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VIRUS-CELL INTERACTIONS

Weak *cis* and *trans* Interactions of the Hemagglutinin with Receptors Trigger Fusion Proteins of Neuropathogenic Measles Virus Isolates

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ABSTRACT Measles virus (MeV) is an enveloped RNA virus bearing two envelope glycoproteins, the hemagglutinin (H) and fusion (F) proteins. Upon receptor binding, the H protein triggers conformational changes of the F protein, causing membrane fusion and subsequent virus entry. MeV may persist in the brain, infecting neurons and causing fatal subacute sclerosing panencephalitis (SSPE). Since neurons do not express either of the MeV receptors, signaling lymphocytic activation molecule (SLAM; also called CD150) and nectin-4, how MeV propagates in neurons is unknown. Recent studies have shown that specific substitutions in the F protein found in MeV isolates from SSPE patients are critical for MeV neuropathogenicity by rendering the protein unstable and hyperfusogenic. Recombinant MeVs possessing the F proteins with such substitutions can spread in primary human neurons and in the brains of mice and hamsters and induce cell-cell fusion in cells lacking SLAM and nectin-4. Here, we show that receptor-blind mutant H proteins that have decreased binding affinities to receptors can support membrane fusion mediated by hyperfusogenic mutant F proteins, but not the wild-type F protein, in cells expressing the corresponding receptors. The results suggest that weak interactions of the H protein with certain molecules (putative neuron receptors) trigger hyperfusogenic F proteins in SSPE patients. Notably, where cell-cell contacts are ensured, the weak *cis* interaction of the H protein with SLAM on the same cell surface also could trigger hyperfusogenic F proteins. Some enveloped viruses may exploit such *cis* interactions with receptors to infect target cells, especially in cell-to-cell transmission.

IMPORTANCE Measles virus (MeV) may persist in the brain, causing incurable subacute sclerosing panencephalitis (SSPE). Because neurons, the main target in SSPE, do not express receptors for wild-type (WT) MeV, how MeV propagates in the brain is a key question for the disease. Recent studies have demonstrated that specific substitutions in the MeV fusion (F) protein are critical for neuropathogenicity. Here, we show that weak *cis* and *trans* interactions of the MeV attachment protein with receptors that are not sufficient to trigger the WT MeV F protein can trigger the mutant F proteins from neuropathogenic MeV isolates. Our study not only provides an important clue to understand MeV neuropathogenicity but also reveals a novel viral strategy to expand cell tropism.

KEYWORDS measles virus, neuropathogenicity, subacute sclerosing panencephalitis, virus receptor, weak interaction

Measles is still an important cause of death worldwide, especially among young children in developing countries (1). Measles virus (MeV), the causative agent of the disease, is an enveloped RNA virus in the family *Paramyxoviridae* and has two envelope glycoproteins, the hemagglutinin (H) and fusion (F) proteins. MeV enters the cell through membrane fusion at the cell surface. The binding of the H protein to a

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cellular receptor triggers the conformational changes of the F protein from the prefusion to the postfusion form, thereby causing the fusion of the virus envelope with the cell membrane and allowing the delivery of the virus genome into the cell (2–6). The H and F proteins are also expressed on the surface of MeV-infected cells, inducing syncytia via cell-cell fusion of infected and neighboring uninfected cells. The signaling lymphocytic activation molecule (SLAM; also called SLAMF1 or CD150) on immune cells and nectin-4 on epithelial cells are known to act as receptors for MeV (7–9).

MeV persists, albeit rarely, in the central nervous system, causing fatal subacute sclerosing panencephalitis (SSPE) several years after acute infection (6). In SSPE patients, MeV mainly propagates in neurons, which express neither SLAM nor nectin-4 (10, 11), but the neuron receptor for MeV has not been identified. Notably, wild-type (WT) MeV isolates from acute measles patients are unable to spread in primary human neurons *in vitro* and do not induce membrane fusion in SLAM- and nectin-4-negative cells (12–14). Although a recent study suggested that *trans*-endocytosis elicited by nectins accounts for the first phase of MeV neural invasion (15), how MeV propagates in the brain is still unclear. Through adaptation to persistence in the brain, MeV isolates from SSPE patients accumulate numerous mutations in their genomes, including the gene encoding the matrix protein involved in virus particle formation (16–18). Since the mutations preclude the production of free MeV particles, it is thought that cell-to-cell transmission accounts for MeV propagation in neurons (6, 19, 20).

Recent studies have demonstrated that specific substitutions in the ectodomain of the F protein are critical for neuropathogenicity of MeV, conferring on the virus the ability to spread in primary human neurons *in vitro* as well as in the brains of experimentally infected mice and hamsters (6, 12–14, 21–26). These substitutions were shown to destabilize the prefusion form of the F protein, rendering it hyperfusogenic. Importantly, the F proteins containing such substitutions can induce membrane fusion in SLAM- and nectin-4-negative cells when expressed together with the WT H protein. The reason why the structurally unstable hyperfusogenic F proteins induce membrane fusion and mediate viral spread in human neurons lacking the known receptors is unknown. Since decreased stability lowers the energy level of the activation barrier required to induce the conformational changes of the F protein, we proposed that even weak interactions of the H protein with particular molecules (other than SLAM and nectin-4) that cannot trigger the WT F protein for the conformational changes are sufficient to trigger structurally unstable mutant F proteins (21). Thus, the H protein may interact only weakly with the putative MeV neuron receptor(s).

To test this idea, here we employed a reverse strategy where so-called receptor-blind MeV H proteins were examined in combination with SLAM and nectin-4. These mutant H proteins have substitutions within the receptor binding sites and fail to use respective receptors, although they retain certain levels of binding affinities to the receptors (2, 27–30). We found that weak interactions between the SLAM-blind H protein and SLAM or between the nectin 4-blind H protein and nectin-4 could support membrane fusion mediated by hyperfusogenic mutant F proteins but not the WT F protein, verifying our hypothesis. Unexpectedly, we also found that the *cis* interaction of the SLAM-blind H protein with SLAM on the same cells could trigger hyperfusogenic F proteins. This finding defies the general notion that the viral attachment protein interacts in *trans* with its receptor. The *cis* interaction may play a role in cell-to-cell transmission of enveloped viruses, where close cell-cell contacts (e.g., neurological synapses and polarized epithelia) exist and *trans* interactions are not essential.

