The EMBO Journal vol.15 no.16 pp.4282–4296, 1996

Identification of a surface glycoprotein on African green monkey kidney cells as a receptor for hepatitis A virus

Gerardo Kaplan[1,2], Atsuko Totsuka[3], Peter Thompson[1], Toshitaka Akatsuka[1,3], Yasuo Moritsugu[3] and Stephen M.Feinstone[1]

[1]Laboratory of Hepatitis Research, Division of Viral Products, Office of Vaccine Review and Research, Center for Biologics Evaluation and Research, Food and Drug Administration, Building 29A, NIH, Bethesda, MD 20892, USA and [3]Department of Viral Disease and Vaccine Control, National Institute of Health, Murayama Annex, 4-7-1 Gakuen, Musashimurayama, Tokyo 208, Japan

[2]Corresponding author

Very little is known about the mechanism of cell entry of hepatitis A virus (HAV), and the identification of cellular receptors for this picornavirus has been elusive. Here we describe the molecular cloning of a cellular receptor for HAV using protective monoclonal antibodies raised against susceptible African green monkey kidney (AGMK) cells as probes. Monoclonal antibodies 190/4, 235/4 and 263/6, which reacted against similar epitopes, specifically protected AGMK cells against HAV infection by blocking the binding of HAV. Expression cloning and nucleotide sequence analysis of the cDNA coding for epitope 190/4 revealed a novel mucin-like class I integral membrane glycoprotein of 451 amino acids, the HAV cellular receptor 1 (HAVcr-1). Immunofluorescence analysis indicated that mouse Ltk$^-$ cells transfected with HAVcr-1 cDNA gained limited susceptibility to HAV infection, which was blocked by treatment with monoclonal antibody 190/4. Our results demonstrate that the HAVcr-1 polypeptide is an attachment receptor for HAV and strongly suggest that it is also a functional receptor which mediates HAV infection. This report constitutes the first identification of a cellular receptor for HAV.

Keywords: HAV/integral membrane glycoprotein/mucin/picornavirus/virus receptor

Introduction

The initial step in the life cycle of a virus is its attachment to a cell surface receptor, the expression pattern of which influences host range and tissue tropism. Some viruses attach to receptors with a restricted distribution and others to widely distributed receptors that allow viruses to infect several cell types and have a wide host range. Examples of this latter case include influenza virus binding to sialic acid (Weis *et al.*, 1988) and sindbis virus binding to the high-affinity laminin receptor (Wang *et al.*, 1992). Very little is known about the cell entry of human hepatitis viruses. Their cellular receptors have been difficult to characterize because of poor *in vitro* viral growth, the association of virions with serum and cell-derived materials which mask genuine virus–receptor interactions leading to cell entry, and the attachment of virions to susceptible and non-susceptible cells. The characterization of cellular receptors for hepatitis A virus (HAV), a hepatotropic picornavirus which causes an acute liver disease in humans (for a review see Gust and Feinstone, 1988), has been elusive. HAV grows in cell cultures of primate (Daemer *et al.*, 1981; Provost *et al.*, 1981) and non-primate (Dotzauer *et al.*, 1994) origin, usually establishing persistent infections. The association of HAV with immunoglobulins (Margolis *et al.*, 1988; Margolis and Nainan, 1990), fibronectin (Seelig *et al.*, 1984), α_2-macroglobulin (Zajac *et al.*, 1991) and other host cell-derived materials (Lemon and Binn, 1985) suggested that HAV could infect cells by a surrogate receptor-binding mechanism via receptors to virion-bound host materials (Zajac *et al.*, 1991). Early work characterizing cellular receptors for HAV showed that HAV infectivity in tissue culture required calcium (Stapleton *et al.*, 1991; Zajac *et al.*, 1991) and was inhibited by treatment of the cells with trypsin, phospholipases and β-galactosidase, suggesting that proteins as well as lipids and sugars were involved in the attachment of HAV to cells (Seganti *et al.*, 1987).

Because cellular receptors for other picornaviruses have been characterized by raising monoclonal antibodies (mAbs) against susceptible cells and selecting those capable of blocking infection (Bass and Greenberg, 1992), we decided to use a similar approach to identify cellular receptors for HAV. Here we report that three mAbs directed against the cell surface protected African green monkey kidney (AGMK) cells against HAV infection. Expression cloning revealed that the protective mAbs reacted against a novel cell surface mucin-like class I integral membrane glycoprotein. Mucins are extended soluble or membrane-bound serine- and threonine-rich heavily O-glycosylated proteins capable of forming mucus gels involved in the maintenance of tissue hydration, lubrication and cell protection against proteases, chemical irritants, extreme pH and biological agents (Neutra and Forstner, 1987). An immunofluorescence analysis of Ltk$^-$ cells transfected with cDNA coding for the 190/4 epitope indicated that cells gained limited susceptibility to HAV infection which was blocked by treatment with mAb 190/4. Here we showed that the cell surface glycoprotein identified by mAb 190/4, which we named the HAV cellular receptor 1 (HAVcr-1), is an attachment receptor for HAV. Our data strongly suggest that HAVcr-1 is also a functional receptor for HAV infection because the transfection of HAVcr-1 cDNA into Ltk$^-$ cells conferred limited susceptibility to HAV infection, and monoclonal antibody 190/4 protected AGMK cells and Ltk$^-$ transfectants against HAV infection.

Table I. Characterization of anti-AGMK cell surface mAbs

Clone No.	Binding to AGMK cell surface[a]	Protection of AGMK cells against HAV infection[b]	Anti-HAV titer[c]	Ig subclass
17/4	+	<100	ND	ND
48/1	+	<100	ND	ND
83/3	+	<100	ND	ND
108/1	+	<100	ND	ND
190/4	+	7132	<100	IgG1
235/4	+	9024	<100	IgG1
263/6	+	18 820	<100	IgG2a

[a]Binding of mAb to the cell surface of AGMK cells was determined by an indirect immunofluorescence assay on unfixed cells using 1/1000 dilution ascites and staining with a 1/500 dilution of FITC-conjugated goat anti-mouse antibody. (+) Strong cell surface staining.
[b]Reciprocal of dilution of mouse ascites which inhibited 50% of growth of the KRM003 strain of HAV in AGMK cells. Growth of HAV was determined by an ELISA of cells infected with a MOI of 30 for 3 days.
[c]ELISA titers expressed as the reciprocal of highest dilution of mouse ascites which reacted with HAV. Plates were coated with rabbit anti-HAV, treated with the purified KRM003 strain of HAV and stained with dilutions of mouse ascites and horseradish peroxidase-conjugated goat anti-mouse antibody. Under similar conditions, a mixture of anti-HAV neutralizing mAbs K3-4C8, K2-4F2 and K3-2F2 had a titer >20 000. ND, not determined.

Results

Anti-AGMK cell mAbs protect AGMK cells against HAV infection

To test whether mAbs raised against the cell surface could protect cells against HAV infection, we immunized BALB/c mice with permissive AGMK cells and generated mAbs. Supernatants of 592 independent hybridoma colonies were tested for their ability to block HAV growth in AGMK cells, as assessed by an ELISA using a rabbit anti-HAV antibody. Supernatants of hybridoma clones 190/4, 235/4 and 263/6 protected AGMK cells against infection with the tissue culture-adapted KRM003 strain of HAV. Mouse ascites from these protective mAbs and four other randomly picked non-protective mAbs were tested for their ability to protect AGMK cells against HAV infection (Table I). Only ascites from mAbs 190/4 (IgG1), 235/4 (IgG1) and 263/3 (IgG2a) protected AGMK cells against infection, which confirmed the protective effect observed previously with the hybridoma supernatants.

Protective mAbs react against an epitope expressed at the cell surface of AGMK cells

Indirect immunofluorescence showed that protective mAbs 190/4, 235/4 and 263/6 were directed against the cell surface of AGMK cells (Table I). In addition, a cell surface ELISA of unfixed cells (Kaplan *et al.*, 1989) showed that these mAbs reacted against the cell surface of AGMK cells, whereas control anti-VPg mAb, directed against the VPg protein of HAV, did not react with the cells (Figure 1A).

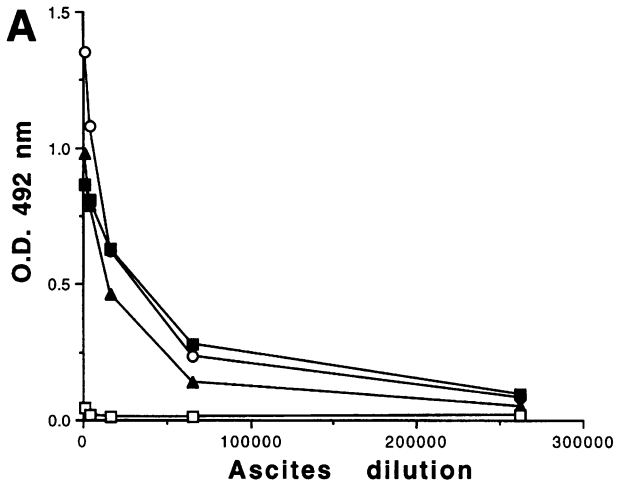
To determine whether the three protective mAbs were directed against the same epitope, horseradish peroxidase-labeled and unlabeled protective mAbs were bound to AGMK. The three protective mAbs competed with each other for binding to the cell surface of AGMK cells (data not shown), indicating that they were directed against the same or overlapping cell surface epitopes. One of the protective mAbs, mAb 190/4, was arbitrarily selected for further studies and purified through protein A–agarose beads. Using a cell surface ELISA, we compared binding to AGMK cells of purified mAb 190/4 and other IgG1 subclass mAbs directed against known cell surface receptors; this confirmed that mAb 190/4 reacted with AGMK cells at levels similar to anti-human α3 integrin mAb P1B5 and anti-poliovirus receptor mAb D171, whereas anti-human αv integrin mAb VNR139 did not react at all (Figure 1B).

The binding of mAb 190/4 to the cell surface of AGMK cells was also analyzed by an *in situ* rosette assay (Kaplan *et al.*, 1989). AGMK cells treated with mAb 190/4 ascites bound anti-mouse immunobeads (Figure 2A), whereas AGMK cells treated with control mouse ascites did not bind immunobeads (Figure 2B), confirming that mAb 190/4 reacted against a cell surface epitope on these cells.

mAb 190/4 protects AGMK cells against infection with different genotypes of HAV

As the three anti-AGMK cell mAbs protected AGMK cells against infection with the KRM003 strain of HAV of genotype 3B (Table I), it was of interest to investigate whether mAb 190/4 could also protect cells against infection with other genotypes of HAV (Robertson *et al.*, 1992). To do so, we utilized indirect immunofluorescence to observe the protection of AGMK cells against infection with a tissue culture-adapted HM175 strain of HAV (genotype 1B). Confluent monolayers of AGMK cells were treated with 100 μg/ml mAb 190/4 or BB7.2 as a negative control and infected with HAV at a multiplicity of infection (MOI) of 1 or mock infected. At 72 h postinfection (p.i.), cells were fixed and stained with human anti-HAV antibody and fluorescein isothiocyanate (FITC)-labeled goat anti-human antibody. HAV-infected cells treated with mAb BB7.2 showed the characteristic intense granular fluorescence of HAV-infected cells (Figure 3A), whereas cells treated with mAb 190/4 showed a very low fluorescence background (Figure 3B), indicating that mAb 190/4 protected cells against infection with the HM175 strain of HAV. As expected, mock-infected AGMK cells treated with mAb BB7.2 (Figure 3C) or mAb 190/4 (data not shown) did not fluoresce, indicating that the fluorescence was HAV specific.

