A residue located at the junction of the head and stalk regions of measles virus fusion protein regulates membrane fusion by controlling conformational stability

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Abstract
The fusion (F) protein of measles virus performs refolding from the thermodynamically metastable prefusion form to the highly stable postfusion form via an activated unstable intermediate stage, to induce membrane fusion. Some amino acids involved in the fusion regulation cluster in the heptad repeat B (HR-B) domain of the stalk region, among which substitution of residue 465 by various amino acids revealed that fusion activity correlates well with its side chain length from the Ca (P<0.01) and van der Waals volume (P<0.001), except for Phe, Tyr, Trp, Pro and His carrying ring structures. Directed towards the head region, longer side chains of the non-ring-type 465 residues penetrate more deeply into the head region and may disturb the hydrophobic interaction between the stalk and head regions and cause destabilization of the molecule by lowering the energy barrier for refolding, which conferred the F protein enhanced fusion activity. Contrarily, the side chain of ring-type 465 residues turned away from the head region, resulting in not only no contact with the head region but also extensive coverage of the HR-B surface, which may prevent the dissociation of the HR-B bundle for initiation of membrane fusion and suppress fusion activity. Located in the HR-B domain just at the junction between the head and stalk regions, amino acid 465 is endowed with a possible ability to either destabilize or stabilize the F protein depending on its molecular volume and the direction of the side chain, regulating fusion activity of measles virus F protein.

INTRODUCTION
Measles virus (MV), a member of the genus Morbillivirus in the family Paramyxoviridae, is an enveloped virus possessing two glycoprotein spikes, the haemagglutinin (H) and fusion (F) proteins [1]. The initial step of MV infection is binding of the H protein to cellular receptors, which successively triggers the conformational change of the F protein promoting membrane fusion between virus envelope and the target cell plasma membrane (virus–cell fusion) and also between the plasma membranes of the infected cells and neighbouring uninfected cells (cell–cell fusion) to spread the virus RNA genome into the cytoplasm [2]. The F protein is a homotrimer protein (Fig. 1b). After synthesis at rough-surfaced endoplasmic reticulum as an inactive precursor, F0, the F protein should be cleaved into active complex of disulfide-linked F1 and F2 subunits by furin protease in the trans-Golgi en route to the cell surface [3], through which the fusion peptide is exposed at the N terminus of the F1 subunit. To play the principal role in membrane fusion, the F protein performs refolding from the thermodynamically metastable prefusion form to the highly stable postfusion form, by way of an energetically activated unstable intermediate state [4, 5]. To overcome this energy barrier, the F protein requires an activation signal (triggering) from the H protein [6–10].

A series of structural changes of paramyxovirus F protein during the membrane fusion has been proposed based on the X-ray structures of the prefusion and postfusion forms of the F proteins derived from parainfluenza virus 5 (PIV5) and human parainfluenza virus 3, respectively [11, 12]. The prefusion form consists of two regions (Fig. 1b): the stalk region is the three-helix bundle of the homotrimer of the F protein including heptad repeat B (HR-B) domain, and the head region is composed of DI, DII and
DIII domains, including HR-A as well as HR-C as parts of the DIII domain. Upon activation by the haemagglutinin-neuraminidase protein (an attachment protein corresponding to the H protein of MV), conformational change of the F protein is initiated. Firstly, the monomers in the HR-B domain dissociate from each other, and the HR-A domain folded within the head region extends to form a long helix bundle that protrudes, inserting the fusion peptide exposed at the N-terminal end of the F1 subunit into a target cell membrane. The transiently formed energetically activated unstable pre-hairpin intermediate F protein may be lodged [7, 16], and two domains, N-terminal region of the HR-B and microdomain in the DIII adjacent to the HR-A, where the initial conformational changes of the F protein molecule are believed to take place [17–23]. In the N-terminal region of the HR-B domain, amino acid substitutions to facilitate fusion activity of the F protein were observed as a cluster at positions 461, 462, 464 as well as 465 (Fig. 1c) [20–24]. Among them, T461I substitution or combined mutations of N462S/N465S (together with S103I) has been proved to correlate with the enhanced fusion activity of a neurotropic MV variant, subacute sclerosing panencephalitis (SSPE) virus [23, 24]. SSPE is a fatal disease of the central nervous system that develops with a very low frequency 7–10 years after recovery from ordinary acute

