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Defining the N-Linked Glycosylation Site of Hantaan Virus Envelope Glycoproteins Essential for Cell Fusion

Feng Zheng[1], Lixian Ma[1,*], Lihua Shao[2], Gang Wang[1], Fengzhe Chen[1], Ying Zhang[1], and Song Yang[1]

[1]Qilu Hospital of Shandong University, Jinan, Shandong province, 250012, China
[2]School of Public Health of Shandong University, Jinan, Shandong province, 250012, China

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The Hantaan virus (HTNV) is an enveloped virus that is capable of inducing low pH-dependent cell fusion. We molecularly cloned the viral glycoprotein (GP) and nucleocapsid (NP) cDNA of HTNV and expressed them in Vero E6 cells under the control of a CMV promoter. The viral gene expression was assessed using an indirect immunofluorescence assay and immunoprecipitation. The transfected Vero E6 cells expressing GPs, but not those expressing NP, fused and formed a syncytium following exposure to a low pH. Monoclonal antibodies (MAbs) against envelope GPs inhibited cell fusion, whereas MAbs against NP did not. We also investigated the N-linked glycosylation of HTNV GPs and its role in cell fusion. The envelope GPs of HTNV are modified by N-linked glycosylation at five sites: four sites on G1 (N134, N235, N347, and N399) and one site on G2 (N928). Site-directed mutagenesis was used to construct eight GP gene mutants, including five single N-glycosylation site mutants and three double-site mutants, which were then expressed in Vero E6 cells. The oligosaccharide chain on residue N928 of G2 was found to be crucial for cell fusion after exposure to a low pH. These results suggest that G2 is likely to be the fusion protein of HTNV.

Keywords: Hantaan virus, cell-cell fusion, glycoproteins, N-linked glycosylation

Hantaan virus (HTNV), a member of the Hantavirus genus, family Bunyaviridae, is the major cause of severe hemorrhagic fever with renal syndrome. Like other viruses in this family, HTNV has a tripartite, single-stranded, negative-sense RNA genome. The small, medium, and large genomic RNA segments encode the nucleocapsid protein (NP), the precursor of the virion envelope glycoproteins (GPC), and virion-associated RNA polymerase, respectively. GPC is co-translationally cleaved in order to generate two proteins designated G1 and G2, which are modified by N-linked glycosylation. The glycoproteins (GPs) contain five sites of N-linked glycosylation: four on G1 (N134, N235, N347, and N399) and one on G2 (N928) (Schmaljohn and Dalrymple, 1983; Schmaljohn, 1996; Shi and Elliott, 2004) (Fig. 1).

In general, the fusion of the viral envelope with the target cell membrane is an essential step in the entry of enveloped viruses into cells. This membrane fusion process results in the release of viral proteins and the RNA genome into the host cell, thus initiating an infectious cycle. Enveloped viruses mediate cell-cell fusion in addition to mediating the fusion between the viral envelope and the host cell membrane. Increasing research indicates that glycoproteins (GPs) on the surface of viruses, such as paramyxovirus F protein and HIV gp160 protein, are responsible for these membrane fusion processes (White, 1990; Weissenhorn *et al.*, 1999). Fusion proteins generally contain a short region called the "fusion peptide," which is essential for membrane fusion (Dutch *et al.*, 2000; Eckert and Kim, 2001). As an enveloped virus, when HTNV infects a host cell, its membrane must fuse with the host cell membrane, thus allowing the contents of the virus to be transferred to the host. Although the cell fusion activities mediated by *Hantavirus* under acidic conditions were discovered over 20 years ago (Arikawa *et al.*, 1985), little is currently known about the mechanism underlying these cell fusion activities. Researchers have recently reported that envelope GPs were the fusogens of HTNV (Ogino *et al.*, 2004) however, the exact location of the fusion peptide remained to be elucidated.

In this study, we reexamined the fusogenic activity of HTNV GPs in Vero E6 cells and confirmed that the GPs are capable of independently mediating cell-cell fusion. We investigated the N-linked glycosylation of HTNV GPs and its role in cell fusion in order to determine the location of the fusion peptide. The results of our study demonstrate that the oligosaccharide chain on residue N928 is crucial

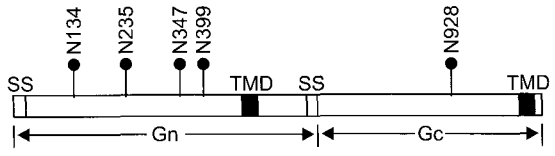

Fig. 1. Schematic diagram of the HTNV GPC and N-linked glycosylation sites. GPC is represented by the bar, with signal sequences (SS) and transmembrane domains (TMD) shown as open boxes and filled boxes, respectively. Lollipops indicate the locations of the N glycosylation sites on G1 and G2 (four on G1 and one on G2).

* To whom correspondence should be addressed.
(Tel) 86-531-8216-9385; (Fax) 86-531-8216-9385
(E-mail) grbkmlx@yahoo.com.cn

for cell fusion. Considering that the N-linked glycosylation of HTNV GPs plays an important role in protein folding and intracellular trafficking, we believe that G2 is likely to be the fusion protein of HTNV.

