Virology 381 (2008) 178–183

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

A hantavirus causing hemorrhagic fever with renal syndrome requires gC1qR/p32 for efficient cell binding and infection

Yun Choi [b,1], Young-Chan Kwon [a,1], Soo-In Kim [a,b], Jung-Min Park [b], Kyung-Hee Lee [a], Byung-Yoon Ahn [a,*]

[a] School of Life Sciences and Biotechnology, Korea University, 5-1 Anamdong, Seoul, 136-701, Republic of Korea
[b] Mogam Research Institute, 341 Pojungdong, Yongin, 449-910, Republic of Korea

ARTICLE INFO

Article history:
Received 29 April 2008
Returned to author for revision 9 June 2008
Accepted 19 August 2008
Available online 2 October 2008

Keywords:
Hantavirus
gC1qR/p32
Viral binding
Infection

ABSTRACT

Hantaan virus (HTNV) is a pathogenic hantavirus that causes hemorrhagic fever with renal syndrome (HFRS). HTNV infection is mediated by αvβ3 integrin. We used protein blots of Vero E6 cell homogenates to demonstrate that radiolabeled HTNV virions bind to gC1qR/p32, the acidic 32-kDa protein known as the receptor for the globular head domain of complement C1q. RNAi-mediated suppression of gC1qR/p32 markedly reduced HTNV binding and infection in human lung epithelial A549 cells. Conversely, transient expression of either simian or human gC1qR/p32 rendered non-permissive CHO cells susceptible to HTNV infection. These results suggest an important role for gC1qR/p32 in HTNV infection and pathogenesis.

Introduction

Hantaan virus (HTNV) is a highly pathogenic hantavirus that causes hemorrhagic fever with renal syndrome (HFRS) in humans (Schmaljohn and Nichol, 2007). Transmission of HTNV to humans occurs by inhalation of the aerosolized excreta of infected rodents. Pathogenic features of the disease include generalized capillary damage leading to drastic alteration in the permeability of the vascular endothelium (Klein and Calisher, 2007). HTNV does not lyse endothelial cells, so it is unclear how HTNV infection changes vascular permeability.

The in vitro study of HTNV replication is limited to a few cultured cell lines (French et al., 1981), and the reason for this restriction remains unknown. HTNV utilizes integrin $\alpha v\beta_3$ during the cell entry process (Gavrilovskaya et al., 1999). Recently, the GPI-anchored protein DAF/CD55 was shown to mediate HTNV entry across the apical membrane of polarized epithelial cells (Krautkramer and Zeier, 2008). Other cellular proteins have also been implicated in HTNV entry (Kim et al., 2002; Mou et al., 2006).

The protein gC1qR/p32 (also called p33 or HABP-1) is a 32-kDa glycoprotein that was initially shown to bind the globular "head" domain of the complement protein C1q (Peerschke et al., 1994). Expressed in many cell types including endothelial cells, dendritic cells, lymphocytes, and platelets, the receptor shows affinity for a broad range of plasma and cellular proteins, including thrombin, vitronectin, high molecular weight kininogen, and hyaluronic acids (Deb and Datta, 1996; Ghebrehiwet and Peerschke, 1998; Herwald et al., 1996). The gC1qR/p32 protein also reportedly interacts with a number of viral proteins, such as the hepatitis C viral core, the rubella viral capsid, IE63 of the herpes simplex virus type-1, EBNA1 of Epstein–Barr virus, adenovirus polypeptide V, and Rev and Tat of HIV-1 (Ghebrehiwet and Peerschke, 2004). The gC1qR/p32 protein is also utilized for the attachment or entry of microbial pathogens such as *Listeria monocytogenes*, *Staphyloccus aureus*, and *Plasmodium falciparum* (Braun et al., 2000; Nguyen et al., 2000; Biswas et al., 2007).

In the present study, we demonstrate that gC1qR binds to HTNV and mediates viral infection in cell culture.

