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Bernard Delmas*, Jacqueline Gelfi*, René L'Haridon*, Lotte Katrine Vogel†, Hans Sjöström‡, Ove Norén† & Hubert Laude‡

Aminopeptidase N is a major receptor for the enteropathogenic coronavirus TGEV

*Unité de Virologie et Immunologie Moléculaires, INRA, Domaine de Vilvert, 78350 Jouy-en-Josas, France
†Department of Biochemistry C, Panum Institute, DK 2200 Copenhagen N, Denmark
‡Correspondence should be addressed.

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FIG. 1 Characterization of anti-receptor monoclonal antibodies. The neutralization activity of six anti-ST cell antibodies (G43 and T35) was measured in four cell systems permissive to TGEV. The neutralizing titre is expressed as the last of serial fivefold dilutions protecting the monolayer against the viral cytopathic effect (CPE). Monoclonal antibody 51.13 is directed against TGEV spike protein S (ref. 17). ST, PDS and Thy are porcine testis, kidney and thyroid cell lines, FCWF is a feline cell line.

METHODS. BALB/c mice were immunized by intraperitoneal injection of 5 × 10⁷ intact ST cells three times at 1-month intervals and boosted by injection of 180 μg ST membranes. The supernatant from 800 hybridoma clones prepared from spleen cells was tested for neutralizing activity using a standard microassay except that the cells were preincubated for 2 h with the antibodies. Six positive hybridomas were cloned and amplified. IgGs from ascites fluid were used as a source of antibodies.

acts as a receptor. First, virions bound specifically to aminopeptidase N that was purified to homogeneity. Second, recombinant expression of aminopeptidase N conferred infectivity by TGEV to an otherwise non-permissive cell line.

To obtain monoclonal antibodies against the TGEV receptor, hybridomas were prepared from a mouse immunized with ST cells, a swine testis cell line highly susceptible to TGEV. Several of the resulting antibodies exhibited, in three different porcine cell systems, a blocking activity comparable to that of a high-titre neutralizing anti-TGEV antibody (Fig. 1). By contrast, no significant protection by the antibodies was observed after virus challenge in a feline cell system, or towards irrelevant viruses (group A bovine rotavirus or vesicular stomatitis virus; data not shown). Therefore, the selected antibodies had the characteristics expected for antibodies recognizing a major TGEV receptor.

The monoclonal antibodies all recognized a polypeptide of relative molecular mass 150,000 (150K) in ST cell extracts, together with a faint band interpreted as the mannose-rich intracellular precursor (Fig. 2, lane 1). When using solubilized brush-border membranes from pig small intestine, three major species of 150K, 95K and 50K were immunoprecipitated (Fig. 2, lane 4). The first 30 amino acids of the 95K species were determined through N-terminal sequencing: NH₂-Ala-Lys-Gly-Phe-Tyr-Ile-Ser-Lys-Ala-Leu-Gly-Ile-Leu-Gly-Ile-Leu-Leu-Gly-Val-Ala-Ala-Val-Ala-Thr-Ile-Ile-Ala-Leu-Ser-Val-COOH. This sequence was identical to the N-terminal sequence (minus the first Met) of porcine aminopeptidase N, deduced from the exon 1 nucleotide sequence.

FIG. 3 Demonstration of a virus-receptor binding with purified components. a. Aminopeptidase N (APN) preincubated with or without purified antibody G43 or a control antibody was incubated in the absence (--) or presence of TGEV or bovine coronavirus (BCV) virions, then centrifuged through a glycerol cushion. The presence of APN-specific bands in the pellets was revealed by western blotting. b. Left panel, soluble APN was incubated on plastic dishes coated with TGEV, BCV or rotavirus virions. Bound APN was revealed by immunoblot. Right panel, APN was preincubated with serial dilutions of either G43 or a control antibody and tested for TGEV binding as above.

METHODS. Anchor-free APN was purified by immunoabsorbent chromatography from porcine intestine microvillar membranes after Triton X-100 solubilization and trypsin treatment. The purity of APN polypeptides was confirmed by SDS–PAGE and Coomassie blue staining. TGEV and BCV virions were purified as described. Rotavirus virions were purified on a CsCl gradient. a APN (0.2 μg) was mixed with a 50 μg virion suspension in cell culture medium. After 1 h at 37 °C, the virions were pelleted through a 10% glycerol cushion by centrifugation at 150,000g for 30 min. One TGEV sample was mixed with APN preincubated with G43 IgG (200 μg ml⁻¹). APN bound to virus was revealed by western blotting using rabbit IgGs directed against denatured APN and a peroxidase conjugate. b. APN (0.5 μg) was added to virus-coated wells (1 μg per well) for 1 h at 37 °C after incubation (or mock incubation) with dilutions of G43 IgGs at 200 μg ml⁻¹. After washing with PBS plus 0.05% Tween 20, bound APN was detected by rabbit IgGs against native APN and a phosphatase conjugate.
Further evidence that the anti-TGEV-receptor antibodies recognized aminopeptidase N was obtained by showing that (1) an antibody raised against rabbit aminopeptidase N\(^2\) reacted with the same three polypeptides in brush-border membrane preparations (Fig. 2, lane 2): 95K and 50K, corresponding to the B (amino) and C (carboxy) subunits of the pig aminopeptidase, and 150K, unreacted aminopeptidase\(^2\); (2) the immunoprecipitated material hydrolysed leucine \(\beta\)-nitroanilide, a chromogenic substrate specific for aminopeptidase (ref. 6; data not shown).