We further verified by radioimmunoassay the protective effect of mAb 190/4 against the infection of AGMK cells with different genotypes of HAV. Confluent monolayers



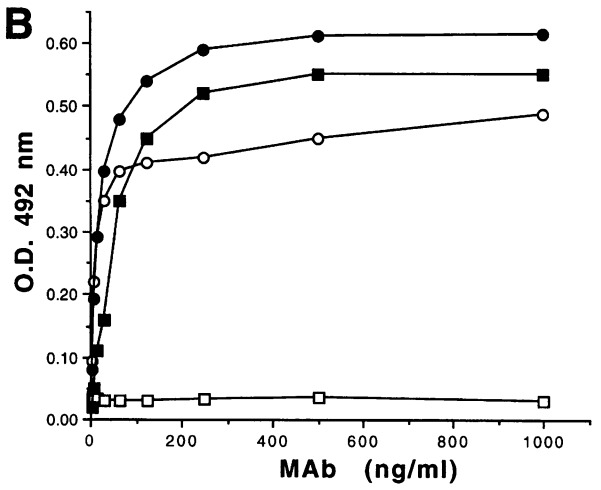


Fig. 1. An ELISA for the detection of epitopes at the cell surface of AGMK cells. AGMK cell monolayers in 96-well plates were treated with different mAbs, washed extensively and stained with peroxidase-conjugated anti-mouse antibody. OPD was used as the substrate and an OD at 492 nm was read. (**A**) Four-fold dilutions of ascites of protective anti-AGMK cell mAbs 190/4 (○), 235/4 (■) and 236/6 (▲), and of anti-HAV-VPg mAb (□), were titrated on the AGMK cell monolayers. (**B**) Two-fold dilutions of purified anti-AGMK mAb 190/4 (○), anti-poliovirus receptor mAb D171 (■), anti-human α3 integrin mAb P1B5 (●) and anti-human integrin αv mAb VNR139 (□) were titrated on the AGMK cell monolayers.

of AGMK cells in 96-well plates were treated with 10 μg/ml mAb 190/4 or P1B5 or mock treated, incubated for 1 h at room temperature and challenged with a MOI of 10, 1 or 0.1 $TCID_{50}$ per cell of HM175, KRM003 or AGM27 (genotype V) strains of HAV. After 72 h of incubation at 35°C, monolayers were fixed with 80% methanol, treated with ^{125}I-labeled human anti-HAV antibody, washed extensively and exposed to X-ray film. While treatment with mAb P1B5 did not affect HAV infection (data not shown), treatment of AGMK cells with mAb 190/4 inhibited infection with the three strains of

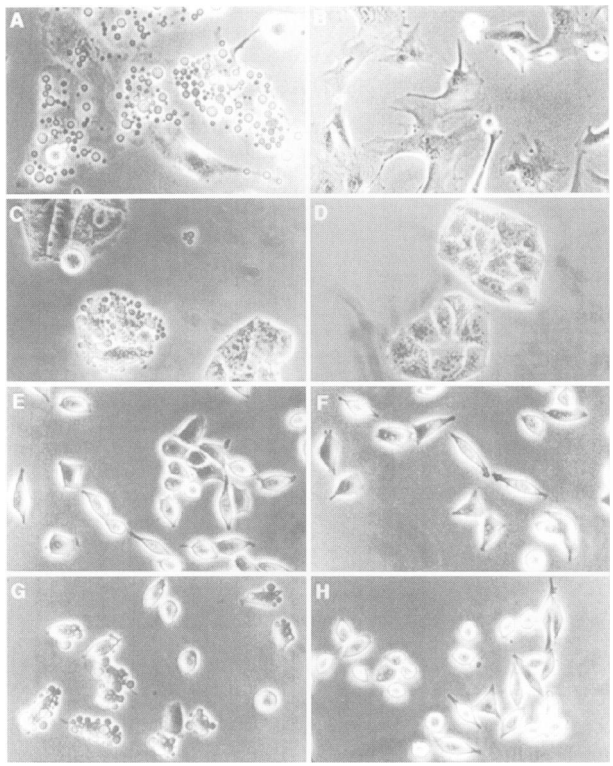


Fig. 2. *In situ* rosette assay for the detection of epitope 190/4 at the cell surface. AGMK (**A** and **B**), E14C (**C** and **D**), LDR2 (**E** and **F**) and Lcr5 (**G** and **H**) cells were treated with ascites of mAb 190/4 (A, C, E and G) or negative control ascites (B, D, F and H). Beads coupled to anti-mouse antibody were used to detect the binding of mAb 190/4 to the cell surface.

HAV (Figure 4A, compare the HAV-specific signal in mAb P1B5-treated cells with the reduced signal in mAb 190/4-treated cells). As expected, the degree of mAb 190/4-mediated protection decreased with increasing amounts of virus used in the challenge and was more noticeable at a MOI of 1 than of 10. At a MOI of 0.1, we only detected replication of the KRM003 strain of HAV which was completely abrogated by treatment with mAb 190/4 (Figure 4A).

Since the radioimmunoassay data (Figure 4A) indicated that mAb 190/4 protected AGMK cells against infection with the three genotypes of HAV, we chose tissue culture-adapted strain HM175 of HAV to further study the protection by a slot blot analysis of total cellular RNA. Confluent monolayers of AGMK cells treated with 10 μg/ml purified mAb 190/4 or control anti-human HLA-A2 mAb BB7.2 were challenged with a MOI of 10, 1 and 0.1 of HAV. Monolayers were incubated for 72 h p.i. at 35°C, and the protective effect of mAb 190/4 was assessed by a slot blot analysis of total RNA extracted from infected cells (Figure 4B). At a MOI of 0.1, HAV-specific RNA was detected in AGMK cells treated with control mAb BB7.2, but was undetectable in AGMK cells treated with mAb 190/4, suggesting that cells were completely protected against HAV infection. The protective effect of mAb 190/4 was also observed at MOI values of 1 and 10, but it diminished with the increasing amount of virus used in the challenge. This MOI-dependent protective effect was also observed in poliovirus-infected HeLa

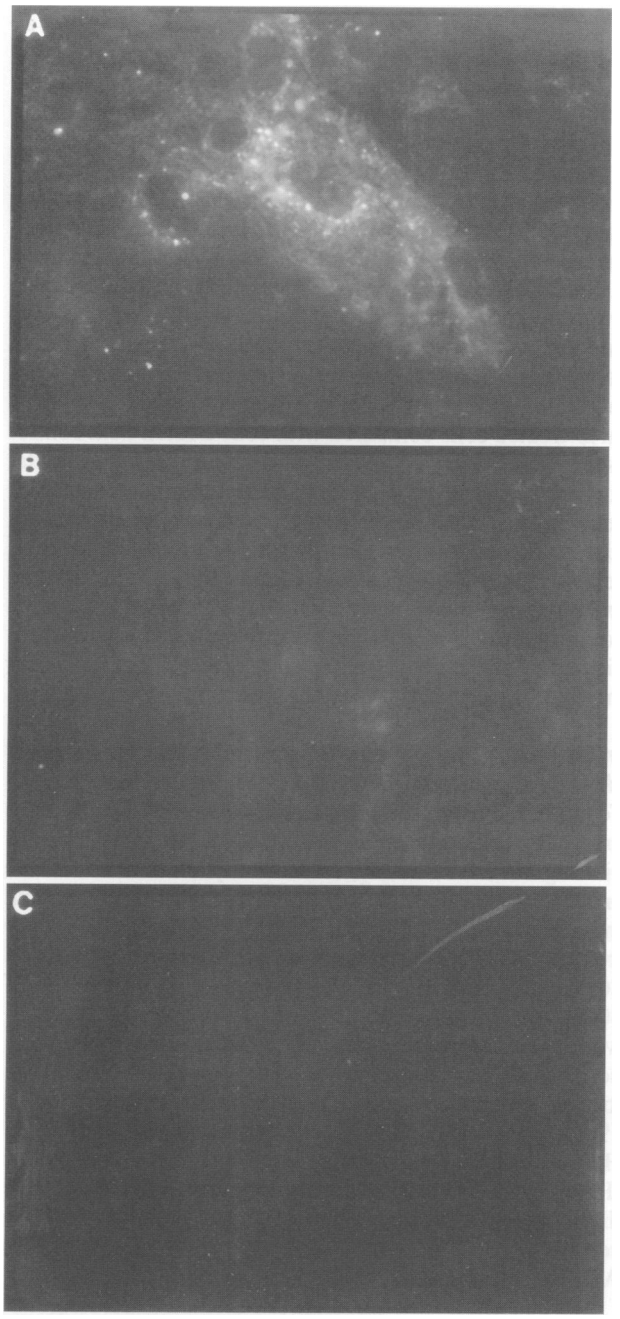


Fig. 3. Immunofluorescence analysis of mAb 190/4-mediated protection of AGMK cells against HAV infection. AGMK cell monolayers in eight-well slides were treated with 100 μg/ml mAb BB7.2 (**A** and **C**) or 190/4 (**B**) for 1 h at room temperature and infected with a MOI of 1 with the tissue culture-adapted strain HM175 of HAV (A and B) or mock infected (C). Monolayers were incubated at 35°C for 72 h, fixed and stained with human anti-HAV and FITC-labeled goat anti-human antibodies. Immunofluorescent micrographs were taken with a Zeiss Axioscope microscope at ×400 magnification with an oil immersion objective. Cells treated with mAb 190/4 and mock infected did not immunofluoresce (data not shown). Cells stained with normal human sera (negative control of HAVAB kit; Abbott) instead of the anti-HAV antisera did not immunofluoresce (data not shown).

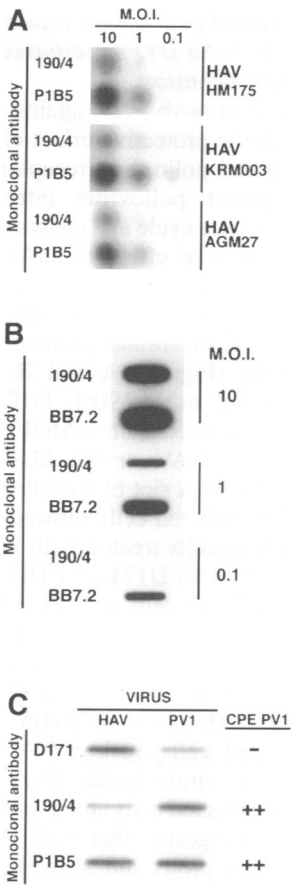


Fig. 4. Radioimmunoassay and slot blot analysis of mAb 190/4-mediated protection of AGMK cells against HAV infection. (**A**) A radioimmunoassay of the protection of AGMK cells against infection with three different genotypes of HAV. AGMK cell monolayers in 96-well plates treated with mAb 190/4 or P1B5 were infected with MOI of 10, 1 and 0.1 with the HM175, KRM003 or AGM27 strain of HAV. After 72 h at 35°C, monolayers were fixed, treated with ^{125}I-labeled anti-HAV antisera, washed extensively and autoradiographed for 12 h. (**B**) A slot blot analysis of the protection of AGMK cells against infection with the HM175 strain of HAV. Confluent monolayers of AGMK cells in six-well plates were treated with mAb 190/4 or BB7.2 for 30 min at room temperature, washed and infected with MOI of 10, 1 or 0.1 with HAV for 1 h at room temperature. Monolayers were washed and incubated at 35°C. Cytoplasmic extracts were prepared at 3 days p.i. and total RNA was extracted. HAV-specific RNA was detected with a ^{32}P-labeled HAV cDNA probe. (**C**) A slot blot analysis of the mAb-mediated protection of AGMK cells against HAV and poliovirus infection. Confluent monolayers of AGMK cells in six-well plates were treated with anti-poliovirus receptor mAb D171, mAb 190/4 or anti-human α3 integrin mAb P1B5 and infected with a MOI of 1 with the HM175 strain of HAV or the Mahoney strain of PV1. Total RNA from PV1-infected cells was probed with ^{32}P-labeled PV1 cDNA; total RNA from HAV-infected cells was probed with ^{32}P-labeled HAV cDNA. Autoradiographs were exposed for 24 h with intensifying screens for HAV-infected cells and for 1 h for PV1-infected cells without screens. Before the preparation of cytoplasmic extracts, the CPE of PV1-infected AGMK cells was assessed under an inverted microscope 12 h p.i. (–, no CPE; ++, 50% CPE).

