

# Measles Fusion Machinery Is Dysregulated in Neuropathogenic Variants

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**ABSTRACT** Paramyxoviruses, including the human pathogen measles virus (MV), enter host cells by fusing their viral envelope with the target cell membrane. This fusion process is driven by the concerted actions of the two viral envelope glycoproteins, the receptor binding protein (hemagglutinin [H]) and the fusion (F) protein. H attaches to specific proteinaceous receptors on host cells; once the receptor engages, H activates F to directly mediate lipid bilayer fusion during entry. In a recent MV outbreak in South Africa, several HIV-positive people died of MV central nervous system (CNS) infection. We analyzed the virus sequences from these patients and found that specific intrahost evolution of the F protein had occurred and resulted in viruses that are “CNS adapted.” A mutation in F of the CNS-adapted virus (a leucine-to-tryptophan change present at position 454) allows it to promote fusion with less dependence on engagement of H by the two known wild-type (wt) MV cellular receptors. This F protein is activated independently of H or the receptor and has reduced thermal stability and increased fusion activity compared to those of the corresponding wt F. These functional effects are the result of the single L454W mutation in F. We hypothesize that in the absence of effective cellular immunity, such as HIV infection, MV variants bearing altered fusion machinery that enabled efficient spread in the CNS underwent positive selection.

**IMPORTANCE** Measles virus has become a concern in the United States and Europe due to recent outbreaks and continues to be a significant global problem. While live immunization is available, there are no effective therapies or prophylactics to combat measles infection in unprotected people. Additionally, vaccination does not adequately protect immunocompromised people, who are vulnerable to the more severe CNS manifestations of disease. We found that strains isolated from patients with measles virus infection of the CNS have fusion properties different from those of strains previously isolated from patients without CNS involvement. Specifically, the viral entry machinery is more active and the virus can spread, even in the absence of H. Our findings are consistent with an intrahost evolution of the fusion machinery that leads to neuropathogenic MV variants.

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Measles virus (MV) infection remains one of the leading causes of death among young children worldwide (1), despite the availability of an effective live-virus vaccine. Measles was thought to be eliminated in the United States in 2000 (defined as an interruption of continuous transmission lasting  $\geq 12$  months) (2); it was considered to be a problem only in developing countries (3). In 2001, the American Red Cross, United Nations Foundation, U.S. Centers for Disease Control and Prevention, UNICEF, and World Health Organization launched a global partnership called the Measles Initiative, whose mission was 2-fold: interruption of MV transmission in large geographic areas and reduction of measles deaths by 90% before 2010 through improved vaccination coverage (1, 4). A 71% reduction in global mortality from measles was achieved between 2000 and 2011. However, there has

been a resurgence of measles disease in the United States, with more cases in 2014 than at any time since 1996.

MV infection starts in the respiratory tract. The alveolar macrophages and dendritic cells are the primary targets (5–7) that express the MV receptor signaling lymphocyte activation molecule (SLAM, also called CD150). Attachment of the MV receptor binding protein hemagglutinin (H) to CD150 leads to infection of these cells, which then transmit the virus to bronchus-associated lymphoid tissues and/or draining lymph nodes. The virus proliferates in CD150-expressing B and T lymphocytes, and viremia ensues (5, 8). The adherens junction protein (PVRL4 or nectin 4) also serves as an MV receptor but is found on the basolateral surface of respiratory epithelial cells; it is implicated in viral transmission at later stages of illness (8–10). In healthy individuals, MV

infection elicits a very strong MV-specific immune response, including transient immune suppression. Viremia is brought under control by cellular immunity within 2 weeks of infection, even though viral genomes can persist for several more months, even in the face of humoral immunity (5, 11, 12).

While MV may spread to the central nervous system (CNS) in up to 50% of infected patients, as evidenced by abnormal electroencephalographic activity (13, 14), the findings generally are transient. However, severe CNS complications may also occur soon after infection, such as with acute encephalomyelitis (AME), or years after infection as a result of viral persistence, such as with subacute sclerosing panencephalitis (SSPE). Even with presumably functional cell-mediated immunity and high antiviral antibody titers, CNS infection is not eradicated in patients suffering from SSPE (15). The third form of MV-induced CNS disease—progressive infectious encephalitis or measles inclusion body encephalitis (MIBE)—occurs in immunosuppressed patients several months following measles infection.

During the initial steps of infection, MV attaches to target cells and enters via the concerted actions of the MV H and fusion (F) proteins, which are present on the surface of the virus. In the infected cell, F is synthesized as a precursor (F<sub>0</sub>) that is cleaved within the cell to yield the prefusion F complex comprising three C-terminal F<sub>1</sub> subunits associated via disulfide bonds with three N-terminal F<sub>2</sub> subunits. On the surfaces of new infectious virions, this trimeric F structure is kinetically trapped in a metastable conformation, primed for fusion activation upon engagement of the H glycoprotein by a target cell surface receptor (either CD150 or nectin 4 for wild-type [wt] MV) (10, 16–18). After receptor engagement by H, the prefusion F protein on the viral surface undergoes a structural transition, extending and inserting its hydrophobic fusion peptide into the target cell. F then refolds into a stable postfusion 6-helix bundle structure, a process that brings the viral and target cell membranes together to initiate fusion. We refer to the H/F pairs of MV as the viral fusion machinery, since these proteins act in concert to bring about entry (19–22). The mechanisms whereby MV infects and spreads in the CNS are still unclear but have been shown to require F protein (23–25) and thus can be inhibited by fusion inhibitors targeting MV F (23, 26).

We characterized the fusion machinery of neuropathogenic MV isolates recovered from the CNS of patients who suffered from MIBE that was fatal and found that in these variant viruses, the fusion machinery is altered so that F is more readily activated. For paramyxovirus fusion molecules, a specific balance between stability and activation is required for fitness *in vivo* (27, 28); overly active fusion machinery seems to be a detriment to viability. Nevertheless, in these CNS MV isolates, the usual balance of F stability versus activation was skewed toward activation. Our data show that F proteins from two MIBE patients contain one specific modification that increases F's ability to mediate fusion and decreases F's thermal stability. We hypothesize that MV underwent intrahost evolution leading to CNS-pathogenic MV variants, possibly facilitated by growth in HIV-infected individuals with impaired cellular immunity.

## RESULTS

**Properties of the H/F fusion machinery of MV isolates from MIBE patients.** MV was isolated from the postmortem brain tissue of two HIV-infected patients who were diagnosed with MIBE via positive MV PCR during a measles epidemic in South Africa.

