Identification of a cell surface 30 kDa protein as a candidate receptor for Hantaan virus

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Cellular receptors play an important role in virus pathogenesis. As a first step in virus infection, viruses attach to specific receptors on the surface of cells; Hantaan virus infects susceptible cells by attaching to a receptor located on the cell surface. To date, the identity of the Hantaan virus host cell receptor remains unknown. To determine the protein on the cell surface to which Hantaan virus binds, a virus overlay protein-binding assay was performed with radiolabelled virus. A 30 kDa (30K) protein was identified as a putative receptor for Hantaan virus. The specificity of virus interactions with this protein was demonstrated with a competition assay using unlabelled Hantaan virus and poliovirus. Unlabelled Hantaan virus inhibited the binding of radiolabelled Hantaan virus to this 30 kDa protein, whereas poliovirus did not. A polyclonal antibody against the 30K protein blocked binding of Hantaan virus to Vero-E6 cells and, consequently, virus infection. Blocking with the anti-30K antibody reduced virus infection of cells by 70%. These data strongly suggest that the 30 kDa surface protein is a putative receptor for Hantaan virus.

Introduction

Hantaan virus, a member of the family Bunyaviridae, is an enzootic virus of wild rodents and causes persistent infection in natural hosts. The virus is spread by aerosolized excreta and not by insect vectors. Human infections occur under conditions of close contact with the rodent host (Lee et al., 1981). The major natural host of Hantaan virus, Apodemus agrarius (striped field mouse), develops an asymptomatic persistent infection and the virus is excreted in urine for months, possibly even years. Hantaan virus is a causative agent of haemorrhagic fever with renal syndrome (HFRS) in humans. The disease is an acute, prostrating febrile illness in which approximately one-third of patients develop haemorrhagic manifestations, with between 10 and 15% of patients exhibiting shock. The mortality rate due to this infection is between 5 and 10% (Cohen, 1982). Virions range from 80 to 120 nm in size (mainly between 92 and 105 nm). Virus particles are spherical or pleomorphic, depending on the method used for fixation (Ito et al., 1979; Martin et al., 1985; Lee, 1982). Hantaan virus contains a single-stranded RNA genome of negative polarity that is divided into three segments: large (L), medium (M) and small (S). L, M and S RNA segments encode a large (L) protein (a viral RNA polymerase), glycoproteins (G1 and G2) and a nucleocapsid (N) protein, respectively.

In order to multiply, a virus must first infect a cell. The host range of a particular virus defines both the type of tissue that the virus infects and the animal species in which it multiplies. To infect a cell, the virion must attach to the cell surface, penetrate the cell and become sufficiently uncoated to make its genome accessible to the virus or host machinery for transcription and translation. The attachment step constitutes specific binding of a viral protein (anti-receptor) to a constituent of the cell surface (receptor). Cellular receptors play an important role in virus pathogenesis. Specific initial interactions with cells largely determine the host range specificities and tissue tropisms of viruses (Fields & Greene, 1982).

The basis for cell and tissue tropism of viruses is often related to the ability of virus attachment proteins (VAPs) to bind a specific cell receptor. Virus binding is followed by membrane fusion, leading to productive infection in susceptible hosts. The envelope glycoproteins (G1 and G2) of members of the family Bunyaviridae (including Hantaan virus) are the major structural proteins exposed on the surface and have been identified as bunyavirus VAPs (Gonzalez et al., 1982; Grady et al., 1983; Kingford et al., 1983). It is presumed that the corresponding envelope proteins (G1 and G2) of Hantaan
Methods

**Cells and virus.** Vero-E6 cells (ATCC, #CRL-1586) were grown at 37 °C in Dulbecco’s modified minimal essential medium (DMEM) supplemented with non-essential amino acids, vitamins, 10% foetal bovine serum (FBS), 2 mM l-glutamine, 50 µg/ml streptomycin, 50 U/ml penicillin and 20 mM HEPES buffer.

