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Characterization of functional domains in the human coronavirus HCV 229E receptor

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Human aminopeptidase N (hAPN or CD13) and porcine aminopeptidase N (pAPN) are functional receptors for human coronavirus (HCV) 229E and porcine transmissible gastroenteritis virus (TGEV), respectively. However, hAPN cannot function as a receptor for TGEV and pAPN cannot function as a receptor for HCV 229E. In this study, we constructed a series of chimeric hAPN/pAPN genes and expressed the corresponding proteins in transfected cells. Subsequently, we identified the chimeric proteins that can function as a receptor for HCV 229E. The results show that replacement of a small region of pAPN sequence (pAPN amino acids 255–348) with the corresponding hAPN sequence (hAPN amino acids 260–353) converts pAPN into a functional receptor for HCV 229E. The region of hAPN that we have defined in this way does not correspond to the region of pAPN that has been identified as essential for the TGEV–receptor interaction. We conclude that although both viruses use a homologous receptor protein, different regions of the protein are required to mediate susceptibility to infection with HCV 229E and TGEV.

Introduction

The human coronavirus (HCV) 229E and the porcine coronavirus transmissible gastroenteritis virus (TGEV) are closely related and are antigenically and genetically distinct from the so-called 'haemagglutinating' coronaviruses, such as murine hepatitis virus (MHV) and bovine coronavirus (BCV), and the avian coronavirus infectious bronchitis virus (IBV) (Siddell, 1995). Therefore, not surprisingly, infection of cells with HCV 229E and TGEV is mediated by the same molecule, the cell-surface receptor protein aminopeptidase N (APN) (Yeager *et al.*, 1992; Delmas *et al.*, 1992). APN (CD13) is a type II glycoprotein and belongs to a family of membrane bound metallopeptidases (Ashmun *et al.*, 1992). The protein is found in vast amounts on the microvillar membrane of the small intestine where it degrades oligopeptides in the course of digestion (Olsen *et al.*, 1988). However, the protein is also present on renal proximal tubule epithelium, lung epithelium, synaptic membranes of the central nervous system and cells of the granulocytic and monocytic lineage (Olsen *et al.*, 1988; Look *et al.*, 1989; Delmas *et al.*, 1994). In cell types of the neuronal and haematopoietic system, APN has been implicated in the metabolism of regulatory peptides (Look *et al.*, 1989). The interaction of a virus with its cellular receptor is one of the major targets of the host immune response during a virus infection. Neutralizing antibodies which interfere with virus–receptor interaction are able to block the infection process *in vitro* and *in vivo* (Dimmock, 1993). However, the virus–receptor interaction is also a possible target for the development of synthetic anti-viral drugs. This is exemplified by the finding that zinc-chelating agents, like 1,10-phenanthroline, which block APN activity concomitantly inhibit infection by HCV 229E (Yeager *et al.*, 1992).

Although human APN (hAPN) and porcine APN (pAPN) have a sequence similarity of 78% at the amino acid level, pAPN cannot serve as a receptor for HCV 229E and vice versa (Delmas *et al.*, 1994; this study). Thus, construction of chimeric hAPN/pAPN genes and expression of the corresponding proteins has been used to define the amino acids in pAPN that are critical for infection of cells with TGEV. In these experiments, a region between pAPN amino acids 716–813 was identified as being necessary to confer susceptibility to TGEV infection (Delmas *et al.*, 1994).

To define the region that determines the species specific interaction between HCV 229E and hAPN, we have performed a similar set of transfection/infection experiments with chimeric hAPN/pAPN proteins. We show here that an amino acid sequence between hAPN amino acids 260–353 is essential for the HCV 229E receptor activity of hAPN. Thus, two

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different regions of the pAPN and hAPN receptor proteins are needed to mediate infection by the related coronaviruses TGEV and HCV 229E.

Methods

■ **Viruses and cells.** MRC-5 (ECACC 84101801), CRFK (ECACC 86093002), DBT (Kumanishi, 1967) and L_{929} (ECACC 85011425) cells were grown at 37 °C in minimal essential medium (MEM) with Earle's salts, supplemented with 10% fetal bovine serum (FBS), non-essential amino acids, glutamine and antibiotics. COS-7 cells (ECACC 87021302) were grown in Dulbecco's modified MEM supplemented with 10% FBS, non-essential amino acids, glutamine and antibiotics. The HCV 229E isolate used in these studies has been described previously (Raabe *et al.*, 1990). Virus was propagated in monolayers of MRC-5 cells at 33 °C.