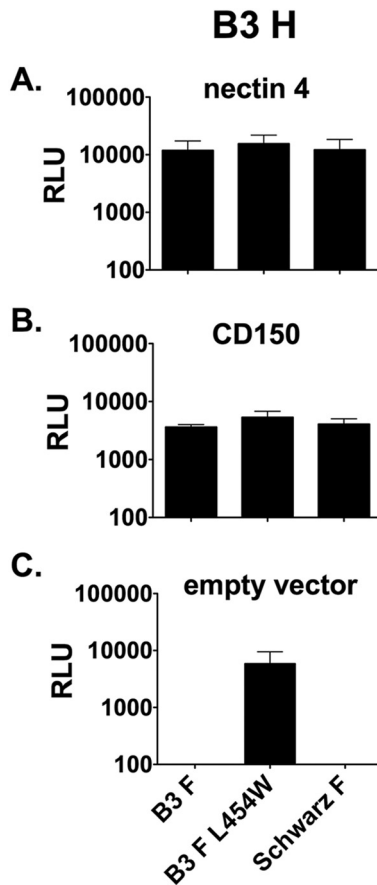
Viral-genome sequencing revealed that in both cases, the MV F gene contained the same nucleotide mutation, one that resulted in a leucine-to-tryptophan substitution at position 454 (L454W) (29). The first patient was a 27-year-old woman who developed MIBE 3 months after her acute measles. Of note, the L454W mutation present in the virus isolated from her brain was not present in virus from the earlier blood samples obtained during her acute MV infection. The second was a 34-year-old woman who developed typical MIBE symptoms 3 weeks after acute MV infection.

To analyze the functional differences between the H/F fusion machinery of wt MV and “CNS-adapted” isolates, H and F genes were obtained from the wt B3 (the endemic genotype in most of the African continent [30]), Schwarz (vaccine strain [31]), and IC323 (a recombinant wt MV strain [32]) MV strains and cloned into the pCAGGS expression vector. The L454W mutation was introduced in different wt-MV backgrounds to compare its functional effects on F. We analyzed the fusion properties of these MV H/F pairs, their requirements for receptor engagement, and their receptor preference in a quantitative fusion assay. Cells coexpressing B3 MV H and the F proteins indicated in Fig. 1 together with the alpha peptide of  $\beta$ -galactosidase ( $\beta$ -Gal) were overlaid with cells expressing the  $\beta$ -Gal omega peptide and either the MV receptor nectin 4 (Fig. 1A), the MV receptor CD150 (Fig. 1B), or an empty vector (Fig. 1C). Upon cell-to-cell fusion, the alpha and omega peptides reconstitute  $\beta$ -Gal activity, which is proportional to the extent of fusion.

Consistently with the fact that nectin 4 is the MV receptor with the strongest affinity for H (17, 18, 35, 36), coexpression of B3 MV H with the F proteins indicated in Fig. 1 effectively promoted fusion in the presence of nectin 4 (12,000 to 15,000 relative luminescence units [RLU]) (Fig. 1A). In addition, all three F proteins (B3 F, B3 F L454W, and Schwarz F) induced similar levels of fusion (note that we did not observe any defects or differences in the processing of these F proteins; see Fig. S2 in the supplemental material). Fusion with cells expressing CD150 was less than fusion with cells expressing nectin 4, consistent with the reported lower affinity of MV for the CD150 receptor (17, 18, 35, 36), but again, the various F proteins were equally fusogenic (Fig. 1B). However, when the target cells were not expressing any known MV receptor, only F bearing the L454W mutation induced fusion when coexpressed with B3 MV H (6,000 RLU) (Fig. 1C).

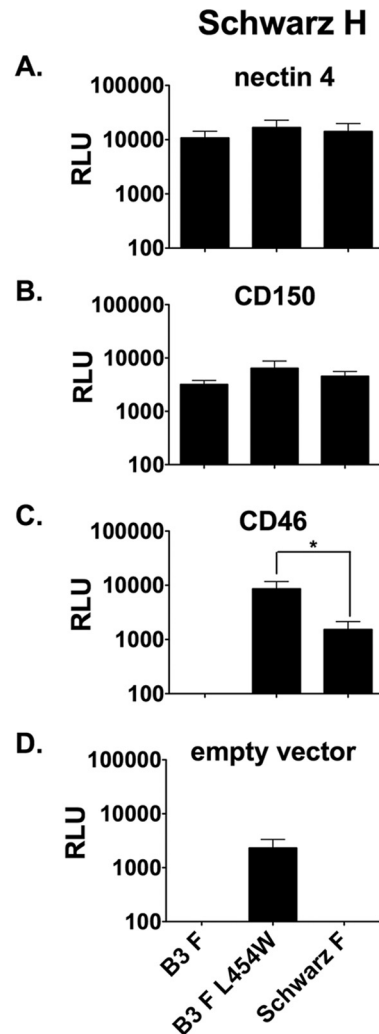
In Fig. 2, we assessed fusion promotion by the three F proteins (as shown in Fig. 1) but in the presence of the H protein of the Schwarz strain (MV vaccine strain [31, 37]). When the target cells expressed either nectin 4 (Fig. 2A) or CD150 (Fig. 2B), the three F proteins induced similar levels of fusion when coexpressed with the Schwarz strain's H. Target cells bearing CD46, which is a functional receptor for the Schwarz strain of MV but not for the wt or field strains, were also tested for fusion with the H/F pairs (Fig. 2C). Schwarz's H failed to activate B3's F despite the availability of CD46, and fusion was observed only with coexpression of Schwarz's F (1,500 RLU) or B3's F bearing the L454W mutation (9,000 RLU). Fusion with cells lacking a known MV receptor was observed only with B3's F L454W (3,000 RLU) (Fig. 2D).

**Functional analysis of F proteins present in CNS-adapted MV strains.** We compared the functional features of F bearing the L454W mutation with those of previously described F proteins from SSPE MV strains bearing T461I and S103I N462S N465S and F protein with laboratory-generated hyper-fusogenic mutations (N462K and S262R) (38). The N462K mutation arose under the



**FIG 1** MV B3 H-mediated fusion. The cell-to-cell fusion of 293T cells coexpressing the indicated MV F protein and MV B3 H with BHK21 cells transfected with MV receptor nectin 4 (A), with CD150 (B), or with an empty vector (C) was assessed by a  $\beta$ -Gal complementation assay as described in Materials and Methods. The values are means (with SEM) of results from triplicate experiments.

selective pressure of a small-molecule fusion inhibitor (39), and S262R (in a region near the heptad repeat domain at the N terminus implicated in fusion triggering) emerged during tissue culture passage of strain IC323-EGFP (40). We introduced the L454W mutation into the background of wt IC323 F; IC323 F was chosen because it was recently used to assess the effect of F mutations found in SSPE patients on neuropathogenicity *in vivo* (40); therefore, all the mutations of interest were studied here in the background of IC323's F. The fusion properties conferred by the specific F mutations in the presence of three different MV receptors were assessed (Fig. 3). For purposes of comparison, we included the human parainfluenza virus type 3 (HPIV3) fusion machinery, comprised of receptor binding hemagglutinin-neuraminidase (HN) and F; for this experiment, we used an HN bearing a mutation (T193A) that enhances fusogenicity (41). HPIV3 HN engages ubiquitous sialic acid molecules, and fusion is expected to be independent of the specific target cell receptor used in this experiment. All the MV F proteins coexpressed with IC323 MV H effectively promoted approximately similar levels of fusion with nectin 4-bearing cells (Fig. 3A). With CD150-expressing target cells, levels of fusion mediated by MV H/F pairs were similar for all F proteins (<1-fold differences) (Fig. 3B) and, as expected based on

