Biomedical Research (Tokyo) **38** (2) 89–97, 2017

The amino acid at position 624 in the glycoprotein of SFTSV (severe fever with thrombocytopenia virus) plays a critical role in low-pH-dependent cell fusion activity

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(Received 11 December 2016; and accepted 19 December 2016)

ABSTRACT

Severe fever with thrombocytopenia syndrome virus (SFTSV) is a novel phlebovirus responsible for causing an emerging zoonotic disease. We previously established subclones from SFTSV strain YG1 based on differences in low-pH-dependent cell fusion activities and found two amino acid substitutions, Y328H and R624W, in the envelope glycoprotein (GP) of high fusion subclones. In this study, we show that transiently expressed GP with the R624W mutation, but not the Y328H mutation, induced cell fusion under acidic conditions. GP possessing either tryptophan, serine, glycine or aspartic acid at position 624 induced cell fusion, whereas GP possessing basic amino acids such as arginine or lysine did not induce cell fusion. These results indicated that the amino acid at position 624 has an important role for inducing low-pH-dependent cell fusion.

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging zoonosis with a high case fatality rate characterized by thrombocytopenia, leukopenia, and fever. SFTS was first reported in a rural area of China in 2005 as a disease of unknown cause, and it was not until 2009 when the causative virus, SFTS virus (SFTSV), was identified from a patient (8, 14, 23, 27, 29). In 2012, SFTSV was also isolated in South Korea and Japan (28, 30, 31). In the latter case, the virus—termed SFTSV strain YG1—was isolated from a patient who died from a febrile illness of unknown etiology, although since the patient had never travelled abroad, she is thought to have been infected in Japan (24). Notably, two cases of a similar febrile illness with thrombocytopenia have been reported in Missouri, USA, and the causative agent, named Heartland virus, was determined to be a novel tick-borne phlebovirus closely related to SFTSV (18, 19).

SFTSV belongs to the genus *Phlebovirus* of the family *Bunyaviridae* (27, 29). SFTSV possesses a negative-strand RNA genome that consists of three segments, designated large (L), medium (M), and small (S). The L segment encodes an RNA-dependent RNA polymerase; the M segment encodes a glycoprotein precursor (GPC) that is co-translationally cleaved into Gn and Gc, which then form a spike complex known as the envelope glycoprotein (GP); and the S segment encodes a nucleocapsid protein and a non-structural protein (3, 29). As is the case for other viruses in the family *Bunyaviridae*, Gn and Gc of SFTSV are involved in virus attachment and entry (6, 12, 25). SFTSV attaches to host cell receptors and is endocytosed in a receptor-mediated manner. Acidification of the endocytic vesicle triggers a conformational change in GP, which then facilitates the fusion of the viral envelope with the vesicle membrane (15, 22, 26). In a recent study, the crystal structure of Gc was solved, and several amino acids were determined to be key factors for

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viral propagation (11, 17). Nevertheless, the molecular mechanism of cell fusion and the function of Gn and Gc of SFTSV remain to be elucidated.

In an effort to better understand the mechanism that mediates SFTSV cell fusion, we previously examined the low-pH-dependent cell fusion of YG1-infected cells (1, 21). A limiting dilution experiment revealed that the original stock of strain YG1 consisted of quasispecies, from which we were able to establish three sub-clones, each with different low-pH-dependent cell fusion activities (20). Two of the three subclones, B7 and A4, induced strong cell fusion under acidic conditions, and compared to the reported nucleotide sequence strain YG1 (GenBank accession number AB817987) had two common amino acid mutations: Y328H in Gn and R624W in Gc. The third subclone, E3, as well as the original stock of strain YG1, exhibited little cell fusion and did not possess the two point mutations in Gn and Gc.

In this study, we focused on the two amino acid mutations identified in Gn and Gc of the subclones that exhibited strong cell fusion activity. To investigate the molecular function of Gn and Gc for viral entry, we determined the amino acid that was responsible for induction of low-pH-dependent cell fusion and evaluated the function for cell fusion.

MATERIALS AND METHODS

Cells. Vero E6 cells were maintained in minimum essential medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 5% fetal bovine serum (Biowest, Riverside, MO, USA), insulin-transferrin-selenium (Gibco), non-essential amino acid (Gibco), penicillin-streptomycin mixture (Sigma, St Louis, MO, USA), and gentamicin (Sigma), which was referred to as growth medium. Cells were cultured at 37°C in 5% CO_2.

