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2 **Previously Unrecognized Amino Acid Substitutions in the Hemagglutinin and**
3 **Fusion Protein of Measles Virus Modulate Cell-Cell Fusion, Hemadsorption, Virus**
4 **Growth and Penetration Rate**

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1 **Abstract**

2 Wild-type measles virus (MV) isolated in B95a cells could be adapted to Vero cells
3 after several blind passages. In this study, we have determined the complete
4 nucleotide sequences of the genomes of wild-type (T11wild) and its Vero cell-adapted
5 (T11Ve-23) MV strain and identified amino acid substitutions, R516G, E271K, D439E
6 and G464W (D439E/G464W), N481Y/H495R and Y187H/L204F, in the nucleocapsid,
7 V, fusion (F), hemagglutinin (H) and large proteins, respectively. Expression of
8 mutated H and F proteins from cDNA revealed that the H495R substitution, in addition
9 to N481Y, in the H protein was necessary for the wild-type H protein to use CD46
10 efficiently as a receptor and that the G464W substitution in the F protein was important
11 for enhanced cell-cell fusion. Recombinant wild-type MV strains harboring the F
12 protein with the mutations D439E/G464W [F(D439E/G464W)] and/or
13 H(N481Y/H495R) proteins revealed that both mutated F and H proteins were required
14 for efficient syncytium formation and virus growth in Vero cells. Interestingly, a
15 recombinant wild-type MV strain harboring the H(N481Y/H495R) protein penetrated
16 slowly into Vero cells, while, a recombinant wild-type MV strain harboring both the
17 F(D439E/G464W) and H(N481Y/H495R) proteins penetrated efficiently into Vero cells,
18 indicating that the F(D439E/G464W) protein compensates for the inefficient penetration
19 of a wild-type MV strain harboring the H(N481Y/H495R) protein. Thus, the F and H
20 proteins synergistically function to assure efficient wild-type MV growth in Vero cells.

Introduction

Measles virus (MV), which belongs to the genus *Morbillivirus* in the family *Paramyxoviridae*, is an enveloped virus with a non-segmented negative-strand RNA genome. The MV genome encodes six structural proteins: the nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin (H), and large (L) proteins. The P gene also encodes two other accessory proteins, the C and V proteins. The C protein is translated from an alternative translational initiation site leading a different reading frame, and the V protein is synthesized from an edited mRNA. MV has two envelope glycoproteins, the F and H proteins. The former is responsible for envelope fusion, and the latter is responsible for receptor binding (12).

Wild-type MV strains isolated in B95a cells and laboratory-adapted MV strains have distinct phenotypes (18). Wild-type MV strains can grow in B95a cells but not in Vero cells, while laboratory-adapted MV strains can grow in both B95a and Vero cells. Wild-type MV strains do not cause hemadsorption (HAd) in African green monkey red blood cells (AGM-RBC), while most of laboratory-adapted MV strains cause HAd. Importantly, wild-type MV strains are pathogenic and induce clinical signs that resemble human measles in experimentally infected monkeys while laboratory-adapted MV strains do not.

One approach to identify amino acid substitutions responsible for these phenotypic differences is the comparison of a wild-type MV strain with a standard laboratory-adapted MV strain such as the Edmonston strain. With regard the H protein, amino acid substitutions important for HAd activity and cell-cell fusion in tissue culture

1 cells were identified by expressing the H proteins in mammalian cells (15, 21).
2 Recently, Tahara et al. revealed that the M, H, and L proteins are responsible for
3 efficient growth in Vero cells by constructing a series of recombinant viruses in which
4 part of the genome of the wild-type MV was replaced with the corresponding sequences
5 of the Edmonston strain (45, 46, 47).

6 Another approach is the comparison of wild-type MV strains with their Vero
7 cell-adapted MV strains. It was reported that Vero cell-adapted MV strains could be
8 obtained by successive blind passages of wild-type MV strains in Vero cells (18, 24, 30,
9 43). Interestingly, in vivo and in vitro phenotypes of Vero cell-adapted MV strains
10 were similar to those of laboratory-adapted standard MV strains (18, 19, 24, 30, 43).
11 Comparison of the complete nucleotide sequences of the genomes of wild-type MV
12 strains with those of Vero cell-adapted wild-type MV strains revealed amino acid
13 substitutions in the P, C, V, M, H and L proteins (27, 42, 48, 53).