RESULTS

Specific substitutions in the ectodomain of the F protein compensate for the binding defect of receptor-blind H proteins. R533A and Y543S substitutions in the receptor-binding sites render the MeV H protein deficient for binding to SLAM and nectin-4, respectively (2, 27–30). Surface plasmon resonance (SPR) analysis confirmed that these substitutions strongly reduce the binding of the MeV H protein to the corresponding receptors (data not shown). Fusion support activities of these mutant H

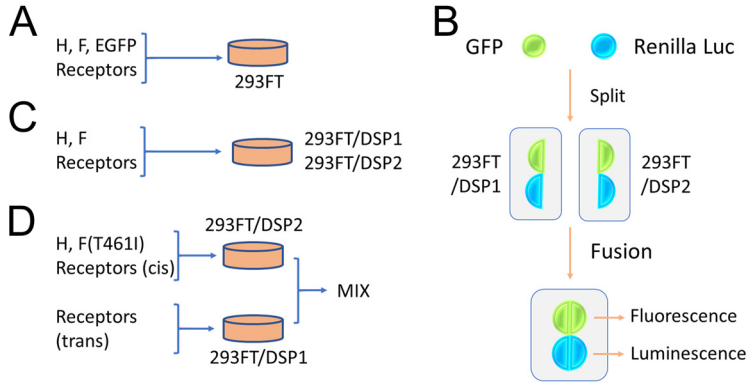

FIG 1 Schematic diagrams of assays used in this study. (A) The fusion assay was performed by expressing all components (MeV H, MeV F, EGFP, and a receptor) together in 293FT cells. Cells were observed under fluorescence microscopy 30 h after transfection. (B) The principle of the DSP assay system. DSP1 and DSP2 are a pair of chimeric reporter proteins, each composed of split Renilla luciferase and split GFP. 293FT/DSP1 and 293FT/DSP2 cells are 293FT cells stably expressing DSP1 and DSP2, respectively. When 293FT/DSP1 and 293FT/DSP2 cells are fused, Renilla luciferase and GFP activities are restored by the association of DSP1 and DSP2. (C) The quantitative fusion assay (the DSP assay) was performed by expressing all components (MeV H, MeV F, and a receptor) in cocultured 293FT/DSP1 and 293FT/DSP2 cells. Renilla luciferase activity was measured 24 h after transfection. (D) The quantitative fusion assay to evaluate *trans*- and *cis*-acting receptor activities. 293FT/DSP2 cells transfected with plasmids expressing MeV H and MeV F proteins and with or without the plasmid expressing a receptor (in *cis*) were mixed with 293FT/DSP1 cells transfected with the control plasmid or that expressing a receptor (in *trans*). Renilla luciferase activity was measured 24 h after the cells were mixed.

proteins were then evaluated by the fusion assay. 293FT cells were transfected with plasmids expressing one of the MeV H proteins [WT-H, H(R533A), or H(Y543S)], the WT-F protein, one of the receptors (SLAM or nectin-4), and enhanced green fluorescent protein (EGFP) (Fig. 1A). H(R533A) and H(Y543S) did not support syncytium formation dependent on SLAM and nectin-4, respectively (Fig. 2A). The T461I substitution in the ectodomain of the F protein is found in multiple MeV isolates from SSPE patients and enables MeV to spread in primary human neurons and brains of mice and hamsters and to induce membrane fusion in cells lacking SLAM and nectin-4 (12, 13, 24). When F(T461I) was used in place of WT-F for the fusion assay, the combination of H(R533A) and SLAM or H(Y543S) and nectin-4 caused syncytium formation as efficiently as that of WT-H and SLAM or WT-H and nectin-4, respectively (Fig. 2B).

We also examined two other F proteins containing substitutions in the ectodomain, F(S103I/N462S/N465S) (designated F-triple) and F(M94V), as well as the F protein missing the cytoplasmic tail (F-Δ30). F-triple and F(M94V) have been shown to possess the same properties as F(T461I) (12, 13). In contrast, F-Δ30 could neither induce syncytia in cells lacking SLAM and nectin-4 nor exhibit strong neuropathogenicity, although its fusion-enhancing effect was evident in SLAM-positive cells (12, 13, 24). F-triple and F(M94V), but not F-Δ30, induced apparent syncytia in 293FT cells when expressed together with H(R533A) and SLAM (Fig. 2C) or with H(Y543S) and nectin-4 (Fig. 2D).

Thus, the F proteins possessing specific substitutions in the ectodomain could compensate for the binding defect of receptor-blind H(R533A) and H(Y543S), indicating that even weak interactions of the H protein with receptors could trigger these hyperfusogenic F proteins.

Receptor activity of SLAM for SLAM-blind H(R533A) differs depending on the conditions of the fusion assay. To quantitate levels of membrane fusion supported by low-affinity interactions between the receptor-blind H proteins and their corresponding receptors, we employed the dual split protein (DSP) assay system (31–33). In this system, a pair of chimeric reporter proteins, DSP1 and DSP2, each comprised of the split Renilla luciferase and split GFP, are used (Fig. 1B). When cell-cell fusion is induced between 293FT cells stably expressing DSP1 and DSP2 (293FT/DSP1 and