**Fig. 1.** Abrogation of MV cell–cell fusion by N465H substitution in the F protein. (a) Cytopathic effect of the cells infected with rMV/F-WT or rMV/F-N465H. B95a cells were infected with EGFP-expressing rMV carrying the F-WT protein or F-N465H protein and fixed with 1 % paraformaldehyde at 72 h post-infection. EGFP expression in the infected cells was observed under a fluorescence microscope. White bars indicate 100 µm. (b) Localization of residue 465 in the prefusion form of MV F protein structure. Surface model of the constructed prefusion form of MV F protein trimer (left) and schematic diagram of the domains of MV F protein (right). Residue 465 is marked in red. (c) A cluster of amino acids involved in the fusion activity in the N-terminal region of the HR-B domain, close-up from the direction of the arrow in (b).
measles [25, 26]. An MV persistently infecting in the brain accumulates mutations to finally cause SSPE, and infectivity in the neuronal cells and neurovirulence of the SSPE viruses are considered to be associated with their hyper-fusogenic character although fusion is notably absent in the central nervous system of the patients [23, 24, 27]. Recently, we identified another mutation at amino acid 465, N465H, as the one that contrarily almost completely diminishes cell–cell fusion activity of the F protein, suggesting that amino acid 465 should play some role in regulating fusion activity of the F protein. In the previous study, we showed that amino acid 465 in the HR-B domain actually regulates fusion activity cooperatively with amino acids in the DIII domain by complementarily adjusting the thermodynamic stability of the F protein. The N465H substitution strongly stabilizes the prefusion structure of the F protein, which compensates for destabilization by N183D, F217L, P219S, I225T or G240R substitution in the DIII domain and maintains the proper conformation of the F protein to express fusion activity [28]. Destabilization by these five amino acid substitutions in the DIII domain was explained either by involvement in the inter-subunit interaction of the F protein trimer or by contact with the fusion peptide directly or indirectly. On a molecular basis, however, how amino acid N465H substitution stabilizes the F protein remains to be elucidated.

In this study, we aimed to elucidate the molecular mechanism of amino acid 465 of MV F protein to control fusion activity and successfully demonstrate that its molecular volume and the direction of the side chain regulate the stability of the prefusion structure and therefore the fusion efficiency of the F protein. Located just at the junction between the head and stalk regions where the bottom edge of the head region touches the three-helix bundle of the stalk region, amino acid 465 is endowed with a possible ability to either stabilize or destabilize the F protein.

RESULTS

Cell–cell fusion activity of the F protein is closely related with the side chain length and the molecular volume of amino acid 465

The N465H substitution of MV F protein was identified as a mutation to cause the defect of cell–cell fusion activity with an MV variant isolated from the clinical yuKE strain (Fig. 1a) [28]. Whereas amino acid 465 is one of the amino acids clustering in the HR-B domain whose substitution enhances fusion activity of the F Protein (Fig. 1c) and was reported to be involved in accelerated fusion activity of SSPE virus to cause fatal infection in the brain [23], the N465H substitution almost completely eliminated cell–cell fusion activity of the F protein, which suggests that amino acid 465 should play a significant role in regulating fusion activity of the F protein.

To examine the characteristics of amino acid 465 controlling fusion activity, Asn465 of the wild-type F (F-WT) protein was substituted by various amino acids (465-mutated F proteins). Each mutant F protein was expressed together with the H protein of the yuKE strain using the pcDNA3 expression vector in Vero cells infected with T7 RNA polymerase-expressing vaccinia virus, and fusion activity was evaluated by counting the number of nuclei in syncytia after overlaying Vero/hSLAM cells. Substitution of Asn465 by Lys, Ile, Gln or Leu increased the fusion activity by approximately two-fold, but that by Phe or Tyr resulted in less than half the fusion activity of the F-WT protein (Fig. 2a). Especially, the F protein with Trp, Pro or His substitution showed a trace of or no fusion activity. The F protein is synthesized as inactive precursor, F0, and activated by furin into disulfide-linked F1 and F2 subunits [3]. The cell surface expressions of the active F1 subunit and total F protein (combined amount of F1 and F0 proteins) of each mutant F protein were not significantly different, which could not explain the change of the fusion activity (Fig. 2b). Here, we noticed that the F proteins demonstrating strong fusion activity carry amino acid 465 with relatively long side chains. As shown in the upper panel of Fig. 2(c), cell–cell fusion activity and the length of the side chain from Cα measured on the constructed model of MV prefusion F protein based on that of PIV5 (see Fig. 5) showed good correlation (P<0.01), and when fusion activity was plotted against Van der Waals volume, an indicator of physical mass calculated based on Van der Waals radius of atoms, the correlation became stronger (P<0.001) only if five amino acids that demonstrated the lowest fusion activity were excluded (Fig. 2c, lower panel). These five amino acids were revealed to carry ring structures (ring-type amino acids): imidazole group of His, pyrrolidine ring of Pro, indole group of Trp, and phenyl group of Tyr and Phe.