Transfections were done by calcium phosphate precipitation as described by Chen & Okoyama (1987). Routinely, 1×10^5 COS-7 cells were seeded per 24-well plate and 8 h later they were transfected with 10 μg of plasmid DNA. Transfection efficiencies were controlled by Southern blotting of DNA isolated from transfected cells, using a 781 bp *Eco*RI–*Bam*HI fragment of the hAPN cDNA as a probe. After a further 24 h, the cells were infected with HCV 229E at an m.o.i. of 5. After incubating for 1 h at 33 °C the virus-containing supernatant was removed and the cells were washed twice with PBS. After a further 16 h at 33 °C, cytoplasmic protein extracts were prepared or mRNA was isolated. All experiments were performed at least three times. For the generation of stable pools of transfected cells, 1 μg of PGK-neo plasmid DNA was added to the transfection mix. After 24 h, the cells were seeded into a 10 cm Petri dish and incubated in medium supplemented with 500 μg/ml G418 for 14 days. The resistant colonies were pooled and subsequently analysed.

■ **Protein analysis.** Cytoplasmic extracts of transfected and infected cells were prepared as described (Grosse & Siddell, 1994). The extracts were separated on a 15% SDS–polyacrylamide gel, transferred to nitrocellulose and analysed by immunostaining with antibody NG12, which is specific for HCV 229E nucleocapsid protein (Ziebuhr, 1995). The immune reactions were developed with the ECL system (Amersham). Protein was extracted with Triton X-100 as described by Delmas *et al.* (1994): 2×10^5 cells were lysed in 100 μl of Triton X-100 buffer (50 mM-Tris–HCl pH 7·3, 1% Triton X-100); aliquots (30 μl) were then incubated with 120 μl potassium phosphate buffer (50 mM, pH 7·4) containing 6 mM-alanine *p*-nitroanilide or 6 mM-leucine *p*-nitroanilide at 37 °C for 16 h. The optical density (OD) of the samples was determined at 405 nm. The OD_{405} of lysates derived from control cells (COS-7 cells transfected with a parental expression vector, without insert) was subtracted from the OD_{405} read for cells transfected with the APN constructs.

■ **RNA analysis.** Polyadenylated RNA was isolated from transfected and infected cells as described (Grosse & Siddell, 1994). One half of the mRNA isolated from a total of 2×10^5 cells was separated on an agarose gel containing 6% formaldehyde. The gel was subsequently dried and hybridized with an oligonucleotide (5′ AGA AAC TTC ATC ACG CAC TGG 3′) complementary to a sequence in the HCV 229E N-gene, in a buffer containing 5 × SSPE, 5 × Denhardt's solution, 0·05% SDS and 100 μg/ml of digested yeast RNA

■ **DNA cloning.** The hAPN cDNA was kindly provided by I. Hickson (ICRF, Oxford, UK). The hAPN coding region was excised as an *Xho*I fragment from the clone provided and recloned into the expression vector pBK-CMV (Stratagene) to generate plasmid pBK-hAPN; this was digested with *Bst*EII and religated to construct plasmid pBK-hAPNdel. A PCR product generated with the primers CD13–1 (5′ GCG CCA TGG AGA ACT GGG GAC TGG TGA CCT ACC 3′) and CD13–2 (5′ TAT GGT CAC CAG GTT CCC GAA CCA CTG GGC GGC GAC CTC ATG A 3′) using pBK-hAPN DNA as template was digested with *Bst*EII and cloned into plasmid pBK-hAPNdel to generate plasmid pBK-$hAPN^{H/A}$. To construct plasmid pAP7, an *Nco*I fragment of hAPN cDNA was excised from plasmid pBK-hAPN and inserted into the *Nco*I site of plasmid pAPN (kindly provided by B. Delmas, INRA, Jouy-en-Josas, France). To generate pAP30, an *Nco*I–*Bbs*I fragment of pBK-hAPN (encoding amino acids 1–259) was ligated with a *Bbs*I–*Bsu*36I fragment of pAPN (encoding amino acids 255–718) and inserted into pAPN digested with *Nco*I and *Bsu*36I. To generate plasmid pAP31 an *Nco*I–*Xmn*I fragment excised from plasmid pAPN (encoding amino acids 1–214) and an *Xmn*I–*Nco*I fragment excised from plasmid pBK-hAPN (encoding amino acids 220–353) were ligated; the ligation product was re-isolated from an agarose gel and ligated with pAPN DNA digested with *Nco*I. To produce plasmid pAP32, an *Nco*I–*Bbs*I fragment of pAPN (encompassing amino acids 1–254) was ligated with a *Bbs*I–*Nco*I fragment of pBK-hAPN (encoding amino acids 260–353) and inserted into pAPN digested with *Nco*I. The plasmids pAP24 and pAP27 were provided by B. Delmas and used without any modification. The proteins encoded by these constructs are shown schematically in Figs 1(*a*), 2(*a*) and 4(*a*).