Two experiments were designed to demonstrate any direct association between aminopeptidase N and the virus. First, soluble aminopeptidase N was centrifuged after incubation in the presence of virions (Fig. 3a). Aminopeptidase N-specific bands were recovered with pelleted TGEV virions only. Second, when the aminopeptidase was incubated in the presence of adsorbed virions (Fig. 3b), it bound to TGEV and not the other entropic viruses. In both assays, earlier incubation with an antibody against aminopeptidase N reduced the binding considerably. Because the two components were purified to homogeneity, it was concluded that the interaction between the aminopeptidase and TGEV occurs in the absence of any other cellular protein.

The gene encoding aminopeptidase N (APN) was expressed in non-permissive cells to see whether it would confer with the capacity to bind TGEV. A pig intestine complementary DNA library was screened by use of a homologous DNA probe derived from the 5' end of APN gene. A full-length cDNA copy was cloned and contained an open reading frame of 2,859 nucleotides encoding a polypeptide 79% identical to human aminopeptidase (data not shown). MDCK cell clones stably transformed with the porcine APN cDNA expressed a polypeptide of 150K which reacted with antibodies against aminopeptidase N (Fig. 4a). The aminopeptidase activity\(^6\) of the transfected clones was about 40-fold higher compared with non-transfected clones. On viral challenge, all of the three independent clones tested seemed to be susceptible to TGEV infection, as proved by extensive destruction of the infected monolayers and synthesis of the viral structural polypeptides (Fig. 4b, c). Earlier incubation with an antibody specific for aminopeptidase N prevented the appearance of viral cytopathic effect. These results show that aminopeptidase N was the only porcine protein necessary to confer susceptibility on canine kidney cells naturally resistant to TGEV. Moreover, the protease function of the molecule did not seem to be involved because it was blocked by bestatin, an inhibitor of aminopeptidase, without preventing the infection (Fig. 4b).

So far, defined receptors include molecules that belong to the immunoglobulin superfamily, such as CD4 for HIV\(^1\), ICAM-1 for rhinovirus\(^5\), poliovirus receptor\(^6\) and a carcinoembryonic antigen for murine hepatitis coronavirus\(^12\), and also a monocarboxylic transporter for murine leukemia retroviruses\(^11\). Our study provides strong evidence that porcine aminopeptidase N serves as a receptor for an enveloped RNA virus, TGEV. This emphasizes the diversity of the membrane-bound proteins that viruses subvert for gaining entry into cells.

Aminopeptidase N is a well-documented ectoenzyme that binds to the membrane through an N-terminal segment\(^12,13\). Human aminopeptidase N is identical to CD13, a surface antigen of many myeloid cells\(^14\). It is a zinc-binding protease that catalyzes the removal of N-terminal, preferentially neutral residues from peptides. It is expressed in many tissues at different levels\(^15\), the highest activity being found in the small intestinal mucosa, where the aminopeptidase represents about 8% of the protein content of the apical membrane of the differentiated enterocytes, and in the kidney brush border. It is also expressed to a lesser extent in liver, lung and colon, where the virus does replicate, but without causing the specific histopathological damage seen in the small intestine\(^16\). In the intestine, the distribution of the receptor and the site of multiplication of TGEV are thus strikingly correlated. This argues for a pivotal role of aminopeptidase N/CD13 in determining the tissue tropism of TGEV. Investigating the nature of the virus interaction with aminopeptidase N could provide a rationale for the design of an antiviral strategy against TGEV and related infections.

