M protein of subacute sclerosing panencephalitis virus, synergistically with the F protein, plays a crucial role in viral neuropathogenicity

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Abstract
Subacute sclerosing panencephalitis (SSPE) is a rare fatal neurodegenerative disease caused by a measles virus (MV) variant, SSPE virus, that accumulates mutations during long-term persistent infection of the central nervous system (CNS). Clusters of mutations identified around the matrix (M) protein in many SSPE viruses suppress productive infectious particle release and accelerate cell–cell fusion, which are features of SSPE viruses. It was reported, however, that these defects of M protein function might not be correlated directly with promotion of neurovirulence, although they might enable establishment of persistent infection. Neuropathogenicity is closely related to the character of the viral fusion (F) protein, and amino acid substitution(s) in the F protein of some SSPE viruses confers F protein hyperfusogenicity, facilitating viral propagation in the CNS through cell–cell fusion and leading to neurovirulence. The F protein of an SSPE virus Kobe-1 strain, however, displayed only moderately enhanced fusion activity and required additional mutations in the M protein for neuropathogenicity in mice. We demonstrated here the mechanism for the M protein of the Kobe-1 strain supporting the fusion activity of the F protein and cooperatively inducing neurovirulence, even though each protein, independently, has no effect on virulence. The occurrence of SSPE has been estimated recently as one in several thousand in children who acquired measles under the age of 5 years, markedly higher than reported previously. The probability of a specific mutation (or mutations) occurring in the F protein conferring hyperfusogenicity and neuropathogenicity might not be sufficient to explain the high frequency of SSPE. The induction of neurovirulence by M protein synergistically with moderately fusogenic F protein could account for the high frequency of SSPE.

INTRODUCTION
Measles virus (MV) is the causative agent of the highly contagious acute disease measles, with characteristic symptoms such as high fever, cough and maculopapular rash [1, 2]. Entering the host by the respiratory route, MV first infects dendritic cells and alveolar macrophages in the respiratory tract and spreads via the bloodstream to lymphoid organs throughout the body using signalling lymphocyte activation molecule (SLAM, also called CD150) expressed on immune cells as a cellular receptor [3, 4]. In the late stage of infection, MV infects respiratory epithelial cells using nectin 4 as a receptor [5, 6], followed by shedding of progeny viral particles via coughing and sneezing to transmit to susceptible new hosts [2, 7, 8]. Very rarely, MV may break into the central nervous system (CNS) and persist independently of SLAM and nectin 4 because neither is expressed in the human CNS [9, 10], causing a rare fatal neurodegenerative disease, subacute sclerosing panencephalitis (SSPE) [1]. SSPE usually occurs 6 to 8 years on average after contraction of acute measles, as a result of accumulated mutations in the viral genome.
MV, a member of the genus *Morbillivirus* in the family *Paramyxoviridae*, is an enveloped virus with a non-segmented negative-sense RNA genome encoding six proteins: nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin protein (H) and large protein (L). The P gene also encodes the additional accessory proteins, V and C [1, 11]. Two envelope glycoproteins, the H and F proteins forming an H/F protein complex [12], have roles in receptor binding and membrane fusion, respectively, to introduce the ribonucleoprotein (RNP) complex, composed of the RNA genome and the N, P and L proteins, into the host cell by fusing the viral envelope with the plasma membrane (envelope fusion) and thus initiating infection [13–17]. The H and F proteins expressed on the surface of the infected cell also elicited fusion of the plasma membrane with that of adjacent cells, forming a multinuclear giant cell, a syncytium, to spread infection (cell-cell fusion) [1]. The M protein promotes formation of viral particles and plays an important role in the assembly of infectious viral particles by interacting with the RNP complex as well as the cytoplasmic tails of the H and F proteins and taking them into the viral particle [11, 18].

MV variants isolated from the brains of SSPE patients, known as SSPE virus, differ from wild-type MV and have specific characteristics: lack of infectious viral particle production, enhanced cell–cell fusion ability, and neurovirulence in rodents [19–23]. In the genome, clusters of mutations occur around the M gene in many SSPE viruses [24–28]. Recombination experiments in which the M gene of MV was replaced by that of an SSPE virus or was deleted from the genome revealed that abrogation of M protein function accounts for the lack of particle formation and the efficient cell–cell fusion ability of SSPE viruses, but not for neurotoxicity. However, M protein might be involved in the establishment of persistent infection in the brain [29, 30]. Recently, such mutations were identified in the ectodomain of the F protein, and enhanced its fusion activity, leading to the formation of syncytia of receptor (SLAM and/or nectin 4)-negative cells, a characteristic related to the neuropathogenicity of SSPE viruses [31, 32].

The Kobe-1 strain of SSPE virus isolated from the brain of a 5-year-old patient only 6 weeks after the onset of SSPE possessed very few mutations from the wild-type MV field isolate prevalent in Japan and from which the Kobe-1 strain possibly originated [33]. It is likely that the Kobe-1 strain was isolated before additional accumulation of mutations and carries the minimum mutations required for the onset of SSPE. In this study, we identified the gene(s)/protein(s) responsible for its neurovirulence and found that the F protein of the Kobe-1 strain did not itself confer neurovirulence and that mutations in the M protein are indispensable for pathogenicity in suckling mice. The mechanism by which M protein and F protein promote the neurovirulence of the Kobe-1 strain is demonstrated, and the role of the M protein in neuropathogenicity is discussed.

**RESULTS**

**The M gene of the SSPE virus Kobe-1 strain is indispensable for neuropathogenicity**

Recent studies have identified the F protein, not the previously suggested M protein, of the SSPE viruses as the viral element responsible for neuropathogenicity [31, 32, 34]. To identify the viral gene(s)/protein(s) that cause neurovirulence of the SSPE virus Kobe-1 strain, the F gene of an EGFP-expressing recombinant MV ICB strain (rMV), the D3 genotype virus, which is close to the parental MV of the Kobe-1 strain, was replaced by that of the Kobe-1 strain (Fig. 1a). The recovered virus rMV/sF, however, showed no lethality when inoculated in the brain of suckling mice, like other chimeric viruses such as rMV/sM and rMV/sH, the recombinant MV ICB strains possessing the M and H genes of the SSPE virus Kobe-1 strain, respectively (Fig. 1b). Next, we introduced the M, F and H genes of the Kobe-1 strain in combination into the genome of the MV ICB strain (Fig. 1a). As shown in Fig. 1b, the chimeric viruses possessing the M and F genes of the SSPE virus Kobe-1 strain, rMV/sMF, were highly lethal and the rMV/sSMFH with the M, F and H genes of the Kobe-1 strain killed mice slightly more slowly. By contrast, chimeric virus carrying the F and H genes, or the M and H genes of the Kobe-1 strain, rMV/sFH or rMV/sMH, did not kill mice. These results demonstrate that the M gene of the SSPE virus Kobe-1 strain is required in addition to the F gene for neurovirulence in mice, and that the H gene of the SSPE Kobe-1 strain might attenuate virulence. The recombinant SSPE virus Kobe-1 strain (rSSPEV) killed mice more slowly than the chimeric virus rMV/sMFH, suggesting that the Kobe-1 strain carries other gene(s) than the H gene that attenuate neurovirulence.