Hantaan virus strain 76-118 was propagated in Vero-E6 cells, as described previously (Elliott et al., 1984).

**Preparation of labelled Hantaan virus.** Radiolabelled Hantaan virus was obtained from infected Vero-E6 cells. Briefly, 1.5 × 10⁵ cells were infected at an m.o.i. of 0.01 p.f.u. per cell. At day 8 post-infection, the medium was replaced with methionine-free DMEM containing 5 mCi [³⁵S]methionine (DuPont) and 10% FBS. Cells were then incubated at 37 °C. After 2 days, the supernatant was obtained by centrifugation at 200 g for 10 min and layered onto a 20% sucrose cushion for further centrifugation at 80 000 g for 2 h. Pellets were suspended in TNE buffer (50 mM Tris–HCl, 150 mM NaCl and 5 mM EDTA, pH 7.4) and incubated overnight at 4 °C. The virus suspension was layered onto a 20–60% sucrose gradient and centrifuged at 80 000 g for 3 h. The opaque band containing virus particles was isolated and dialysed overnight against TNE buffer at 4 °C. Virus material was pelleted at 100 000 g for 1 h and stored at −70 °C in TNE buffer. The virus titre was determined with a focus-forming assay using Vero-E6 cells, as described previously (Kariwa et al., 1994). As a control, a labelled sample from uninfected cells was prepared using the same protocol as that described above.

**Binding assay.** Direct binding assays were performed to characterize the attachment of Hantaan virus to Vero-E6 cells. For these studies, 12-well plates with 1 × 10⁵ Vero-E6 cells per well were placed at 4 °C for 2 h before incubation with different amounts of radiolabelled Hantaan virus. The experiment was performed at 4 °C to avoid virus penetration of cells. After 2 h, the medium was removed, cells were washed twice with fresh medium and lysed with lysis buffer (10 mM PBS, 150 mM NaCl, 1% Triton X-100 and 0.1% SDS) (Anderson et al., 1992). Radioactivity in both the medium and cells was measured in a scintillation counter. Assays were conducted in triplicate. For calculation of non-specific binding in the saturation experiment, cells were preincubated with different concentrations of unlabelled Hantaan virus prior to the addition of 8 × 10⁶ c.p.m. of labelled Hantaan virus. Binding assays were performed twice. Each point was determined in duplicate and values from independent experiments varied from 3 to 5% from the average.

For trypsin treatment, 5 × 10⁴ Vero-E6 cells were incubated with a 0.075% solution of trypsin for 30 min at 37 °C (Daughaday et al., 1981), followed by incubation for 45 min in fresh medium supplemented with 10% FBS to inactivate the trypsin. Finally, cell monolayers were washed twice with fresh medium and a binding assay was performed, as described above.

**Preparation of cell membrane protein.** To obtain cell membrane proteins, phase partitioning with Triton X-114 was performed (Bordier, 1981). Vero-E6 cells were washed three times with TBS (10 mM Tris–HCl and 150 mM NaCl, pH 7.5). Cells were lysed in 4 cell pellet vols of 1% Triton X-114 in TBS in the presence of the protease inhibitor cocktail (Sigma) at 4 °C and centrifuged at 4000 g for 30 min. The supernatant was incubated overnight at −20 °C and for 10 min at 37 °C and centrifuged at 60 g for a further 15 min. The pellet was resuspended in the same volume of TBS in the presence of protease inhibitors and incubated at 4 °C for 30 min. To ensure efficient recovery of membrane proteins, the procedure was repeated, starting with incubation at 37 °C. Acetone precipitation was performed to eliminate detergent and the protein was quantified by the Micro BCA method (Bio-Rad).

Trypsin treatment was performed as described above, with an increased incubation time of 1 h. Following incubation, cells were washed three times with cold PBS and cell extracts were prepared as described above.