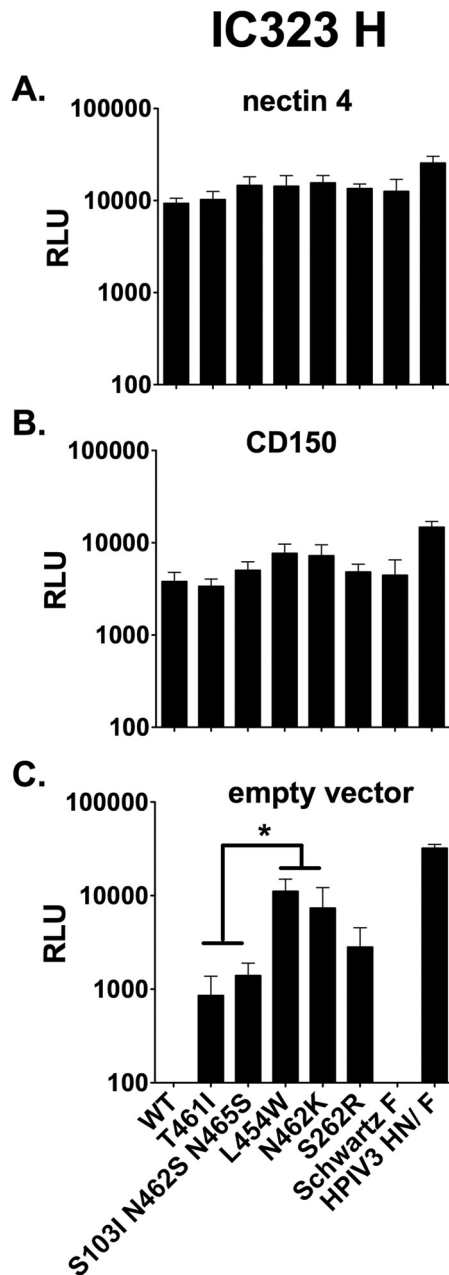


**FIG 2** MV Schwarz H-mediated fusion. The cell-to-cell fusion of 293T cells coexpressing the indicated MV F protein and MV Schwarz H with BHK21 cells transfected with MV receptor nectin 4 (A), with CD150 (B), with CD46 (C), or with an empty vector (D) was assessed by a  $\beta$ -Gal complementation assay as described in Materials and Methods. The values are means (with SEM) of results from triplicate experiments. \*,  $P < 0.05$  (one-tailed, unpaired *t* test).

the results shown in Fig. 1, were less than levels of fusion with nectin 4-expressing cells for all the F proteins.

In the absence of a known receptor, no fusion was induced by wt IC323's F or Schwarz's F, but fusion was induced by all the mutant F proteins (Fig. 3C). F bearing the L454W mutation induced more fusion than the SSPE-derived F proteins. The fusion induced by F L454W is similar to that induced by hyper-fusogenic F bearing N462K. While all the mutated F proteins promoted fusion when coexpressed with the receptor binding protein MV H in the absence of a known MV receptor, the extent of fusion mediated by F bearing the SSPE mutations was less than that mediated by MIBE L454W F and the lab-adapted F proteins (N462K F and S262R F). Taken together, these data suggest that coexpression of MV H with the variant neuropathogenic F proteins is sufficient to mediate fusion, even without known MV receptors.

**Regulation of fusion promotion by the MV receptor binding protein.** The finding that variant F proteins mediate fusion in the



**FIG 3** Functional analysis of F proteins from CNS-adapted MV strains. The cell-to-cell fusion of 293T cells coexpressing the indicated MV F protein and MV IC323 H with BHK21 cells transfected with MV receptor nectin 4 (A), with CD150 (B), or with an empty vector (C) was assessed by a  $\beta$ -Gal complementation assay as described in Materials and Methods. The values are means (with SEM) of results from triplicate experiments. \*,  $P < 0.05$  (two-tailed, unpaired  $t$  test).

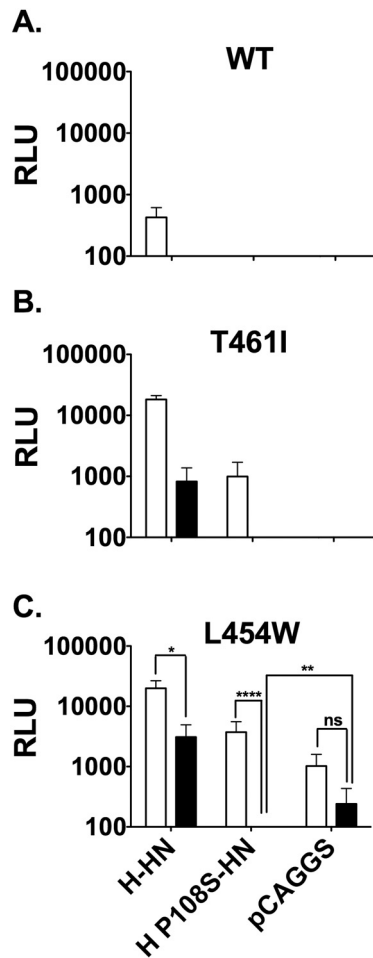
absence of known receptors for H (Fig. 1C, 2D, and 3C) raised the question of whether the variant F proteins can activate independently of receptor-engaged H or whether they respond to H engagement by an as-yet-unknown receptor. To address this question, we used chimeric receptor binding proteins that permit us to experimentally modulate the activities of H, including receptor engagement and F activation. The use of the globular head of a sialic acid-binding paramyxovirus (HPIV3) and the stalk of MV H

in these chimeras allows for independent analysis of the functions of the stalk of H. The HPIV3 head allows us to use a small sialic acid analog (zanamivir) to modulate receptor engagement and thereby separate the functions of receptor engagement and F triggering. Zanamivir blocks receptor binding so that the independent role of the stalk domain can be assessed when the molecule is not receptor engaged, as we have shown previously for other chimeric receptor proteins (33, 34, 42) and HPIV3 HN variants (43).

The two chimeric receptor binding proteins for this experiment were comprised of the MV IC323 H stalk domain and the HPIV3 HN globular head (H-HN). One of the two H-HN proteins bears the mutation P108S in the stalk region ( $H_{P108S}$ -HN), which decreases activation of MV F by the MV H stalk (34). The two H-HN proteins bound to sialic acid-bearing red blood cells (RBCs), as expected, and promoted the ability of neuropathogenic MV F proteins to mediate fusion (Fig. S2). The fusion properties of the chimeric receptor binding proteins were quantified with the  $\beta$ -galactosidase complementation assay used in Fig. 1 to 3. Effector cells were cotransfected with H-HN/F pairs comprising either H-HN,  $H_{P108S}$ -HN, or the empty vector pCAGGS and one of the following fusion proteins, wt MV F (Fig. 4A), F T461I (Fig. 4B), or F L454W (Fig. 4C), along with the beta-galactosidase alpha peptide. The effector cells were also transfected with uncleaved influenza virus hemagglutinin (HA), a nonhomologous sialic acid binding protein present solely for its tethering function (to bring the two cell populations into appropriately close contact), so that all experimental conditions were tested in the presence of equivalent tethering. The cells were overlaid with target cells expressing the omega peptide in the absence (white bars) or presence (black bars) of 5 mM zanamivir, a small sialic acid analog that disengages the HPIV3 globular domain from its sialic acid receptor (44). Uncleaved influenza virus HA mediates cell-to-cell contact even in the presence of zanamivir, allowing independent analysis of the effect of cell tethering versus specific-receptor engagement, but does not cause fusion by itself or activate F (43, 45, 46).