To examine the propagation of the neurovirulent viruses carrying the genes of the SSPE virus Kobe-1 strain in human neuronal cells, SH-SY5Y neuroblastoma cells were infected with the chimeric viruses. rMV/sF spread slightly more efficiently than rMV, which was significantly facilitated by the introduction of the M gene of the Kobe-1 strain (rMV/sMF) (Fig. 1c). When rMV/sSMFH was compared with rMV/sFH, rMV/sSMFH spread more efficiently, indicating that the Kobe-1 M gene strongly promotes viral growth in neuronal cells. By contrast, replacement of the M gene of the viruses with the MV ICB F gene (e.g. rMV and rMV/sH) by the M gene of the Kobe-1 strain enhanced viral replication slightly (rMV/sM and rMV/sSMH, respectively). rMV/sFH and rMV/sSMFH replicated less efficiently than rMV/sF and rMV/sSMF, respectively, suggesting that the H gene of the SSPE Kobe-1 strain suppresses viral growth in neuronal cells. The neurovirulence of the chimeric viruses in mice is correlated with their ability to grow in neuronal cells: rMV/sMF and rMV/sSMFH and rSSPEV (in that order) spread efficiently in SH-SY5Y cells. The F gene of the SSPE virus Kobe-1 strain encodes an F protein with nine amino acid substitutions as well as a shortened cytoplasmic tail, and the M gene carries three amino acid substitutions compared with the MV ICB strain, respectively [33]. These results indicate that some mutations in the F protein and M protein of the SSPE Kobe-1 strain
Fig. 1. Requirement of the M gene for neurovirulence by the SSPE virus Kobe-1 strain. (a) Schematic diagram of the genomes of the EGFP-expressing recombinant chimeric viruses. The protein-coding regions from the measles virus ICB strain (rMV) are shown as blue boxes, and those from the SSPE virus Kobe-1 strain (rSSPEV) as red boxes. (b) Mortality of suckling mice intracerebrally inoculated with chimeric viruses. Six suckling mice were infected with 7×10^2 p.f.u. of virus and monitored for 10 days. (c) Spread of infection in neuronal cells. SH-SY5Y cells were infected with chimeric virus at an m.o.i. of 0.001. After incubation at 37 °C for 7 days, EGFP-expressing infected cells were observed under a fluorescence microscope. Magnification, ×200.
are indispensable for viral propagation in the brain and neurovirulence.

**The M protein of the SSPE virus Kobe-1 strain significantly enhances the fusion activity of Kobe-1 F protein**

The neurovirulence of some SSPE viruses correlates with elevated viral cell–cell fusion ability acquired from mutation(s) in the F protein, conferring hyperfusogenicity on the F protein [31, 32, 34]. Neurotoxicity of the SSPE virus Kobe-1 strain, however, cannot be achieved simply by mutation(s) in the F protein but requires additional mutation(s) in the M protein. Therefore, the contribution of these proteins of the Kobe-1 strain to viral fusion was evaluated in Vero/SLAM cells. Fig. 2a shows a typical syncytium formed by each chimeric virus (Fig. 1a), and Fig. 2b indicates the fusion ability estimated by enumerating nuclei. The higher fusion ability of rMV/sF compared to rMV demonstrates that the F protein of the Kobe-1 strain [F/SSPEV(Kobe) protein] acquired increased fusion activity compared with that of the MV ICB strain [F/MV(ICB) protein]. In contrast, the H protein of the Kobe-1 strain [H/SSPEV(Kobe) protein] slightly suppressed viral fusion (compare rMV/sH with rMV or rMV/sFH with rMV/sF). Replacement of the M protein of the MV ICB strain [M/MV(ICB) protein] by that of the SSPE Kobe-1 [M/SSPEV(Kobe) protein] enlarged the syncytia formed by rMV/sM, rMV/sMF, rMV/sMH, or rMV/sMFH, demonstrating the fusion upregulating activity of the M/SSPEV(Kobe) protein. rMV/sMF and rMV/sMFH, which had neurovirulence in mice (Fig. 1b), formed large syncytia. rMV/sMF showed greater fusion ability than rMV/sMFH, probably because of restriction of fusion by the H/SSPEV(Kobe) protein. rSSPEV demonstrated lower fusion ability than rMV/sMFH, indicating the involvement of viral element(s) other than the M, F and H proteins in viral fusion.

Infectious viral particle production by the chimeric viruses was also assayed (Fig. 2c). The M/SSPEV(Kobe) protein strongly suppressed release of infectious viral particles: trace amount by rMV/sM and rMV/sMH, or under the detectable amount by rMV/sMF and rMV/sMFH. By contrast, viruses carrying the M/MV(ICB) protein produced infectious virus, depending on the F protein. While rMV and rMV/sH had a high titre of cell-free infectious particles, rMV/sF and rMV/sFH [F protein was replaced by the F/SSPEV(Kobe) protein] showed limited release, an inverse correlation with cell–cell fusion ability. Therefore, the defect of the chimeric viruses in infectious particle production is caused by the M/SSPEV(Kobe) protein and in part by the F/SSPEV(Kobe) protein.

The highly fusogenic F proteins of SSPE viruses support spread of viral infection to receptor-negative cells by cell–cell fusion, a marker of neurovirulence enabling viral propagation in the brain [31, 32, 34], where the MV receptors, SLAM and nectin 4, are not expressed [9, 10]. Next, the ability of the chimeric viruses to induce cell–cell fusion was estimated in receptor-negative Vero cells. As shown in Fig. 3a, while rMV and rMV/sH did not spread, rMV/sF formed small syncytia. Next, the M/SSPEV(Kobe) protein was introduced to the chimeric viruses. While rMV/sM and rMV/sMH showed threefold increased fusion ability compared with rMV and rMV/sH, respectively, rMV/sMF and rMV/sMFH acquired six- to sevenfold increased fusion compared to rMV/sF and rMV/sFH, respectively (Fig. 3b). Therefore, the fusion activity of the F/SSPEV(Kobe) protein in Vero cells was significantly increased by the M/SSPEV(Kobe) protein. The neurovirulence of the chimeric viruses coincided with their fusion ability in Vero cells because only rMV/sMF, rMV/sMFH and rSSPEV (in that order) were neurovirulent (Fig. 1b). The results in Figs. 2 and 3 indicate that the fusion activity of the F/SSPEV(Kobe) protein, although higher than that of the F/MV(ICB) protein, is not sufficiently increased to support efficient growth of the virus in receptor-negative cells, and that the M/SSPEV(Kobe) protein compensates for the deficit. Viral neurovirulence paralleled the acceleration of viral cell–cell fusion and growth in receptor-negative cells in a manner that was dependent on both the F protein and the M protein.