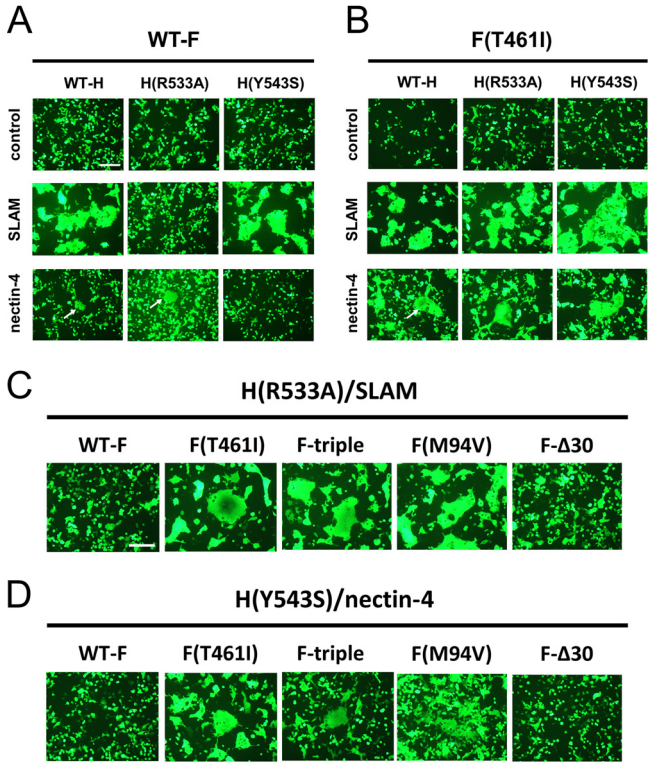

FIG 2 Cell-cell fusion supported by receptor-blind H proteins in combination with WT and mutant F proteins. (A and B) 293FT cells were transfected with different combinations of plasmids expressing MeV H [WT-H, H(R533A), or H(Y543S)], MeV F [WT-F (A) or F(T461I) (B)], a receptor (SLAM, nectin-4, or control vector), and EGFP. Arrows indicate small-sized syncytia. (C and D) 293FT cells were transfected with plasmids expressing MeV H [H(R533A) (C) or H(Y543S) (D)], MeV F [WT-F, F(T461I), F-triple (S103I/N462S/N465S), F(M94V), or F-Δ30], a receptor (SLAM [C] or nectin-4 [D]), and EGFP. Cells were observed under fluorescence microscopy 30 h after transfection. Scale bar, 200 μm.

293FT/DSP2 cells), Renilla luciferase and GFP activities are restored by the association of DSP1 and DSP2.

293FT/DSP1 and 293FT/DSP2 cells were cocultured and transfected with plasmids expressing MeV H, MeV F, and SLAM or nectin-4 (Fig. 1C), and Renilla luciferase activity was measured 24 h after transfection. Cell-cell fusion was detected upon expression of SLAM or nectin-4, but the other members of the nectin family (nectin-1, nectin-2, and nectin-3) did not induce membrane fusion with this assay using WT H and F(T461I) (data not shown). Thus, induction of cell-cell fusion under this experimental condition requires a specific receptor, either SLAM or nectin-4. When WT-H and WT-F were expressed with SLAM or nectin-4 in the assay, luciferase activities were significantly increased compared with that of the receptor-negative control (Fig. 3A). In contrast, H(R533A) and H(Y543S), in combination with WT F, were unable to induce luciferase activities with SLAM and nectin-4, respectively. However, when F(T461I) was transfected in place of WT-F, H(R533A) and H(Y543S) exhibited significant luciferase activities with SLAM and nectin-4, respectively. The levels of fusion induced by the combination of H(R533A) and F(T461I) with SLAM and that of H(Y543S) and F(T461I) with nectin-4 were comparable to those induced by the combination of WT-H and WT-F with the corresponding receptors. Thus, the results with the quantitative DSP assay are consistent with the observations with the fusion assay under fluorescence microscopy (Fig. 2A and B and 3A).

Although transfecting cells with plasmids expressing MeV envelope proteins and receptors together is a convenient method to evaluate cell-cell fusion (the first protocol) (Fig. 1A and C), it may not reflect real situations where the MeV envelope proteins and host receptors are usually expressed on separate membranes. Therefore, the