Results

HTNV binding of gC1qR/p32

In order to identify the cellular proteins that interact with HTNV, membrane proteins were isolated from Vero E6 cells, separated by denaturing gel electrophoresis, blotted onto a nitrocellulose membrane, and incubated in a solution of [^{35}S]-labeled HTNV virions. A protein band corresponding to a molecular weight of ~32 kDa was visualized by autofluorography (Fig. 1A). The exact identities of the proteins forming this band could not be determined from this sample due to insufficient material. More material was resolved using a 2-D gel electrophoresis technique, and the virion-binding assay was repeated. A spot corresponding to a molecular weight of ~32 kDa with a pI ~4.7 was visualized by autofluorography (Fig. 1B). The gel was stained, and the 32-kDa protein was extracted, trypsinized, and subjected to MS/MS analysis. Sequencing of a unique peptide fragment

* Corresponding author. School of Life Sciences and Biotechnology, Korea University, 5-1 Anamdong, Seoul, 136-701, Republic of Korea. Fax: +82 2 923 9923.
E-mail address: ahnbyung@korea.ac.kr (B.-Y. Ahn).
[1] Authors who contributed equally to this work.

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doi:10.1016/j.virol.2008.08.035

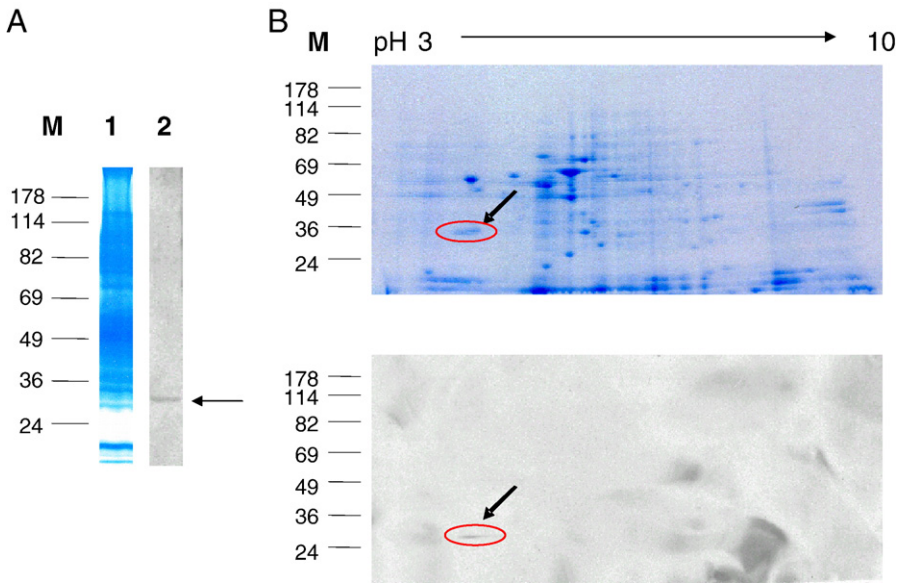

Fig. 1. Identification of HTNV binding proteins. (A) Vero E6 cell membrane proteins (~40 μg) were resolved by electrophoresis on a denaturing 4–20% polyacrylamide gel. The Coomassie Blue-stained gel (lane 1) and the autofluorograph of the nitrocellulose membrane strips probed with [^{35}S]-methionine-labeled HTNV (lane 2) are shown with protein size markers on the left. (B) Cell membrane proteins (~185 μg) were resolved by isoelectrofocusing followed by denaturing gel electrophoresis. The Coomassie Blue-stained gel (upper panel) and an autofluorograph of a virus-bound blot (lower panel) are shown. The position of the 32-kDa HTNV-bound protein is indicated.

(^{208}EVSFQSSGESEWK^{220}) identified the protein as gC1qR/p32, a highly acidic and ubiquitous protein known as the receptor for the globular "head" domain of complement protein C1q. We confirmed the results of the virion-binding assay by repeating the 2-D gel separation and then immunoblotting the gel with a gC1qR/p32 antibody. The antibody specifically recognized the 32-kDa protein in the 2-D gel blot at the corresponding position of the virion-binding assay (data not shown). We also confirmed that the gC1qR/p32 protein in the blot does not bind [^{35}S]-labeled mock fractions prepared from uninfected cells.