Results

In order to identify determinants in hAPN which are necessary for its function as a receptor for HCV 229E, we analysed the receptor function of hAPN, pAPN and chimeric hAPN/pAPN molecules in a transfection/infection assay. Infection of cells was assayed by detection of HCV 229E N protein in cell lysates using monoclonal antibody NG12. As is evident in Fig. 1(*b*), this monoclonal antibody also cross-reacts with a COS-7 cellular protein of about 85 kDa. The control cells we used in our assays were MRC-5 and CRFK cells, which are both susceptible to HCV infection (lanes 1 and 2, Fig. 1*b*) and COS-7 cells transfected with pBK-CMV, which are not infected by HCV 229E (lane 6, Fig. 1*b*).

In a first set of experiments we analysed the receptor function of the proteins hAPN, pAPN, AP24 and AP27. The results of this experiment (summarized in Fig. 1*a* and shown in Fig. 1*b*) show that COS-7 cells transfected with a plasmid expressing hAPN were susceptible to HCV 229E infection (lane 7), whereas COS-7 cells transfected with a plasmid expressing pAPN were not susceptible (lane 3). Cells transfected with a plasmid expressing the chimeric protein AP24, which contains pAPN amino acids 717–813 grafted onto its human homologue (Delmas *et al.*, 1994) were also infected with HCV 229E (lane 4). The same chimera also confers susceptibility to TGEV (Delmas *et al.*, 1994). Cells transfected with a plasmid expressing the chimeric protein AP27, which contains hAPN amino acids 646–917 on a pAPN backbone (Delmas *et al.*, 1994), did not confer susceptibility to HCV 229E infection (lane 5). These results suggest that two different protein domains in pAPN and hAPN mediate infection by TGEV and HCV 229E, respectively.