Viruses. SFTSV strain YG1 was kindly provided by Dr. Ken Maeda, Yamaguchi University (24). Subclones of SFTSV strain YG1 (E3 and B7) were selected by the limiting dilution method with low-pH-dependent cell fusion activity as an indicator (20).

Plasmids construction. Total RNA was extracted from cells infected with strain YG1 or subclone B7. cDNA was synthesized from extracted RNA using SuperScript II Reverse Transcriptase (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) with random primers (Invitrogen). A cDNA fragment containing the entire coding region of GPC was amplified from cDNA of strain YG1 and was inserted into the pCAGGS-MCS plasmid vector (pCSFTSV-GP). Two cDNA fragments containing each amino acid substitution site of Gn or Gc were amplified from cDNA of B7 and cloned into pCSFTSV-GP using specific restriction enzyme sites. Other amino acid substitutions R624S, R624G, R624K and R624D were introduced into pCSFTSV-GP by site-directed mutagenesis. The plasmid sequence was confirmed by Sanger sequencing prior to use.

Cell fusion and fusion index assay. Vero E6 cells were transfected with plasmids using TransIT-LT1 and incubated at 37°C for 48 h. The supernatants were replaced with 50 mM acetate-buffered saline or phosphate buffered saline with a pH range from pH 4.8 to 7.0. After incubation for 2 min at room temperature, the buffer was removed and fresh growth medium was added. After incubation for 16 to 24 h, cells were fixed with acetone (Kanto Chemical Inc., Tokyo, Japan) and stained with Giemsa's solution (Merck, Darmstadt, Germany). To calculate the fusion index, cells were fixed with acetone and stained by indirect immunofluorescence assay using a mixture of anti-Gn and Gc antibodies and Alexa Fluor 488 goat anti-rabbit IgG secondary antibody. Fusion index was calculated by the following formula: 1 − (number of GP-expressing cells/number of nuclei) (1). One to two hundreds of transfected cells from selected representative fields were counted for each experiment.

Flow cytometry. Vero E6 cells were transfected with plasmids using TransIT-LT1 and incubated at 37°C for 48 h. The cells were removed from the tissue culture plate by pipetting and suspended in flow cytometry buffer (PBS with 0.5% BSA and 5 mM EDTA). The cells were then stained for 1 h with anti-Gn and Gc antibodies and Alexa Fluor 488-labeled anti-rabbit IgG secondary antibody. After washing twice in flow cytometry buffer, the stained cells were diluted in flow cytometry buffer to the appropriate concentration and detected by FACS Verse Flow cytometer (BD Bioscience, Franklin Lakes, NJ, USA).

Indirect immunofluorescence assay. Vero E6 cells were transfected with plasmids using TransIT-LT1 (Mirus Bio LLC, Madison, WI, USA) and incubated at 37°C for 48 hours (h) according to the manufacturer's protocol. The cells were washed with PBS and fixed with 10% neutral buffered formalin (Nakarai, Kyoto, Japan). Cells fixed with 10% formalin were permeabilized with 1% Triton X-100 in PBS for 10 min. Gn and Gc expressed in the cells were

stained by an anti-Gn or anti-Gc antibody (#6647; rabbit polyclonal antibody was raised against a 19 amino acid synthetic peptide near the amino terminus of human SFTS Virus HB29 membrane glycoprotein or #6653, rabbit polyclonal antibody was raised against a 15 amino acid synthetic peptide near the carboxy terminus of human SFTS Virus HB29 membrane glycoprotein; ProSci Inc., CA, USA) and Alexa Fluor 488 goat anti-rabbit IgG secondary antibody (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA).

Structure modeling. The pre-fusion structure of SFTSV Gc was generated by homology modeling based on that of Rift Valley fever virus (RVFV) Gc (PDB code: 4HJ1). After 100 models of pre-fusion SFTSV Gc were generated using MODELLER 9v6 (7), the model with the best score for probability density function was chosen. The model was evaluated using Profiles-3D (16). The coordinates of the post-fusion structure of SFTSV Gc were taken from the crystal structure (PDB code: 5G47).

Statistics. Statistical analyses were performed with Welch's *t* test. *P* values of less than 0.05 were considered significant.

Biosafety statements. All studies with SFTSV were performed in the BSL-3 facility at the Graduate School of Medicine, Hokkaido University under the hazardous agents protocol approved by the Institutional Biosafety Committee of the Graduate School of Medicine, Hokkaido University.

RESULTS

A single amino acid residue plays a critical role for induction of low-pH-dependent cell fusion

In the previous study, we established subclones from SFTSV strain YG1 with different virological characteristics (20). Subclone B7, which had two amino acid differences in Gn and Gc (Y328H and R624W), induced strong cell fusion in infected cells under acidic (pH 5.6) conditions. In contrast, subclone E3 possesses 328Y and 624 R in Gn and Gc did not induce obvious cell fusion in infected cells (20).