Roles of gC1qR/p32 for HTNV infection

In order to investigate the interaction between HTNV and gC1qR/p32 during the viral infection process, we used siRNA to suppress gC1qR/p32 expression. We have chosen human lung epithelial A549 cells for this experiment since respiratory epithelium is the primary target of HTNV and this cell line supports HTNV infection as efficiently as Vero E6 cells in vitro. Two independent cell clones were established, each stably expressing siRNA targeting the gC1qR/p32 mRNA sequence at either the junction of exons 1 and 2 or exon 3. Viral binding was tested by incubating pre-chilled cells with HTNV at a multiplicity of infection (MOI) of 1 for 2 h at 4 °C. After several washes, the extent of viral adsorption was subsequently assessed by immunoblotting for the viral nucleocapsid N protein. A substantial amount of virus remained

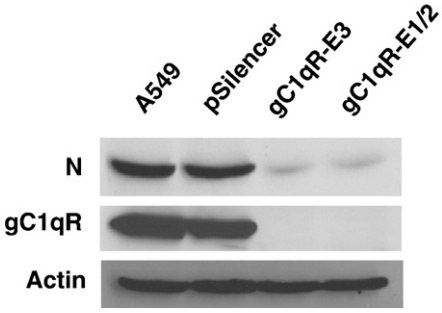

Fig. 2. HTNV binding of gC1qR/p32-siRNA cells. Human A549 cells expressing gC1qR/p32 siRNA (targeting exon 3 or the junction of exon 1 and 2) were pre-chilled and incubated with HTNV (MOI=1) for 2 h at 4 °C in serum-free medium. After washing several times at 4 °C, cell-bound virus was quantitated by immunoblotting of viral N protein. Also probed were gC1qR/p32, and actin as a loading control. Cells expressing either no siRNA or an unrelated siRNA (pSilencer) were also analyzed for comparison.

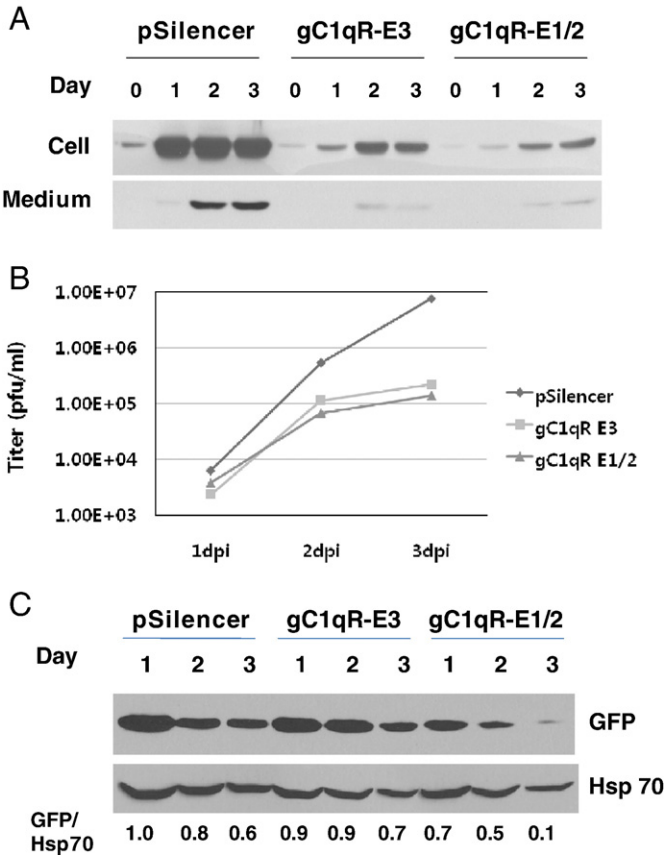

Fig. 3. HTNV infection of gC1qR/p32-siRNA cells. (A) N protein levels in HTNV-infected A549 cells (MOI=1) and in the culture medium were determined by immunoblotting at the times indicated. (B) HTNV titer in the culture medium at the times indicated was determined by plaque assay with Vero E6 cells. (C) Infection of NDV in A549 cells (MOI=10) was determined by immunoblotting of cell lysate for recombinant NDV-encoded GFP expression. Hsp70 expression was used as a control.