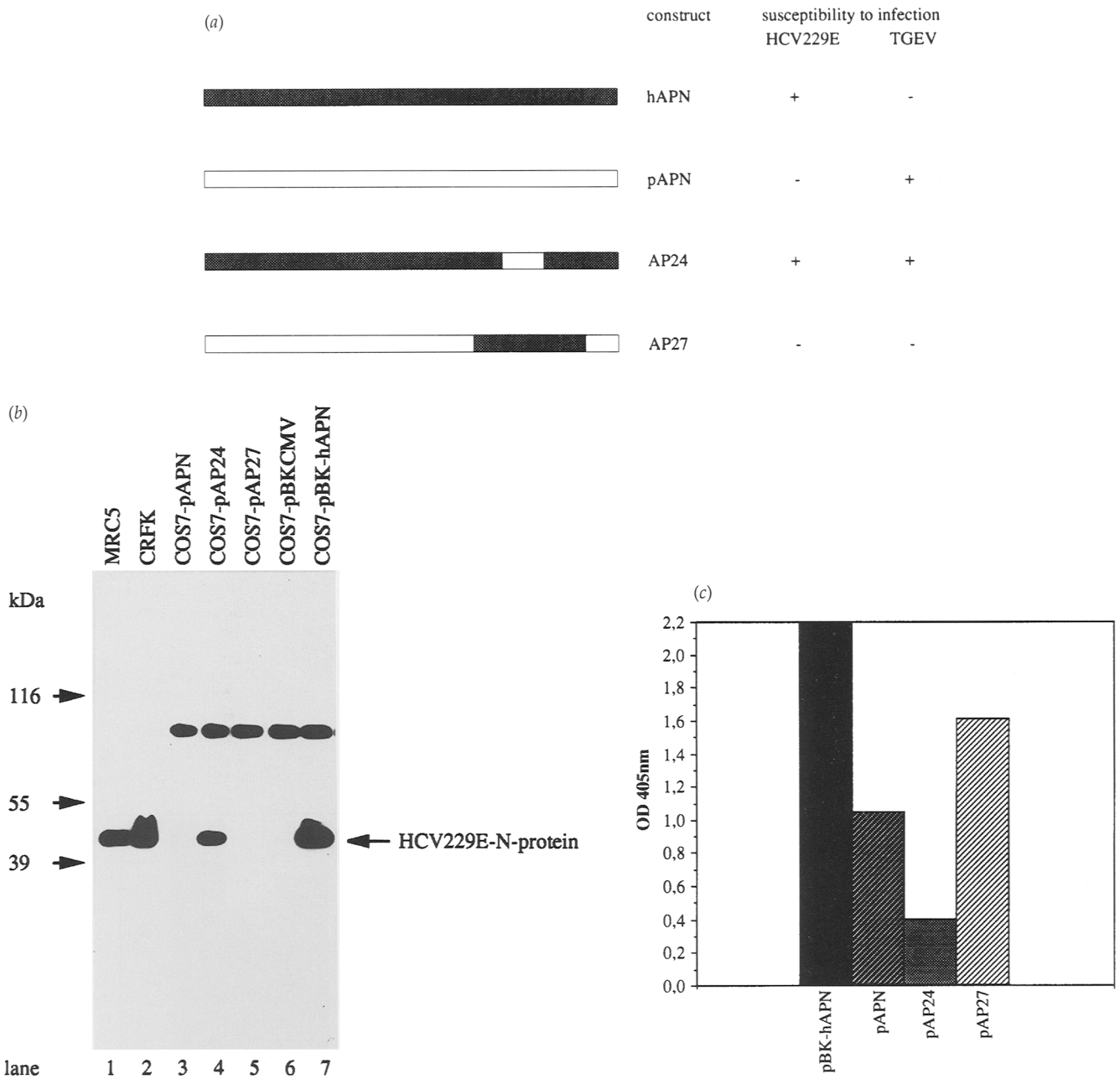

Fig. 1. (*a*) Schematic representation of hAPN, pAPN and the chimeric proteins AP24 and AP27. hAPN sequences are represented by shaded boxes, pAPN sequences are represented by open boxes. The susceptibility of transfected COS-7 cells to infection with HCV 229E is indicated. The indicated susceptibility of transfected MDCK cells to infection with TGEV is taken from Delmas *et al.* (1994). (*b*) Western blot analysis of cytoplasmic extracts of transfected and infected cells. Lane 1, MRC5 cells inoculated with HCV 229E; lane 2, CRFK cells inoculated with HCV 229E; lane 3, COS-7 cells transfected with pAPN and subsequently inoculated with HCV 229E; lane 4, COS-7 cells transfected with pAP24 and subsequently inoculated with HCV 229E; lane 5, COS-7 cells transfected with pAP27 and subsequently inoculated with HCV 229E; lane 6, COS-7 cells transfected with pBK-CMV and subsequently inoculated with HCV 229E; lane 7, COS-7 cells transfected with pBK-hAPN and subsequently inoculated with HCV 229E. The position of the N-protein is indicated. (*c*) Aminopeptidase activity in Triton extracts of transfected cells as measured by hydrolysis of alanine *p*-nitroanilide.

In order to monitor expression of the APN proteins in transfected cells, we measured the aminopeptidase activity in Triton X-100 extracts (Delmas *et al.*, 1994). Detectable enzymatic activity implies that the APN proteins are expressed and correctly folded. Aminopeptidase activity was detected for all of the APN proteins tested (Fig. 1*c*) which suggests that, for example, the failure of AP27 to act as a receptor for HCV 229E is not due to trivial reasons.