In this study, to determine the amino acid responsible for the fusion activity, we constructed expression plasmids of SFTSV-GPC with either one or both amino acid mutations and evaluated their low-pH-dependent cell fusion activities in transfected cells (Fig. 1A). Cells transfected with each plasmid were treated with pH 5.6 acetate-buffered saline at 48 h post-transfection. As shown in Fig. 1B, GP with a tryptophan substitution at position 624 (GP-Y/W and GP-H/W) clearly induced cell fusion in transfected cells. On the other hand, GPs with an arginine at position 624, such as the one derived from subclone E3 (GP-Y/R), as well as mutant GP-H/R, did not induce cell fusion (Fig. 1B). To compare the fusion activities of GPs, we determined the fusion index in transfected cells. GP-Y/W showed the highest fusion activity (Fig. 1C). GP-H/W showed some cell fusion activity but it was significantly reduced compared with that of GP-Y/W. These results indicated that the amino acid substitution R624W was critical for induction of low-pH-dependent cell fusion, whereas the Y328H substitution in Gn potentially interfered with low-pH-dependent cell fusion. We next compared the pH ranges needed for induction of cell fusion in infected and transfected cells. Formation of syncytia was detected under pH 5.0 to 6.2 in GP-Y/W-expressing cells (Fig. 1D), which was similar in subclone B7 infected cells (data not shown). On the other hand, GP-Y/R did not cause cell fusion in transfected cells in the pH range tested. We also tested buffers with below pH 4.8 but the cells were not viable under these conditions.

Expression levels of GPs were similar in all mutants

To determine the reason for enhanced cell fusion activity of mutants, expression levels of GP on the cell surface were compared. We evaluated GP expression on transfected cells using flow cytometry with a mixture of anti-Gn and anti-Gc antibodies (Fig. 2A). No significant difference in GP expression was observed for any of the GP constructs possessing amino acid substitution R624W. To evaluate the overall expression of GP in transfected cells, cells were permeabilized with 0.1% saponin followed by staining with antibodies. Similar to the cell surface expression, no difference in total GP expression was observed among the transfected cells (data not shown). To confirm the levels of Gc expression on the cell surface, we detected Gc on non-permeabilzed cells. Immunofluorescence analysis revealed a small amount of Gc expression on the cell surface compared with cells transfected with empty plasmid (Fig. 2B). Moreover, in permeabilized cells, little co-localization of Gn and Gc with a plasma membrane marker was observed, and no difference was observed among the different GP mutants (Fig. 2C). Both Gn and Gc were observed to be peri-nuclear, and co-localized with a maker of endoplasmic reticulum or Golgi-complex, as has been previously demonstrated (data not shown, (26)), although a slightly