Materials and Methods

Cells and viruses

The Vero E6 cell line was provided by the Institute for Viral Disease Control and Prevention (IVDC), Chinese Center for Disease Control and Prevention. Previous research has shown that infected Vero E6 cells do not show low pH-dependent cell-cell fusion after more than 150 subcultures (Ogino *et al.*, 2004). Therefore we selected cells that had undergone 20-30 passages. The growth medium used was Eagle's minimal essential medium (EMEM), supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine adjusted to the appropriate pH (7.2) with 1.0 M NaOH; the supplemented medium was designated G-EMEM. The binding medium was EMEM with 10% FBS, 2 mM L-glutamine containing 10 mM hydroxyethylpiperazineethane sulfonic acid, and 10 mM morpholinoethane-sulfonic acid buffer adjusted to the appropriate pH (5.8) with 1.0 M NaOH; this was designated B-EMEM. HTNV strain A9 was provided by the IVDC and was propagated in Vero E6 cells.

Antibodies

Human recombinant monoclonal antibodies (MAbs) against HTNV envelope GPs and NP (Koch *et al.*, 2003) were provided by the IVDC. Human anti-HTNV serum was provided by the Center for Disease Control and Prevention of Shandong, China. Goat anti-human immunoglobulin G conjugated with fluorescein isothiocyanate (FITC) and a streptavidin-horseradish peroxidase conjugate were purchased from Sigma, USA.

Plasmid construction and site-directed mutagenesis

The GP- and NP-encoding sequences of HTNV strain A9 were amplified by RT-PCR, subcloned into the expression plasmid pcDNA3.0 under the control of a CMV promoter, and were designated pcA9M and pcA9S, respectively. The plasmid pcA9M was used as the template for site-directed PCR mutagenesis. One (for a single mutation) or two (for a double mutations) of the asparagine (N) residues at the N-linked glycosylation sites on G1 and G2 were replaced with alanine (A) in order to construct the N-glycosylation site mutants. The five single N-glycosylation site mutants were designated N134A, N235A, N347A, N399A, and N928A according to the position of the substitution. Three double glycosylation site mutants were designated N235/928A, N347/928A, and N399/928A. The coding regions of the mutants were also cloned into the expression plasmid pcDNA3.0 and designated pcA9MMN134A, pcA9MMN235A, pcA9MMN347A, pcA9MMN399A, pcA9MMN928A, pcA9MMN235/928A, pcA9MMN347/928A, and pcA9MMN399/928A, respectively. All of the molecular biology procedures were performed according to the standard protocols (Sambrook and Russell, 2001). Beijing Sunbiotech Corporation certified that all of the plasmids contained the correct sequences.

Transient gene expression and examination

The Vero E6 cells were prepared on 6-well glass slides and incubated for approximately 12 h prior to transfection. When the cell monolayers reached 70% confluence, the recombinant GPs and NP of HTNV were transfected into the cells, alone or together, using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol. The GP gene mutants were simultaneously transfected into Vero E6 cells, and the plasmid pcDNA3.0 was also transfected into cells as a control. An immunofluorescence assay (IFA) and immunoprecipitation (Cellular Labeling and Immunoprecipitation Kit. Roche. c11647) were performed approximately 60 h after transfection in order to examine the expression of envelope GPs, NP, and mutant proteins in the Vero E6 cells. The IFA was performed as previously described (Dafalla, 1972) using MAbs against GPs and NP as the primary antibodies, respectively, and MAb against human immunoglobulin G labeled with FITC, which emits a characteristic green fluorescence under a fluoromicroscope, as the secondary antibody. Immunoprecipitation was performed according to the manufacturer's protocol using human anti-HTNV serum as the primary antibody.

Flow-cytometric analysis

Flow-cytometric analyses were performed approximately 60 h after transfection, as previously described (Ogino *et al.*, 2004). The cells were detached from the culture dishes using 0.1% trypsin and 0.02% EDTA in phosphate-buffered saline (PBS) and were subsequently transferred to microcentrifuge tubes. The cell suspension was adjusted to a concentration of 10^5 cells with a fluorescence-activated cell sorter (FACS) solution and fixed with 1% paraformaldehyde in PBS for 10 min at room temperature. The fixed cells were initially reacted with human anti-HTNV serum for 30 min and then stained with FITC-conjugated goat anti-human immunoglobulin G for 30 min at room temperature. After being washed with FACS solution, the cells were suspended with FACS solution and analyzed using a FACS Calibur system (Becton Dickinson).

Cell fusion experiments

Cell fusion experiments were performed approximately 60 h after transfection as previously described (Ogino *et al.*, 2004). One chamber of the slide was washed twice with PBS and replaced with prewarmed B-EMEM (pH 5.8) for 30 min. 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 h at 37°C in a humidified atmosphere containing 5% CO_2. The other chamber of the slide, which 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 min at room temperature. The methanol was then discarded and the cells were stained with Giemsa Stain Solution (Sigma) for 30 min at room temperature. Cell fusion was measured by polykaryon formation and expressed as the fusion index (FI). The FI was calculated as [1 - (number of cells/number of nuclei)]. Approximately 100 nuclei per field were counted and the average FI of five different fields was calculated under a light microscope under a magnification of ×200.