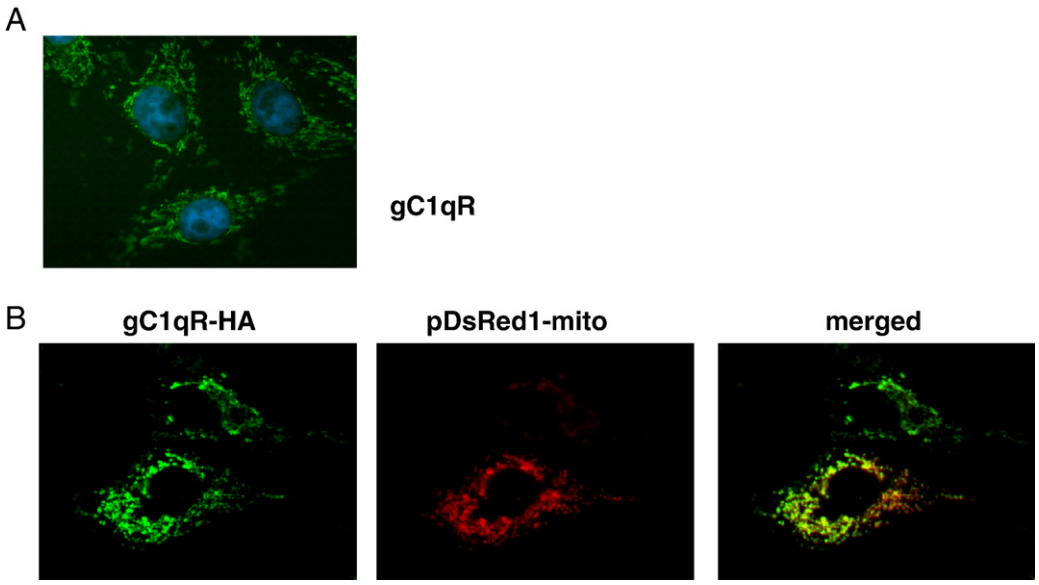

Fig. 4. Immunostaining of gC1qR/p32. (A) Vero E6 cells fixed with paraformaldehyde were incubated with gC1qR/p32 antibody followed by FITC-conjugated secondary antibody. The cells were stained with 0.1 μg/ml of DAPI and observed under a fluorescence microscope. (B) Vero E6 cells co-transfected with human gC1qR/p32 and pDsRed1-mito were probed with anti-HA antibody and FITC-conjugated secondary antibody. The two images are combined on the right.

bound to the surfaces of both normal A549 cells and control cells expressing an unrelated siRNA. In contrast, viral binding was barely detectable on the surface of both cell lines expressing gC1qR/p32 siRNA (Fig. 2). Expression of gC1qR/p32 was almost completely suppressed in those cells while that of actin was unaffected.

In order to further investigate the role of gC1qR/p32 in HTNV infection, control A549 cells and the two cell lines expressing gC1qR/p32 siRNA were infected with HTNV. A rapid synthesis of N protein was observed in the control cells after 24 h. In contrast, the levels of N protein in siRNA-expressing cell lines were ~5% those of control cells (Fig. 3A). The viral titer in the culture media of the siRNA-expressing cell lines was lower than that of control cells by ~10-fold at 48 h and ~100-fold at 76 h, respectively (Fig. 3B). These results demonstrate that gC1qR/p32 is required for efficient HTNV infection.