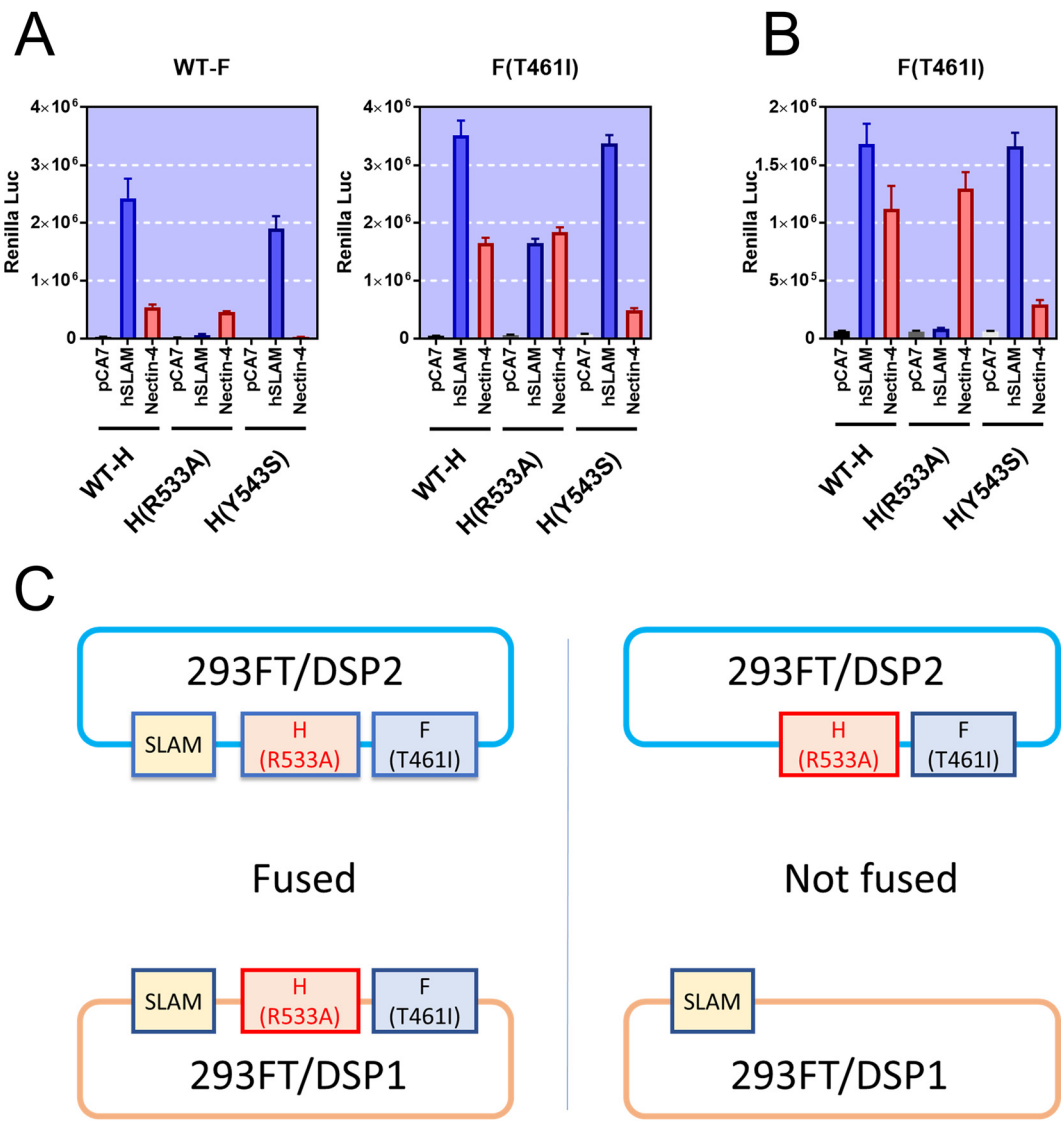

FIG 3 Receptor activities of SLAM and nectin-4 tested under two different conditions. (A) MeV H [WT-H, H(R533A), or H(Y543S)), MeV F (WT-F or F(T461I)], and a receptor (SLAM, nectin-4, or control [vector plasmid]) were expressed together in cocultured 293FT/DSP1 and 293FT/DSP2 cells as shown in Fig. 1C ($n = 3$, means ± standard deviations [SD]). (B) 293FT/DSP1 cells were transfected with a plasmid expressing SLAM, nectin-4, or the control. The cells were mixed with 293FT/DSP2 cells transfected with plasmids expressing MeV H [WT-H, H(R533A), or H(Y543S)] and F(T461I) proteins as shown in Fig. 1D ($n = 3$, means ± SD). (C) Schematic diagrams showing results obtained with different protocols. The diagram on the left corresponds to Fig. 1C and panel A of this figure, and the one on the right corresponds to Fig. 1D and panel B of this figure.

protocol was modified to evaluate *trans*-acting receptor activities of SLAM and nectin-4 for the receptor-blind H proteins (the second protocol) (Fig. 1D). 293FT/DSP1 cells were transfected with the plasmid expressing SLAM or nectin-4, while 293FT/DSP2 cells were transfected with plasmids expressing H and F(T461I) proteins. The transfected 293FT/DSP1 cells were mixed with the transfected 293FT/DSP2 cells 3 to 4 h later, and Renilla luciferase activity was measured 24 h after the cells were mixed. In this protocol, the combination of H(R533A) and SLAM in *trans* was unable to induce luciferase activity even with F(T461I), while luciferase activity was detected with the combination of H(Y543S) and nectin-4 (Fig. 3B). The result with the combination of H(R533A) and SLAM in *trans* was unexpected. Figure 3C schematically shows the difference between the two protocols. In the first protocol, both 293FT/DSP1 and 293FT/DSP2 cells express all of H, F, and SLAM. In the second protocol, 293FT/DSP2 cells express MeV envelope proteins, while 293FT/DSP1 cells express only SLAM. The decreased binding affinity of

H(R533A) to SLAM might not be sufficient to trigger membrane fusion between the cells; thus, bidirectional low-affinity interactions between H(R533A) on 293FT/DSP1 cells and SLAM on 293FT/DSP2 cells as well as between H(R533A) on 293FT/DSP2 cells and SLAM on 293FT/DSP1 cells might be required to support cell-cell fusion with F(T461I). Alternatively, SLAM might interact in *cis* with H(R533A) to trigger F(T461I).

SLAM exhibits *cis*-acting receptor activity for SLAM-blind H(R533A) in the copresence of E-cadherin. To determine how the receptor-blind H proteins interact with receptors to trigger F(T461I), SLAM and nectin-4 were expressed in *trans*, in *cis*, or both in *trans* and in *cis* with regard to H and F proteins in the DSP assay (Fig. 4A, a to c). H(R533A) could support membrane fusion only when SLAM was expressed both in *trans* and in *cis* (Fig. 4B), whereas H(Y543S) could do so when nectin-4 was expressed in *trans* (Fig. 4C). Since fusion occurred between 293FT/DSP2 cells expressing H(R533A), F(T461I), and SLAM and 293FT/DSP1 cells expressing SLAM (Fig. 4A, c, and B), the possibility that bidirectional H(R533A)/SLAM interactions are necessary for the induction of fusion could be excluded (293FT/DSP1 cells did not express H and F proteins). Thus, we are left with the possibility that SLAM interacts in *cis* with H(R533A). The importance of homophilic SLAM/SLAM binding in immune responses has been well established (34–36). We hypothesized that in addition to the *cis* interaction between H(R533A) and SLAM on 293FT/DSP2 cells, the homophilic binding of SLAM on 293FT/DSP1 and SLAM on 293FT/DSP2 cells is necessary for cell-cell fusion, because the binding affinity of H(R533A) to SLAM is not sufficient to bring the two membranes to be fused into close apposition. This interpretation would nicely explain the findings that H(R533A) supports fusion when SLAM is expressed on both 293FT/DSP1 and 293FT/DSP2 cells but not when it is expressed on either type of cells.