The cell fusion inhibition assay was performed as follows. The cells that had been co-transfected with GPs and NP were incubated at 37°C for 30 min with twofold serial dilutions of the MAbs in 5% FBS in PBS at pH 7.2 prior to treatment with B-EMEM. After washing twice with PBS to remove the free antibody, cell fusion experiments were

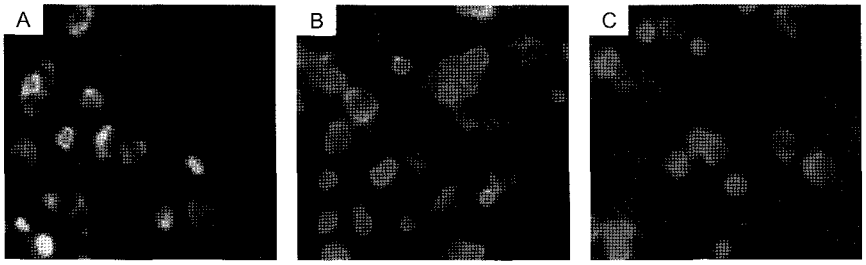

Fig. 2. Expression of recombinant envelope GPs and NP in Vero E6 cells examined by IFA. IFA was performed 60 h after transfection to confirm the expression of HTNV GPs and NP. The characteristic green fluorescence was observed in cells transfected with GPs (A) and NP (B). Control cells transfected with the empty plasmid pcDNA3.0 (C) showed no characteristic green fluorescence. (×200 magnification.)

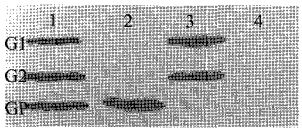

Fig. 3. Expression of recombinant envelope GPs and NP examined by immunoprecipitation. Immunoprecipitation was performed after transfection of cells with the following plasmids, using human anti-HTNV serum as the primary antibody. Lane 1: Co-transfection with GPs and NP; lane 2: transfection with NP; lane 3: transfection with GPs; lane 4: transfection with control plasmid pcDNA3.0.

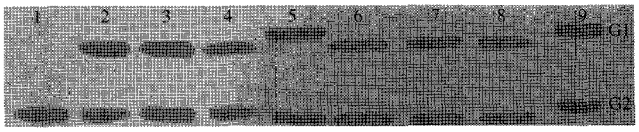

Fig. 4. Expression of mutated GPs examined by immunoprecipitation. Immunoprecipitation was performed after the transfection of cells with the following plasmids, using human anti-HTNV serum as the primary antibody. Lane 1: N134A; lane 2: N235A; lane 3: N347A; lane 4: N399A; lane 5: N928A; lane 6: N235/928A; lane 7: N347/928A; lane 8: N399/928A. GPs without mutations were also analyzed as a control (lane 9).

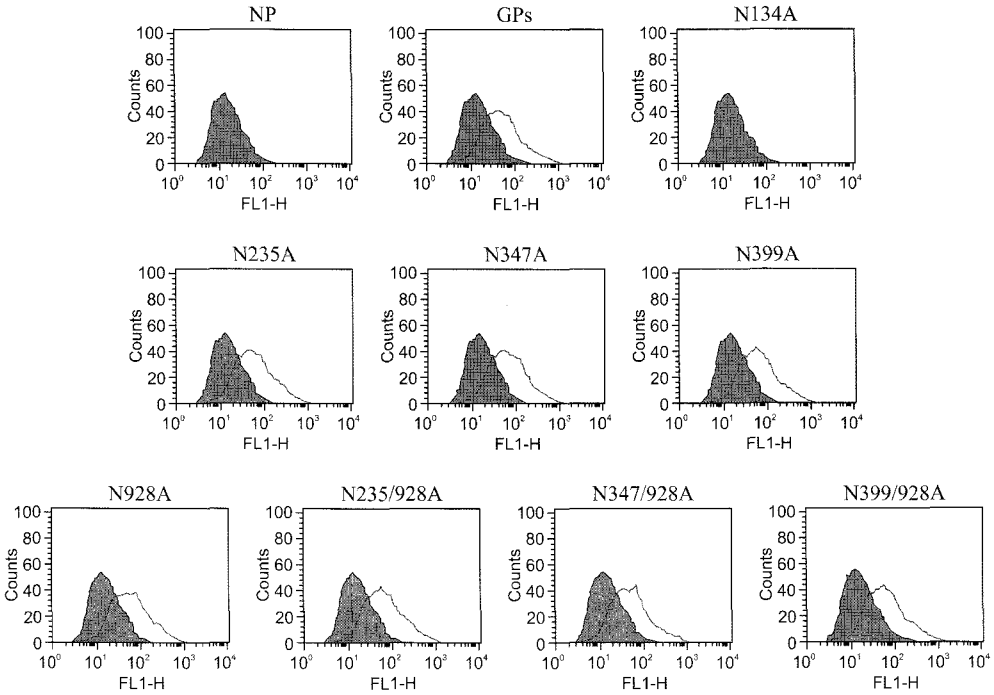

Fig. 5. Flow cytometric analysis. Human anti-HTNV serum was used to detect the surface expression of GPs, NP, and mutants. The identity of GPs, NP, and each mutant is listed above the figures. The level of polyclonal antibody binding is indicated by a shift of the open histogram (with human anti-HTNV serum) to the right of the solid control histogram (FITC-conjugated goat anti-human immunoglobulin G only).