To estimate the specificity of gC1qR/p32 for HTNV, we tested the infection of New Castle Disease Virus in the A549 cells carrying gC1qR/p32 siRNA. NDV infection assessed by the expression of recombinant virus-encoded GFP (Park et al., 2003) was not affected by gC1qR/p32 knockdown (Fig. 3C). It is of note that HTNV binding of the immobilized gC1qR/p32 was not affected by co-incubation with polio virus preparation in our previous study (Kim et al., 2002).

Cellular distribution of gC1qR/p32

Present in a wide range of cell types including lymphocytes, neutrophils, hepatocytes, and endothelial cells, gC1qR/p32 has been found in mitochondria, in the nucleus, at the cell membrane, and in the extracellular matrix (Ghebrehiwet et al., 1995; Peterson et al., 1997). Cellular distribution of gC1qR/p32 in Vero E6 cells was examined by immunofluorescence assay. The assay showed the pattern typical of mitochondrial localization for the endogenous gC1qR/p32 (Fig. 4A), as reported by other investigators (Dedio et al., 1998). Vero E6 cells transfected with a full-length human gC1qR/p32 cDNA also showed a pattern of mitochondrial localization of the recombinant protein, virtually overlapped with that of the co-transfected marker protein, pDsRed1-mito (Fig. 4B). These results indicate that the majority of gC1qR/p32 is localized in the mitochondria. In order to assess the distribution of gC1qR/p32 on the cell surface Vero E6 cells were treated with sulfo-NHS-LC-biotin, a membrane-impermeable compound that attaches biotin moieties to accessible lysine residues on the cell surface. Biotinylated proteins were extracted, affinity-purified, and immunoblotted with antibody against gC1qR/p32. Biotin-labeled gC1qR/p32 was detected in the affinity-purified fraction, whereas no gC1qR/p32 was purified from unlabeled cells. From the intensity of bands affinity-purified gC1qR/p32 was estimated to be ~2% of the total gC1qR/p32 present in the cell lysate (Fig. 5).

Expression of gC1qR/p32 in CHO cells

The effect of gC1qR/p32 expression on viral infection was investigated through transient expression of heterologous gC1qR/p32 in HTNV-non-permissive CHO cells. CHO cells were transfected with either simian or human gC1qR/p32 constructs, incubated for 24 h, and infected with HTNV. At 48 h post-infection, cells expressing either the human or the simian gC1qR/p32 constructs produced 36 to 50% more N protein than control cells (Fig. 6A). Viral titer in the culture media of gC1qR/p32-expressing cells at 48 h post-infection

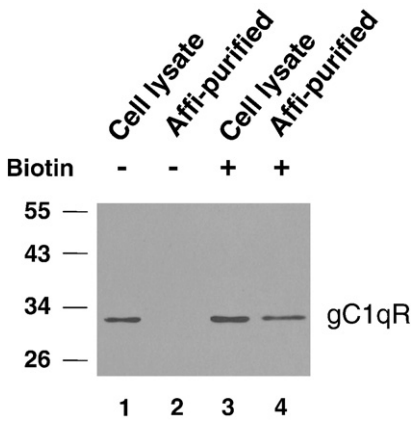

Fig. 5. Biotinylation of gC1qR/p32. Biotin-labeled Vero E6 cells were lysed and membrane proteins were extracted and affinity-purified with streptavidin agarose beads. Eluted proteins were separated by gel electrophoresis and immunoblotted with gC1qR/p32 antibody (lane 4). Proteins from cells without biotin labeling were affinity-purified as a control (lane 2). Cell lysate prior to the affinity step (~5% of total lysate) was loaded for comparison: unlabeled (lanes 1); biotin-labeled (lane 3).

