Cell Fusion Activities of Hantaan Virus Envelope Glycoproteins

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Received 2 February 2004/Accepted 18 May 2004

Hantaan virus (HTNV)-infected Vero E6 cells undergo cell fusion with both infected and uninfected cells under low-pH conditions. Flow cytometry and fluorescence microscopy of HTNV-infected Vero E6 cells showed that envelope glycoproteins (GPs) were located both on the cell surface and in the cytoplasm. Neutralizing monoclonal antibodies (MAbs) against the G1 and G2 envelope GPs inhibited cell fusion, whereas nonneutralizing MAbs against G1 or G2 and MAbs against the nucleocapsid protein (NP) did not. Transfected Vero E6 cells that expressed GPs but not those that expressed NP fused and formed syncytia. These results indicate that HTNV GPs act as fusogens at the cell surface. No fusion activity was observed either in infected Vero cells that were passaged more than 150 times or in BHK-21 cells, although GPs appeared to localize to the cell surface. This variability in fusion induction suggests the involvement of host cell factors in the process of cell membrane fusion.

Viruses of the genus *Hantavirus* in the family *Bunyaviridae* possess a negative-sense RNA genome that consists of three segments, designated the large (L), medium (M), and small (S) segments. The S segment encodes the nucleocapsid protein (NP), the M segment encodes a glycoprotein (GP) precursor that is cotranslationally cleaved into envelope GPs G1 and G2, and the L segment encodes the viral RNA polymerase (10). Hantaviruses cause two severe human diseases: hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome. In addition, some hantaviruses are apathogenic for humans. Hantaviruses are maintained in the rodent reservoir and are transmitted to humans via contaminated excreta.

Hantavirus GPs induce neutralizing antibodies (4) and agglutinate goose erythrocytes (24). G1 and G2 seem to be type 1 transmembrane proteins that form heterodimers in the endoplasmic reticulum (ER) during the intracellular assembly of virus particles (1). As is the case for other members of the *Bunyaviridae* family, hantavirus GPs are retained in the Golgi membrane, and mature virus particles bud into the Golgi compartment (1, 9).

Tissue culture cells that are infected with hantaviruses related to hemorrhagic fever with renal syndrome, such as Hantaan virus (HTNV) and Seoul and Puumala viruses, undergo cell-cell fusion under conditions of low pH (5, 14). In general, enveloped viruses fuse their envelopes with the endosomal membrane under acidic conditions and cause low-pH-dependent fusion of infected cells (fusion from within), which is mediated by GPs (7, 11). In addition, it has been reported that lysosomotropic agents inhibit hantavirus entry (12). Thus, hantavirus GPs are considered fusogens, although there is no direct evidence that they show this activity.

To solve the question “Are hantavirus GPs fusogens?” in

this study, we show that cell fusion is induced by the expression of GPs from their respective cDNA sequences. Furthermore, we evaluate the expression of GPs on the cell surface by fluorescence analysis and by using fusion inhibition assays with monoclonal antibodies (MAbs). This is the first experimental demonstration that GPs promote fusion-inducing activities on cell surfaces.

MATERIALS AND METHODS

Cells and virus. The Vero E6 cell line (ATCC C1008; CRL 1586) was grown and maintained in Eagle's minimal essential medium (EMEM) (Invitrogen, Grand Island, N.Y.), which was supplemented with 10% fetal bovine serum (FBS), a mixture of nonessential amino acids (Invitrogen), and 2 mM L-glutamine the supplemented medium was designated G-EMEM. The Vero E6 cell line that was used in these experiments was within five subcultures of the stock that was obtained from ATCC. We found previously that after >150 subcultures, infected Vero E6 cells did not show low-pH-dependent cell-cell fusion. Therefore, we selected one of the high-passaged Vero E6 cell lines and designated it Vero E6-H. The Vero E6-H and BHK-21 cell lines were grown and maintained in EMEM supplemented with 5% FBS and 2 mM L-glutamine. P388D1 cells originating from DBA/2 mice were purchased from the American Type Culture Collection (TIB63) and grown in RPMI 1640 medium (Flow Laboratories, Inc., McLean, Va.), supplemented with 5% FBS, 4×10^{-5} M 2-mercaptoethanol, and 2 mM L-glutamine. P388D1 cells continuously infected with HTNV were prepared as antigen-presenting cells as was done previously (2). HTNV strain 76118, clone 1 (8, 23) was propagated in Vero E6 cells. The culture supernatant was collected 10 days after inoculation and stored at -80°C for use as the stock virus.

