

Envelope glycoproteins of hantavirus can mediate cell-cell fusion independently

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SUMMARY

Hantaviruses (HTVs) are enveloped viruses and can induce low PH-dependent cell fusion. In this report we molecularly cloned viral glycoproteins (GPs) cDNA and nucleocapsid (NP) cDNA of two strains of *Hantaan virus* and one strain of *Seoul virus* and expressed in Vero E6 cells under control of a CMV promoter. The examinations of viral gene expressions were carried out by IFA and immune-precipitation. After treatment with low PH (PH 5.8) medium the syncytium were observed in the cells transfected with the GPs clones while in the cells transfected with the NP clones we did not find this phenomenon. Furthermore cotransfection of the NP and GPs did not enhance fusion activity. Treatment with anti-GP monoclonal antibodies could inhibit fusion activity whereas the antibodies against NP could not. These results indicated that GPs can mediate cell-cell fusion independently.

KEY WORDS: Hantavirus, cell-cell fusion, envelope glycoproteins

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INTRODUCTION

Hantaviruses (HTVs) as one genus of the family *Bunyaviridae*, are the only viral hemorrhagic fever (VHF) viruses with a worldwide distribution, with documented clinical cases on the Eurasian landmass and the American continent. *HTVs* can cause hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS).

At present at least three HFRS causing *hantaviruses* have been described: *Hantaan virus (HTNV)*, *Puumala virus (PUUV)*, and *Seoul virus (SEOV)*. Like other viruses in this family, *HTVs* has a tripartite, single-stranded negative sense RNA genome. The small (S), medium (M), and large (L) genomic RNA segments encode the

nucleocapsid protein (NP), the precursor to the virion envelope glycoproteins (GPC), and the virion-associated RNA polymerase, respectively. GPC is cotranslationally cleaved to generate two proteins designated G1 and G2 which are anchored in the viral envelope membrane by their C-terminal transmembrane regions (Lee *et al.*, 1978; Schmaljohn and Hjelle, 1997; Schmaljohn and Dalrymple, 1983; Spiropoulou, 2001).

In general, the fusion of viral membranes with target-cell membranes is an essential step in the entry of enveloped viruses into cells. This membrane fusion process leads to the release of viral proteins and the RNA genome into the host cell, initiating an infectious cycle.

Enveloped viruses not only can mediate fusion between the viral envelope and host cellular membrane but can also mediate cell-cell fusion. In recent years much research has indicated that glycoproteins on the surface of the viruses, such as *paramyxovirus* F protein and the *HIV* gp160 protein, are responsible for these membrane fusion processes (Weissenhorn *et al.*, 1999; White, 1990; Dutch *et al.*, 2000). Although the cell

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fusion activities mediated by *HTVs* under acidic conditions had been found over twenty years ago (Arikawa *et al.*, 1985), little is known about the mechanism of this phenomenon. More recently researchers reported that envelope glycoproteins (GPs) of *HTNV* 76-118 strain can induced cell-cell fusion and considered that GPs is the fusogen of *HTNV* (Ogino *et al.*, 2004). However there is no evidence to show this is a common characteristic in other strains and types viruses of *HTVs*.

To solve this problem the following viruses were used to investigate cell-cell fusion activities induced by *HTVs*: *HTNV* strain A9, *HTNV* strain 84FLI and *SEOV* strain L99. We constructed recombinant plasmid vectors containing the coding sequences for the M and S fragments of these viruses and expressed them in Vero E6 cells. Furthermore, the cell fusion experiments and inhibition assays were performed to clarify that GPs are fusogens of *HTVs*.

MATERIALS AND METHODS

Cells and viruses

The Vero E6 cell line (ATCC c1008; CRL 1586) was provided by the Institute for Viral Disease Control and Prevention (IVDC), Chinese Center for Disease Control and Prevention (CCDC). Previously research reported that after 150 subcultures, infected Vero E6 cells did not show low-pH-dependent cell-cell fusion (Ogino *et al.*, 2004) so we selected the cells passaged 20-30 times. The growth medium was Eagle's minimal essential medium (EMEM) (Invitrogen, Grand Island, N.Y.), which was supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine adjusted to the appropriate pH (7.2) with 1.0M NaOH and the supplemented medium was designated G-EMEM.