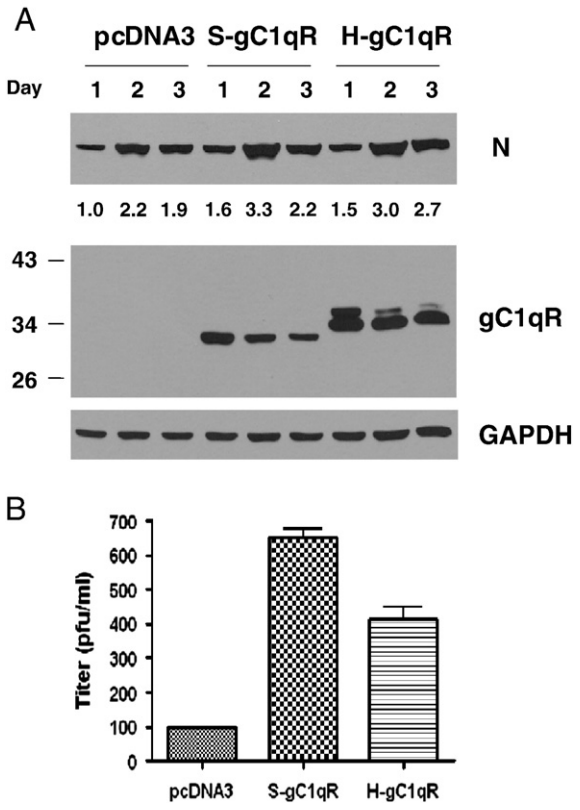

Fig. 6. HTNV infection of CHO cells. (A) CHO cells transfected with simian (S) or human (H) gC1qR/p32 were infected with HTNV (MOI=1), and cell-associated N protein was analyzed by immunoblotting at the times indicated. Transiently expressed gC1qR/p32 was also measured by immunoblotting with anti-HA antibody. Cellular GAPDH was used as a loading control. (B) HTNV titer in the culture medium at 48 h post-infection was determined by plaque assay.

was 4 to 7-fold higher than that of control cells (Fig. 6B). Thus, expression of simian or human gC1qR/p32 rendered CHO cells susceptible to HTNV infection. However, despite gC1qR/p32 expression N protein expression in CHO cells was transient: it increased on day 2 but declined on day 3. Viral titer in the culture media also peaked on day 2. This result reflects the non-permissive nature of CHO cells for HTNV replication. The effect of gC1qR/p32 expression was more significant in the early stage of infection, probably in the viral uptake process. We observed a similar effect in CHO cells stably expressing gC1qR/p32 (data not shown), further suggesting that this is not due to the transient expression of gC1qR/p32 in this cell.

Discussion

In this study, we demonstrated that efficient HTNV binding and infection depends on gC1qR/p32 expression. To our knowledge, this is the first investigation to report gC1qR/p32 binding to intact virions of enveloped viruses. In the previous study we reported the binding of radiolabeled HTNV virions with a ~30-kDa Vero E6 cell protein (Kim et al., 2002). Although specificity of the virus and cell interaction was demonstrated in the study, we were unable to determine the identity of the protein due to insufficient materials and limited resolution of regular gel electrophoresis. In the present study, we identify that the ~30-kDa protein is the gC1qR/p32 through MS/MS analysis of a 2-D gel and immunoblotting with a specific antibody.

Our immunostaining showed that gC1qR/p32 is mainly localized in the cytoplasm, particularly in the mitochondria. However, the result of our biotin labeling was consistent with previous investigations showing localization of some gC1qR/p32 on the cell surface (Eggleton et al., 1995; Gupta et al., 1991). Although gC1qR/p32 has no transmembrane domain, it is thought to locate on the cell surface through interactions with other membrane-bound proteins or components. For example, the lateral association of gC1qR/p32 with β_1 integrin has been suggested in C1q-mediated cell adhesion and spreading (Feng et al., 2002).