Antibodies. Eleven different MAbs against the envelope GPs G1 and G2 and against the NP were prepared as mouse ascitic fluids by inoculating hybridoma cells into mice. The MAbs were purified from the ascites with the Affi-gel Protein A MAPS II kit (Bio-Rad Laboratories, Hercules, Calif.). The concentrations of the antibodies were adjusted to 500 $\mu\text{g}/\text{ml}$ of phosphate-buffered saline (PBS) and used as stock solutions. The MAbs raised against G2 (5B7) and the NP (ECO2) were labeled with Alexa Fluor 488 and Alexa Fluor 546 dyes, respectively, with a Monoclonal Antibody Labeling kit (Molecular Probes, Inc., Eugene, Oreg.) and adjusted to a concentration of 100 $\mu\text{g}/\text{ml}$ as the stock solution.

Cell fusion and fusion inhibition assays. The cell fusion assay was performed as described previously, with some modifications (5). The infected cells were exposed to prewarmed (37°C) EMEM, which was adjusted to pH 5.8 (this medium was designated low-EMEM) for 1 to 5 min. The medium was subsequently replaced with G-EMEM and incubated at 37°C for 4 to 16 h. The cells were then washed with PBS, fixed, and stained with Giemsa (E. Merck, Darmstadt, Germany). The fusion index was calculated as $[1 - (\text{number of cells}/$

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FIG. 1. Low pH-triggered syncytium formation of HTNV-infected Vero E6 cells. Vero E6 cells were infected with HTNV at an MOI of 0.1. Infected and uninfected Vero E6 (mock-treated) cells were treated with low-EMEM (pH 5.8) for 5 min and then incubated with G-EMEM for 1 h. The cells were fixed with methanol and stained with Giemsa. Infected cells treated with low-EMEM (A), infected cells not treated at low pH (B), and mock-infected cells treated with low EMEM (C) are shown.

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

The cell fusion inhibition assay was performed as follows. The infected cells were incubated at 37°C for 30 min, with twofold serial dilutions of the MAb in 5% FBS into PBS at pH 7.2. The anti-NP MAb (C16D11) (26) was used as the control. The cells were then washed to remove free antibody and exposed to prewarmed low-EMEM for 3 min. After eliminating the low-EMEM, the medium was replaced with G-EMEM and incubated at 37°C for 1 h. Finally, the cells were fixed with methanol and stained with Giemsa, and the fusion index was calculated as described above.

Cell fusion in cocultures of infected and uninfected cells. Two different fluorescent dyes were used to distinguish uninfected cells from infected cells during the coculture experiment. Uninfected cells were labeled with the fluorochrome probe CellTracker Orange CMTMR {5-(and-6)-[(4-chloromethyl) benzoyl amino] tetramethylrhodamine}, and the infected cells were labeled with the fluorochrome probe CellTracker Green CMFDA (5-chloromethylfluorescein diacetate; both from Molecular Probes), according to the manufacturer's protocol. Briefly, the cells were incubated for 45 min at 37°C in EMEM that contained 1 μ g of CMTMR or CMFDA/ml, washed extensively with EMEM to remove the unincorporated dye, and then incubated for an additional 30 min at 37°C in EMEM. The infected and uninfected cells were subsequently trypsinized and mixed together in a 1:1 ratio. The suspension of mixed cells was seeded onto eight-well Teflon-coated glass slides (Cell Line; Erie Scientific Co., Portsmouth, N.H.). After 2 h of incubation, the medium was replaced with PBS (pH 5.8) that was prewarmed to 37°C for 1 min and further incubated with G-EMEM (pH 7.2). The cells were fixed in 3% paraformaldehyde, and the fluorescence was examined and photographed with a confocal microscope (Axiophot; Zeiss, Oberkochen, Germany). CellTracker Green CMFDA emits green fluorescence, whereas CellTracker Orange CMTMR emits red fluorescence. This distinguishes infected and uninfected cells, as well as fused cells, which emit yellow fluorescence due to dual labeling.