**The M protein of the SSPE Kobe-1 strain lost its fusion-suppressing activity**

How does the M/SSPEV(Kobe) protein upregulate the fusion activity of the F protein? To answer this question, using a protein expression system, we investigated the behaviour of the M protein while the H/F protein complex executes cell–cell fusion. First, the fusion activity of the F/MV(ICB) protein and the F/SSPEV(Kobe) protein co-expressed with the H/MV(ICB) or H/SSPEV(Kobe) protein was estimated in the absence of the M protein. The F/SSPEV(Kobe) protein exhibited greater fusion activity than the F/MV(ICB) protein (Fig. 4a, right). The fusion activities were slightly lower when expressed with the H/SSPEV(Kobe) protein than with the H/ MV(ICB) protein. The additionally expressed M/MV(ICB) protein suppressed the fusion activities of the F proteins by half those in the absence of the M protein for all combinations of F and H proteins (Fig. 4a, left). In the presence of the M/SSPEV(Kobe) protein, the fusion activities of the F proteins were similar to those in the absence of the M protein, indicating abolition of the suppressive effect by the M/MV(ICB) protein (Fig. 4a, middle). The high fusion activity of the F/SSPEV(Kobe) protein in the presence of the M/SSPEV(Kobe) protein is consistent with the high fusion ability of the neurovirulent viruses, rMV/sMF and rMV/sMFH, which carry the F and M proteins of the SSPE virus Kobe-1 strain.

The M protein of MV plays an important role in transporting H and F proteins on the surface of infected cells into the viral particle by binding to their cytoplasmic tail, interfering with cell–cell fusion of infected cells with adjacent cells [18]. To explore the molecular mechanism by which the M/SSPEV(Kobe) protein abolished the suppressive effect of the M/MV(ICB) protein on the fusion activity of the F protein, the interaction of the M protein with the H/F protein complex was investigated. Pulldown of the H protein precipitated the F protein, verifying the formation of a complex by the two proteins (Fig. 4b, right). When the M/MV(ICB) protein
Fig. 2. Growth characteristics of chimeric viruses. (a) Syncytium formation by chimeric viruses in Vero/hSLAM cells. Vero/hSLAM cells were infected with each chimeric virus in Fig. 1 at an m.o.i. of 0.001 and incubated at 37 °C. At 36 h p.i., EGFP-expressing infected cells were observed under a fluorescence microscope. Magnification, ×200. (b) Quantification of cell–cell fusion. Infected Vero/hSLAM cells shown in (a) were fixed, permeabilized and incubated with Hoechst 33342. Cell–cell fusion is displayed as the number of nuclei in an EGFP-expressing syncytium. Nuclei in five syncytia were counted under a fluorescence microscope and are presented as average values with standard deviations (mean±sd). Statistical analysis was performed by unpaired Student’s t-test: *, P<0.05. (c) Infectious cell-free virus production by the chimeric viruses. B95a cells were infected with virus at an m.o.i. of 0.001. After incubation at 37 °C for 4 days, viruses in the culture fluid were titrated in Vero/hSLAM cells. Data from five independent experiments are presented as mean±sd.
was co-expressed with the F and H proteins, the M protein co-precipitated with the H/F protein complex, confirming the interaction of the M/MV(ICB) protein with the H/F protein complex (Fig. 4b, left). The co-expressed M/SSPEV(Kobe) protein was not detected in the precipitate of the H/F protein complex (Fig. 4b, middle). Therefore, the M/SSPEV(Kobe) protein lost its ability to bind to the H/F protein complex, explaining the enhanced cell–cell fusion activity in the presence of the M/SSPEV(Kobe) protein. The M/SSPEV(Kobe) protein has no effect on the fusion activity of the F protein, resulting in abolition of the fusion-suppressing activity of the M/MV(ICB) protein.

The F/SSPEV(Kobe) protein has a shortened cytoplasmic tail, a feature typical of many SSPE viruses [25, 27, 35], but the interaction of the H/F protein complex composed of the F/SSPEV(Kobe) protein with the M/MV(ICB) protein was not impaired (Fig. 4b, left). If the H/F protein complex

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**Fig. 3.** Enhanced cell–cell fusion by neurovirulent chimeric viruses in the receptor-negative Vero cells. (a) Syncytium formation by chimeric viruses in Vero cells. Vero cells were infected with the chimeric viruses in Fig. 1 at an m.o.i. of 0.001. At 72 h p.i., EGFP-expressing infected cells were observed under a fluorescence microscope. Magnification, ×200. (b) Quantification of Vero cell–cell fusion. Infected Vero cells shown in (a) were fixed, permeabilized and incubated with Hoechst 33342. Nuclei in EGFP-expressing cells were enumerated under a fluorescence microscope. Cell–cell fusion is displayed as the number of nuclei in a syncytium. Data from five infected syncytia are presented as mean±s.d. Statistical analysis was performed by unpaired Student’s t-test: *, P<0.05.
Fig. 4. Cell–cell fusion suppression by the M protein of wild-type MV, which is abolished by the M protein of SSPE virus Kobe-1 strain. (a) Cell–cell fusion by H and F proteins co-expressed with the M protein. Vero/hSLAM cells were transfected with plasmids expressing myc-tagged H and F proteins of the MV ICB strain (m) or the SSPE virus Kobe-1 strain (s) together with that expressing the M protein of each virus or pCA7 vector for the without-M-protein condition (−). After incubation at 37 °C for 15 h, the cells were fixed and stained with crystal violet, and syncytia and nuclei per syncytium were enumerated under a microscope. Cell–cell fusion activity is shown as the product of two values and relative cell–cell fusion activity was calculated as described in the Methods section. n=3, mean±sd. Statistical analysis was performed by unpaired Student’s t-test: ns, not significant. (b) Interaction of M protein with the H/F protein complex. 293 T cells were transfected with plasmids expressing the myc-tagged H and F proteins of the MV ICB strain (m) or the SSPE virus Kobe-1 strain (s) together with that expressing the M protein of each virus or pCA7 vector as the control (−) and incubated at 37 °C. The cells were lysed 48 h after transfection and the lysates (input) were subjected to immunoprecipitation (IP) with an anti-myc antibody, followed by Western blotting using anti-myc, anti-F, anti-M and anti-β-actin primary antibodies, as described in the Methods section.
is transported into rMV/sF and rMV/sFH particles by the M/MV(ICB) protein, the infectivity of the released particles would not be reduced compared with that of rMV and rMV/sH. It is possible that the F/SSPEV(Kobe) protein induces cell–cell fusion before viral particle release due to the higher fusion activity than the F/MV(ICB) protein (Fig. 2c).