The H-HN chimeric protein promoted fusion mediated by wt MV F (Fig. 4A), as we have seen previously with a chimeric protein that bears the globular head of Newcastle disease virus (NDV) and the stalk domain of MV H (34). Fusion was abolished in the presence of the triggering-defective  $H_{P108S}$ -HN chimeric protein. No fusion was observed in the presence of zanamivir with either chimeric protein, indicating that activation of wt F by the receptor binding protein requires receptor engagement. For F T461I from an SSPE MV strain, H-HN effectively promoted fusion (18,280 RLU); in contrast to wt F, this F protein also promoted some fusion in the presence of zanamivir to disengage H-HN from the receptor and when it was coexpressed with triggering-impaired  $H_{P108S}$ -HN (995 RLU), although its fusion was much less than when it was coexpressed with receptor-engaged H-HN. Without expression of any specific binding protein, fusion was negligible (65 RLU).

The fusion of F L454W in the presence of the chimeric receptor-binding proteins is shown in Fig. 4C. F L454W mediates fusion when either H-HN or  $H_{P108S}$ -HN is also expressed, though much less in the presence of  $H_{P108S}$ -HN with a defective stalk (3,725 RLU compared to 19,957 RLU). F L454W mediates some fusion in the absence of a specific receptor binding protein (1,021 RLU), though fusion is less than when either of the H-HN chimeric proteins is also expressed. In the presence of zanamivir to disengage the chimeric receptor binding proteins from the sialic acid



**FIG 4** Regulation of fusion promotion by receptor engagement and by the stalk domain of the receptor binding protein. The cell-to-cell fusion of 293T cells coexpressing uncleaved influenza virus HA, the indicated chimeric binding proteins (*x* axis), and wt MV F (A), MV F T461I (B), or MV F L454W (C) with BHK21 cells transfected with an empty vector was assessed in the absence (white bars) or presence (black bars) of 5 mM zanamivir. Fusion was assessed by a  $\beta$ -Gal complementation assay as described in Materials and Methods. The values are means (with SEM) of results from triplicate experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.005$  (two-tailed, unpaired *t* test).

receptor, F L454W mediates some fusion (3,092 RLU) when it is coexpressed with H-HN but less than when receptor-engaged H-HN is present, showing that F is H responsive. When coexpressed with receptor-disengaged H<sub>P108S</sub>-HN (with a defective stalk [34]), F L454W, like F T461I, mediates almost no fusion (8 RLU; less than with no H at all). Thus, both F L454W and F T461I require a functional H stalk and receptor engagement to be maximally activated, and in the presence of a functional receptor binding protein, disengagement from the receptor decreases the fusion properties of the L454W F protein, indicating that this mutant F is still functionally responsive to the stalk activity of the receptor binding protein. The finding that the chimeric H-HN protein with a triggering-defective stalk, when free of the receptor, actually decreases fusion mediated by L454W F compared with that with no H stalk at all may suggest that H can exert a suppressive role on fusion triggering, as we have proposed for HPIV3 (43).

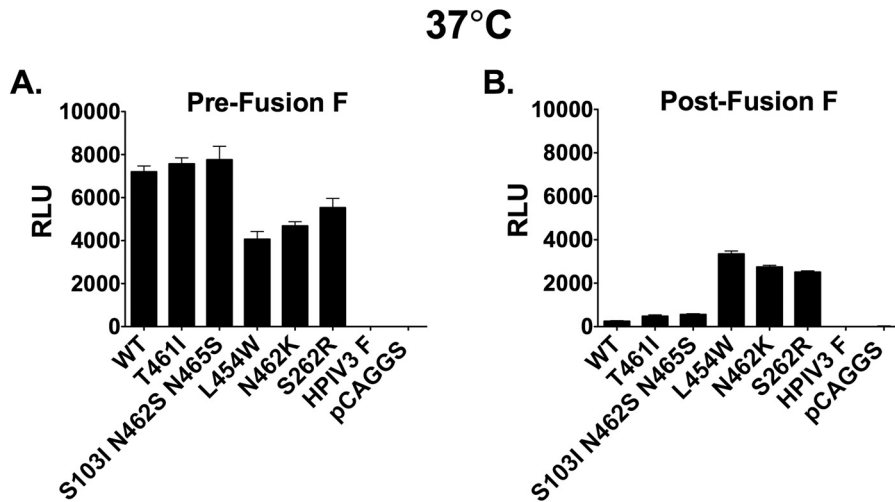
**Analysis of the thermal stability of the F proteins from IC323 and CNS-adapted MV strains.** In light of the enhanced fusion

mediated by F L454W—whether in the presence of H-HN or in the presence of the stalk-defective H<sub>P108S</sub>-HN engaged with the receptor—we investigated the thermal stability of the MV F proteins to determine whether the L454W mutation may confer instability accompanied by a propensity for activation. Cells expressing IC323-derived F proteins bearing either L454W, the SSPE-derived mutations (T461I and S103I N462S N465S), or the hyper-fusogenic mutations (N462K and S262R) or HPIV3 F were incubated overnight (O/N) at 37°C, and stained with previously characterized monoclonal antibodies (MAbs) that distinguish between prefusion and postfusion F (47). Control cells were transfected with the empty vector pCAGGS. Binding of the MAbs was quantitated (RLU) with a secondary antibody conjugated to  $\beta$ -galactosidase (34). The prefusion antibody, which recognizes F before fusion activation, bound similarly to wt F and the SSPE-derived F protein that had been incubated at 37°C but less to F L454W and to F bearing the known hyper-fusogenic mutations N462K and S262R (Fig. 5A). The postfusion MAb, which recognizes F after fusion activation, showed only negligible binding to the wt F and the SSPE-derived F proteins, but significant postfusion signal was observed for the F proteins bearing L454W, N462K, and L262R (Fig. 5B). This result suggested that even at 37°C, the L454W mutation reduced thermal stability compared to that of wt F.

In order to assess the thermal stability of each F protein in its prefusion state, the expressing cells were incubated O/N at 32°C instead of 37°C to prevent activation of any F during the O/N incubation. Cell protein synthesis was then synchronized using cycloheximide (see Materials and Methods), and the cells were transferred to the temperatures and times indicated in Fig. 6 and probed with the prefusion antibody. At 4°C (Fig. 6A), without any opportunity for activation, the prefusion signals were similar for all the F proteins. After incubation at 50°C for 10 min, only background levels of prefusion F were detected for the L454W, N462K, and S262R F proteins (Fig. 6B). In contrast, both SSPE F variants had prefusion levels similar to that of wt F. After incubation at 50°C for 30 min, only the wt and SSPE T461 F proteins still had any signal for prefusion F, although much less than they had 20 min earlier (Fig. 6C). As previously reported (47), at 60°C after 10 min, none of the F proteins had a detectable portion in the prefusion state. These data indicate that MIBE F L454W (like the F proteins bearing the known hyper-fusogenic mutations) is less thermally stable than the F proteins bearing SSPE-derived mutations (T461I and S103I N462S N465S), offering a potential basis for its fast-triggering phenotype.