14 At present, these phenotypic differences are explained mainly by the receptor usage
15 of MV. Wild-type MV strains can use signaling lymphocyte activation molecule
16 (SLAM; also called CD150), but not CD46, as a cellular receptor, whereas
17 laboratory-adapted MV strains can use both SLAM and CD46 as cellular receptors (7,
18 10, 16, 29, 56, 60).

19 However, receptor usage per se cannot explain all of the phenotypic differences (20,
20 25, 48, 53). For example, recombinant Edmonston strains expressing wild-type H
21 proteins can grow in Vero cells to some extent (17, 54). Several reports suggested the
22 presence of the third MV receptor on Vero cells (14, 44, 54, 60). Other reports

1 indicated the contribution of the M protein on cell-cell fusion and growth of MV in Vero
2 cells (4, 27, 47). Recently, the unidentified epithelial cell receptor for MV was
3 predicted in primary culture of human cells (1, 55) and several epithelial cell lines (23,
4 51). However, the identity of the third receptor on Vero cells and the unidentified
5 epithelial cell receptor is not clear yet. Thus, the mechanism of Vero cell adaptation of
6 wild-type MV is not completely understood.

7 In order to understand the molecular mechanism of these phenotypic changes of
8 wild-type MV strains during adaptation in Vero cells, we determined the complete
9 nucleotide sequences of the genomes of the wild-type (T11wild) and its Vero
10 cell-adapted (T11Ve-23) MV strains (43) and examined the effect of individual amino
11 acid substitutions using a mammalian cell expression system and reverse genetics. We
12 show here that previously unrecognized new amino acid substitutions in the H and F
13 proteins are important for MV adaptation and HAd activity.

14

15

Materials and Methods

16 Cells and viruses

17 Vero cells (AGM kidney) were maintained in minimal essential medium (MEM)
18 supplemented with 10% fetal bovine serum (FBS). B95a cells (an adherent marmoset
19 B-cell line transformed with Epstein-Barr virus) (18) were maintained in Dulbecco's
20 modified essential medium (DMEM) supplemented with 10% FBS. CHO cells
21 (Chinese hamster ovary) constitutively expressing human SLAM (56) were maintained
22 in RPMI 1640 medium supplemented with 10% FBS and 500 µg of G418 per ml. The

1 T11wild strain isolated by using B95-8 cells from throat swab of measles patient (43)
2 was propagated in B95a cells. Strain T11Ve-20 (T11 strain passaged in Vero cells 20
3 times) (43) was passaged in Vero cells three times to obtain the T11Ve-23 strain.
4 Vaccinia virus vTF7-3 encoding T7 RNA polymerase was obtained from B. Moss.

5

6 **Sequencing of the T11wild and T11Ve-23 genomes**

7 The viral RNAs of the T11wild and T11Ve-23 strains were extracted from B95a cells
8 infected with the T11wild or T11Ve-23 strains using an RNeasy kit (QIAGEN, Hilden,
9 Germany). Overlapping cDNA fragments spanning the entire length of the genome
10 were synthesized by reverse transcription-PCR (RT-PCR) using an RT/Platinum *Taq* Kit
11 (Invitrogen, Carlsbad, CA) according to the instruction of the manufacturer. The 5'
12 termini of negative- and positive-sense genomes were reverse transcribed and amplified
13 using a 5' Full RACE Core Set (TaKaRa, Kyoto, Japan). Nucleotide sequences of
14 cDNA fragments were determined using ABI Prism 3100 Avant Genetic Analyzer
15 (Applied Biosystems, Carlsbad, CA) and MV-specific primers.