(Nobis *et al.*, 1985) and AGMK (data not shown) cells protected with anti-poliovirus receptor mAb D171. AGMK cells treated with purified mAb BB7.2, P1B5, VNR139 or D171, or mock treated and infected with HAV, had similar levels of HAV-specific RNA (data not shown), indicating that these mAbs had no effect against HAV infection. Taken together, our protection data obtained by immunofluorescence (Figure 3), radioimmunoassay (Figure 4A) and slot blot analysis (Figure 4B) confirmed that mAb 190/4 protected AGMK cells against infection with different genotypes of HAV, whereas no other mAb tested affected HAV infection.

mAb 190/4-mediated protection against HAV infection parallels mAb D171-mediated protection against poliovirus infection

The protective effect of mAb 190/4 against HAV infection was compared with the protective effect of mAb D171, a well-characterized anti-poliovirus receptor mAb (Nobis *et al.*, 1985), against poliovirus infection. Because poliovirus has a short life cycle and causes a lytic infection in AGMK, the protective effect of mAb D171 against poliovirus infections was assessed 12 h p.i., whereas the protective effect of mAb 190/4 against HAV infection was assessed 72 h p.i., as in similar protection experiments described previously (Figure 4A and B). AGMK cells treated with 10 μg/ml mAb 190/4, D171 or P1B5, or mock treated, were infected with a MOI of 1 with either the HM175 strain of HAV or the Mahoney strain of poliovirus type 1 (PV1). A slot blot analysis of total RNA extracted from HAV-infected cells showed lower levels of HAV-specific RNA in cells treated with mAb 190/4 than in cells treated with mAb D171 or P1B5, and indicated that mAb 190/4 protected cells against HAV infection. A slot blot analysis of total RNA extracted from AGMK cells infected with PV1 showed a lower level of PV1-specific RNA in cells treated with mAb D171 than in cells treated with mAb 190/4 or P1B5, indicating that mAb D171 protected cells against PV1 infection. The similar low levels of viral-specific RNA found in mAb 190/4-treated HAV-infected cells and in mAb D171-treated PV1-infected cells suggested that both mAbs conferred comparable levels of protection against viral infection. Levels of HAV-specific RNA in AGMK cells treated with mAbs D171 and P1B5 were similar to those found in mock-treated cells (data not shown). Similarly, levels of PV1-specific RNA in AGMK cells treated with mAbs 190/4 and P1B5 were comparable with those found in mock-treated cells (data not shown). Therefore, the treatment of AGMK cells with mAbs D171 and P1B5 did not affect HAV infection, and treatment with mAbs 190/4 and P1B5 did not affect PV1 infection. It should be pointed out that viral-specific RNA was not detected in total RNA extracted from mock-infected cells treated with any of the above-mentioned mAbs (data not shown). To control for RNA loading, membranes were stripped and reprobed with ^{32}P-labeled β-actin cDNA, which showed that similar amounts of total RNA were loaded in each slot (data not shown), indicating that the differences in levels of viral-specific RNA were caused by the effect of the protective mAbs.

Before cytoplasmic extracts were prepared for the slot blot analysis, an examination of PV1-infected cells under the inverted microscope showed no cytopathic effect (CPE) in cells treated with mAb D171 and an ~50% CPE in cells treated with mAb 190/4 or P1B5 (Figure 4C). The lack of a CPE in mAb D171-treated cells correlated with low levels of PV1-specific RNA, while a 50% CPE in mAb 190/4- and mAb P1B5-treated cells correlated with higher levels of PV1-specific RNA (Figure 4C). Because our HAV strains do not cause a CPE in AGMK cells, we were unable to correlate the CPE with levels of HAV-specific RNA. However, it could be argued that the low levels of HAV-specific RNA found in mAb 190/4-treated cells could not have induced a CPE in a HAV lytic system, just as the low level of PV1-specific RNA found in mAb D171-treated PV1-infected cells did not cause an apparent CPE.

mAbs 190/4, 235/4 and 263/6 protect AGMK cells specifically against HAV infection

A slot blot analysis of total RNA showed that mAb 190/4 protected AGMK cells against HAV infection but not against PV1 infection (Figure 4C). To characterize further the specificity of the protection conferred by the anti-AGMK cell mAbs against HAV infection, AGMK cells were treated with mAbs 190/4, 235/4 and 263/6 and infected with different picornaviruses which are known to cause a CPE in AGMK cells. Protection against viral infection was assessed by the appearance of a CPE in mAb-treated cells compared with cells treated with control ascites (Nobis *et al.*, 1985). Under similar experimental conditions, mAbs 190/4, 235/4 and 263/6 protected AGMK cells against HAV infection, as assessed by an ELISA (data not shown), and mAb D171 protected AGMK cells against PV1 infection (Table II). A microscopic examination showed that mAbs 190/4, 235/4 and 263/6 did not protect AGMK cells against infection with coxsackie B viruses, echoviruses or polioviruses (Table II), demonstrating that protection was specific against HAV infection.

mAbs 190/4, 235/4 and 262/6 block the binding of HAV to the cell surface of AGMK cells

To study the mechanism by which anti-AGMK cell mAbs protected cells against HAV infection, we analyzed the binding of HAV to AGMK cells by an ELISA in the presence and absence of protective mAbs. Confluent monolayers of AGMK cells grown in 96-well plates were treated with dilutions of ascites of mAbs 190/4, 235/4 and 263/6, and infected with a HAV strain KRM003 (MOI of 3000) for 1 h at 37°C. After washing three times, cells were fixed and stained with rabbit anti-HAV antibody and horseradish peroxidase-labeled anti-rabbit antibody. At the lowest dilution assayed (1:256), treatment with mAb 190/4, 235/4 and 263/6 ascites reduced the binding of HAV to AGMK cells by 93.6, 94.5 and 97.3%, respectively (Figure 5A). The inhibition of binding decreased with the dilution of the mAb and, as expected, treatment with a control ascites did not inhibit the binding of HAV to AGMK cells.

To quantitate the mAb 190/4-mediated blockage of binding of HAV to AGMK cells, monolayers of AGMK cells in 96-well plates were treated with decreasing amounts of purified mAb 190/4 or control mAb BB7.2 (which does not bind to AGMK cells, data not shown) and infected with HAV strain KRM003 (MOI of 5000) for 1 h at room temperature. Monolayers were washed extensively and HAV bound to cells was detected with ^{125}I-labeled human anti-HAV antibody and autoradiography. This radioimmunoassay further confirmed that treatment with mAb 190/4 blocked the binding of HAV to AGMK cells (Figure 5B, compare HAV-specific signal in AGMK cells treated with mAbs 190/4 and BB7.2). A densitometric analysis of the autoradiography revealed that the 50% binding of HAV strain KRM003 to AGMK cells was inhibited by ~1 μg/ml mAb 190/4 (Figure 5C). Similar results were obtained when mAb P1B5 (which binds to the cell surface of AGMK cells; see Figure 1B) or mAb anti-mouse CD71 (which does not bind to AGMK

Table II. Protection of AGMK cells against enteroviral infection by treatment with anti-cell surface mAbs

Virus	CPE 24 h p.i.					CPE 48 h p.i.				
	Control[a]	190/4	235/4	263/6	D171[b]	Control	190/4	235/4	263/6	D171
Coxsackievirus B1	+	+	+	+	ND	+++	+++	+++	+++	ND
Coxsackievirus B3	+	+	+	+	ND	++++	++++	++++	++++	ND
Coxsackievirus B5	+	+	+	+	ND	++++	++++	++++	++++	ND
Echovirus 5	+	+	+	+	ND	++++	++++	++++	++++	ND
Echovirus 6	+	+	+	+	ND	++++	++++	++++	++++	ND
Echovirus 7	+	+	+	+	ND	++++	++++	++++	++++	ND
Echovirus 9	++	++	++	++	ND	++++	++++	++++	++++	ND
Poliovirus 1	+++	+++	+++	+++	–	++++	++++	++++	++++	+
Poliovirus 2	++	++	++	++	ND	++++	++++	++++	++++	ND
Poliovirus 3	+	+	+	+	ND	+++	+++	+++	+++	ND

Confluent monolayers of AGMK cells in 96-well plates were treated with 50 µl/well hybridoma cell culture fluid for 1 h at room temperature. Duplicate wells were infected with 100 $TCID_{50}$/well of corresponding picornavirus for 1 h at room temperature. Monolayers were washed three times and incubated for 48 h at 37°C. The CPE was assessed under an inverted microscope 24 and 48 h p.i.; duplicated wells gave a similar degree of CPE. (+, ++, +++, ++++) Positive CPE of increasing level; (–) no CPE; ND, not determined.

[a]Control hybridoma culture fluid came from hybridoma cell lines producing anti-AGMK cell antibody which reacted with the cell surface of AGMK cells and did not protect AGMK cells against HAV infection (see Table I).

[b]Protective anti-poliovirus receptor mAb was purified through protein A–agarose beads and used at a concentration of 10 µg/ml.