Large molecular volume of non-ring-type amino acid 465 lowers the energy barrier for refolding of the F protein, whereas ring-type amino acids stabilize the F protein

Fusion activity of MV F protein is enhanced by N462S or N462K substitution in the HR-B helix by destabilizing the molecule [21]. Since amino acid Asn465 is located just one turn away (about 4 Å) of Asn462 from the head region (Fig. 1c), fusion activity of each 465-mutated F protein was analysed at 30 °C to examine whether the substitution affects the stability of the F protein (Fig. 3a). It was found that the cell–cell fusion activities of the 465-mutated F proteins relative to that of the F-WT protein increased more prominently at 30 °C as the length of the side chain became longer (Fig. 3b). The ratio of the relative fusion activity at 30 °C to that at 37 °C is in good correlation especially with Van der Waals volume (P<0.01), which indicates that the F proteins possessing amino acid 465 of larger volume induce cell–cell fusion more readily at low temperature (Fig. 3c). The result suggests that large molecular volume of amino acid 465 lowers the energy barrier for the conformational change of the F protein to induce cell–cell fusion.
Five 465-mutated F proteins with a ring-type amino acid were excluded from the fusion analyses at 30°C as their fusion activities were extremely restricted and independent of Van der Waals volume (Fig. 2c). Then, fusion assay of these F proteins was performed at higher temperature. The F-N465W, F-N465P and F-N465H proteins which hardly expressed cell–cell fusion activity at 37°C formed tiny syncytia at 40°C (Fig. 3d, upper panel). Relative fusion activities of these five mutant F proteins normalized to that of the F-WT protein were reduced as temperature decreased, contrarily to the F-N465K protein whose relative activity was stimulated, demonstrating that they require elevated energy to exert fusion activity (Fig. 3d, lower panel). The result suggests that these five ring-type amino acids at position 465 stabilize the F protein and raise the energy barrier for conformational change of the F protein.