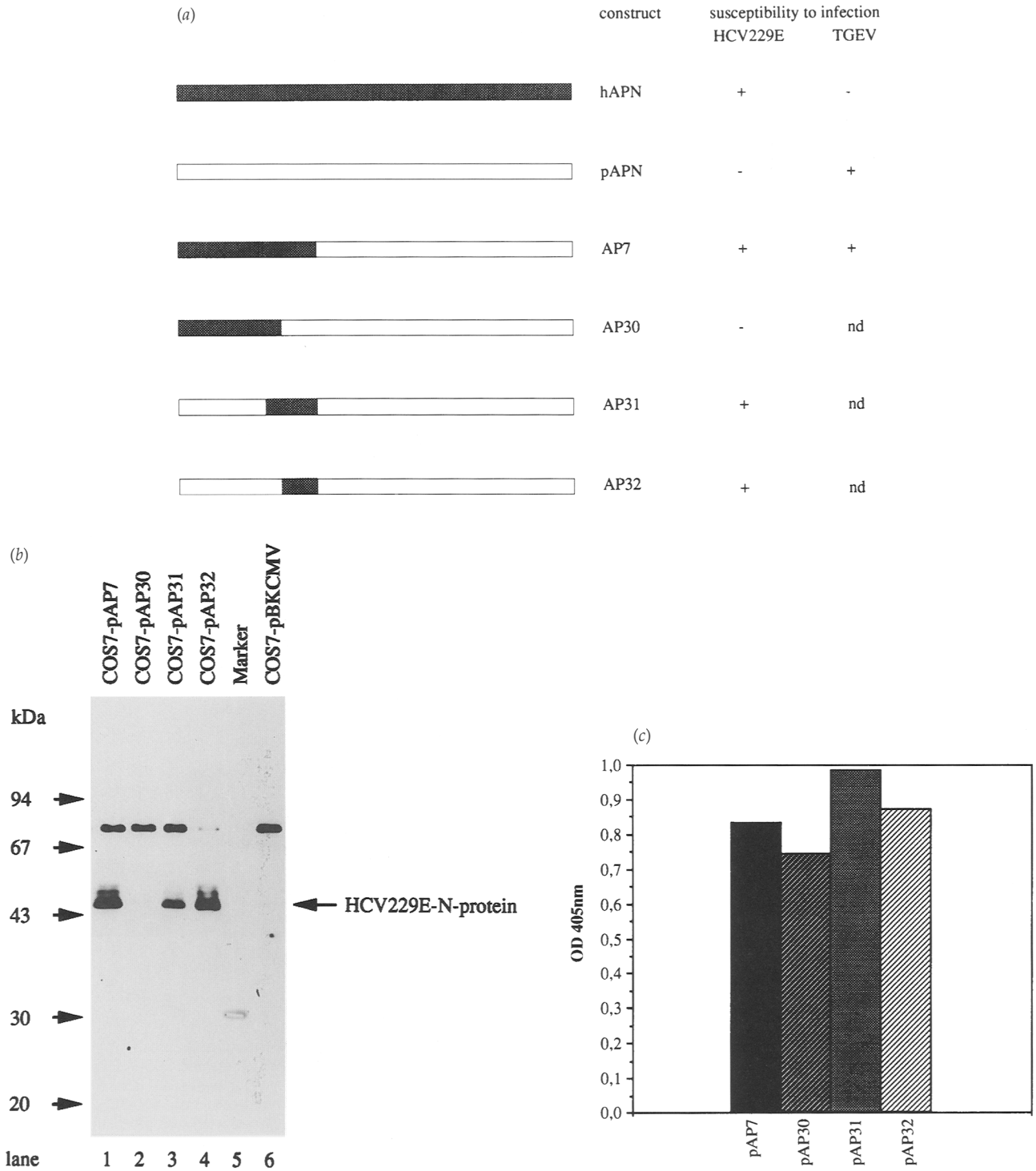

Fig. 2. (a) Schematic representation of hAPN, pAPN and the chimeric proteins AP7, AP30, AP31 and AP32. hAPN sequences are represented by shaded boxes, pAPN sequences are represented by open boxes. The susceptibility of transfected COS-7 cells to infection with HCV 229E is indicated. The susceptibility of transfected MCDK cells to infection with TGEV is taken from Delmas *et al.* (1994). nd, Not determined. (*b*) Western blot analysis of cytoplasmic extracts of transfected and infected cells. Lane 1, COS-7 cells transfected with pAP7 and subsequently inoculated with HCV 229E; lane 2, COS-7 cells transfected with pAP30 and subsequently inoculated with HCV 229E; lane 3, COS-7 cells transfected with pAP31 and subsequently inoculated with HCV 229E; lane 4, COS-7 cells transfected with pAP32 and subsequently inoculated with HCV 229E; lane 5, Protein molecular mass marker; lane 6, COS-7 cells transfected with pBK-CMV and subsequently inoculated with HCV 229E. The position of the N-protein is indicated. (*c*) Aminopeptidase activity in Triton extracts of transfected cells as measured by hydrolysis of alanine *p*-nitroanilide.