Expression of recombinant envelope GPs G1 and G2 in Vero E6 cells and cell fusion experiments. The cDNAs that contain the coding sequences for the HTNV GPs G1 and G2 and the NP were subcloned into the expression plasmid pCAGGS/MCS (15) and designated pCHTNM (16) and pCHTNS (27), respectively. Vero E6 cell monolayers at 80% confluency were prepared in eight-chamber LabTek Chamber Slides (Nunc, Inc., Naperville, Ill.) 1 day before transfection. Plasmid DNA (0.2 μ g) was transfected with Trans IT-LT1 (Pan-Vera, Madison, Wis.) according to the manufacturer's protocol. After 48 h of incubation, two chambers of the slide were washed once with PBS and replaced with prewarmed low-EMEM (pH 6.0) for 2 min. The medium in the two chambers of the slide was then replaced with G-EMEM (pH 7.2). The cells were incubated in G-EMEM for 4 to 24 h at 37°C. The two chambers of the slide that were not treated with low-EMEM were used as the controls. The cells were then fixed with 10% formaldehyde in PBS for 10 min and treated with 0.1% Triton X-100 in PBS at room temperature for 5 min. After being washed with PBS, the cells in the first chamber were stained with the anti-G2 MAb 5B7, which was labeled with Alexa Fluor 488, and the cells in the second chamber were stained with Giemsa.

Focus reduction neutralization test (FRNT). The FRNT test was performed as described previously (3). Briefly, 100 μ l of serial twofold dilutions of the purified MAbs was mixed with an equal volume of the virus suspension, which contained 200 focus-forming units of virus, at 37°C for 1 h. Then, 50 μ l of each mixture was inoculated onto a Vero E6 cell monolayer on a 96-well plastic plate and incubated at 37°C for 1 h in a CO₂ incubator. After removal of the inoculum, the cells

were overlaid with medium that contained 1.5% carboxymethyl cellulose. After incubation for 7 days, the monolayers were fixed with acetone/methanol (1:1), dried, and stored at -80°C until used. Infected cell foci were detected immunohistochemically with anti-NP monospecific polyclonal antibodies. The FRNT titer was expressed as the highest antibody dilution that resulted in a reduction of >80% in the number of infected cell foci.

Fluorescence assay (FA) for the detection of envelope GPs and NP at the cell surface and in the cytoplasm. Cells were prepared on 24-well glass slides 1 day before virus inoculation. One day after incubation, the cells were washed with PBS containing 1 mM Ca²⁺, Mg²⁺, and 0.1% NaN₃ [designated PBS (+)] at 4°C. For cell surface staining, the cells were fixed with 2% paraformaldehyde in PBS. For intracellular staining, the cells were fixed with acetone. The fixed cells were incubated for 1 h at 37°C with a mixture of the Alexa Fluor 488-labeled anti-G2 MAb 5B7, which fluoresces green, and the Alexa Fluor 546-labeled anti-NP MAb ECO2, which fluoresces red, at a dilution of 1:500. For nuclear counterstaining, the infected cells were incubated for 30 min with 0.1 μ g of Hoechst 33342 (Molecular Probes)/ml, which emits blue fluorescence. After being washed with PBS (+), the cells were cultured for 1 h. The samples were examined under a fluorescence microscope (Nikon model E600) with UV2A, B-2A, and G-2A filters, which detect the colors blue, green, and red, respectively. Specific fields were photographed with a digital camera.