Fig. 2. Correlation of cell–cell fusion activity with the side chain length and the molecular volume of amino acid 465 in the F protein. (a) Cell–cell fusion activities of the 465-mutated F proteins at 37°C. Vero cells were transfected with a plasmid expressing one of the 465-mutated F proteins together with the H-protein-expressing plasmid and incubated at 37°C. At 24 h posttransfection, the cells were overlaid with Vero/hSLAM cells in the presence of 100 µM cycloheximide and further incubated at 37°C for 12 h. Cell–cell fusion activity was determined as described in Methods. A representative result of three repeated experiments is shown. WT applies to the F-WT protein possessing N465. (b) Cell surface expression and cleavage efficiency of the 465-mutated F proteins. Vero cells were transfected with the 465-mutated F-protein-expressing plasmids, biotinylated at 24 h posttransfection and lysed. Biotinylated cell surface proteins were precipitated with streptavidin-coated sepharose beads and subjected to immunoblot analysis using rabbit antibody against the F protein, followed by determination of the intensity of each F protein band using ImageJ software (http://rsbweb.nih.gov/ij/index.html). Total amounts (combined amount of the F1 subunit and uncleaved F0 protein) of the cell surface-expressed 465-mutated F proteins are shown relative (%) to that of the F-WT protein. Cleavage efficiency was evaluated as the ratio of the F1 subunit to the total amount of the F protein and are also shown relative value (%) to that of the F-WT protein. (c) Correlation of cell–cell fusion activity of the F protein with the side chain length and the molecular volume of residue 465. Cell–cell fusion activity of the 465-mutated F protein at 37°C in (a) was plotted against the length of the side chain (upper panel) or Van der Waals volume (lower panel) of residue 465. Correlations were evaluated by Spearman’s rank correlation for the non-ring-type amino acids (solid circles). Linear regression line is shown as a black line with the regression coefficient (r). Five ring-type amino acids were excluded from the evaluation (open circles).
Fig. 3. Temperature dependence of fusion function of the 465-mutated F proteins. (a) Cell–cell fusion activities of the 465-mutated F proteins at 37 and 30 °C. Vero cells were transfected with a plasmid expressing one of the 465-mutated F proteins and incubated at 37 °C as described for Fig. 2(a). After overlay with Vero/hSLAM cells, the cells were further incubated at 37 °C for 12 h, or transferred to 30 °C and incubated for 18 h in the presence of 100 µM cycloheximide, and cell–cell fusion activity was determined. (b) Relative cell–cell fusion activities of the 465-mutated F proteins to that of the F-WT protein. Cell–cell fusion activities of the 465-mutated F proteins at 37 and 30 °C in (a) were normalized to the values for the F-WT protein at 37 and 30 °C, respectively. (c) Correlation of the enhanced relative cell–cell fusion activities of the 465-mutated F proteins at 30 °C with the molecular volume of amino acid 465. Ratio of relative cell–cell fusion activities of the F-465 mutant proteins at 30 °C to those at 37 °C in (b) was plotted against the length of the side chain (upper panel) and Van der Waals volume (lower panel) of amino acid 465. Correlations were evaluated by Spearman’s rank correlation. The linear regression is shown as a black. Ring-type amino acids were excluded from the evaluation. (d) Stimulation of cell–cell fusion activities of the 465-mutated F proteins possessing a ring-type amino acid at higher temperature. Vero cells were transfected with a plasmid expressing a 465-mutated F protein carrying a ring-type amino acid with the H-protein-expressing plasmid and incubated at 37 °C. At 24 h posttransfection, Vero/hSLAM cells were overlaid onto the Vero cells, then continuously incubated at 30 °C for 18 h (blue), at 37 °C (green) or at 40 °C (red) for 12 h, in the presence of 100 µM cycloheximide. Cell–cell fusion activity was quantified as described in Fig. 2(a) (upper panel) and normalized to the value of the F-WT protein at respective temperature as in (b) (lower panel). Relative cell–cell fusion activities of the 465-mutated F proteins with ring-type amino acids were enlarged (inset to the lower panel).
Thermodynamic stability of the 465-mutated F proteins evaluated by the protein conformation using monoclonal antibody

In a previous report, Ader et al. [10] successfully identified the conformation of MV F protein using mAbs that allow the discrimination between the prefusion and postfusion forms and evaluated the thermodynamic stability of the F protein. Whereas anti-MV F mAb 186CA recognizes only prefusion conformation of the F protein, mAb 16AG5 binds specifically to an epitope present only in the postfusion (triggered) F structure. Since mAbs specific for the prefusion structure including 186CA are no longer available and could not be obtained, we studied the thermodynamic stability of the 465-mutated F proteins by detecting the triggered postfusion form using mAb 16AG5 referred to as anti-triggered F mAb. For detection of the F protein equally under all conditions, the bioactive F proteins harbouring an engineered FLAG tag in the ectodomain [10], the linear epitope of which is recognized by an anti-FLAG mAb M2, was employed. To trigger F refolding and drive the prefusion conformation to that of postfusion, the cells transfected with one of the F-protein-expressing plasmids were subjected to heat treatment at 45, 50 or 55 °C for 45 min. Then the reactivity against the anti-triggered F mAb 16AG5 or anti-FLAG tag mAb M2 was observed by immunofluorescence analysis. The remaining cell−cell fusion activity after the heat treatment was also estimated.

The anti-triggered F mAb 16AG5 detected none of the F-N465K, F-N465T, F-WT(Asn465), F-N465G and F-N465F proteins after exposure to 45 °C as well as after holding at 37 °C without heat treatment (Fig. 4a). When the cells expressing one of these F proteins were treated at 50 or 55 °C, the F proteins obtained reactivity to the mAb 16AG5, which shows that their conformation were converted to those of the triggered F protein, the postfusion form. The number of cells detected by anti-triggered F mAb in Fig. 4(a) was counted under a fluorescence microscope and graphically demonstrated in Fig. 4(b). It is clearly shown that the F-N465K protein was most easily converted to the triggered conformation and that the number of anti-triggered F mAb positive cells decreased as the molecular volume of the amino acid 465 was reduced to the smallest count of the F-N465F protein, a ring-type amino acid. The result is in good correlation proportionately with the cell−cell fusion activity shown in Fig. 2(a). As a control, cells were stained with the anti-FLAG mAb recognizing a linear epitope, which returned indistinguishable staining under all conditions confirming that the protein level of the F proteins after heat treatment was unchanged. Furthermore, the cell−cell fusion activities of the 465-mutated F proteins remaining after the heat treatment were examined (Fig. 4c). Fig. 4(d) shows relative cell−cell fusion activity of these five F proteins normalized to that without heat treatment. Whereas the F-N465K protein lost most of the cell−cell fusion activity after exposure to the temperature of 50 °C and about half the activity even at 45 °C, the F-N465G and F-N465F proteins exhibited fusion activity after the heat treatment at 55 °C. These two F proteins maintained nearly whole cell−cell fusion activity after the treatment at 45 °C. These results ensure that amino acid 465 with a large molecular volume lowers, whereas ring-type amino acid raises, the energy barrier for conformational change of the F protein.