**Fusion and thermal stability in the presence of influenza virus HA.** The relative thermal instability of MIBE F (L454W), shown in Fig. 6, taken together with the finding that expressed F L454W can mediate fusion without a homotypic receptor binding protein (Fig. 4C) raises the question of whether H-independent F activation for MIBE F or any of the other F proteins under investigation can lead to functional fusion. We explored this question by assessing H-independent fusion in parallel with thermal activation at a range of temperatures for F L454W and compared results to those for F T461I, an SSPE-derived F protein that is as thermally stable as wt F (Fig. 6). The target receptor-bearing cells in this assay were RBCs that tolerate a range of incubation temperatures and provide precise quantitation of fusion (43).

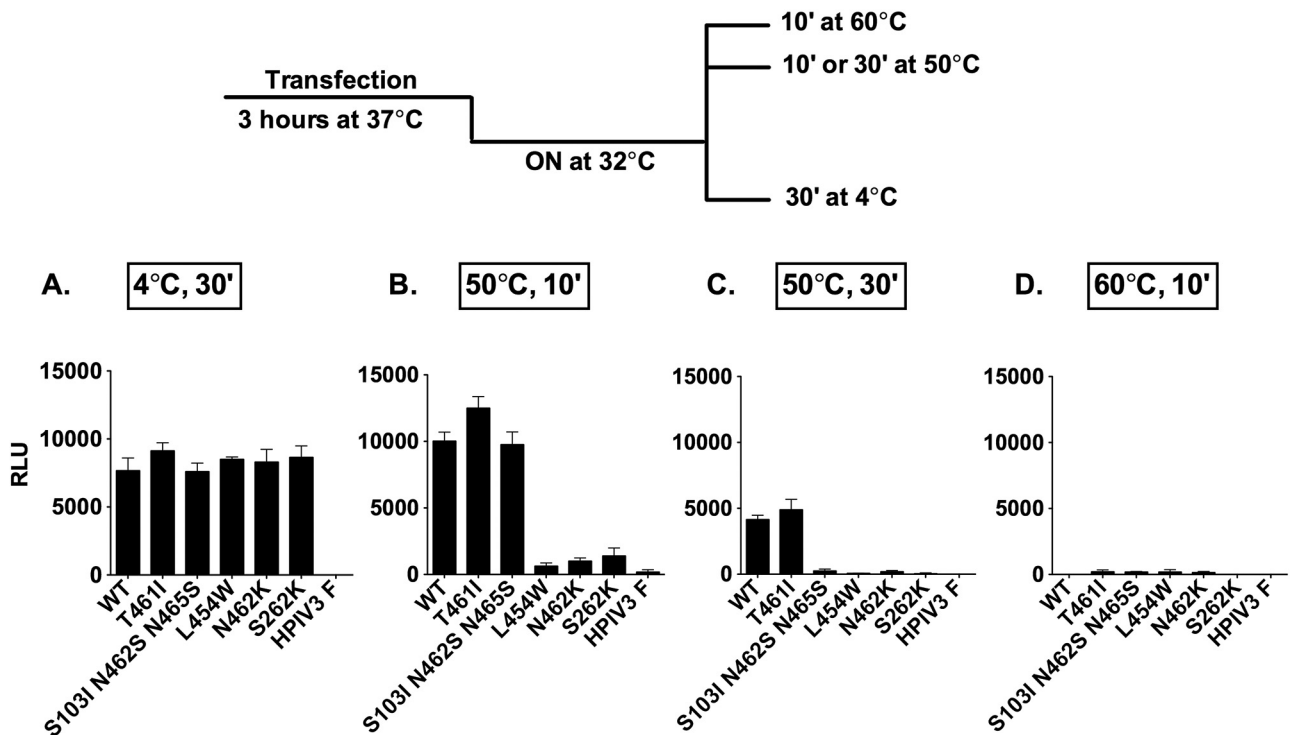
Cells expressing the F proteins indicated in Fig. 7 (or the empty vector pCAGGS as a control) together with uncleaved influenza



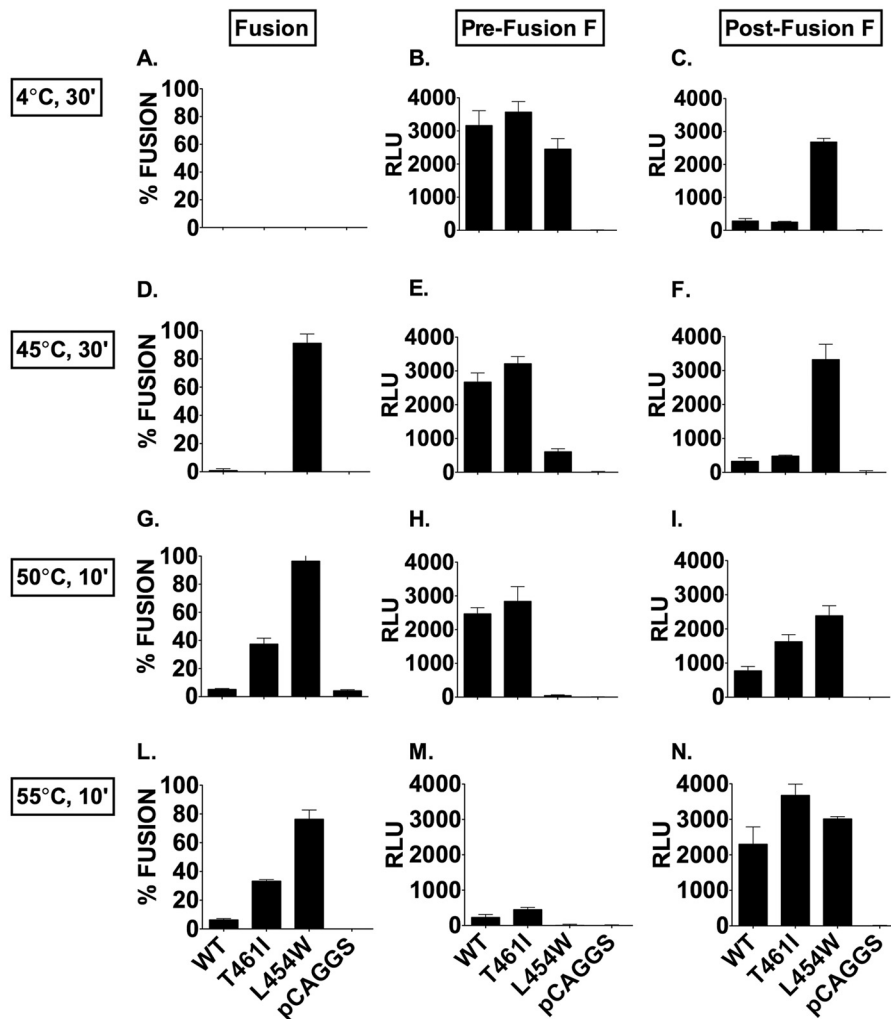
**FIG 5** Binding of conformation-specific monoclonal antibodies to wt and mutant IC323 F proteins. 293T cells expressing the indicated MV F proteins, HPIV3 F, and the empty vector pCAGGS (x axis) were incubated overnight at 37°C. Cellular protein expression was synchronized by a 1-h cycloheximide incubation. Cells were incubated at 4°C with mouse MAbs recognizing either the prefusion (A) or the postfusion (B) state of MV F and stained as described in Materials and Methods. The values on the y axis represent the relative luminescence units (RLU) of the stained cells and are averages from triplicate samples (with standard deviations [SD]) from a representative experiment, repeated three times.