16

17 **Construction of expression plasmids and DNA transfection**

18 Amino acid substitutions (in the H protein, N481Y and H495R; in the F protein:
19 D439E and G464W) were introduced either independently or in combination into the H
20 or F gene open reading frames by site directed mutagenesis using complementary
21 primer pairs and the p(+)-MV323 plasmid encoding the antigenomic full-length cDNA
22 of the IC-B strain of MV (49) as a template, and the amplified H and F genes were

1 subcloned into pCAGGSP7 vector (31). pCAGGSP7 plasmid expressing the
2 Edmonston strain H protein was previously described (54). Vero and B95a cells in
3 six-well cluster plates were cotransfected with equal amounts (1.0 µg) of the H and F
4 expression plasmids containing respective mutations along with an enhanced green
5 fluorescent protein (EGFP)-expressing plasmid, pCA-EGFP, which was added to detect
6 small-cell-cell fusion, by using GeneJuice (Novagen, Gibbstown, NJ) and
7 Lipofectamine 2000 reagent (Invitrogen), respectively. At 24 and 48 h transfection,
8 cells were photographed under a fluorescent microscope. In some experiments, 100
9 µg of fusion-inhibiting peptide (FIP; Z-D-Phe-Phe-Gly) (Peptide Institute Inc., Osaka,
10 Japan) per ml was added to medium to inhibit cell-cell fusion (37).

11

12 **Construction of full-length cDNAs and reverse genetics**

13 The mutated F genes were digested with BstEII and PacI and introduced into
14 p(+)-MV323-EGFP (14) between the BstEII and PacI sites. The mutated H genes were
15 digested with PacI and SpeI and introduced into p(+)-MV323-EGFP between the PacI
16 and SpeI sites. Recombinant MV strains were generated from mutated
17 p(+)-MV323-EGFP plasmids by using CHO cells constitutively expressing human
18 SLAM cells and vaccinia virus vTF7-3 as reported previously (50). Generated MV
19 strains were propagated in B95a cells, and virus stocks at three to four passages in B95a
20 cells were used for experiments.

21

22 **HAd assay**

1 Vero cells transfected with the H protein expression plasmids were overlaid with
2 0.5% AGM-RBC. After incubation for 1 h at 37°C, the monolayers were gently
3 washed three times with DMEM and once with phosphate-buffered saline to remove
4 nonadsorbed AGM-RBC. Then adsorbed AGM-RBC were lysed with H₂O, and
5 released hemoglobin was measured at 540 nm using a spectrophotometer.

6

7 **Flow cytometry**

8 Vero cell suspensions transfected with the H or F gene expression plasmids were
9 incubated with the anti-H monoclonal antibody B5 or the anti-F monoclonal antibody
10 C527 (40), respectively, and fluorescein isothiocyanate-conjugated anti-mouse
11 immunoglobulin G (IgG); cell suspensions were fixed with 3.7% paraformaldehyde and
12 analyzed by a FACSCalibur instrument (Becton Dickinson, San Jose, CA).

13

14 **Measurement of infection efficiency**

15 Vero cells in 12-well cluster plates were infected with approximately 100 times the
16 50% tissue culture infective dose (TCID₅₀), which was measured on B95a cells, of each
17 EGFP-expressing MV strain, incubated at 37°C in a 5% CO₂ atmosphere for 1 h,
18 washed with DMEM two times, and overlaid with 0.8% agarose-containing medium.
19 After 1 to 2 days post infection (p.i.), the number of EGFP-expressing syncytia or cells
20 expected to be derived from single EGFP-expressing MV particle was counted under a
21 fluorescence microscope.

22

1 **Penetration assay**

2 Penetration rate of the recombinant viruses was measured as described previously
3 (57) with some modification. Briefly, Vero or B95a cell monolayers in 12-well cluster
4 plates were inoculated with 100 μ l of medium containing approximately 100
5 fluorescence-forming units per well of each virus and incubated at 37°C for indicated
6 times in Fig. 9. Viruses that had not penetrated into the cells were then inactivated by
7 treatment with 1 ml per well of citric buffer (40 mM citric acid [pH 3.0], 10 mM KCl,
8 135 mM NaCl) at room temperature for 1 min. After two washes with DMEM, the
9 cells were overlaid with 0.8% agarose-containing medium and incubated at 37°C for 48
10 h, and the number of syncytia or cells expected from single EGFP-expressing MV
11 particle was counted under a fluorescence microscope. Experiments without acid
12 treatment were set to 100%.