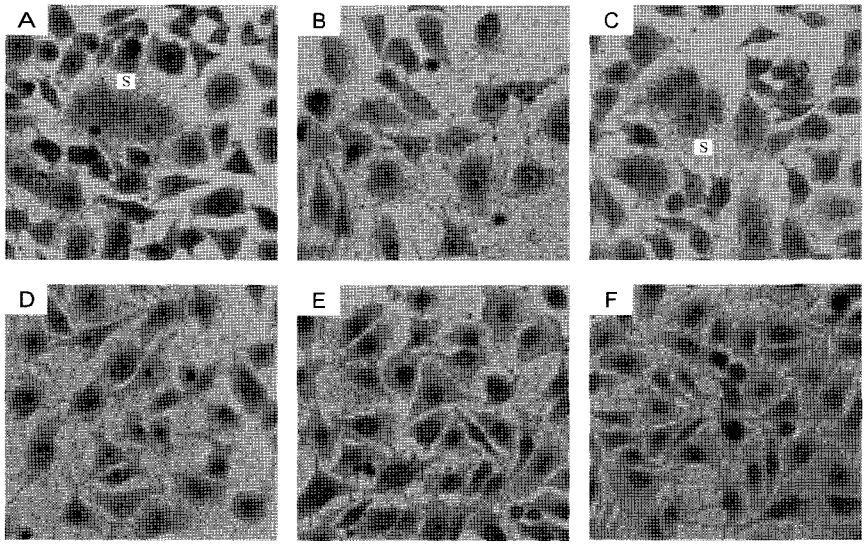

Fig. 6. Cell fusion induced by HTNV GPs. 60 h after transfection, Vero E6 cells were treated with B-EMEM (pH 5.8) for 30 min and then incubated with G-EMEM for 24 h. The cells were fixed with methanol and stained with Giemsa. Cells transfected with GPs alone (A, E); cells transfected with NP alone (B); cells co-transfected with GPs and NP (C); cells transfected with plasmid pcDNA3.0 (D, F); cells treated with B-EMEM (A, B, C, D); cells not treated with B-EMEM (E, F). S: syncytium. (×200 magnification.)

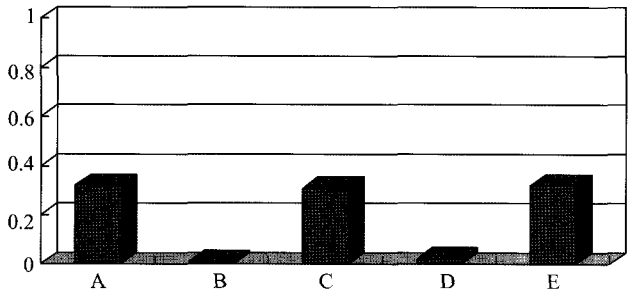

Fig. 7. FI of cell fusion induced by GPs. The cells were treated with B-EMEM and the FI was calculated after the expression of the following proteins. A: GPs; B: NP; C: co-expression of GPs and NP. Cells transfected with GPs and NP together were treated with MAbs prior to treatment with B-EMEM in the cell fusion inhibition assay. D: MAbs against GPs; E: MAb against NP.

performed as described above and the FI was calculated.

Results

Expression of recombinant envelope GPs and NP in Vero E6 cells

IFA was performed approximately 60 h after transfection of the recombinant vectors into the cells. The characteristic green fluorescence was detected in the cells transfected with GPs (Fig. 2A) and in the cells transfected with NP (Fig. 2B). No fluorescence was observed in the cells transfected with the control plasmid pcDNA3.0 (Fig. 2C). These observations indicate that the recombinant envelope GPs and NP of HTNV were expressed in the cells. Immunoprecipitation was performed to separately confirm the expression and co-expression of GPs and NP. GPs are translated as a single precursor that is cleaved into G1 and G2 with molecular weights of approximately of 68 kDa and 55 kDa, respectively (Schmaljohn *et al.*, 1987; Schmaljohn, 1996). There were two bands in the cells transfected with GPs alone (Fig. 3, lane 3). There was only one band of about 50 kDa in the cells transfected with NP alone (Schmaljohn *et al.*, 1986; Schmaljohn, 1996) (Fig. 3, lane 2), and there were three bands in the cells transfected with both GPs and NP (Fig. 3, lane 1). There were no characteristic bands in the cells transfected with the control plasmid pcDNA3.0 (Fig. 3, lane 4). The expression of GPs and NP was thus confirmed.

