected by TGEV at different multiplicities of infection. No infection could be proved through immunofluorescence analysis (Table 2). To see whether the nonsusceptibility of the two bovine cell lines could be associated either with a lack of receptor recognition or with a possible block after the receptor binding step, the following experiments were performed. (i) Flow cytometric analysis failed to detect any binding of TGEV to MDBK cells with porcine ST cells used as a positive control (not shown). (ii) MDBK and EBTr cells transfected with a plasmid encoding the pAPN were infected with TGEV. Newly synthesized viral antigens were observed in the EBTr-transfected cells (Table 2), indicating that these cells are competent for TGEV replication.

Conclusions. In this study, we showed that the APN receptor can be engineered to modulate the specificity of recognition by TGEV, CCV, and FIPV, three viruses antigenically closely related. The chimeras consisted of hAPN in which an approximately 200-amino-acid domain was replaced by its counterpart in the APN from a heterologous species. Their respective receptor activity was examined by stable expression in otherwise refractory cells. Strikingly, the canine chimera (AP18-hch) conferred susceptibility to infection by all three viruses. It thus exhibited a wider spectrum of virus recognition than the porcine (AP18-hph) and bovine (AP18-hbh) chimeras, both recognized by TGEV only (Table 1).

TABLE 1. Virus susceptibility of cells expressing wild-type and chimeric APN[a]

Cell type	APN expressed by cells	Infection by virus[b]: CCV	TGEV	FIPV Strain 79-1146	FIPV Strain TN406	BCV
BHK		−	−	−	−	(−)[c]
	hAPN	−	−	−	−	(−)
	pAPN	−	+	−	−	(−)
	AP18-hch	+	+	+	−	(−)
	AP18-hbh	−	+	−	−	(−)
	AP18-hph	−	+	−	−	(−)
MDCK		−	−	−	ND[d]	−
	hAPN	ND	−	ND	ND	ND
	pAPN	ND	+	ND	ND	ND
	AP18-hbh	−	+	−	ND	−
	AP18-hph	ND	+	ND	ND	ND

[a] Susceptibility was assayed by detection of viral antigens by indirect immunofluorescence using appropriate antibodies. Cells susceptible to the different viruses were infected in parallel as positive control.

[b] Cells were infected at a multiplicity of infection of 2. + and − indicate >10% infected cells and no infected cells, respectively.

[c] Presence of some infected cells (fewer than 10 per 10^6 cells).

[d] ND, not done.