13

14 **Nucleotide sequence accession numbers**

15 The complete nucleotide sequences of the genomes of the T11wild and T11Ve-23
16 strains were deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases
17 under accession numbers AB481087 and AB481088, respectively.

18

19

Results

20 **Nucleotide sequences of the genomes of the T11wild and T11Ve-23 strains**

21 We identified a total of 10 nucleotide differences between the genomes of the
22 T11wild and T11Ve-23 strains (Table 1). Among these, eight nucleotide substitutions

1 resulted in amino acid substitutions: R516G, E271K, D439E and G464W
2 (D439E/G464W), N481Y/H495R and Y187H/L204F, in the N, V, F, H and L proteins,
3 respectively. Two nucleotide substitutions did not cause amino acid substitutions.
4 When the nucleotide sequence of the T11wild strain was compared with that of the IC-B
5 strain (53), a total of 41 nucleotide differences were identified. Among these, nine
6 nucleotide differences resulted in 12 amino acid differences (three nucleotide
7 differences in the P protein open reading frame resulted in six amino acid differences in
8 the P and overlapping C and V proteins). The T11wild strain is closely related to the
9 IC-B strain and belongs to the genotype D3.

10

11 **Effect of amino acid substitutions in the wild-type H protein on cell-cell fusion in** 12 **Vero cells**

13 We first examined amino acid substitutions found in the H protein in a comparison of
14 the T11wild and T11Ve-23 strains because the H protein mediates receptor binding and
15 is most important for determining the cell specificity of MV. To evaluate the effect of
16 N481Y/H495R substitutions in the wild-type H protein, the wild-type H proteins
17 containing individual mutations were expressed in Vero (SLAM deficient [SLAM-] and
18 CD46+) cells along with the F protein to examine cell-cell fusion. For this purpose,
19 we used the wild-type H protein expression plasmid based on the IC-B strain (54).
20 There is an amino acid difference (E235G) in the H protein of the T11wild strain
21 relative to that of the IC-B strain. The same amino acid difference (E235G) is also
22 found in the H protein of the Edmonston relative to the IC-B strain. However, this

1 amino acid difference, which exists in the β 1-sheet in the H protein, has no effect on
2 cell-cell fusion in Vero cells (46). The amino acid sequence of the F protein of the
3 T11wild strain was completely identical to the sequence of the F protein of the IC-B
4 strain (53). The wild-type H protein along with the F protein did not induce cell-cell
5 fusion (Fig.1) as described previously (54), because the wild-type H protein cannot use
6 CD46 on Vero cells. The wild-type H protein containing the mutation N481Y
7 [H(N481Y)], in the presence of the F protein induced moderate cell-cell fusion (Fig. 1)
8 because the H(N481Y) protein can use CD46 weakly (42, 46). The H(H495R) protein
9 in combination with the F protein did not induce cell-cell fusion (Fig. 1). In contrast,
10 the H(N481Y/H495R) protein in combination with the F protein induced extensive
11 cell-cell fusion (Fig. 1). Thus, the introduction of the H495R substitution in addition
12 to the N481Y mutation in the wild-type H protein strongly enhances the CD46-binding
13 activity of the wild-type H protein.

14

15 **Effect of amino acid substitutions in the F protein on cell-cell fusion in Vero cells**

16 We next examined amino acid substitutions in the F protein because the F protein
17 mediates virus-cell as well as cell-cell fusion and is important for virus infection and
18 virus dissemination. The F(D439E) protein in combination with the wild-type H
19 protein did not induce cell-cell fusion (Fig.1). In contrast, the F(G464W) protein in
20 combination with the wild-type H protein induced moderate cell-cell fusion (Fig. 1),
21 suggesting that the F(G464W) protein can induce cell-cell fusion even if the binding
22 between the H protein and unidentified receptor on Vero cells is weak. The

1 F(D439E/G464W) protein in combination with the wild-type H protein induced
2 moderate cell-cell fusion, which was slightly greater than that of F(G464W) protein (Fig.
3 1), indicating that the D439E substitution has little effect on cell-cell fusion.
4 Importantly, when the F(D439E/G464W) protein was expressed along with the
5 H(N481Y/H495R) protein, huge and rapid cell-cell fusion was induced, and almost all
6 cells were detached from dishes at 48 h post transfection (Fig. 1). These results
7 indicate that the F protein with the amino acid substitution G464W is highly fusogenic
8 and enhances the cell-cell fusion mediated by the F and H proteins. The F(G464W)
9 protein alone did not induce cell-cell fusion in Vero cells (data not shown).