Expression of mutated GPs in Vero E6 cells

Since most of the MAbs against HTNV GPs do not react, or react poorly, with denatured and misfolded proteins (Schmaljohn *et al.*, 1987; Wang *et al.*, 1993), we performed an immunoprecipitation to examine expression of mutants using human anti-HTNV serum as the primary antibody. We found that the site-directed mutation of N-linked glycosylation resulted in a significant electrophoretic mobility shift of G1 and G2, which corresponded to the apparent molecular weight losses of approximately of 3000 Da for the single-site mutants and 6000 Da for the double-site mutants (Fig. 4). These differences are consistent with the loss of one or two N-linked oligosaccharide chains (Shi and Elliott, 2004). Only one band, corresponding to G2, was observed in mutant N134A (Fig. 4, lane 1), which suggests that the mutation of the oligosaccharide chain on residue N134 resulted in the misfolding of G1.

Demonstration of cell surface expression of GPs with or without mutation

The surface localization of GPs was examined by flow cytometry. The surface expression of GPs, but not of NP, was observed, as shown in Fig. 5. Furthermore, all of the

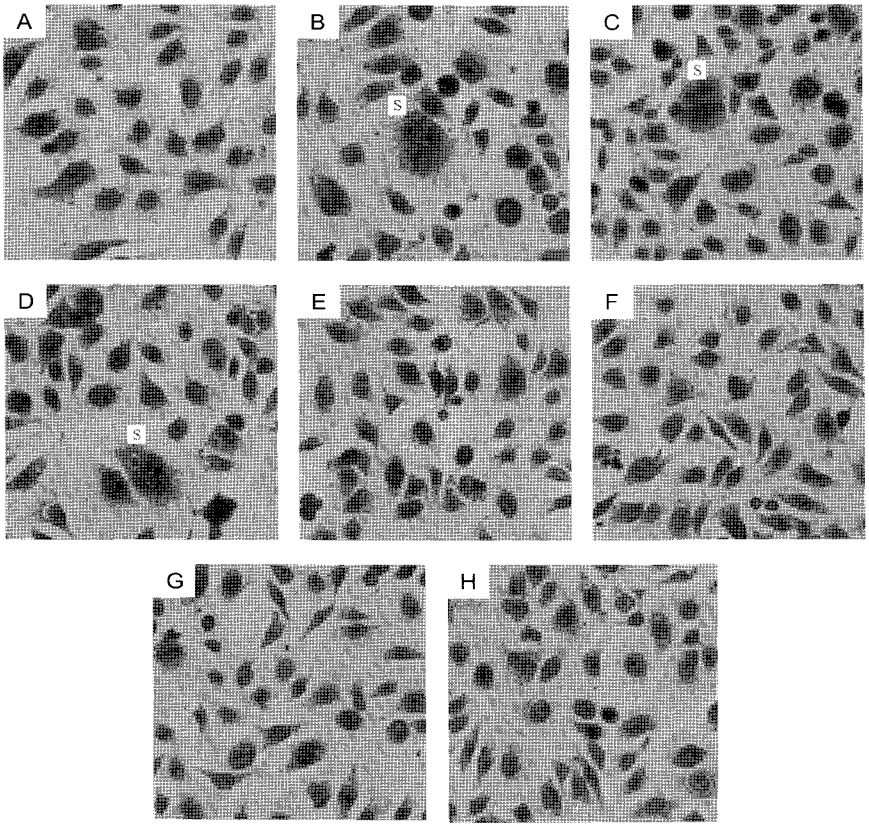

Fig. 8. Cell fusion activity of glycosylation-defective mutant GPs. 60 h after transfection with the following mutants, Vero E6 cells were treated with B-EMEM (pH 5.8) for 30 min and then incubated with G-EMEM for 24 h. The cells were fixed with methanol and stained with Giemsa. A: mutant N134A; B: mutant N235A; C: mutant N347A; D: mutant N399A; E: mutant N928A; F: mutant N235/928A; G: mutant N347/928A; H: mutant N399/928A. S: syncytium. (×200 magnification.)

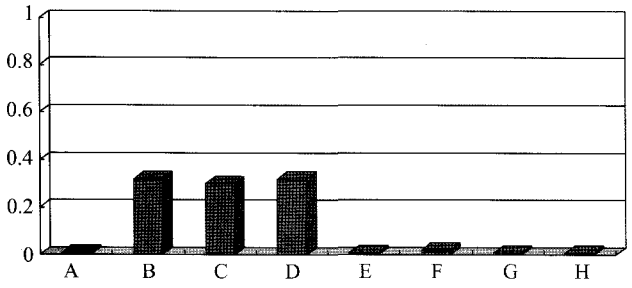

Fig. 9. The FI of cell fusion induced by glycosylation-defective mutant GPs. The cells were treated with B-EMEM and the FI was calculated after transfection with the following mutants. A: mutant N134A; B: mutant N235A; C: mutant N347A; D: mutant N399A; E: mutant N928A; F: mutant N235/928A; G: mutant N347/928A; H: mutant N399/928A.

mutated GPs were expressed on the cell surface, except for N134A (Fig. 5).