To substantiate this hypothesis, E-cadherin was exogenously expressed on cells (Fig. 4A, d to f, and D). E-cadherin is a calcium-dependent cell adhesion molecule, and it preferentially interacts with itself to form the adherens junction (37). When 293FT cells were transfected with plasmids expressing WT H, F(T461I), and E-cadherin, some syncytia were formed, but they were much fewer in number and smaller in size than those formed by the expression of H(R533A), F(T461I), and SLAM or by the expression of H(Y543S), F(T461I), and nectin-4 (Fig. 4D). Expression of E-cadherin on both 293FT/DSP1 and 293FT/DSP2 cells without receptors also induced membrane fusion to some extent in the DSP assay (Fig. 4B, d, and C, d). However, the additional expression of SLAM in *cis*, but not in *trans*, with respect to MeV envelope proteins (on 293FT/DSP2 cells) increased the level of membrane fusion (Fig. 4B, e and f). The level of membrane fusion induced by the expression of SLAM in *cis* in the presence of E-cadherin was comparable to that by the expression of SLAM both in *trans* and in *cis* (Fig. 4B, c and f).

Taken together, the results indicate that the induction of membrane fusion mediated by H(R533A) and F(T461I) has two requirements: first, the *cis* interaction between H(R533A) and SLAM, and second, cell-cell contact mediated by homophilic SLAM-SLAM or homophilic E-cadherin–E-cadherin binding. Although a certain level of fusion may be induced by the mere close proximity of two membranes, as previously suggested in another experimental system of MeV-mediated membrane fusion (38), the full level of fusion appears to demand the interaction between the H protein and its receptor that triggers the F protein. In contrast, expression of E-cadherin did not affect the level of fusion induced by the *trans* interaction between H(Y543S) and nectin-4 (Fig. 4C). Furthermore, the level of fusion induced by the *cis* interaction between them was negligible, as it was comparable to that induced in the presence of E-cadherin alone (without nectin-4). Thus, H(Y543S) triggers F(T461I) by interacting in *trans* with nectin-4.

The *cis* interaction of WT H with SLAM cannot trigger the F protein. We then examined whether the *cis* interaction also occurs between WT H and SLAM. As expected, expression of SLAM or nectin-4 in *cis* with respect to WT H could not trigger F(T461I) to cause membrane fusion in the DSP assay (Fig. 5A), because sufficient cell-cell contact did not exist between 293FT/DSP1 and 293FT/DSP2 cells. The additional

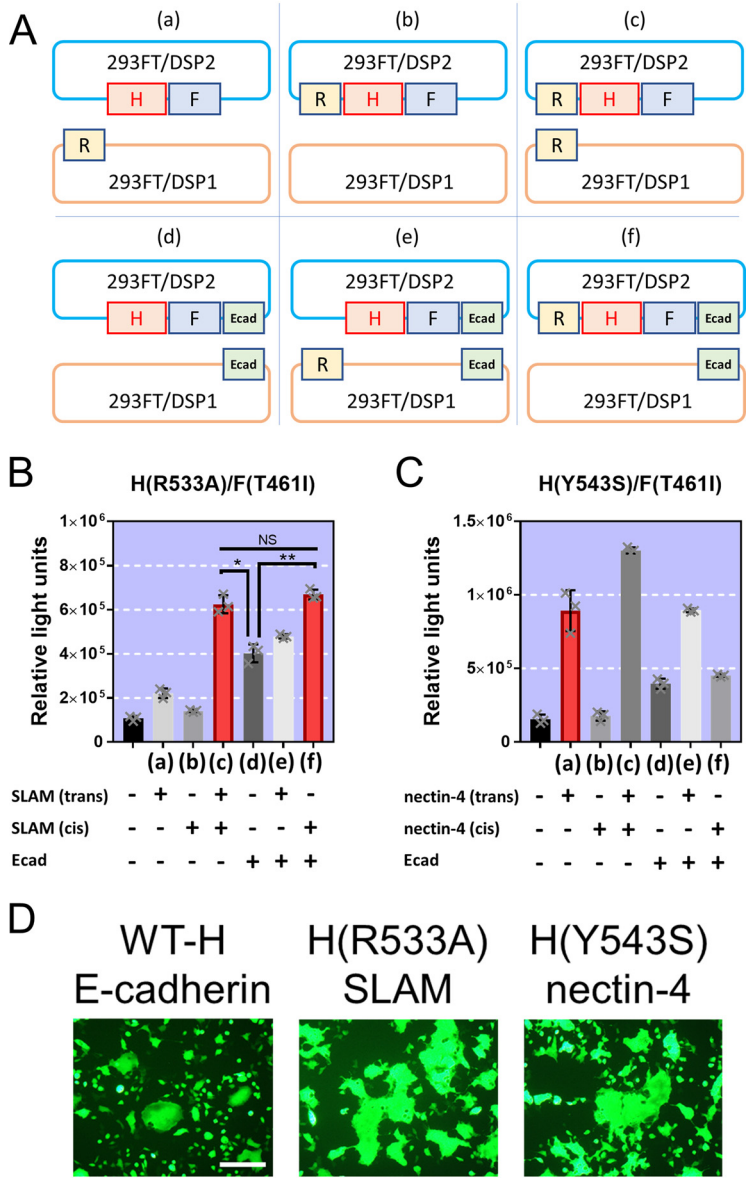

FIG 4 *cis*- and *trans*-acting receptor activities of SLAM and Nectin-4. (A) Schematic diagrams of the experimental conditions used in panels B and C. (a to c) SLAM and nectin-4 (designated R) were expressed in 293FT/DSP1 cells (in *trans*), 293FT/DSP2 cells (in *cis*), or both (in *cis* and in *trans*). (d to f) E-cadherin (Ecad) was also coexpressed in both 293FT/DSP1 and 293FT/DSP2 cells. (B) The results of the quantitative fusion assay with the combination of H(R533A) and SLAM ($n = 3$, means ± SD). (C) The results with the combination of H(Y543S) and nectin-4 ($n = 3$, means ± SD). Results were analyzed by the unpaired two-tailed Student *t* test. *, $P = 0.0026$; **, $P = 0.0006$; ns, $P = 0.18$. (D) A limited level of syncytium formation induced by E-cadherin. Expression of E-cadherin, together with WT-H and F(T461I), in 293FT cells induced syncytia, but they were fewer in number and smaller than those induced by a combination of H(R533A) and SLAM or H(Y543S) and nectin-4. Scale bar, 200 μm.