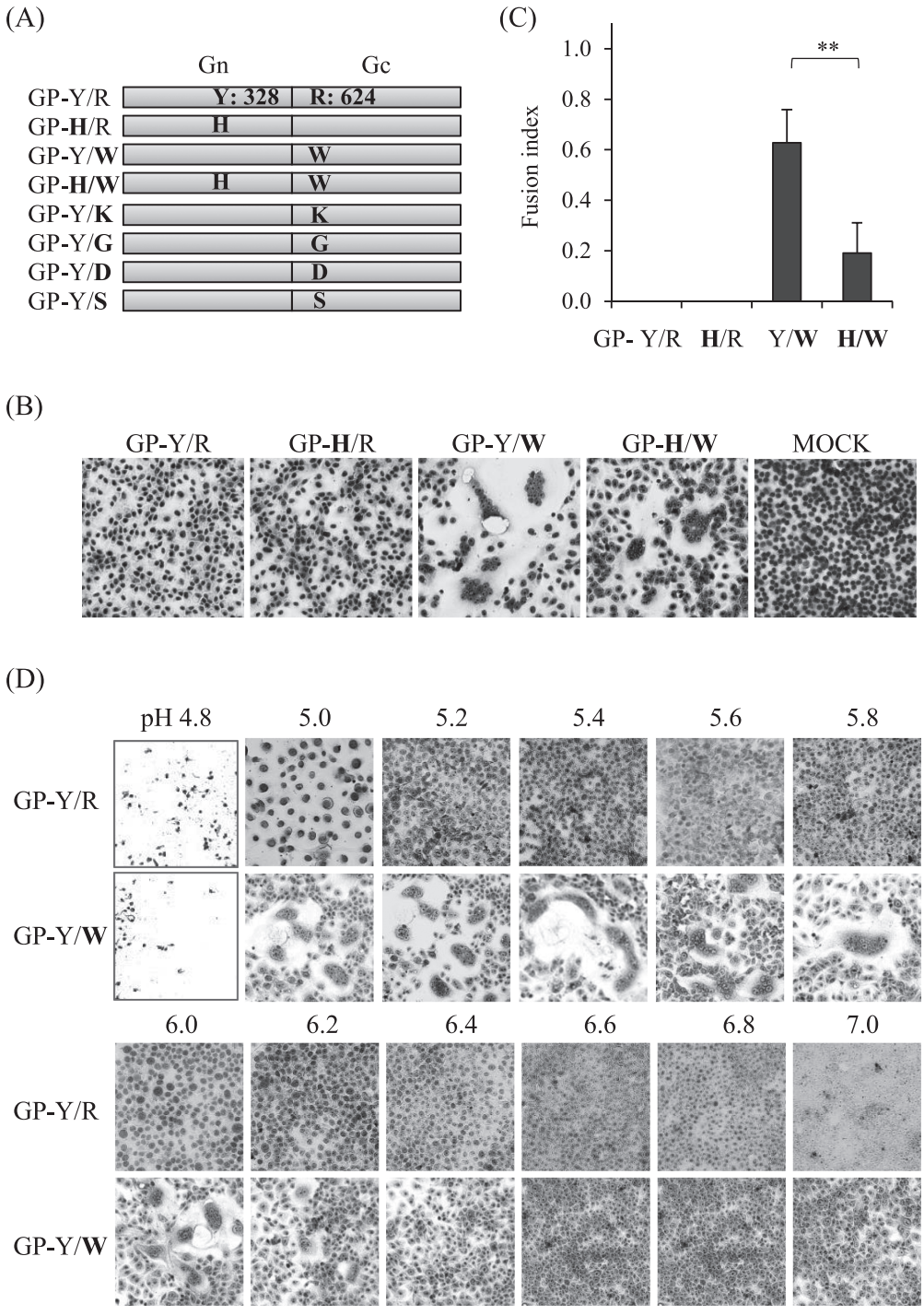

Fig. 1 Determination of responsible mutation in GP for induction of pH-dependent cell fusion. (**A**) Schematic diagram of GPs indicates the site of amino acid substitutions. Amino acids substituted from original sequence are shown in bold font. (**B**) Vero E6 cells were transfected with pCSFTSV-GP-Y/R, H/R, Y/W, H/W or mock empty plasmid. Cells were treated with pH 5.6 buffer for 2 min. At 24 h after treatment, cells were fixed by acetone and stained by Giemsa staining. (**C**) Vero E6 cells were transfected with pCSFTSV-GP-Y/R, H/R, Y/W or H/W and incubated at 37°C for 48 h. The cells were treated with pH 5.6 buffer for 2 min. At 24 h after treatment, cells were fixed by acetone and stained by indirect immunofluorescence assay. The number of cells and nuclei of Gc-expressing cells were counted, and the fusion index was calculated. The index is shown as an average of triplicate with standard deviation (S.D.). $^{**}P < 0.01$ (**D**) Vero E6 cells were transfected with pCSFTSV-GP-Y/R or GP-Y/W. Cells were treated with buffers from pH 4.8 to 7.0 for 2 min. The cells were fixed by acetone and stained by Giemsa staining.