virus HA (as in Fig. 4C, a nonhomologous sialic acid binding protein present solely for its tethering function) were incubated O/N at 32°C as described for Fig. 6. Cell protein synthesis was then synchronized using cycloheximide, and the expressing cells were also neuraminidase (NA) treated to ensure that the expressed HA

would interact exclusively with target cell receptors without interference from sialic acid moieties on the F/HA-expressing effector cells (43). In panels A, D, G, and L of Fig. 7, the cells were then allowed to bind to receptor-bearing cells (i.e., RBCs) at 4°C and then subjected to the temperatures indicated in Fig. 7 for the noted



**FIG 6** Thermal stability of the wt and mutant IC323 F proteins. 293T cells expressing the indicated MV F proteins or HPIV3 F (x axis) were incubated overnight at 32°C. Cellular protein expression was synchronized by a 1-h cycloheximide incubation. The cells were then incubated at either 4°C for 30 min (30') (A), 50°C for 10 min (B), 50°C for 30 min (C), or 60°C for 10 min (D) and then at 4°C with mouse MAbs recognizing the prefusion state of MV F. Cells were stained as described in Materials and Methods. The values on the y axis represent the relative luminescence units (RLU) of the stained cells and are averages of results for triplicate samples (with SD) from a representative experiment repeated 3 to 4 times.

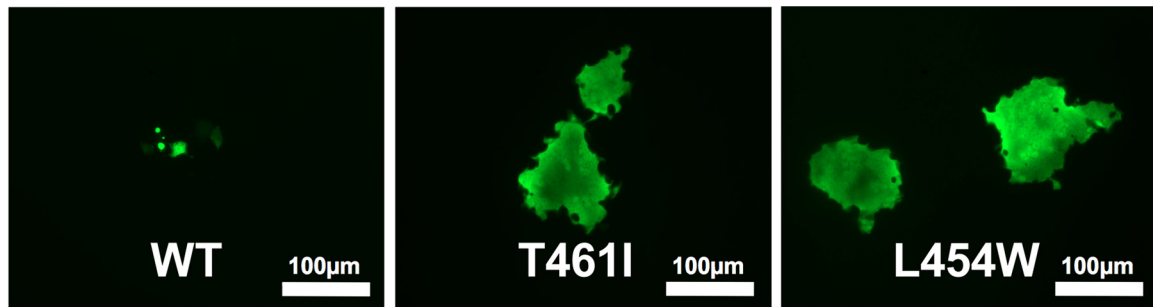


**FIG 7** Temperature-induced fusion and thermal stability of the wt and mutant IC323 F L454W and T461I proteins. 293T cells coexpressing uncleaved influenza virus HA and the indicated MV F proteins or the empty vector pCAGGS (*x* axis) were incubated overnight at 32°C. Cellular protein expression was synchronized by a 1-h cycloheximide incubation. Cells were allowed to bind to receptor-bearing RBCs at 4°C before being subjected to the indicated temperatures and then transferred to three different conditions. For panels A, D, G, and L (fusion), cells were then incubated at either 4°C for 30 min (A), 45°C for 30 min (D), 50°C for 10 min (G), or 55°C for 10 min (L). The values on the *y* axis reflect quantification of RBCs that were fused. For panels B, E, H, and M (prefusion signal), the cells were incubated at 4°C with MAb recognizing the prefusion state of MV F and stained as described in Materials and Methods; the values on the *y* axis represent the relative luminescence units (RLU) of the stained cells, reflecting quantification of prefusion MAb bound. For panels C, F, I, and N (postfusion signal), the cells were incubated at 4°C with MAb recognizing the postfusion state of MV F and stained as described in Materials and Methods, and the values on the *y* axis represent the relative luminescence units (RLU) of the stained cells, reflecting quantification of postfusion MAb bound. The data are averages of results for triplicate samples (with SD) from a representative experiment repeated 3 times.

times. Fusion with RBCs was quantified and is expressed as a percentage of total bound RBCs (*y* axis) (43). The cells expressing F L454W fused with the target cells (100%) in the absence of MV H at a temperature of 45°C, but F T461I (which is the SSPE-derived F protein that rendered the IC323 virus most lethal *in vivo* in a previous report [40]) did not promote fusion at 45°C but did promote partial fusion even at the higher temperatures of 50°C and 55°C. wt F did not mediate fusion in the absence of MV H, as previously noted (47), even at the temperature at which it was seen (Fig. 6) to transition out of its prefusion state.

In direct parallel with the fusion experiment, we assessed the thermal stability of F L454W compared to those of F T461I and wt F (with the empty vector pCAGGS as a control). After overnight incubation at 32°C, protein synthesis was synchronized with cy-

cloheximide and the cells were transferred to the temperatures indicated in Fig. 7. Figure 7B, C, E, F, H, I, M, and N show cells stained with the MAbs that distinguish between prefusion and postfusion F (as in Fig. 6), and the *y* axis (RLU) reflects the prefusion signal for the three indicated F molecules. At 4°C, without any opportunity for activation, the prefusion signals were similar for all F proteins (Fig. 7B). However, the postfusion signal (Fig. 7C) was substantial for F L454W while negligible for F T461I and wt F. At 45°C, the prefusion MAb (Fig. 7E) bound to wt and T461I F, indicating the presence of substantial prefusion F, but there was very little signal for F L454W. The prefusion state signal for F L454W approached the level of the negative control, indicating that at this temperature there was very little F L454W remaining in the prefusion state. Almost all the expressed F L454W was present



**FIG 8** Plaque formation by recombinant viruses bearing variant F proteins. Vero cells (without the MV receptor) were infected with the indicated viruses and incubated at 37°C for 48 h.

in the postfusion state (Fig. 7F), as recognized by the postfusion MAb.

Even at 50°C, a portion of F T461I and wt F were still present in the prefusion state; at this temperature, F L454W was all in the postfusion state, with negligible prefusion signal (Fig. 7H). At 55°C, the prefusion MAb no longer detected any of the 3 F proteins. In fact, all three were detected by the postfusion MAb, indicating that at this temperature all the F proteins had been activated to their postfusion state.