The binding medium was EMEM with 10% FBS, 2 mM L-glutamine containing 10mM Hydroxyethyl-piperazineethane sulfonic acid (HEPES) and 10 mM Morpholinoethane-sulfonic acid (MES) buffer adjusted to the appropriate pH (5.8) with 1.0M NaOH and was designated B-EMEM. *HTNV* strain A9, *HTNV* strain 84FLI and *SEOV* strain L99 were provided by IVDC, CCDC and propagated in Vero E6 cells. The culture supernatant was collected 7-10 days after inoc-

ulation and stored at -70°C for use as the stock viruses.

Antibodies

The monoclonal antibodies (MAbs) against envelope GPs and against nucleocapsid protein (NP) were provided by IVDC, CCDC. Mouse sera infected with *HTNV* or *SEOV* were provided by Center for Disease Control and Prevention of Shandong province in China. The monoclonal antibody against mouse immunoglobulin G labeled with fluorescein isothiocyanate (FITC) and streptavidin-horseradish peroxidase conjugate was purchased from SIGMA CORPORATION of AMERICA.

Expression of GPs and NP of *HTVs* in *vero* E6 Cells

The GPs and NP-encoding sequences of *HTNV* strain A9, *HTNV* strain 84FLI and *SEOV* strain L99 were RT-PCR amplified and subcloned into the expression plasmid pcDNA3.0 and designated pcA9M, pcA9S, pc84M, pc84S, pcL99M, and pcL99S respectively.

The process of construction of the recombinant vectors performed as described previously (Sambrook and Russell, 2001). Before transfection the Vero E6 cells were prepared on 6 - well glass slides and incubated about 12 hours. After the cell monolayers overspread 70% confluency of the slides the recombinant GPs and NP of each strain virus were transfected into Vero E6 cells with Lipofectamine 2000 (INVITROGEN c11668) respectively or together according to the manufacturer's protocol. At the same time the empty vector pcDNA3.0 was also transfected into Vero E6 cells as control. 60 hours after transfection, IFA and Immunoprecipitation (Cellular Labeling and Immunoprecipitation Kit. ROCHE.c11647) were carried out to examine the expressions of envelope GPs and NP in Vero E6 cells. IFA was done as described previously (Dafalla, 1972) and the MAbs against GPs and NP as the primary antibody, the monoclonal antibody against mouse immunoglobulin G labeled with FITC as the secondary antibody which can emit characteristic green fluorescence in fluoromicroscope. Immunoprecipitation was performed according to the manufacturer's protocol and the serum of mouse infected with *HTNV* or *SEOV* as the primary antibody.

Cell fusion experiments and inhibition

Cell fusion experiments were performed about 60 hours after transfection, as described previously (Ogino *et al.*, 2004). One chamber of the slide was washed twice with phosphate-buffered saline (PBS) and replaced with prewarmed B-EMEM (pH 5.8) for 30 minutes, the medium in the chamber of the slide was then replaced with G-EMEM (pH 7.2). The cells were incubated in G-EMEM for 16-24 hours at 37°C in a humidified atmosphere containing 5 percent CO₂. The other chamber of the slide that was not treated with B-EMEM was used as the control. The cells were then washed twice with PBS and treated with methanol for 5-10 minutes at room temperature. After discarding the methanol the cells were stained with Giemsa Stain Solution (SIGMA c51811) diluted in 1:10 for 30 minutes at room temperature. Then the syncytium could be observed by optical microscope and the fusion index (FI) calculated. The FI was calculated as [1 - (number of cells/number of nuclei)].

Approximately 100 nuclei per field were counted at a magnification of $\times 200$, and the average FI of five fields was calculated.

The cell fusion inhibition assay was performed as follows. Before treatment with B-EMEM the cells cotransfected GPs and NP were incubated at 37°C for 30 minutes, with twofold serial dilutions of the MAbs in 10% FBS into PBS at pH 7.2. After washing twice with PBS to remove free antibody the cell fusion experiments were performed as described above and then the FI were calculated.

RESULTS

Expression of recombinant vectors examined by IFA

After the recombinant vectors were transfected into the cells and incubated for about 60 hours IFA were performed. The characteristic green fluorescence was detected in the cells transfected with the GPs clones (Figure 1a) and in the cells transfected with the NP clones (Figure 1b). As control the empty plasmid pcDNA3.0 was also transfected into Vero E6 cells and the characteristic fluorescence was not observed (Figure 1c). These observations indicated that the recombinant envelope GPs and NP of HTVs had been expressed in the cells.