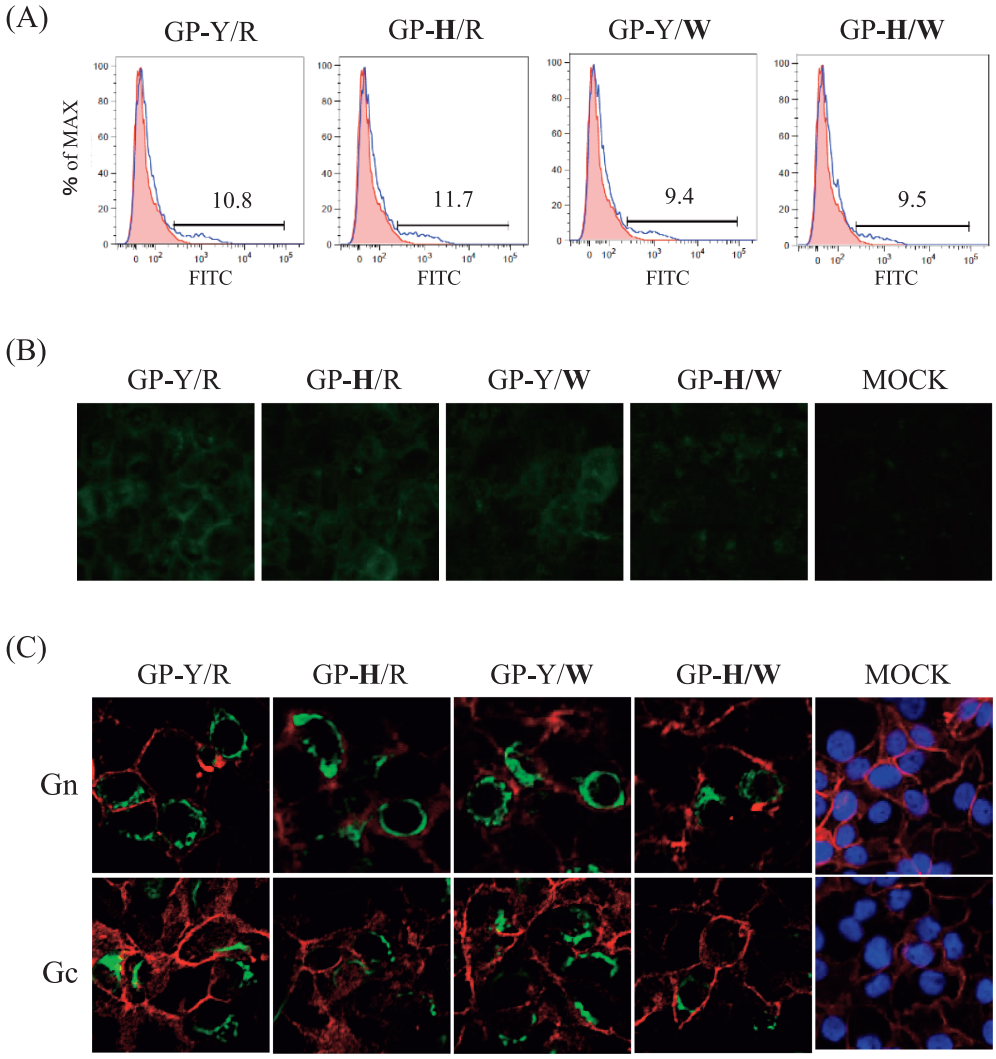

Fig. 2 Expression of GP in transfected cells. (**A**) Vero E6 cells were transfected with pCSFTSV-GP-Y/R, H/R, Y/W or H/W and incubated at 37°C for 48 h. The cells were collected in flow cytometry buffer and stained with anti-Gn and anti-Gc antibodies and with Alexa Fluor 488 goat anti-rabbit IgG secondary antibody. The red histogram line indicates mock cells transfected with an empty vector, and the blue histogram line indicates cells expressing GPs. (**B**) Cells transfected with pCSFTSV-GP-Y/R, H/R, Y/W, H/W or empty plasmid were fixed with 10% formalin/PBS at 48 h post-transfection. The cells were stained with anti-Gc antibody. (**C**) Cells transfected with pCSFTSV-GP-Y/R, H/R, Y/W, H/W or empty plasmid were fixed with 10% formalin and permeabilized with 1% TritonX-100 at 48 h post-transfection. The cells were stained with anti-Gn or Gc antibody (green) and anti-alpha1 sodium potassium ATPase antibody (red) as a plasma membrane marker.

higher background of fluorescence was observed by anti-Gn antibody.

Positively charged amino acid is important for SFTSV GP

Aside from subclone B7, the arginine at position 624 in Gc is highly conserved in SFTSV strains recorded in the database. However, strain SPL100A (GenBank accession number: AB985313, Japanese isolate) possesses another basic amino acid, lysine, at position 624, and Heartland virus possesses a serine, despite sharing high amino acid identity with SFTSV in the surrounding sequence (Fig. 3A). The more distantly related Rift Valley Fever virus (RVFV) and Uukuniemi virus (UUKV) have a serine and aspartic acid, respectively, at the site corresponding to position 624 in SFTSV. To evaluate the importance of the amino acid at position 624 for the induction of cell fusion, we analyzed the fusion activities of GPs with several different amino acids at this position (Fig. 1A). As shown in Fig. 3B, transfected cells expressing GP-Y/G or GP-Y/S, which have a glycine