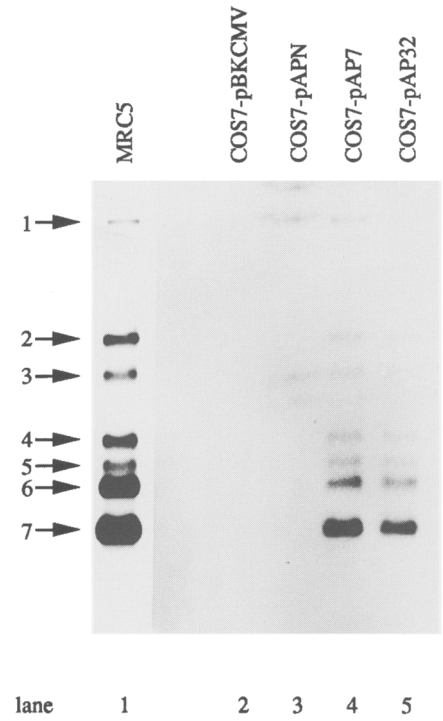

Fig. 3. Northern blot analysis of poly(A)$^+$ RNA isolated from transfected and infected cells. Lane 1, MRC5 cells inoculated with HCV 229E; lane 2, COS-7 cells transfected with pBK-CMV and subsequently inoculated with HCV 229E; lane 3, COS-7 cells transfected with pAPN and subsequently inoculated with HCV 229E; lane 4, COS-7 cells transfected with pAP7 and subsequently inoculated with HCV 229E; lane 5, COS-7 cells transfected with pAP32 and subsequently inoculated with HCV 229E. The positions of the HCV 229E RNA species are indicated.

To define more exactly the region in hAPN which is critical for its receptor activity, we analysed a number of additional constructs that express chimeric proteins containing hAPN sequences grafted onto a pAPN backbone. This experiment (summarized in Fig. 2*a* and shown in Fig. 2*b*) shows that the chimeric proteins AP7, AP31 and AP32 are able to confer on COS-7 cells susceptibility to HCV 229E infection (lanes 1, 3 and 4). The chimeric protein AP30 did not function as an HCV 229E receptor in COS-7 cells (lane 2). All the chimeric proteins have comparable aminopeptidase activity (Fig. 2*c*) indicating that they are all expressed and folded correctly.

In order to confirm the result described above, at the level of viral RNA synthesis, we analysed the mRNA fraction isolated from transfected/infected cells by hybridization with an oligonucleotide complementary to HCV 229E mRNA 7. An RNA ladder, representing HCV 229E mRNAs 1–7 and indicative of viral replication, was detected in COS-7 cells transfected with plasmids that expressed AP7 and AP32, but not in COS-7 cells transfected with a plasmid that expressed pAPN or with the control plasmid pBK-CMV (Fig. 3). The same results were obtained with stable pools of transfected COS-7 cells (data not shown). This experiment proves conclusively that the chimeric protein AP32 can act as a functional receptor for HCV 229E and defines a region between hAPN amino acids 260–353 as being essential for receptor activity.

The region of the hAPN protein defined above is in close proximity to the catalytic domain of hAPN (Fig. 4*a*). Yeager *et al.* (1992) have shown that some monoclonal antibodies which inhibit enzymatic activity of hAPN also block its receptor function, and that deletion of 39 amino acids encompassing the catalytic centre of hAPN eliminates receptor activity. However, the enzymatic activity of hAPN did not appear to be required for the infection process because competitive aminopeptidase inhibitors did not interfere with receptor function (Yeager *et al.*, 1992). To test directly if the enzymatic activity of hAPN was required for its receptor function, we transfected COS-7 cells with a plasmid that expressed hAPN$^{H/A}$, a mutant hAPN in which one of the catalytic histidine residues was replaced by alanine (Fig. 4*a*). This mutant protein did not show any aminopeptidase activity as evidenced by the lack of alanine *p*-nitroanilide hydrolysis in cell extracts from transfected cells (Fig. 4*c*), but was still able to mediate infection with HCV 229E (lane 1, Fig. 4*b*). This result clearly shows that hAPN enzymatic activity is not a prerequisite for receptor function and implies that the effects observed by Yeager *et al.* (1992) were most likely due to steric hindrance by bound antibody and significant structural changes in the mutated protein.