Side chain of the ring-type amino acid 465 takes the reverse orientation of that of non-ring-type amino acids

To understand the effect of amino acid substitution at 465 on the conformation of the F protein, a structure model of prefusion MV F protein was built using the X-ray structure of PIV5 F protein (PDB: 2B9B) [12]. In the model structure of the prefusion F protein, Asn465 in the HR-B domain is located on the surface of the three-helix bundle of the stalk region (Fig. 5a, d). Then mutations of residue 465 were undertaken in the structure model, in which energetically preferred conformations of mutated side chains were explored. Since the F protein is a homotrimer protein, all of the three positions of residue 465 were mutated. As a result, a difference between ring- and non-ring-type residues, excluding Gly, Ala and Pro, was found in their side chain orientations towards the head region of the prefusion form (Fig. 5b). Preferred χ1 conformations of ring- and non-ring-type side chains were trans and gauche*, respectively. Out of three mutated sites, one for each chain, all of the three χ1 angles took trans for Phe, Tyr, Trp and His. All of the three χ1 angles took gauche* for Arg, Lys, Ile, Gin, Leu, Thr, Val, Asp, Cys, Met and Asn; two gauche* and one trans for Ser. These differential preferences of χ1 conformations among ring- and non-ring-type side chains were consistent with the statistical analysis of χ1 angles of residues located in helices of known protein structures [29]. Fig. 5(c) demonstrates that Lys465 extends the side chain towards Leu354 and Asn462, while the imidazol group of His465 is directed away apart from the head region in close proximity to the helix in the HR-B domain (Fig. 5e).

In the model structure, ring-type side chains of residue 465 with the trans confirmation of χ1 angle extensively cover the surface of the other residues in the helix bundle, particularly that of Lys469 in the same subunit and that of Val467 in the neighbouring subunit (Table 1). The side chains of Val467 and ring-type residues at 465 (His465) are so close to each other to such an extent that water molecules cannot penetrate between them (Fig. 6).

DISCUSSION

The F protein of MV executes refolding from the metastable prefusion form to the highly stable postfusion form to exert its fusion function. Upon activation by the H protein, the monomers forming the three-helix bundle of the HR-B domain dissociate from each other and the HR-A domain folded within the DIII domain in the head region extends to form a long helix bundle, which protrudes towards a target cell membrane to initiate the F protein refolding. Recently, we reported that amino acid 465 in the HR-B domain regulates fusion activity.
cooperatively with amino acids in the DIII domain by complementarily adjusting the thermodynamic stability of the F protein [28]. In this study, we found the definitive difference between the non-ring- and ring-type residues at amino acid 465 in regulating the stability of the F protein and therefore its fusion activity.

In the case of non-ring-type residues, excluding Gly and Ala, their side chain $\chi_1$ angles preferentially take the gauche$^+$ conformation, which makes the bodies of side chains turn towards the head region of the prefusion form, making contacts with Leu354 and Asn462. Prussia et al. [21] demonstrated that mutation at 462 could disrupt the hydrophobic interaction between the stalk and head regions, decreasing the energy barrier for dissociation of the HR-B domain. Since Leu354 takes part in this hydrophobic interaction along with Thr314, Leu353, Ala367, Ile452, Leu454, Leu457 and Thr461 (Fig. 5a), amino acid 465 may cause some influence via contacts with Leu354 and Asn462. Longer side chains can penetrate more deeply into the head region and make such contacts more extensive (Fig. 5c, d),