expression of E-cadherin in both 293FT/DSP1 and 293FT/DSP2 cells did not increase the level of fusion supported by WT H and SLAM (Fig. 5B). Rather, it was even lower than that obtained when E-cadherin was expressed without SLAM. To understand why the expression of E-cadherin did not help the combination of WT H and SLAM to support membrane fusion, WT H and H(R533A) were transiently expressed with SLAM in 293FT cells, and cell surface expression levels of the H proteins were examined by flow cytometry (Fig. 5C). Both the percentage and the mean fluorescent intensity of WT H-positive cells were decreased by coexpression of SLAM, while those of H(R533A)-positive cells were not affected (Fig. 5C). The results suggest that the high-affinity *cis*

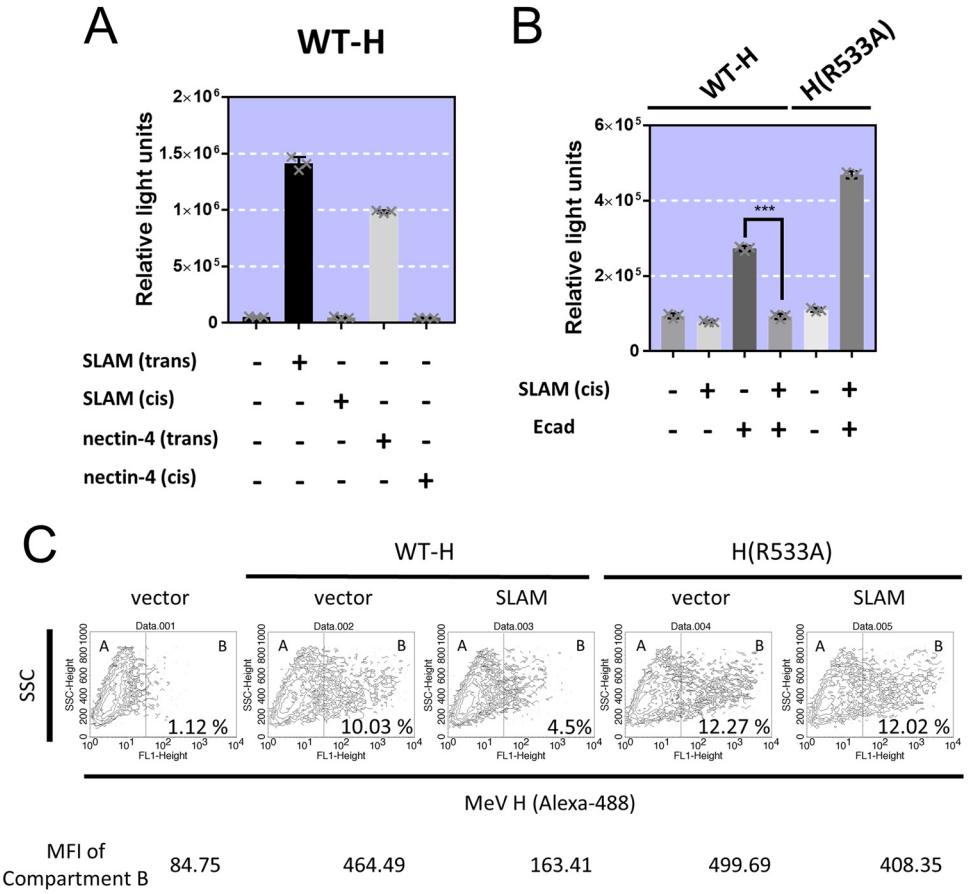

FIG 5 Fusion-inhibitory effect of SLAM expressed in *cis* with WT H. (A) 293FT/DSP1 cells were transfected with the plasmid expressing SLAM, nectin-4, or none. The cells were mixed with 293FT/DSP2 cells transfected with plasmids expressing WT-H, F(T461I), and a receptor (SLAM, nectin-4, or none). Luciferase activity was measured 24 h after the cells were mixed ($n = 3$, means ± SD). (B) The quantitative fusion assay (in *cis*) was performed by expressing MeV H [WT-H or H(R533A)] and F(T461I), together with or without SLAM, in 293FT/DSP2 cells ($n = 3$, means ± SD). In addition, E-cadherin was expressed in both 293FT/DSP1 and 293FT/DSP2 cells. Results were analyzed by the unpaired two-tailed Student *t* test. ***, $P = 0.0000053$. (C) 293FT cells were transfected with the plasmids expressing MeV H protein [WT-H or H(R533A)] and a receptor (SLAM or control). Surface expression of the MeV H proteins was analyzed by flow cytometry. Percentages and mean fluorescent intensities (MFI) of the H protein-positive population in compartment B are shown.

interaction between WT H and SLAM cannot trigger the F protein for membrane fusion because of downregulation of the H protein from the cell surface, whereas the low-affinity *cis* interaction between H(R533A) and SLAM hardly downregulates the H protein, causing membrane fusion.

DISCUSSION

In this study, we demonstrated that hyperfusogenic F proteins, but not the WT F protein, are triggered by the low-affinity interaction of SLAM-blind H(R533A) with SLAM or that of nectin-4-blind H(Y543S) with nectin-4. These receptor-blind H proteins possess much lower binding affinity to the respective receptors than the WT H protein (29, 30). Thus, the results confirmed our hypothesis that weak interactions of the H protein with particular molecules that cannot trigger the WT F protein may be sufficient to trigger structurally unstable hyperfusogenic F proteins. It remains to be determined whether the putative MeV neuron receptor exhibits much lower affinity to the H protein than SLAM and nectin-4, but the attempts to identify the receptor should take this possibility into account.