10

11 **Cell-cell fusion in B95a cells and the effect of fusion inhibitor**

12 A cell-cell fusion assay was also performed in B95a (SLAM+ CD46-) cells. In
13 B95a cells, all combinations of the H and F proteins induced large amounts of cell-cell
14 fusion, but the H(H495R) protein promoted somewhat weaker cell-cell fusion (Fig. 2).
15 These results suggest that the introduction of the H495R and/or N481Y substitution in
16 the H protein does not severely compromise its ability to use SLAM as a receptor.

17 It has been reported that mutant F proteins with amino acid substitutions at positions
18 94, 367, and 462 were resistant to fusion inhibitors such as FIP (8, 35, 36). Since
19 position 464 is in close proximity to position 462, we examined the effect of FIP on
20 cell-cell fusion induced by the mutant F and wild-type H proteins. FIP inhibited
21 cell-cell fusion induced by the F and F(D439E) proteins but not the F(G464W) and
22 F(D439E/G464W) proteins (Fig. 2). These results indicate that the G464W

1 substitution in the F protein confers resistance to FIP on the F protein.

2

3 **HAd activity of mutant and wild-type H proteins**

4 Since Shibahara et al. reported that T11Ve-20 strain gained the ability to agglutinate
5 AGM-RBC (43), we examined HAd activity of mutant and wild-type H proteins.
6 When the H proteins were expressed in Vero cells, the wild-type H, H(N481Y), and
7 H(H495R) proteins did not show any detectable HAd activity in this experimental
8 condition (Fig. 3). In contrast, the H(N481Y/H495R) and Edmonston strain H
9 proteins showed marked HAd activity (Fig. 3). These results indicate that the
10 wild-type H protein with two amino acid substitutions (N481Y/H495R) has strong
11 CD46-binding activity that is almost equivalent to that of the Edmonston strain H
12 protein. The H(N481Y) protein promoted moderate cell-cell fusion with the wild-type
13 F protein but did not show HAd activity. These results suggest that the H(N481Y)
14 protein is similar to some paramyxovirus attachment protein mutants (5) that lack
15 receptor binding activity but still promote cell-cell fusion.

16

17 **Cell surface expression of the H and F proteins**

18 To verify that mutant H and F proteins were properly expressed on the cell surface,
19 flow cytometric analysis was performed.

20 When Vero cells were transfected with plasmids expressing the wild-type H or
21 H(N481Y/H495R), both proteins were expressed on the cell surface of Vero cells (Fig.
22 4). The H(N481Y/H495R) protein was more strongly stained by the antibody against

1 the H protein (Fig. 4). These results may reflect higher synthesis rate or higher
2 stability of the H(N481Y/H495R) protein in Vero cells than the wild-type H protein.

3 When Vero cells were transfected with plasmids expressing the F or
4 F(D439E/G464W) proteins, both proteins were expressed on the cell surface of Vero
5 cells (Fig. 5). The cell surface expression level of the F protein was slightly higher
6 than that of the F(D439E/G464W) protein in Vero cells (Fig. 5).

7 These results indicate that the H(N481Y/H495R) and F(D439E/G464W) proteins
8 were properly expressed on the cell surface of Vero cells although expression levels
9 were somewhat different from those of wild-type proteins.