**The M protein of the MV ICB strain stabilizes the F protein**

MV F protein induces membrane fusion by a conformational change from the prefusion to the postfusion form, which is regulated by the stability of the prefusion molecule [36–38]. We estimated the fusion activity of the F protein in the presence of the M protein at 40°C to examine the effect of the M protein on the stability of the F protein. The F protein with the T461I mutation (F/T461I) was included for comparison, because the T461I mutation in some SSPE viruses causes hyper fusogenicity and, therefore, neurovirulence in rodents [31, 39]. At 37°C in the absence of the M protein, the F/SSPEV(Kobe) protein showed higher fusion activity than the F/MV(ICB) protein, but the F/T461I protein demonstrated the highest activity (Fig. 5a, left). Because the fusion activities of the F/SSPEV(Kobe) and F/T461I proteins relative to that of the F/MV(ICB) protein were lower at 40°C than at 37°C, these two F proteins were destabilized compared with the F/MV(ICB) protein (Fig. 5a, right). The smaller ratio (0.4-fold)
between the values of the F/T461I protein at 37 °C and at 40 °C, compared to that of the F/SSPEV(Kobe) protein indicated that the F/T461I protein is more destabilized than the F/SSPEV(Kobe) protein. Next, fusion activity was assayed in the presence of the M protein at 37 and 40 °C (Fig. 5b, upper panels) and evaluated as the relative fusion activity, setting the value of each F protein in the absence of the M protein at 1 (Fig. 5b, lower panels). When the M/MV(ICB) protein was co-expressed, the relative fusion activity of each F protein at 40 °C increased compared with that at 37 °C, indicating that the F protein was stabilized by the M/MV(ICB) protein. The magnitudes of the increases – 1.6-fold for the F/MV(ICB) protein, 1.4-fold for the F/SSPEV(Kobe) protein and 1.2-fold for the F/T461I protein – showed that the stabilizing effect of the M/MV(ICB) protein on the F protein correlated with the initial stability of the F protein (Fig. 5a, right). The F/SSPEV(Kobe) protein is stabilized more strongly than the F/T461I protein by the M/MV(ICB) protein, suppressing the fusion activity of the F/SSPEV(Kobe) protein. By contrast, no difference was found in the relative fusion activities at 37 and 40 °C with co-expression of the M/SSPEV(Kobe) protein, indicating that the M/SSPEV(Kobe) protein did not affect the stability of the F protein.

DISCUSSION

SSPE viruses are very rarely isolated from the brain of patients with SSPE. Genetically, the most striking feature of the isolates is the numerous mutations, including the frequent adenine-to-guanine or uracil-to-cytosine biased hypermutation, which often occur, especially around the M gene [25, 40–44]. The M protein of MV plays a central role in producing infectious viral particles by constituting their structure and by binding to the cytoplasmic tail of the H/F protein complex as well as to the viral RNP to transport them into the particle. The highly accumulated mutations around the M gene of SSPE viruses should impair M protein function [44–50]. The recombinant MVs whose M gene was replaced by that of an SSPE virus or was deleted from the genome demonstrated defects in the release of infectious viral particles and enhanced cell–cell fusion ability, showing that loss of M protein function switches the mode of propagation of MV. These highly assembly-defective fusogenic recombinant MVs slowed viral spread in the brain, penetrated more deeply into the brain parenchyma, and caused a prolonged chronic CNS infection, resulting in delayed death in mice compared to the parental MV [29, 30]. This observation differed from the fact that SSPE viruses are highly lethal and drive the infected rodents promptly to death [31, 32]. Therefore, these experiments did not prove the functionally defective M protein to be responsible for neurovirulence, although it may play a role in establishing persistent infection in the brain. In contrast, Ayata et al. for the first time reported that a recombinant MV (ICB strain) whose F gene was replaced by that of the SSPE Osaka-2 strain demonstrated neurovirulence in hamsters and that the T461I mutation in the F protein was responsible for pathogenicity [31]. In the same recombination experiment, the ICB strain acquired no neurovirulence from exchange of the M protein with that of the Osaka-2 strain. The F protein performs membrane fusion via a conformational change from the metastable prefusion form to the stable postfusion form to initiate infection by introducing RNP into target cells. It is generally accepted that mutations in the F protein destabilizing the prefusion form confer hyperfusogenicity on the F protein and increase cell–cell fusion and propagation in the brain, transforming an MV into a neurotropic SSPE virus [32, 34, 39, 51–55]. The chimeric MV ICB strain, rMV/sF, whose F gene was exchanged for that of the SSPE Kobe-1 strain, however, did not display neurovirulence and at first we did not find the F gene to be involved in pathogenicity of the Kobe-1 strain. However, the chimeric MV, rMV/sMF, whose M and F genes were replaced by those of the SSPE Kobe-1 strain, was lethal in mice. In the SSPE Kobe-1 strain, the F and M proteins synergistically function in neurovirulence. To our knowledge, this is the first report of the involvement of the M protein in SSPE virus neurovirulence. We identified the G301W mutation in the F protein as destabilizing the F protein in the presence of M/SSPEV(Kobe) protein (manuscript in preparation).

Our results demonstrate that the M protein of the wild-type MV, M/MV(ICB) protein, interferes with the fusion activity of the F protein by binding to the H/F protein complex (Fig. 4). The neuropathogenicity of a recombinant MV ICB strain possessing a single amino acid substitution, T461I in the F protein [31, 34], could be explained by the fusion activity of the hyperfusogenic F/T461I protein even with the M/MV(ICB) protein, because it is too labile to be stabilized by M/MV(ICB) protein. The F protein of the SSPE virus Kobe-1 strain, F/SSPEV(Kobe) protein, is more stable than the hyperfusogenic F/T461I protein, although it is actually less stable and shows higher fusion activity than the wild-type F/MV(ICB) protein (Fig. 5a). The moderately destabilized F/SSPEV(Kobe) protein was stabilized by the M/MV(ICB) protein (Fig. 5b), which suppresses cell–cell fusion of the chimeric virus rMV/sF and interferes with its spread in the brain. Thus, rMV/sF did not exhibit neurovirulence. By contrast, the F/SSPEV(Kobe) protein in the chimeric virus, rMV/sMF, retained fusion activity in the presence of the M/SSPEV(Kobe) protein because it had lost its ability to stabilize F protein, allowing rMV/sMF propagation in the brain and neurovirulence. It might be desirable to introduce the M gene and F genes of an SSPE virus into wild-type MV to investigate pathogenicity without the confounding effect of the wild-type MV M protein.

While MV persists in the brain by escaping from the immune system, mutant viruses possessing a defect in viral particle release may have a selection advantage [56, 57]. The results in Fig. 2 demonstrate that infectious particle release related inversely to cell–cell fusion ability: the lower the particle release, the greater the cell–cell fusion. The M/SSPEV(Kobe) protein was not transported to the plasma membrane and was localized throughout the cytosol [23]. The absence of the M protein in the plasma membrane might explain not only the limited particle release but also the increased cell–cell fusion. This is because the F protein-stabilizing effect of the M protein
was abolished by the lack of interaction with the H/F protein complex in the plasma membrane (Fig. 4b). The M protein of the SSPE Biken strain was detected in the soluble cytosolic fraction but not in the plasma membrane fraction [49]. If SSPE viruses selected in the brain as particleless viruses carry the M protein of the aberrant transport character, such an M protein may enhance the cell–cell fusion of SSPE viruses. Although SSPE has a very low frequency, around 1 case per 100,000 cases of measles [58–60], more recent studies have estimated a higher risk of 22:100,000 [61], and 1:1367 or 1:1700 to 1:3300 in children who acquired measles under the age of 5 years [62, 63]. Like the T461I mutation, mutations in the F protein in the domains responsible for stability and conformational change, the HR-B domain in the stalk region near the junction with the head region (amino acid 456–495) or the DIII domain in the head region (amino acid 53–296) [17, 36, 64], confer neuropathogenicity, leading to SSPE. The probability, however, of those mutations is not sufficient to explain the high frequency of SSPE. During persistent infection, if a particleless SSPE virus carries transport-defective M protein, the M protein will not suppress cell–cell fusion. Such M proteins will ensure sufficient cell–cell fusion for growth in the brain of virus with a moderately destabilized F protein, like the F(SSPEV(Kobe)) protein, increasing the likelihood of SSPE. Investigation of the cooperative roles of the F and M proteins in neuropathogenicity is underway with SSPE strains other than Kobe-1.