X-ray crystallography studies indicated that gC1qR/p32 protein possesses a trimeric doughnut-shaped structure with a central channel approximately 20 Å in diameter (Jiang et al., 1999). The highly acidic N-terminus of gC1qR/p32 contains a binding site for C1q and mediates binding of the heparin-binding form of vitronectin (Lim et al., 1996). Our attempt to block HTNV binding of gC1qR/p32 with soluble C1q or gC1qR/p32 antibodies (such as clone 60.11, which binds aa 76–93 or clone 74.5.2, which binds aa 203–218 of gC1qR/p32) did not inhibit HTNV infection (data not shown). Although these results indicate that the HTNV binding site is distinct from that of C1q binding, elucidation of the gC1qR/p32 domains responsible for HTNV binding and viral proteins involved requires further study.

Virus binding to the cell surface may also trigger signaling cascades in the host cell. Hepatitis C virus core protein has been proposed to modulate host immune responses by binding to gC1qR/p32 expressed on T-cells and activating the ERK/MEK signaling pathway (Yao et al., 2001). Given the hemorrhagic characteristic of HTNV infection, it is tempting to speculate that the binding of HTNV to gC1qR/p32 is involved in dysregulation of the complement pathway. Although our results demonstrate that gC1qR/p32 mediates HTNV binding and infection, whether and how the protein is involved in the pathogenesis of HFRS remains a challenging issue.

Materials and methods

Cell and virus samples

African green monkey kidney Vero E6 cells (CRL 1586) were grown in DMEM (Sigma). Human lung carcinoma A549 (CCL 185) and Chinese hamster ovary (CHO) (CRL 9096) cells were grown in RPMI 1640 (Sigma), supplemented with 10% FBS, 100 μg/ml streptomycin, and 100 U/ml penicillin (Invitrogen). For viral infections, cells were incubated in the presence of HTNV (76–118) in serum-free medium for 1.5–2 h, after which the medium was replaced with fresh medium containing 10% FBS. Viral titer was measured by plaque assay using Vero E6 cells (Kang et al., 1999). HTNV virions were labeled with [^{35}S]-methionine and purified from culture media by sucrose gradient centrifugation as previously described (Kim et al., 2002).

Viral binding to cellular protein blots

Vero E6 cells (~1.5×10^8) were harvested and lysed with 15 strokes of a Dounce homogenizer at 4 °C in 10 ml of buffer [20 mM Tris–HCl (pH 7.5), 100 mM NaCl, 10 mM KCl, 1.5 mM $MgCl_2$, 10 mM HEPES, and 1% protease inhibitor cocktail (Roche)]. After removal of cell nuclei by centrifugation at 100 g for 5 min, the microsomal and membrane fractions were separated by centrifugation at 20,000 *g* for 30 min at 4 °C. The resulting pellet was resuspended in ReadyPrep rehydration/sample buffer (Bio-Rad) (8 M urea, 2% CHAPS, 50 mM DTT, and 0.2% Bio-Lyte 3/10 ampholytes). For 2-D gel analysis, ~185 μg of protein was applied to a ReadyStrip pH-gradient strip (pH 3–10) at room temperature, incubated overnight, and subjected to isoelectric focusing in a Protean IEF cell (Bio-Rad) in which the following cycles were applied: 250 V for 20 min, 8000 V in a linear ramp for 2.5 h, and 8000 V for 5.3 h. The electrofocused strip was placed on duplicate 4–20% polyacrylamide gels and separated by standard electrophoresis under denaturation conditions. One gel was blotted onto a nitrocellulose membrane (Schleicher and Schuell) for the viral binding assay, and the other was stained with Coomassie Blue R-250.

For the viral binding assay, the membrane was soaked overnight at 4 °C in PBS supplemented with 4% BSA and blocked with 5% skim milk in PBS for 1 h. After three washes with PBS, the membrane was incubated overnight at room temperature with [^{35}S]-labeled HTNV (~1×10^6 c.p.m.) in MEM supplemented with 10% FBS. After three, 15-min washes in PBS supplemented with 2% BSA and one wash in PBS supplemented with 0.1% NP-40, the membrane was dried and viral binding was visualized by autofluorography. Proteins showing virus binding were trypsinized, and peptide sequences were determined by ESI-MS/MS analysis (In2gen, Korea). Homologous protein sequences present in the NCBI database were searched using the MASCOT program.