**Virus overlay protein-binding (VOPB) assay.** To identify cell polyepitides involved in virus binding, a VOPB assay was carried out. This assay was performed as described by Jin et al. (1994), Ludvig et al. (1996), Salas-Benito & del Angel (1997) and Crane et al. (1991), with some modifications. Briefly, 200 µg total or membrane proteins from the cell line were subjected to SDS–PAGE and transferred onto nitrocellulose membranes using a semi-dry blotting apparatus in transfer buffer (48 mM Tris, 29 mM glycine and 20% methanol) (Smith & Wright, 1985). After overnight renaturation of transferred proteins with 4% BSA in PBS at 4 °C, membranes were blocked for 1 h at room temperature with 5% skimmed milk (Gibco-BRL) in PBS and re-washed three times with PBS. Membranes were incubated overnight with radiolabelled (1 × 10⁶ c.p.m.) Hantaan virus in MEM supplemented with 10% FBS at room temperature with gentle rocking. After washing four times for 15 min with 2% BSA in PBS and once with 0.1% Nonidet P-40 in PBS at room temperature, membranes were dried and autoradiographed.

To determine the specificity of the virus–cell protein interactions, VOPB assays were performed under conditions of high salt concentrations. Briefly, before incubation with virus, membranes were washed once for 5 min with PBS and 1% skimmed milk and once in high-salt washing solution (PBS, 1% skimmed milk and 220 mM NaCl). Incubation with virus was performed under the same conditions as described above but in the presence of high-salt washing solution. Finally, membranes were washed three times with high-salt solution prior to exposure to X-ray film (Marianneau et al., 1996).

**Protein purification.** Total cell membrane proteins were separated by 1–20% non-reducing SDS–PAGE. A protein with a molecular mass of 30 kDa was electroeluted from the gel at 140 V for 20 min in electrophoresis apparatus (Whole Gel Eluter, Bio-Rad). Protein concentrations were estimated with the Micro BCA method (Bio-Rad).

**Production of polyclonal antibody.** BALB/c mice were immunized five times intraperitoneally with 20 µg soluble 30 kDa (30K) protein obtained by electroelution, emulsified in Freund’s complete adjuvant for primary immunization and in Freund’s incomplete adjuvant
Results and Discussion

Hantaan virus specifically binds Vero-E6 cells

To determine the receptor–ligand interactions during attachment of Hantaan virus to Vero-E6 cells, a series of virus-binding experiments was performed. Initially, constant numbers of cells were incubated with different concentrations of $^{35}$S-labelled Hantaan virus (Fig. 1a). Bound virus was quantified in triplicate and counts were averaged. The counts of cell-bound virus increased in proportion to virus input and then reached a plateau, indicating saturation of cellular binding sites. Therefore, binding of Hantaan virus to Vero-E6 cells was dose-dependent and saturable. To determine the specificity of cell–virus binding, competition experiments were performed using unlabelled Hantaan virus (Fig. 1b). Different amounts of unlabelled Hantaan virus were preincubated with Vero-E6 cells, followed by the addition of labelled virus. After the incubation of cells with unlabelled virus, dose-dependent binding was observed, suggesting specific interactions between Hantaan virus and Vero-E6 cells. A control sample was obtained from uninfected cells using the same protocol employed to purify labelled virus. Protein was not detected in this sample and the maximum radioactivity observed (50 c.p.m.) was comparable to the background buffer value (30 c.p.m.). This minute increment (20 c.p.m.) may be due to traces of protein from culture supernatant or media containing the isotope. Since the radioactivity in the control was low enough to be discounted, we assume that the numbers generated were due to inhibition of binding only. Our results show that Hantaan virus binds Vero-E6 cells in a dose-dependent and saturable manner characteristic of specific receptor–ligand interactions, suggesting the presence of a specific receptor for the virus on the surface of Vero-E6 cells.