Expression of recombinant vectors examined by immunoprecipitation

To confirm separately the expression and coexpression of GPs and NP of HTVs immunoprecipitation was performed. The GPs were translated as integral and then cleaved into G1 and G2 with molecular weights approximately of 68 kilodalton (KD) and 55 KD respectively. So there were two bands in the results that transfected with GPs separately (Figure 2 Lane 3). In the results that transfected with NP separately there was only one binding at about 50KD (Schmaljohn *et al.*, 1987; Schmaljohn *et al.*, 1986, Elliott, 1990) (Figure 2, Lane 2) and consequently there were three bands in the results that transfected with GPs and NP together (Figure 2, Lane

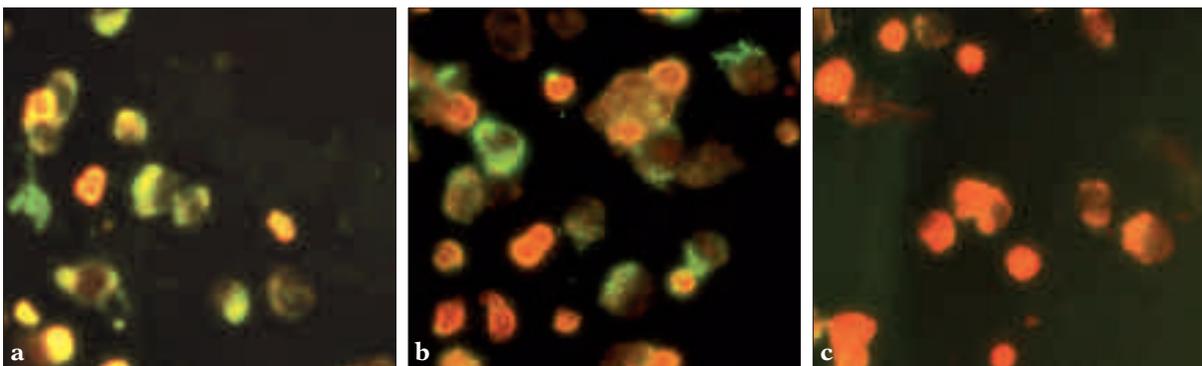


FIGURE 1 - Expression of recombinant vectors examined by IFA. After 60 hours of transfection IFA were carried out to testify the expression of GPs and NP of HTVs. In the cells transfected with GPs a) and NP b) of HTNV A9 strain and the other two strains (data not shown), the characteristic green fluorescence was observed. As control in the cells transfected with empty plasmid pcDNA3.0 c) no characteristic green fluorescence was observed (Magnification $\times 200$).

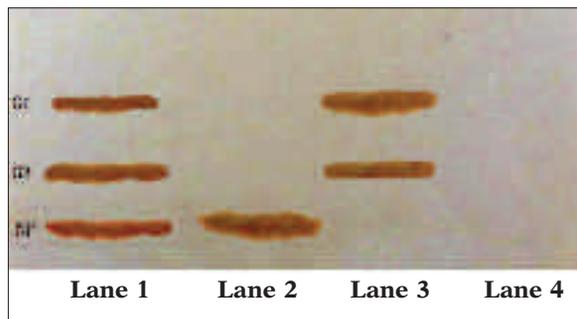


FIGURE 2 - Expression of recombinant vectors examined by Immunoprecipitation. Lane 1: coexpression of GPs and NP of HTNV A9 strain in Vero E6 cells. Lane 2: expression of NP of HTNV A9 strain in Vero E6 cells. Lane 3: expression of GPs of HTNV A9 strain in Vero E6 cells. Lane 4: the cells transfected with empty plasmid pcDNA3.0 as control. The results were the same in HTNV strain 84FLI and SEOV strain L99 (data not shown).

1). We can clearly observe the results from the figure shown below and as the control there was no characteristic binding observed in the result transfecting the empty plasmid pcDNA3.0 (Figure 2, Lane 1). According to these results the expression of GPs and NP was confirmed.