Flow cytometry. The cells were dispersed with 0.1% trypsin (1:250 dilution; Difco) and 0.02% EDTA in PBS and washed with fluorescence-activated cell sorter (FACS) solution (0.5% BSA plus 0.01% NaN₃ in PBS). The cell suspension was adjusted to a concentration of 10⁵ cells in a 100 μ l of FACS solution, and fixed with 1% paraformaldehyde in PBS for 10 min at room temperature. After the cells were washed with FACS solution, 1:100 dilutions of the purified MAbs in FACS solution were added, and the mixture was incubated for 30 min. After cells were washed with FACS solution, fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin G antibodies were added, and the mixture was incubated for 30 min. After being washed with FACS solution, the cells were suspended in 500 μ l of FACS solution and analyzed with a FACSCalibur system (Becton Dickinson). The data were evaluated with Becton Dickinson FACSscan research software, CellQuest version 3.0.1.f.

RESULTS

Low-pH-triggered syncytium formation by HTNV-infected Vero E6 cells. Syncytium formation by infected Vero E6 cell monolayers appeared at low pH (Fig. 1A) but not at neutral pH (Fig. 1B). Uninfected monolayers did not form syncytia under low-pH conditions (Fig. 1C). To confirm that the infected cells could fuse with uninfected cells, we employed a coculture system with infected and uninfected cells, which were labeled with different fluorescence markers. As shown in Fig. 2A, infected (green) and uninfected (red) cells were clearly distinguishable. Syncytia appeared after treatment at low pH (Fig. 2B). The colors of the syncytia varied from reddish yellow to greenish yellow. Since color tone is considered to reflect the ratio of the numbers of infected and uninfected cells, these

FIG. 2. Infected Vero E6 cells fuse with uninfected cells. Cell fusion was examined in cocultures of infected and uninfected Vero E6 cells. The cells were infected with hantavirus at an MOI of 0.05. Eleven days after infection, the cells were labeled with CellTracker Green CMFDA (green fluorescence). The uninfected cells were labeled with CellTracker Orange CMTMR (red fluorescence). The cells were trypsinized, and equal numbers of infected and uninfected cells were mixed and seeded onto eight-well glass slides. Two hours after being seeded, the cells were treated with low-EMEM (A) or left untreated (B). After the low-EMEM was replaced by G-EMEM, the cocultures were incubated for 16 h and then fixed with 3% paraformaldehyde. The fluorescence microscopy photographs taken with FITC and rhodamine filters are overlaid.

results show that HTNV-infected cells are able to fuse with both infected and uninfected cells.

Cell fusion mediated by recombinant GPs. To obtain direct evidence that HTNV GPs induce low-pH-dependent cell fusion, recombinant GPs were expressed transiently in Vero E6 cells by cloning the cDNA sequences into the pCAGGS mammalian expression vector. Furthermore, the cell fusion activities of the transfected cells were examined. The expression of recombinant GPs was confirmed by a FA with the anti-GP MABs (Fig. 3A). After treatment at low pH, the cells that expressed GPs formed syncytia (Fig. 3B and D). These results clearly show that the HTNV GPs function as fusogens. The syncytia were formed, presumably, by the fusion of GP-expressing cells with neighboring cells that did not express GPs, since the syncytia (Fig. 3B) appeared larger than the infected cell foci (Fig. 3A). This observation is consistent with the results of the coculture experiment with infected and unin-

FIG. 3. Expression of recombinant envelope GPs and cell fusion. GPs were expressed in Vero E6 cells by transfection with pCHTNM. After 48 h of transfection, the cells were treated with low-EMEM (B and D) or left untreated (A and C). Fluorescent antibody staining for envelope GPs (A and B) and Giemsa staining (C and D) of cells are shown.

fected cells, in that infected or GP-bearing cells were able to induce cell fusion with normal cells.