A 30K protein on the surface of Vero-E6 cells is a Hantaan virus-binding protein

To determine the molecule(s) on Vero-E6 cells that bind Hantaan virus, $[^{35}]$S)methionine-labelled viruses were incubated with proteins, subjected to SDS–PAGE and transferred onto nitrocellulose membranes. Under isotonic conditions, labelled Hantaan virus bound a protein of approximately 30 kDa from Vero-E6 cell lysates (Fig. 2a, lane 2). When labelled proteins
from uninfected cells were incubated with transferrred proteins from Vero-E6 cells, no reaction was observed (Fig. 2a, lane 1). Moreover, the presence (Fig. 2b, lane 1) or absence (Fig. 2b, lane 2) of β-mercaptoethanol did not alter the binding pattern. More stringent conditions with 220 mM NaCl to reduce non-specific binding (Fig. 2c, lane 1) had no effect on virus binding. These results suggest that the 30K membrane protein (which does not appear to consist of disulfide-linked subunits) specifically binds Hantaan virus. No additional protein bands that bound Hantaan virus were identified.

**Binding of Hantaan virus to the 30K protein is specific**

The specificity of virus binding to the 30K protein was demonstrated by competition assays with unlabelled Hantaan virus and poliovirus. The overlay mixture was incubated with 0, 560 or 280 µg of unlabelled Hantaan virus (Fig. 3a, lanes 1–3) or with 280 µg (lane 4) of poliovirus before incubation with labelled Hantaan virus. Unlabelled Hantaan virus competed with labelled virus for binding in contrast to poliovirus, indicating specific interactions between Hantaan virus and the 30K protein. To analyse further the specificity of the virus–30K protein interactions, a binding assay was conducted; this assay involved preincubation of labelled Hantaan virus with the electroleulated 30K protein. The observed reduction in the binding of labelled Hantaan virus to Vero-E6 cells was dose-dependent on the concentration of the protein used (Fig. 3b). Preincubation of Hantaan virus with soluble 30K protein prevented infection of Vero-E6 cells by 60% (Fig. 3c). The specificity of the virus interactions with the 30K protein was demonstrated further by competition assays with unlabelled Hantaan virus and poliovirus. Unlabelled Hantaan virus inhibited binding of the labelled virus to the 30K protein, whereas poliovirus did not.

To characterize the 30K protein, we purified the protein, in soluble form, by gel electrodution from cell membrane proteins. The soluble protein specifically bound Hantaan virus in solution and inhibited virus infection of Vero-E6 cells. Our results indicate that the 30K protein is involved in virus infection.

**Antibodies against the 30K protein block Hantaan virus binding**

To investigate the particular function of the 30K protein in virus infection, a polyclonal antibody obtained after immunization of mice with the electroleulated 30K protein was generated. This antibody was employed to detect the protein in Western blot assays (Fig. 4a, lane 2). No bands were observed after incubation with preimmune serum (Fig. 4a, lane 1). To determine whether the antibodies recognized the same protein that bound Hantaan virus, a binding assay was performed. Polyclonal serum against the 30K protein blocked the binding of labelled virus to Vero-E6 cells by 50%, while preimmune serum did not affect virus binding (Fig. 4b). To analyse whether the antibody inhibited virus binding to the
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30K protein in cells, a blocking assay with anti-30K protein antibody was performed. Preincubation of Vero-E6 cells with anti-30K protein antibody inhibited Hantaan virus infection of cells by a maximum of 70% (Fig. 4c), compared to an average of 60%. In contrast, preimmune serum did not inhibit virus infection. These results collectively suggest that the 30K protein plays a role as a Hantaan virus receptor.

A number of viruses use more than one distinct attachment receptor. For example, human immunodeficiency virus (HIV) uses CD4 as its main attachment receptor but also interacts with glycolipid galactosyl ceramide to mediate infection. The polyclonal anti-30K antibody blocked virus binding to the 30K protein of Vero-E6 cells and, consequently, virus infection of the cell; blocking with the anti-30K antibody substantially decreased virus infection (by 70%). However, upon addition of excess antibody, 100% reduction was not observed. Therefore, it is possible that Hantaan virus attachment and infection is mediated by yet another unidentified receptor, albeit inefficiently. Alternatively, another explanation for this result is that Hantaan virus attaches to a subset of surface molecules that are not capable of mediating Hantaan virus infection.