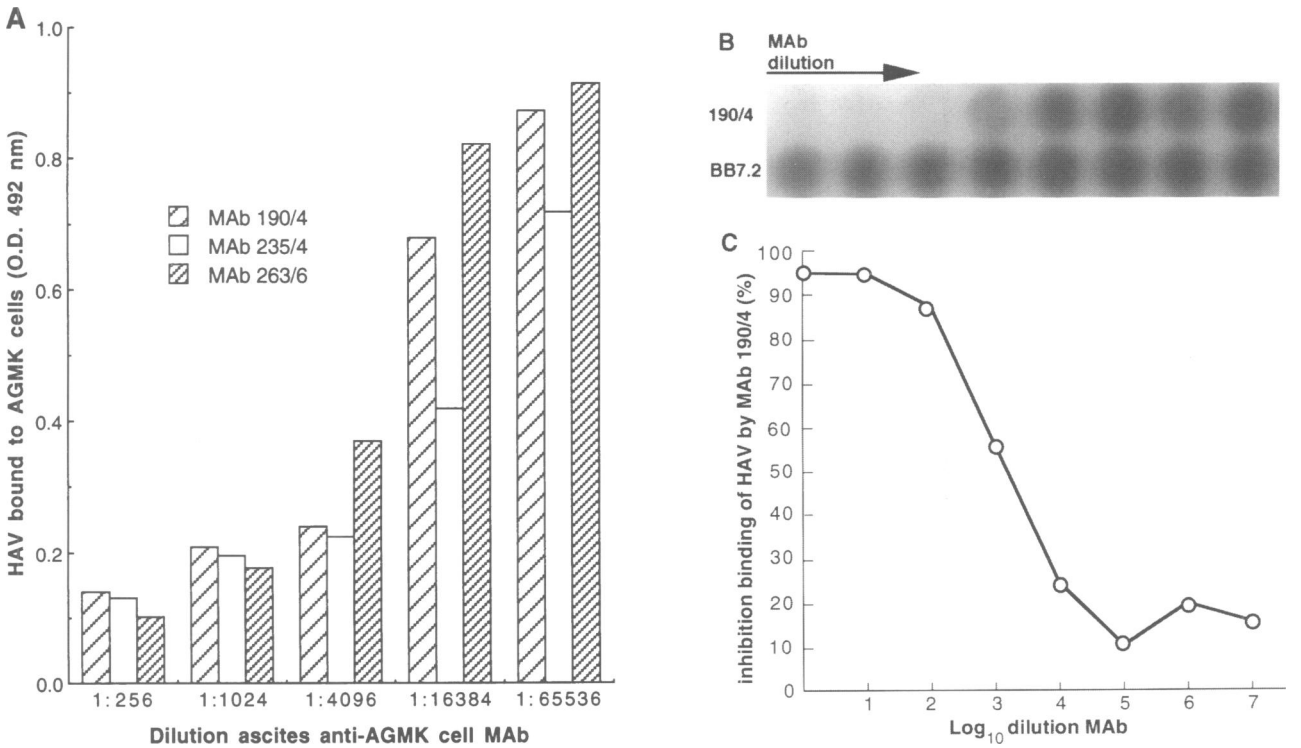


Fig. 5. Protective anti-AGMK cell mAbs block the binding of HAV to AGMK cells. (**A**) mAb 190/4, 235/4 and 263/6 ascites block the binding of HAV to AGMK cells. AGMK cell monolayers in 96-well plates were treated with 4-fold dilutions of protective mAb 190/4, 235/4 and 263/6 ascites for 1 h at 37°C and infected with a MOI of 3000 with the KRM003 strain of HAV for 1 h at 37°C. Monolayers were washed, fixed and stained with rabbit anti-HAV antibody and horseradish peroxidase-labeled anti-rabbit antibody. (**B**) Purified mAb 190/4 blocks the attachment of HAV to AGMK cells. AGMK cell monolayers in 96-well plates were treated with 10-fold dilutions of mAb 190/4 or BB7.2 and infected with the KRM003 strain of HAV. Monolayers were washed, fixed, treated with ^{125}I-labeled human anti-HAV antibody and autoradiographed. (**C**) The percentage of binding inhibition of HAV to the cell surface was calculated by a densitometric analysis of the autoradiography shown in (B), and was repeated at least three times within an experimental error of ~10%.

cells; data not shown) was used instead of mAb BB7.2 to determine the rate of inhibition of binding (data not shown).

Molecular cloning of the cDNA coding for epitope 190/4

To molecularly clone the cDNA coding for epitope 190/4, we devised an expression cloning strategy based on an improved Epstein–Barr virus (EBV) shuttle vector system (Murphy *et al.*, 1992; Swirski *et al.*, 1992). Briefly, an AGMK cell cDNA library cloned into the shuttle vector pDR2 (which confers hygromycin resistance to eukaryotic cells) was transfected into HeLa cells which expressed the EBV nuclear antigen EBNA-1 (E14C cells). Hygromycin-resistant cells were selected and panned to polystyrene Petri dishes (Aruffo and Seed, 1987; Seed and Aruffo,

1987) coated with affinity-purified goat anti-mouse antibody and mAb 190/4. The expression of epitope 190/4 at the cell surface was confirmed by an *in situ* rosette assay, which showed that panned E14C cells treated with mAb 190/4 were decorated with immunobeads (Figure 2C), whereas the same cells treated with control ascites (Figure 2D) and E14C cells, or E14C cells transfected with vector pDR2, did not react with the immunobeads (data not shown). *E.coli* were transformed with episomal DNA extracted from E14C cells expressing the 190/4 epitope. The resulting rescued plasmids were retransfected into E14C cells. An *in situ* rosette assay of hygromycin-resistant cells revealed that at least two plasmids containing identical cDNA inserts of ~2000 bp, pDR2–GL37/5 and pDR2–GL37/9, encoded the 190/4 epitope.

The molecule encoding epitope 190/4 is a novel membrane-bound mucin-like cell surface receptor

The protein identified by mAb 190/4 and encoded in pDR2–GL37/5 was named HAVcr-1. The nucleotide sequence of HAVcr-1 cDNA (Figure 6A) has 2093 bp, a poly(A) tail at its 3′ end and encodes a putative HAVcr-1 polypeptide of 451 amino acids starting at nucleotide 196 and ending at nucleotide 1549. The HAVcr-1 has the typical features of a type I integral membrane glycoprotein. An analysis of the hydrophobicity plot (Figure 6B) according to the method of Kyte and Doolittle (1982) showed two distinctive hydrophobic regions: a putative 17 amino acid signal sequence with a hydrophobic core following the initiating methionine, and a putative transmembrane domain of 22 residues between amino acids 370 and 391 which is the major hydrophobic region of the protein. A cysteine residue (Cys376) found within the transmembrane domain is probably used for the addition of fatty acids which may stabilize attachment of the mucin to the membrane (Magee *et al.*, 1989). Between the signal sequence and the transmembrane domain there is a predicted extracellular domain of 354 residues which can be divided into two distinctive domains: a cysteine-rich (Cys-rich) N-terminal region of ~109 residues, and a C-terminal segment of ~243 residues containing a high frequency of threonine, serine and proline (T/S/P-rich region). Although Cys-rich regions have been found in several secreted mucins such as human MUC2 and MUC5 (Meerzaman *et al.*, 1994), it is not characteristic of membrane-bound mucins (Strous and Dekker, 1992). The T/S/P-rich region includes 27 consecutive and almost identical repeats of the six-amino acid consensus sequence PTTTTL (Figure 6C), followed by an array of probable degenerate repeats. The large number of threonine residues present in the inferred HAVcr-1 sequence may serve as sites for O-linked glycosylation. There are two potential sites (Asn–X–Ser/Thr) for the attachment of N-liked carbohydrates in the Cys-rich region, and two in the T/S/P-rich region. The intracellular domain has 60 residues and bears a single consensus recognition site for protein kinase C at position 448.

A search of non-redundant Protein Data Bank + SwissProt + PIR + SPUpdate + GenPept + GPUpdate databases using the BLAST program (Altschul *et al.*, 1990) indicated that HAVcr-1 was not identical to any registered sequence. Homology of HAVcr-1 to a variety of Thr-rich mucin-like glycoproteins could be attributed to the number of short and low complexity repeats (four out of six residues of the repeats are a Thr). For instance, portions of the T/S/P-rich region of HAVcr-1 have up to 50% similarity with human (Gerard *et al.*, 1990) and monkey (An *et al.*, 1994) tracheal mucin, 44% similarity with human intestinal mucins (Toribara *et al.*, 1991; Gum *et al.*, 1994), 50% similarity with fruit fly glue protein (Garfinkel *et al.*, 1983; Swida *et al.*, 1990), 46% similarity with surface antigen of *Leishmania* (Murray and Spithill, 1991), 53% similarity with mucin-like surface proteins of *Trypanosome* (Reyes *et al.*, 1994), 53% similarity with the A-agglutinin attachment protein of yeast (Roy *et al.*, 1991), and 43 and 53% similarity to prepropasmolysin (Hoffmann, 1988) and integumentary mucin (Hauser and Hoffmann, 1992) of *Xenopus*.

The Cys-rich region of HAVcr-1 shares some homology with members of the immunoglobulin superfamily (IgSF)

Surprisingly, an analysis of only the Cys-rich region of HAVcr-1 using the BLAST program showed homology with variable domains of members of the IgSF (Figure 7). An analysis of the Cys-rich region using the MOTIFS program (Wisconsin Genetics Computer Group), which searched for patterns defined in the PROSITE database, revealed that sequences surrounding Cys105 followed the pattern [FY]–x–C–x–[VA]–x–H, defined as the signature of the immunoglobulins and major histocompatibility complex proteins and found in IgSF constant domains. Interestingly, a naked eye analysis of the five remaining cysteines indicated that amino acids around Cys36, which is the most N-terminal Cys residue, had the pattern L/I/V–x–L/I–x–C occurring around conserved disulfide bonds in members of the IgSF (Barclay *et al.*, 1993). Therefore, it is possible that Cys36 and Cys105 form an Ig-type disulfide bond. The distribution of the remaining four cysteines (residues 46, 52, 57 and 104) did not follow any recognizable consensus pattern and might, in conjunction with Cys36 and Cys105, form a novel type of domain. The Cys residues in Cys-rich regions of secreted mucins, like MUC2, including the Cys–Cys dipeptides such as Cys104–Cys105 of HAVcr-1, are believed to form intra- and intermolecular disulfide bonds responsible for the polymerization of mucins and the formation of mucus gels (Devine and McKenzie, 1992; Strous and Dekker, 1992). Therefore, Cys residues found in HAVcr-1, which are atypical of membrane-bound mucins, could also be involved in forming intermolecular disulfide bonds. It should be pointed out that database searches of the 5′ and 3′ end non-coding sequences and other domains of HAVcr-1 did not reveal any significant homology with known sequences.