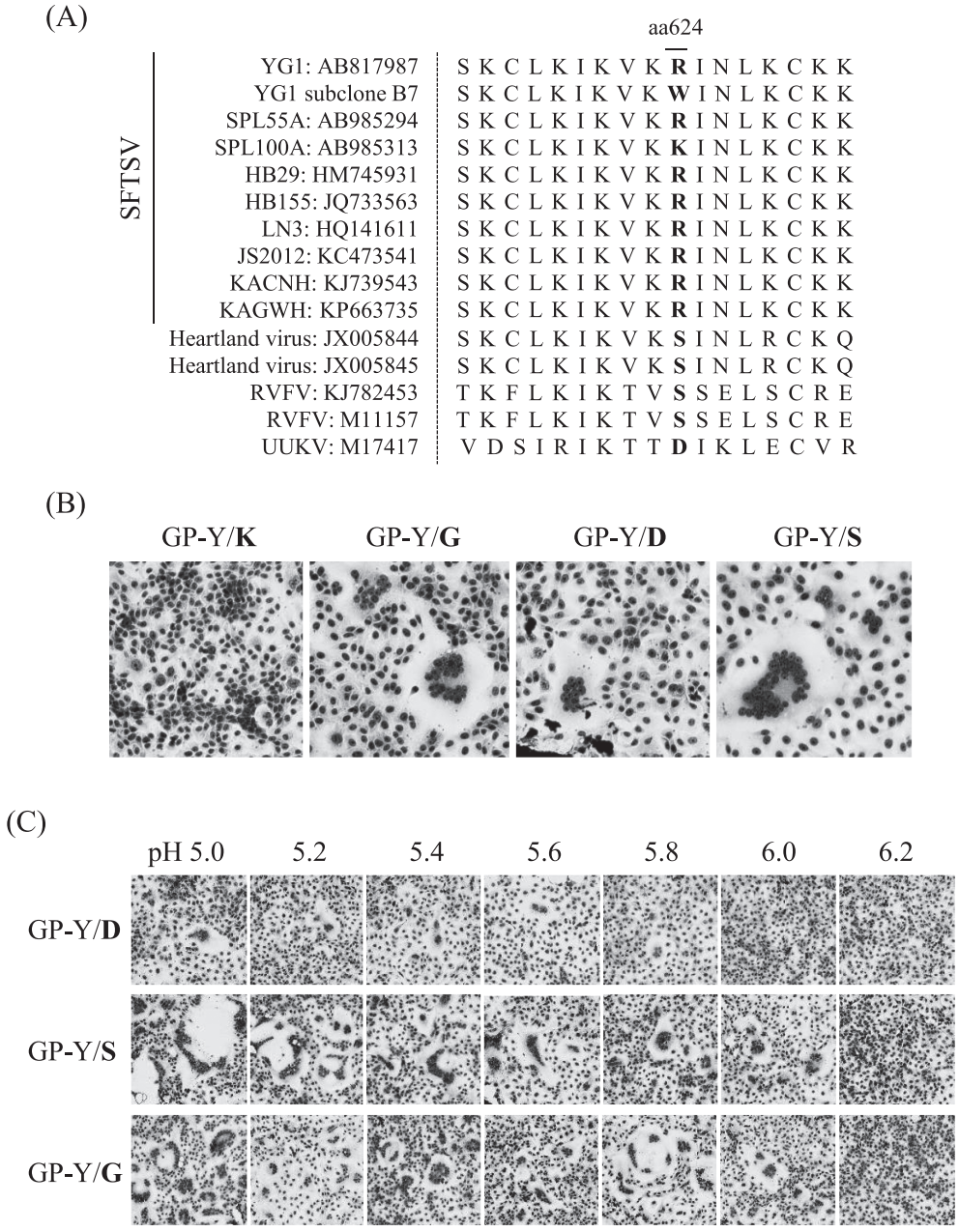

Fig. 3 Evaluation of fusion activities associated with amino acid at position 624. (**A**) Amino acid sequence alignment of SFTSV, Heartland virus, RVFV, and UUKV at amino acid positions 615 to 631. (**B**) Vero E6 cells were transfected with pCSFTSV-GP-Y/K, Y/D, Y/G orY/S and incubated at 37°C for 48 h. The cells were treated with pH 5.6 buffer for 2 min. At 24 h after treatment, cells were fixed by acetone and stained by Giemsa staining. (**C**) Vero E6 cells were transfected with pCS-FTSV- GP-Y/K, Y/D, Y/G or Y/S. Cells were treated with buffers from pH 5.0 to 6.4 for 2 min. The cell were fixed by acetone and stained by Giemsa staining.

and a serine, respectively, at position 624, showed formation of syncytia after pH 5.6 buffer treatment, as did GP-Y/W-expressing cells. On the other hand, a lysine at position 624 (GP-Y/K) did not cause cell fusion, despite similar levels of Gc expression. GP-Y/D, which has an acidic aspartic acid at position 624, induced cell fusion, although the syncytia were smaller than those formed by GP-Y/W (Fig. 3B). GP-Y/G and GP-Y/S also showed the same pH threshold for fusion as did GP-Y/W, and fusion was triggered below pH 5.8 in GP-Y/D expressing cells (Fig. 3C).