Fig. 4. Evaluation of thermodynamic stability of the 465-mutated F proteins using anti-triggered F structure (anti-trig F) mAb. (a) Detection of the triggered structure of the F protein. Vero cells were transfected with the plasmid expressing one of the 465-mutated F proteins and incubated at 37°C. At 24 h posttransfection, transfected cells were heat treated at 45, 50 or 55°C, or kept at 37°C for 45 min. Subsequently, unfixed and unpermeabilized cells were stained with anti-trig F or anti-FLAG mAb for 1 h at 4°C followed by incubation with Alexa Fluor 488-conjugated secondary antibody. Images of the positive cells were captured with a fluorescence microscope. (b) The number of cells reactive to the anti-trig F mAb. Positive cells in (a) were counted (five fields each) under a fluorescence microscope. A representative result of three repeated experiments is shown. (c) Cell–cell fusion activities of the 465-mutated F proteins remaining after the heat treatment. Vero cells were transfected with the plasmid expressing one of the 465-mutated F proteins together with the H-protein-expressing plasmid and incubated at 37°C. At 24 h posttransfection, the transfected cells were heat treated at the indicated temperature for 45 min and were overlaid with Vero/hSLAM cells in the presence of 100 µM cycloheximide. After further incubation at 37°C for 12 h, cell–cell fusion activity was quantified as described in Fig. 2(a). (d) Relative cell–cell fusion activities of the 465-mutated F proteins. Cell–cell fusion activities in (c) were normalized to the value of each F protein treated at 37°C.
which may disturb the hydrophobic interaction between the stalk and head regions and cause destabilization of the molecule by lowering the energy barrier for refolding and conferring the F protein enhanced cell–cell fusion activity.

In the case of ring-type residues, excluding Pro, their side chain $\chi_1$ angles preferentially take the trans conformation, which makes the bodies of side chains turn away from the head region of the prefusion form, resulting in no contact with Leu354 and Asn462 (Fig. 5e). Compared with the F protein with Gly or Ala which showed the lowest cell–cell fusion activity among the non-ring-type residue mutants due to the short side chain to reach Leu354 or Asn462, F protein mutants with ring-type residues demonstrated further restricted fusion activities and highly increased thermodynamic stability. The phenomena could be effectively explained by analysing the structure model of the prefusion form. In the model structure, ring-type side chains of residue 465 with the trans conformation of $\chi_1$ angle extensively cover the surface of Lys469 in the same subunit and Val467 in the neighbouring subunit (Table 1). The side chains of Val467 and ring-type residues at 465 are positioned close to each other, preventing the penetration of water molecules between them (Fig. 6), which may hamper the dissociation of the helices from each other. In addition, Lys469 can form a salt bridge with Glu471 in the neighbouring subunit and existence of bulky ring-type residues at 465 may restrict the movement of the side chain of Lys469, facilitating the maintenance of the inter-helical salt bridge with Glu471 (Fig. 6). These effects to stabilize the helix bundle may hinder the conversion of the F protein from the prefusion to postfusion form. Zokarkar et al. [30] showed that an engineered

![Diagrams](image-url)

**Fig. 5.** Orientation of mutated side chains at position 465 in the structure model of MV F protein prefusion form. (a) Location of Asn465. Main chains depicted in ribbon model are coloured by subunit. Asn462 and Asn465 are depicted in stick model, and Thr314, Leu353, Leu354, Ala367, Ile452, Leu454, Leu457 and Thr461 in space-filling model are coloured by atom. Residues shown in space-filling model constitute the hydrophobic cluster between the stalk and head regions of the prefusion form. (b) Frequencies of side chain $\chi_1$ rotamers of mutated amino acid 465. Out of the three mutation sites, one for each subunit in the homotrimer F protein, the number of such sites that take the gauche+ (blue) or trans (red) conformation as the most energetically preferred $\chi_1$ angle in the model structure is shown for each mutant. (c–e) Conformation of the side chain at position 465. Main chains depicted in ribbon model are coloured by subunit, and Leu354, Asn462 and Asn465 are in stick model. The most energetically preferred conformation is shown for each side chain. The preferred $\chi_1$ rotamers for Lys465, Asn465 and His465 are gauche+, gauche+ and trans, respectively. The nearest distances (Å) between side chains from residue 465 to Leu354 and Asn462 are shown in green.
Table 1. Decrease of the solvent accessible surface area (Å²) of Val467 and Lys469 by mutating residue 465 from Ala to each of Asn, His, Phe, Try and Trp