HAV binds to HAVcr-1 expressed at the surface of mouse Ltk⁻ cell transfectants

To verify that the cDNA coding for epitope 190/4 encoded a functional receptor for HAV, non-susceptible mouse Ltk⁻ cells were transfected with pDR2–GL37/5 and infected with HAV. Hygromycin-resistant Ltk⁻ cells transfected with pDR2–GL37/5 and expressing the 190/4 epitope were selected by panning and named Lcr5 cells. Hygromycin-resistant Ltk⁻ cells transfected with vector pDR2 (LDR2

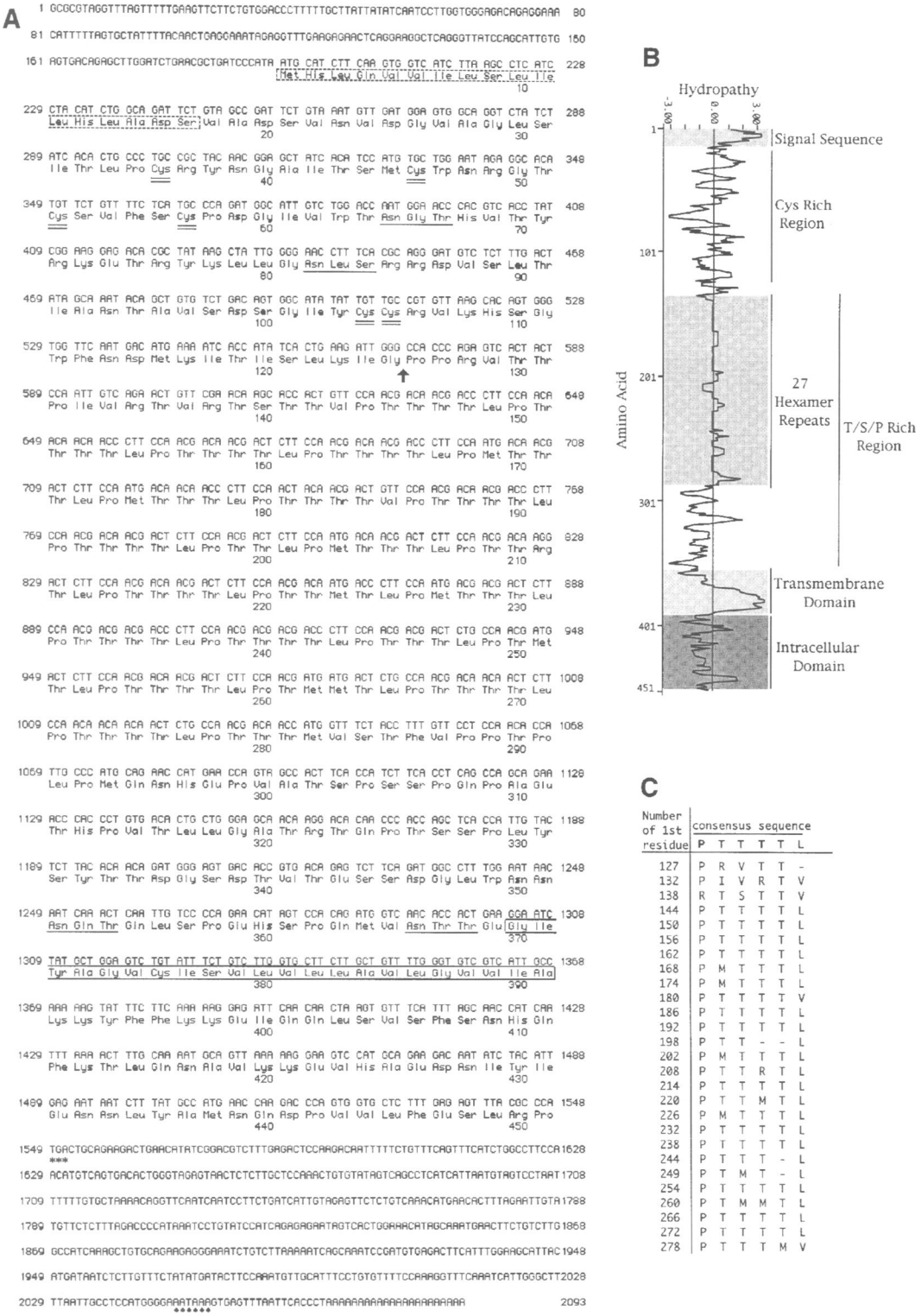


Fig. 6. cDNA sequence, the predicted amino acid composition and a protein hydropathy plot of human HAVcr-1. The nucleotide sequence of the HAVcr-1 cDNA contained in pDR2–GL37/5 was determined on both strands by dideoxy chain termination (Sanger *et al.*, 1977) using specific oligonucleotides. (**A**) Nucleotide sequence and inferred amino acid sequence of HAVcr-1. The dashed box indicates the putative signal sequence determined by the method of von Heijne (1986) using a public domain program developed by Ned Mantei (Department of Biochemistry, Swiss Federal Institute of Technology, ETH-Zentrum, Zurich, Switzerland), which calculated scores of 6.56 for Ser17 and 6.38 for Ala19. Although we cannot rule out the possibility that the signal sequence could be cleaved at Ala19, it is most likely that the cleavage occurs at Ser17 because of its higher score and more N-terminal position. The six cysteines of the Cys-rich region are double underlined. Potential N-linked glycosylation sites are underlined. The arrow indicates the putative beginning of the T/S/P-rich region. The putative transmembrane domain is boxed. The termination codon and a canonical polyadenylation signal are indicated by asterisks and dots, respectively. (**B**) Hydropathic plot of HAVcr-1 according to the method of Kyte and Doolittle (1982). Various regions are identified to the right of the figure. (**C**) Alignment of the consecutive 27 hexameric peptide repeats of the T/S/P-rich region starting at Pro127. Gaps made in the sequence to optimize the alignment are indicated by dashes. GenBank accession number: X98252.

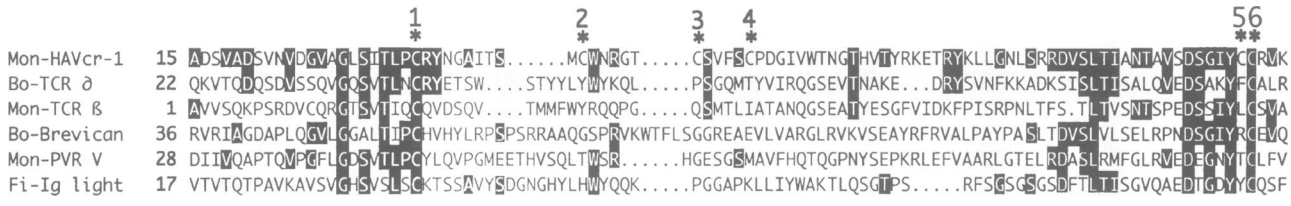


Fig. 7. Alignment of first domain of HAVcr-1 protein with the first domains of members of the IgSF. The Cys-rich region of HAVcr-1 (Mon-HAVcr-1) is aligned with the immunoglobulin-like domains of bovine T cell receptor V δ-chain (B0-TCR δ; N.Ishiguro, Y.Aida, T.Shinagawa and M.Shinagawa, unpublished results), monkey T cell receptor V β-chain (Mon-TCR β; E.E.M.Jaeger, R.E.Bontrop and J.S.Lanchbury, unpublished results), bovine brevican first domain (Bo-brevican; Yamada *et al.*, 1994), monkey receptor for poliovirus V domain (Mon-PVR V; Koike *et al.*, 1992) and fish Ig V light chain (Fi-Ig light; Daggfeldt *et al.*, 1993) using the PILEUP program (Wisconsin Genetics Computer Group) and optimized further. Gaps introduced in the sequences for the alignment are indicated by dots; numbers of the first residues are indicated. Identical amino acids to the HAVcr-1 sequence are in white on a black background. The six Cys residues present in HAVcr-1 are indicated by asterisks.

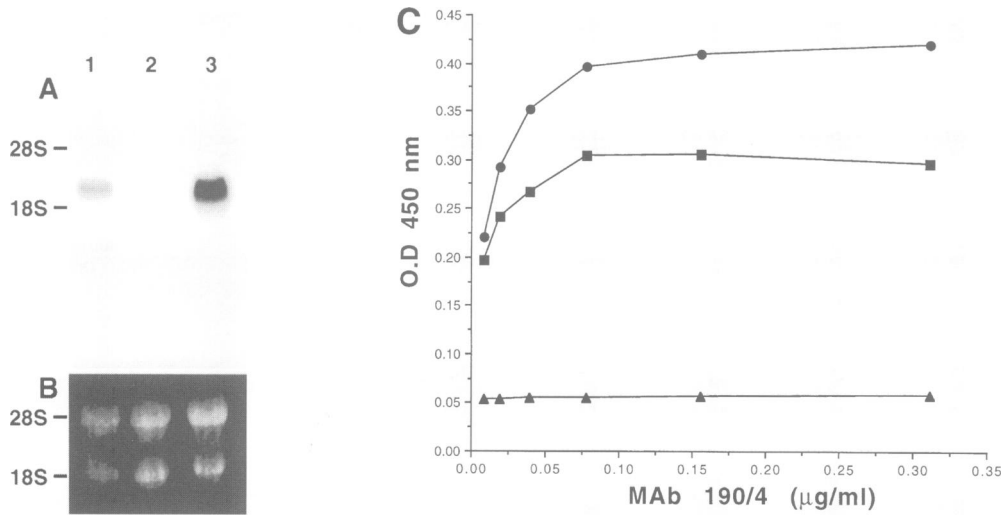


Fig. 8. Expression of HAVcr-1 in Ltk⁻ cell transfectants. (**A**) A Northern blot analysis. Total RNA (20 µg) extracted from Lcr5 (Ltk⁻ cells transfected with HAVcr-1 cDNA in pDR–GL37/5; lane 1), LDR2 (Ltk⁻ cells transfected with vector pDR2; lane 2) and AGMK cells (lane 3) was fractionated on a 1% agarose–formaldehyde gel and transferred to a nylon membrane (Zeta-probe; Bio-Rad) in 10× SSC. A full-length HAVcr-1 cDNA probe cut from pDR2–GL37/5 with *Xba*I and *Bam*HI was labeled with ^{32}P by random priming, hybridized to the membrane in 50% formamide–5× SSC at 42°C, washed with 2× SSC–0.1% SDS at 65°C and autoradiographed for 48 h. (**B**) Ethidium bromide staining of agarose gel before transferring to control for RNA loading. Ribosomal 28S and 18S RNAs contained in the total RNA samples were used as molecular weight markers. (**C**) An ELISA to detect the expression of epitope 190/4 at the cell surface. Monolayers of AGMK (●), Lcr5 (■) and LDR2 (▲) cells in 96-well plates were treated with 2-fold dilutions of purified mAb 190/4 by duplicates and stained with peroxidase-conjugated anti-mouse antibody. OPD was used as the substrate and the average OD at 492 nm for each mAb dilution was plotted.

cells) were used as a negative control for receptor expression and susceptibility to HAV infection. The *in situ* rosette assay showed that at least 70% of Lcr5 cells were decorated with immunobeads and, therefore, expressed the 190/4 epitope at the cell surface (Figure 2G), whereas LDR2 cells were not decorated with immunobeads and, therefore, did not express the 190/4 epitope at the cell surface (Figure 2E). As expected, neither LDR2 nor Lcr5 cells treated with control ascites reacted with the immunobeads (Figure 2F and H respectively). A Northern blot analysis of total RNA (Figure 8A) confirmed that a specific 190/4 transcript of ~2000 bases was expressed in both Lcr5 (lane 1) and AGMK (lane 3) cells but not in LDR2 cells (lane 2). The expression of epitope 190/4 in Lcr5 cells was further confirmed by a cell surface ELISA. Purified mAb 190/4 reacted only with Lcr5 cells in a dilution-specific manner and did not react with LDR2 cells (Figure 8C), whereas purified control anti-mouse CD71 mAb C2 directed against the mouse transferrin receptor reacted similarly with both cell lines (data not shown). These results clearly show that HAVcr-1 and its 190/4 epitope were expressed at the cell surface of Lcr5 cells.

To analyze the binding of HAV to Ltk⁻ transfectants, Lcr5, LDR2 and control AGMK cells were infected with HAV at a MOI of 10 for 4 h at 35°C. Unbound virus was washed extensively, and cell-bound HAV was released by three freeze–thaw cycles. Because there is no clear eclipse of the input HAV, infectious HAV can be isolated from cells at all times p.i. (Anderson *et al.*, 1988). The three freeze–thaw cycles used to release a significant amount of infectious poliovirus adsorbed to cells at 2°C (Mandel, 1962) were also effective in releasing HAV adsorbed to AGMK cells and other cell lines (unpublished results). The released infectious virus was titrated in FRhK/4 cells by an endpoint dilution assay, which showed that Lcr5 cells bound 10 times more HAV than LDR2 cells, and control AGMK cells bound 100 times more HAV than LDR2 cells (Figure 9A). These data suggested that the 10-fold increase in binding of HAV observed in Lcr5 cells was caused by the expression of HAVcr-1 at the cell surface.