The hypothesis about the low-affinity receptor was based on the following observations and reasoning. Neurons, main target cells of MeV in SSPE, do not express the

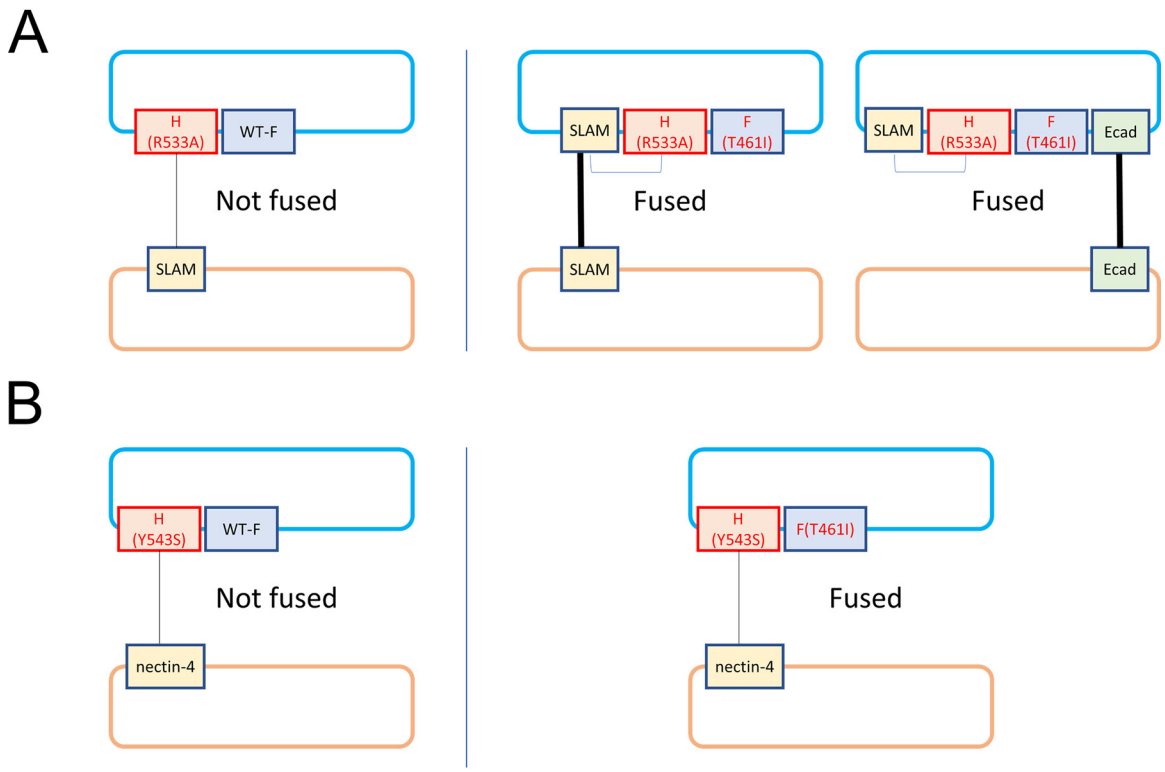

FIG 6 Schematic diagrams of membrane fusion supported by low-affinity interactions between the MeV H proteins and receptors. (A) The conditions under which the combination of H(R533A) and SLAM could and could not support cell-cell fusion. Three requirements have to be met for successful membrane fusion. First, the F protein is structurally unstable and hyperfusogenic. Second, cell-cell contact is ensured by adhesion molecules, such as E-cadherin or SLAM itself. Third, SLAM is expressed in *cis* with H(R533A). (B) The combination of H(Y543S) and nectin-4 can induce cell-cell fusion when the F protein is hyperfusogenic and nectin-4 is expressed in *trans*, not in *cis*, with H(Y543S).

known MeV receptors, SLAM and nectin-4. On the other hand, MeV neuropathogenicity is critically mediated by hyperfusogenic F proteins containing substitutions in the ectodomain, which are almost invariably found in MeV isolates from SSPE patients (12–14, 21, 22, 24, 25). Since these hyperfusogenic F proteins still require the H protein to induce membrane fusion and anti-H protein monoclonal antibodies inhibit membrane fusion mediated by hyperfusogenic F proteins, there must be a specific neuron receptor(s) interacting with the H protein (14). Furthermore, these hyperfusogenic F proteins are structurally unstable and more readily triggered for the conformational changes (13, 21–23, 25).

Unexpectedly, the present study also revealed that SLAM can exhibit *cis*-acting receptor activity for the SLAM-blind H protein where cell-cell contact is maintained by E-cadherin or SLAM itself (Fig. 6A). Since MeV enters the cell through membrane fusion at the cell surface, MeV receptors have to fulfill two functions by interacting with the H protein: bringing two membranes to be fused into close apposition and triggering the conformational changes of the F protein. These two requirements also apply to receptors of other enveloped viruses that similarly enter the cell. However, where the proximity of the two membranes is structurally assured, the first requirement may not be necessary. Our study indeed showed this, but the receptor SLAM was found to act in *cis*, not in *trans*, on the SLAM-blind H protein to trigger hyperfusogenic F(T461I). In contrast, nectin-4 was able to interact in *trans* with the nectin-4-blind H protein to trigger F(T461I) without assistance by E-cadherin (Fig. 6B), just as it does with the WT H protein to trigger the WT and hyperfusogenic F proteins. The reason why the expression of E-cadherin alone (without receptors) slightly induces membrane fusion is currently unclear. Bringing two membranes into close apposition might allow receptor-

independent fusion or engagement of an unidentified *cis*-acting receptor endogenously expressed in 293FT cells.

It is currently unknown why the *cis*, but not *trans*, interaction of the SLAM-blind H protein with SLAM triggers F(T461I) and the *trans*, but not *cis*, interaction of the nectin-4 blind H protein with nectin-4 does so. Some undefined structural difference between the H-SLAM and H–nectin-4 interactions may affect the process of bringing two membranes into close apposition and triggering hyperfusogenic F proteins, accounting for the different findings with the two receptors. Furthermore, the *cis* interaction of the WT H protein with SLAM could not trigger F(T461I), probably because the high-affinity interaction between the WT H protein and SLAM downregulated the molecules from the cell surface, where they have to act on the F protein. Thus, the *cis*-acting receptor activity is observed only for SLAM in conjunction with the SLAM-blind H protein and hyperfusogenic F proteins. Nevertheless, such *cis* interactions with receptors could be exploited by viruses where cell-cell contact is assured.