Demonstration of cell surface expression of GPs. To examine further the role of GPs in cell fusion, surface localization of GPs was examined by flow cytometry and immunofluorescent antibody staining of HTNV-infected Vero E6 cells. As shown in Fig. 4A, surface expression of GPs (G1 and G2), but not of the NP, was observed by flow cytometry. As further confirmation, we showed the localization of GPs and NP in Vero E6 cells by immunofluorescent antibody staining. In the results shown in the lower panels of Fig. 4B, the cells were fixed with acetone for intracellular staining. GPs and the NP were visualized by the Alexa Fluor 488-labeled anti-GP (green fluorescence) and Alexa Fluor 546-labeled anti-NP (red fluorescence) MABs, respectively. For counterstaining purposes, nuclear DNA was stained with Hoechst 33342 (blue fluorescence). The cells shown in Fig. 4B, upper panels, were fixed with paraformaldehyde for surface staining, and those shown in the lower panels were fixed with acetone for the assessment of intracellular distribution. The same field is shown in all three images. GPs were detected by the paraformaldehyde fixation method. The GP distribution patterns on the cell surface were diffuse. In contrast, intracellular GPs were localized around the perinuclear region, ER, and Golgi compartment. No surface staining was observed for the anti-NP MAB, although the NP was detected intracellularly, where it was more widely distributed than the GPs. These results indicate that HTNV GPs are able to localize to the cell surface, where they potentially mediate pH-dependent cellular fusion. In addition, the surface localization of recombinant GPs that were expressed in Vero E6 cells via pCHTNM transfection was confirmed by the same assay that was used for infected cells (Fig. 4C and data not shown).

Inhibition of cell fusion by anti-GP MABs. To further evaluate the fusion activities of the GPs and their localization, we screened a panel of MABs for their ability to bind to the cell surfaces of HTNV-infected Vero E6. Although all of the anti-GP MABs bound to surface-localized GPs, as shown by flow cytometry, only certain anti-GP MABs (Table 1) blocked cell fusion activity (Table 1). The 3D5, HCO2, and 11E10 MABs, which are directed against either G1 or G2, displayed neutralizing activities and inhibited syncytium formation. These results support the notion that surface-localized GPs are responsible for the low-pH-dependent cell fusion of HTNV-infected cells. In addition, the G1b, G2a, and G2c epitopes, which are involved in cell fusion, are also associated with neutralization of the infecting virus (Table 1).

Cell line dependency of HTNV-mediated cell fusion activity. We examined several cell lines for the ability to induce pH-dependent cell syncytium formation following infection (Table 2). All the cell lines were infected with HTNV at a multiplicity of infection (MOI) of 0.1 and incubated for 8 days, after which all the cells were confirmed as infected by FA staining with MABs against the GPs and the NP. Of the cell lines examined, only the Vero E6 cells that were purchased from the American Type Culture Collection and subcultured a maximum of five times showed low-pH-dependent cell fusion (Table 2). The Vero E6-H subline, which was subcultured >150 times, did not form syncytia. Moreover, the BHK-21 and P388D1 cells did

FIG. 4. Vero E6 cell surface expression of HTNV proteins. (A) Flow cytometric analysis. MAbs against HTNV NP (C16D11) and envelope GPs G1 (3D5) and G2 (HCO2) were used to detect the surface expression of the respective proteins. The level of MAb binding is indicated by the open histogram (with MAb) shift to the right from the solid control histogram (FITC-conjugated goat anti-mouse immunoglobulin G only). (B) Comparison of surface expression and intracellular expression of proteins in infected cells. MAbs 5B7-Alexa Fluor 488 and ECO2-Alexa Fluor 546 were used for the detection of the viral proteins GP and NP, respectively. Nuclei were visualized by staining with Hoechst 33342. (C) The surface expression of GPs in transfected Vero E6 cells that expressed GPs. Vero E6 cells were transfected with pCHTNTM; 48 h after transfection, cells were fixed for cell surface staining described in Materials and Methods. The MAbs 5B7-Alexa Fluor 488 was used for the detection of the viral GPs. (D) Production of GP and NP in p388D1 cells continuously infected with HTNV. The cells were fixed for inner-cell staining as described in Materials and Methods. The GPs and NP were detected as described in the legend to panel A.