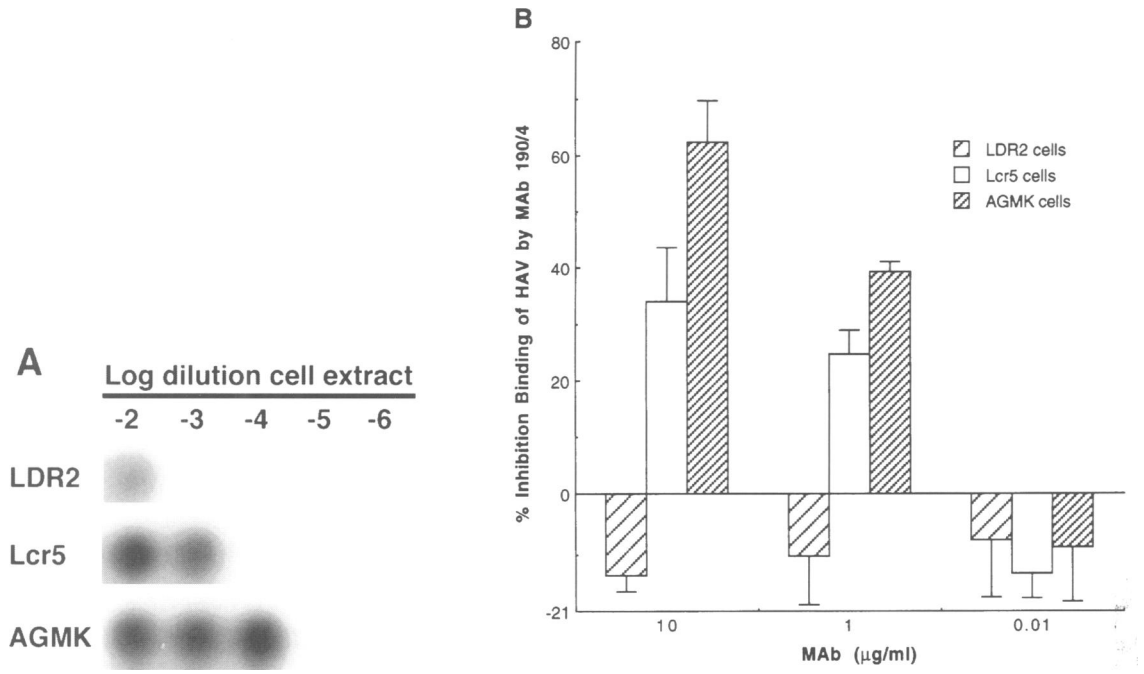


Fig. 9. Binding of HAV to Ltk⁻ cell transfectants. (**A**) Increased binding of HAV to Lcr5 cells. Confluent AGMK, Lcr5 and LDR2 cell monolayers in six-well plates were infected with a MOI of 10 with the KRM003 strain of HAV for 4 h at 35°C. Unbound virus was washed extensively with EMEM containing 10% FBS; bound virus was released by three freeze–thaw cycles. Cell debris was pelleted, and supernatant was titrated on FRhK/4 cells. After 2 weeks, cells were fixed, treated with ^{125}I-labeled human anti-HAV and autoradiographed. (**B**) mAb 190/4-mediated inhibition of binding of HAV to Lcr5 cells. Monolayers of AGMK, Lcr5 and LDR2 cells in 96-well plates were treated with 10, 1 or 0.01 μg/ml mAb 190/4 or C2 (anti-mouse CD71 mAb) and infected with a MOI of 1 with HAV. Unbound virus was washed extensively and bound virus was detected with ^{125}I-labeled human anti-HAV antibody and autoradiography. The inhibition of binding of HAV was determined by a densitometric analysis of the autoradiographs. Values correspond to the mean of three experiments and the standard deviation is shown as a line.

To demonstrate further that HAV attached to Lcr5 cells via the HAVcr-1 receptor, the binding of HAV to Lcr5 was inhibited by treatment with mAb 190/4. A radioimmunoassay of the binding of HAV to LDR2, Lcr5 and AGMK cells (Figure 9B) showed that treatment with 10 μg/ml mAb 190/4 inhibited the binding of HAV to Lcr5 cells by ~35% and to AGMK cells by ~60%. This inhibition of binding was dilution dependent and undetectable at a concentration of 10 ng/ml mAb 190/4 (Figure 9B). Although HAV binds non-specifically to mouse L cells (Zajac *et al.*, 1991), the treatment of LDR2 cells with mAb 190/4 did not affect the binding of HAV when compared with treatment with mAb C2 (Figure 9B) or mock treatment (data not shown). The lower rate of mAb 190/4-mediated inhibition of binding of HAV to Lcr5 cells compared with AGMK cells could be attributed to several factors, such as the non-specific background of binding of HAV to Ltk⁻ cell transfectants, the lower level of expression of HAVcr-1 in Lcr5 cells (Figure 8), and the 10-fold lower binding of HAV to Lcr5 cells (Figure 9A). In conclusion, our data clearly show that mAb 190/4 specifically inhibits the binding of HAV to Lcr5 cells and further confirm that HAVcr-1 is an attachment receptor for HAV.

Transfection of HAVcr-1 into mouse Ltk⁻ cells confers susceptibility to HAV infection

The susceptibility of the Ltk⁻ cell transfectants to HAV infection was studied by an immunofluorescence analysis using anti-HAV neutralizing mAb 813. Lcr5 cells infected with HAV showed the characteristic HAV-specific cytoplasmic fluorescence (Figure 10A), whereas LDR2 cells infected with HAV showed a diffuse fluorescence background (Figure 10B); fluorescence was not observed in mock-infected Lcr5 and LDR2 cells (data not shown). HAV-specific immunofluorescence in HAV-infected Lcr5 cells was not observed at 0 h p.i., and increased during the first 24–72 h of infection (data not shown), suggesting that the *de novo* synthesis of HAV antigens rather than internalization of the input virus occurred in Lcr5 cells.

To further confirm that HAV infected Lcr5 cells via the HAVcr-1, HAV infection was blocked by treatment with mAb 190/4. Control AGMK cells treated with mAb BB7.2 developed the characteristic immunofluorescence of HAV-infected cells (Figure 11A), whereas AGMK cells treated with mAb 190/4 developed a low background fluorescence (Figure 11B) and therefore were protected against HAV infection. As expected, HAV-infected LDR2 cells treated with either mAb BB7.2 (Figure 11C) or 190/4 (Figure 11D) did not show any HAV-specific fluorescence. On the other hand, the protective effect of mAb 190/4 was quite evident in HAV-infected Lcr5 cells: mAb BB7.2-treated cells showed the characteristic intense green granular fluorescence of HAV-infected cells (Figure 11E), whereas mAb 190/4-treated cells showed only a low background fluorescence (Figure 11F). The infection of Lcr5 cells with HAV was limited, and the HAV-specific immunofluorescence faded out within 1 week. HAV-infected Lcr5 cells showed a <10-fold increase in HAV titers and a <2-fold increase in HAV-specific RNA (data not shown), which could be attributed to the limited infection of Lcr5 cells. Attempts to further adapt HAV to grow in Lcr5 cells

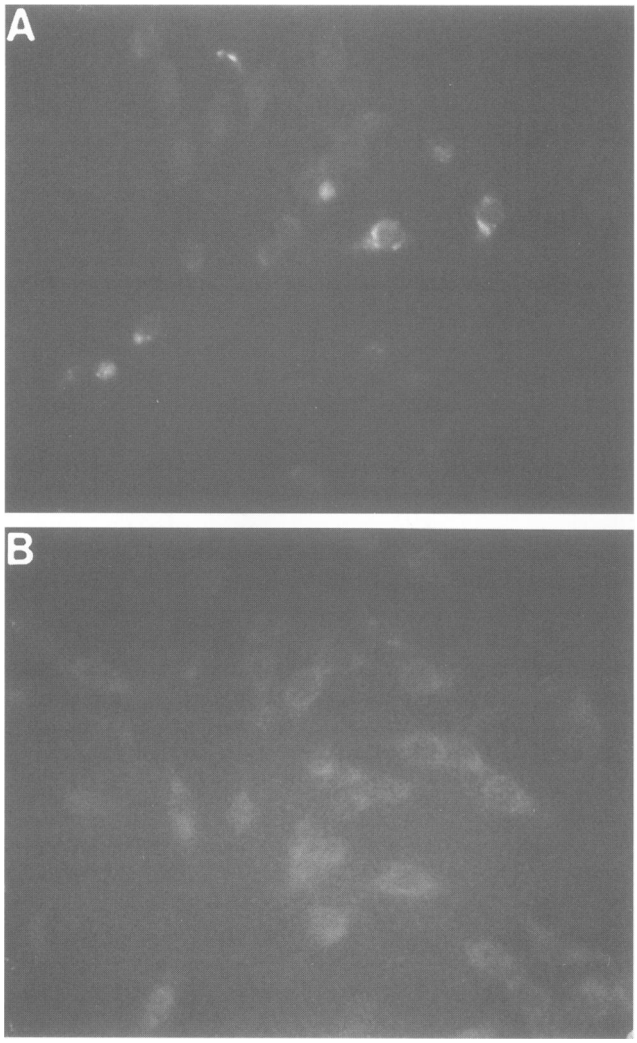


Fig. 10. An immunofluorescence analysis of the susceptibility of Ltk⁻ transfectants to HAV infection. Lcr5 and LDR2 cell monolayers in eight-well slides were infected with a MOI of 10 with HAV for 72 h at 35°C, fixed and stained with biotinylated anti-HAV neutralizing mAb 813 and FITC-labeled avidin. Mock-infected cells did not immunofluoresce (data not shown).

by serial passaging and multiple reinfections have been unsuccessful. Therefore, our results suggest that HAVcr-1 is a functional cellular receptor for HAV because the limited susceptibility to HAV conferred upon transfection of pDR2–GL37/5 into Ltk⁻ cells was blocked by treatment with mAb 190/4.

Discussion

We have identified a cellular receptor for HAV using mAb 190/4 which blocked HAV infection in AGMK cells. mAb 190/4 was raised against the cell surface of AGMK cells and specifically protected AGMK cells against infection with HAV (Figures 3 and 4, and Table I) but not against infection with other picornaviruses (Figure 4C and Table II). Treatment of AGMK cells with 10 μg/ml mAb 190/4 conferred the complete protection of AGMK cells against HAV infection at a MOI of 0.1 (Figure 4B). Although there are major differences in the life cycle of PV1 and HAV regarding the length of the replication cycle and the ability to cause a lytic infection in AGMK cells, the degree of protection against HAV infection conferred by mAb 190/4 resembled the level of protection against poliovirus infection conferred by the well-characterized mAb D171 (Figure 3C). This similarity suggested that the mechanism by which mAb 190/4 blocked HAV infection was equivalent to the mechanism by which mAb D171 blocked poliovirus infection, and was confirmed by the finding that mAb 190/4 blocked the binding of HAV to the cell surface of AGMK cells (Figure 5).