The cell-cell fusion activities mediated by GPs

After treatment with acidic medium the syncytium appeared in the cells transfected with GPs separately (Figure 3a) and in the cells cotransfected with GPs and NP (Figure 3c) and there was no distinct difference in FI between them (Figure 4). No syncytium was observed in the cells transfected with NP separately (Figure 3b) or in the cells transfected with empty plasmid pcDNA3.0 (Figure 3d). No syncytium appeared in any of the cells without treatment with B-EMEM (Figure 3e, f). To further evaluate the role of GPs in the fusion activities the cell fusion experiments were carried out in the cells cotransfected with GPs and NP after treatment with superfluous MAbs against GPs and NP respectively. As a result, no syncytium was observed after treatment with superfluous MAbs against GPs while it can still be observed after treatment with superfluous MAbs against NP. And there was no dramatic change in the efficiency of cell fusion after treatment with superfluous MAbs against NP (Figure 4). These

results showed that GPs could induce cell-cell fusion in low PH and this process was independent of other viral components (e.g., the NP) and replication. From the figure shown in below (Figure 4) we found that although the efficiency of cell fusion differed, all of three virus strains showed similar results. So we can conclude that envelope GPs as fusogens may be a common characteristic of the genus *HTVs*.

DISCUSSION

A large body of data for viruses such as *orthomyxoviruses*, *togaviruses*, *rhabdoviruses*, *bunyaviruses*, and some *retroviruses* indicates that, following attachment of the virus to a cell surface receptor, the viruses are endocytosed and transported to a low-pH endosomal compartment where the low pH triggers the fusion of the viral envelope with the cellular plasma membrane, releasing the viral genome into the cytoplasm (White, 1990; Horvath and Lamb, 1992).

To obtain direct evidence that envelope GPs are responsible for the fusion activity we adopted transfected Vero E6 cells to perform cell fusion experiments. In previous research GPs and NP of *HTNV* were expressed by vaccinia virus and baculovirus recombinants (Schmaljohn *et al.*, 1990, Pensiero *et al.*, 1988). But a cytopathic effect was quite evident in the vaccinia virus-infected cells which result in the apparent morphological changes and influence the observation of the syncytium.

Baculovirus only infected insect cells such as *spodopterafrugiperda* (SF-9) cells and we did not observe the syncytium in it (data not shown). Instead of these two expression systems described above we adopted the expression plasmid pcDNA3.0 vector system. In all three virus strains we obtained similar results. The syncytium formed in the cells transfected with GPs regardless of the transfection of NP and no enhancement of the fusion phenomenon was observed after transfection of them together. Furthermore we carried out the cell fusion inhibition assay and found that only the antibodies against GPs can inhibit the cell fusion activities while the antibodies against NP could not. These results provided direct evidence that GPs of *HTVs* can mediate cell-cell fusion and this process is