During the course of this study, we also transfected a number of the plasmids encoding APN proteins into murine DBT (astrocytoma) and L_{929} (connective tissue fibroblast) cells. In all cases in which the HCV 229E receptor was functional in COS-7 cells, we observed it to be functional in the murine cell lines (data not shown). This suggests that replication of HCV 229E in tissue culture cells is dependent upon the availability of a functional receptor protein on the cell surface but not on the availability of species-specific factors needed, for example, for viral RNA or protein synthesis. The maturation of viral particles, however, appears to depend (at least in part) on cell specific factors, as permanently growing cell lines which are permissive to HCV 229E infection (like CK or HeLa cells) fail to produce detectable titres of infectious virus (data not shown), whereas cell lines with a limited life span (like MRC5 or WI38 cells) support virus multiplication. Viral proteins, however, are synthesized at a comparable rate in HeLa and MRC5 cells (J. Herold, personal communication). Therefore, permanently growing cell lines which have been transfected with a functional receptor molecule cannot be used as a simple cell culture system to propagate HCV 229E.

Discussion

In this study, we have defined the amino acid sequences of hAPN that are required for its function as an HCV 229E receptor. Taken together with the study of Delmas *et al.* (1994),

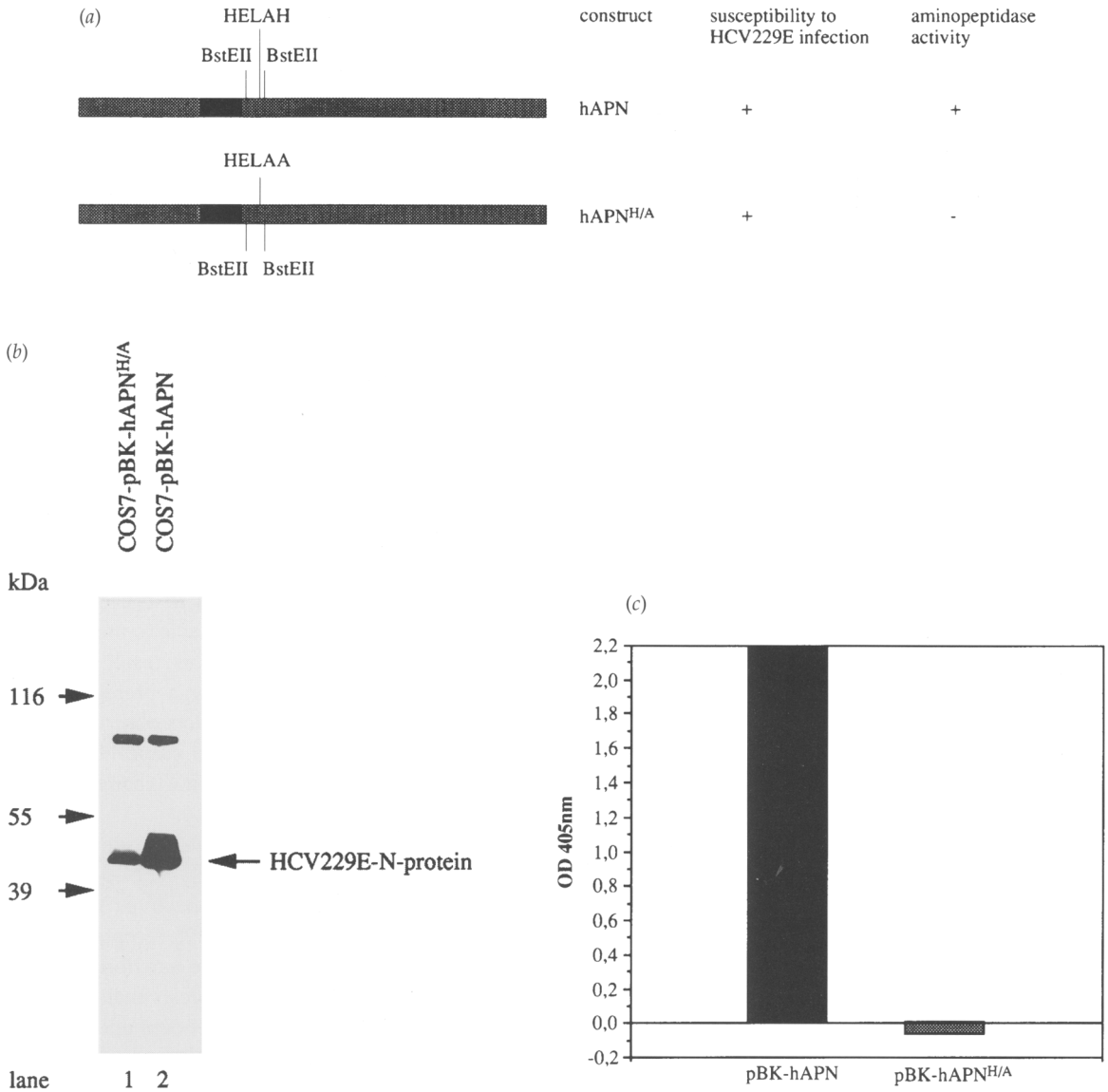

Fig. 4. (*a*) Schematic representation of hAPN and the mutant hAPN$^{H/A}$. The positions of the catalytic centre (HELAH) and the *Bst*EII restriction sites are indicated. The amino acid segment required for hAPN receptor function is marked as a black box. The susceptibility of transfected COS-7 cells to infection with HCV 229E and the detectable aminopeptidase activities are indicated. (*b*) Western blot analysis of cytoplasmic extracts of transfected and infected cells. Lane 1, COS-7 cells transfected with pBK-hAPN$^{H/A}$ and subsequently inoculated with HCV 229E; lane 2, COS-7 cells transfected with pBK-hAPN and subsequently inoculated with HCV 229E. The position of the N-protein is indicated. (c) Aminopeptidase activity in Triton extracts of transfected cells as measured by hydrolysis of alanine *p*-nitroanilide.

our results indicate that, although HCV 229E and TGEV use a homologous receptor protein, different regions of the protein are required to mediate infection with the two viruses. Furthermore, we have confirmed that the catalytic activity of hAPN is not required for its receptor function. And finally, in the cell lines we have examined, we could not detect any specific factor, other than the availability of a functional receptor molecule, that governs susceptibility to virus infection.