10

11 **Syncytium formation of recombinant MV strains in Vero and B95a cells**

12 To characterize the contribution of the H(N481Y/H495R) and F(D439E/G464W)
13 proteins in the context of MV replication, the EGFP-expressing recombinant MV strains
14 with the H(N481Y/H495R) and/or F(D439E/G464W) proteins were generated using a
15 reverse genetics system of the IC-B strain (49). MV-wild corresponds to the IC323
16 strain expressing EGFP and has the original H and F proteins (14). MV-mH, MV-mF,
17 MV-mF/mH viruses have H(N481Y/H495R), F(D439E/G464W), and both
18 F(D439E/G464W) and H(N481Y/H495R) proteins, respectively. We first examined
19 syncytium formation induced by recombinant MV strains in Vero and B95a cells. In
20 Vero cells, MV-wild did not induce syncytium, and MV-mF induced fused cells
21 consisting of only a few cells, while MV-mH and MV-mF/mH induced moderate and
22 large syncytia, respectively (Fig. 6). Thus, the H(N481Y/H495R) protein is primarily

1 required for syncytium formation by wild-type MV in Vero cells, and the
2 F(D439E/G464W) protein strongly enhances syncytium formation. In B95a cells, all
3 recombinant MV strains induced large syncytium (Fig. 6).

4

5 **Growth of recombinant MV strains in Vero and B95a cells**

6 We then examined the growth kinetics of recombinant MV strains in Vero and B95a
7 cells. In Vero cells, MV-wild and MV-mF hardly grew under this experimental
8 condition, and MV-mH grew slowly (Fig. 7). In contrast, MV-mF/mH grew very
9 rapidly inducing extensive syncytia. The growth of MV-mF/mH declined after 48 h
10 p.i., because MV-mF/mH induced extensive syncytia, and infected cells were detached
11 from dishes. These results indicate that the H(N481Y/H495R) protein is primarily
12 required for growth in Vero cells and that the F(D439E/G464W) protein strongly
13 enhances the virus growth in Vero cells. In B95a cells, all recombinant MV strains
14 grew well but to different extents (Fig. 7). MV-wild and MV-mF/mH grew efficiently,
15 while MV-mF and MV-mH grew somewhat slowly. The growth of MV-mF/mH
16 declined after 48 h p.i., because of extensive syncytium formation and subsequent cell
17 death.

18

19 **Infection efficiency of recombinant MV in Vero cells**

20 We next examined infection efficiency of recombinant MV strains in Vero cells.
21 Since FIP did not block the second round of infection of MV-mF and MV-mF/mH,
22 agarose-containing medium was overlaid after infection, and then the number of

1 EGFP-expressing syncytia or cells expected to be derived from single EGFP-expressing
2 MV particle was counted (Fig. 8). MV-wild and MV-mF did not efficiently bind to
3 and enter into Vero cells under this experimental condition. In contrast, MV-mH and
4 MV-mF/mH bound to and entered into Vero cells efficiently. These results indicate
5 that the H(N481Y/H495R) protein is primarily important for efficient infection of
6 wild-type MV to Vero cells.

7

8 **Penetration rate of recombinant MV strains in Vero and B95a cells**

9 To further characterize the effect of the F(D439E/G464W) protein on MV infection,
10 we determined the penetration rate of recombinant MV strains in Vero cells by
11 inactivating virus that was attached but had not yet achieved cell entry by using low-pH
12 buffer. Although Vero cells were almost equally infected with both MV-mH and
13 MV-mF/mH (Fig. 8), MV-mF/mH penetrated more quickly into Vero cells than MV-mH
14 (Fig. 9). We next examined the penetration rate of recombinant MV strains in B95a
15 cells. MV-mF penetrated more quickly into B95a cells than MV-wild, and
16 MV-mF/mH penetrated more quickly into B95a cells than MV-mH (Fig. 9). These
17 results indicate that the F(D439E/G464W) protein enhances penetration rate and that the
18 F(D439E/G464W) protein compensates for the inefficient penetration of MV-mH in
19 Vero as well as B95a cells.