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A disulfide bond in the membrane-proximal external region located in the stalk of PIV5 F protein inhibits the fusion activity. Without artificial bonding between the stalks, our data confirmed that dissociation and opening of the HR-B bundle should be the first requirement for conformational change of paramyxovirus F protein. Side chains of Gly and Ala at position 465 are too short to take part in the interaction not only with Leu354 and Asn462 for destabilization of the hydrophobic cluster but also with Lys469 and Val467 for stabilization of the helix bundle, suggesting that Gly and Ala at position 465 cause neither enhancement nor suppression of cell–cell fusion activity of the F protein. As for Pro, since Pro is often observed to cause a kink in helix, the presence of Pro may change the orientation of helix axes in the three-helix bundle stalk and may change interactions between the stalk and head of the prefusion form. It is actually impossible to get a picture of the F protein structure at the energetically activated unstable intermediate stage on the way from the prefusion to the postfusion form. Estimation, therefore, of how an amino acid affects the reforming procedure would be quite difficult. In order to seek other possible structural features of amino acid 465 related with fusion activity of the F protein, we examined correlations of the fusion activity data with various amino acid indices (Table S1, available in the online Supplementary Material). While no significant correlation was seen with secondary structure propensities of amino acids, high correlation coefficients of 0.5 to 0.8 were found with amino acid indices related with side chain flexibility [31–34]. Correlations of the fusion activity with side chain flexibility could indicate that such amino acid residues as can easily alter their side chain conformations when their environments (surrounding residues, molecules and solvents) change have less possibility to prevent the F protein from passing through the intermediate states on the way to large structural change, suggesting that amino acid 465 with flexible side chain should be favourable for conformational change of the F protein. Although why such a flexible side chain is required at position 465 remains to be solved, amino acid 465 should modulate fusion activity of the F protein depending on the orientation, the length and the flexibility of its side chain.

METHODS

Cells and viruses

Vero cells constitutively expressing human signalling lymphocyte activation molecule (Vero/hSLAM) (a gift from Y. Yanagi, Kyushu University) [35] and Vero cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 7 % FBS. Cells of a marmoset B-cell line transformed with Epstein–Barr virus (B95a) were maintained in high-glucose DMEM supplemented with 10 % FBS and BHK cells constitutively expressing T7 RNA polymerase (BHK/T7-9) (a gift from N. Ito and M. Sugiyama, Gifu University) [36] were maintained in RPMI 1640 medium supplemented with 8 % FBS and 0.6 mg ml⁻¹ hygromycin B.

The yuKE strain of MV (genotype H1) isolated from a measles patient was passaged once in B95a cells followed by 10 times in Vero/hSLAM cells. The cloned viruses of the yuKE strain, syncytium-forming clone 1 possessing Asn465 in the F protein and clone 2 lacking syncytium formation with His in the same position of the F protein, and recombinant MVs (rMVs) carrying Asn465 (rMV/F-WT) and His465 (rMV/F-N465H) in the F protein, respectively, were described previously [28]. T7 RNA polymerase-expressing vaccinia virus

After the fusion activity of the rMV/F-WT and rMV/F-N465H clonal recombinant MVs was examined, the syncytium index was measured in Vero/hSLAM cells, where syncytium formation is induced by MV in a secondary fashion. The yuKE strain and recombinant MVs were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 7 % FBS.
(vTF7-3) [37] was a gift from B. Moss (National Institutes of Health, USA).

**Plasmid construction**

cDNA of the F protein mRNA of the yuKE strain clone 1 was synthesized by reverse-transcription PCR (nucleotide sequence; GenBank: AB968383). The *Hpa*–*Pst*I fragment (nt 6362 to 7867 according to the IC-B strain genome sequence; GenBank: AB016162) of the p(+)MV323c72-EGFP plasmid [38] derived from p(+)MV323-EGFP (a gift from Y. Yanagi) [39] was replaced by the corresponding fragment of clone 1, which generated the full-length genome plasmid p(+)MV323c72-EGFP/F-WT(H1) carrying the WT-F gene of the yuKE strain. In amino acid substitutions, N465H was introduced into the p(+)MV323c72-EGFP/F-WT(H1) to obtain the full-length genome plasmid p(+)-MV323c72-EGFP/F-N465H for clone 2.

For expression of proteins using plasmid, cDNA of the WT and mutant F genes as well as the H gene of the yuKE strain were cloned into pcDNA3 plasmid (Invitrogen Life Technologies). To express the bioactive F proteins harbouring an engineered FLAG tag in the ectodomain [10], FLAG tag sequence (DYKDDDDK) was inserted in the C-terminal region of the F2 subunit between Val104 and Thr105.

