FURTHER CHARACTERIZATION OF AMINOPEPTIDASE-N AS A RECEPTOR FOR CORONAVIRUSES

Bernard Delmas,[1] Jacqueline Gelfi,[1] Hans Sjöström,[2]
Ove Noren,[2] and Hubert Laude[1]

[1]Unité de Virologie et Immunologie Moléculaires
Institut National de la Recherche Agronomique
78352 Jouy-en-Josas, France
[2]Department of Biochemistry C, Panum Institute
University of Copenhagen, DK2200 Denmark

ABSTRACT

We recently reported that porcine aminopeptidase-N (pAPN) acts as a receptor for transmissible gastroenteritis virus (TGEV). In the present work, we addressed the question of whether TGEV tropism is determined only by the virus-receptor interaction. To this end, different non-permissive cell lines were transfected with the porcine APN cDNA and tested for their susceptibility to TGEV infection. The four transfected cell lines shown to express pAPN at their membrane became sensitive to infection. Two of these cell lines were found to be defective for the production of viral particles. This suggests that other factor(s) than pAPN expression may be involved in the production of infectious virions. The pAPN-transfected cells were also tested for their susceptibility to several viruses which have a close antigenic relationship to TGEV. So far, we failed to evidence permissivity to the feline infectious peritonitis coronavirus FIPV and canine coronavirus CCV. In contrast, we found clear evidence that porcine respiratory coronavirus PRCV, a variant of TGEV which replicates efficiently in the respiratory tract but to a very low extent in the gut, may also utilise APN to gain entry into the host cells. This suggests that the switch between TGEV and PRCV tropisms in vivo may involve other determinant(s) than receptor recognition.

INTRODUCTION

Coronaviruses are characterized by a restricted host range and tissue tropism[1]. Thus, TGEV replicates selectively in the enterocytes covering the villi of the small intestine[2], whereas human coronavirus 229E (HCV-229E) multiplies in the upper respiratory tract[3]. It was recently reported that APN/CD13 acts as a receptor for these two viruses[4,5]. APN/CD13 is expressed in a large variety of tissues and cells -notably epithelial and myeloid cells- (6,7 and references therein). The highest APN activity is associated with the brush border membrane of the enterocytes and of the renal proximal tubule cells. The distribution of APN and the site of multiplication of TGEV in the intestine are strongly correlated. In other organs like liver, lung, kidney and in cells of the myeloid lineage where APN is expressed, TGEV may replicate, but without causing the histological damages observed in the intestine[8]. HCV-

Coronaviruses, Edited by H. Laude and J.F. Vautherot
Plenum Press, New York, 1994

229E multiplies in the epithelium of the trachea where APN is assumed to be expressed, but in contrast to TGEV, HCV-229E enteric infections have not been clearly identified[3]. Taken together, these observations strongly suggest that APN expression is a prerequisite to allow virus multiplication, but is not the sole determinant of the tissue tropism in vivo. In the first part of this paper, we report data from in vitro experiments which suggest that other factor(s) than APN expression might modulate the susceptibility to TGEV.

The second part deals with experiments aiming to establish whether APN expression would confer susceptibility to three viruses antigenically related to TGEV: the feline infectious peritonitis virus FIPV, the canine coronavirus CCV and the porcine respiratory coronavirus PRCV, the respiratory variant of TGEV. Both FIPV and CCV have been reported to replicate in the small intestine of experimentally infected piglets[9], thus suggesting that they might use porcine APN as a receptor. In contrast, PRCV replicates selectively in the lung alveolar epithelium and at a very low level in the small intestine enterocytes. The fact that PRCV spike gene encodes a truncated protein has led to the hypothesis that the altered tropism of PRCV could relate to an impaired interaction with the APN molecule[10].

MATERIAL AND METHODS

Viruses. The isolate RM4 of PRCV, the CCV strain K378/20 (both supplied by Rhône-Mérieux, Lyon) and the FIPV strain 79.1146 (supplied by M. Horzinek, Utrecht) were used as a source of virus.

Cell transfections. The cDNA encoding the pAPN was subcloned downstream of the ubiquitin promotor in the BamHI site of the pTEJ4 expression vector[4]. MDCK, HRT-18, BHK-21 and Vero cells were cotransfected with this construct and pSV2neo by $CaPO_4$ precipitation (MDCK cell line) or by lipofection (other cell lines). Cell clones resistant to the neomycin analogue G418 were selected and assayed for APN expression by measuring a APN activity (MDCK cell clones) or by TGEV susceptibility acquisition (other cell clones).