The 30K protein is located on the surface of Vero-E6 cells

To examine the location of the 30K protein within the cell, Vero-E6 cells were preincubated with trypsin and VOPB and binding assays were then performed. VOPB assays with membrane proteins of Vero-E6 cells treated with trypsin demonstrated that trypsinization removed the 30K protein from the surface of Vero-E6 cells (Fig. 5a, lane 2). Trypsin digestion for 30 min reduced binding by 80% after 1 h of incubation with labelled viruses (Fig. 5b), indicating a peptidic and surface nature of the 30K protein. However, after 3 h of incubation, an increase in binding was observed (c.p.m. values increased by up to twofold).
the 30K protein on the cell membrane. Trypsin treatment of the cells inhibited virus binding. The susceptibility of the virus-binding protein to protease treatment was confirmed in these assays, where the addition of trypsin prevented recognition of the 30K proteins by labelled Hantaan virus. The presence of the 30K protein on the surface of Vero-E6 cells and the observation that electroeluted protein and its antibody inhibit Hantaan virus binding strongly support the theory that the protein is a putative receptor, or part of a receptor complex, for Hantaan virus.

In conclusion, we demonstrate that binding and infection of cells by Hantaan virus is sensitive to (i) competitive inhibition with a soluble electroleut 30K putative receptor protein, (ii) enzymatic removal of this protein from the cell surface and (iii) blockage with an antibody to the 30K protein.

The cellular entry of hantaviruses constitutes specific interactions between the virus and cells mediated by $\beta_3$ integrins (Gavrilovskaya et al., 1998, 1999). However, there is no direct evidence that Hantaan virus binds to $\beta_3$ integrins on the cell surface. According to Gavrilovskaya et al. (1999), infection of cells by HFRS-causing hantaviruses (including Hantaan virus) was partially inhibited by antibodies to $\beta_3$ integrin and the integrin ligand vitronectin. As stated above, it is possible that additional interactions exist between the virus and the host cells. It is currently unclear whether $\beta_3$ integrin is a virus-binding protein (or site) on cells or whether it is involved in the internalization of virus only. Our data indicate that it is unlikely that $\beta_3$ integrin is related to the 30K protein. The two proteins differ considerably in terms of relative molecular mass ($\beta_3$ integrin has a molecular mass of about 105 kDa). Moreover, in additional experiments performed in our laboratory, the binding of the 30K protein to its virus counterpart was not inhibited by either vitronectin or fibronectin (ligands for integrin) and did not interact with a monoclonal antibody to $\beta_3$ integrin. We are currently in the process of sequencing the 30K protein, following which, its relationship with $\beta_3$ integrin will be ascertained.

A wide variety of cell surface molecules serve as virus attachment receptors. Such receptors range from cell-specific transmembrane proteins of well-defined receptor superfamilies (such as CD4 as a receptor for HIV) to the more ubiquitous cell surface-associated carbohydrate moieties (the common carbohydrate moiety sialic acid for influenza virus). To date, the mechanism by which Hantaan virus attaches to its host cell has not been elucidated. Despite growing interest in the putative receptor for this virus, the events that govern the initial attachment of Hantaan virus remain poorly understood.

We aim to completely characterize the DNA and amino acid sequences of the novel 30K protein to confirm that it is a Hantaan virus receptor. Structural nuclear magnetic resonance or X-ray crystallography studies may be required to facilitate the characterization of protein–protein interactions between Hantaan virus and the cellular receptor. If the 30K protein is indeed a specific receptor for Hantaan virus, it would be of interest to determine its distribution in susceptible and resistant cell lines. Further characterization and sequencing of the receptor protein will inevitably aid in determining its normal cellular functions and understanding its interactions with Hantaan virus.

References


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