Subcloning of gC1qR/p32 cDNA and siRNA

The gC1qR/p32 cDNA was amplified, respectively, from a HeLa cell cDNA library and Vero E6 cell mRNA using primers based on the sequences of the genes from humans (AF338439) and monkeys (X75913) (Beatch and Hobman, 2000; Ghebrehiwet et al., 1994). For transient expression, the entire protein coding sequence (282 aa for HeLa or 281 aa for Vero E6 cells) and a C-terminal HA epitope tag (LEPYDVPDY) subcloned in the pcDNA3 (Invitrogen) was transfected into cells using Lipofectamine PLUS (Invitrogen). An A549 cell line expressing gC1qR/p32 siRNA was established by transfection with pSilencer 2.1-U6 puro (Ambion) containing one of two distinct 21-bp antisense sequences targeting the gC1qR/p32 mRNA (NM_001212). One antisense sequence targeted the junction between exons 1 and 2 (5′-aaagct ttgtct ccgtcg gtg) and the other targeted exon 3 (5′-tcaaat gttggt gggatg ctg). Independent colonies were isolated after incubation for 3 weeks in medium containing 1 μg/ml puromycin, and gC1qR/p32 expression was analyzed by immunoblotting.

Immunofluorescence and immunoblotting assays

To verify the expression of gC1qR/p32, cells grown on a chamber slide were fixed with 3.7% paraformaldehyde in PBS for 15 min at room temperature. After blocking for 10 min in PBS supplemented with 1% BSA and 0.1% Tween 20, cells were treated for 1 h with gC1qR/p32 antibody (Santa Cruz), washed three times in PBS, and incubated for 30 min with FITC-conjugated secondary antibodies (Sigma). The cells were mounted with Fluoroguard antifade solution (Bio-Rad) and observed under an Axioskop2-Plus microscope (Zeiss). Cellular image was processed using IPLab (Scanalytics).

For immunoblotting, cellular proteins were separated electrophoretically on a denaturing polyacrylamide gel and subsequently transferred to a nitrocellulose membrane. The membrane was incubated with appropriate antibodies in TBST buffer [10 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.05% Tween-20] containing 5% skim milk. The target protein bands were visualized on an X-ray film by using PicoWest ECL reagents (Pierce) and the signal intensity was determined by densitometry with TINA software (Raytest). The following antibodies were also used: HTNV N (this laboratory), HA epitope (Sigma), actin (Santa Cruz), GFP (Santa Cruz), GAPDH (Santa Cruz), and HRP-conjugated IgG (Amersham).

Biotinylation of cell surface proteins

Cells (~3×10^7) were washed three times with cold PBS and incubated for 20 min on ice in a solution of 5 mM sulfosuccinimidyl-6-(biotinamido) hexanoate (sulfo-NHS-LC-biotin) (Pierce) in cold PBS (pH 8.0). After washing the cells three times with cold PBS containing 100 mM glycine to block free biotin, cells were lysed for 10 min on ice in modified RIPA buffer [50 mM Tris–HCl (pH 8.0), 120 mM NaCl, 0.5% NP40, protease inhibitor cocktail (Roche)]. Cell nuclei and insoluble debris were removed by centrifugation at 850 *g* for 15 min at 4 °C, and the biotin-conjugated proteins remaining in the supernatant were incubated in 20 μl of streptavidin–agarose (Pierce) for 1 h at 4 °C. The beads were washed three times with PBS, and the biotinylated proteins were eluted with 6 M guanidine–HCl (pH 1.5), dialyzed, and immunoblotted for gC1qR/p32.

Acknowledgments

This work was supported by the Korea Science and Education Foundation (R01-2002-000-00214-0 to B.Y.A.). Y.C.K. and K.H.L were supported by the BK21 Graduate Fellowship from the Ministry of Education, the Republic of Korea. We thank P. Palese (Mount Sinai) for providing NDV-GFP.

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