METHODS

Cells and viruses

Vero cells constitutively expressing human SLAM (Vero/hSLAM) (a gift from Y. Yanagi, Kyushu University) [4] and Vero cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 8% foetal bovine serum (FBS). 293T cells and cells of a marmoset B-cell line transformed with Epstein–Barr virus (B95a) were maintained in high-glucose DMEM supplemented with 10% FBS and 1% MEM non-essential amino acid solution (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan). BHK cells constitutively expressing T7 RNA polymerase (BHK/T7-9) (a gift from N. Ito and M. Sugiyama, Gifu University) [65] were maintained in RPMI 1640 medium supplemented with 10% FBS and 0.6 mg ml⁻¹ hygromycin B. Isolation of SSPE virus Kobe-1 strain was described previously [33]. Recombinant MVs (rMVs) were generated according to Seki et al. [66] as described previously [67] using MV full-length genome plasmids. T7 RNA polymerase-expressing vaccinia virus (vTF7-3) [68] was a gift from B. Moss (National Institutes of Health, USA).

Plasmid construction

The cDNA of the genome of the SSPE virus Kobe-1 strain (GenBank: AB254456) was synthesized by reverse-transcription PCR. The Sall-SacII fragment [nucleotides (nt) 3365–4921 according to the ICB strain genome sequence (GenBank: AB016162) of the p(+)MV323c72-EGFP plasmid [67] derived from the full-length genome plasmid of the MV ICB strain, p(+)MV323-EGFP (a gift from Y. Yanagi) [69], was replaced by the corresponding region of the Kobe-1 strain, generating a plasmid with the full-length genome of the ICB strain carrying the M gene of the SSPE virus Kobe-1 strain [p(+)MV323-EGFP/M]. The SacII-Pocal fragments (nt 4922–7243) of p(+) MV323c72-EGFP and p(+)MV323-EGFP/sM were replaced by the corresponding fragment of the Kobe-1 strain, which generated p(+)MV323-EGFP/sF carrying the F gene of the Kobe-1 strain and p(+)MV323-EGFP/sMF carrying the M and F genes of the Kobe-1 strain, respectively. To exchange the ICB strain H gene for that of the Kobe-1 strain, the Pacl-Spel fragments (nt 7243–9175) of p(+)MV323c72-EGFP, p(+)MV323-EGFP/sM, p(+)MV323-EGFP/sF and p(+)MV323-EGFP/sMF were replaced by the corresponding fragment of the Kobe-1 strain, yielding p(+)MV323-EGFP/sh, p(+)MV323-EGFP/sM, p(+)MV323-EGFP/sFH and p(+)MV323-EGFP/sMF, respectively. The plasmid p(+)MV323-EGFP/SSPEV containing all genes of the Kobe-1 strain was constructed by replacing the Small-Sall fragment (nt 839–3364) and the Spel-Eco47III fragment (nt 9176–15767) of p(+)MV323-EGFP/sMF with the corresponding regions of the Kobe-1 strain.

For protein expression, the M gene (nt 3438–4445), the F gene (nt 5458 to 7110) and the H gene (nt 7271 to 9124) were amplified by PCR using p(+)MV323c72-EGFP or the cDNA of the SSPE virus Kobe-1 strain as the template. The M and F genes were cloned into the pCA7 vector or pcDNA3 vector (Invitrogen, Carlsbad, CA, USA), generating pCA7-M/MV(ICB), pCA7-M/SSPEV(Kobe), pCA7-F/MV(ICB), pCA7-F/SSPEV(Kobe), pcDNA-M/MV(ICB), pcDNA-M/SSPEV(Kobe), pcDNA-F/MV(ICB) and pcDNA-F/SSPEV(Kobe), respectively. The amplified H genes were cloned into the pCA7-myc vector or pcDNA3-myc vector prepared by inserting the myc-tag fragment downstream of the multiple cloning site of the pCA7 and pcDNA3 vectors, generating pCA7-H-myc/MV(ICB), pCA7-H-myc/SSPEV(Kobe), pcDNA-H-myc/MV(ICB) and pcDNA-H-myc/SSPEV(Kobe), respectively. To introduce the Thr to Ile mutation at residue 461 (T461I) of the F protein, the ICB F gene carrying a C to T substitution at nt 6839 was cloned into the pcDNA3 vector.

Virus titration

Monolayers of Vero/hSLAM cells in 24-well plates were infected with serially diluted virus samples. After 1 h of incubation at 37°C, the virus samples were removed, and the cells were overlaid with DMEM containing 2% FBS and 2.5% methylcellulose. After 2 days, plaque-forming units (p.f.u.) were determined by enumerating plaques expressing EGFP under a fluorescence microscope (Axioskop; Zeiss, Jena, Germany).

Virus challenge

BALB/c suckling mice purchased from CLEA Japan, Inc. (Tokyo, Japan) were used prior to 3 weeks old after passing a medical inspection. Mice were under anaesthesia when inoculated intracerebrally with 7×10⁷ p.f.u. of each recombinant
chimeric virus in a 20 µl suspension of B95a cells. After inoculation, clinical symptoms were observed daily, and moribund mice were euthanized.

**Cell–cell fusion assay (syncytium formation by recombinant viruses)**

Vero cells or Vero/hSLAM cells cultured in 24-well plates were infected with rMVs at a multiplicity of infection (m.o.i.) of 0.001 and incubated at 37°C for 36 or 72 h. The cells were fixed in 1% paraformaldehyde, permeabilized with 1% Triton X-100, and stained with Hoechst 33342 (Sigma-Aldrich, St Louis, MO, USA). The number of nuclei in an EGFP-expressing syncytium was counted under a fluorescence microscope.

**Cell–cell fusion assay (syncytium formation by expressed F, H and M proteins)**

Subconfluent monolayer cultures of Vero/hSLAM cells in 24-well plates were transfected with 0.5 µg of the F protein-expressing plasmid [pCA7-F/MV(ICB) or pCA7-F/SSPEV(Kobe)] and 0.5 µg of the H protein-expressing plasmid [pCA7-H-myc/MV(ICB) or pCA7-H-myc/SSPEV(Kobe)] together with 1 µg of the M protein-expressing plasmid [pCA7-M/MV(ICB) or pCA7-M/SSPEV(Kobe)] or pCA7 vector and incubated at 37°C. At 15 h post-transfection, the cells were fixed and stained with crystal violet, and syncytia and nuclei per syncytium were enumerated under a microscope. Cell–cell fusion activity was shown as the product by multiplying the two values. To estimate the effect of the M protein, relative cell–cell fusion activity was calculated by dividing the cell–cell fusion activity of each combination of F and H proteins in the presence of M protein by that in the absence of the M protein.