Cell fusion activity induced by GPs

Cell fusion experiments were performed using cells transfected with GPs and NP alone or together to confirm that the HTNV GPs are responsible for cell fusion. After treatment with acidic medium, syncytia appeared in cells transfected with GPs alone (Fig. 6A) and in the cells co-transfected with GPs and NP (Fig. 6C). There was no distinct difference between their FIs, which were 0.32 and 0.31, respectively (Fig. 7). No syncytia formed in the cells transfected with NP alone (Fig. 6B) or in the cells transfected with plasmid pcDNA3.0 (Fig. 6D). No syncytia appeared in any of the cells that were not treated with B-EMEM (Fig. 6E and F). Cell fusion experiments were performed using cells co-transfected with GPs and NP after treatment with MAbs against GPs and NP in order to further evaluate the role of GPs in cell fusion. No syncytia appeared after the treatment with the MAb against GPs; however, syncytia were observed with the MAb against NP. No dramatic change was observed in the efficiency of cell fusion following treatment with the MAb against NP (Fig. 7). These results show that GPs are able to independently induce low pH-dependent cell fusion.

Cell fusion activity of glycosylation-defective mutant GPs

We performed cell fusion experiments in cells transfected with GP gene mutants to clarify the exact location of the active domain involved in the cell fusion of HTNV. We found that syncytia formed in cells transfected with pcA9M MN235A, pcA9MMN347A, or pcA9MMN399A (Fig. 8B-D), and that there was no distinct difference in the FI between them (Fig. 9). No syncytia formed in the cells transfected with pcA9MMN134A (Fig. 8A), pcA9MMN928A, pcA9MMN

235/928A, pcA9MMN347/928A, or pcA9MMN399/928A (Fig. 8E-H). These results suggest that a mutation of N-linked glycosylation at residues N134 and N928 can result in the loss of cell fusion.

Discussion

Previous research showed that GPs and NP of HTNV were expressed by vaccinia virus and baculovirus recombinants (Pensiero *et al.*, 1988; Schmaljohn *et al.*, 1990). However, a cytopathic effect that resulted in apparent morphological changes was evident in the vaccinia virus-infected cells, while the baculovirus can only infect insect cells such as spodopterafrugiperda cells in which no syncytia were observed (data not shown.). We, however, used the expression plasmid pcDNA3.0 vector system. Syncytia formed in the cells expressing GPs regardless of NP expression, and no increase in fusion was observed with the co-expression of GPs and NP, as shown in Fig. 6. A cell fusion inhibition assay revealed that only antibodies against GPs inhibited cell fusion and that the antibody against NP did not. These results are consistent with a previously published report (Ogino *et al.*, 2004) and strengthen the finding that GPs are the fusogens of HTNV.

The fusion reaction may occur via two different mechanisms: a) the fusion between the viral envelope and the host cell plasma membrane, and b) the fusion of the endosomal membrane with the viral envelope after viral particle internalization by receptor-mediated endocytosis. In both scenarios, membrane fusion is mediated by viral fusion proteins that undergo structural reorganization to convert from a nonfusogenic to a fusogenic conformation. Partly based on their molecular architecture, two classes of viral fusion proteins have been described (Jardetzky and Lamb, 2004). The fusion domains of the class I fusion proteins of viruses, such as the influenza virus and the sendai virus, are exposed as fusion peptides after protease digestion (Durell *et al.*, 1997). In contrast, class II fusion proteins possess internal fusion domains that act as fusogens in the absence of specific cleavage by proteases (Peisajovich and Shai, 2003). In other genera of the family *Bunyaviridae*, studies have shown that G1-containing liposomes are unable to induce fusion, whereas the viral particles that have had most of their G1 removed by protease treatment, but still contain G2, are able to fuse with liposomes (Pobjecky *et al.*, 1989). These results indicate that the primary active fusion domain may be located on G2.

Although there are some reports of low pH-dependent cell fusion induced by HTNV, little is known about the exact location of the fusion peptide or the mechanism involved. Therefore, we investigated the N-linked glycosylation of HTNV GPs and its role in cell fusion in order to address these problems. N-linked oligosaccharide chains can affect protein functions in many ways, including the promotion of proper folding, maintenance of protein conformation and stability, protection of proteins from proteolysis, and the modulation of the biological activities of proteins (Rademacher *et al.*, 1988). Previous studies on the roles of oligosaccharide chains attached to the fusion proteins of other viruses have shown that the loss of these chains leads to a deficiency or loss of cell fusion (Hiroaki *et al.*, 2000; Zimmer *et al.*, 2001).

The biological roles of N-linked oligosaccharide chains are often assessed using tunicamycin, which is a potent inhibitor of N-glycosylation (Schwarz *et al.*, 1976; Mottet *et al.*, 1986). However, tunicamycin treatment, which inhibits the synthesis of dolichol intermediates, causes a total loss of N-linked oligosaccharide chains and is prone to artifacts including the formation of nonspecific aggregates of misfolded unglycosylated proteins (Machamer and Rose, 1988; Ng *et al.*, 1989; Malvoisin and Wild, 1994) Site-directed mutagenesis at specific N-glycosylation sites would be a useful alternative for analyzing the roles of specific oligosaccharide chains.