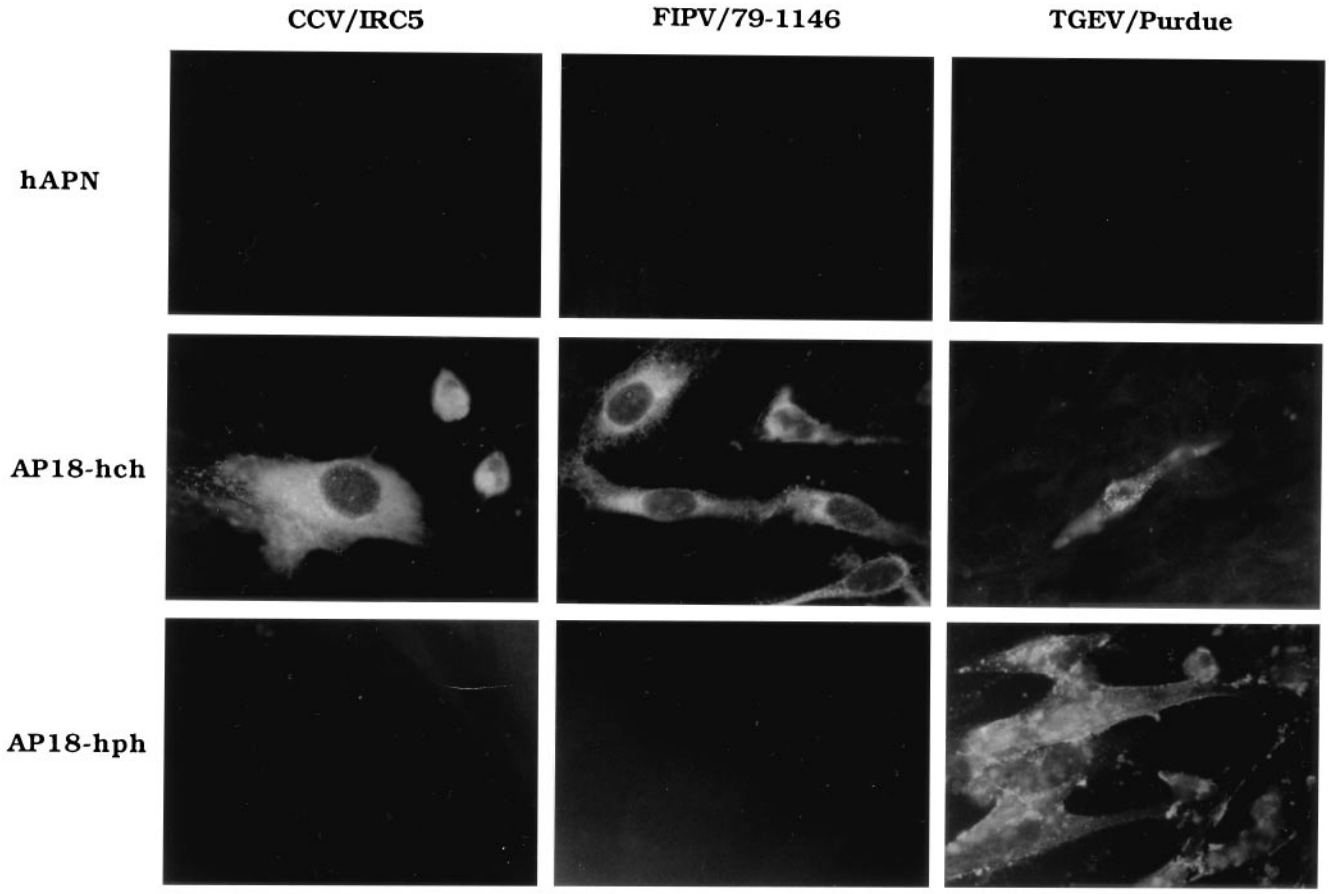

FIG. 2. Receptor activity of different APN chimeras. BHK cell clones expressing the chimeras AP18-hch and AP18-hph were infected with either CCV, FIPV serotype II, or TGEV, and virus antigens were detected 20 h after virus inoculation by immunofluorescence using the antispike antibody 48.1 (9).

It was also found that the ability of these viruses to use (or not) the different APN chimeras generally reflects their capacity to replicate in cells from heterologous species in vitro. Thus, TGEV is able to replicate in canine cells, whereas CCV and FIPV appear to be unable to infect porcine cells (Table 2). Taken together, our findings provide strong evidence that, like TGEV and HCV-229E, CCV and FIPV use APN as a receptor to enter cells from their natural host. Cloning of the canine and feline APNs would be of interest to examine to what extent the receptor/spike protein binding efficiency contributes to the species specificity.

TABLE 2. Permissivity of cultured cell lines originating from different species for CCV, FIPV, and TGEV[a]

Cell line	Species origin	CCV	FIPV strain		TGEV
			79-1146	TN-406	
A72	Canine	+	+	−	+
FCWF	Feline	+	+	+	+
ST	Porcine	−	−	−	+
MDBK	Bovine	ND[b]	ND	ND	−
EBTr	Bovine	ND	ND	ND	−
EBTr-pAPN		ND	ND	ND	+

[a] Susceptibility was assayed by detection of viral antigens by immunofluorescence.
[b] ND, not done.