not show syncytium formation. We attempted to detect the surface GPs that were responsible for cell fusion by flow cytometry analysis. A positive shift relative to the control histogram, which was obtained with the FITC-conjugated secondary antibody, was observed in infected Vero E6, Vero E6-H, and BHK-21 cells. However, surface GPs were not detected on HTNV-infected P388D1 cells, which was certainly infected with HTNV, and both N and GPs were being produced in the cytoplasm (Fig. 4D). These results indicate that HTNV GPs are presented on the cell surfaces of certain cell lines. Furthermore, cell fusion activity was not observed in Vero E6-H or BHK-21 cells. These results show that host-derived factors, other than GPs, are required for HTNV-induced cell fusion.

DISCUSSION

In this report, we used infected and transfected Vero E6 cells to provide the first experimental evidence that HTNV GPs act as fusogens. This fusion activity was independent of other viral components (e.g., the NP) and replication. Furthermore, the results suggest that both the surface transport of GP and GP-mediated fusion activities are cell line dependent.

Various enveloped viruses induce the fusion of the membranes of virus envelopes and cells through interaction with virus envelope GPs, although the precise mechanism underlying this phenomenon remains unclear (11). Nevertheless, it is generally accepted that the binding of GPs to their specific

TABLE 1. Inhibition of HTNV-induced cell fusion by MAbs against G1 and G2

Epitope ^a	MAB	Cell surface ^b	Fusion inhibition ^c	FRNT ^d
G1a1	8B6	ND ^d	—	—
G1a2	6D4/10F11	+	—	—
G1b	3D5	+	>64	>64
G2a1	HCO2	+	4	2
G2c	11E10	+	2	16
G2d	5B7	ND	—	—
G2e	20D3	+	—	—
G2f1	8E10	+	—	—
G2f2	7G6	+	—	—
N	ECO2	—	—	—
N	C16D11	—	—	—

^a As determined by the competitive binding assay (4).

^b ND, not determined; +, MAB bound to cell surface; —, MAB did not bind to cell surface.

^c Highest MAB dilution that resulted in a reduction of approximately 50% in the fusion index of the pH 5.8-treated HTNV-infected cells. The starting MAB concentration was 0.5 mg/ml.

^d Highest MAB dilution that resulted in a reduction of approximately 80% in the number of infected cell foci. The starting MAB concentration was 0.5 mg/ml. —, <1.

receptors on the cell surface is an important stage in establishing close contact between the two membranes. In this scenario, specific regions of the GPs make contact with the cell membrane, with or without conformational changes to the GPs, followed by the initiation of membrane fusion. In this study, we clearly showed that HTNV-infected cells fuse with each other under conditions of low pH. In addition, the coculture experiments with infected and uninfected cells showed that infected cells are able to fuse with uninfected cells with the same efficiency as infected cells fuse with each other (Fig. 2). The surface localization of the GPs was detected by flow cytometry analysis and fluorescent antibody analysis (Fig. 4). In addition, several MAB clones inhibited membrane fusion when targeted to the cell surface (Table 1). These results strongly suggest that HTNV GPs locate to the surface of the infected cell, where they interact with the cell membranes of other cells, probably via specific receptors.

In this study, we induced pH-dependent cell fusion by expressing recombinant GPs in transfected Vero cells. This is the first direct evidence that HTNV GPs are fusogens. In contrast to the expression systems described in previous reports (21), the expression vector used in our study did not require the provision of the polymerase from other vectors. Our system permits longer incubation periods without the cytopathic effect and allows the induction of syncytium formation to the same

extent as that seen in authentic virus-infected cells. Cell fusion was not observed in NP-expressing cells. No enhancement of the fusion phenomenon was observed after the coexpression of GPs and the NP (data not shown). Therefore, it appears that only the GPs are fusogens. As shown in Fig. 3B, GP-expressing cells appeared to fuse with nontransfected cells, since the syncytia were apparently larger than the GP-expressing cell foci. These results provide evidence that HTNV GPs interact with normal cell membranes.