HTNV GPs possess five N-linked glycosylation sites (Fig. 1), four of which are conserved among all *Hantaviruses* (at residues N134, N347, N399, and N928). The oligosaccharide chain on residue N134 has been found to be crucial for protein folding, whereas single mutations at the other glycosylation sites are better tolerated (Shi and Elliott, 2004). Consistent with these findings, our data show that all glycosylation-defective mutants were expressed on the cell surface, except for N134A, which only expressed G2. A previous report indicated that G1 and G2 were retained in the endoplasmic reticulum and did not transport to the cell surface when individually expressed (Shi and Elliott, 2002). Since our fusion assay was dependent on protein expression at the cell surface, no cell fusion was observed with mutant N134A. We found that a mutation of the second (N235), third (N347), or fourth (N399) glycosylation site on G1 had no effect on cell fusion, while a mutation of the single site on G2 (N928) resulted in a loss of cell fusion. We also examined cell fusion in three double N-linked glycosylation site mutants. The patterns of cell fusion of the three mutants containing one change on G1 (N235A, N347A, or N399A) and one on G2 (N928A) were similar to those with GPs containing single mutations on G2. These results indicate that a lack of N-linked glycosylation at site N928 on G2 is sufficient to prevent cell fusion, which provides direct evidence that the fusion peptide of HTNV is on G2 and not on G1.

Computational proteomic analysis has recently provided evidence that the Gc of *Hantaviruses* and other members of the family *Bunyaviridae* can be classified as class II viral fusion proteins (Garry and Garry, 2004; Tischler *et al.*, 2005). Since HTNV possesses sequences or structural/functional motifs common to other class II fusion proteins, they may share a common mechanism of viral membrane fusion. Exposure to the acidic pH of endosomes triggers a major conformational change on the virion's surface, involving the dissociation of native protein complexes and the formation of homotrimers of the fusion proteins. The energy released during this transition from metastable dimers at the viral surface to stable target membrane-inserted homotrimers is used to drive the merging of the viral envelope and cell membranes (Heinz and Allison, 2000; Kielian *et al.*, 2000; Eckert and Kim, 2001). Considering that the N-linked glycosylation of GPs plays an important role in protein folding, it is likely that the mutagenesis of N-linked glycosylation of G2 results in conformational changes of G2 and

thus a failure to trigger its structural reorganization resulting in the loss of cell fusion.

In summary, our studies confirmed that only N-linked glycosylation at site N928 on G2 is crucial for cell fusion. These results provide direct evidence that the fusion peptide of HTNV is likely to be on G2. Cell-cell fusion mediated by fusion proteins is the most important step in the entry of viruses into host cells, and the elucidation of this process would allow for the emergence of drugs and vaccines to block viral infection. Further study must be conducted in order to achieve this objective.

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References

Arikawa, J., I. Takashima, and N. Hashimoto. 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, 303-313.

Dafalla, A.A. 1972. The indirect fluorescent antibody test for the serodiagnosis of strongyloidiasis. *J. Trop. Med. Hyg.* 75, 109-111.

Durell, S.R., I. Martin, J.M. Ruysschaert, Y. Shai, and R. Blumenthal. 1997. What studies of fusion peptides tell us about viral envelope glycoprotein-mediated membrane fusion. *Mol. Membr. Biol.* 14, 97-112.

Dutch, R.E., T.S. Jardetzky, and R.A. Lamb. 2000. Virus membrane fusion proteins: biological machines that undergo a metamorphosis. *Biosci. Rep.* 20, 597-612.

Eckert, D.M. and P.S. Kim. 2001. Mechanisms of viral membrane fusion and its inhibition. *Annu. Rev. Biochem.* 70, 777-810.

Garry, C.E. and. R.F. Garry. 2004. Proteomics computational analyses suggest that the carboxyl terminal glycoproteins of *Bunyaviruses* are class II viral fusion protein (beta-penetrenes). *Theor. Biol. Med. Model.* 1, 10.

Heinz, F.X. and S.L. Allison. 2000. Structures and mechanisms in *flavivirus* fusion. *Adv. Vir. Res.* 55, 231-269.

Hiroaki, S., J.T. Yamashita, M. Kawakita, H. Taira, and J. Bwchem. 2000. Functional analysis of the individual oligosaccharide chains of Sendai virus fusion protein. *J. Biochem.* 128, 65-72,

Jardetzky, T.S. and R.A. Lamb. 2004. Virology: a class act. *Nature* 427, 307-308.

Kielian, M., P.K. Chatterjee, D.L. Gibbons, and Y.E. Lu. 2000. Specific roles for lipids in virus fusion and exit: examples from the *alphaviruses*. *Subcell. Biochem.* 34, 409-455.

Koch, J., M. Liang, I. Queitsch, A.A. Kraus, and E.K.F. Bautza. 2003. Human recombinant neutralizing antibodies against Hantaan virus G2 protein. *Virology* 308, 64-73.