**Immunoprecipitation and Western blot analyses**

Subconfluent monolayer cultures of 293T cells in six-well plates were transfected with 0.5 µg of the F protein-expressing plasmid [pCA7-F/MV(ICB) or pCA7-F/SSPEV(Kobe)] and 0.5 µg of the H protein-expressing plasmid [pCA7-H-myc/MV(ICB) or pCA7-H-myc/SSPEV(Kobe)] together with 1 µg of the M protein-expressing plasmid [pCA7-M/MV(ICB) or pCA7-M/SSPEV(Kobe)] or pCA7 vector and incubated at 37°C. At 48 h post-transfection, cells were suspended in 0.1 M HEPES/NaOH (pH 7.5) containing cross-linking reagent dithiobis(succinimidyl propionate) (DSP; Thermo Fisher Scientific, Waltham, MA, USA) followed by incubation for 2 h, at 4°C, and the cross-linking reaction was stopped by 10 mM Tris/HCl (pH 7.5). The cells were solubilized with 1 ml of lysis buffer consisting of 1.0% Triton X-100, 10 mM Tris/HCl (pH 7.5) and 5 mM NaCl [23]. The cell extracts were subjected to immunoprecipitation after centrifugation at 13 000 g for 10 min at 4°C.

A small amount (28 µl) of cell extract was mixed with SDS loading buffer as a total cell extract sample. The rest of the extract was incubated for 1 h, at 4°C, with protein G-conjugated magnetic beads (Bio-Rad, Hercules, CA, USA), which had been preincubated with an anti-myc rabbit polyclonal antibody (Cell Signaling Technology, Danvers, MA, USA) for 1 h at room temperature. Immune complexes were obtained by magnetization and washed with lysis buffer according to the instructions for the magnetic beads, and the magnetized proteins and total cell extracts were subjected to SDS-PAGE in 10% polyacrylamide gels followed by electroblotting onto PVDF membranes. Proteins were detected by incubating the membranes with a mouse monoclonal antibody against MV M protein (Merck Millipore, Burlington, MA, USA), rabbit polyclonal antibody against MV F protein [70], rabbit polyclonal antibody against myc-tag (GeneTex, Irvine, TX, USA), or mouse monoclonal antibody against β-actin (Cell Signaling Technology), followed by incubation with an HRP-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Dallas, TX, USA), or goat anti-rabbit IgG (Cell Signaling Technology) secondary antibody. Proteins were visualized using the ECL Plus Western blotting detection system (GE Healthcare, Chicago, IL, USA) by exposure to an autoradiography film.

**Evaluation of F protein stability**

Thermodynamic stability of F protein was evaluated based on the temperature dependence of the cell–cell fusion activity relative to that of standard F protein, as reported previously [70–72]. The relative cell–cell fusion activity of F protein was calculated by dividing the cell–cell fusion activity at 37°C or 40°C by that of the F/MV(ICB) protein at the same temperature. The effect of M protein on the thermodynamic stability of F protein was evaluated in the same way. The cell–cell fusion activity of the F protein in the presence of the M protein at 37°C or 40°C was divided by that in the absence of the M protein at the same temperature to estimate relative cell–cell fusion activity. F protein stability was assessed as follows: destabilized when relative cell–cell fusion activity was higher at 37°C than at 40°C, and stabilized when relative activity at 40°C was higher than that at 37°C.

**Funding information**

This work was supported by Grants-in-Aid for Young Scientists (grant number 19K16678) and Grants-in-Aid for Scientific Research (grant numbers 16K09112 and 19K08941) from the Japan Society for the Promotion of Science (JSPS).

**Acknowledgements**

We would like to thank Yusuke Yanagi (Kyushu University) for providing Vero/hSLAM cells, p(+)MV323-EGFP and pCA7, Makoto Takeda (National Institute of Infectious Diseases) for providing pCITE-IC-N, pCITE-IC-PΔC and pCITEko9301B-L, Naoto Ito and Makoto Sugiyama (Gifu University) for providing the BHK/T7-9 cells, and Bernard Moss (National Institute of Allergy and Infectious Diseases, National Institutes of Health) for providing tRF-7-3. We also thank Jun-ichi Miyazaki (Osaka University) for permission to use the CAG promoter of pCA7. Author Mr Daichi Nishikawa was not available to confirm co-authorship, but the corresponding author Dr Masae Itoh affirms that author Mr Daichi Nishikawa contributed to the paper by performing the research and vouches for author Mr Daichi Nishikawa’s co-authorship status.

**Author contributions**

H.H. and M.I. designed the research; Y.S., K.H., D.N., H.W., M.K., K.S. and D.P.J. performed the research; Y.K., B.G., H.H. and M.I. interpreted the results; Y.S. and M.I. wrote the paper; and M.I. directed the research.

**Conflicts of interest**

The authors declare that there are no conflicts of interest.
Ethical statement
The animal experiment was approved by the Committee of the Institute for Experimental Animals, Kobe University Graduate School of Medicine (permit number 23-67) and all the procedures were performed in accordance with the guidelines.