The crystal structure of the post-fusion form of

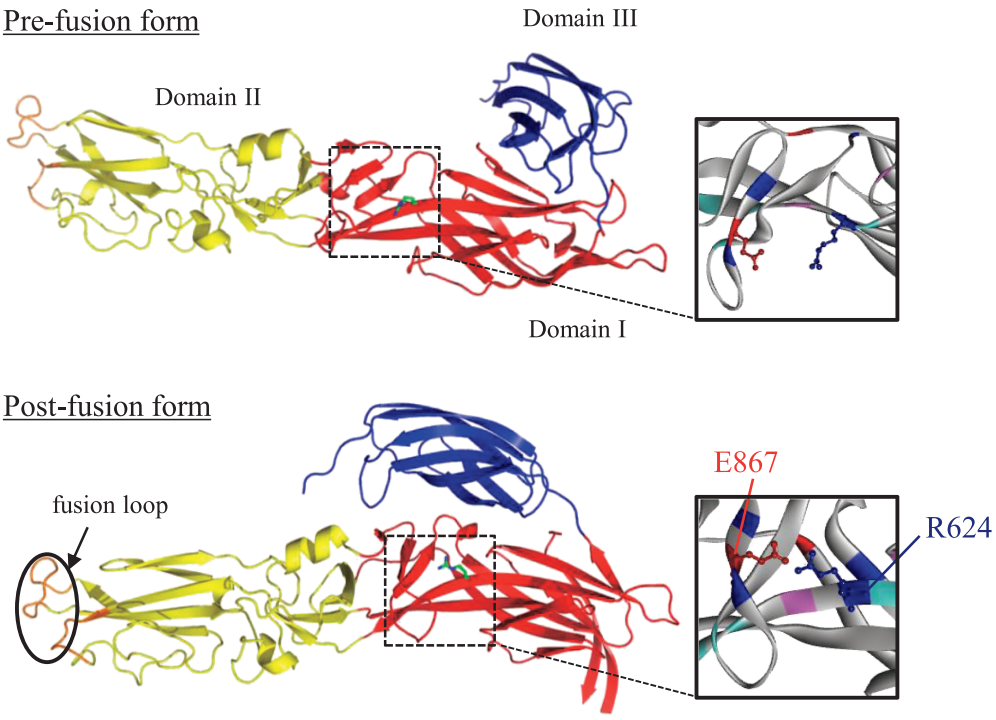

Fig. 4 Structure models of SFTSV Gc. Structural rearrangement of SFTSV Gc from pre-fusion to post-fusion structures. The green amino acid indicates the amino acid at position 624. Domain I is in red, domain II is in yellow, domain III is in blue, and the fusion loop in domain II is in orange. Magnified images of domain I are shown in the box to the right. Blue indicates arginine, light blue indicates lysine, red indicates glutamic acid and pink indicates aspartic acid.

SFTSV Gc was determined in a recent study (11). Using this structure, we generated pre- and post-fusion structural models of SFTSV Gc in an effort to identify the structural contribution made by the amino acid at position 624 (Fig. 4). Position 624 was located in the 4th β-strand in domain I, which was present on the outside of the structure. A glutamic acid at position 867 located in the 10th β-strand of domain I was contiguous to the R624 in both the pre- and post-fusion structural models. Notably, both side chains of R624 and E867 were spatially closely arranged. In the post-fusion model, more basic and acidic amino acids were located around the amino acid at position 624 compared with the pre-fusion model.

DISCUSSION

Structural analysis of SFTSV and other phleboviruses demonstrated that Gc is a class II viral fusion protein and that the membrane fusion process is induced by structural rearrangement triggered by acidic conditions (2, 5, 11, 17). This fusion process could be evaluated as low-pH-dependent cell fusion caused by Gn and Gc expressed on the cell surface (9, 21). Similar to other phleboviruses, it was reported that SFTSV infection was inhibited by treatment with lysosomotropic agents and that formation of syncytia was induced under acidic conditions (12, 26). We also confirmed that SFTSV strain YG1 induced the formation of syncytia under acidic conditions and we then established subclones based on their ability to facilitate cell fusion (20). Subclones with high fusion activity isolated from strain YG1 had two amino acid mutations in Gn and Gc, and the amino acid at position 624 in Gc played a critical role in the induction of low-pH-dependent cell fusion.