Hantavirus-induced, pH-dependent cell fusion has been reported previously for HTNV and Seoul and Puumala viruses (5, 14). Thus, low-pH-dependent cell fusion may be a common characteristic of GPs from viruses in the genus *Hantavirus*, as seen for viruses in *Influenzavirus*, *Togavirus*, *Flavivirus*, *Rhabdovirus*, *Bunyavirus*, and *Arenavirus* (11). Therefore, the low-pH-dependent cell fusion caused by hantavirus GPs may act via the same mechanism as that which effects fusion between virion and endosome membranes during virus entry, as reviewed previously (11). As shown in Table 1, the fusion related-epitopes G1b, G2a, and G2c overlapped completely with neutralization-related epitopes. In general, either the neutralization of virus infectivity is caused by the inhibition of virus absorption to the receptor or it occurs during the uncoating steps. Jin et al. have reported the partial inhibition of hantavirus entry by a lysosomotropic agent (12). This observation suggests that hantavirus uncoating results from the fusion of the virion envelope with the endosome membrane (12). Therefore, the neutralization of virus infectivity seen with the MAbs used in the present study may be due to the inhibition of cell fusion.

The domains on HTNV GPs that direct cell fusion remain to be identified. The fusion domains of certain viruses, such as influenza virus and Sendai virus, are exposed as fusion peptides after protease digestion (7). On the other hand, some viruses possess internal fusion domains that act as fusogens in the absence of specific cleavage by proteases (17). The HTNV GPs appear to have internal fusion domains, as there has been no report to indicate that proteolysis is essential for HTNV infectivity. To confirm the protein responsible for fusion, we expressed the G1 and G2 proteins from their cDNA sequences and examined their fusion-inducing activities. However, since neither the G1 nor the G2 protein alone localized to the cell surface, and no cell fusion was observed, the potential role of the hydrophobic domains could not be confirmed. On the other hand, cell fusion was observed in cells that coexpressed G1 and G2 (data not shown). These observations indicate that both G1 and G2 are necessary for the collective transportation of GPs. Figure 5 shows a schema of HTNV GPs. The hydrophobic regions in the GPs were predicted from the amino acid sequences and are shown to include two signal peptides (S1 and S2) and possible transmembrane regions (H1, H2, H3, H4, H5, and H6). The two signal peptides must be removed by signal peptidase (19). However, the C terminal of the G1 protein has not yet been determined. The schema shows that G2 protein is a typical type 1 membrane protein. In addition, anti-G2 antibody was induced predominantly, rather than anti-G1 antibody, despite the difference in the molecular weight. Most of the anti-G2 MAbs possess hemagglutination inhibition activity (4). This information suggests that the G2 protein locates more externally than G1 and has a role in binding to

TABLE 2. Cell surface detection of GPs and cell fusion activities of various infected cells^a

Cell line	Detection of GPs on cell surface	Fusion at pH 5.8
Vero E6	+	+
Vero E6-H	+	—
BHK-21	+	—
P388D1	—	—

^a The cell fusion assay and FACS analysis were performed 8 days after infection.

FIG. 5. Schema for HTNV GPs. Signal peptide and transmembrane regions were predicted with the SOSUI program (<http://sosui.proteome.bio.tuat.ac.jp/sosui/frame0.html>) and the SignalP server, version 3.0 (<http://www.cbs.dtu.dk/services/SignalP/>). Signal peptides were designated S1 and S2, and possible transmembrane regions were shown as black or gray boxes labeled H1 to H6. Black boxes represent primary transmembrane regions, and gray boxes represent secondary transmembrane regions. N-terminal regions of G1 and G2 were shown in a previous published study (19). A total of five possible N-glycosylation sites were also demonstrated previously (20). Open circles indicate amino acids which were mutation sites in viruses eluding FRNT MABs (13, 25).

the cell surface. By contrast, the structure of G1 is difficult to predict. Three out of six anti-G1 MABs (2D5, 3D5, and 16D2) (4) had FRNT activity equivalent to fusion inhibition activity. Furthermore, the hydrophobic regions H1 through H5 are candidates to be the internal fusion domain of G1. Therefore, G1 may be a protein responsible for fusion. However, the structures of G1 and G2 are interdependent, because many FRNT MAB-related mutations are found in G1, along with two mutations in G2. The FRNT and fusion regions should be discontinuous structures in both G1 and G2. Alternative approaches, such as the introduction of single mutations into the GP sequences, are needed to elucidate the regions of G1 and G2 that are responsible for cell fusion.