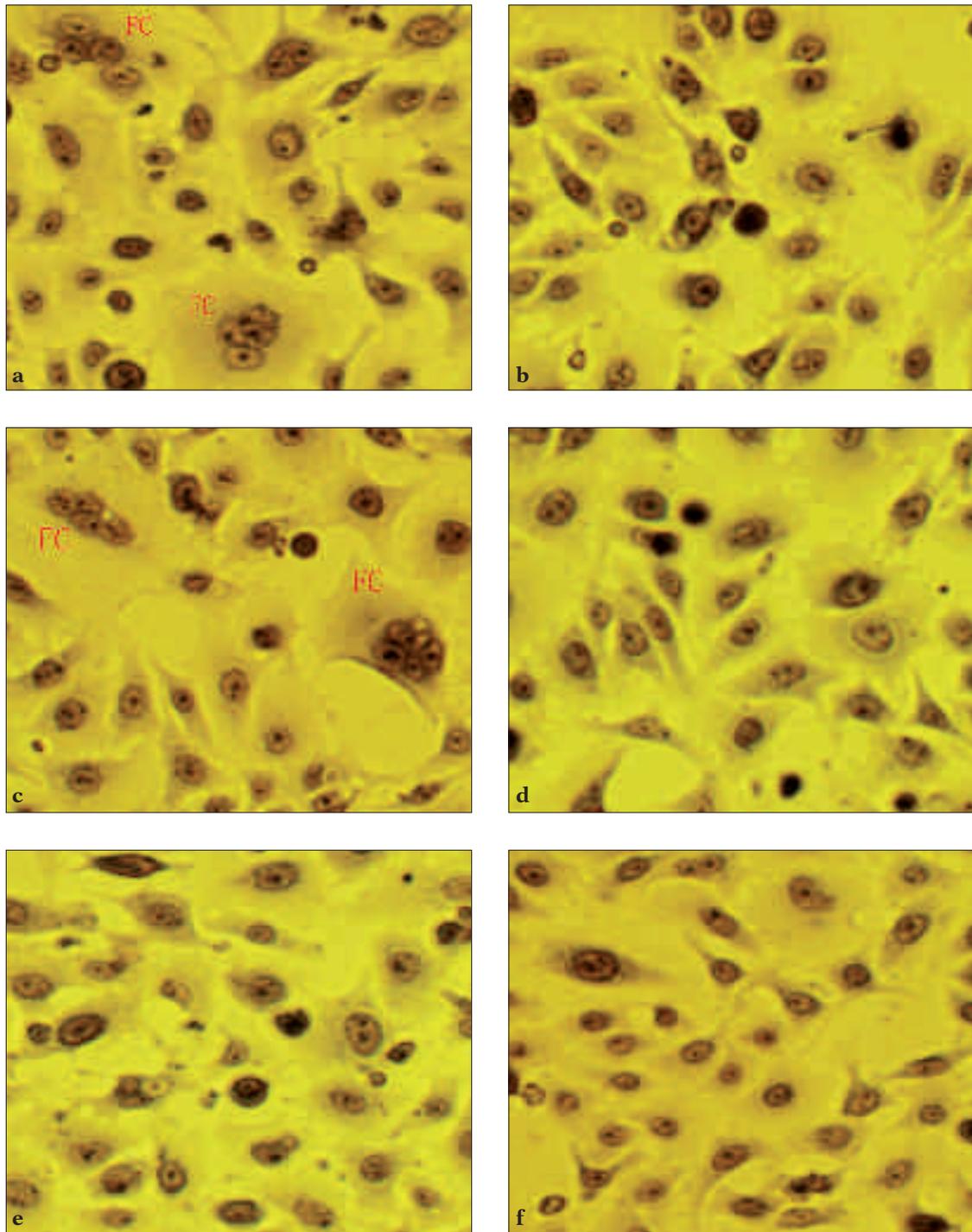


FIGURE 3 - The cell fusion activities mediated by GPs of HTNV strain A9. After 60 hours of transfection the Vero E6 cells were treated with B-EMEM (pH 5.8) for 30 minutes and then incubated with G-EMEM for 24 hours. The cells were fixed with methanol and stained with Giemsa. The cells transfected with GPs separately (a, e), the cells transfected with NP separately (b), the cells transfected with GPs and NP together (c), the cells transfected with empty plasmid pcDNA3.0 (d, f). The cells treated with B-EMEM (A, B, C, D) and the cells without treatment with B-EMEM (e, f) are shown (Magnification $\times 200$). FC: Syncytium. There were similar results in HTNV strain 84FLI and SEOV strain L99 (data not shown).

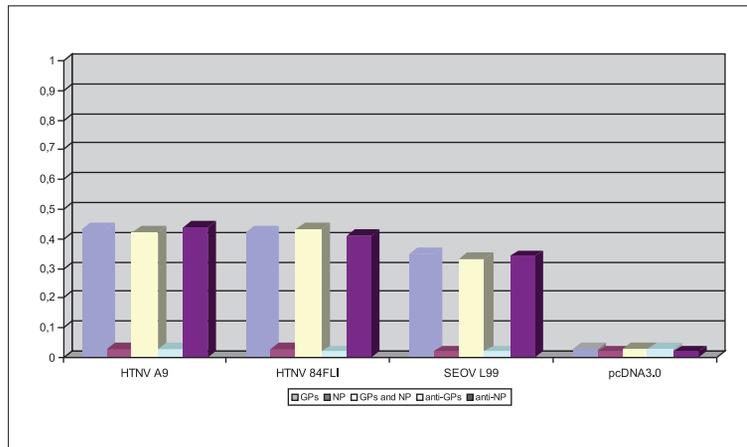


FIGURE 4 - The results of the fusion experiments and its inhibition assay in the cells transfected with recombinant vectors of three virus strains and empty plasmid pcDNA 3.0. GPs: FI of cell fusion experiments in the cells transfected with GPs of HTVs. NP: FI of cell fusion experiments in the cells transfected with NP of HTVs. GPs and NP: FI of cell fusion experiments in the cells cotransfected with GPs and NP of HTVs. Anti-GPs: FI of cell fusion experiments in the cells cotransfected with GPs and NP of HTVs after treatment with MAbs against GPs. Anti-NP: FI of cell fusion experiments in the cells cotransfected with GPs and NP of HTVs after treatment with MAbs against NP.

independent of other viral components (e.g., the NP) and replication.