20

21

Discussion

22

We found two amino acid substitutions (N481Y/H495R) in the H protein in

1 comparison of the T11wild and T11Ve-23 strains; Shibahara et al. found only one amino
2 acid substitution (N481Y) in the H protein in comparison of the T11wild and T11Ve-20
3 strains and suggested that a protein(s) other than the H protein contributed to HAd
4 activity (43). However, as we confirmed that the wild-type H protein with
5 N481Y/H495R substitutions induced strong HAd activity, contribution of a viral
6 protein(s) other than the H protein is less likely. We think that MV RNA containing a
7 nucleotide substitution (A to G at position 8754) resulting in the H495R substitution
8 was still a minor population in the T11Ve-20 stock, and M13 phage clones containing
9 the mutated cDNA were not selected in three M13 clones used for sequencing. The
10 additional three passages of the T11Ve-20 in Vero cells to obtain the T11Ve-23 strain
11 may expand the population of mutant MV containing the H495R substitution in the H
12 protein.

13 The results of cell-cell fusion assay and HAd assay indicate that the wild-type H
14 protein with N481Y/H495R substitutions is fully capable of using CD46 as an
15 alternative receptor. Previous studies have shown that specific amino acid
16 substitutions (N481Y and S546G) in the H protein of the wild-type strain relative to
17 laboratory-adapted MV strains were critical in determining the use of CD46 as a cellular
18 receptor (2, 11, 15, 21, 24, 25, 30, 32, 38, 39, 41, 42, 43, 48, 59). Subsequent
19 systematic analyses identified amino acids or regions in the H protein that are important
20 for CD46 binding (26, 33, 46, 58). However, amino acid position 495 was not
21 mentioned in these papers. Therefore, this is the first report indicating the importance
22 of the amino acid position 495 in the wild-type H protein for CD46 binding. In the

1 crystal structure of the H protein (13), both positions 481 and 495 are in the β 4-sheet of
2 the side face of the head domain (data not shown).

3 We found that the F protein with the G464W substitution enhanced cell-cell fusion in
4 Vero cells. This result indicates that not only CD46 binding but also fusion activity of
5 the F protein affects cell-cell fusion in Vero cells. In a predicted three-dimensional
6 structure model for the F protein, position 464 is mapped in the C-terminal heptad
7 repeat (HR-B) prefusion stalk (22, 36, 61). It was reported that amino acid
8 substitution at residue 462 in the HR-B domain affected prefusion stability of the F
9 protein and conferred resistance to fusion inhibitors on the F protein (8). Likewise, the
10 G464W substitutions may decrease in the dissociation barrier between the HR-B/HR-B
11 linker domain and the head of the fusion protein and enhance fusion activity, as is the
12 case for amino acid substitutions at residue 462 (36).

13 Previous reports indicated that the P, C, V, M, H, and L proteins are important for
14 efficient growth of wild-type MV in Vero cells (9, 27, 45, 46, 48, 53, 54). However,
15 present results clearly indicate that the F protein is also important for the regulation of
16 cell specificity of MV. Since MV-mF did not grow in Vero cells, we speculate that
17 amino acid substitutions might be first introduced in the H protein and that subsequent
18 mutations might then be introduced into the F protein to grow efficiently in Vero cells.

19 Interestingly, MV-mH did not penetrate efficiently into B95a cells. One possibility
20 is that the H(N481Y/H495R) protein has weak SLAM-binding activity compared to the
21 wild-type H protein. Another possibility is that the H(N481Y/H495R) protein might
22 have some defects in functional interaction with the F protein. Several reports

1 indicated that the formation of the F-H protein complex influences the fusion function
2 of MV (6, 28, 34). Experiments are now underway to examine the formation of the
3 F-H protein complex to elucidate this point.

4 In this study, we did not examine the effect of amino acid substitutions in the N, V
5 and L proteins and a nucleotide substitution in the noncoding region of the F protein on
6 virus growth in Vero cells. Although these mutations might have some effect on virus
7 growth in Vero cells as reported previously (3, 45, 52), our results indicate that amino
8 acid substitutions in the F and H proteins are major determinants for Vero cell
9 specificity of wild-type MV strains.

10 Kobune et al. reported that MV strains isolated or passaged in Vero cells were
11 apathogenic in monkey models (18). Infection of monkeys with recombinant
12 wild-type MV strains constructed in this study might provide clues to the effect of
13 amino acid substitutions in the envelope proteins on pathogenicity and attenuation of
14 MV.