MeV isolates from SSPE patients usually do not produce virus particles because of mutations but retain the ability to spread in a cell-to-cell manner through membrane fusion (6). It is thought that MeV nucleocapsids (ribonucleoprotein complexes) spread in neurons *trans*-synaptically via membrane fusion between the pre- and postsynaptic membranes (19, 20). The average size of the synaptic cleft is about 20 nm, and the contact between pre- and postsynaptic membranes is maintained by various synaptic adhesion molecules (39, 40). Thus, neurological synapses may obviate the requirement for cell-cell contact during MeV-induced membrane fusion and be ideal for not only the *trans* but also the *cis* interaction of the MeV H protein with receptors to trigger the F protein. Accordingly, the putative MeV neuron receptor may be present on the same synaptic membrane as and/or on a different synaptic membrane from the one on which the H protein is expressed. Identification of a putative neuron receptor, which is a future challenge, is required to provide direct evidence for the contribution of weak *cis* and *trans* interactions of the H protein with the receptor to MeV neurotropism.

In addition to neurological synapses, virus cell-to-cell transmission has been shown to occur at virological synapses by human immunodeficiency virus 1 (41–44) and at polarized epithelia by respiratory viruses (45, 46). The *cis* interaction with receptors may play a role in cell-to-cell transmission at these sites.

To expand cell tropism and host ranges, viruses may adapt to utilize new receptors. Usually, their receptor-binding attachment proteins may undergo changes through mutations so that they acquire the ability to bind new receptor molecules. An illustrative example is that substitutions in the H protein such as H(N481Y) allow MeV vaccine strains to utilize ubiquitously expressed CD46 as a receptor (47, 48). However, it is not the changes in the receptor-binding H protein but those in the F protein that confer on MeV isolates from SSPE patients the ability to utilize other host molecules than SLAM and nectin-4 as a receptor. This is a novel strategy of virus evolution by which a virus adapts to the environment lacking cells with authentic receptors.

MATERIALS AND METHODS

Cells. 293FT cells (R70007; Invitrogen) were maintained in Dulbecco's modified Eagle medium (DMEM) (FUJIFILM Wako Pure Chemical Corporation, Japan) supplemented with 10% fetal bovine serum (FBS). 293FT cells stably expressing DSP1 and DSP2 (31–33), kindly provided by Z. Matsuda, the University of Tokyo, were maintained in DMEM supplemented with 10% FBS and 1 μg/ml puromycin (InvivoGen, San Diego, CA).

Plasmids. The eukaryotic expression vector pCA7 (49) is a derivative of pCAGGS (50). The pCA7 plasmids expressing the MeV H protein (the IC-B strain), MeV F protein (the IC-B strain), and human SLAM were described previously (7, 12, 13, 27). The cDNA for the nectin-4 gene was inserted into the EcoRI and NotI sites in the pCA7 plasmid.

Fusion assay. 293FT cells cultured in 24-well plates were transfected with different combinations of pCA7 plasmids expressing the MeV H protein [WT-H, H(R533A) or H(Y543S)], MeV F protein [WT-F, F(T461I), F-triple, F(M94V) or F-Δ30], EGFP, and MeV receptor (SLAM, nectin-4, or control [vector plasmid only]) using Lipofectamine LTX (Thermo Fisher Scientific). The induction of cell-cell fusion was evaluated 30 h after transfection by fluorescence microscopy (Fig. 1A). For quantification of cell-cell fusion, 293FT/DSP1 and 293FT/DSP2 cells were cocultured in 24-well plates and then transfected with pCA7 plasmids expressing the MeV H protein [WT-H, H(R533A) or H(Y543S)], MeV F protein [WT-F or F(T461I)],

and MeV receptor (SLAM, nectin-4, or control). The Renilla luciferase activity in the transfected cells was analyzed 24 h after transfection using a Renilla luciferase assay system (Promega, Madison, WI) (Fig. 1C). To evaluate the *cis*- and *trans*-acting abilities of MeV receptors, 293FT/DSP1 cells and 293FT/DSP2 cells were cultured in 24-well plates and 6-well plates, respectively. 293FT/DSP1 cells were transfected with the pCA7 plasmid expressing the MeV receptor (SLAM, nectin-4, or control) (for *trans* evaluation). 293FT/DSP2 cells were transfected with the pCA7 plasmids expressing the MeV H protein [WT-H, H(R533A), or H(Y543S)], MeV F protein [F(T461I)], and a MeV receptor (SLAM, nectin-4, or control) (for *cis* evaluation). For some experiments, both 293FT/DSP1 and 293FT/DSP2 cells were also transfected with the plasmid expressing E-cadherin. The transfected 293FT/DSP2 cells were detached and overlaid onto the transfected 293FT/DSP1 cells 3 to 4 h after transfection. The Renilla luciferase activity in the cells was analyzed 24 h after mixing the cells, using a Renilla luciferase assay system (Promega, Madison, WI). Chemiluminescence was measured using a Mithras LB940 plate reader (Berthold Technologies, Pforzheim, Germany) (Fig. 1D). All measurements were taken from distinct samples.

Flow cytometry analysis. 293FT cells were transfected with the plasmid expressing the MeV H protein and with that expressing SLAM or the control. The cells were incubated with human polyclonal antibody against MeV (51) 48 h after transfection, followed by Alexa Fluor 488-conjugated anti-human IgG (Molecular Probes, Inc.). The cells were then analyzed on a FACSCalibur HD flow cytometer using BD CellQuest Pro, version 5.2.1, software. Target cell populations were first distinguished from cell debris by forward scatter (FSC) and side scatter (SSC) gating, followed by detection of the MeV H-positive populations (Fig. 5C).

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