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Human aminopeptidase N is a receptor for human coronavirus 229E

Curtis L. Yeager*, Richard A. Ashmun†‡, Richard K. Williams*, Christine B. Cardellincho*, Linda H. Shapiro†, A. Thomas Look†‡§ & Kathryn V. Holmes*‡

HUMAN coronaviruses (HCV) in two serogroups represented by HCV-229E and HCV-OC43 are an important cause of upper respiratory tract infections1. Here we report that human aminopeptidase N, a cell-surface metalloprotease on intestinal, lung and kidney epithelial cells3–4, is a receptor for human coronavirus strain HCV-229E, but not for HCV-OC43. A monoclonal antibody, RBS, blocked HCV-229E virus infection of human lung fibroblasts, immunoprecipitated aminopeptidase N and inhibited its enzymatic activity. HCV-229E-resistant murine fibroblasts became susceptible after transfection with complementary DNA encoding human aminopeptidase N. By contrast, infection of human cells with HCV-OC43 was not inhibited by antibody RBS and expression of aminopeptidase N did not enhance HCV-OC43 replication in mouse cells. A mutant aminopeptidase lacking the catalytic site of the enzyme did not bind HCV-229E or RBS and did not render murine cells susceptible to HCV-229E infection, suggesting that the virus-binding site may lie at or near the active site of the human aminopeptidase molecule.

To develop a monoclonal antibody against the HCV-229E receptor, we produced hybridomas against deoxycholate-solubilized membrane proteins of two HCV-229E-susceptible human cell lines (WI38 lung fibroblasts and HL60 myeloid leukemia cells). A monoclonal antibody designated RBS protected WI38 and RD human cell lines from HCV-229E-induced cytopathic effects and protected WI38 cells from virus infection (Fig. 1a–c). RBS pretreatment reduced the number of HCV-229E-infected WI-38 cells at 10 h post-infection by 96%, compared with cells pretreated with control mouse ascites. By contrast, RBS did not inhibit replication of HCV-OC43 in WI38 or RD cells, indicating that the receptor specificities of HCV-OC43 and HCV-229E are different.

Susceptibility to HCV-229E infection in mouse–human somatic cell hybrids depends on a gene located on human chromosome 15 (ref. 6). A promising candidate for the HCV-229E receptor is human aminopeptidase N (hAPN; EC 3.4.11.2), a cell-surface glycoprotein encoded by a gene on bands q25–q26 of human chromosome 15 (ref. 7) and expressed on human lung, renal and intestinal epithelial cells, fibroblasts and nerve synapses3–4. This exopeptidase removes amino-terminal residues to complete the digestion of short peptides in the gut and helps break down neurotransmitter peptides in the brain3,5,6,8. hAPN is identical to CD13, a glycoprotein identified on granulocytes, monocytes and their bone marrow progenitors7,9,10. Porcine aminopeptidase N is a receptor for transmissible gastroenteritis virus, a porcine coronavirus in the same serogroup as HCV-229E (ref. 11). Because aminopeptidase N from humans, pigs and other mammals are structurally similar5,7,12–14, we investigated whether HCV-229E and RBS would bind specifically to hAPN and whether expression of hAPN by murine cells would make them susceptible to infection with HCV-229E.

<table>
<thead>
<tr>
<th>Monoclonal antibodies</th>
<th>Inhibition of enzyme activity (%)</th>
<th>Binding to hAPN&lt;sub&gt;mur&lt;/sub&gt;-3T3†</th>
<th>Inhibition of HCV-229E infection‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>WM15</td>
<td>91</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>RBS</td>
<td>90</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MY7</td>
<td>42</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Chemical inhibitors‡</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actininor</td>
<td>100</td>
<td>NA</td>
<td>–</td>
</tr>
<tr>
<td>Bestatin</td>
<td>100</td>
<td>NA</td>
<td>–</td>
</tr>
<tr>
<td>1,10-Phenanthroline</td>
<td>100</td>
<td>NA</td>
<td>–</td>
</tr>
<tr>
<td>2,2'-Dipyridyl</td>
<td>100</td>
<td>NA</td>
<td>+</td>
</tr>
</tbody>
</table>

* The inhibition of hAPN activity was determined as described in the legend to Fig. 2d.
† Binding of antibodies to hAPN<sub>mur</sub>-3T3 cells was measured by flow cytometry, as outlined in the legend to Fig. 3. The mutant lacks peptidase activity; thus, assays for chemical inhibition were not applicable (NA).‡ Confluent monolayers of WI38 cells in 96-well plates were pretreated with dilutions of antibodies or inhibitors in medium for 1 h, and then challenged with 1 × 10<sup>5</sup> p.f.u. per well of HCV-229E. After 1 h of adsorption, the inoculum was removed, and the cells were incubated with fresh medium containing antibodies or inhibitors for 48 h, at which time the monolayers were examined for virus-induced cytopathic effects. Such effects were evident in HCV-229E-infected controls pretreated with normal serum, but not in mock-infected controls. Plus signs, HCV-229E-induced cytopathic effects were inhibited by antibodies up to a dilution of 1:200. All incubations were at 37 °C.
§ Inhibitors were tested at the following concentrations: bestatin, 1 mg ml<sup>–1</sup>; 1,10-phenanthroline, 1.5 mM; 2,2'-dipyridyl, 2.5 mM; actininor, 2.7 mM. Antibodies were tested at concentrations that saturated available binding sites in flow cytometric assays.

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