PRCV-induced cytopathic effect. The assays were performed 16h after infection at a multiplicity of 0.1 PRCV plaque-forming units (p.f.u.) per cell. Monolayers were fixed and stained with a crystal violet solution (10% alcohol). The dye associated to intact cells was quantified by optical absorbance after solubilization in acetic acid[11].

RESULTS AND DISCUSSION

ANOTHER FACTOR THAN AMINOPEPTIDASE-N EXPRESSION INVOLVED IN TGEV PERMISSIVITY ?

Clones derived from four cell lines (MDCK, HRT-18, BHK-21 and Vero cells) and expressing recombinant pAPN were selected. They were assayed for their susceptibility to TGEV infection by several approaches: detection of viral antigens synthesized in infected cells by immunofluorescence or immunoprecipitation assays, measurement of the cytopathic effect (c.p.e.) induced by the infection, titration of infectious viral progeny and quantification of viral particles produced. All the pAPN-expressing clones derived from the four cell lines studied became TGEV-sensitive as determined by the synthesis of viral antigens and by the c.p.e. observed 12 to 18h post infection (p.i.), after infection at a multiplicity of 10-20 p.f.u. per cell (not shown). BHK- and Vero-derived clones produced infectious virions in the same range than the swine testis cell line ST, which is highly permissive to TGEV (5.8×10^6 to 1.4×10^7 p.f.u./ml). In contrast, infectivity titers recovered from MDCK- and HRT-derived clones 20h p.i. did not differ significantly from the titer of the virus measured after viral adsorption (Fig. 1). Thus, MDCK and HRT cell clones failed to produce infectious particles. To confirm this observation, MDCK cell monolayers were infected at different multiplicities of infection

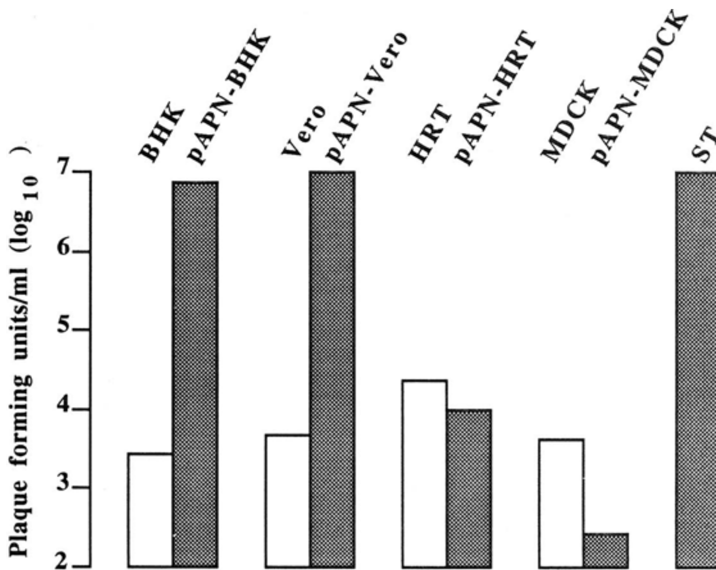

Figure 1. Infectious virus production in different cell lines expressing constitutively porcine aminopeptidase-N. Cell cultures were infected with TGEV, inoculum was rinsed 1h after adsorption and the total infectivity titer was determined 20h post infection by plaque assay on ST cells.

Table 1. TGEV cytopathic effect[a] as a function of the multiplicity of infection in two pAPN-expressing cell lines.

Cell clone	Multiplicity of infection				
	10	1	0.1	0.01	0.001
pAPN-BHK	++[b]	++	++	++	+
pAPN-MDCK	++	+	-	-	-

[a] Observed 48h post infection.

[b] Degree of lysis of the cell sheets: >90% (++); between 90 and 10% (+); <10% (-).

(m.o.i.) and the viability of the cells was measured 48 h p.i. so as to allow several cycles of virus multiplication. Table 1 shows that MDCK monolayers became destroyed only at a high m.o.i. (>1), in contrast to BHK monolayers, which were lysed at a markedly lower m.o.i. In the same way, we tried to identify formation of viral particles by metabolic ^{35}S-labeling of infected MDCK cells followed by PAGE analysis of the labeled material obtained through high-speed sedimentation of cell culture supernatants. We failed to detect labeled virus between 5 to 11h p.i. whereas viral particles were detected 6h p.i. in a control infection of ST cells (not shown).