The amount of GP on the cell surface is an important factor for inducing low-pH-dependent cell fusion because GP expressed on the cell surface will initiate cell membrane fusion by acidic buffer treatments (21). Since GP of bunyaviruses is mostly retained in the Golgi apparatus, making it difficult to detect GP at the cell surface, overexpression can be used to increase the overall levels of GP and thereby increase the proportion expressed on the plasma membrane (9). SFTSV YG1 (GP-Y/R) showed little expression of GP on the cell surface, and did not induce cell fusion in the transfected cells. However, GP possessing an amino acid substitution at position

624 induced strong cell fusion in transfected cells without any detectable increase in Gn and Gc expression on the cell surface or within the cell. Although a very small amount of GP expression was detected by flow cytometry, no clear co-localization with the plasma membrane was found by indirect immunofluorescence assay. These results suggest that other factors including enhanced sensitivity to low pH conditions are implicated in cell fusion activity. Indeed, GP-Y/W expression induced formation of syncytia over a wide pH range below pH 6.2. In addition, cell fusion activity was enhanced by substitution with not only a tryptophan at position 624, but also a glycine, serine, and aspartic acid. Only substitution with arginine or lysine had no affect on cell fusion activity.

Since tryptophan is an aromatic amino acid, substitution to tryptophan was expected to affect the structural rearrangement. However, we demonstrated that an amino acid substitution to glycine (GP-Y/G), which is structurally the simplest amino acid, also caused cell fusion. On the other hand, substitution to lysine induced no or very little syncytium formation, suggesting that enhanced fusion activity is derived from the sensitivity for acidic conditions, not by structural inhibition. In addition, GP-Y/S and GP-Y/D also induced cell fusion in transfected cells. RVFV and UUKV have a serine and an aspartic acid, respectively, at the site corresponding to position 624 in SFTSV, and both viruses also induce formation of syncytia over a pH range below pH 6.2 or 5.8, respectively (9, 15). The pH ranges for cell fusion of RVFV and UUKV were the same as those for SFSTV GP-Y/S and GP-Y/D, suggesting that the amino acid at position 624 plays a role in determining the sensitivity of phlebovirus GPs to low pH conditions. The amino acid at position 624 is located in the β-strand of domain I in Gc. A comparison of the pre- and post-fusion structural models of Gc indicated that a large structural rearrangement occurs post-fusion in domain I, including secondary structure modifications. Although the 4th β-strand of domain I did not move dramatically, an amino acid difference might affect the conformational change. In the post-fusion structural model, basic and acidic amino acids, in particular the side chain of E867, were located near R624, making potential ionic contact between these amino acids in low-pH conditions. Although further analysis is required to determine the interactions among these amino acids, we hypothesize that a post-fusion structure stabilized by an interaction between the amino acids at positions 624 and 867 would be critical for inducing cell fusion.

Viruses possessing amino acid differences at both positions 328 and 624 were only initially identified as high fusion clones in our previous study (20); however, we reported that the amino acid substitution Y328H slightly reduced cell fusion. In general, the some histidine residues in Gc promote pH-dependent protonation (10, 13). De Boer *et al.* reported that mutation of a single histidine (857H) in RVFV Gc completely abrogated virus entry as well as acid-induced Gc oligomerization (4). The histidine residue at position 940 (940H) of SFTSV Gc is also important for virus replication (11). Conversely, the structure and function of SFTSV Gn during virus entry remains unclear. It is possible that this mutation Y328H affects cell fusion activity at the step of pH-triggered conformational change of GP. To clarify the mechanism of virus-cell membrane fusion, the conformational changes that affect the interaction between Gn and Gc need to be determined.

In this study, we demonstrated that transiently expressed SFTSV GP with substitutions at amino acid position 624 induced strong cell fusion under acidic conditions. We also provided evidence indicating the importance of low-pH sensitivity and stability as well as GP expression levels to the induction of low-pH dependent cell fusion. Our study has provided an important insight into the mechanisms of SFTSV entry.

Acknowledgements

We thank Dr. Ayato Takada, Dr. Wakako Furuyama and Ms. Aiko Ohnuma (Research Center for Zoonosis Control, Hokkaido University) for their support to perform the experiments. This work was supported by Research Program on Emerging and Re-emerging Infectious Diseases from the Japan Agency for Medical Research and Development (AMED).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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