Because the fusion assay and the pre- and postfusion states were carried out in parallel, these data indicate that the presence of F L454W in the postfusion state (Fig. 7E, F, H, and I) correlates with induction of H-independent fusion by this F molecule (Fig. 7D and G). However, even when significant conversion of F T461I and wt F to the postfusion state has occurred (Fig. 7I and N), these F molecules fail to mediate significant H-independent fusion (Fig. 7G and L).

**Recombinant viruses bearing CNS-adapted F proteins.** To observe the properties of the mutated glycoproteins in the context of infectious viruses, we cloned the SSPE F (T461I) and MIBE F (L454W) genes in the backbone of the MV IC323 DNA clone and generated recombinant viruses that express enhanced green fluorescent protein (EGFP) for direct visualization of infected cells (24, 40, 48). Recombinant viruses bearing F L454W were placed *in vitro* in the presence of sera from MV-vaccinated individuals, and the sera neutralized wt MV and the recombinant virus at the same titer (data not shown), meaning that this recombinant virus could be studied under biosafety level 2 (BSL2) conditions.

Using the recombinant viruses, we asked whether the viruses spread without receptors, as suggested by the results with expressed glycoproteins indicating that SSPE F (T461I) and MIBE F (L454W) mediate fusion even in the absence of known receptors. Viruses bearing T461I F have been shown to spread in the absence of a known receptor (40), and we proposed that if the L454W F-bearing virus could spread from cell to cell in the absence of known receptors, this might correlate with its CNS adaptation. In Fig. 8 (and Fig. S3), recombinant MV viruses expressing EGFP and wt F, T461I F, or L454W F were used to infect Vero cells (with or without SLAM receptors) at a multiplicity of infection (MOI) of 0.01 at 32°C or 37°C. Cells were lysed, and the N gene copy number was determined at the time points indicated in Fig. S3. In Vero cells without known entry receptors for MV, at 37°C, all viruses entered cells. While wt virus appears to enter, there is no increase in genome replication over time (Fig. S3) and no syncytium formation (Fig. 8). In contrast, the T461I F and L454W F viruses

showed significant viral replication (Fig. S3) and syncytium formation (Fig. 8), with the virus bearing L454W F forming the most syncytia. In Vero cells bearing receptors (Vero-SLAM), all viruses grew similarly at 32°C, but the L454W F gene started with significantly more copies, perhaps suggesting more-efficient entry. At 37°C, all three viruses grew at similar levels in cells bearing SLAM receptors. These experiments with live viruses indicate that the mutations in F that confer independence from a receptor indeed permit the spread of virus in receptor-lacking cells.

## DISCUSSION

The myriad mutations observed in the genomes of SSPE-derived MV viruses have made it difficult to identify a single connection between CNS-adapted F proteins and the progression of neuropathology (49, 50). Recently, neuropathology was induced *in vivo* by recombinant viruses bearing SSPE-derived mutations in F (T461I or S103I N462S N465S) or the laboratory-generated hyper-fusogenic mutations (N462K and S262R) (40), suggesting that F is involved in this process. The role of F in CNS invasion has been experimentally shown in a murine model (26), but the pathogenesis of SSPE remains poorly understood to date (38, 51–53). The isolation of viruses bearing F L454W from two fatal cases of MV encephalitis during the South African outbreak (29, 54) supported the notion that mutations in the viral fusion machinery are associated with MV neuropathology. We identified functional differences between the H/F fusion machinery of wild-type (wt) MV and CNS-adapted MV isolates from HIV-positive patients and propose that these fusion properties play an important role in the clinical manifestation of the disease.

The experiments shown in Fig. 1 and 2 reveal that the F protein from viruses isolated from MIBE patients (F L454W) mediated fusion when coexpressed with MV H, even in the absence of a known MV receptor. This surprising finding suggests that the L454W mutation markedly increases the fusogenicity of the F protein. In fact, as shown in the experiments of Fig. 3, the F proteins of the several neuropathogenic strains induced fusion in the absence of a known MV receptor; F bearing the L454W mutation induced more fusion than the SSPE-derived F proteins, as well as fusion similar to that induced by the known hyper-fusogenic F protein bearing N462K. Despite its ability to fuse without a specific receptor, it is clear that in the presence of MV receptors, the fusion mediated by F L454W is modulated by the activating signal from receptor-engaged H. The H/F pairs mediated more fusion in the presence of higher-affinity receptors (i.e., nectin 4) than in the



presence of lower-affinity receptors, highlighting the contribution of receptor-engaged H to this process.

Even in the absence of a known receptor, fusion promotion by F L454W was modulated by the receptor binding protein (H); when it was paired with B3 H (Fig. 1C) (~6,000 RLU), more fusion was induced than when it was paired with Schwarz H (Fig. 2D) (~2,000 RLU). Strikingly, in the absence of receptor engagement, H bearing a defective stalk (H<sub>P108S</sub>-HN) fails to activate F L454W to fuse, even though it can fuse without the presence of any receptor-engaged binding protein (Fig. 4C). A possible explanation for this finding invokes the notion that prior to receptor engagement, the receptor binding protein plays a role in stabilizing F and preventing its untimely activation (43). The H stalk that is defective in terms of triggering F may, conversely, be more stabilizing to F so that F paired with this H protein does not activate. If future experiments support this interpretation, MV H, like HPIV3 HN, may exert an F-stabilizing effect and switch to triggering mode when it reaches the target cell.

To explore the biochemical consequences of the F L454W mutation, which may inform its phenotype, F L454W was compared to previously characterized neuropathogenic F proteins by assessing activation by heat (Fig. 5 and 6). F L454W, like laboratory-selected hyper-fusogenic F proteins (N462K and S262R), could be activated by heat even in the absence of a homotypic receptor binding protein. In fact, F L454W was the least thermally stable of the variant F proteins that we studied. SSPE-derived F T461I, however, required the presence of a homotypic receptor binding protein, consistent with its greater stability and less promiscuous activation. A previous report suggested that while heat could induce wt F to transition from its pretriggered to its posttriggered state, heat alone was not sufficient to activate F for fusion promotion (47); the heat-triggered F proteins were not capable of mediating fusion. However, the F L454W protein alone can be induced to promote fusion when activated by heat (Fig. 7), suggesting that a labile F protein can, in fact, mediate fusion once activated. The fact that this variant F emerged from *in vivo* evolution of the MV fusion machinery suggests that such a labile F protein has biological significance.

We have previously proposed that ongoing engagement of paramyxovirus receptor binding proteins is required for fusion promotion. In that paradigm, the first step of triggering is not sufficient for subsequent fusion (55), a notion that would be consistent with the reported failure of heat to activate MV F for fusion promotion (47). However, as shown here, the F L454W MV fusion machinery appears capable of functioning in a “spring-loaded” fashion without a requirement for ongoing receptor binding protein engagement, a propensity that would likely be restricted in most wt viruses (27, 28, 56, 57).