Altogether, these results suggest that the presence of APN on the cell surface is not sufficient by itself to allow a complete cycle of replication. The defectiveness in viral production of MDCK clones seems to involve a late event in TGEV replication cycle since accumulation of structural viral proteins was clearly evidenced in infected cells. There are several hypotheses to explain such results. MDCK cell restriction could be associated to the presence (or the absence) of a cellular factor. The fact that all the porcine kidney cell lines checked in our laboratory are permissive to TGEV indicate that this putative factor would be species-specific. Another hypothesis is based on the fact that the pAPN-MDCK clones were selected on their capacity to express large amounts of pAPN: 50 to 100 fold the basal expression of APN in the MDCK cell line. It is possible that during the process of virus maturation, the newly synthetized particles are captured, via the viral spikes, by the APN molecules present in large amount in the endoplasmic reticulum, thus resulting in a block of virion maturation and/or transport. To explore this hypothesis, we plan to obtain clones of ST cells, naturally permissive to TGEV replication and expressing large quantities of APN in order to examine their capacity to produce viral particles.

PORCINE RESPIRATORY CORONAVIRUS UTILIZES PIG AMINOPEPTIDASE-N AS A RECEPTOR

To show if FIPV and CCV can use pig APN as a receptor, we performed infections of BHK cells expressing recombinant pig APN. No c.p.e. could be observed in these cells after infection with FIPV or CCV (not shown). These results would indicate that neither FIPV nor CCV are able to infect pAPN-expressing BHK clones. They are consistent with the fact that our attempts to infect any of the pig cell lines available in the laboratory with FIPV and CCV have met with no success. Nevertheless, the possibility remains that FIPV and CCV still may bind porcine APN, thus implying other factor(s) than receptor recognition could modulate the viral infection. In this respect, we observed that MDCK-derived clones expressing human APN (hAPN) were not susceptible to the human coronavirus 229E, whereas hAPN-3T3 clones became sensitive to HCV-229E (5 and data not shown). Alternatively, it is tempting to speculate that FIPV and CCV recognize feline and canine APN in a species-specific manner, as it seems to be the case for TGEV and HCV-229E.

To establish if PRCV can use pAPN as a receptor, PRCV infections of MDCK and BHK cells expressing pAPN were carried out. As shown in Fig. 2, a specific c.p.e. was observed in a pAPN-BHK cell clone whereas BHK cells behave as unsensitive cells. A similar result was obtained with MDCK-derived cell clones (not shown). Preincubation of the pAPN-BHK cell clone with the anti-APN antibody G43 resulted in a complete block of TGEV infection. In addition, we analyzed the synthesis of the PRCV nucleoprotein N in infected cells by immunofluorescence assay. A cytoplasmic fluorescence was observed in pAPN-BHK cells only (not shown). To confirm these results, ST cells were incubated with dilutions of the six anti APN antibodies before infection. Table 2 shows that these antibodies are able to block PRCV infection in the same range than they do with TGEV.

Altogether, these results provide strong evidence that the respiratory virus PRCV, like the enteropathogenic virus TGEV, recognize pAPN on target cells. We previously demonstrated, using two different binding assays, that TGEV binds directly to pig APN[4]. To complete these studies, we performed a binding assay between TGEV and pure pAPN preincubated with the anti-S neutralizing antibody 48.1. This antibody was able to block binding between virions and APN, at the same extent as an anti-receptor antibody did

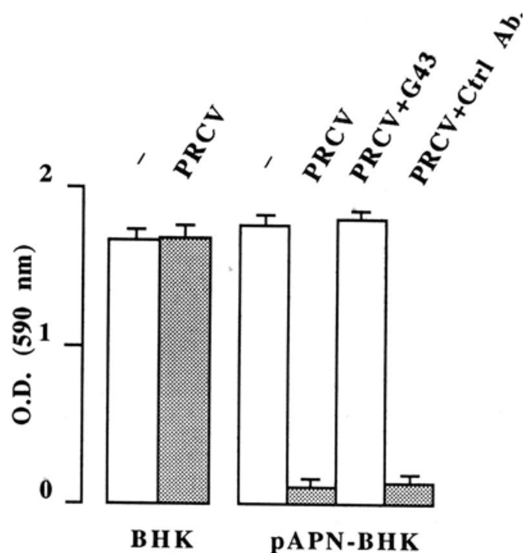

Figure 2. Colorimetric quantification of pAPN-BHK and BHK cells survival after infection or mock-infection (-) by PRCV. The dye incorporated in surviving cell monolayers was measured by optical absorbance. In two assays, G43 or a control antibody were added before infection. The data are given as mean values + s.d.m. (n=4).