**Quantitative cell–cell fusion assay**

Vero cells seeded in 24-well plates were transfected with 0.5 μg each of the F-protein- and the H-protein-expressing plasmids using polyethyleneimine, infected with vTF7-3 at a multiplicity of infection (m.o.i.) of 2 and incubated at 37°C. At 24 h posttransfection, the cells were overlaid with Vero/hSLAM cells and further incubated at 30°C for 18 h, or at 37 or 40°C for 12 h in the presence of 100 μM cycloheximide. The cells were fixed, stained with crystal violet and numbers of syncytia and nuclei per syncytium were counted under a microscope. Cell–cell fusion activity is shown as the product of the two values.

**Evaluation of the F protein stability**

Thermodynamic stability of the F protein mutants was evaluated based on temperature dependence of the cell–cell fusion activity relative to that of the wild-type F (F-WT) protein according to the method reported previously [21, 40]. Relative cell–cell fusion activity was calculated by dividing the cell–cell fusion activity of each F protein mutant at...
30, 37 and 40 °C by that of the F-WT protein at the same temperature, respectively. Stability of the mutant F protein was assessed as follows: destabilized when the relative cell-cell fusion activity was higher at 30 °C than at 37 °C and stabilized when the relative activity at 40 °C was higher than that at 37 °C.

Cell surface biotinylation and Western blotting analysis
Vero cells in 24-well plates were transfected with 0.5 μg of the F-protein-expressing plasmid using Lipofectamine LTX (Invitrogen), infected with vTF7-3 at an m.o.i. of 2 and incubated at 37 °C for 24 h. Then cells were incubated with 0.25 mg of EZ-Link Sulfo-NHS-SS-Biotin (Thermo Scientific) at room temperature for 30 min followed by lysis (150 mM NaCl, 1.0 % Triton X-100, 50 mM Tris/HCl, pH 8.0) at 4 °C for 1 h. The lysate clarified by centrifugation was mixed with Streptavidin Sepharose High Performance (GE Healthcare Bio-Science) at 4 °C for 120 min. The adsorbed biotinylated proteins were subjected to SDS-PAGE and electroblotting onto PVDF membranes. The F proteins were detected using rabbit polyclonal antibody against MV F protein as the first antibody as described previously [28].

Detection of conformational change of the F protein using a monoclonal antibody by indirect immunofluorescence staining
Vero cells were transfected with 0.5 μg of the F-protein-expressing plasmid using Lipofectamine LTX and incubated at 37 °C. At 24 h posttransfection, the transfected cells were transferred to 45, 50 or 55 °C and kept for 45 min. Subsequently, un固定ed and unpermeabilized cells were washed twice with ice-cold PBS and stained with anti-trig F mAb 16AG5, which binds specifically to an epitope present only in the postfusion (triggered) F structure (a gift from M. Ehnlund, Karolinska University, Sweden) [10, 41] or anti-FLAG mAb M2 (Sigma-Aldrich) followed by incubation with Alexa Fluor 488-conjugated secondary antibody. The number of positive cells was counted under a fluorescence microscope.

Structure modelling of the F protein
Homology-based structure model of prefusion MV F protein was built using the X-ray structure of PIIV5 F protein (PDB: 2B9B) [12] with the MOE software (Chemical Computing Group). Although sequence identity between the target and template proteins was 29.9 %, there were few gaps in the sequence alignment, resulting in few template-free residues in the model building. The model structure was evaluated with the 3D profile method [42] implemented in the Discovery Studio software (BIOVIA; Dassault Systèmes). Mutations at position 465 were carried out with the Rotamer Explorer function in MOE, which explored energetically preferred conformations for mutated side chains in the presence of surrounding residues.

Correlation of fusion activity data with various amino acid indices
Pearson’s correlation coefficients between the fusion activity values of the 465-mutated F proteins at 37 and 30 °C, and various amino acid indices were calculated. The amino acid indices represent biochemical and physico-chemical properties of amino acids, including secondary structure propensities, hydrophobicity and structural flexibility. The AAindex database [43], which contains 544 amino acid indices such as preferences of helix, sheet, turn and coil, hydrophobicities and amino acid compositions of various sets of proteins, was employed. In addition, other amino acid indices related to side chain flexibility [32–34], backbone flexibility [44] and the 3D-1D scores [45] were also employed.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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