Three points in the presented data need a specific comment. Firstly, in this and our previous study (3), the canine MDCK cell line, known to express endogenous APN at its surface (14), was nevertheless fully refractory to infection by any of the three viruses. The apparent lack of receptor activity of canine MDCK APN may be explained by some polymorphism of the canine APN gene or by a mutation acquired during passaging of this line. Secondly, cells expressing chimeric or authentic canine APN could not be infected by type I FIPV (strain TN506), unlike that seen with type II FIPV (strain 79-1146). This observation is consistent with the recent proposal that type II virus might have originated from a recombination between type I virus and CCV (15). Thirdly, although the bovine APN chimera was revealed to act as a receptor for TGEV, we (and others) failed at infecting bovine cells with the latter. Pairwise alignment between the bovine and porcine stretches introduced in the chimeras showed a higher level of amino acid identity compared to that for the canine and porcine stretches (82 and 71%, respectively). Thus, a possible explanation would be that TGEV binds authentic bovine APN as well and that the infection is restricted at a subsequent step. Such a situation is likely to account for the observed inability of HCV-229E to infect hAPN-expressing MDCK cells (5). However, we favor an alternative view in which TGEV infection of bovine cells is restricted at the receptor level. This is supported by the finding that bovine cells expressing pAPN were fully competent for TGEV infection. If really so, this would imply that a bovine

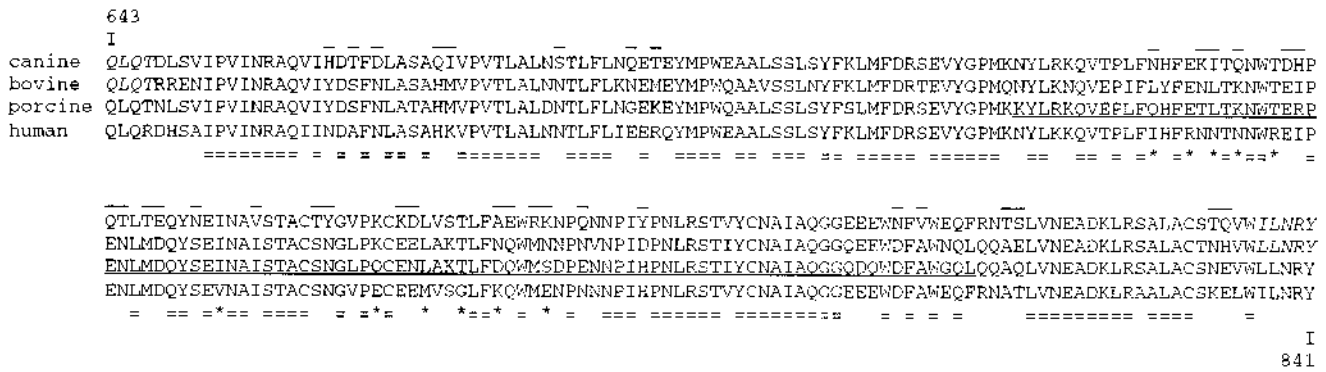

FIG. 3. Multialignment of the canine, bovine, porcine, and human homolog domains of the AP18 constructs. The single-letter amino acid code is used throughout. Amino acids derived from the primer sequence are in italics. Amino acids conserved in the four sequences are reported below the alignment (equality sign). The underlined porcine stretch represents the minimal pAPN domain able to confer TGEV permissivity (amino acids 717 to 813) in the AP24 chimera previously described (5). Stars indicate human amino acids which are specific to the human sequence between amino acids 717 and 813; overlined amino acids are specific to the canine sequence.

APN domain expressed in a human context can be better used by TGEV than this domain expressed in the homologous molecule, and/or that a region(s) of the APN molecule other than the 643- to 841-amino-acid stretch can modulate the TGEV-APN interaction.

The present data strengthen the view that the same region of the carboxy-terminal domain of APN is important in governing the species specificity of virus recognition by the three serologically related viruses FIPV, CCV, and TGEV. Binding to chimeric APN most probably involves crucial amino acids within the 643 and 841 stretch. Multialignment of the relevant APN domains revealed a 56% amino acid identity among the four sequences and a complete conservation of proline (10) and cysteine (4) residues, whereas potential Asn-linked glycosylation sites vary in number (one to three) and location (Fig. 3). The TGEV binding site was previously shown to implicate the 717 to 813 region, where the porcine and human sequences diverge by 28 amino acids (5). A pairwise alignment of the same APN region using the four sequences now available revealed that only 11 amino acids are specific to hAPN (Fig. 3). These amino acids are likely to play an essential role in the species specificity of APN recognition by TGEV.

This study represents an additional step toward a better understanding of the relationships between coronaviruses and their receptors identified as APN. It lends further support to the view that molecular constraints at the level of receptor recognition determine at least in part their species specificity.

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