The most interesting question that arises from this study is why different regions of the hAPN and pAPN molecules determine whether or not they are able to serve as functional receptors for HCV 229E or TGEV, respectively. Evidently, these regions are needed to mediate, directly or indirectly, a specific interaction with their respective ligands (i.e. the HCV 229E and TGEV surface glycoproteins) but it is not clear how this specificity is introduced. If specificity is at the level of receptor–ligand recognition, then this could be tested by using recombinant proteins in either a VOPBA (virus overlay protein blot assay; Boyle *et al.*, 1987) or ELISA type assay. If the

specificity of the interaction is governed by, for example, the ability of the receptor protein to induce conformational changes in its ligand, then it may be possible to analyse this event by using monoclonal antibodies specific for different forms of the S protein. In either case, it will also be important to determine whether the two regions that have been identified (hAPN amino acids 260–353 and pAPN amino acids 716–813) are really independent of each other. For example, it would be interesting to know if the chimeric protein AP32 can simultaneously function as a receptor for HCV 229E and TGEV. In the long term, detailed analysis of the hAPN–HCV 229E S protein interaction should provide the information needed for the development of a rational intervention strategy.

The chimeric hAPN/pAPN protein AP32 is comprised of hAPN amino acids 260–353 grafted onto a pAPN backbone. In this region of the APN molecule, 17 residues differ between the porcine and the human proteins. Noticeably, between amino acids 268–275, there are six amino acid differences and these residues fall into a region which is predicted to have a high hydrophilicity, a high surface probability and to fold into an α-chain in hAPN but not in the pAPN protein. We suggest that these residues may play a critical role in the hAPN/HCV 229E surface glycoprotein interaction and a more detailed analysis, using site-directed mutagenesis, is in progress to test this prediction.

Interestingly, our experiments have shown that feline kidney cells can be infected with HCV 229E, indicating that they also express a functional receptor for this virus. The same cell type has also been demonstrated to be susceptible to infection with TGEV (Klumperman *et al.*, 1994). HCV 229E, TGEV and the feline coronavirus feline infectious peritonitis virus (FIPV) are grouped into the same genetic cluster (Siddell, 1995) and it is likely that FIPV also utilizes APN as a receptor. If this is the case, the feline APN molecule is sufficiently homologous to the porcine and also to the human APN to allow for infection of CRFK cells with HCV 229E and TGEV. Thus, at least theoretically, cats may be a species in which TGEV, HCV 229E and FIPV replication can take place and in which virus recombinants could arise.

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