Table 2. Neutralizing activity of anti-APN antibodies towards TGEV and PRCV in ST cells.

	Neutralization titer [a]	
Antibody	TGEV	PRCV
G 3	160	<20
G 18	5,000	7,500
G 43	20,000	40,000
I 31	320	20
T 35	160	<20
W 26	20,000	20,000

[a] Expressed as the reciprocal of the last dilution protecting against the viral cytopathic effect.

binding between virions and APN, at the same extent as an anti-receptor antibody did (unpublished results). Thus, the viral S protein binds specifically to APN. The S gene of different PRCV strains is deleted of 672 (or 681) nucleotides, resulting in a shortening of 224 (or 227) amino acid stretch in the N terminal part of the protein[10]. Since PRCV is able to infect BHK cells expressing pAPN, we conclude that this amino acid stretch missing in the S PRCV protein is not directly involved in the binding of the S protein on APN.

In conclusion, it appears that the difference of tropism between PRCV and TGEV cannot be simply explained by the unability of PRCV to interact with APN. PRCV replicates selectively in the respiratory tract : in the alveolar epithelial cells, which are assumed to express APN and in the alveolar macrophages where CD13, a marker of the myeloid lineage shown to be identical to APN[7], is also expressed. It is thus conceivable than PRCV uses APN to gain entry in these cells. The fact that the respiratory virus HCV-229E also uses human APN as a receptor also favors this view. Several hypotheses can be made to explain the defect of productive PRCV infection in the intestine, such as an instability of PRCV spike in the digestive tract or a low capacity of PRCV spike protein to induce membrane fusion between viral particles and enterocyte membranes. Finally, the conversion of ORF3a to a pseudogene in PRCV genome has also to be considered as possibly contributing to its altered tropism.

Acknowledgments

B.D. was supported in part by a research grant from the Commission of the European Communities.

REFERENCES

1. S. Siddell, H. Wege and V. Ter Meulen, The biology of coronaviruses, *J. gen. Virol.* 64:761 (1983).
2. M. Pensaert, E.O. Haelterman and T. Burstein, Transmissible gastroenteritis of swine: virus-intestinal cell-interactions, *Arch. Gesamte. Virusforch.* 31:321 (1970).
3. K. McIntosh, in *Virology* 2nd edn (eds Fields , D.N. *et al.*) pp857, Raven, New York (1990).
4. B. Delmas, J. Gelfi, R. L'Haridon, L. K. Vogel, H. Sjöström, O. Noren and H. Laude, Aminopeptidase N is a major receptor for the enteropathogenic coronavirus TGEV, *Nature* 357:417 (1992).
5. C.T. Yeager, R.A. Ashmun, R.K. Williams, C.B. Cardellichio, L.H. Shapiro, A.T. Look and K.V. Holmes, Human aminopeptidase N is a receptor for human coronavirus 229E, *Nature* 357:420 (1992).
6. A.J. Kenny and S. Maroux, Topology of microvillar membrane hydrolases of kidney and intestine, *Physiol. Rev.* 62:91 (1982).
7. A.T. Look, R.A. Ashmun, L.H. Shapiro and S.C. Peiper, Human myeloid plasma membrane glycoprotein CD13 (gp150) is identical to aminopeptidase N, *J. clin. Invest.* 83:1299 (1989).
8. E.O. Haelterman, On the pathogenesis of transmissible gastroenteritis of swine, *J. Am. vet. med. Assoc.* 160:534 (1972).
9. R.D. Woods, N.F. Cheville and J.E. Gallagher, Lesions in the small intestine of newborn pigs inoculated with porcine, feline and canine coronaviruses, *Am. J. vet. Res.* 42:1163 (1981).
10. H. Laude, K. Van Reeth and M. Pensaert, Porcine respiratory coronavirus: molecular features and virus-host interactions, *Vet. Res.* 24:125 (1993).
11. W. Kueng, E. Silber and U. Eppenberger, Quantification of cells cultured on 96-well plates, *Analyt. Biochem.* 182:16 (1989).