References
1. Griffin DE. Measles virus. In: Knipe DM, Howley PM, Cohen JI, Griffin DE and Lamb RA (eds). Fields Virology, 6th edn, Vol. 1. Philadelphia, PA: Lippincott Williams & Wilkins; 2013. pp. 1042–1069.
2. Rota PA, Moss WJ, Takeda M, de Swart RL, Thompson KM, et al. Measles. Nat Rev Dis Primers 2016;2:16049.
3. Tatsu H, Ono N, Tanaka K, Yanagi Y. SLAM (CDw150) is a cellular receptor for measles virus. Nature 2000;406:893–897.
4. Ono N, Tatsu H, Tanaka K, Minagawa H, Yanagi Y. V domain of human slam (CDw150) is essential for its function as a measles virus receptor. J Virol 2001;75:1594–1600.
5. Mühlebach MD, Mateo M, Sinn PL, Pruffer S, Uhlig KM, et al. Adherence junction protein nectin-4 is the epithelial receptor for measles virus. Nature 2011;480:530–533.
6. Noyce RS, Bondre DG, MN H, Lin LT, Sisson G, et al. Tumor cell marker PVRL4 (nectin 4) is an epithelial cell receptor for measles virus. PLoS Pathog 2011;7:e1002240.
7. Leonard VHJ, Sinn PL, Hodge G, Miest T, Devaux P, et al. Measles virus blind to its epithelial cell receptor remains virulent in rhesus monkeys but cannot cross the airway epithelium and is not shed. J Clin Invest 2008;118:2448–2458.
8. Noyce RS, Richardson CD. Nectin 4 is the epithelial cell receptor for measles virus. Trends Microbiol 2012;20:429–439.
9. Reymond N, Fabre S, Lecocq E, Adelaïde J, Dubreuil P, et al. Nectin4/PRR4, a new adafin-associated member of the nectin family that trans-interacts with nectin1/PRR1 through V domain interaction. J Biol Chem 2001;276:43205–43215.
10. McQuaid S, Cosby SL. An immunohistochemical study of the distribution of the measles virus receptors, CD46 and SLAM, in normal human tissues and subacute scarring paramyxoviral hepatitis. Lab Invest 2002;82:403–409.
11. Lamb RA, Parks GD. Paramyxoviridae. In: Knipe DM, Howley PM, Cohen JI, Griffin DE and Lamb RA (eds). Fields Virology, 6th edn, Vol. 1. Philadelphia, PA: Lippincott Williams & Wilkins; 2013. pp. 957–995.
12. Brindley MA, Chaudhury S, Plemper RK. Measles virus glyco- protein complexes preassemble intracellularly and relax during transport to the cell surface in preparation for fusion. J Virol 2015;89:1220–1241.
13. Hashiguchi T, Ose T, Kubota M, Maita N, Kamishikiryo J, et al. Structure of the measles virus hemagglutinin bound to its cellular receptor SLAM. Nat Struct Mol Biol 2011;18:135–141.
14. Plemper RK, Brindley MA, Iorio RM. Structural and mechanistic studies of measles virus influence paramyxovirus entry. PLoS Pathog 2011;7:e1002058.
15. Bosse S, Jardetzky TS, Lamb RA. Timing is everything: Fine-tuned molecular machines orchestrate paramyxovirus entry. Virology 2015;479–480:518–531.
16. Platet P, Alves L, Herren M, Aguilar HC. Measles virus fusion protein: Structure, function and inhibition. Viruses 2016;8:E112.
17. Hashiguchi T, Fukuda Y, Matsuoka R, Kuroda D, Kubota M, et al. Structures of the prefusion form of measles virus fusion protein in complex with inhibitors. Proc Natl Acad Sci USA 2018;115:2496–2501.
18. Tahara M, Takeda M, Yanagi Y. Altered interaction of the matrix protein with the cytoplasmic tail of hemagglutinin modulates measles virus growth by affecting virus assembly and cell-cell fusion. J Virol 2007;81:6827–6836.
19. Thorham H, Arnesen K, Mehta PD. Encephalitis in ferrets caused by a nonproductive strain of measles virus (D.R.) isolated from patient with subacute scarring panencephalitis. J Infect Dis 1977;136:229–238.
20. Makino S, Sasaki K, Nakagawa M, Saito M, Shinozaka Y, et al. Isolation and biological characterization of a measles virus-like agent from the brain of an autopsied case of subacute scarring panencephalitis (SSPE). Microbiol Immunol 1977;21:193–205.
21. Homma M, Tashiro M, Konno H, Ohara Y, Hino M, et al. Isolation and characterization of subacute scarring panencephalitis virus (Yamagata-1 strain) from a brain autopsy. Microbiol Immunol 1982;26:1195–1202.
22. Sugita T, Shiraki K, Ueda S, Iwa N, Shoji H, et al. Induction of acute myoclonic encephalopathy in hamsters by subacute scarring panencephalitis virus. J Infect Dis 1984;150:340–347.
23. Jiang DP, Ide Y, Nagano-Fujii M, Shoji I, Hotta H. Single-point mutations of the M protein of a measles virus variant obtained from a patient with subacute scarring panencephalitis critically affect solubility and subcellular localization of the M protein and cell-free virus production. Microbes Infect 2009;11:467–475.
24. Cattaneo R, Schmid A, Billeter MA, Sheppard RD, Udem SA. Multiple viral mutations rather than host factors caused effective measles virus gene expression in a subacute scarring panencephalitis cell line. J Virol 1988;62:1388–1397.
25. Cattaneo R, Schmid A, Eschle D, Baczko K, Meulen ter, et al. Biased hypermutation and other genetic changes in defective measles viruses in human brain infections. Cell 1988;55:255–265.
26. Ayata M, Hirano A, Wong TC. Structural defect linked to nonrandom mutations in the matrix gene of Biken strain subacute scarring panencephalitis virus defined by cDNA cloning and expression of chimeric genes. J Virol 1989;63:1162–1173.
27. Schmid A, Spieelhofer P, Cattaneo R, Baczko K, Meulen ter, et al. Subacute scarring panencephalitis is typically characterized by alterations in the fusion protein cytoplasmic domain of the persisting measles virus. Virology 1992;188:910–915.
28. Moulin E, Beal V, Jeantet D, Horvat B, Wild TF, et al. Molecular characterization of measles virus strains causing subacute scarring panencephalitis in France in 1977 and 2007. J Med Virol 2011;83:1614–1623.
29. Cathomen T, Mriak B, Sphehn D, Drillien R, Naef R, et al. A matrix-less measles virus is infectious and elicits extensive cell fusion: consequences for propagation in the brain. EMBO J 1998;17:3899–3908.
30. Patterson JB, Cornu TI, Redwine J, Dailes S, Lewicki H, et al. Evidence that the hypermutated M protein of a subacute scarring panencephalitis measles virus actively contributes to the chronic progressive CNS disease. Virology 2001;291:215–225.
31. Ayata M, Takeuchi K, Takeda M, Ohgimoto S, Kato S, et al. The F gene of the Osaka-2 strain of measles virus derived from a case of subacute scarring panencephalitis is a major determinant of neurovirulence. J Virol 2010;84:11189–11199.
32. Ayata M, Tanaka M, Kameoka K, Kuwamura M, Takeuchi K, et al. Amino acid substitutions in the heptad repeat A and C regions of the F protein responsible for neurovirulence of measles virus Osak-a-1 strain from a patient with subacute scarring panencephalitis. Virology 2010;404:141–149.
33. Hotta H, Nihei K, Abe Y, Kato S, Jiang DP, et al. Full-length sequence analysis of subacute scarring panencephalitis (SSPE) virus, a mutant measles virus, isolated from brain tissues of a patient shortly after onset of SSPE. Microbiol Immunol 2006;50:525–534.
34. Watanabe S, Shirogane Y, Suzuki SO, Ikegame S, Koga R, et al. Mutant fusion proteins with enhanced fusion activity promote measles virus spread in human neuronal cells and brains of suckling hamsters. J Virol 2013;87:2648–2659.
35. Ning X, Ayata M, Kimura M, Komase K, Furukawa K, et al. Alterations and diversity in the cytoplasmic tail of the fusion protein of subacute scarring panencephalitis virus strains isolated in Osaka, Japan. Virus Res 2002;86:129–131.
36. Doyle J, Prussia A, White LK, Sun A, Liotta DC, et al. Two domains that control prefusion stability and transport competence of the measles virus fusion protein. J Virol 2008;80:1524–1536.
37. Avila M, Alves L, Khorasavi M, Ader-Ebert N, Drigg F, et al. Molecular determinants defining the triggering range of pre fusion F complexes of canine distemper virus. J Virol 2014;88:2951–2966.

38. Satoh Y, Yonemori S, Hirose M, Shogaki H, Wakimoto H, et al. A residue located at the junction of the head and stalk regions of measles virus fusion protein regulates membrane fusion by controlling conformational stability. J Gen Virol 2017;98:143–154.

39. Watanabe S, Shirogane Y, Sato Y, Hashiguchi T, Yanagi Y. New insights into measles virus brain infections. Trends Microbiol 2019;27:164–175.

40. Cattaneo R, Schmid A, Spielhofer P, Kaelin K, Baczko K, et al. Mutated and hypermutated genes of persistent measles viruses which caused lethal human brain diseases. Virology 1989;173:415–425.

41. Wong TC, Ayata M, Hirano A, Yoshikawa Y, Tsuruoka H, et al. Generalized and localized biased hypermutation affecting the matrix gene of a measles virus strain that causes subacute scarring panencephalitis. J Virol 1989;63:5464–5468.