As shown in Table 2, the multiple passaged Vero E6 cell subline, Vero E6-H, did not show fusion activity under low-pH conditions, although the Vero E6-H cells expressed levels of G1 and G2 or $\beta 1$ and $\beta 3$ integrin molecules on the cell surface that were comparable to those observed with Vero E6 cells (data not shown). Therefore, we speculate that cellular factors are also involved in the regulation of low-pH-dependent cell fusion. HTNV-induced cell fusion was observed in the original Vero E6 cells used in a previous study (5). In that study, the cells were passaged many times using EMEM (Nissui, Tokyo, Japan) without supplemental nonessential amino acids. After about 150 passages, the shape of the cells was similar to that of ordinary Vero cells, rather than Vero E6 cells. During passage, the mutant line converted back to the original cell line because it was able to grow faster than the original cells. Unfortunately, no intermediate-passaged cells remained, such as those after 50 or 100 passages. The difference between the original and Vero E6-H cells is unclear.

We have also found that one cell line (BHK-21) is able to transport GP to the cell surface, while another cell line (P388D1) is not (Table 2). These results show that the transportation of GPs in infected cells varies by cell type. Interestingly, the GPs in P388D1 cells were distributed as focal dots, which might have been limited to the Golgi complex and did not completely correspond with NP distribution (Fig. 4D). P388D1 is an effective antigen-presenting cell that is derived

from the mouse. Previously, it was suggested that HTNV persistence in the mouse was related to the suppression of CD8 T cells (2). In addition, cell-dependent differences in the behavior of GPs might partially explain the difference in pathogenesis in humans and the reservoir rodent. In a recent study, Spiropoulou et al. showed that Sin Nombre virus GPs localized to the cell surface only at the late stage of infection (22). Similarly, our study demonstrates that HTNV GPs can localize to the cell surface but only in certain combinations of viruses and cell lines (Fig. 4 and Table 2).

A previous study of HTNV stability at various pH levels showed that the virus titer decreased abruptly at pH 6.5 and that the virion was completely inactivated at pH 6.3 (18). In the present study, cell fusion occurred at pH 6.3 and, similar to the results of the previous report, conformational changes appeared to occur in the HTNV GPs at around pH 6.3. We propose that low-pH virus inactivation is induced by irreversible conformational changes in the viral GPs.

As is the case with other member viruses of the family *Bunyaviridae*, it is generally accepted that hantaviruses mature in the ER and bud into the Golgi apparatus. The transportation of GPs to the cell surface and fusion ability varied, depending on the cell line; therefore, it may be that surface localization of GPs occurs only under particular conditions. Moreover, the role of the GPs on the cell surface remains to be clarified. Nevertheless, the characterization of the cell fusion activities of GPs provides important information regarding the structure-function relationships of GPs. In addition, the discovery of cellular factors that inhibit hantavirus-induced cell fusion may lead to drugs that block viral infection, such as those developed for the treatment of infections involving respiratory syncytial virus (6).

ACKNOWLEDGMENTS

We thank C. S. Schmaljohn for the hybridomas and cDNA clones of the HTNV M and S segments. We also thank A. Lundkvist (Swedish Institute for Infectious Disease Control, Sweden) for discussion of, and comments on, the manuscript. We acknowledge Textcheck (English consultants) for the grammatical revision of the final draft of the paper.

M.O. was a Research Fellow of the Japanese Society for the Promotion of Science (JSPS) and was supported by JSPS Research Fellowships for Young Scientists. This work was also supported, in part, by Grants-in-Aid for Scientific Research and the Development of Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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