To clarify which one is responsible for fusion we subcloned the cDNAs containing the coding sequences for the G1 and G2 fragments of HTNV A9 strain into the expression plasmid pcDNA 3.0/MCS respectively and transfected into Vero E6 cells separately. But in order to become transport competent G1 and G2 have to interact with each other in the endoplasmic reticulum. Neither G1 nor G2 can properly target the Golgi complex when transfected separately and no functional protein was synthesized (Ruusala *et al.*, 1992). So we did not detect them with IFA and immunoprecipitation and no fusion activity appeared. Although lacking direct evidence, some studies had been done to elucidate the definite location of the active domain on fusion. In another genus of family *Bunyaviridae* previous research showed that MAbs against G1 can reduced the efficiency of cell fusion and changed the requirement for a lower PH (Scarano *et al.*, 1985). However, other research reported that G1 containing liposomes were unable to induce fusion whereas virus particles that had most of their G1 removed by protease treatment but still contained G2 were able to fuse liposomes (Pobjecky *et al.*, 1989). More recently, proteomics computational analyses suggested that similar sequences or common structural/functional motifs are collinearly located in *Bunyavirus* Gc and *Alphavirus* E1. Features common to other class II fusion proteins, including an internal fusion peptide, a carboxyl terminal transmembrane domain and regions with a high propen-

sity to interface with bilayer membranes, are conserved and in similar locations in Gc of viruses in each genus of the *Bunyaviridae* (Garry and Garry, 2004; Tischler, 2005). These results indicated that the primary active domain of fusion may be located on G2 and the function of G1 is likely to recognize and bind with the receptor of host cells.

From all of the above, we considered that the fusion mechanisms mediated by HTVs are as follows. Initially the fusion peptide is deeply buried in an interior region of G2. When the processed protein is exposed to acid conditions G1 becomes more hydrophobic and exposes the domain that can recognize and bind with the receptor which is likely the β_3 integrin (Song *et al.*, 2005) on the host cell surface. Then followed by further conformational changes in both G1 and G2 and resulting in exposing the fusion peptide and the transient prehairpin intermediate is formed. Because the fusogenic structure is extremely stable and presumably represents the most thermodynamically favorable form of the molecule, the progression from the transient intermediate to the fusogenic conformation brings the two membrane proximal regions closer (Eckert and Kim, 2001). Ultimately, both transmembrane regions of the fusion glycoprotein (the fusion peptide and the original transmembrane helix) occupy the same membrane as the viral and cellular membranes become one. When the cells were treated with superfluous MAbs against GPs it is likely that the binding domain or the fusogenic domain had been blocked out and the conformational changes did not occur. Thus the

transient prehairpin intermediation would not come into being and cell fusion would be inhibited.

Although GPs as fusogens had been proved in this study the specific domain of the fusion peptide and the details of fusion mechanism are still unknown. The cell-cell fusion activity mediated by fusion protein is the most important step that the virus enters the host cells and explicitly shows this process would be in favor of the emergence of drugs and vaccines to block viral infection. To achieve this objective more work must be done.