42. Wong TC, Ayata M, Ueda S, Hirano A. Role of biased hypermutation in evolution of subacute scarring panencephalitis virus from progenitor acute measles virus. J Virol 1991;65:2191–2199.

43. Baczko K, Lampe J, Liebert UG, Brinckmann U, Meulen ter et al. Clonal expansion of hypermutated measles virus in a SSPE brain. Virology 1993;197:188–195.

44. Ayata M, Komase K, Shinag M, Matsunaga I, Katayama Y, et al. Mutations affecting transcriptional termination in the P gene end of subacute scarring panencephalitis viruses. J Virol 2002;76:13062–13068.

45. Sheppard RD, Raine CS, Bornstein MB, Udem SA. Measles virus matrix protein synthesized in a subacute scarring panencephalitis cell line. Science 1985;228:1219–1221.

46. Cattaneo R, Schmid A, Rebmann G, Baczko K, Meulen ter et al. Accumulated measles virus mutations in a case of subacute scarring panencephalitis: interrupted matrix protein reading frame and transcription alteration. Virology 1986;154:97–107.

47. Cattaneo R, Rebmann G, Schmid A, Baczko K, Meulen ter et al. Altered transcription of a defective measles virus genome derived from a diseased human brain. EMBO J 1987;6:681–688.

48. Yoshikawa Y, Tsuruoka H, Matsumoto M, Haga T, Shioida T, et al. Molecular analysis of structural protein genes of the Yamagata-1 strain of defective subacute scarring panencephalitis virus. II. Nucleotide sequence of a cDNA corresponding to the P plus M plus 5' non-cis intrinsic mRNA. Virus Genes 1990;4:151–161.

49. Hiran A, Wang AH, Gombart AF, Wong TC. The matrix proteins of neurovirulent subacute scarring panencephalitis virus and its acute measles virus progenitor are functionally different. Proc Natl Acad Sci USA 1992;89:8745–8749.

50. Hiran A, Ayata M, Wang AH, Wong TC. Functional analysis of matrix proteins expressed from cloned genes of measles virus variants that cause subacute scarring panencephalitis reveals a common defect in nucleocapsid binding. J Virol 1993;67:1848–1853.

51. Ader N, Brindley M, Avila M, Orvell C, Horvat B, et al. Mechanism for active membrane fusion triggering by morbillivirus attachment protein. J Virol 2013;87:314–326.

52. Jurgens EM, Mathieu C, Palermo LM, Hardie D, Horvat B, et al. Measles fusion machinery is dysregulated in neuropathogenic variants. mBio 2015;6:e02528.

53. Watanabe S, Shinji O, Yuta S, Suzuki SO, Koga R, et al. Measles virus mutants possessing the fusion protein with enhanced fusion activity spread effectively in neuronal cells, but not in other cells, without causing strong cytopathology. J Virol 2015;89:2710–2717.

54. Sato Y, Watanabe S, Fukuda Y, Hashiguchi T, Yanagi Y, et al. Cell-to-cell measles virus spread between human neurons is dependent on hemagglutinin and hyperfusogenic fusion protein. J Cell 2018;92:e02166–17.

55. Angius F, Smuts H, Rybkina K, Stelitano D, Eley B, et al. Analysis of a subacute scarring panencephalitis genotype B3 virus from the 2009-2010 south african measles epidemic shows that hyperfusogenic F proteins contribute to measles virus infection in the brain. J Virol 2019;93:e01700–18.

56. Carter MJ, Meulen ter. Subacute Sclerosing Panencephalitis: Are Antigenic Changes Involved in Measles Virus Persistence? Prog Brain Res 1983;59:163–171.

57. Norrb Y, Kristenson K, Brzosko WJ, Kaspersen JG. Measles virus matrix protein detected by immune fluorescence with monoclonal antibodies in the brain of patients with subacute scarring panencephalitis. J Virol 1985;56:337–340.

58. Centers for Disease Control. Subacute Sclerosing Panencephalitis surveillance. Morb Mortal Weekly Rep 1982;31:585–588.

59. Okuno Y, Nakao T, Ishida N, Konno T, Mizutani H, et al. Incidence of subacute scarring panencephalitis following measles and measles vaccination in Japan. Int J Epidemiol 1989;18:684–689.

60. Campbell H, Andrews N, Brown KE, Miller E. Review of the effect of measles vaccination on the epidemiology of SSPE. Int J Epidemiol 2007;36:1334–1348.

61. Bellini WJ, Rota JS, Lowe LE, Katz RS, Dyken PR, et al. Subacute scarring panencephalitis: more cases of this fatal disease are prevented by measles immunization than was previously recognized. J Infect Dis 2005;192:1686–1693.

62. Schönberger K, Ludwig M, W. Wildner M, Weissbrich B. Epidemiology of subacute scarring panencephalitis (SSPE) in Germany from 2003 to 2009: a risk estimation. PLoS One 2013;8:e68909.

63. Wendorf KA, Winter K, Zipprich J, Schechter R, Hacker JK, et al. Subacute Sclerosing Panencephalitis: the Devastating Measles Complication that Might be More Common than Previously Estimated: a risk estimation. Clin Infect Dis 2017;65:226–232.

64. Plumper RK, Comans RW. Mutations in the putative HR-C region of the measles virus F2 glycoprotein module syncytium formation. J Virol 2003;77:4181–4190.

65. Ito N, Takayama-Ito M, Yamada K, Hosokawa J, Sugiyama M, et al. Improved recovery of rabies virus from cloned cDNA using a vaccinia virus-free reverse genetics system. Microbiol Immunol 2003;47:613–617.

66. Seki F, Yamada K, Nakatsu Y, Okamura K, Yanagi Y, et al. The SI strain of measles virus derived from a patient with subacute scarring panencephalitis possesses typical genome alterations and unique amino acid changes that modulate receptor specificity and reduce membrane fusion activity. J Virol 2011;85:11871–11882.

67. Wakimoto H, Shimodo M, Satoh Y, Kitagawa Y, Takeuchi K, et al. F-actin modulates measles virus cell-cell fusion and assembly by altering the interaction between the matrix protein and the cytoplasmic tail of hemagglutinin. J Virol 2013;87:1974–1984.

68. Fuerst TR, Earl PL, Moss B. Use of a hybrid vaccinia virus-T7 RNA polymerase system for expression of target genes. Mol Cell Biol 1987;7:2538–2544.

69. Hashimoto K, Ono N, Tatsuo H, Takeda M, Takeuchi K, et al. SLAM (CD150)-independent measles virus entry as revealed by recombinant virus expressing green fluorescent protein. J Virol 2002;76:6743–6749.

70. Satoh Y, Hirose M, Shogaki H, Wakimoto H, Kitagawa Y, et al. Intramolecular complementation of measles virus fusion protein stability confers the fusion activity at 37°C. FEBS Lett 2015;589:152–158.

71. Prussia AJ, Plumper RK, Snyder JP. Measles virus entry inhibitors: a structural proposal for mechanism of action and the development of resistance. Biochemistry 2008;47:13573–13583.

72. Plattet P, Langedijk JP, Zipperle L, Vandevelde M, Orvell C, et al. Conserved leucine residue in the head region of morbilliviruses fusion protein regulates the large conformational change during fusion activity. Biochemistry 2009;48:9112–9121.