A nucleotide sequence analysis showed that the epitope 190/4 cDNA coded for a novel class I integral membrane chimeric receptor, the HAVcr-1, which has an extracellular globular first domain followed by a mucin-like and probably heavily O-glycosylated extended structure. By analogy to other picornaviruses such as poliovirus and rhinovirus, which bind to first domains of immunoglobulin-like receptors (Nomoto, 1992), it is possible that HAV will also bind to the globular domain of HAVcr-1. Homology of the HAVcr-1 first domain with immunoglobulin-like domains (Figure 7) further suggests that HAV could interact with the first domain of HAVcr-1. Mutagenesis of the HAVcr-1 cDNA will help to identify the binding site for HAV and determine which sequences are important for binding and viral entry. Because calcium ions are known to form complexes with mucins (Strous and Dekker, 1992) and are required for the infectivity of HAV in tissue culture (Stapleton *et al.*, 1991; Zajac *et al.*, 1991), it is possible that association with calcium ions alters HAVcr-1 in such a way that it exposes or stabilizes the HAV binding site or modifies the virus–receptor interaction to allow viral cell entry. It is likely that the T/S/P-rich region extends the globular domain of HAVcr-1 well above the cell surface, like the 'lollipop on a stick' model (Jentoft, 1990), which will make the first domain more accessible for interactions with extracellular macromolecules. Although the natural function of the HAVcr-1 is not known, it is possible that carbohydrate structures of the extended O-glycosylated T/S/P-rich domain could be recognized by lectin-like receptors, and that the first globular domain could also have a function in cell–cell interactions. Because of the presence of a protein kinase C phosphorylation site in the intracellular domain, it is also possible that HAVcr-1 is involved in signal transduction pathways.

Non-susceptible mouse Ltk⁻ cells transfected with HAVcr-1 cDNA (Lcr5 cells) expressed the 190/4 epitope at the cell surface (Figures 2 and 8C). Lcr5 cells bound at least 10-fold more infectious HAV than Ltk⁻ cells transfected with vector pDR2 (Figure 9A), which suggested that the expression of HAVcr-1 at the cell surface was responsible for such an increase in binding activity. The mAb 190/4-mediated inhibition of binding of HAV to Lcr5 cells clearly confirmed that HAV bound to Lcr5 cells via the HAVcr-1 receptor (Figure 9B). Because mAb 190/4 inhibited the binding of HAV to both AGMK and Lcr5, we conclude that the HAVcr-1 cell surface protein is an attachment receptor for HAV.

Our immunofluorescence data showed that Ltk⁻ cells transfected with HAVcr-1 cDNA (Lcr5 cells) gained susceptibility to HAV infection and that treatment with mAb 190/4 protected those cells against HAV infection (Figure 10). However, because of the limited nature of the HAV infection in Lcr5 cells, we did not detect a

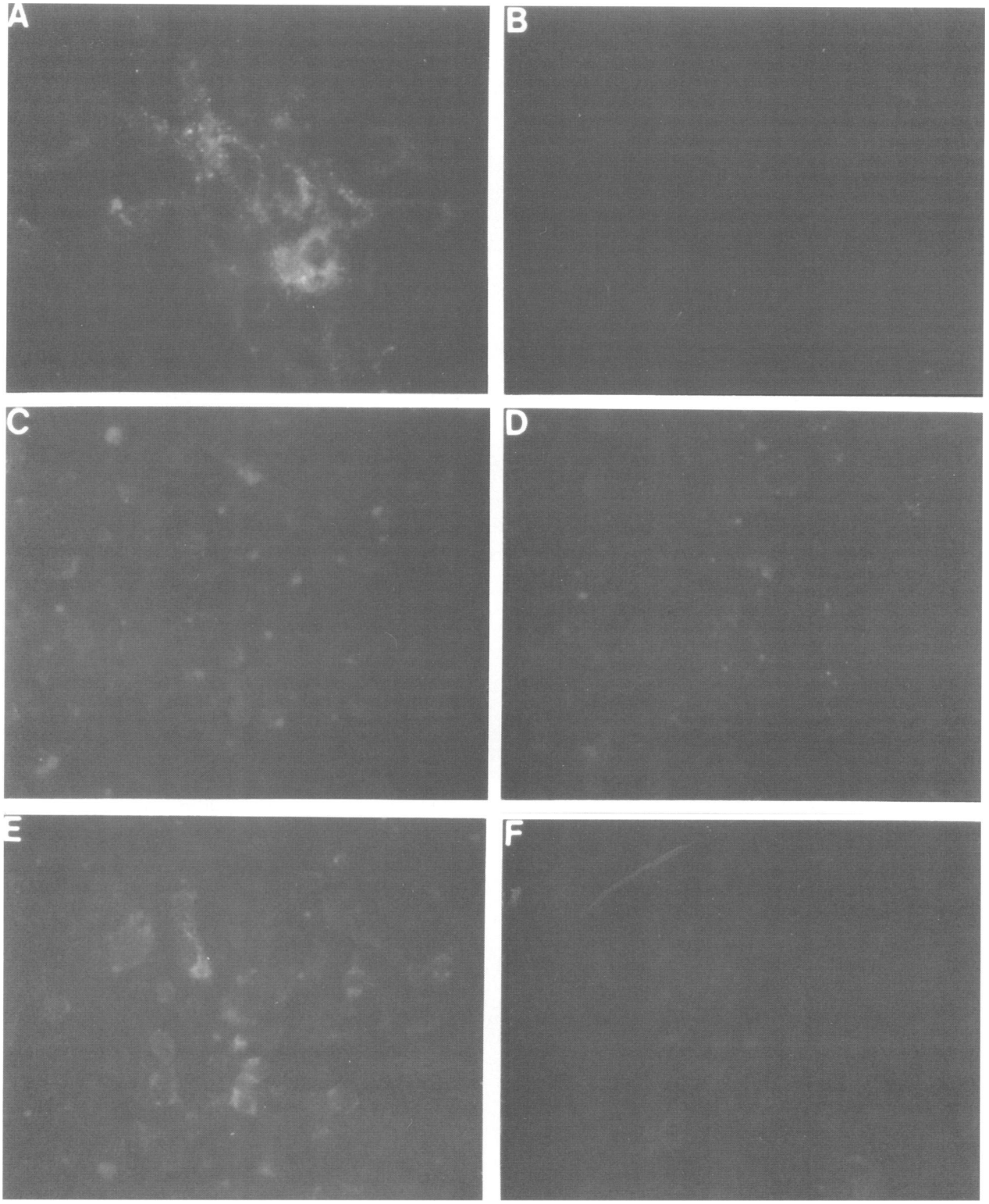


Fig. 11. Immunofluorescence analysis of mAb 190/4-mediated protection of Lcr5 cells against HAV infection. Confluent monolayers of clone GL37 of AGMK (**A** and **B**), LDR2 (**C** and **D**) and Lcr5 (**E** and **F**) cells in eight-well slides were treated with 0.1 ml (100 μg/ml) mAb 190/4 (B, D and F) or mAb BB7.2 (A, C and E) diluted in PBS–5% FBS for 1 h at room temperature. Monolayers were infected with HAV at a MOI of 10 or mock infected for 24 h at 34°C, washed four times with EMEM–10% FBS, fixed with cold acetone, and stained with human anti-HAV antibody and FITC-labeled goat anti-human antibody. Cells stained with normal human sera instead of the anti-HAV antisera did not immunofluoresce (data not shown).

significant increase in HAV titers and HAV-specific RNA (data not shown). Previously we showed that although some non-primate cells were susceptible to HAV infection, Ltk$^-$ cells were neither susceptible to HAV infection nor supported HAV replication upon transfection of virion RNA (Dotzauer *et al.*, 1994). The latter suggested that Ltk$^-$ cells had intracellular block(s) to HAV replication. Therefore, the limited susceptibility of Lcr5 cells to HAV infection could be explained by the high MOI ($\geq$10) and long infection time (several hours) required to trigger the

infection. It is most likely that the transfection of virion RNA did not deliver enough genomes into the cytoplasm of Ltk$^-$ cells to induce the limited HAV infection observed in Lcr5 cells upon infection with a high MOI of HAV. The strong intracellular block(s) to HAV replication were not overcome by serial passaging and multiple reinfections of Lcr5 cells with HAV. Further studies will be required to identify the nature of this block. HAV infection of other non-susceptible cells of different origin (dog, pig, rat, etc.) transfected with HAVcr-1 cDNA mimicked the limited susceptibility to HAV infection observed in Ltk$^-$ cells (data not shown), and indicated that intracellular block(s) to HAV replication are widespread in mammalian cells, as suggested previously (Dotzauer *et al.*, 1994). The involvement of cellular host factors other than cell surface receptors in permissiveness to viral infection has been documented extensively. For instance, mouse cells expressing the HIV receptor CD4 are not permissive to HIV replication (Maddon *et al.*, 1986), and mouse and Chinese hamster cells expressing decay-accelerating factor CD55 are not susceptible to infection with echovirus 7 and related viruses (Ward *et al.*, 1994). Therefore, the limited susceptibility of Lcr5 cells to HAV infection is not surprising at all.

Two strong lines of evidence support the suggestion that HAVcr-1, besides being an attachment receptor for HAV, is also a functional receptor which mediates HAV infection. First, mAbs 190/4, 235/4 and 263/6 protected susceptible AGMK cells specifically against infection with three different genotypes of HAV (Table I and Figures 3 and 4), suggesting that the attachment of HAV to the HAVcr-1 protein is the interaction which triggers the cell entry of HAV. Secondly, our immunofluorescence data showed that the limited susceptibility of Lcr5 to HAV infection was blocked by treatment with mAb 190/4, strongly suggesting that HAVcr-1 is indeed a functional receptor for HAV. Further corroboration that HAVcr-1 is a functional receptor for HAV awaits the identification of non-susceptible cells which could fully support HAV replication upon transfection of HAVcr-1 cDNA; such cells have not yet been identified.

Further characterization of the HAVcr-1, its distribution and interaction with the HAV virion will help us to better comprehend the mechanisms of replication and pathogenesis of this unique picornavirus. The availability of the cDNA and gene coding for the monkey HAVcr-1 and human homolog(s) could allow the development of antiviral agents, which block the virus–receptor interaction, and transgenic models to study liver disease caused by hepatitis viruses.

Materials and methods

Cells, antibodies and viruses

The continuous clone GL37 of AGMK cells was selected for supporting optimal growth of HAV (Totsuka and Moritsugu, 1994). Mouse Ltk$^-$ cells were obtained from ATCC. Both cell lines were grown in EMEM containing 10% fetal bovine serum. mAbs D171 (mouse IgG_1) directed against the poliovirus receptor (Nobis *et al.*, 1985), BB7.2 (mouse IgG_1) directed against HLA-A2 (Parham and Brodsky, 1981), P1B5 (mouse IgG_1) directed against human α3 integrin (Gibco BRL), VNR139 (mouse IgG_1) directed against human αv integrin (Gibco BRL), and C2 (rat IgG_1) directed against mouse transferrin receptor CD71 (Pharmingen) were purified through protein A– or protein G–agarose beads. Rabbit anti-HAV antibody was produced by immunizing animals with highly purified virions of the KRM003 strain of HAV. FITC-conjugated goat anti-human antibody (Accurate) and affinity-purified peroxidase-conjugated anti-rabbit and peroxidase-conjugated anti-mouse (KPL Laboratories) antibodies were used as recommended by the manufacturers. Normal human sera, convalescent human anti-HAV sera and ^{125}I-labeled anti-HAV antibodies were obtained from the HAVAB kit (Abbott). Biotinylated anti-HAV neutralizing mAb 813 (Accurate Labs.) (Crevat *et al.*, 1990) and FITC-labeled Avidin (Vector Laboratories) were used in immunofluorescence studies. Control mouse ascites fluid was obtained from BALB/c mice bearing the NS-1 myeloma (Sigma Chemical Company). Ascites from anti-VPg mAb (Y.Moritsugu *et al.*, unpublished results), directed against VPg protein of HAV, was produced in parallel with the anti-AGMK cell mAbs.