15

16

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Figure legends

16 Fig. 1. Cell-cell fusion induced by mutated H and F proteins expressed from cDNA in
17 Vero cells. Vero cells were cotransfected with various H and F protein expression
18 plasmids. An EGFP expression plasmid was also cotransfected to detect
19 small-cell-cell fusion. At 48 h posttransfection, the cells were observed under a
20 fluorescence microscope.

21

22 Fig. 2. Cell-cell fusion induced by mutated H and F proteins expressed from cDNA in

1 B95a cells. B95a cells were cotransfected with various H and F protein expression
2 plasmids in the presence (+) or absence of FIP. An EGFP expression plasmid was also
3 cotransfected to detect small-cell-cell fusion. At 48 h posttransfection, the cells were
4 observed under a fluorescence microscope.

5

6 Fig. 3. HAd activity of mutated H proteins. Vero cells were transfected with various
7 H protein expression plasmids. At 48 h posttransfection, AGM-RBC were added to
8 cells and incubated for 1 h at 37°C. After a washing step, adsorbed AGM-RBC were
9 lysed in H₂O, and released hemoglobin was measured at 540 nm. Ed-H, Edmonston H
10 protein; Abs, absorbance.

11

12 Fig. 4. Synthesis and cell surface expression of the H protein in Vero cells. Cell
13 suspension of transfected Vero cells were fixed with paraformaldehyde and incubated
14 with the anti-H monoclonal antibody and fluorescein isothiocyanate-conjugated
15 anti-mouse IgG, and cell surface expression of the H protein was analyzed by a
16 FACSCalibur.

17

18 Fig. 5. Synthesis and cell surface expression of the F protein in Vero cells. Cell
19 suspension of transfected Vero cells were fixed with paraformaldehyde and incubated
20 with the anti-F monoclonal antibody and fluorescein isothiocyanate-conjugated
21 anti-mouse IgG, and cell surface expression of the F protein was analyzed by a
22 FACSCalibur.

1

2 Fig. 6. Syncytium formation of recombinant MV strains in Vero and B95a cells.
3 Vero and B95a cells were infected with MV-wild, MV-mH, MV-mF, or MV-mF/mH at
4 multiplicities of infection of 0.1 to 0.01. At 48 h p.i., EGFP autofluorescence in
5 MV-infected cell monolayer was observed under a fluorescence microscope. Panels
6 show representative images.

7

8 Fig. 7. Replication kinetics of recombinant MV strains in Vero and B95a cells. Vero
9 and B95a cells were infected with MV-wild (open triangles), MV-mH (open circles),
10 MV-mF (solid triangles), or MV-mF/mH (solid circles) at a multiplicity of infection of
11 0.01 per cell. Cells were harvested with culture medium at the indicated time point,
12 and TCID₅₀s were determined in B95a cells.

13

14 Fig. 8. Infection efficiencies of recombinant MV strains on Vero cells. Vero cells in
15 12-well cluster plates were infected with 100 TCID₅₀s of MV-wild, MV-mH, MV-mF, or
16 MV-mF/mH and overlaid with 0.8% agarose-containing medium. After 48 h p.i., cell
17 infectious units were counted under a fluorescence microscope. The averages and
18 standard deviations of the results of three experiments are shown.

19

20 Fig. 9. Penetration rates of recombinant MV strains in Vero and B95a cells. Vero
21 and B95a cells were inoculated with MV-wild (light gray bars), MV-mH (open bars),
22 MV-mF (black bars), or MV-mF/mH (dark gray bars) and incubated for various time at

1 37°C, and viruses that had not penetrated into the cells were then inactivated by
2 treatment with citric buffer (pH 3.0) at room temperature for 1 min. After two washes
3 with DMEM, the cells were overlaid with 0.8% agarose-containing medium and
4 incubated at 37°C for 48 h, and cell infectious units were counted under a fluorescence
5 microscope. The number of cell infectious units on Vero and B95a cells incubated
6 with viruses for 60 min and without citric buffer treatment were set to 100%.

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9