Machamer, C.E. and J.K. Rose. 1988. Influence of new glycosylation sites on expression of the vesicular stomatitis virus G protein at the plasma membrane. *J. Biol. Chem.* 263, 5948-5954.

Malvoisin, E. and F. Wild. 1994. The role of N-glycosylation in cell fusion induced by a vaccinia recombinant virus expressing both measles virus glycoproteins. *Virology* 200, 11-20.

Mottet, G., A. Portner, and L. Roux. 1986. Drastic lmmunoreactavity changes between the immature and mature forms of the Sendai virus HN and Fo glycoproteins. *J. Virol.* 59, 132-141.

Ng, D.T., S.W. Hiebert, and R.A. Lamb. 1989. Different roles of individual N-linked oligosacchande chains in folding, assembly, and transport of the simian virus 5 hemagglutinin-neuraminidase. *Mol. Cell Biol.* 10, 1989-2001.

Ogino, M., K. Yoshimatsu, H. Ebihara, K. Araki, B.H. Lee, M. Okumura, and J. Arikawa. 2004. Cell fusion activities of Hantaan virus envelope glycoproteins. *J. Virol.* 78, 10776-10782.

Peisajovich, S.G. and Y. Shai. 2003. Viral fusion proteins: multiple regions contribute to membrane fusion. *Biochem. Biophys. Acta.* 1614, 122-129

Pensiero, M.N., G.B. Jennings, C.S. Schmaljohn, and J. Hay. 1988. Expression of the Hantaan virus M genome segment by using a vaccinia virus recombinant. *J. Virol.* 62, 696-702.

Pobjecky, N., N. Nathanson, and F.G. Scarano. 1989. Use resonance energy transfer assay to investigate the fusion function of La Crosse virus. p. 24, *In* Kolakofsky D., and B.W. Mahy (eds), Genetics and pathogenicity of negative strand viruses. Amsterdam, New York, USA

Rademacher, T.W., R.B. Parekh, and R.J.L. Dwek. 1988. Glycobiology *Annu. Rev. Biochem.* 57, 785-838.

Sambrook, J. and D.W. Russell. 2001. Molecular Cloning A Laboratory Manual, 3rd (ed), Cold Spring Harbor Laboratory Press, New York, USA

Schmaljohn, C.S. 1996. Molecular biology of *hantavirus*, p. 63-90. *In* R.M. Elliott. (ed.), The *Bunyaviridae*. Plenum Press, New York, N.Y., USA

Schmaljohn, C.S., Y.K. Chu, A.L. Schmaljohn, and J.M. Dalrymple. 1990. Antigenic subunits of Hantaan virus expressed by baculovirus and vaccinia virus recombinants. *J. Virol.* 64, 3162-3170.

Schmaljohn, C.S. and J.M. Dalrymple. 1983. Analysis of Hantaan virus RNA: Evidence for a new genus of *Bunyaviridae*. *Virology* 131, 482-491.

Schmaljohn, C.S., G.B. Jennings, J. Hay, and J.M. Dalrymple. 1986. Coding strategy of the S genome segment of Hantaan virus. *Virology* 155, 633-643.

Schmaljohn, C.S., A.L. Schmaljohn, and J.M. Dalrymple. 1987. Hantaan virus MRNA: Coding strategy, nucleotide sequence, and gene order. *Virology* 157, 31-39.

Schwarz, R.T., J.M. Rohrschneider, and M.F. Schmidt. 1976. Suppression of glycoprotein formation of Semliki forest, influenza, and avian sarcoma virus by tunicamycin. *J. Virol.* 19, 782-791.

Shi, X.H. and R.M. Elliott. 2002. Golgi localization of hantaan virus glycoproteins requires coexpression. *Virology* 300, 31-38.

Shi, X.H. and R.M. Elliott. 2004. Analysis of N-Linked glycosylation of Hantaan virus glycoproteins and the role of oligosaccharide side chains in protein folding and intracellular trafficking. *J. Virol.* 78, 5414-5422.

Tischler, N.D., A. Gonzalez, T.A. Perez, M. Rosemblatt, and P.D.T. Valenzuela. 2005. *Hantavirus* Gc glycoprotein: evidence for a class II fusion protein. *J. Gen. Viyol.* 86, 2937-2947.

Wang, M., D.G. Pennock, K.W. Spik, and C.S. Schmaljohn. 1993. Epitope mapping studies with neutralizing and non-neutralizing monoclonal antibodies to the G1 and G2 envelope glycoproteins of Hantaan virus. *Virology* 197, 757-766.

Weissenhorn, W., A. Dessen, L.J. Calder, S.C. Harrison, J.J. Skehel, and D.C. Wiley. 1999. Structural basis for membrane fusion by enveloped viruses. *Mol. Membr. Biol.* 16, 3-9.

White, J.M. 1990. Viral and Cellular Membrane Fusion Proteins. *Annu. Rev. Physio.*, 52, 675-697.

Zimmer, G., I. Trotz, and G. Herrler. 2001. N-Glycans of F protein differentially affect fusion activity of human respiratory syncytial virus. *J. Virol.* 75, 4744-4751.