Human tissue culture-adapted strain HM175 of HAV (genotype 1B) was derived from infectious cDNA (Cohen *et al.*, 1987) and grown in FRhK/4 cells. Human tissue culture-adapted strain KRM003 (genotype 3B) (Totsuka and Moritsugu, 1994) and monkey wild-type strain AGM27 (genotype V) (Tsarev *et al.*, 1991) of HAV were grown in AGMK cells. The Mahoney strain of poliovirus type 1 was grown in HeLa cells. Coxsackie B viruses, echoviruses and polioviruses were obtained from ATCC.

Production and isolation of protective anti-AGMK cell mAbs

BALB/c mice were primed by the intraperitoneal injection of clone GL37 of AGMK cells (Totsuka and Moritsugu, 1994) (3×10^7 cells/injection) adjuvanted with 10^9 *Bordetella pertussis* fixed cells, boosted 3 months later in a similar fashion but without adjuvant, and killed 3 days later. Immune splenocytes were fused with Sp2/0–Ag14 cells (ATCC) and hybridomas were selected in HAT medium. mAbs capable of protecting AGMK cells against HAV infection were screened by an ELISA. Monolayers of AGMK cells grown in 96-well tissue culture plates (3×10^4 cells/well) were treated with 0.05 ml hybridoma supernatant per well at 37°C for 1 h, infected with the KRM003 strain of HAV (10^6 $TCID_{50}$/well) for 30 min at room temperature, washed three times and incubated for 3 days at 37°C. Monolayers were fixed with 80% methanol at 4°C for 1 h, and replication of HAV was detected by staining with rabbit anti-HAV antibody and horseradish peroxidase-labeled anti-rabbit antibody.

Immunochemical detection of 190/4 epitope

For the *in situ* rosette assay (Kaplan *et al.*, 1989), cell monolayers were treated with a 1:200 dilution of mAb 190/4 ascites or negative control ascites. Binding of antibody to the cell surface of live cells was detected with anti-mouse immunobeads (Bio-Rad). For a cell surface ELISA (Kaplan *et al.*, 1989), unfixed cells grown in 96-well plates were treated with dilutions of mAbs (purified or ascitic fluid) for 1 h at room temperature, washed extensively and treated with a 1:1000 dilution of affinity-purified peroxidase-labeled anti-mouse antibody for 1 h at room temperature. OPD (Sigma Chemical Company) and TMB (KPL) substrates were used as suggested by the manufacturers, and absorbance was read at 492 and 450 nm, respectively.

Immunochemical detection of HAV

HAV was titrated on 96-well plates containing subconfluent monolayers of FRhK/4 cells (Dotzauer *et al.*, 1994). 100 µl of 10-fold dilutions of HAV were applied in duplicate wells and incubated at 35°C. After 2 weeks, cells were fixed with 80% methanol and treated with ^{125}I-labeled human anti-HAV antibody. Titers were calculated as the highest dilution at which HAV could be detected by direct autoradiography of the 96-well plate.

The protection of AGMK cells against HAV infection was visualized by indirect immunofluorescence. Confluent monolayers of AGMK cells in eight-well slides (Nunc) were treated with 0.1 ml 100 µg/ml 190/4 or BB7.2 mAb diluted in PBS and 5% fetal bovine serum (FBS) for 1 h at room temperature, infected with a MOI of 1 with the tissue culture-adapted HM175 strain of HAV or mock infected for 30 min at room temperature, washed four times with EMEM–10% FBS, and incubated with the same media at 35°C in a CO_2 incubator. After 72 h p.i., cells were fixed with cold acetone and stained with a 1:1000 dilution of human anti-HAV antibody (positive control HAVAB kit) and a 1:300 dilution of FITC-labeled goat anti-human antibody (Accurate). Immunofluorescent micrographs were taken with a Zeiss Axioscope microscope at ×400 with an oil immersion objective. The protection of Lcr5 cells against HAV infection was assayed similarly, but confluent monolayers of AGMK, LDR2 and Lcr5 cells treated with mAb 190/4 or mAb BB7.2 for 1 h at room temperature were infected with a MOI of 10 with HAV for 24 h at 35°C.

The susceptibility of Ltk$^-$ cell transfectants to HAV infection was also visualized by immunofluorescence. LDR2 and Lcr5 cell monolayers in eight-well slides (Nunc) were infected with HAV at a MOI of 10 or mock infected for 72 h at 35°C, washed four times with EMEM–10% FBS, fixed with cold acetone and stained with a 1:200 dilution of biotinylated anti-HAV neutralizing mAb 813 and a 1:50 dilution of FITC-labeled avidin (Vector Laboratories). Immunofluorescent micrographs were taken as described above.

The binding of HAV to AGMK cells was quantitated by an ELISA. Confluent monolayers of AGMK cells grown in 96-well plates were treated with 4-fold dilutions of mAb 190/4, 235/4 and 263/6 ascites for 1 h at 37°C and infected with a MOI of 3000 of tissue culture-adapted strain KRM003 for 1 h at 35°C. After washing three times, cells were fixed with 80% methanol. HAV attached to the cells was stained with a 1:2000 dilution of rabbit anti-HAV antibody and horseradish peroxidase-labeled anti-rabbit antibody. The inhibition of binding was calculated with respect to the binding of HAV to AGMK cells treated with dilutions of a negative control ascites (100% binding).

The binding of HAV to AGMK, Lcr5 and LDR2 cells was also quantitated by a radioimmunoassay. Confluent monolayers of AGMK cells grown in 96-well plates were treated with 50 µl of 10-fold dilutions (1 mg/ml–100 pg/ml) of purified mAb 190/4 or control monoclonal BB7.2 for 1 h at 37°C and infected with tissue culture-adapted strain KRM003 of HAV at a MOI of 5000 $TCID_{50}$/cell for 1 h at 37°C. Monolayers were washed three times, fixed with 80% methanol, blocked with 5% bovine serum albumin in PBS, incubated with ^{125}I-labeled human anti-HAV antibody from the HAVAB kit (Abbott Laboratories), washed four times with PBS and exposed to X-ray film (XAR-2; Kodak) with an intensifying screen for 24–96 h. A densitometric analysis of the autoradiography was performed on a Macintosh Quadra950 computer using the public domain NIH Image program (written by Wayne Rasband at the US National Institutes of Health). The percentage of inhibition caused by the binding of HAV to the cell surface was calculated for each antibody dilution by comparing the density of binding of HAV to monolayers treated with mAb 190/4 with negative control antibody.

The protection of AGMK cells against infection with three different genotypes of HAV was also analyzed by a radioimmunoassay. AGMK cell monolayers in 96-well plates were treated with 50 µl (10 µg/ml) mAb 190/4 or P1B5 for 1 h at room temperature and infected with MOI of 10, 1 and 0.1 with strains HM175, KRM003 or AGM27 of HAV. After a 72 h incubation under CO_2 at 35°C, monolayers were fixed with 80% methanol, treated with 50 µl ^{125}I-labeled anti-HAV antiserum, washed extensively and autoradiographed for 12 h.

Slot blot analysis

Confluent monolayers of AGMK cells in six-well plates were treated with 1 ml (10 µg/ml) mAbs in PBS and 5% FBS for 30 min at room, washed three times and infected with a MOI of 1, 0.1 or 0.01 with the HM175 strain of HAV or PV1 for 1 h at room temperature. Monolayers were washed three times and incubated at 35°C in a CO_2 incubator. Cytoplasmic extracts were prepared at 12 h p.i. for PV1-infected cells and 72 h p.i. for HAV-infected cells. Total cell RNA was extracted with phenol–chloroform–1% SDS. Equal amounts were applied to nitrocellulose using an S&S slot blotter, baked at 80°C for 2 h and hybridized with ^{32}P-labeled HAV cDNA (Dotzauer *et al.*, 1994) or PV1 cDNA (Kaplan *et al.*, 1989).

Expression cloning of cDNA coding for epitope 190/4

Methods for constructing pDR2-based cDNA libraries and rescuing episomal DNA (Murphy *et al.*, 1992; Swirski *et al.*, 1992) were followed with very few modifications. HeLa cells, which do not express the 190/4 epitope, were transfected with the EBNA-1 gene of EBV. The single-cell clone E14C, which had a 10–100 times higher transfection efficiency than parental HeLa cells, was isolated. E14C cells were transfected with an AGMK cDNA library and transfectants were selected with 250 µg/ml hygromycin. Hygromycin-resistant cell transfectants were detached from culture flasks using 0.5 mM EDTA in PBS and panned to polystyrene Petri dishes (Aruffo and Seed, 1987; Seed and Aruffo, 1987) coated with purified 190/4 mAb. Non-adsorbed cells were removed by extensive washing. Adherent cells were dislodged by pipetting, grown in 75 cm^2 flasks and panned twice more. The expression of the 190/4 epitope at the cell surface of panned E14C cells was confirmed by an *in situ* rosette assay. Episomal DNA was extracted from a confluent monolayer of epitope 190/4-expressing E14C cells grown in a 75 cm^2 flask. *E.coli* DH10 cells were transformed with episomal DNA, and ampicillin-resistant colonies were selected. Plasmid DNA was extracted from 48 independent ampicillin-resistant colonies, digested with restriction enzymes *Bam*HI and *Xba*I, which releases the cDNA insert from the pDR2 plasmid, and analyzed by electrophoresis in a 0.8% agarose gel containing ethidium bromide. Plasmids were transfected back into E14C cells, and hygromycin-resistant cells were selected and analyzed for the expression of 190/4 epitope.

Northern blot analysis of expression of HAVcr-1

Total cell RNA (20 µg) prepared by the guanidine thiocyanate–CsCl technique (Maniatis *et al.*, 1982) from Lcr5 (Ltk$^-$ cells transfected with HAVcr-1 cDNA in pDR–GL37/5), LDR2 (Ltk$^-$ cells transfected with vector pDR2) and AGMK cells were fractionated on a 1% agarose–formaldehyde gel, stained with ethidium bromide and transferred to a nylon membrane (Zeta-probe; Bio-Rad) in 10× SSC. A full-length HAVcr-1 cDNA probe cut from pDR2–GL37/5 with *Xba*I and *Bam*HI was labeled with ^{32}P by random priming, hybridized to the membrane in 50% formamide–5× SSC at 42°C, washed with 2× SSC–0.1% SDS at 65°C, and autoradiographed for 48 h.

Acknowledgements

We thank Dr Hira Nakhasi, Dr Barry Falgout, Dr Michael Norcross, Dr Gabriela Dveksler, Dr Pravina Mattoo, Dr Marian Major and Dr Loris McVittie for helpful discussions about the manuscript. The studies in Japan were supported by a grant-in-aid from the Ministry of Health and Welfare. We thank Dr S.Emerson for providing the AGM27 strain of HAV.

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Received on February 26, 1996; revised on May 8, 1996