The HIV-infected patients with MV CNS manifestations identified in the South African epidemic, from whom the variant viruses were isolated, all had low CD4<sup>+</sup> T-cell lymphocyte counts, suggesting that those who developed fatal encephalitis had impaired cellular immunity and failed to clear the viral infection in the usual time frame (1, 11, 12). As another explanation for why immune clearance may have failed, it is noteworthy that alterations in MV H, F, or M proteins have been shown to permit MV to escape neutralizing human anti-MV sera *in vitro* (58), and some of these mutations were located within several residues of L454W (e.g., L457W). It is feasible that even in the presence of humoral immunity, F L454W may have arisen as an antibody escape variant

and replicated without cellular immunity surveillance, despite not being a predominant species in the blood of one of the patients during acute measles; however, the fact that the L454W F-bearing recombinant virus is neutralized by human serum makes this less likely. An interesting possibility is that mutants like L454W F exist within circulating viruses and that the selective pressure of growth in the CNS may have expanded this virus into the brains of the two patients. The virus isolated from the blood of one patient was wt (29); however, it is possible that mutant viruses were present below the limit of detection.

It is not possible at this time to determine whether the MV bearing F L454W entered the CNS via the circulation or evolved from the parental strain in the CNS. In either case, an important question is whether viruses bearing the CNS-adapted F proteins can infect the lung, which is MV's portal of entry, and, thus, may spread between hosts. Initial infection by wt MV does not involve the airway epithelium because the apical surfaces lack wt MV receptors. However, at the end of the infection cycle, MV reaches the basolateral surface of the airway epithelium and the infection is spread via droplet transmission. Wild-type MV strains do not infect when they are applied apically to the respiratory epithelium (59), and it will be of interest to determine whether the less-receptor-specific CNS-adapted MVs described here may infect via the apical surface and, if so, whether these viruses cause different diseases and/or alter viral transmission. The neutralization of the L454W F-bearing virus by the sera of vaccinated persons suggests that the existing vaccine can lead to protective immunity. Future work with this and other viruses bearing CNS-adapted mutations will require careful analysis of pathogenesis *in vivo* and broader analysis of the neutralizing activity of sera from vaccinated people.

The diagnosis of CNS disease in HIV patients during the recent outbreak in South Africa provided a unique opportunity to study the viral features that may correlate with MV adaptation to the CNS. The MV fusion machinery, like HPIV3's fusion machinery and that of other paramyxoviruses (27), seems to be tightly regulated in order to activate fusion only at the right place and time. The finding that the fusion machinery of isolates from the CNS of MIBE patients is altered to render F more readily activated suggests that this feature was advantageous in these particular hosts. Under selective pressure, the usual balance of F stability versus activation was skewed toward activation, and the neuropathogenic variants differ from the wt in their regulation of fusion. Based on our finding that promiscuous fusion by another paramyxovirus (HPIV3 bearing a mutation in the binding/triggering site of HN) is detrimental for fitness in human tissues and *in vivo* (27, 28, 60), we predicted that the properties that allow these variant viruses to spread in the CNS (overactive fusion machinery) will render these viruses deficient in transmission between hosts. However, the recombinant viruses bearing L454W F infects and spreads at 37°C, even without known receptors. With the recombinant viruses in hand (Fig. 8 and S3), we are now poised to address the question of whether interhost spread of neuropathogenic variants is likely.

## MATERIALS AND METHODS

**Plasmids and reagent.** The genes of MV IC323, B3, and Schwarz H and F proteins were codon optimized, synthesized, and subcloned into the mammalian expression vector pCAGGS. Plasmids encoding nectin 4, CD150, and CD46 were commercially acquired.

**Transient expression of the receptor binding proteins and F genes.** Transfections were performed in 293T cells according to the protocols of the Lipofectamine 2000 manufacturer (Invitrogen).

**Cells.** 293T (human kidney epithelial cells) and BHK (baby hamster kidney cells) were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco; Invitrogen) supplemented with 10% fetal bovine serum (FBS) and antibiotics in 5% CO<sub>2</sub>.

**$\beta$ -Gal complementation-based fusion assay.** The  $\beta$ -Gal complementation-based fusion assay was performed as described previously (61, 62). Briefly, 293T cells transiently transfected with the constructs indicated above and the omega reporter subunit were incubated with cells coexpressing viral glycoproteins and the alpha reporter subunit (63).

**$\beta$ -Gal assay for cell immunity stationing with F-conformation-specific MABs.** Monolayers of 293T cells were transiently transfected with viral glycoprotein constructs. Eighteen to 20 h posttransfection, cells were transferred to the temperatures indicated in the figures. Following those time periods, cells were incubated with mouse monoclonal pre-fusion MV F and postfusion MV F antibodies (1:1,000) for 1 h on ice. Cells were washed with phosphate-buffered saline (PBS) and then incubated for 1 h on ice with anti-mouse secondary antibody conjugated with biotin (1:500; Life Technologies). Cells were washed with PBS and then fixed for 10 min on ice with 4% paraformaldehyde (PFA). Following fixation, cells were washed twice, blocked for 20 min on ice with 3% bovine serum albumin (BSA) in PBS, washed with PBS, and then incubated for 1 h on ice with streptavidin conjugated with  $\beta$ -galactosidase (1:1,000). Cells were washed again with PBS, the  $\beta$ -galactosidase substrate (1:50, Applied Biosystems) was added, and luminescence was measured using a SpectraMax M5 (Molecular Devices) microplate reader.

**Recombinant virus production and analysis.** MV IC323-EGFP (64) is a recombinant virus expressing the EGFP gene. All proteins with the mutations T461I, S103 N462S N465S (from plasmids kindly provided by Yanagi, Kyushu University, Fukuoka, Japan), N462K, and L454W were generated in the MV IC323-EGFP background by reverse genetics. MV IC323 recombinant viruses were rescued in 293-3-46 cells as previously described (65). MV recombinants were titrated by plaque assay on Vero/human SLAM cells.

**Kinetic analysis of the virus infection.** Vero cells (not bearing the MV receptor) or Vero-SLAM cells were infected at an MOI of 0.01 for 90 min either at 32°C or at 37°C in Opti-MEM (Gibco) with 0% FCS. After 90 min, cells were washed twice, medium was replaced by DMEM (Gibco)–3% FCS, and cells were incubated at the temperatures indicated in the figures for 4 h, 24 h, or 48 h after infection. After the incubation period, the medium was removed and cells were lysed using RA1 lysis buffer (Macherey-Nagel)–1% beta-mercaptoethanol. RNA was isolated from cells using a NucleoSpin RNA extraction kit (Macherey-Nagel) as per the manufacturer's instructions. Reverse transcription was performed on 0.5  $\mu$ g of total RNA using oligo(dT) and random-hexamer oligonucleotide primers (iScript cDNA synthesis kit; Bio-Rad), amplified on a Biometra Tpersonal PCR device, and cDNAs were diluted 1/10. Quantitative PCR was performed as previously described (48, 66). Results are means  $\pm$  standard errors of the means (SEM) ( $n = 3$ ).

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02528-14/-DCSupplemental>.

Figure S1, TIF file, 0.6 MB.

Figure S2, TIF file, 0.3 MB.

Figure S3, TIF file, 7 MB.

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