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REFERENCES

- ARIKAWA, J., TAKASHIMA, I., AND HASHIMOTO, N., (1985). Cell fusion by haemorrhagic fever with renal syndrome (HFRS) virus and its application for titration of virus infectivity and neutralizing antibody. *Arch. Virol.* **86**: 303-13.
- DAFALLA, A.A., (1972). The indirect fluorescent antibody test for the serodiagnosis of strongyloidiasis. *J. Trop. Med. Hyg.* **75** (6): 109-11.
- DUTCH, R.E., JARDETZKY, T.S, AND LAMB, R.A., (2000). Virus membrane fusion proteins: biological machines that undergo a metamorphosis. *Biosci Rep.* **20** (6): 597-612.
- ECKERT, D.M., AND KIM, P.S. (2001). Mechanisms of viral membrane fusion and its inhibition. *Annu. Rev. Biochem.* **70**: 777-810.
- ELLIOTT, R.M. (1990). Molecular biology of Bunyaviridae. *J. Gen. Virol.* **71**: 501-22.
- GARRY, C.E., AND GARRY, R.F. (2004). Proteomics computational analyses suggest that the carboxyl terminal glycoproteins of Bunyaviruses are class II viral fusion protein (beta-penetrines). *Theor. Biol. Med. Model.* **1**: 10.
- HORVATH, C.M. AND LAMB, R.A. (1992). Studies on the fusion peptide of a paramyxovirus fusion glycoprotein: roles of conserved residues in cell fusion. *J. Virol.* **66** (4): 2443-55.
- LEE, H.W., LEE, P.W., JOHNSON, K.M. (1978). Isolation of the etiologic agent of Korean hemorrhagic fever. *J. Infect. Dis.* **137** (3): 298.
- OGINO, M., YOSHIMATSU, K., EBIHARA, H., ARAKI, K., LEE, B.H., OKUMURA, M., AND ARIKAWA, J. (2004). Cell fusion activities of hantaan virus envelope glycoproteins. *J. Virol.* **78** (19), 10776-82.
- PENSIERO, M.N., JENNINGS, G.B., SCHMALJOHN, C.S., AND HAY, J. (1988). Expression of the Hantaan virus M genome segment by using a vaccinia virus recombinant. *J. Virol.* **62** (3): 696-702
- POBJECKY, N., NATHANSON, N. AND SCARANO, F.G. (1989). Use resonance energy transfer assay to investigate the fusion function of La Crosse virus. In: Kolakofsky, D, and Mahy BW (eds). Genetics and pathogenicity of negative strand viruses. Amsterdam, New York 24-32.
- RUUSALA, A., PERSSON, R., SCHMALJOHN, C.S. AND PETTERSSON, R.F. (1992). Coexpression of the membrane glycoproteins G1 and G2 of Hantaan virus is required for targeting to the Golgi complex. *Virology.* **186**: 53-64.
- SAMBROOK, J. AND RUSSELL, D.W. (2001). Molecular cloning. A laboratory manual. 3rd ed. Cold Spring Harbor Laboratory Press, New York.
- SCARANO, F.G., JANSSEN, R.S., NAJJAR, J.A., POBJECKY, N. AND NATHANSON, N. (1985). An avirulent G1 glycoprotein variant of La Crosse bunyavirus with defective fusion function. *J. Virol.* **54** (3): 757-63.
- SCHMALJOHN, C.S., CHU, Y.K., SCHMALJOHN, A.L. AND DALRYMPLE, J.M. (1990). Antigenic subunits of Hantaan virus expressed by baculovirus and vaccinia virus recombinants. *J. Virol.* **64** (7): 3162-70.
- SCHMALJOHN, C.S. AND DALRYMPLE, J.M. (1983). Analysis of hantaan virus RNA: evidence for a new genus of bunyaviridae. *Virology.* **131**: 482-91.
- SCHMALJOHN, C.S. AND HJELLE, B. (1997). Hantaviruses: a global disease problem. *Emerg. Infect. Dis.* **3** (2): 95-104.
- SCHMALJOHN, C.S., JENNINGS, G.B., HAY, J. AND DALRYMPLE, J.M. (1986). Coding strategy of the S genome segment of Hantaan virus. *Virology.* **155**: 633-43.
- SCHMALJOHN, C.S., SCHMALJOHN, A.L., AND DALRYMPLE, J.M. (1987). Hantaan virus MRNA: coding strategy, nucleotide sequence, and gene order. *Virology.* **157**: 31-9.
- SONG, J.W., SONG, K.J., BAEK, L.J., FROST, B., PONCZ, M., AND PARK, K.S. (2005). In vivo characterization of the integrin β_3 as a receptor for Hantaan virus cellular entry. *Exp. Mol. Med.* **37** (2): 121-7.
- SPIROPOULOU, C.F. (2001). Hantavirus maturation. *Curr. Top. Microbiol. Immunol.* **256**: 33-46.
- TISCHLER, N.D., GONZALEZ, A., PEREZ-ACLE, T., ROSEMBLATT, M. AND VALENZUELA, P.D.T. (2005). Hantavirus Gc glycoprotein: evidence for a class II fusion protein. *J. Gen. Virol.* **86** (Pt 11): 2937-47.
- WEISSENHORN, W., DESSEN, A., CALDER, L.J., HARRISON, S.C., SKEHEL, J.J. AND WILEY, D.C. (1999). Structural basis for membrane fusion by enveloped viruses. *Mol. Membr. Biol.* **16** (1): 3-9.
- WHITE, J.M. (1990). Viral and cellular membrane fusion proteins. *Annu. Rev. Physiol.* **52**: 675-97.

